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Cellular and Organismal Ramifications of de novo Purine Synthesis Dysregulation

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Abstract

Purines are a class of nitrogenous bases and are essential small molecules to life. Purines are used within the cell as genetic information carriers, energy currency, signaling molecules, and cofactors for multiple processes. They are formed through *de novo* and salvage pathways found in cells across the phylogenetic tree. The substrates of enzymes within *de novo* purine synthesis are known to influence other processes within the cell, such as energy homeostasis. In humans, *de novo* purine synthesis disorders are rare, with around 100 people identified. These patients exhibit a range of phenotypes, with varying degrees of mental retardation, seizure activity, facial and body dysmorphic features, autistic features, respiratory failure, and congenital blindness. To date, the explanation of phenotypes associated with these disorders remains elusive and as such, no effective therapeutic has been identified. Rare disorders are often caused by a single genetic mutation and studying rare disorders can providing key insight into processes regulated by that specific enzyme. In this body of work, I use transcriptomic profiling techniques to provide cellular and organismal process characterization of a novel cellular model of *de novo* purine deficiency in three CRISPR generated HeLa cell lines. I examine the *de novo*purine synthesis enzymes ADSL, GART, and ATIC. Processes identified influenced by *de novo*purine dysregulation identified are focused around neural, embryonic, organ, and placental development, epithelial to mesenchymal transition, fatty acid and inflammatory response, muscle function, tumorigenesis, oxidative stress responses, as well as TGFβ/SMAD signaling among others. Metabolomic profiling was employed to bolster transcriptomic findings, with aberrations of metabolic pathways involved in energy production, vitamin B6 and B5 metabolism, oxidative stress responses, lipids, amino acids, among others. My findings highlight areas in which *de novo* purine synthesis enzymes influence cellular processes responsible for cellular and organismal function and represent novel avenues of continued research.

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Purines are a class of nitrogenous bases and are essential small molecules to life. Purines are used within the cell as genetic information carriers, energy currency, signaling molecules, and cofactors for multiple processes. They are formed through *de novo* and salvage pathways found in cells across the phylogenetic tree. The substrates of enzymes within *de novo* purine synthesis are known to influence other processes within the cell, such as energy homeostasis. In humans, *de novo* purine synthesis disorders are rare, with around 100 people identified. These patients exhibit a range of phenotypes, with varying degrees of mental retardation, seizure activity, facial and body dysmorphic features, autistic features, respiratory failure, and congenital blindness. To date, the explanation of phenotypes associated with these disorders remains elusive and as such, no effective therapeutic has been identified. Rare disorders are often caused by a single genetic mutation and studying rare disorders can providing key insight into processes regulated by that specific enzyme. In this body of work, I use transcriptomic profiling techniques to provide cellular and organismal process characterization of a novel cellular model of *de novo* purine deficiency in three CRISPR generated HeLa cell lines. I examine the *de novo* purine synthesis enzymes ADSL, GART, and ATIC. Processes identified influenced by *de novo* purine dysregulation identified are focused around neural, embryonic, organ, and placental development, epithelial to mesenchymal transition, fatty acid and inflammatory response, muscle function, tumorigenesis,

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Chapter One: Introduction

Overview

Purines are a class of nitrogenous bases and are vital to cellular and organismal functions. Defects in purine synthesis have clinical implications in patients and are extremely rare; the processes by which they affect patients are not well understood. There is known relevance of DNPS in energy homeostasis, developmental, and tumor biology. The study of rare disorders often reveals vital information of biological significance. In this dissertation, I explore the potential processes that are affected by purine synthesis dysregulation and metabolite intermediate accumulation.

Nitrogenous bases and their nucleoside and nucleotide conjugates

Nitrogenous bases are essential small molecules to life. These nitrogenous bases can be conjugated with a pentose sugar to form a nucleoside, and further conjugated with one to three phosphate groups to form nucleotides (Figure 1.1A). Nucleotides can then be polymerized into nucleic acids, in the form of deoxy- and ribonucleic acid (DNA and RNA respectively). There are five common identities of nitrogenous bases, grouped into one of two classes. These two classes of nitrogenous bases are purines and pyrimidines. The pyrimidine class is comprised of cytosine, uracil, and thymine while purine class is comprised of adenine and guanine (Figure 1.1B) (Figure 1.1A, B adapted from (Watson et al. 2008)). Nitrogenous bases can be made via two types of pathways, *de novo* and salvage. Salvage pathways typically use degradation products of nucleic acid catabolism

as an input to generate nucleotides with minimal steps while the *de novo* synthesis pathways use small molecule precursors from other biochemical pathways over many steps to form purines and pyrimidines (Roy et al. 2016; Moffatt and Ashihara 2002).

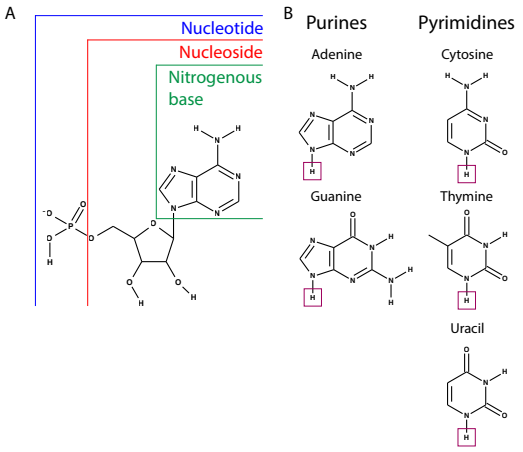


Figure 1.1: Chemical structure of nitrogenous bases and their classes. Nitrogenous bases can be further conjugated into nucleosides and nucleotides. Adenosine triphosphate with nitrogenous base, nucleoside, and nucleotide (triphosphate) conjugation identified (A). Chemical structures of nitrogenous bases composing the two major classes, purines and pyrimidines. Dark red boxes on purine and pyrimidines indicate site of sugar conjugation making nucleosides from nitrogenous bases (B). Chemical structure accessed from Molecular Biology of the Gene 6th edition and skeletal drawings made using PubChem Sketcher 2.4 (PubChem).

Purine metabolism

Purines are a class of nitrogenous bases containing a six-atom ring bound to a five-carbon ring with one of two side group identities dependent on it being adenine or guanine base (Figure 1.1A). The two types of pathways capable of generating purines are the salvage and *de novo. De novo* purine synthesis (DNPS) uses the small molecule phosphoribosyl pyrophosphate (PRPP) and generates inosine monophosphate (IMP) via ten or eleven steps (Jinnah, Sabina, and Van Den Berghe 2013; Kappock, Ealick, and Stubbe 2000) (Figure 1.2A). DNPS, the focus of this doctoral work, utilizes six enzymes in higher eukaryotes, a singular trifunctional enzyme, two bifunctional, and three monofunctional. Salvage synthesis (Figure 1.2B) commonly utilizes the small molecules adenine (Ade), guanine (Gua), or hypoxanthine (Hx) and within one step can be synthesized into AMP, GMP, or IMP, depending on the precursor molecule. IMP is converted into AMP or GMP, each requiring two more sequential steps. Typically, salvage synthesis relies on the small molecule input from

purine catabolism of nucleic acids and diet. The commonly used salvage inputs are Gua, Ade, and Hx as previously mentioned. For DNPS dysregulation, Ade is a unique salvage input. Ade is able to be synthesized into AMP directly, and subsequently converted to IMP by AMP deaminase, which may then be further processed to generate GMP. Hx is converted directly into IMP and then able to be synthesized into either GMP or AMP, however in the conversion of IMP to AMP, the DNPS enzyme ADSL is bifunctional and required to convert SAMP to AMP. Gua is synthesized into GMP, but there is no mechanism in mammalian cells to convert GMP to IMP. Purine catabolism is essential for normal cellular function and breaks down purines to their eventual product of uric acid in humans, which may then be secreted by the body (Figure 1.2C) (Jinnah, Sabina, and Van Den Berghe 2013). Figure adapted from (Jinnah, Sabina, and Van Den Berghe 2013).

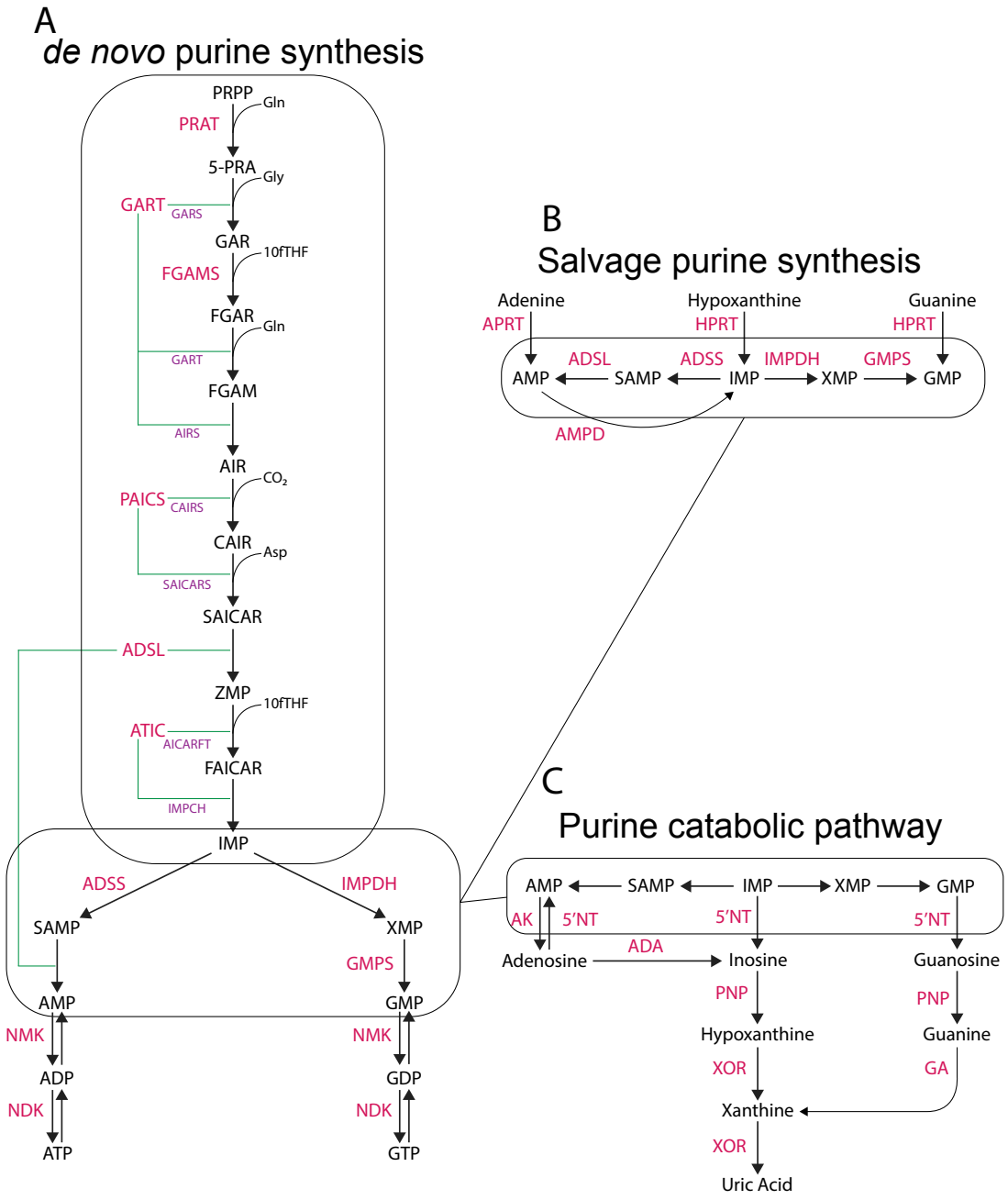


Figure 1.2: Purine synthesis pathways and catabolism. Purines can be synthesized via *de novo* and salvage pathways. DNPS uses the small molecule PRPP as input through ten sequential steps to IMP. Certain steps require other substrates as identified. IMP may then be synthesized into AMP or GMP and phosphorylated to ATP or GTP, these steps are not within DNPS (A). Salvage purine synthesis utilizes the small molecules (among others) adenine, guanine, and hypoxanthine to directly make AMP, IMP, or GMP in one single step. Adenine and hypoxanthine are the only salvage inputs capable of making AMP and GMP (A). Purine catabolism breaks AMP, IMP, and GMP into uric acid in humans for secretion (C). Small molecules/metabolites/intermediates are found as black text, enzymes are pink, functional domains of multifunctional proteins are purple with green lines indicating which steps the multifunctional enzymes catalyze. Figure adapted from Jinnah 2013. Abbreviation list as follows:

Small molecule/Metabolite/Intermediate (Black):
ZMP (AICAR): aminoimidazolecarboxamide ribotide
ADP: adenosine diphosphate
AMP: adenosine monophosphate
ATP: adenosine triphosphate
GDP: guanosine diphosphate
GMP: guanosine monophosphate
GTP: guanosine triphosphate
IMP: inosine monophosphate
PRPP: phosphoribosylpyrophosphate
SAICAR: succinyl- aminoimidazolecarboxamide ribotide
SAMP: succinyl-AMP or adenylosuccinate
XMP: xanthine monophosphate
PRPP: phosphoribosyl pyrophosphate
5-PRA: phosphoribosylamine
GAR: glycineamide ribonucleotide
FGAR: phosphoribosyl-N-formylglycineamide
FGAM: 5'-phosphoribosyl formylglycinamidine
AIR: 5'-phosphoribosyl-5-aminoimidazole
CAIR: 5'phosphoribosyl-4-carboxy-5-aminoimidazole
FAICAR: 5-formamidoimidazole-4-carboxamide ribotide
Asp: aspartate
10fTHF: 10-formyltetrahydrofolate
Gly: glycine
Gln: glutamine
Ade: adenine
Gua: guanine
Hx: hypoxanthine

Enzyme (Pink):
5′NT: 5′-nucleotidase
PRAT: Phosphoribosyl amidotransferase
GART: Phosphoribosylglycinamide Formyltransferase/Phosphoribosylglycinamide Synthetase/Phosphoribosylaminoimidazole Synthetase
FGAMS: phosphoribosyl formylglycinamidine synthase
PAICS: Phosphoribosylaminoimidazole Carboxylase/Phosphoribosylaminoimidazolesuccinocarboxamide Synthase
ADSL: adenylosuccinate lyase
ATIC: 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase/IMP Cyclohydrolase
ADA: adenosine deaminase
AK: adenosine kinase
ADSL: adenylosuccinate lyase
AMPD: adenylate deaminase
PRAT: amidophosphoribosyltransferase
APRT: adenine phosphoribosyltransferase
ADSS: adenylosuccinate synthetase
ATIC: AICAR-transformylase/IMP-cyclohydrolase
GA: guanase
GS: GMP-synthase
HPRT: hypoxanthine-guanine phosphoribosyltransferase
IDH: IMP-dehydrogenase
NDK: nucleoside diphosphate kinase
NMK: nucleoside mono-phosphate kinase
XOR: xanthine oxidoreductase

Enzyme domains (purple):
GARS: phosphoribosylglycinamide synthetase
GART: phosphoribosylglycinamide formyltransferase
AIRS: phosphoribosylaminoimidazole synthetase
CAIRS: phosphoribosylaminoimidazole carboxylase
SAICARS: phosphoribosylaminoimidazolesuccinocarboxamide synthetase
AICARFT: 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase
IMPCH: IMP cyclohydrolase

De novo purine synthesis requires small molecule inputs from multiple connected pathways and processes

In order to understand the implications of DNPS, we must first understand the interconnectedness of multiple biochemical pathways resulting in IMP generation. Glucose is transformed into pyruvate via ten steps. The glycolytic intermediates glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and glyceraldehyde-3-phosphate (G3P), can be funneled into the pentose phosphate pathway, generating ribose-5-phosphate (R5P). R5P is converted into PRPP via phosphoribosyl pyrophosphate synthase (Dayton, Jacks, and Vander Heiden 2016). Once PRPP is synthesized, it may then be utilized for DNPS. The six enzymes necessary to complete the ten sequential steps in DNPS to convert PRPP to IMP in higher eukaryotes require other substrates to contribute atoms in the generation of the inosine moiety (Figure 1.3). DNPS utilizes atom donor substrates in various steps of the pathway to incorporate atoms and moieties into its final IMP structure. These substrates are glycine, glutamine, aspartate, and 10-formyltetrahydrofolate (Figure 1.2A). Amino acid synthesis/transport processes, glutathione-gammaglutamyl amino acid cycle and the folate cycle are all therefore critical for the synthesis of IMP via DNPS (Figure 1.4). Adenosine monophosphate (AMP) or guanosine monophosphate (GMP) are then generated via two

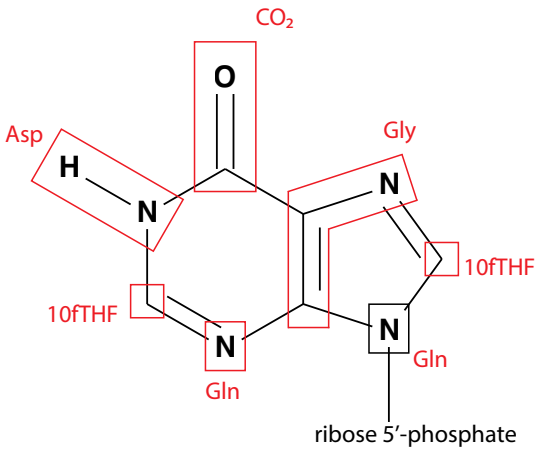


Figure 1.3: Inosine ring substrate contributions. Inosine is synthesized via contributions of substrates within DNPS, each contributing atom is highlighted in red boxes with their respective contributing substrate.

additional steps from IMP (Figure 1.2A). AMP and GMP can then be further processed into products based on cellular need, such as via phosphorylation to generate adenosine triphosphate (ATP) or guanosine triphosphate (GTP).

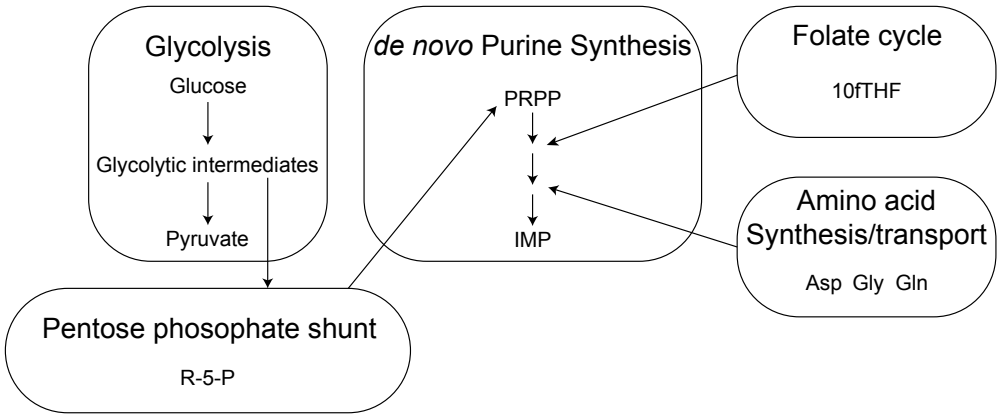


Figure 1.4: DNPS requires input derived from multiple pathways. Glucose is funneled through the Glycolytic metabolic pathway. Certain glycolytic intermediates are then used within the Pentose phosphate shunt pathway which generates R-5-P. R-5-P is then converted into PRPP which is the initial small molecule input for DNPS. Molecules from amino acid synthesis and transport as well as the folate cycle are used as substrates within DNPS.

DNPS evolution

Evolution is classically thought as the gain of favorable adaptative features in a species population to the surrounding environment over time through the process of natural selection, and that all life shares an ancient common ancestor (Darwin 1859). This idea has been implemented in myriad subfields of biology, prompting the idea of enzyme evolution and metabolic pathway evolution (Caetano-Anolles, Kim, and Mittenthal 2007; Caetano-Anollés et al. 2009). Enzyme evolution occurs from processes such as gene duplication (Ohno 1970), rearrangement of gene fragments (Vogel et al. 2004), or horizontal gene transfer (Pál, Papp, and Lercher 2005). The DNPS pathway is highly conserved, found across all three cellular domains, *Bacteria*, *Archaea*, and *Eukarya*

(Armenta-Medina, Segovia, and Perez-Rueda 2014). In microorganisms, such as bacteria, DNPS enzymes are monofunctional (Kappock, Ealick, and Stubbe 2000). Moving higher in cell and organism complexity, some DNPS enzymes adopt multifunctionality within the pathway, with higher eukaryotes (e.g. humans) utilizing six enzymes to complete the ten steps (Pedley and Benkovic 2017). Interestingly, the DNPS enzyme ADSL was found to have a conserved evolutionary characteristic as recent as the transition from Neanderthal to modern humans, with a single nucleotide point mutation that resulted in decreased ADSL stability in modern humans (Stepanova et al. 2020). The ubiquity of DNPS in living organisms across the phylogenetic tree highlights the importance of a functioning pathway necessary for the maintenance of life.

Purines fulfill multiple roles in cellular and organismal functions

Purines fulfill many roles within the cell. As previously stated, nucleotides can be polymerized into genetic information material in the form of RNA and DNA. The monomeric form of ATP is used as a major energy currency. Purines are also critical as primary and secondary signaling molecules. Purines are also used as cofactors for various cellular processes (Pedley and Benkovic 2017).

Extracellular signaling activities have been identified for purines. Purinergic signaling has been implicated in cell proliferation, migration, cellular differentiation, embryonic death, wound healing, inflammation, and others (Burnstock 2009). Indeed, ATP has long since been proposed to act as a neurotransmitter (Burnstock, Dumsday, and Smythe 1972) and myriad functions in nervous system have been found, such as neuroprotection, control over autonomic function, neural-glial interaction, pain and mechanosensory transduction, and physiology of senses (Burnstock 2006).

Cyclic AMP (cAMP) is a classic example of a secondary messenger, employed as such in organisms ranging from amoebas, plants, to humans (Hofer and Lefkimmiatis 2007) mediating processes such as memory, metabolism, gene regulation, and immune function (Beavo and Brunton 2002). Upon cellular signal, ATP will be converted to cAMP, which in turn mediates response (Serezani et al. 2008).

Adenine base nucleotides are components of many coenzymes such as nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, and coenzyme A (Roy et al. 2016; Pedley and Benkovic 2017). The nicotinamide and flavin adenine dinucleotides are essential for the tricarboxylic acid cycle (TCA cycle, commonly known as Krebs or Citric acid cycle) and are used for redox reactions between oxidative phosphorylation and the TCA cycle, in the generation of energy in the form of ADP to ATP conversion (Martínez-Reyes and Chandel 2020). Coenzyme A and its derivatives have myriad cellular roles, such as use in synthesis and oxidation of fatty acids, synthesis of acetylcholine, acetylation of histones (Martinez, Tsuchiya, and Gout 2014), as well as oxidation of pyruvate for TCA cycle input (Martínez-Reyes and Chandel 2020).

Guanine base nucleotides are most notable for their role in G protein-coupled receptors, expressed across most life forms, mediating a majority of cellular responses to external stimuli (Weis and Kobilka 2018). G protein couple receptors utilize guanosine nucleotides, binding and exchanging between GDP and GTP to recognize and incorporate extracellular signals (Weis and Kobilka 2018; Hanlon and Andrew 2015).

DNPS pathway intermediates have roles outside of DNPS

DNPS intermediates are small molecule products and substrates of the enzymes found in the DNPS pathway. Some intermediates found in the pathway are relatively short-lived under cellular conditions. Notably, 5-PRA, the first substrate for the trifunctional enzyme GART, is broken down under cellular conditions within 5 seconds (Rudolph and Stubbe 1995). However, SAICAR and ZMP, the first substrates for the enzymes ADSL and ATIC respectively have been shown to regulate processes outside of DNPS. SAICAR has been recently shown as a binding partner of the glycolytic pathway enzyme PKM2 and is of keen interest for cancer research. ZMP is a known and well characterized activator AMPK, the master regulator of energy homeostasis and mTOR activity. (Figure 1.5)

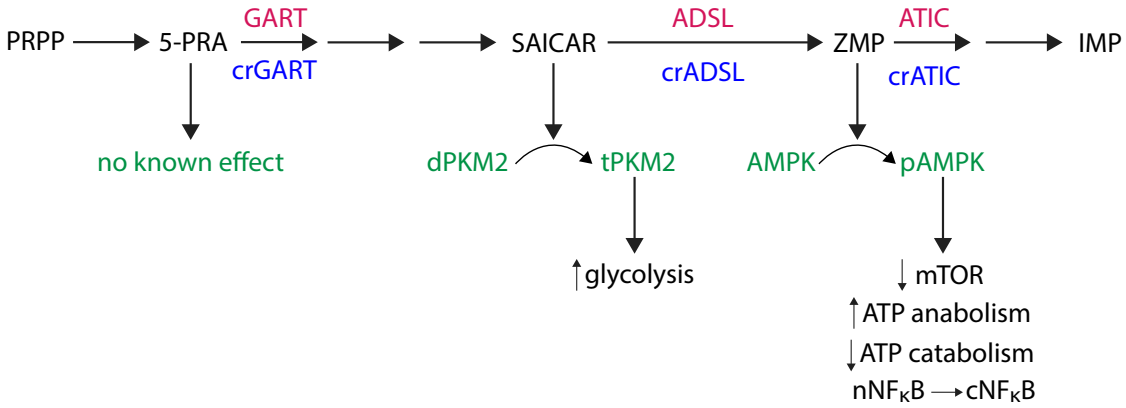


Figure 1.5: DNPS intermediate effects. Multiple intermediates found within the DNPS pathway have off pathway effects. SAICAR was found to tetramerize PKM2 which increases glycolysis and may moonlight as a protein kinase. ZMP is a well characterized to phosphorylate AMPK, which inhibits the mTOR pathway, altering ATP metabolism, and remove nuclear translocation of NFkB. The intermediate 5-PRA is short lived under cellular conditions and has no known off pathway effect.

Glycolysis is a ten sequential step pathway taking glucose and forming pyruvate, directly forming two ATP and two reduced nicotinamide adenine dinucleotide molecules per glucose input. The last step catalyzes phosphoenol pyruvate into pyruvate through the pyruvate kinase (PK) enzyme family and is irreversible. There are four isoforms of PKs,

PKL, PKR, PKM1, and PKM2 (Dayton, Jacks, and Vander Heiden 2016), however we will only be discussing PKM2 as this isoform plays an important role in regulation of transcription and protein phosphorylation in addition to its role in metabolism. Normal aerobic cellular energy production relies slightly on glycolysis to produce pyruvate and form acetyl-CoA in the cytosol, which is then translocated into the mitochondria to produce high energy electrons in the form of nicotinamide and flavin adenine dinucleotide (NADH and FADH, the reduced forms) through the TCA cycle. These high energy electron carriers are then used in oxidative phosphorylation, using oxygen as the electron acceptor in the electron transport chain (ETC) which phosphorylates ADP to ATP creating vast amounts of usable energy per unit input. Anaerobic energy production is used during exposure to oxygen poor environments, unable to support proper ETC function and thus favoring glycolysis. Here pyruvate is shunted to lactic acid formation using the enzyme lactate dehydrogenase and nicotinamide adenine dinucleotide as an electron donor, generating small amounts of energy per unit input in the form of ATP.

When cells become tumorigenic, a change in metabolism and energy production usually occurs. The Warburg Effect is based in an observation that most solid tumors produce lactic acid in an oxygen rich environment (Warburg and Minami 1923). This finding was rather curious, as lactic acid was typically thought solely as a byproduct of glycolytic metabolism in oxygen deficient environments. As these tumors were producing lactic acid in an oxygen rich environment, the Warburg effect became known as aerobic glycolysis. Tumor cells bypass checkpoints in the cell cycle designed to induce a vegetative state after division and as such, are uncontrollably dividing. The Warburg effect hypothesis speculates that due to tumor cells unchecked proliferation, an elevated

need exists for nucleotide bases; as the glycolytic intermediates can be funneled into the pentose phosphate pathway to make PRPP, necessary for the formation of purines and pyrimidines. The cell can therefore synthesize a large pool of necessary nitrogenous bases and therefore DNA and RNA for division events. PKM2 is the most easily manipulatable of the PK isoforms, with multiple regulators molecules and proteins commonly found in proliferating cells, permitting or halting its pyruvate kinase activity (Dayton, Jacks, and Vander Heiden 2016). This control allows selective signaling for glycolytic intermediates to build up for nucleotide synthesis or allowing pyruvate to form for lactate-based energy production. Tumor cells also utilize glutaminolysis pathway for energy production, providing an alternative input for TCA cycle (Zahra et al. 2020). It has been found that most tumors selectively express PKM2 over other isoforms of PKs giving credence to this hypothesis (Mazurek 2011).

PKM2 exists as an active homotetramer (tPKM2), inactive homodimer (dPKM2), or and monomer (Dayton, Jacks, and Vander Heiden 2016) and is typically expressed in fat tissue, lung, pancreas, as well as highly proliferative cell types such as embryonic cells and tumors (Mazurek 2011). As previously stated, multiple avenues exist to modulate PKM2 activity, typically achieved through stabilizing or destabilizing its tetramer (Dayton, Jacks, and Vander Heiden 2016). The glycolytic intermediates phosphoenol pyruvate (PEP) and fructose-1,6-bisphosphate (FBP) as well as serine activates PKM2, while alanine, thyroid hormone T3, and ATP, deactivate PKM2 (Zhang et al. 2019). PKM2 activity is also influenced by protein-based regulators such as by ERK1/2 signaling, tyrosine kinases FGFR1, and 0-GlcNAcetylation (Zhang et al. 2019). SAICAR was recently found to bind PKM2, possibly stabilizing its tetramer (Figure 1.5,

tPKM2) (Keller, Tan, and Lee 2012), and possibly allowing an aberrant moonlighting function by adopting a protein kinase activity (Keller et al. 2014). Although the potential protein kinase activity of PKM2 has been questioned (Hosios et al. 2015), the preponderance of data suggests that the protein kinase moonlighting activity of PKM2 is real (Zhang et al. 2019). The findings that suggest SAICAR has a role in modulating PKM2 activity presents a novel research area in tumor biology, and therefore accumulation of SAICAR in within the cell may be used to study PKM2 activity. SAICAR however, cannot be fed to cells directly as mechanisms have not been found and it is difficult to synthesize, so more circuitous methods need to be applied. Researchers have employed glucose starvation methods (Keller et al. 2014) as well as siRNA against ADSL (Keller, Tan, and Lee 2012). Sugar starvation is less than ideal, owing to the potential total cellular metabolism effects of energy deprivation as well as pyruvate kinase activity directly being affected by the starvation. Stable transfection of anti-ADSL shRNA in HeLa cells have shown ~80% reduction of cellular ADSL protein and permitting SAICAR accumulation (Keller, Tan, and Lee 2012) but still allowing low levels of ADSL and DNPS activity.

ZMP is also a small molecule of particular interest as it is a known AMP mimetic (Hardie 2011; Garcia and Shaw 2017; Douillet et al. 2019). AMP is a known allosteric effector of multiple enzymes, notably the cellular metabolism master regulator protein AMP-activated protein kinase (AMPK). AMPK is a heterotrimer, composed of an α, β, and γ subunit. AMPK senses the AMP/ATP ratio, upon increase in this ratio, AMP binds AMPK and promotes the phosphorylation of AMPK α subunit at Thr172 (pAMPK), activating AMPK activity (Hardie 2011). Activation of AMPK promotes ATP anabolism

and hamstrings ATP catabolism. ZMP is shown to be a potent AMPK activator using the same mechanisms of AMP, specifically it promotes AMPK phosphorylation (Hawley et al. 1995), inhibition of AMPK dephosphorylation (Davies et al. 1995), and allosteric activation of phosphorylated AMPK (Corton et al. 1995). pAMPK is a known effector of the mammalian target of rapamycin complex 1 and 2 (mTORC1 and mTORC2) (Saxton and Sabatini 2017; Huang et al. 2008). The mTOR pathways regulate vital cellular metabolic processes involved in lipid synthesis, glycolysis, mitochondrial and lysosomal biosynthesis, apoptosis, glucose metabolism, cytoskeletal rearrangement, and cell migration (Linke et al. 2017; Saxton and Sabatini 2017). pAMPK inhibits mTORC1 pathway via phosphorylation of its regulatory protein component Raptor and indirectly via activation by phosphorylation of tuberous sclerosis complex 1/2, a GTPase complex that generates an inhibitor of mTORC1 (Inoki, Kim, and Guan 2012). pAMPK has been shown to affect processes seemingly outside of cellular metabolism. It can reverse the nuclear translocation of the proinflammatory transcription factor, NFκB, in response to inflammatory stimuli (Xiang et al. 2019), regulates inflammatory suppression (Jeon 2016), and restriction of interferon-γ signaling (Meares et al. 2013). In addition to AMPK interactions, ZMP was shown to have 74 different enzyme interacting partners in yeast (Douillet et al. 2019), highlighting that ZMP effects within the cell are multiple and complex.

Previous work used AICAriboside (the dephosphorylated form of ZMP) to promote phosphorylation of pAMPK. In these experiments, AICAriboside is administered in cell culture via media supplementation (Corton et al. 1995) or in animal models via injection (Xiang et al. 2019). Upon cellular import, AICAriboside becomes

phosphorylated via adenyl kinase (AK) into its active ZMP form and can then induce phosphorylation of AMPK (Hardie 2011). ATIC is a homodimer and is active only in its dimeric state (Spurr et al. 2012). The drug Compound 14 was recently discovered, blocking the dimerization of ATIC which allows the accumulation directly of the active form of ZMP without relying on cellular import and phosphorylation events (Spurr et al. 2012).

Clinical relevance of DNPS dysregulation

DNPS is highly conserved and critical for cellular and organismal development. To date, only three enzymes are found to have mutations that result in decreased activity within the DNPS pathway in *H. sapiens.* These enzymes are ADSL, ATIC, and PAICS and deficiency is extremely rare.

ADSL deficiency is the most common of DNPS defects. Currently, less than 100 patients have been identified having this disorder (Jurecka et al. 2015). These patients are grouped into three classes depending on phenotype, neo-natal fatal, Type I severe, and Type II mild to moderate. The phenotype is a continuum, with degrees of psychomotor retardation, seizures, visual impairment, speech impairment, and craniofacial dysmorphic features (Jurecka et al. 2015). Multiple mutations have been identified leading to a mutation dependent decrease in ADSL enzymatic activity (Zikanova et al. 2010).

PAICS deficiency is the second most common, with two patients (siblings from two separate pregnancies) identified in the Faroe Islands. The deficiency stems from an amino acid point mutation resulting in Lys53Arg leading to a 75% reduction in enzyme activity. These patients presented with multiple phenotypes such as small body,

brachycephaly, lung malformation, craniofacial dysmorphism, vertebral malformations, among others. Both infants died within three days of birth (Pelet et al. 2019).

AICAribosiduria, also known as ATIC deficiency, has only been identified in a single patient. The patient was found to have one allele with a frameshift in exon two which resulted in unstable mRNA, and a point mutation of Lys426Arg. This mutation showed no detectable AICAR-TF activity and a 60% reduction in IMPCH activity of the ATIC enzyme (the first and second reaction mediated by ATIC respectively). This patient presented with profound mental retardation, epilepsy, brachycephaly, congenital blindness, and facial dysmorphic features, among others. Last available record of record patient check-up was performed at 4 years of age and no treatment was reported directly for AICAribosiduria (Marie et al. 2004).

So far, all patients with defective DNPS have residual enzyme activity. It may be that complete lack of activity is lethal in embryos. Evidence for this includes the difficulty in generating mouse knockouts, to date, a singular mouse line has been made lacking a DNPS enzyme, FGAMS ("C57BL/6NJ-Pfasem1(IMPC)J/Mmjax" n.d.). Our laboratory in fact obtained mice with a heterozygous knockout of ADSL. Breeding and genotyping of several hundred matings of these mice never resulted in a homozygous ADSL knockout mouse.

GART is implicated in Down syndrome (DS), however not due to a deficiency in enzymatic activity. DS is an aneuploidy caused by a triplication in Hsa21. The GART gene is located on 21q22 (Patterson 1987) and is therefore triplicated in DS. The DS phenotype is characterized by cognitive deficits in the form of intellectual and learning disabilities, hypotonia, craniofacial abnormalities, and cardiac malformations (Brodsky et

al. 1997). DS accounts for approximately 1 in 700 live births in the United States (Mai et al. 2019). DS shows unique spatiotemporal expression of the GART enzyme with one study showing cerebellar GART levels precipitously drop post-partum in control while in DS patients showed detectable levels as long as 49 days post-partum (Brodsky et al. 1997). Two laboratories, ours and that of John Gearhart, produced mice transgenic for human GART and therefore triplicated for GART (two mouse copies and one human). These mice showed no detectable phenotype, with the possible exception of a slight hearing deficit (Knox 2006).

It is therefore clear that any disorder affecting DNPS presents as developmental disorder. These clinical phenotypes show the importance for well controlled DNPS in embryogenesis. This is perhaps unsurprising as DNPS is critical for rapidly dividing cells, a feature of embryogenesis. Purinergic components have been of recent interest for pharmacological intervention for congenital neurological defects (Fumagalli et al. 2017). Taken together, full characterization of DNPS dysregulation is of particular importance to understanding embryogenesis as well as neurological defects.

-omics

The central dogma of molecular biology is that DNA is transcribed into RNA, which is then translated into protein. Proteins can then go on to perform countless cellular functions, from anchoring the cell to the extracellular matrix, importing or exporting cargo, trafficking, metabolizing small molecules, modifying the activity of other proteins, acting as a transcription factor, among myriad others. DNA is read and synthesized into RNA via transcription, while RNA is read and made into protein sequences through translation. Proteins may or may not directly alter metabolites, which are typically

thought of as small molecules under 1 kDa in size. The entire DNA sequence is known as the genome, while total RNA is known as the transcriptome, the entire protein content is known as the proteome, and all metabolites are known as the metabolome (Figure 1.6). Each of these "-omes" presents a novel set of data with their advantages and disadvantages. As such, integration of multiple -omics techniques may be required to gain a complete picture of response to stimuli or stress (Riekeberg and Powers 2017).

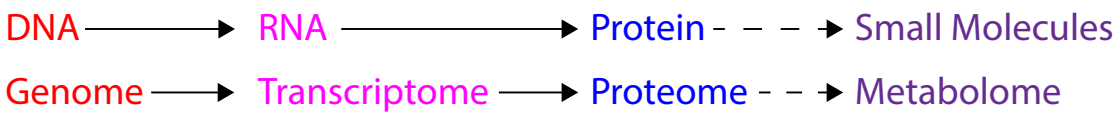


Figure 1.6: Schema of molecular biology dogma. DNA is transcribed to make RNA, which is translated to protein, some proteins can synthesize small molecules. The collective DNA, RNA, protein, or small molecules within a sample represent the genome, transcriptome, proteome, or metabolome respectively.

The genome comprises information about the DNA sequences found within the cell, comprising of coding (gene) and non-coding stretches. Through probing genomics, information can be attained about the root or underlying cause in disease (International Human Genome Sequencing Consortium 2004). However, as cells multiply, errors in genomic replication can occur. Population based genetic drift and inheritance can also allow errors to occur and propagate. These errors manifest in various ways such as single nucleotide variations and can cause cells to function aberrantly. Single nucleotide variations in coding regions may alter protein sequence or in non-coding regions may influence gene expression or splicing (Metzker 2010). Genomics can inform a direct root cause of a disorder, however, gives scant information on how a cell is responding to that root cause or a stimulus. Due to this issue, genomic profiling is ineffective for

understanding cellular responses. Nucleic acids present a major advantage in -omics research however, as minute amounts of DNA can be amplified and read/analyzed through polymerization reactions.

The transcriptome represents all RNA in the cell, including any exon splice variations and coding single nucleotide polymorphism within the genome. Transcriptomics is a technique where cellular RNA is sequenced to gain information into what gene product is to be made as well as the RNA sequences quantified. This information taken together can explain differential expression of specific genes. When cells are exposed to a stimulus, a specific gene or groups of genes can be differentially expressed when compared to control conditions. Owing to the identity and concentration gathering component to transcriptomics, this technique can prove quite powerful. However, there are a few drawbacks. Transcriptomics, while representing what the cell is trying to accomplish in response to a stimulus, does not always track with its respective proteome (Schwanhäusser et al. 2011). This is to say that levels of transcript of a certain gene are not always in agreement with expression levels of that protein. This could be attributed to myriad reasons ranging from RNA and protein stability issues to post translational modifications. Transcriptomics has a similar benefit to genomics in that RNA sequences are able to be amplified using PCR based techniques. This allows trace amounts of RNA to be read and quantified giving an accurate snapshot of cellular response. Transcriptomic data can be generated through a process called RNA sequencing (RNA-seq).

Proteomics is the study of the entire set of proteins within the sample and is rife with problems. The large barrier to entry is the excessively high level of complexity in

proteomics. While genomics and transcriptomics utilize a four nucleotide code with three nucleotide per codon system, proteomics utilize a code of 20 amino acids with over 200 (Deribe, Pawson, and Dikic 2010) potential post translational modifications (e.g. phosphorylation, glycosylation, malonation, lipidation, acetylation, acylation, etc.) with each of these post translational modifications representing a potential change in function for each protein (Duan and Walther 2015). Examples of post translational modification altering protein activity are AMPK activates upon phosphorylation at the single residue Thr172 (Hardie 2011) while the protein CRMP2 is host to 4 nearly sequential phosphorylation sites with altered activity based on not only quantity of sites phosphorylated, but also order in which they are phosphorylated (J. Yu et al. 2019; Yamashita et al. 2012). In addition, cellular localization can confer protein activity. NFκB is a transcription factor typically sequestered in the cytoplasm, signaling induces release from sequestration and can translocated to the nucleus initiating transcriptional activity (Papa 2004). Alternative splicing as well as multimerization can alter protein function as well. There are scalability issues with proteomics owing to the fact that more protein cannot be made similar to that used in transcriptomics and genomics. A popular technique used for to proteomics is mass spectrometry (MS), which poses additional problems. MS data is biased towards peptide sequences with higher concentration, is susceptible to fragmentation differences, and contamination issues. Incomplete databases stemming from data complexity also pose an inherent issue when studying the proteome. As such, protein studies at the true -omics scale currently cannot be performed (Manzoni et al. 2018).

Metabolomics is the study of the entire set of small molecules produced by biological reactions within a sample. Metabolite changes represent the combination of genetics as well as environmental exposures (Manzoni et al. 2018), as such a metabolomic approach is an advantageous diagnostic tool as well as relevant to understanding molecular pathway aberrations related to specific disorders or stimuli. Metabolomic data can be generated, for example, via LC-MS techniques. MS fingerprints are then probed against databases assigning identity and concentration. Metabolomics is not without its drawbacks. Metabolome reference databases show no homogeny in their metabolite accession numbers and metabolites identified by their database, causing difficulty in probing multiple databases. Reference metabolomes are incomplete, in addition the biological role is not always clearly understood for all detectable metabolites. Metabolites also have a variety of functions, for example ATP is used as an energy currency as well as signaling molecule. Detection limitations also dictate total metabolites identified in metabolomic studies, which are often a fraction of the cellular metabolic profile. For these reasons, metabolomics is also currently incompatible with a true -omics scale (Riekeberg and Powers 2017; Manzoni et al. 2018; Min Yan and Xu 2018).

Transcriptomic data generation and file conversion

For RNA applications TriReagent can be used to extract total cellular RNA from multiple types of samples. This approach yields high quality RNA for downstream applications such as RNA-seq. Considerations must be made for sample replicates. Current industry standards for cell culture applications, generally speaking, dictate a replicate of three with diminishing return above six replicates.

RNA-seq allows comprehensive, broad discovery studies and provides information on how stimulus or genotype based gene expression patterns, gaining insight into the dynamics of cellular processes and its potential influence in health and disease (Manzoni et al. 2018).

Dependent on the answer looking for, there are multiple RNA-seq methodologies. We will only be exploring one of these applications relevant to this dissertation. Coding gene RNA-seq requires total RNA to be enriched for messenger RNA (mRNA) and subsequent cDNA library preparation. For coding RNA-seq, total RNA is subjected to poly(A) tail enrichment, a typical feature found in mature mRNA and then fragmented. Modern sequencing instrumentation has sequence size limitation and therefore must be fragmented; due to simplicity, this step is typically applied to the enriched mRNA although the necessary cDNA libraries can be prepared using full length mRNA and then fragmented. cDNA is generated off of fragmented mRNA using random and oligo d(T) primers through first and second strand synthesis steps, ensuring total sequence coverage, generating the necessary cDNA required for sequencing. Blunt ends are created and then adapters are ligated. These adapters contain functional elements necessary for sequencing and can contain a barcode region, a specific sequence within the adapter that allows the cDNA library prepared to have a portion with the same sequence within each preparation, increasing RNA-seq workflow and efficiency by allowing multiplexing of various samples of prepared cDNA libraries within the same sequencing run. The adapted and barcoded cDNA is then amplified and is now ready for sequencing (Figure 1.7A, B) Figures from (“Universal Plus MRNA-Seq with NuQuant User Guide” 2019; Corney

2013). Sequencing conditions must be considered and includes end reads, sequence length, as well as sequencing depth.

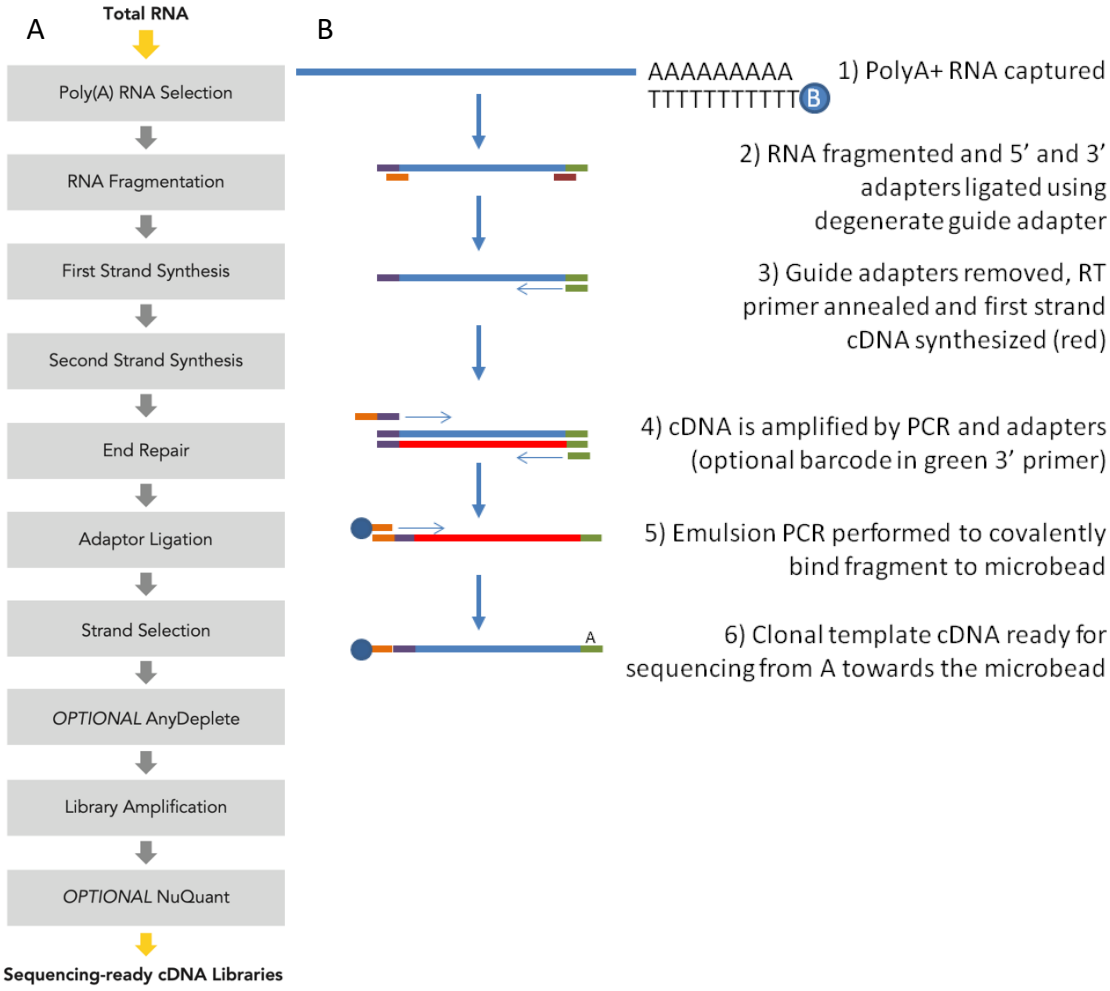


Figure 1.7: Schema of basic RNA-seq workflow for mRNA coding sequencing. mRNA is enriched from total RNA by polyA tail capturing. mRNA is fragmented and then cDNA is synthesized using primer and adapters and cDNA template is prepared for sequencing. representation of workflow, figure from NuQuant Illumina guide (A). Visual representation of workflow figure from Corney 2013 (B).

Sequencing of prepared cDNA can be done via single or paired end reads. For differential gene expression profiling, users can employ single end reads with appropriate read lengths of 50-75 base pairs; this minimizes reading across gene splice junctions while still allowing counting of all mRNAs from the sample. Identification of novel

sequences/splice variants is facilitated by paired end reads with 75 base pairs, allowing more complete coverage of the transcripts with information from 5' and 3' ends. Sequencing depth is a necessary metric for RNA-seq applications, understood as the total number of reads within a sample typically from 5-200 million reads. These read depths are adjusted based upon abundance of transcript and type of RNA-seq desired (i.e. low abundance transcript requires more read depth as to ensure validity of results). These read quantities are targets and are rarely met exactly leading to considerations for downstream processing.

Sequencer output files are converted from raw format into FASTQ format. The FASTQ is an inherently simple format, adapting the FASTA file format and assigning a numeric quality score to each nucleotide in a sequence; and thus has emerged as a standard file format for sequencing information (Cock et al. 2010). FASTQ files are used as direct input for downstream processing applications. Multiple routes exist to process these FASTQ files such as the TopHat-Cufflinks (Trapnell et al. 2012) or Salmon-DeSeq2 (Love, Huber, and Anders 2014) workflows.

Typically, for mRNA coding gene analysis, sequences from FASTQ files are processed to form gene counts for expression quantification and compared amongst all samples processed giving a normalized count. In large datasets, counts of individual identities are valuable to understand changes in abundance from between samples and conditions. These changes in abundance can be represented by a few terms. Fragments per kilobase of transcript per million mapped reads (FPKM) is a normalized estimation of gene expression. Since longer transcripts are expected to generate more reads than shorter ones (due to having more fragments) as well as differences in sequencing depth between

samples, the FPKM value accounts for these discrepancies and normalizes the counts. FPKM must be further processed to make it ideal for DEG analysis, which is accounted for in the CuffLinks processing suite (Trapnell et al. 2012). This total workflow makes FPKM a good metric for understanding differential gene expression indicating a relative concentration of specific gene transcript comparing control to experimental conditions. Normalization of counts through the DESeq2 processing suite utilizes a median-of-ratios methodology for generating DEGs. This is based upon modeling the read counts to a geometric mean value per gene scaled by a program calculated normalization factor and is unbiased by factors such as GC content and allowing DEG analysis (Love, Huber, and Anders 2014).

Changes in abundance for RNA-seq applications are typically displayed as ratios or log(2) fold change. Log(2) values evenly represents the change between samples/conditions of each specific gene reflected over zero on a number line (i.e. if a gene transcript is twice as abundant in one condition, but another gene transcript is half as abundant, the log(2)fold change will be 1 and -1 for the first and second gene transcript respectively, rather than 2 and. 0.5). These are now known as a differentially expressed gene, or DEG. Significance is then calculated between the comparison groups. For software analysis, gene accession numbers offer ease of use, however for downstream application such as Gene Ontology analysis, accession numbers are translated into gene symbols. Applying cutoffs is common practice, used to remove low statistical count anomalies in genes associated with normalized gene count. The final list is now prepared for downstream applications.

Transcriptomic data interpretation through database probing

Once lists of DEGs are generated, multiple databases exist to probe significance from them. In this study, Gene Ontology and Reactome were employed.

Gene Ontology is a manually and digitally curated database sorting genes into three families, biological process, cellular component, and molecular function. Each of these families seeks to group genes into more specific terms upon specific criteria: biological process includes genes that contribute to completion of a biological objective, cellular component refers to gene product localization, while molecular function refers to the biochemical activity of the product. Not only are genes in GO grouped into three large families, GO families are also formed as functional hierarchies of terms, with increasing specificity of function/location/activity of term with each higher level (Figure 1.8) Figure adapted from (Bindea and Mlecnik 2019). Terms at every level have associated genes (Ashburner et al. 2000).

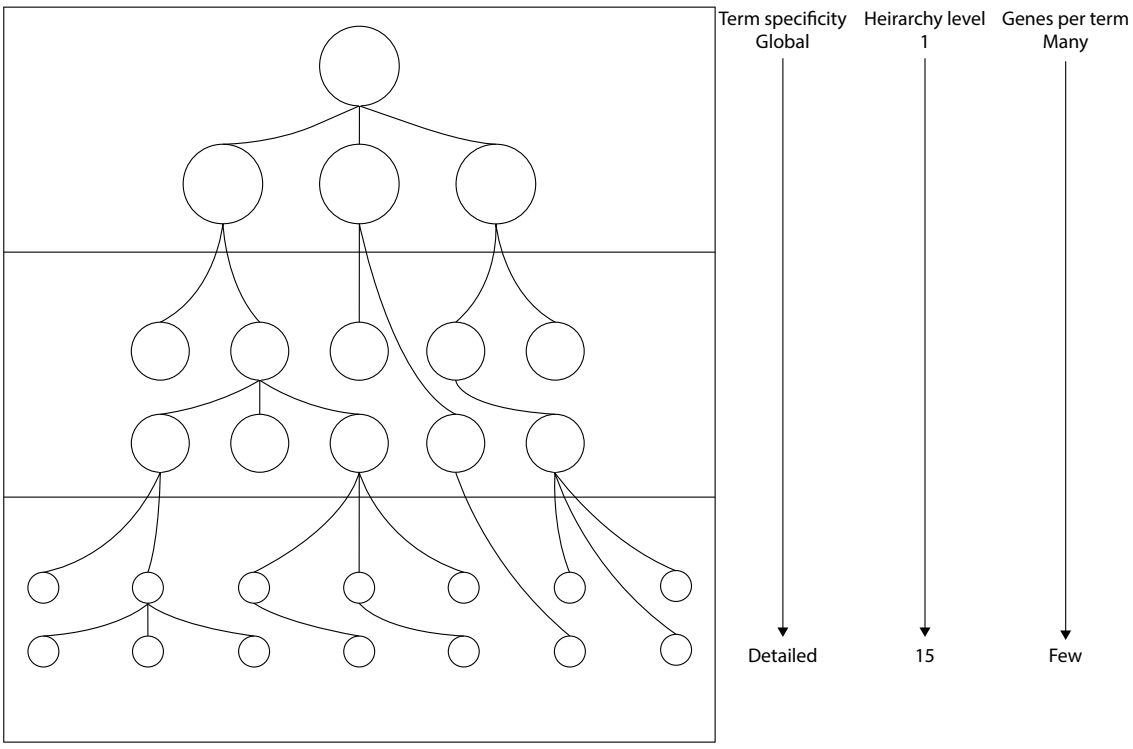


Figure 1.8: Example Gene Ontology Term hierarchy. Gene Ontology terms are broken down by levels, with higher level values representing increased specificity. Higher levels are grouped into lower levels based upon relationships. Figure adapted from Bindea 2012.

Reactome Knowledgebase is a large list of manually curated genes falling into two main categories, Pathways and Reactions (Fabregat et al. 2018). Our system employs the use of enzyme knockouts (KO) of the DNPS therefore of genes involved in biochemical pathways, responsible for the reactions involved in purine synthesis, therefore Reactome provides a suitable complement to the GO families.

Database probing programs exist, each one functioning slightly differently but overall concept remains constant. In essence, DEG lists are entered into the program, database choice is selected, then significant terms associated with the gene list are generated. Two versions of gene inputs typically exist, weighted and unweighted. In a weighted gene list, fold change of FPKM are taken into account while unweighted only

utilizes the gene identity. Overrepresentation is a popular method for unweighted analysis that requires a preselected differentially expressed gene list (Rahmatallah, Emmert-Streib, and Glazko 2014). In overrepresentation, hypergeometric analysis reveals how many genes are predicted to have their expression randomly changed per term. If there are more genes in the DEG list belonging to a specific term than what is predicted to have randomly changed, the term is therefore important for the analysis and p-values are calculated based on the difference between observed and predicted (Mlecnik, Galon, and Bindea 2018; Maere, Heymans, and Kuiper 2005). For exploratory analysis, the overrepresentation unweighted analysis system is ideal as large amounts of data can be generated with a relatively simple yet powerful analysis. The terms generated through overrepresentation analysis can be grouped into larger categories based on hierarchal overlap to be explored more in depth through targeted experimentation.

ClueGO is an example of a database probing program (Mlecnik, Galon, and Bindea 2018). ClueGO can draw off multiple databases and is designed for smaller lists of DEGs. This is accomplished by applying cut offs based on log2(normalized fold change) between experimental and control conditions, using as input the most positive and negative changed genes. A large benefit of the ClueGO programing is the ability to probe different tiers of levels in the GO databases.

ClueGO can probe GO levels 1-15 (least to most specific terms) based upon user defined parameters. Lower level probing yields terms due to the high amount of genes found within those terms, terms are essentially meaningless due to generality (e.g. Developmental Process GO:0032502), while high level terms presents a similar problem in reverse, that is to say terms are hyper-specific with very limited number of genes

comprising the term making it unlikely to show any enrichment in DEG lists. In order to obtain results, users must balance the rate of term return (as seen in low level probing) and term usefulness (as seen in high level probing). For initial assessment, mid-hierarchy assessment is incredibly useful as it allows for terms to be identified and for large groupings of terms to be assessed. That is to say if multiple terms are found within the lower level hierarchal group, it could be posited that this grand theme is affected by the experimental condition (Figure 1.8).

Selection of specific KOs and cell line

Owing to the points previously outlined, the effects related to the three enzymes (GART, ADSL, and ATIC) are a beneficial starting point to understanding generalized DNPS deficiency, congenital disorders of known enzyme deficiency, and metabolic intermediate accumulant effects. Murine models are not ideal for this type of study due to the difficulty of inducing mutations in DNPS enzymes for viable mouse models.

This laboratory pioneered the study of the genetics of DNPS using Chinese hamster ovary (CHO) cells. This included isolation of mutants in each gene of the DNPS pathway including GART, ADSL, and ATIC. In particular, we collaborated with the laboratory of Dr. Stanislav Kmoch (Charles University, Prague, Czech Republic) to characterize the AdeI ADSL deficient mutant as a possible cell culture model of ADSL deficiency (Duval et al. 2013). However, this model has the obvious weakness that it is not a human model.

The HeLa cell line was derived from cervical cancer in a patient in 1951 (Scherer, Syverton, and Gey 1953) and has been in culture since. It has been widely used

experimentally. There are over 107,000 manuscripts listed in PubMed using HeLa cells. Therefore, a great deal is known about essentially all aspects of HeLa cell biology.

The immortalized HeLa cells have several key advantages. First, they are immortal and robust, being able to withstand and show proliferative growth in a wide range of conditions. The immortality of the cell line bypasses ethical issues with animal or patient tissue primary cell culture (Kaur and Dufour 2012) although the HeLa cell line specifically is rife with controversy (Beskow 2016). However, immortal lines suffer from not always replicating the behavior of their derived tissue (Kaur and Dufour 2012). In -omics based experiments, this may perhaps be an advantage. Gross processes identified can be implemented and assessed for their veracity in tissue specific processes. Primary cell lines that reveal enrichment in processes in no way associated with the derived tissue would be received as suspect.

The HeLa line has been used extensively in the search for the purinosome. Due to the fast catabolism of early pathway intermediates, the idea of a functional proteinaceous superstructure was therefore attractive (Smith et al. 1980). In 2009, the first images of cellular compartmentalization, wherein transfected HeLa cells expressing DNPS enzymes tagged to eGFP were shown to aggregate upon purine starvation and disassemble upon purine supplementation (An et al. 2008). Multiple reports have since been published by the Benkovic lab characterizing this cellular structure (Deng et al. 2012; Chan et al. 2015; Zhao et al. 2013; 2015).

CRISPR is a recently developed genomic editing technique that provides greater efficiency and specificity over previous techniques (Ran et al. 2013). One of the most basic forms of CRISPR techniques is knocking out a gene via non-homologous end joining (NHEJ). CRISPR utilizes a guide RNA sequence linked to a Cas9 endonuclease, the guide RNA targets the complex to specific sequences within the genome while the Cas9 is capable of breaking DNA. After the break in DNA is induced, native DNA repair mechanisms repair the break and can create insertions and deletions of nucleotides into the genome at the break site generating a frameshift mutation (Figure 1.9). Figure from ("CRISPR Guide" n.d.). When an early exon of the gene is targeted, the resultant frame shift mutations generated by insertion/deletion errors creates non-sense coding and likely introduces an early stop codon (Ran et al. 2013). Subcloning is performed to isolate cells of a specific break/repair lineage and screening is done to understand the mutation and efficacy of the knock-out. This process results in the genomic knock out and null expression of a targeted, specific gene.

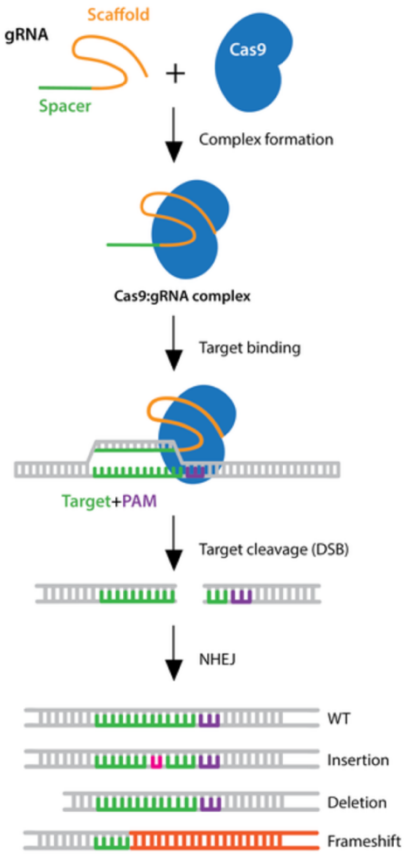


Figure 1.9: Schema of CRISPR based NHEJ mutations. Guide RNA and Cas9 form a complex which targets sequence specific genomic DNA inducing a double strand break which may be repaired with insertions and deletions of nucleotides resulting in a frameshift mutation and subsequent silencing of the targeted gene. Image from addgene.org/guides/crispr/

In 2016, our collaborators produced CRISPR-Cas9 generated knock outs of DNPS

enzymes in the HeLa cell line, they have termed crXXXX, where XXXX is the enzyme that is knocked out (Baresova et al. 2016). They have characterized the cell lines using immunoblotting, qPCR, as well as MS techniques (Mádrová et al. 2018). With exception of the crADSL line, all show no transcript or immunoreactivity for their knocked-out enzyme. crADSL, shows only 1.7% activity compared to WT (Baresova et al. 2016). The Kmoch laboratory has graciously provided our laboratory with the crGART, crADSL, and crATIC HeLa cell mutants. We continue to collaborate with this group to the present.

I chose to explore the HeLa cell based DNPS KOs crGART, crADSL, and crATIC. As previously described, substrates of both ATIC and ADSL have off-target effects, and both proteins have clinical significance as enzyme deficiency were identified in patients (Marie et al. 2004; Jurecka et al. 2015). GART does not accumulate any substrate (Mádrová et al. 2018) and no GART deficient patients have been identified to date, therefore the crGART cell line likely represents effects of generalized DNPS deficiency. GART is also found on Hsa21 and is therefore triplicated in DS and could provide insight into GART involvement in the DS phenotype.

Hypotheses and aims

DNPS deficits have clinical implications, although are uncommonly rare. The phenotypes of DNPS deficiency are not well understood, highlighting the dearth in our current knowledgebase of the cellular and organismal ramifications of DNPS dysregulation. Rare disorders are oft overlooked, however their study many times leads to discoveries of important biological roles. The intermediates of DNPS were previously shown to have roles outside of DNPS in energy homeostasis as well as tumor biology.

Targeted therapeutics show little to no efficacy in DNPS dysregulated patients. These therapies have all been based on sound hypotheses however have shown no success. This implies that the phenotype for these disorders and therefore processes affected by DNPS dysregulation are largely unknown. In purine auxotroph cell lines, we are able to rescue the survival/proliferation phenotype by supplying an exogenous source of purine usually in the form of adenine. This intervention is similar to therapies tried for ADSL deficiency, in which some have been aimed at using salvage pathways or ramping up DNPS to overcome the decreased enzyme activity; these patients did not improve. Therefore, an untargeted approach must be employed to identify and characterize these disorders and processes influenced by DNPS.

A broad scale, transcriptomic and metabolomic analysis of the DNPS-KO HeLa cell lines of crGART, crATIC, and crADSL represents a first step in understanding how DNPS affects cellular and organismal activity. DNPS has long been thought influential in embryonic and nervous system development, however the identification of processes and genes directly involved remained unclear. The processes elucidated will inform future experimentation in specific aspects of biological function and form under the influence of DNPS.

Chapter Two: Transcriptomic Characterization of crGART

Introduction

De novo purine biosynthesis (DNPS) is one of the oldest and most fundamental biochemical pathways (Caetano-Anollés et al. 2009). 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 (Zhao et al. 2015; Chan et al. 2015) and is critical during cellular division (Fridman et al. 2013), 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 membranes is inefficient, DNPS is critical in mammalian development, including embryonic development. In mammals, the trifunctional GART enzyme catalyzes steps 2, 3, and 5 of DNPS (Figure 2.1). 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 (Brodsky et al. 1997). 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 (Pelet et al. 2019). 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 (Jurecka et al. 2015). AICAribosiduria is characterized by mental retardation, blindness, epilepsy, and craniofacial and body dysmorphic features (Marie et al. 2004). The siblings identified with PAICS deficiency died within three days of birth and exhibited craniofacial abnormalities and body dysmorphic features (Pelet et al. 2019). The vast majority of these mutations are amino acid point mutations resulting in decreased enzymatic activity. Thus far, no functional mutations in

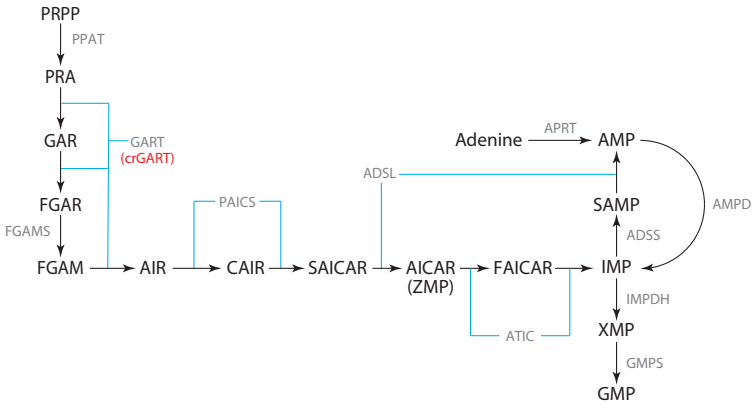


Figure 2.1. De novo purine synthesis pathway. DNPS mediates the conversion of PRPP to IMP. IMP is subsequently converted to AMP or GMP. The HeLa GART KO, crGART, is indicated. Figure generated by Dr. Guido Vacano.

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 (Baresova et al. 2016). 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 (Mazzarino et al. 2019; 2020). 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. 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.

Materials and Methods

Methods were performed according to (Mazzarino et al. 2019), differences are outlined.

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 (and R version 3.5.3).

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).

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. 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.

Results

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

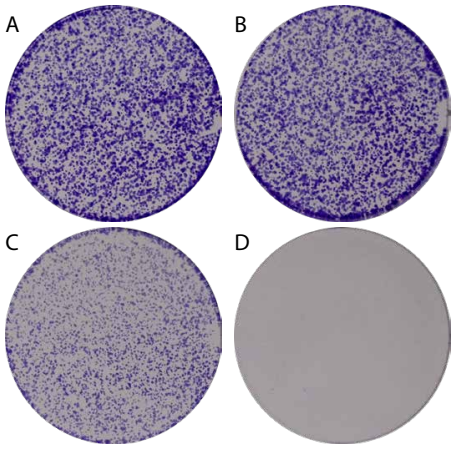


Figure 2.2: Adenine is required for proliferative growth of crGART cell line. WT HeLa cells (A, C) and crGART HeLa (B, D) were cultured in DMEM supplemented with 10% FCM with (A, B) or without (C, D) 100 µM adenine then fixed and stained with crystal violet. Entire cell growth area was imaged.

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 2.2).

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 and crATIC cells accumulate substrate when cultured in adenine free media (Mazzarino et al. 2019; 2020). 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 (Mádrová et al. 2018) as 5-PRA is highly unstable and breaks down in approximately five seconds under cellular conditions (Rudolph and Stubbe 1995). Since it apparently does not accumulate substrate, this suggests that crGART is likely useful as a model of general DNPS deficiency.

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. 4218 genes were significantly differentially expressed by cell type. This represents a log(2)counts fold change range of 10.93 to -13.94 (Figure 2.3A). 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 2.3B). Positive values represent enrichment in crGART and negative log2 values represent enrichment in HeLa. Principal component analysis shows clustering by cell type and supplementation (Figure 2.4).

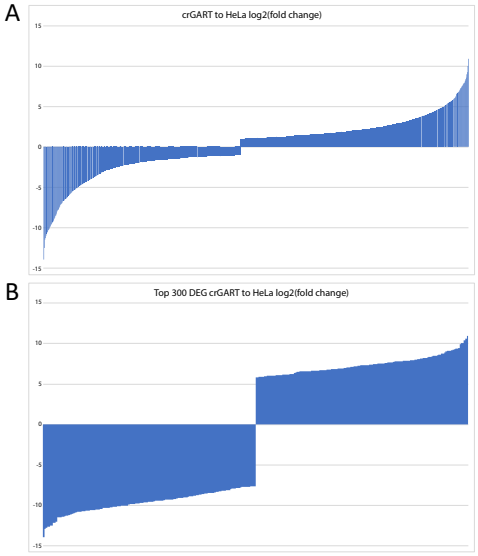


Figure 2.3: log(2) fold change of DEGs in cell lines under experimental conditions. All DEGs that satisfy cutoff constraints between crGART and HeLa (A). 150 most positively and 150 most negatively changed DEGs between crGART to HeLa (B).

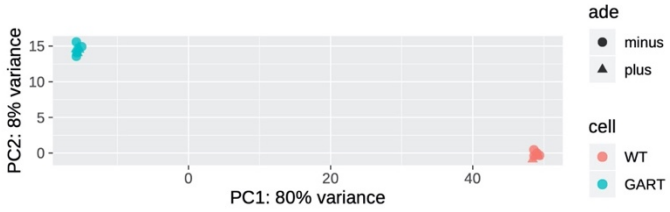


Figure 2.4: Principle component analysis of crGART and HeLa replicates. PCA shows a robust difference by cell type. Figure generated by Dr. Guido Vacano.

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 large number of terms but will focus only on terms centered around special interest to our laboratory (Figure 2.5). All ontology network maps, term, and grouping visualization figures are located after the text of this chapter.

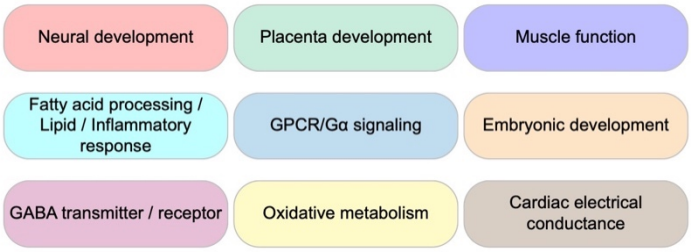
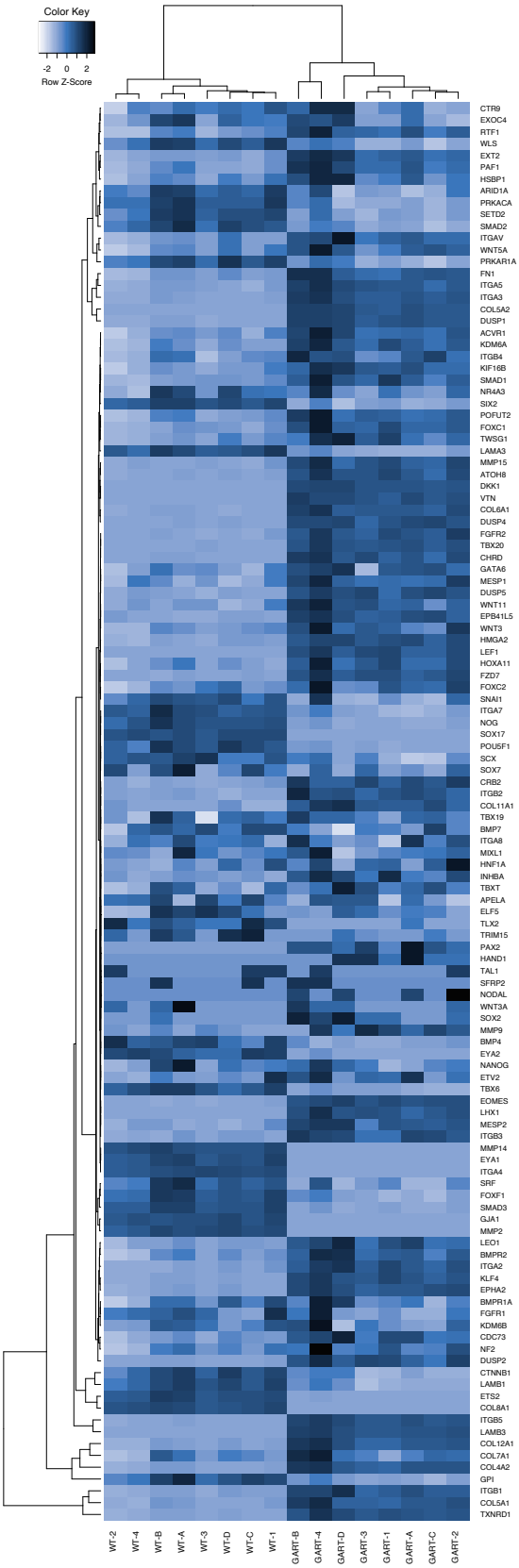


Figure 2.5: Prominent GO groups from ClueGO

For the GO terms associated with Biological process, 70 GO groups were identified with 548 GO terms (Figures 2.8-2.12). 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

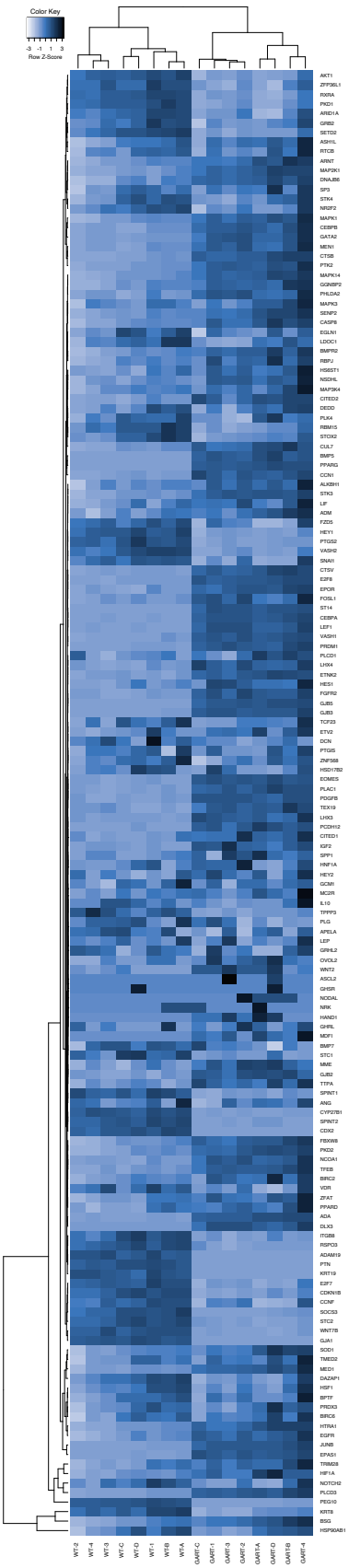
phosphatase activity, blood vessel morphogenesis, epithelial cell differentiation, bone development, endoderm, etc. Placental development included labyrinthine development and female pregnancy. For muscle function terms, we obtained smooth muscle function, myotubes and sarcomere organization, vasoconstriction, 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 gene enrichment for formation of primary germ layer (GO:0001704) and placental development terms (GO:0001890) (Figures 2.6, 2.7) 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 (Figures 2.13, 2.14). 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 (Figures 2.15, 2.16). Term categories included neuronal and ion transmembrane function, development, phospholipid activity, cytokine, etc. In the neuronal category, neuropeptide, acetylcholine receptor, potassium ion gate channels, 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

Figure 2.6: Gene expression heat map of Gene Ontology Primary Germ Layer. Genes identified differentially expressed in our data set that map to the Gene Ontology term Formation of Primary Germ Layer (GO:0001704) by log(2)fold change. Cell types are defined as "WT" for HeLa and "GART" for crGART, "1-4" for adenine supplemented and "A-D" for adenine deficient. Heat map dendrogram shows robust groupings along cell type. Adenine supplementation shows little effect. Genes not satisfying previously define threshold counts are removed. Figure generated by Dr. Guido Vacano.



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 (Figures 2.17, 2.18). Categories include diseases associated with O-glycosylation of proteins, peptide hormone metabolism, rhodopsin-like receptors, TFAP-2, 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 (Figures 2.19, 2.20). Term

Figure 2.7: Gene expression heat map of Gene Ontology Placenta Development. Genes identified differentially expressed in our data set that map to the Gene Ontology term Placenta Development (GO: 0001890) by log(2)fold change. Cell types are defined as "WT" for HeLa and "GART" for crGART, "1-4" for adenine supplemented and "A-D" for adenine deficient. Heat map dendrogram shows robust groupings along cell type. Adenine supplementation shows little effect. Genes not satisfying previously define threshold counts are removed. Figure generated by Dr. Guido Vacano.

categories included proteoglycans and GPI anchors, GPCR, GEF, and Gi activated ligands. Keratin filaments was also noted.

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.

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 and crATIC vs HeLa comparisons (Mazzarino et al. 2019; 2020). 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. 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. This result illustrates the importance of DNPS in multiple aspects of cell development and function.

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 (Silva et al. 2008). Adenine supplementation effectively shuts down DNPS (Holmes et al. 1973; Tu and Patterson 1978). Finally, AMP can be converted to IMP by AMP deaminase, and then converted to GMP by IMP dehydrogenase (IMPDH, converts IMP into XMP) and GMP synthase (converts XMP into GMP) (Watts 1974).

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 (Knox 2006). 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 (Keller, Tan, and Lee 2012; Corton et al. 1995; Meares et al. 2013). 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 (Brodsky et al. 1997). 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 PPAT (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 (Hove-Jensen et al. 2017). 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 (Oates, Vannais, and Patterson 1980). 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 (Holmes et al. 1973). Increased AMP favors the (inactive) dimer form of the enzyme rather than the (active) tetramer form (Holmes, Wyngaarden, and Kelley 1973).

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 (Zhao et al. 2015; Chan et al. 2015), is critical in embryogenesis which is marked by rapid cellular division, and that purines are essential in development (Fumagalli et al. 2017) especially in neural development (Rodrigues, Marques, and Cunha 2019). 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. 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 (Lott 2012a), congenital heart malformation, disease of pulmonary circulation, cardiac arrest, hypotension, infantile spasm, epilepsy, OSA (sleeping disorders), intellectual disabilities, dementia, hypothyroidism, and obesity (Alexander et al. 2016). 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 (Turco and Moffett 2019), is derived from fetal and maternal components, and is essential for *in utero* development (Burton and Jauniaux 2015). 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 (Gude et al. 2004). 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 (Perez-Garcia et al. 2018).

In trisomy 21 placenta, villus abnormalities, hypovascularity and placental hypoplasia (Debieve 2001; Qureshi et al. 1997) have been observed. Labyrinth layers in trisomy 21 placenta were noted to weigh less and exhibit signs of delayed development (Adams, Guedj, and Bianchi 2020). 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 (Freeman et al. 2008). This may be due to impaired placentation (Ramachandran et al. 2015; Radhakrishna et al. 2019). Owing to the term enrichment associated with placental formation and function, GART poses a potential lens to investigate an embryonic contribution to neurological and cardiac defects associated with DS.

DS is most commonly associated with intellectual disability (Alexander et al. 2016). Previous work has demonstrated that defects in purine metabolism and/or purine concentration in the developing brain are related to intellectual disabilities (Brodsky et al. 1997; Fumagalli et al. 2017). 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 (Contestabile, Magara, and Cancedda 2017). Purinergic signaling regulates axon guidance and growth as well as establishment of correct synaptic contacts (Fumagalli et al. 2017) and is crucial for CNS development (Jinnah, Sabina, and Van Den Berghe 2013). 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 (Brodsky et al. 1997).

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) (Sebastián-Serrano et al. 2015). In studies of murine CNS development, TNAP was found to be associated with the neural tube (Narisawa et al. 1994) and is highest in early embryonic development associating with neural precursor and progenitor cells (Langer et al. 2007). TNAP expression has also been observed during synaptic formation and maturation (Fonta et al. 2005), promoting axonal growth

(Díez-Zaera et al. 2011). A non-canonical role of TNAP is hydrolysis of extracellular nucleotides (Fumagalli et al. 2017; Zimmermann, Zebisch, and Sträter 2012). Extracellular ATP was found to induce migration of neural progenitor cells (Striedinger, Meda, and Scemes 2007), indicating that TNAP may play a role in regulating extracellular ATP pools for migratory events. Another TNAP function is found in the metabolism of the Vitamin B6, which is a cofactor for enzymes involved in neurotransmitter (e.g. GABA) synthesis (Calderón-Ospina and Nava-Mesa 2020). 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 (Contestabile, Magara, and Cancedda 2017; Deidda, Bozarth, and Cancedda 2014). 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 (Huo et al. 2018). DS patients exhibit an increased incidence of epileptic seizures (Lott and Dierssen 2010), children exhibit sleep disturbances (Carter et al. 2009) and hyperactivity (Pueschel, Bernier, and Pezzullo 1991): 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 (Contestabile, Magara, and Cancedda 2017; Deidda, Bozarth,

and Cancedda 2014; Begenisic et al. 2014), and learning and memory deficits (Costa and Grybko 2005; Kleschevnikov 2004). 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 (L. Chen et al. 2018). 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 (L. Chen et al. 2018). Fatty acid derivatives, specifically lipoxins (derived from ω-6 via phospholipaseA2 and lipoxygenase catalysis of arachidonic acid) (Sugimoto et al. 2016) and ω-3 metabolites such as maresins, resolvins, and protectins (Serhan et al. 2015) 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 (Hanna and Hafez 2018). Coincidentally, extracellular ATP signaling plays a crucial role in inflammation through the purinergic P2 and P1 receptors (Kominsky, Campbell, and Colgan 2010). DNPS and GART levels may play important roles in these processes.

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, and purine levels are significantly altered in DS. 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.

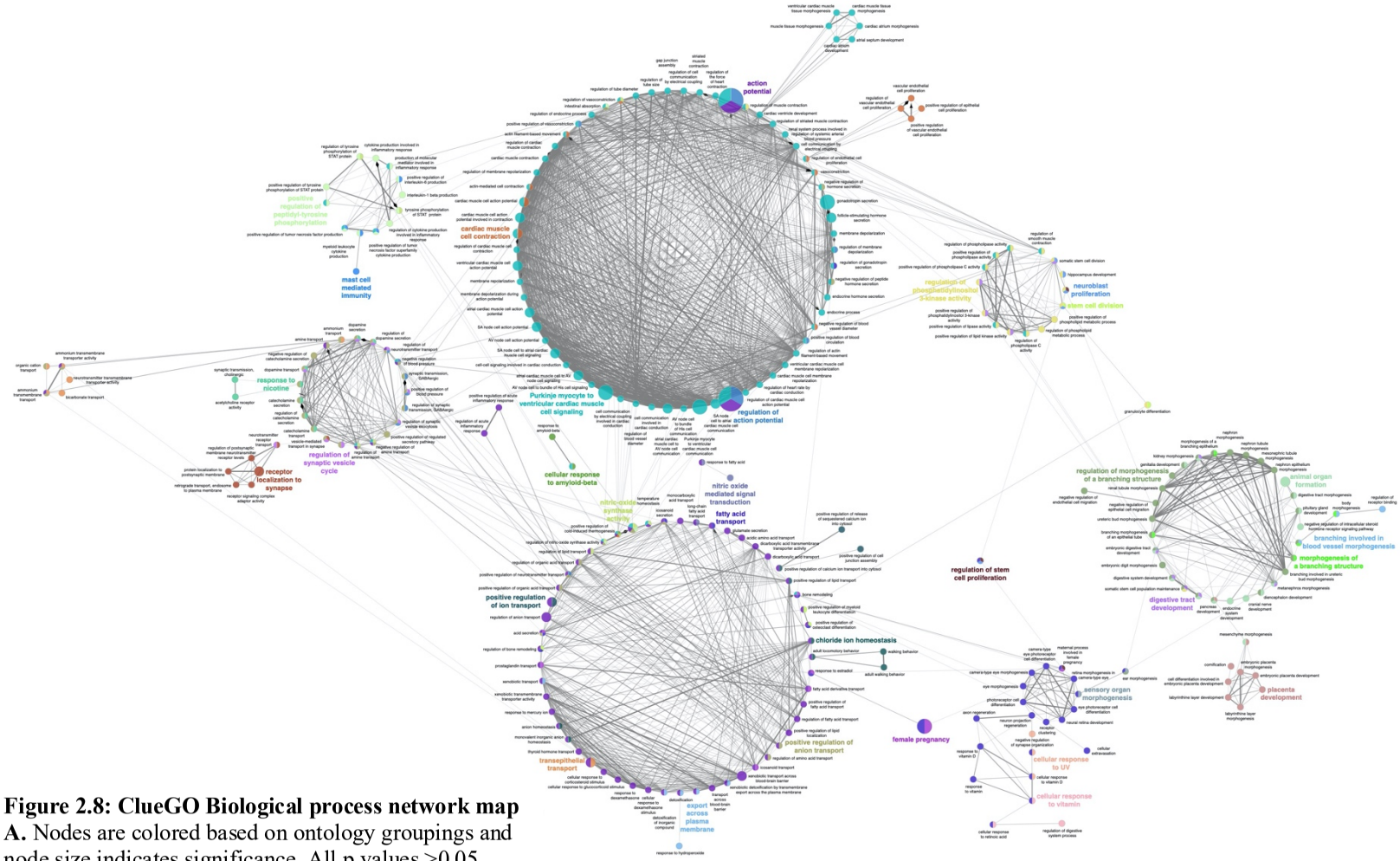


Figure 2.8: ClueGO Biological process network map
A. Nodes are colored based on ontology groupings and node size indicates significance. All p values >0.05. Figure generated by Dr. Guido Vacano.

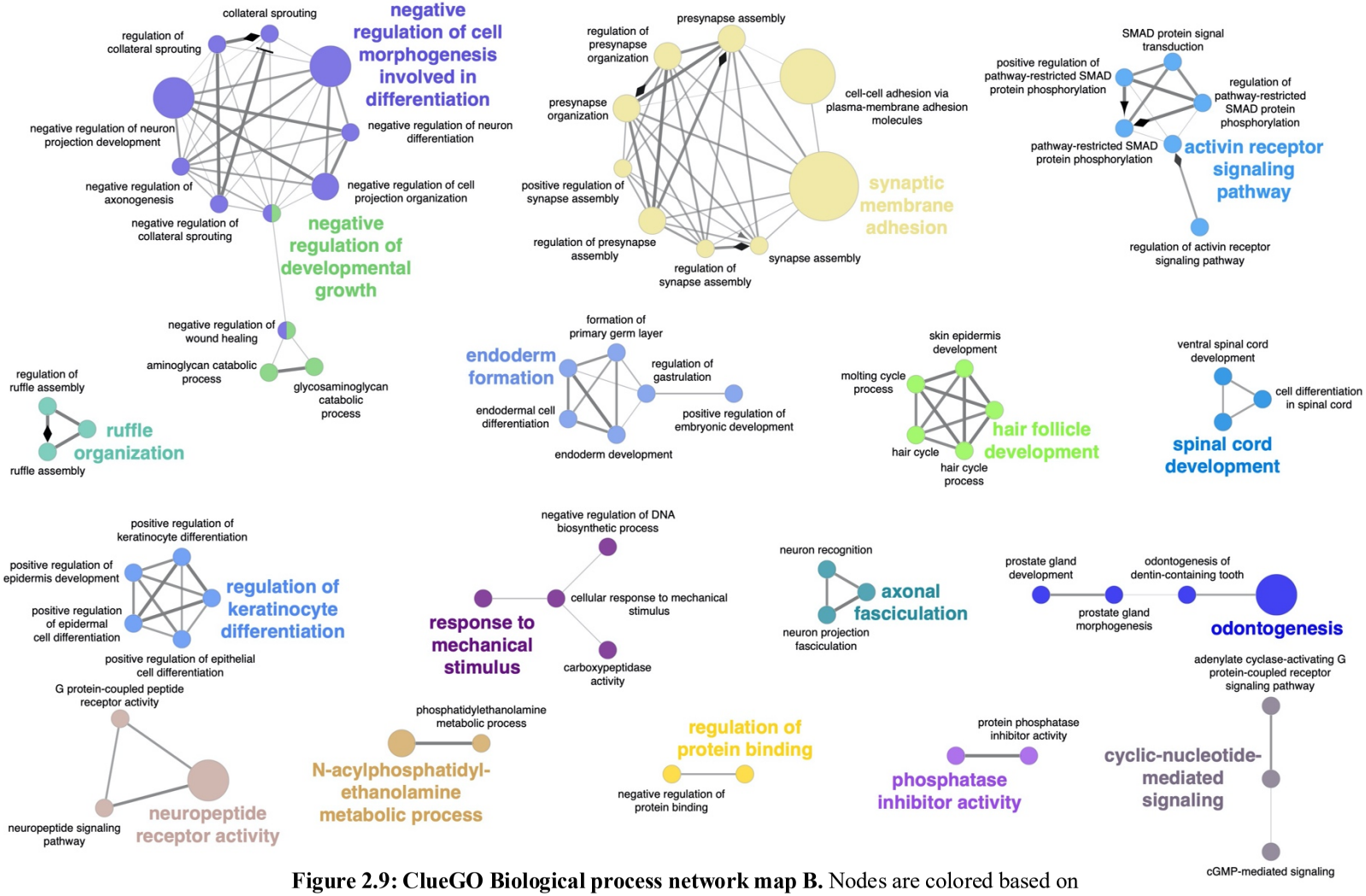


Figure 2.9: ClueGO Biological process network map B. Nodes are colored based on ontology groupings and node size indicates significance. All p values >0.05. Figure generated by Dr. Guido Vacano.

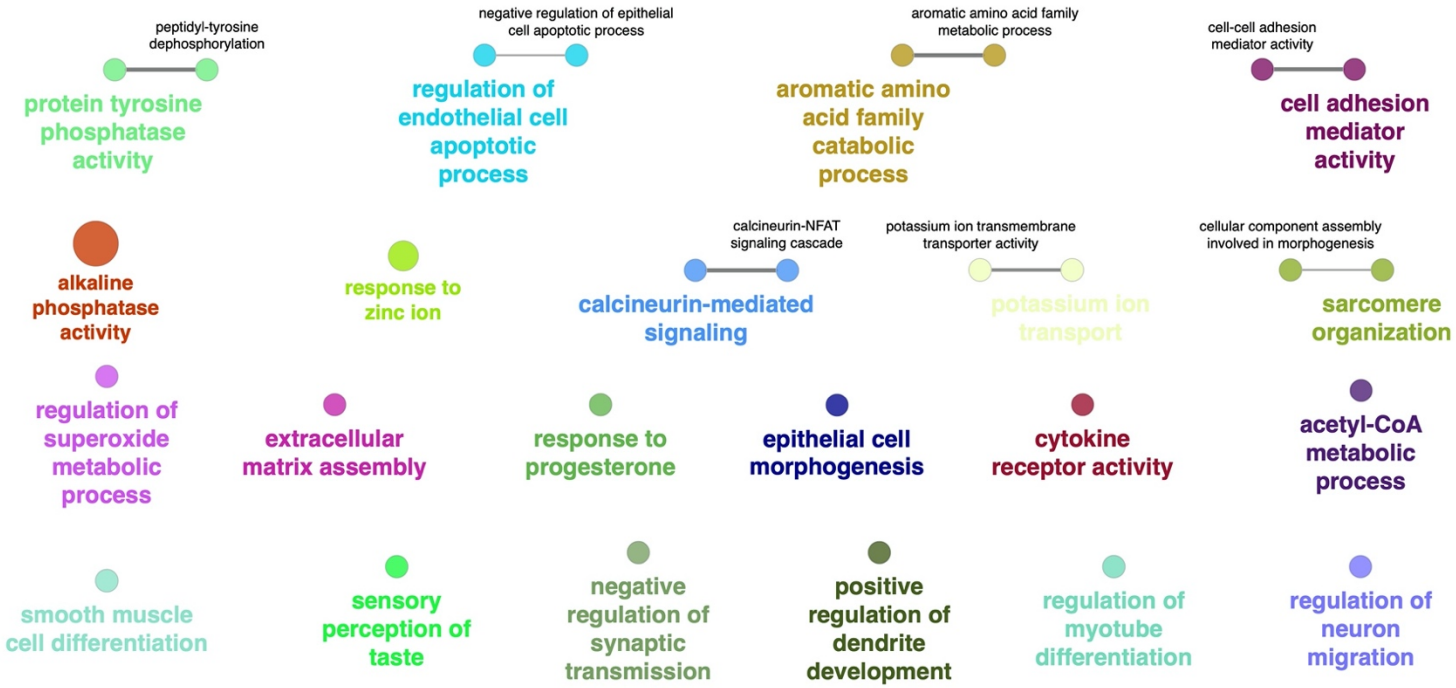


Figure 2.10: ClueGO Biological process network map C. Nodes are colored based on ontology groupings and node size indicates significance. All p values >0.05. Figure generated by Dr. Guido Vacano.

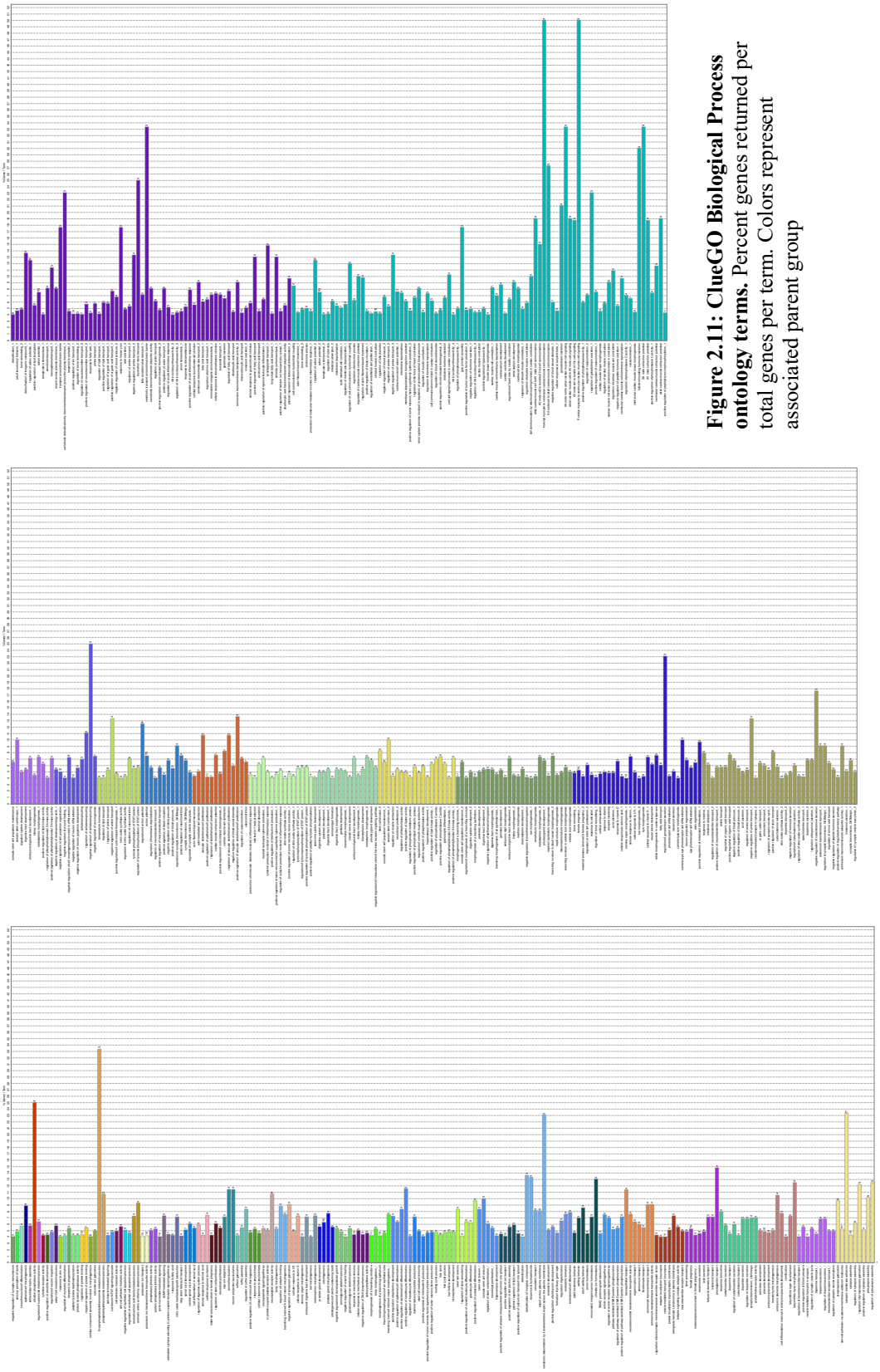


Figure 2.11: ClueGO Biological Process ontology terms. Percent genes returned per total genes per term. Colors represent associated parent group

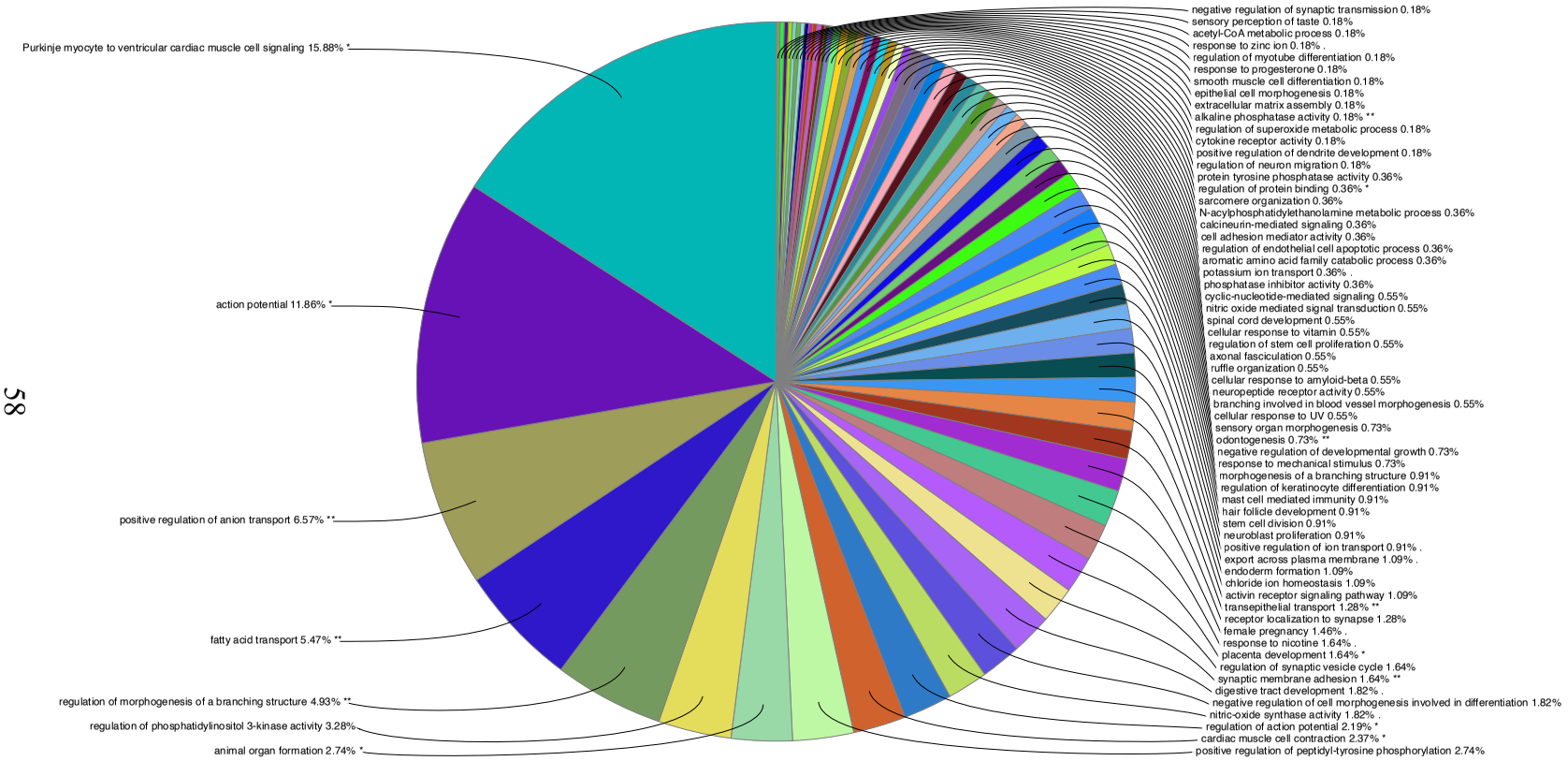


Figure 2.12: ClueGO Biological Process parent ontology groupings. Percent representation of total terms found within representative parent group. Colors represent associated parent group.

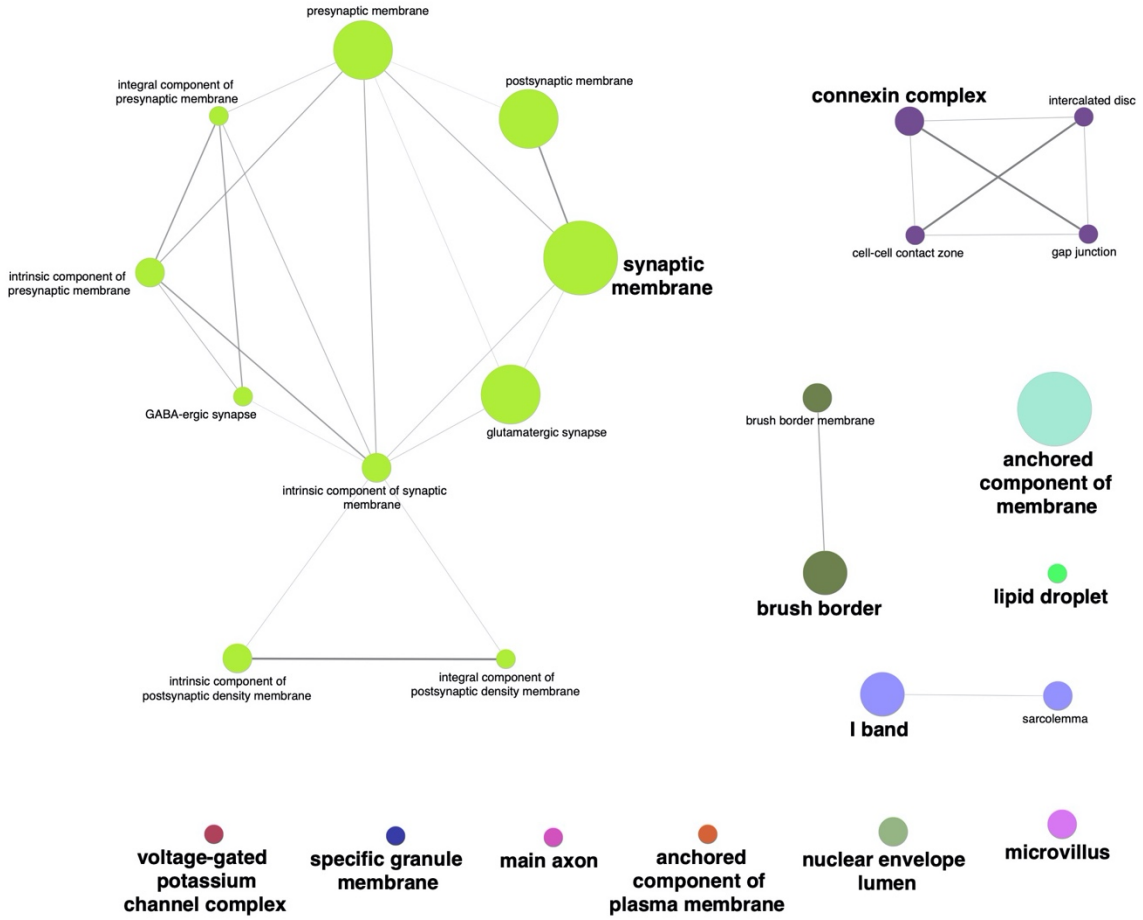


Figure 2.13: ClueGO Cellular Component network map. Nodes are colored based on ontology groupings and node size indicates significance. All p values >0.05.

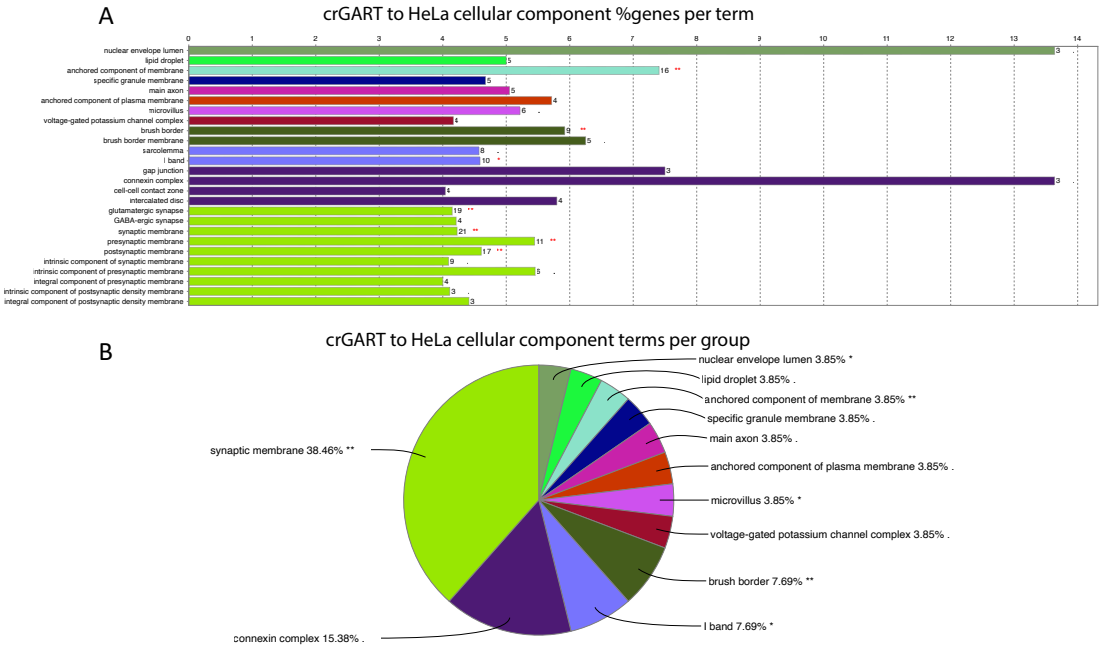


Figure 2.14: ClueGO Cellular Component terms and groupings. Percent genes returned per total genes per term (A). Percent representation of total terms found within representative parent group (B). Colors represent associated parent group

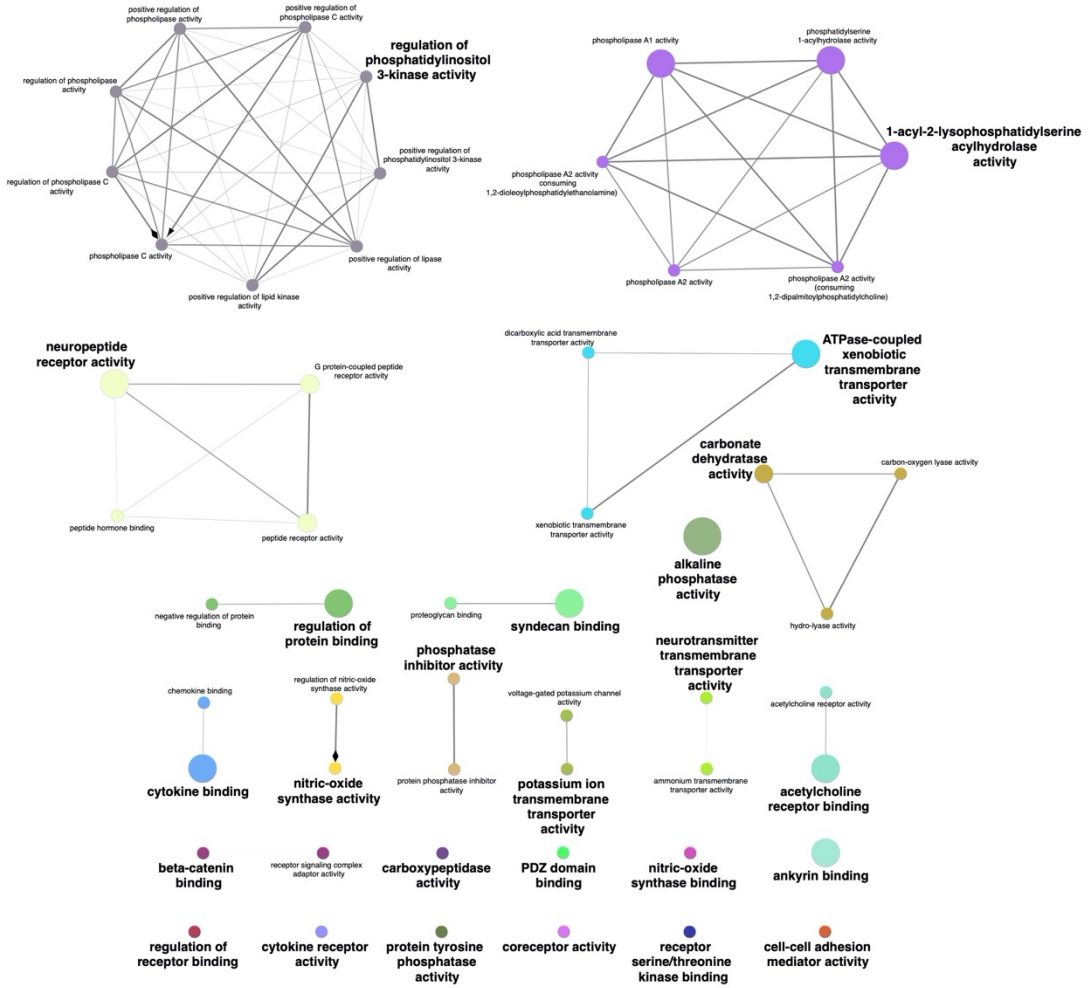


Figure 2.15: ClueGO Molecular Function network map. Nodes are colored based on ontology groupings and node size indicates significance. All p values >0.05.

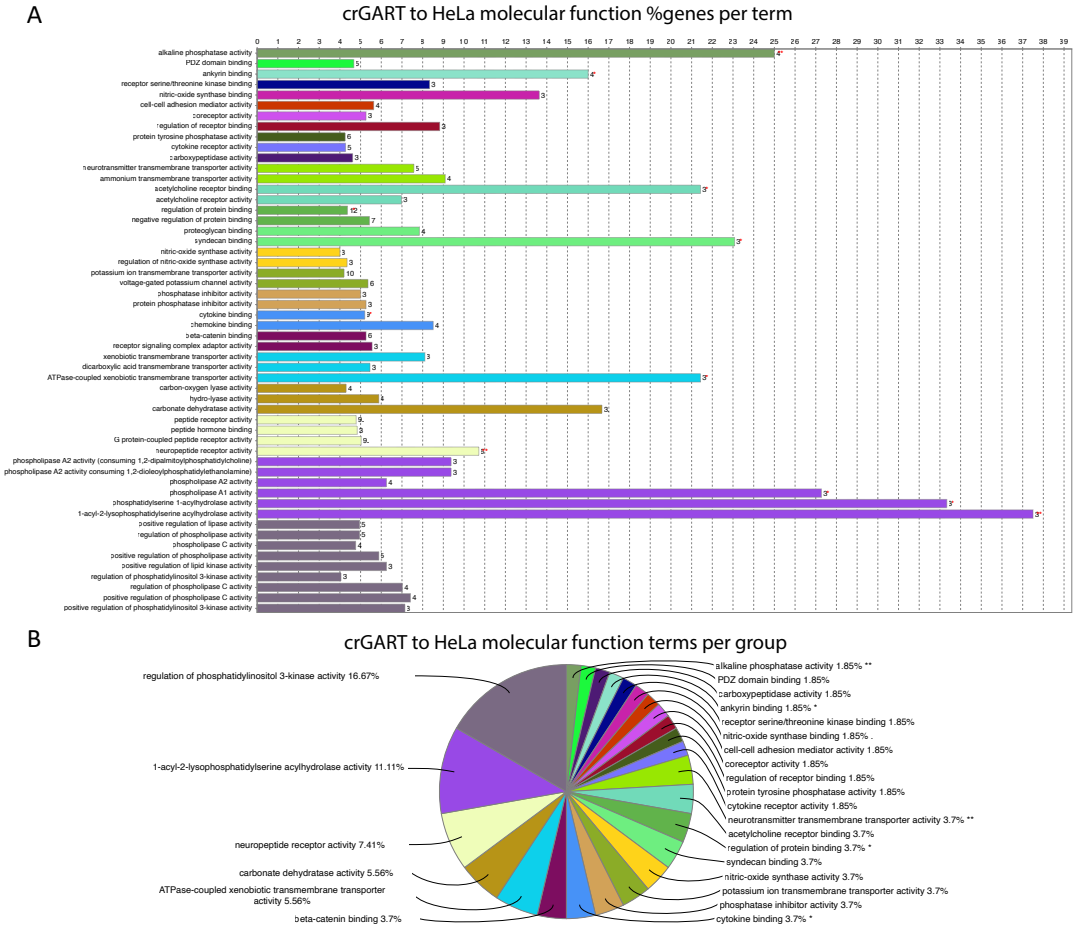


Figure 2.16: ClueGO Molecular Function terms and groupings. Percent genes returned per total genes per term (A). Percent representation of total terms found within representative parent group (B). Colors represent associated parent group.

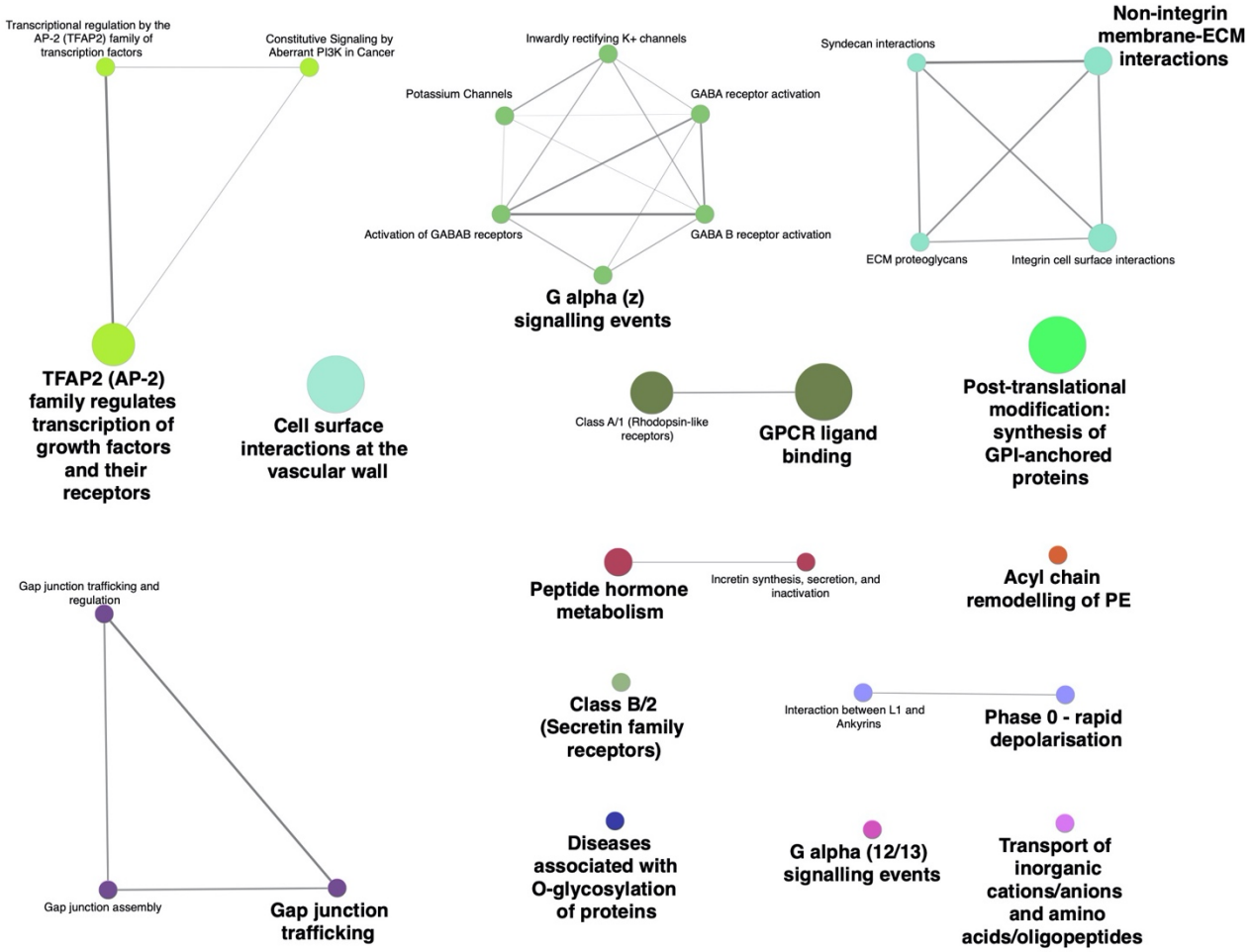


Figure 2.17: ClueGO Reactome Pathways network map. Nodes are colored based on ontology groupings and node size indicates significance. All p values >0.05.

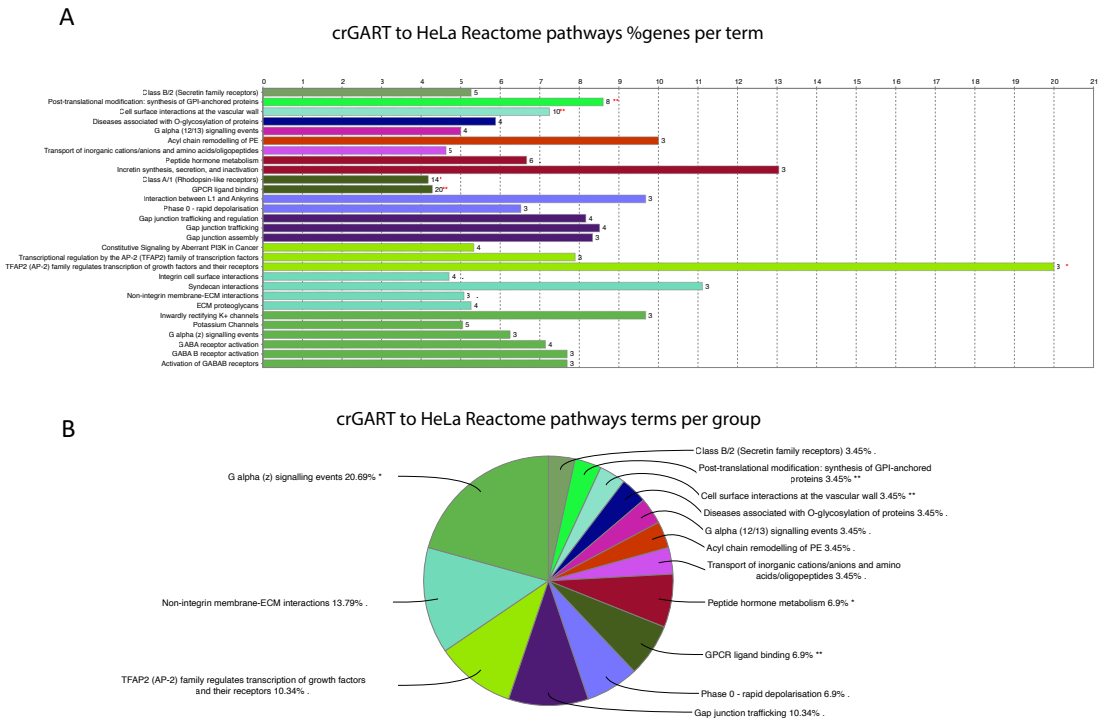


Figure 2.18: ClueGO Reactome Pathways terms and groupings. Percent genes returned per total genes per term (A). Percent representation of total terms found within representative parent group (B). Colors represent associated parent group

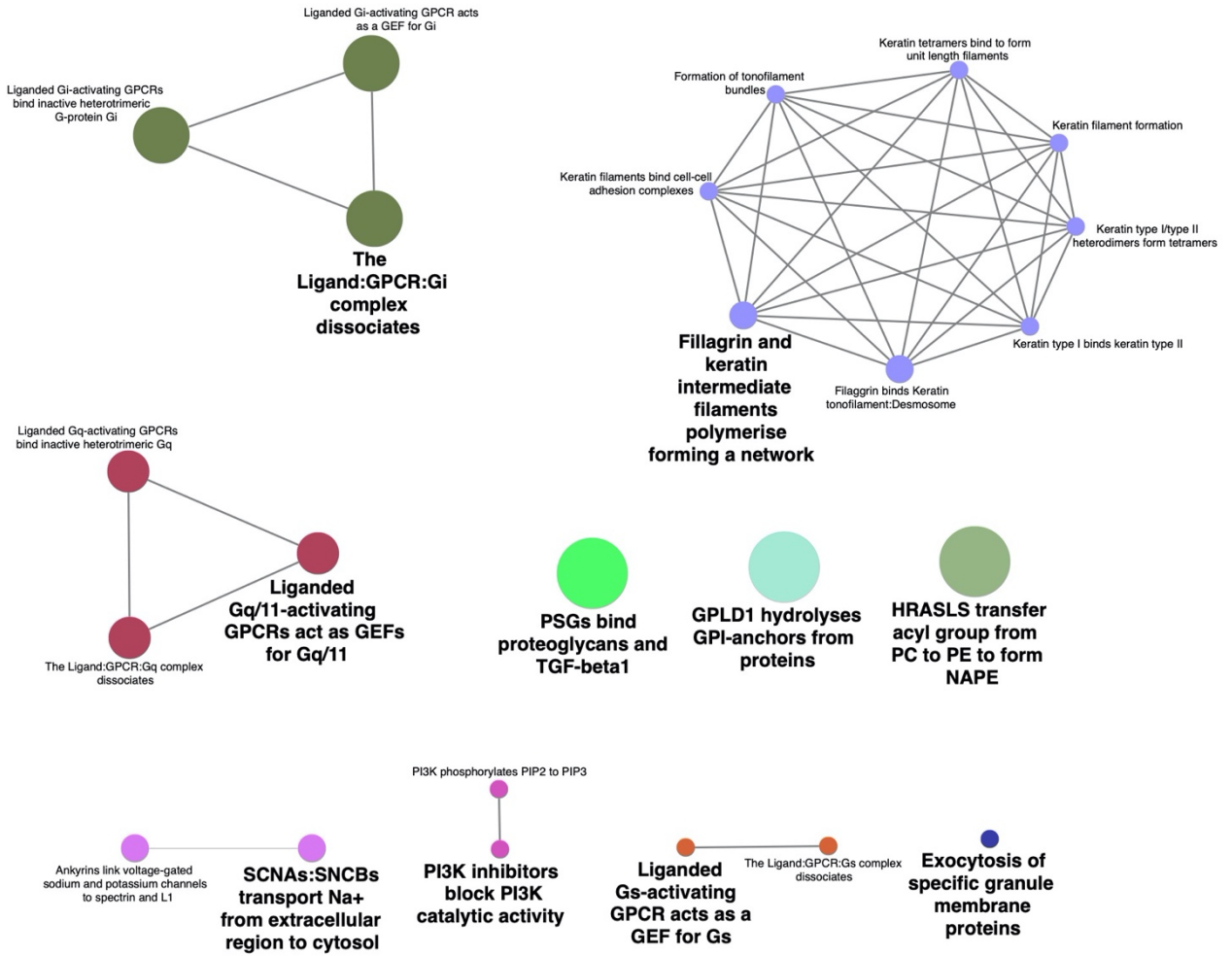


Figure 2.19: ClueGO Reactome Reactions network map. Nodes are colored based on ontology groupings and node size indicates significance. All p values >0.05.

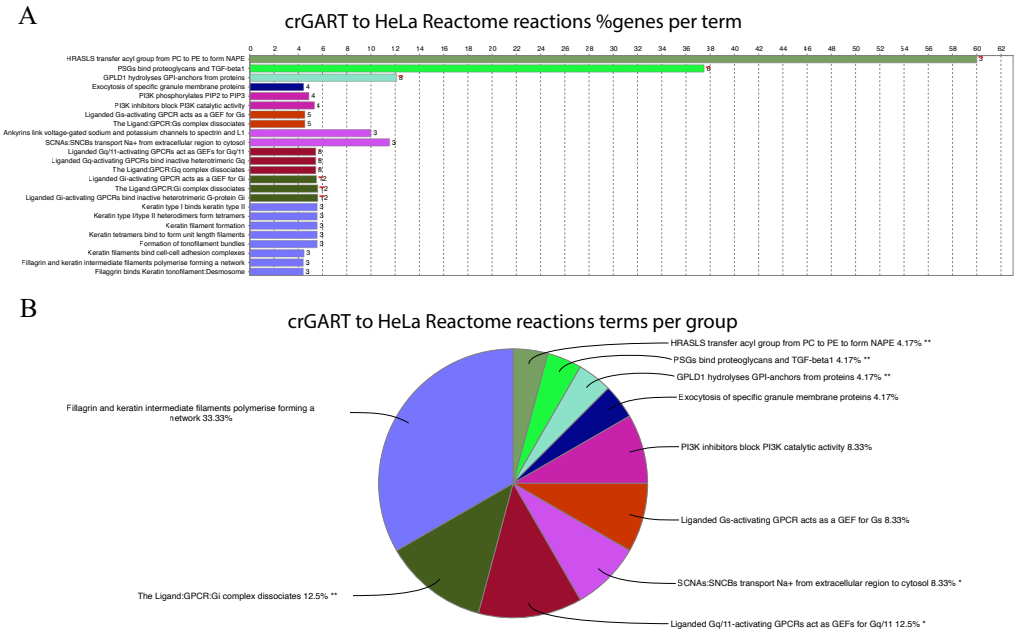


Figure 2.20: ClueGO Reactome Reactions terms and groupings. Percent genes returned per total genes per term(A). Percent representation of total terms found within representative parent group (B). Colors represent associated parent group

Chapter Three: Transcriptomic Characterization of crADSL

Introduction

Defects in *de novo* purine synthesis (DNPS) can cause inborn errors of metabolism. Of particular interest, adenylosuccinate lyase (ADSL) deficiency, an autosomal recessive inborn error of metabolism, has been observed in approximately 80 individuals to date (Jurecka et al. 2015) and is typically diagnosed by SAICA-riboside accumulation in biofluids (Donti et al. 2016). With advances in genomic sequencing and reduction in cost, it is likely that the number of diagnosed ADSL deficient patients will increase in the future. The phenotype of ADSL deficiency is variable and affects multiple systems, presenting as fatal neonatal, severe, or mild to moderate forms including features such as seizures, autistic traits, psychomotor retardation, respiratory failure, and microcephaly. This implies that there are significant alterations in gene expression in ADSL deficiency. The ADSL enzyme is a homotetramer and mutations in ADSL can result in altered tetramer stability or active site disruption resulting in reduced levels of enzyme activity (Zikanova et al. 2010). In the most severe cases, enzyme activity may be reduced by as much as 75%.

DNPS is one of the most ancient biochemical pathways (Caetano-Anollés et al. 2009). In mammalian DNPS, phosphoribosyl pyrophosphate (PRPP) is converted to inosine monophosphate (IMP) in ten enzymatic steps by six different enzymes. IMP is converted to either adenosine monophosphate (AMP) or guanosine monophosphate

(GMP) via two additional enzymatic steps (Figure 3.1). ADSL is a bifunctional homotetrameric enzyme that catalyzes the eighth step, forming aminoimidazole carboxamide ribonucleotide (AICAR) from phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR). It is also responsible for the final step in the conversion of IMP to AMP, specifically cleaving succinyladenosine monophosphate (SAMP) into AMP (Figure 3.1). ADSL is thought to be an enzyme with straightforward and defined functions, however recent evidence suggests a more complicated cellular role.

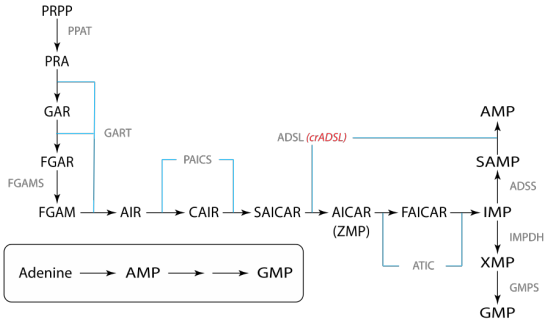


Figure 3.1: de novo purine synthetic pathway (DNPS). PRPP is the input small molecule and is converted by six enzymes in ten steps to IMP and is further processed to either AMP or GMP via two additional steps. ADSL is responsible for SAICAR to AICAR and SAMP to AMP. crADSL is the CRISPR generated HeLa cell line used in this study that lacks ADSL enzyme. Figure generated by Dr. Guido Vacano.

The DNPS intermediates and products have multiple functions within the cell including energy production/regulation via ATP, GTP, and cAMP, cellular signaling, growth, and regulation of other pathways. Recently, the intermediate SAICAR was found to allosterically bind pyruvate kinase M isoform 2 (PKM2) (Keller, Tan, and Lee 2012), the dominant isoform present in tumors. PKM2 catalyzes the last and only irreversible step of glycolysis, forming pyruvate from phosphoenol pyruvate. The dominance of this isoform in tumors is thought to be the root of the Warburg Effect: the production of lactic acid and consequent metabolic reprogramming via aerobic glycolysis in tumor cells (Yang and Lu 2013). PKM2 was also found to act as a co-regulator of transcription (Keller et al. 2014) by binding transcription factors. SAICAR binding to PKM2 is

thought to activate a “moonlighting” PKM2 protein kinase activity that acts to directly alter transcription (Keller et al. 2014). However, this hypothesis is controversial (Hosios et al. 2015). SAICAR was also shown to bind both the dimeric and tetrameric forms of PKM2 (Ming Yan et al. 2016) although recent work suggests that the binding of SAICAR to the dimer promotes its pyruvate kinase activity (Ming Yan et al. 2016). PKM2 was found to induce the hypoxia inducible factor 1 subunit alpha (HIF1α) protein upon nuclear translocation of PKM2 (Luo et al. 2011) which initiates angiogenic and tumorigenic-related events in the cell. SAICAR also accumulates in glucose starved conditions (Keller et al. 2014) although the mechanism for this is not well understood.

Recently, CRISPR-Cas9 was used to generate knock-outs of the enzymes involved in DNPS including ADSL (designated crADSL) (Baresova et al. 2016). The crADSL cells have approximately 1.7% ADSL activity compared to wild type cells (Baresova et al. 2016), accumulate SAICAR (Mádrová et al. 2018) and fail to grow in the absence of adenine (Baresova et al. 2016). To investigate the utility of crADSL as a model for ADSL deficiency, RNA-seq was employed to determine transcriptome differences between crADSL and wild type HeLa (WT) cells cultured for 10 hours in the presence/absence of adenine. RNA-seq is a tool that can provide a snapshot of global transcriptional activity and can aid in characterizing cellular response to mutations, nutrients, stressors, etc. Changes in gene transcription can be rapidly identified and parsed into various groupings, such as gene ontologies or characterized pathways and reactions. As an initial step in understanding alterations in gene expression in ADSL deficiency, we used RNA-seq to compare gene expression in wt HeLa and crADSL cells in the presence and absence of SAICAR accumulation.

Materials and methods

Cell Culture

crADSL was constructed as described previously (Baresova et al. 2016). HeLa cells (CCL-2) were purchased from ATCC (Manassass, Virginia, USA). Cells were grown on 60 mm TPP plates (Techno Plastic Products, AG, Switzerland) with regularly refreshed Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS), 30 μM adenine, and normocin (Invivogen). For purine deprivation experiments (hereafter referred to as starvation), complete media with 30 μM adenine was exchanged two days and one day before starvation. Adenine is used as a nutritional supplement for DNPS experiments as it can be converted to AMP and GMP via enzymes not affected by DNPS knockouts (Kondo et al. 2000). Ten to twelve hours prior to starvation, media were exchanged to DMEM 10% FCS with 100 μM adenine, a concentration of adenine that completely inhibits DNPS (Tu and Patterson 1978). To induce starvation or control conditions, once plates reached ~50-70% confluence, medium was changed to DMEM supplemented with 10% FCM (fetal calf macroserum is FCS dialyzed against saline using a 3.5 kDa barrier), normocin, with or without 100 μM adenine. For cell colony staining experiments, cells were plated in complete growth media and then media were exchanged for DMEM 10% FCM, normocin, with or without 30 μM adenine. Cells were fixed in 10% ethanol / 3.5% acetic acid solution then stained using 0.1% crystal violet solution.

HPLC analysis of SAICAR metabolite accumulation

At each time point, cell culture media was aspirated and cells were washed once with 1 ml cold (4 ºC) 1X PBS and then extracted with 500 μl cold (-20 ºC) 80% EtOH.

Plates were then thoroughly scraped and the cell material was transferred to microfuge tubes and centrifuged at 14,000 x g for 15 minutes at 4 ºC. The supernatant was collected and stored at -80 ºC. Samples were dried using a Speedvac, then resuspended in 300 µl freshly prepared mobile phase (50 mM lithium acetate, 5 mM tetra butyl ammonium phosphate, 2% acetonitrile, pH 4.1). Cellular debris was pelleted by two rounds of centrifugation at 14,000 x g for 20 minutes. Supernatant was frozen at -20 °C until analysis. Samples were transferred to HPLC vials and loaded in an autosampler kept at 10 °C over the course of the runs. Separation of SAICAR was achieved by HPLC–EC analysis similar to our previously described method (Duval et al. 2013). Briefly, separation was obtained using reverse phase HPLC–EC with a TSKgel ODS-80Tm C-18 column (250 mm × 4.6 mm ID, 5 µM) protected by Tosoh Bioscience TSKgel guard cartridge. A column temperature of 33 °C was maintained throughout the analysis. Mobile phase was delivered at a flow rate of 0.7 ml/min. Sample extracts and standards were kept at 10 °C and a 30 µl aliquot of each sample was injected using an ESA autosampler (model 542) using a 30 µl partial loop. After injection and separation, analytes were detected using a CoulArray HPLC system (model 5600A, ESA) with three electrochemical detector modules (four flow-through coulometric detectors in series per module for a total of twelve detectors). EC channels were set to a range of potentials from 0 to 900 mV in 100 mV increments, then 1200 mV and 0 mV to oxidize and detect SAICAR. Autosampler temperature was kept at 10 ºC over the course of runs. Sum of primary peaks area was used to measure analyte accumulation.

RNA-seq

Cells were plated on the same day into 60 mm TPP dishes. Four biological replicates were cultured in purine rich or purine free (starvation) media conditions for 10 hours as previously described in 2.1, and total RNA was extracted using TRIzol reagent (Sigma) according to the manufacturer's protocol. Final purification was performed via spin columns following the manufacturer's protocol (Machery Nagel), with 50 µl total volume DEPC treated water (Sigma). RNA was quantified by NanoDrop One (Thermo Scientific) and frozen at -80° C. RNA quality assessment and RNA-seq was performed by The Genomics and Microarray Core Facility at the University of Colorado, Denver. mRNA libraries were constructed using the Nugen Universal Plus mRNA-Seq + UDI kit (cat # 9144-96), and 50 bp single read sequencing was performed employing the Illumina HiSEQ4000. Conversion of .bcl to FASTQ files was done using CASAVA 2.0.

Processing

Computation was done on a Dell Precision T1700 computer with an Intel Core i7-4790 3.60 GHz CPU and 32 GB RAM running Linux Mint. The RNA-seq sequences, provided in FASTQ format by the Genomics and Microarray Core Facility at the University of Colorado, Denver, were aligned using hisat2 version 2.1.0 to the "genome_snp_tran" indexed human genome [H. sapiens, GRCh38 (ftp://ftp.ccb.jhu.edu/pub/infphilo/hisat2/data/grch38_snp_tran.tar.gz)]. Samtools 1.6 (Li et al. 2009) was used to sort entries in the sam file output from hisat2 and convert to bam format. The bam files were processed using the Cufflinks suite version 2.2.1 (Trapnell et al. 2013) with the "advanced" Cufflinks workflow: Cufflinks → Cuffmerge → Cuffquant → Cuffdiff. The Cuffdiff output was processed using CummeRbund 2.24.0 and various R

and bash scripts. For each mutant vs. WT comparison, the gene_exp.diff file was filtered for significant entries where FPKM values were FPKM ≥ 1 and log2 fold change values were log2 ≥ 1 or log2 ≤ -1 (i.e., 2-fold or greater). The 100 DEGs with the highest absolute log2 values (positive and negative) were combined to generate lists of 200 genes for subsequent ClueGO analyses. Comparisons of crADSL to WT in conditions lacking adenine (MM, or "minus to minus" comparison) and in adenine supplemented conditions (PP, or "plus to plus" comparison) were performed.

ClueGO analysis

ClueGO is a Cytoscape app that extracts representative functional biological information for large lists of genes or proteins (Mlecnik, Galon, and Bindea 2018). ClueGO analyses were performed using Cytoscape version 3.7.0 and ClueGO 2.5.2. The GO and Reactome releases were Homo Sapiens_GO-EBI-UniProt-GOA_17.12.2018 and Homo Sapiens_REACTOME_17.12.2018. Data sets were run pairwise using the crADSL plus adenine vs. WT plus adenine comparison (PP) and the crADSL minus adenine vs. WT minus adenine comparison (MM) with the DEG lists described above. Analyses were performed using default settings: the evidence code was set to "all", and network specificity was set to "representative" with a 3 gene/term cut off, approximating GO levels 3-11. Terms and groups were divided into three categories (MM, PP, or Shared) based on whether most genes defining a term or group were enriched in the MM or PP comparisons, or an equal number of significant genes was obtained from both groups (Shared). Further, "slightly enriched" denotes enrichment by one gene, while "heavily enriched" denotes enrichment by two or more genes.

BiNGO analysis

BiNGO 3.0.3 is a Cytoscape app that analyzes a total gene list and performs GO term enrichment (Biological Process, Cellular Component, and Molecular Function) (Maere, Heymans, and Kuiper 2005). Gene lists were analyzed, and ontologies evaluated by False Discovery Rate (FDR). Nodes are colored according to their associated p-value.

qPCR validation of DEGs

qPCR was performed to validate the reliability of the RNA-seq analysis. Total RNA was prepared as described above and concentrations obtained by NanoDrop (ThermoFisher). cDNA was prepared using iScript cDNA synthesis kit (BioRad #1708890) using 500 ng total RNA per reaction according to the manufacturer's protocol. Candidate genes were selected and primers ordered from IDT using PrimeTime service. The primers are TGFβI (Hs.PT.58.40018323), ALPP (Hs.PT.56a.38602874.g), Twist1 (Hs.PT.58.18940950), IQGAP2 (Hs.PT.58.28018594), GATA3 (Hs.PT.58.19431110), β-Actin (Hs.PT.39a.22214847), OASL (Hs.PT.58.50426392), TUSC3 (Hs.PT.58.3740957), and DPYSL3 (Hs.PT.58.39796068). qPCR was performed on IQ5 (BioRad) with 1 µl of cDNA using IQ Sybr Green Supermix (BioRad #170-8880) and the program: 95 °C for 5 minutes followed by 45 cycles of 95 °C for 10 seconds and 60 °C elongation. Samples were read 30 seconds. C_t values were obtained, normalized to β-Actin, and used for further analysis.

Results

crADSL requires adenine for proliferative growth

Cell growth and purine requirement were assessed for crADSL and WT HeLa cells. When starved long term for purines, crADSL cells showed detachment from the

plate and cell death in adenine depleted (purine free) media. In adenine supplemented media, we observed that crADSL takes more time to attach after re-plating and grows more slowly than WT HeLa. WT HeLa showed proliferative growth in both adenine-supplemented and non-supplemented media (Figure 3.2). These results confirm a requirement for purine supplementation for crADSL for proliferative growth.

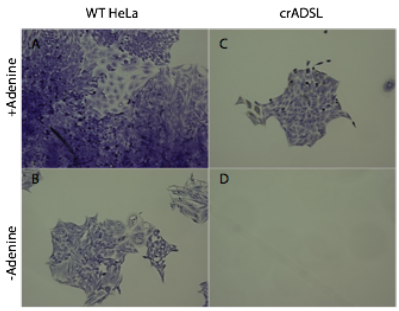


Figure 3.2: Adenine is required for proliferative growth of crADSL line. WT HeLa cells (A, B) and crADSL HeLa (C, D) were in DMEM supplemented with 10% FCM with (A, C) or without (B, D) 100 µM adenine then fixed and stained with crystal violet.

SAICAR accumulates in crADSL but not WT HeLa cells.

HPLC-EC analysis of metabolites from starved crADSL and WT cells was performed to characterize SAICAR accumulation (Figure 3.3). In crADSL, the ADSL substrate SAICAR eluted at 50.1 minutes (Figure 3.4) with detectable accumulation at 6 hours in starvation medium, but not in adenine supplemented medium, and continued until 10 hours, the last time point measured. The ADSL product AICAR, which elutes at 24 minutes, was not observed and SAMP, the second substrate of ADSL, was not observed. This result is consistent with the DNPS pathway block due to ADSL inactivation.

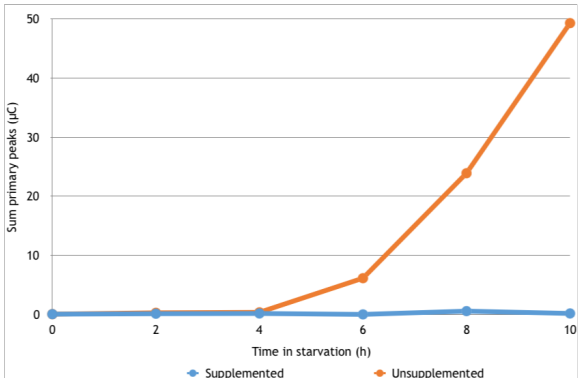


Figure 3.3: SAICAR accumulates in crADSL cells in starvation conditions without adenine. crADSL cells were cultured in 100 µM adenine supplemented (blue) or adenine-free (orange) DMEM with 10% FCM for 10 hours. Metabolites were analyzed on HPLC-EC. The plot indicates

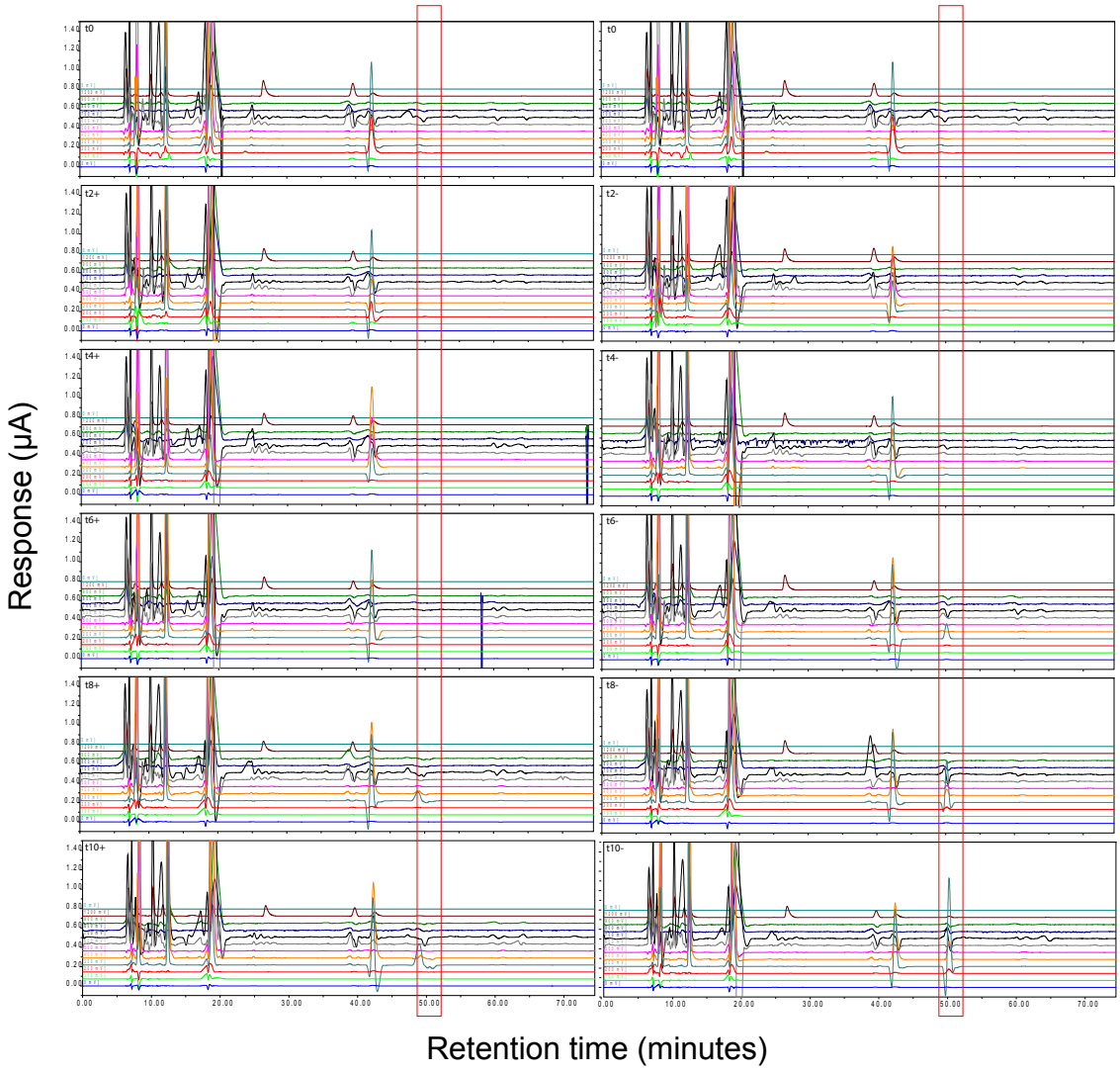


Figure 3.4: HPLC-EC traces of crADSL cells. Cells starved in FCM supplemented media with adenine (left traces) and without adenine (right traces) in two-hour increments. Red square indicates elution time of SAICAR. Primary peak in channel 3 (300mV).

crADSL and WT show DEGs in both adenine rich and depleted conditions

In the experiments described below, purine deprived cells are labeled M and cells in supplemented media are labeled P. RNA-seq analysis was performed to detect transcriptome changes by cell type and adenine supplementation. Entries with FPKM (fragments per kilobase of exon per million reads mapped) values less than 1 were dropped (which removes low count statistical anomalies), and only ≥ 2-fold FPKM changes (log2 ≥ 1 or log2 ≤ -1) were retained with a p-value cut off of 0.05 using Benjamini-Hochberg correction in Cufflinks (Trapnell et al. 2013). Comparison of crADSL to WT in conditions lacking adenine (MM, or "minus to minus" comparison) returns 1659 DEGs. A list of the 100 most positive and 100 most negative DEG log2 values was prepared and encompasses log2 value ranges of 8.823 to 2.311 and -2.593 to -8.788, respectively. Comparison of crADSL to WT in adenine supplemented conditions (PP, or "plus to plus" comparison) returns 1426 DEGs. A list of 100 most positive and 100 most negative DEG log2 values was prepared and encompasses log2 value ranges of 8.577 to 2.266 and -2.526 to -8.289, respectively (Figure 3.5). For our generated full list of DEGs, there were 1144 shared genes and 282 (19.8%) and 515 (31.6%) unique identified genes respectively from the PP and MM comparisons. When the list was reduced to the top 200 DEGs used for further analysis, 153 genes were shared between the

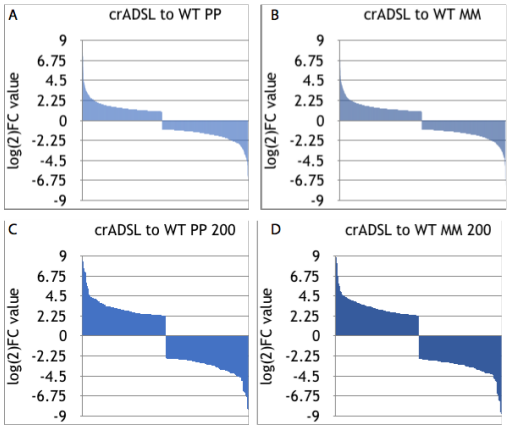


Figure 3.5: log(2) fold change of DEGs in cell lines under experimental conditions. A and B: all DEGs that satisfy cutoff constraints between PP comparison (A) and MM comparison (B). C and D: 100 most positively and 100 most negatively changed DEGs in PP comparison (C) and MM comparison (D).

two comparison groups and 47 unique genes for each PP and MM comparison (Table 3.1). Our analysis was limited to genes from the lists derived from each MM and PP comparison.

Table 3.1: Shared and unique DEGs between comparison groups. Total gene counts that satisfied previously defined cutoffs in the total gene list in the top 200 genes that were used for ClueGO analysis parsed into the PP and MM comparison or shared between the two comparison groups.

	Total DEGs	Top 200 DEGs
Shared	1144	153
Unique PP	282	47
Unique MM	515	47

crADSL and WT HeLa DEGs: enrichment in ontology terms and groupings in supplemented and starvation conditions

Gene Ontologies (GO) are divided into three categories: biological process, cellular component, and molecular function. Each category has a specific aim: biological process includes genes that contribute to completion of a biological objective, cellular component refers to gene product localization, and molecular function refers to the biochemical activity of gene products (Ashburner et al. 2000). The Reactome knowledgebase systematically maps gene products into pathway and reaction networks (or metabolic maps) (Fabregat et al. 2018). In our discussion of ontology and Reactome enrichment in the gene sets, term will be used for a singular ontology or Reactome annotation that shows enrichment due to genes associated with that specific component, pathway, process, reaction, or function. It is important to note that terms may be enriched in a single comparison; that however does not indicate that the term was not significant in the other comparison. All ontology network maps, term, and grouping visualization figures are located after the text of this chapter. Notable findings are compiled (Figure 3.6).

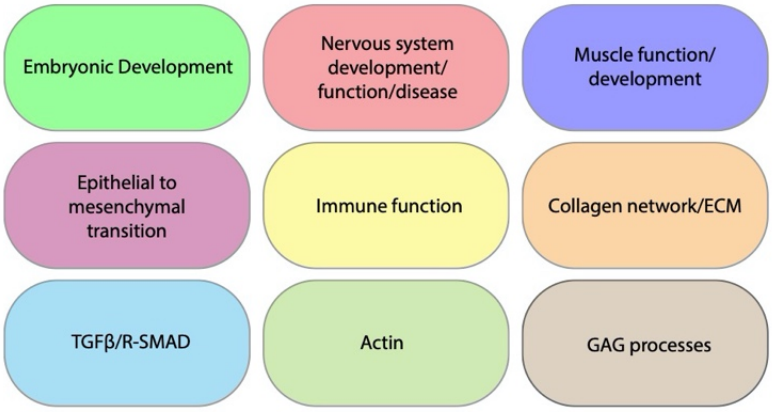


Figure 3.6: Notable findings from RNA-seq/GO analysis.

In GO Biological Process, DEGs mapped to 95 shared terms in 27 groups (Figures 3.8, 3.9) The most notable among these are epithelial to mesenchymal transition with 29.65% of terms related to this group, amyloid fibril formation with 12.06% of terms associated, and actin nucleation at 7.04%. Other notable shared terms involve processes such as smooth muscle cell and heart structure development, neuron or brain associated function/development, bone mineralization, hormone processing, as well as interleukins, Wnt signaling, and tumor necrosis factor. In the PP comparisons, we see many overlaps within the shared groupings, however it should be noted that exit from mitosis, and transcription regulatory region DNA binding were heavily enriched terms. For the MM comparisons, we see mass overlap of terms associated with larger parent groups in the shared list, however Glycosaminoglycan catabolic process as well as some actin/myosin-based terms were heavily enriched while terms associated with TGFβ were mildly enriched.

The Cellular Component ontology produced 6 shared terms in 4 groups (Figures 3.10, 3.11). Extracellular matrix, platelet alpha granule, as well as peroxisomal

membrane were shared. Mild enrichment was noted in the PP comparison lamellipodium membrane and filopodium, while for the MM comparison with 4 terms in 4 groups centered around plasma membrane, golgi, and photoreceptors.

Molecular Function ontology showed 9 shared terms in 8 groups (Figures 3.12, 3.13) in RNA binding at 22.22%, integrin binding at 11.11% Cell-cell adhesion, Laminin binding, and protein self-association type groupings. Mild enrichment in the PP showed 3 terms in 3 groups in Histone acetyltransferase activity, HMG box domain binding, and R-SMAD binding activity while MM has term enrichment in Aβ and TGFβ binding and lipoprotein particle receptor binding.

The Reactome Pathway results showed 10 shared terms in 4 groups (Figures 3.14, 3.15) involved with kinase activity as well as antiviral mechanism by IFN-stimulated genes at 7.41% gene term association term and complement cascades. Collagen type terms were also strongly enriched in shared pairings due to COL15A1, COL25A1, COL3A1, and COL4A4 being present in both lists analyzed. There were no strong preferential associations in the PP list with 5 terms in 4 groups, however slight enrichment in the PP terms were seen in scavenger receptor ligands, EPH-ephrin repulsion, and signaling by PDGF. Immune system terms OAS antiviral response and IFNG response (with 33.33% and 5.43% gene term association respectively) were seen in the PP comparison. The MM comparison showed more interesting enrichment with 19 terms in 6 groups. Due to the TMOD1 and TNNT1 gene differential expression, striated muscle contraction was heavily enriched. HS-GAG metabolism and other terms associated with GAG were heavily enriched in the MM comparison due to differential expression of GPC5 and HSPG2. For genes slightly enriched in the MM comparison over

the PP comparison, collagen related terms such as integrin, ECM proteoglycans, and NCAM, CRMPs and semaphorin, as well as post-translational modification were also slightly enriched in the MM comparison. Various interferon and antiviral terms were slightly enriched in the MM comparison.

The Reactome Reactions results showed similar patterns as Pathways, with strong enrichment in the MM comparison over the PP comparison. Twenty terms in three groups were shared (Figures 3.16, 3.17) and centered around collagen with 90.0% term enrichment, granule membrane proteins, and platelet alpha granule with 5.0% each. The singular enriched PP term associated with IFNG was only slightly enriched. MM comparison revealed 17 terms in 6 groups. Heavily enriched terms in the MM comparison were associated with muscle contraction due to the TMOD1 and TNNT1 gene. GAG based tetrasaccharide linker terms were heavily enriched due to the presence of GPC5 and HSPG2. For terms that were slightly enriched in the MM comparison, we see once again interferon and immune-based terms as well as phosphorylation of CRMPs related terms due to the presence of DPYSL3 (CRMP4) in the MM gene list.

crADSL and WT HeLa showed similar enrichment patterns using BiNGO analysis

To validate our ClueGO findings, we chose to analyze gene sets using the BiNGO app in Cytoscape. Network maps, term and grouping visualizations are shown at end of text for this chapter (Figures 3.18-3.23). While there are new terms that appear in our data sets such as Response to Endoplasmic Reticulum Stress, on the whole this secondary analysis system complements our ClueGO findings.

Validation of gene expression patterns by qRT-PCR

Candidate gene transcripts were selected that showed robust expression patterns and qPCR was performed to assess whether the expression pattern was maintained using a different analysis system. Primers for DPYSL3 (CRMP4), Twist1, TUSC3, TGFβI, IQGAP2, GATA3, ALPP, and OASL showed ΔC_t values in similar expression patterns to the RNA-seq data log2 values (Figure 3.7), demonstrating the validity of our RNA-seq data set in both P and M conditions.

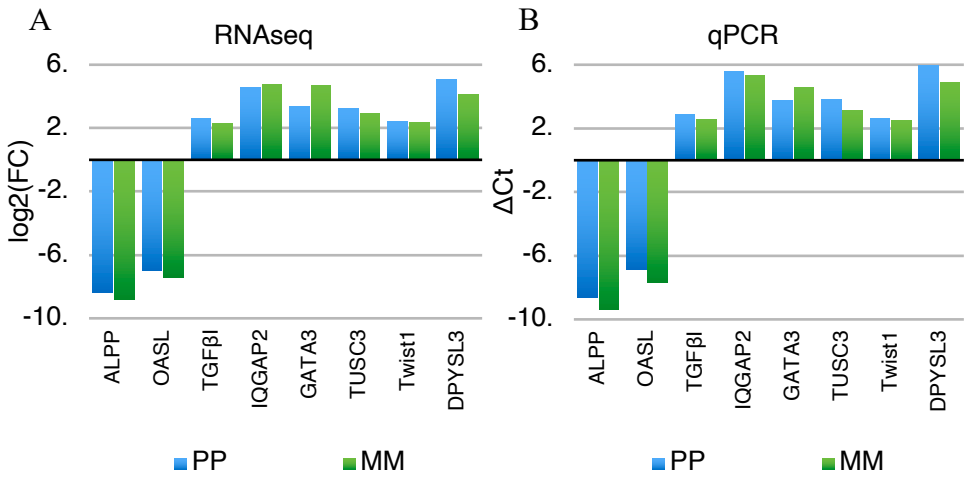


Figure 3.7: Candidate gene verification using qPCR. Difference of crADSL against WT HeLa cells of biological quadruplicates in both PP and MM of RNA-seq (A) and qPCR (B) were normalized to β-Actin. Expression patterns show consistency.

Discussion

In this study, we evaluated the dietary requirements and metabolite accumulation during purine starvation for crADSL, and we performed RNA-seq to compare the crADSL and WT HeLa transcriptomes in purine supplemented and starved conditions. Further, we performed qPCR to verify our RNA-seq results. Our results demonstrate that crADSL requires purine (adenine) supplementation for proliferative growth, SAICAR

accumulates over a time course of ten hours during purine starvation, and we obtain many DEGs both by cell type and adenine supplementation.

Previous results (Keller et al. 2014) support the hypothesis that SAICAR accumulation should produce robust transcriptome changes via the "moonlighting" PKM2 protein kinase activity. It is important to note that their methods are limited to gene chip experiments and not evaluation of viable cells in culture. We observed changes in both PP and MM (SAICAR accumulating) conditions, which suggests that SAICAR accumulation and general ADSL deficiency both mediate transcriptome alteration. Our results show a greater number of DEGs in the MM versus PP comparison, which suggests that SAICAR may be regulating transcription activity, either by a PKM2-SAICAR co-regulation activity or some other mechanism. If PKM2 does act as a co-regulator of transcription, the PKM2-SAICAR complex may potentially activate a select subset of targets for this activity. Our current experiments suggest a SAICAR derived effect on transcription.

In GO Biological Process, we obtained a robust group of terms including and related to epithelial to mesenchymal transition (EMT) and transforming growth factor beta (TGFβ). EMT refers to the process by which polar epithelial cells undergo biochemical changes that convert them to mesenchymal cells, which exhibit increased resistance to apoptosis and enhanced migratory and invasiveness properties (Kalluri 2009). EMT is important in embryogenesis, specifically during primitive streak formation as well as during neural crest formation. EMT in embryogenesis is orchestrated by the Wnt signaling pathway (Kalluri 2009). Consistent with the EMT related terms, we also observed Wnt signaling pathway in our shared comparison. Disruption in EMT might

play an important role in the developmental and neurological phenotypes associated with ADSL deficiency (discussed below). EMT is also important in inflammation and cell migration. Upon trauma to basement membranes, epithelial cells can undergo EMT in response to inflammation from injury (Kalluri and Weinberg 2009). Our results show changes in genes in inflammation/interferon pathways, such as interferon signaling, and IL-1β secretion. Terms associated with interferon, inflammation, and immunity were present in the MM comparison, which suggests that these transcriptional changes are the result of SAICAR accumulation and supports the tentative hypothesis that ADSL deficiency may be an immunological disorder. It also suggests that ADSL deficiency and/or DNPS deficiency may play an important role in immune dysfunction in cancer.

In our GO and Reactome analyses, many shared terms mapped to development associated groups. Since ADSL deficiency is a developmental disorder with phenotypes including dysmorphic features, cognitive deficits, seizures, and psychomotor retardation, it is possible that these terms may be relevant to a plausible explanation of phenotype. In addition, we observed an interesting enrichment in many muscle and movement type terms and groups. This is consistent with the high level of expression of ADSL in muscle cells (Van Den Berghe and Jaeken 1986; Brand and Lowenstein 1978) and may be informative to the psychomotor retardation phenotype observed in ADSL deficiency. Our GO and Reactome results indicate that disruption to DNPS (and ADSL specifically) alters energy production and energy levels and sensing needs, which affect force generation in muscle cells.

Cancer is characterized, among other features, by constitutive cellular division, alterations in cellular metabolism (Warburg Effect) (Liberti and Locasale 2016), collagen

network restructuring (Fang et al. 2014), metastasis and infiltration, and changes in immune function (Iommarini et al. 2017; Krzywinska and Stockmann 2018). Our results show enrichment for these and related terms, which suggests important roles for DNPS deficiency and/or SAICAR accumulation in these cancer-related processes. In addition to the collagen, integrin, EMT, actin, and ECM terms, our GO cellular component analysis showed enrichment for lamellipodia and filopodia. Filopodia employ integrins to produce finger-like protrusions preparatory to cellular migration. Filopodia formation is an important mechanism for cell migration and infiltration during tumor cell metastasis (Arjonen, Kaukonen, and Ivaska 2011). Recently, the role of ADSL in certain cancer types was probed suggesting a potential link in aggressive phenotypes (Park et al. 2018).

While the scope of this manuscript is focused on the ontologies associated with DNPS and ADSL knockout, several individual genes were identified as of specific interest. Aberrations in ALPP, a placental alkaline phosphatase, have been implicated in spontaneous abortions (Vatin et al. 2014), and in some forms of cancers (Fishman et al. 1968; Fishman 1987). Twist1 is a transcription factor important for craniofacial and organ development during embryogenesis, most likely from mesoderm derived tissues, and has been identified in multiple types of tumors and involved in cancer metastasis, resistance to chemotherapy, and it can over-ride oncogene induced apoptosis (Qin et al. 2012). TUSC3 (tumor suppressor candidate 3) is associated with multiple functions including Mg^{2+} uptake, glycosylation and embryonic development, in addition to its tumor suppression function (X. Yu et al. 2017). IQGAP2 integrates Rho GTPase and Ca^{2+}/calmodulin signals for cellular adhesion and cytoskeleton reorganization and was recently found to act as a tumor suppressor (Xie et al. 2012). DPYSL3 (CRMP4) is

primarily a neuronal protein expressed during development and adult stages and is responsible for various tasks including cell migration, differentiation, neurite extension, and axonal regeneration (Alabed et al. 2007). It also has been found to play a role in some non-neuronal cancers in migration and metastasis, although the exact role is still being investigated (Matsunuma et al. 2018). GATA3 is a transcription factor and regulator of numerous developmental pathways and has been found heavily associated with breast cancer (Chou, Provot, and Werb 2010). OASL is a gene associated with viral response and immunity, with activation carried out by interferons (Choi et al. 2015). TGFβI is a ubiquitously secreted ECM protein with plausible participation in morphogenic, embryonic developmental, adhesive/migratory, tumorigenic, wound healing, and inflammatory processes (Thapa, Lee, and Kim 2007). It is apparent with ontologies and gene variability that DNPS and ADSL provide an important context for the study of developmental and cancer biology.

Individuals with ADSL deficiency have a mutated form of ADSL with reduced enzymatic activity. This implies reduced (but not halted) conversion of SAICAR to AICAR and is consistent with results from patient studies (Jurecka et al. 2015; Zikanova et al. 2010). Due to a reduced rate of conversion of SAICAR to AICAR, we would expect transcriptome alteration due to persistent elevation in SAICAR and a reduction in the rate of DNPS. Here we present results detailing transcriptome changes in crADSL due to elimination of ADSL enzyme activity. Future studies will investigate differences between mutant forms of ADSL, and may employ specific cell lines to assess the effects of ADSL dysregulation in developmental, tumor, vascular and muscle biology. These cells and

cells transfected with mutant forms of ADSL should provide an invaluable cellular model of ADSL deficiency.

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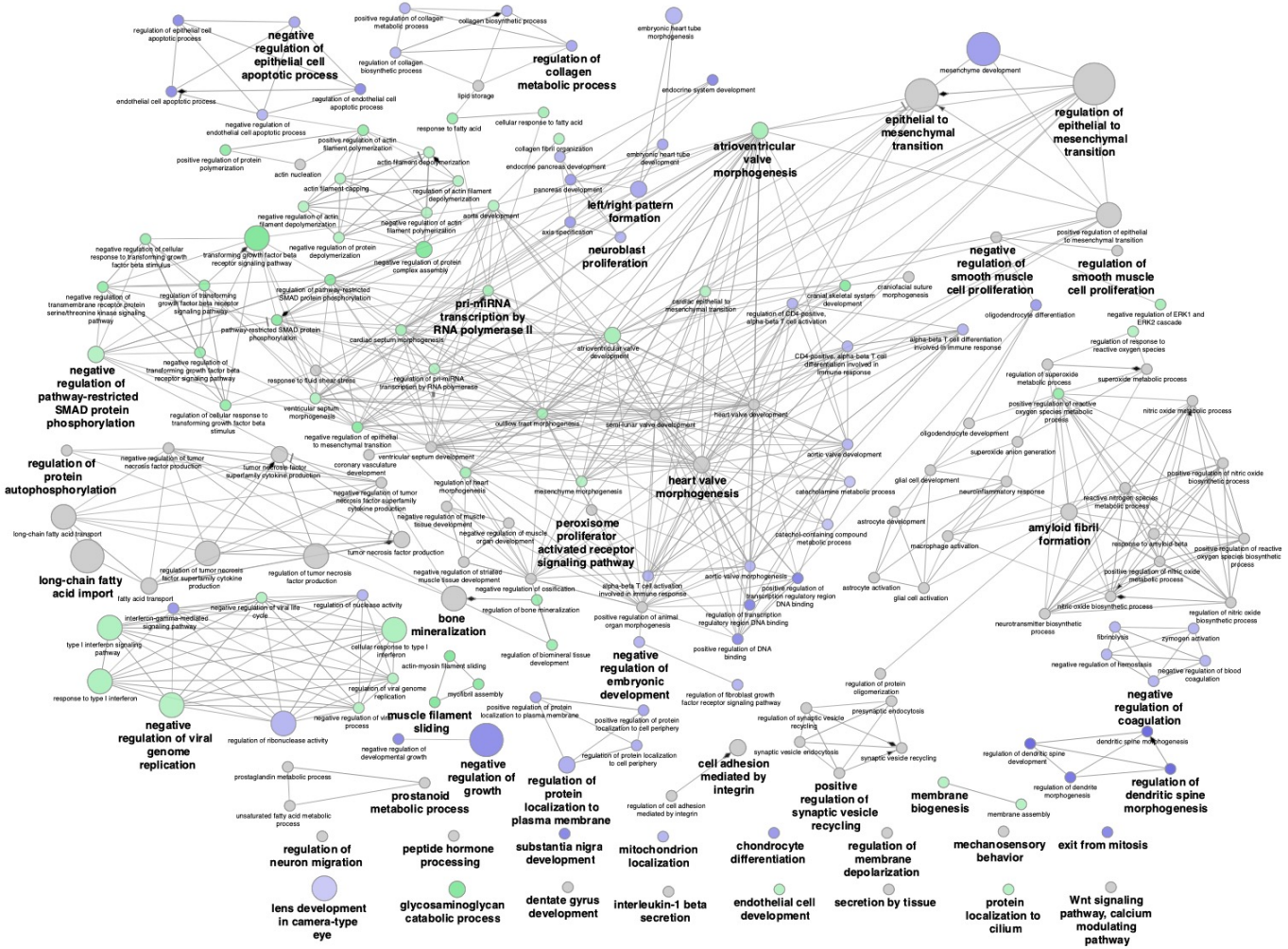


Figure 3.8: ClueGO Biological Process network map. Grey nodes denote shared terms while blue nodes denote enriched terms in PP comparison while green nodes denote enriched terms in MM comparison

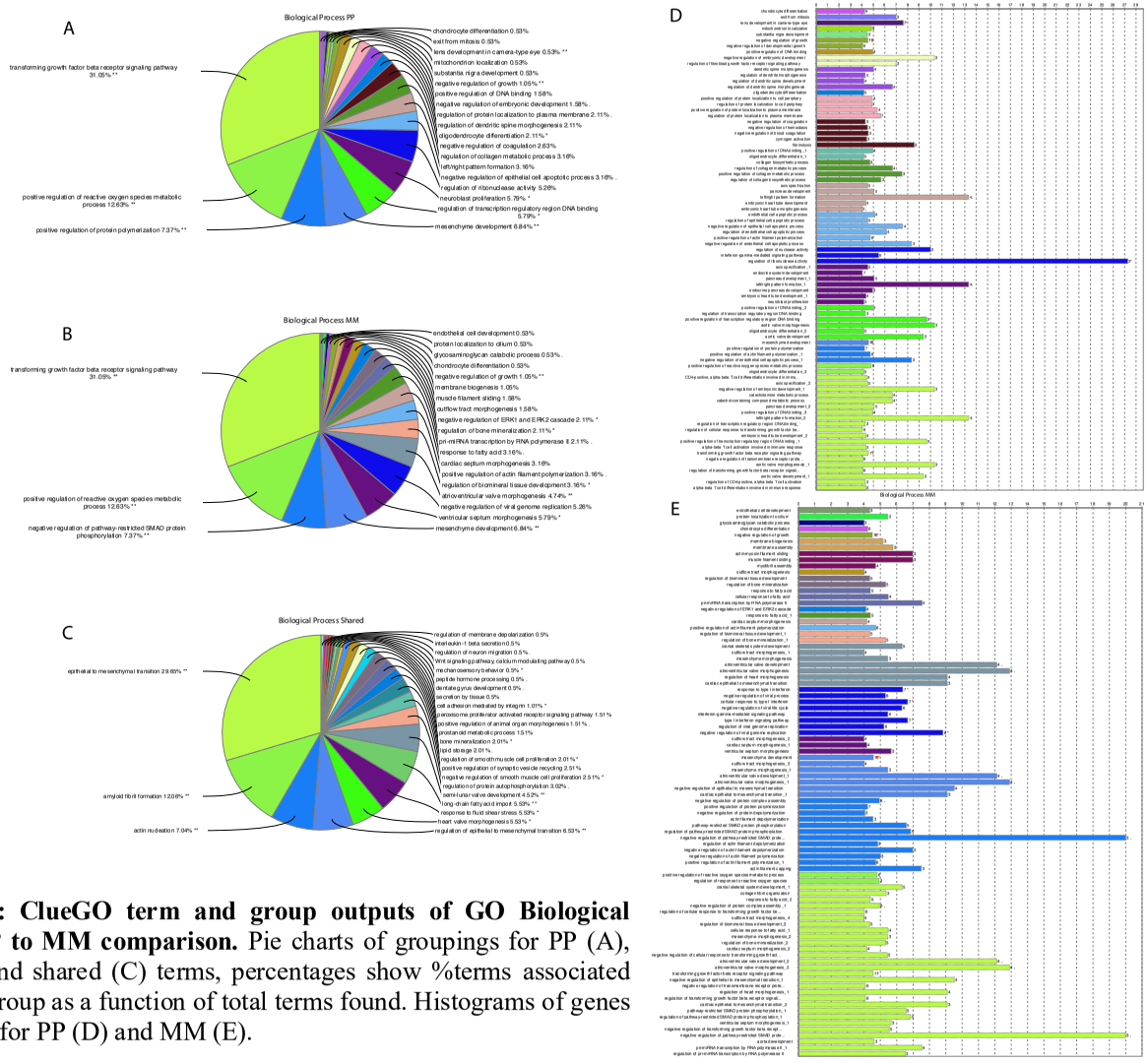


Figure 3.9: ClueGO term and group outputs of GO Biological Process PP to MM comparison. Pie charts of groupings for PP (A), MM (B), and shared (C) terms, percentages show %terms associated with each group as a function of total terms found. Histograms of genes found/term for PP (D) and MM (E).

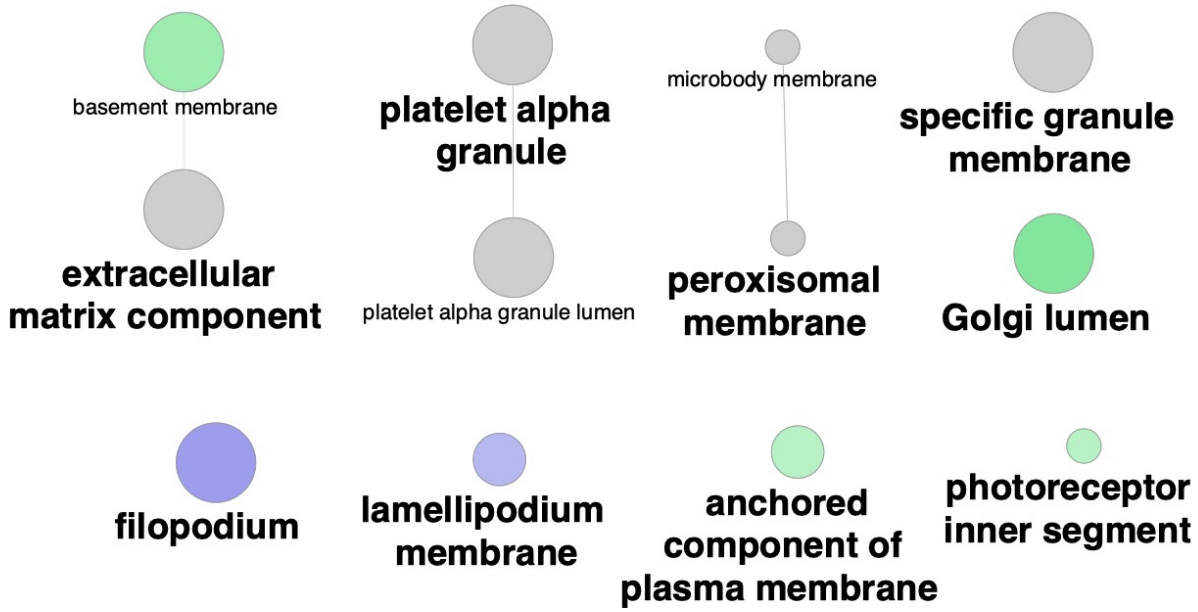


Figure 3.10: ClueGO Cellular Component network map. Grey nodes denote shared terms while blue nodes denote enriched terms in PP comparison while green nodes denote enriched terms in MM comparison

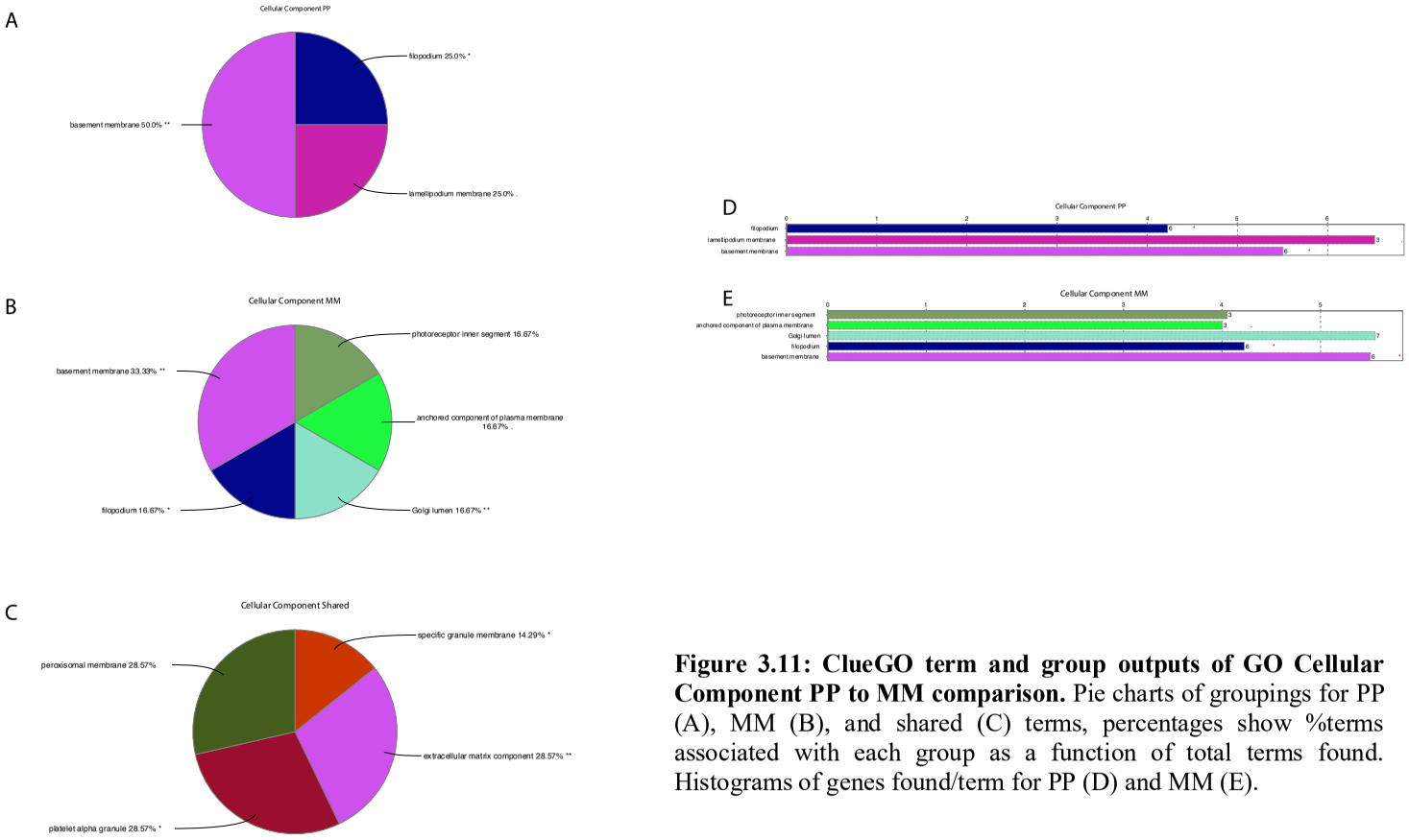


Figure 3.11: ClueGO term and group outputs of GO Cellular Component PP to MM comparison. Pie charts of groupings for PP (A), MM (B), and shared (C) terms, percentages show %terms associated with each group as a function of total terms found. Histograms of genes found/term for PP (D) and MM (E).

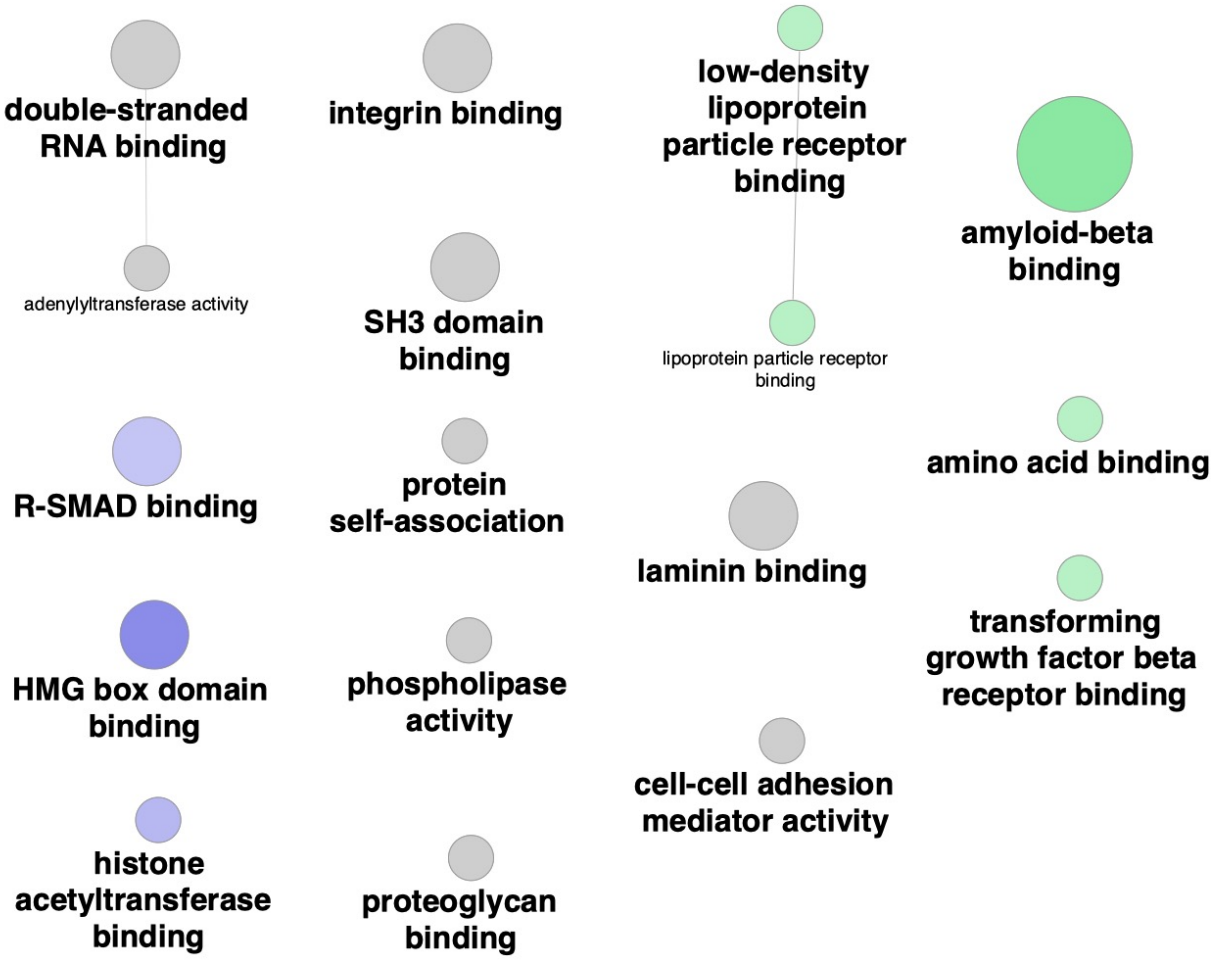
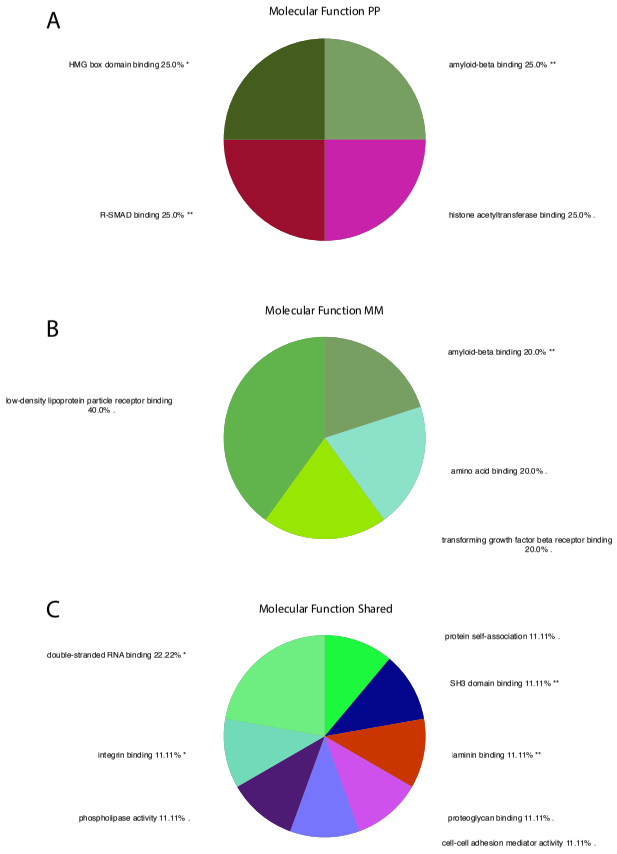


Figure 3.12: ClueGO Molecular Function network map. Grey nodes denote shared terms while blue nodes denote enriched terms in PP comparison while green nodes denote enriched terms in MM comparison



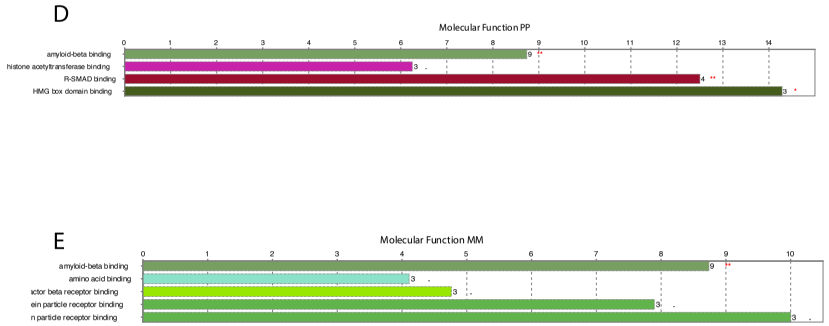


Figure 3.13: ClueGO term and group outputs of GO Molecular Function PP to MM comparison. Pie charts of groupings for PP (A), MM (B), and shared (C) terms, percentages show %terms associated with each group as a function of total terms found. Histograms of genes found/term for PP (D) and MM (E).

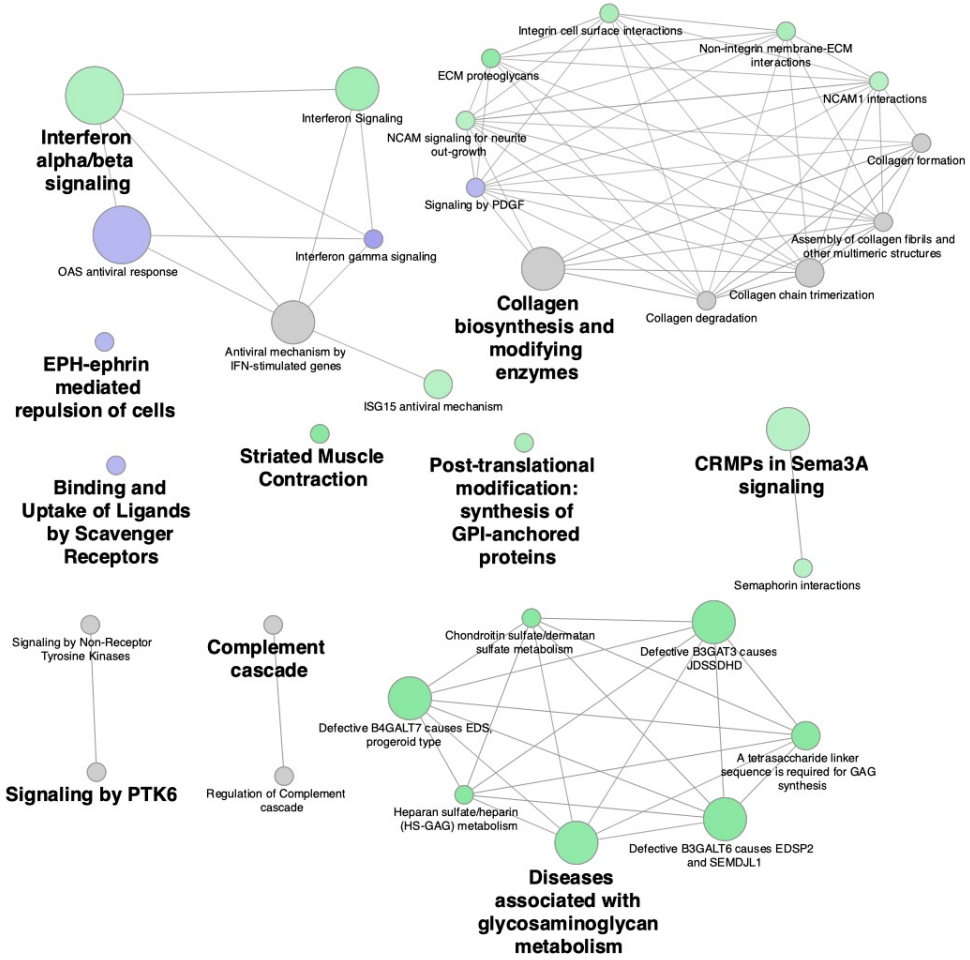


Figure 3.14: ClueGO Reactome Pathways network map. Grey nodes denote shared terms while blue nodes denote enriched terms in PP comparison while green nodes denote enriched terms in MM comparison

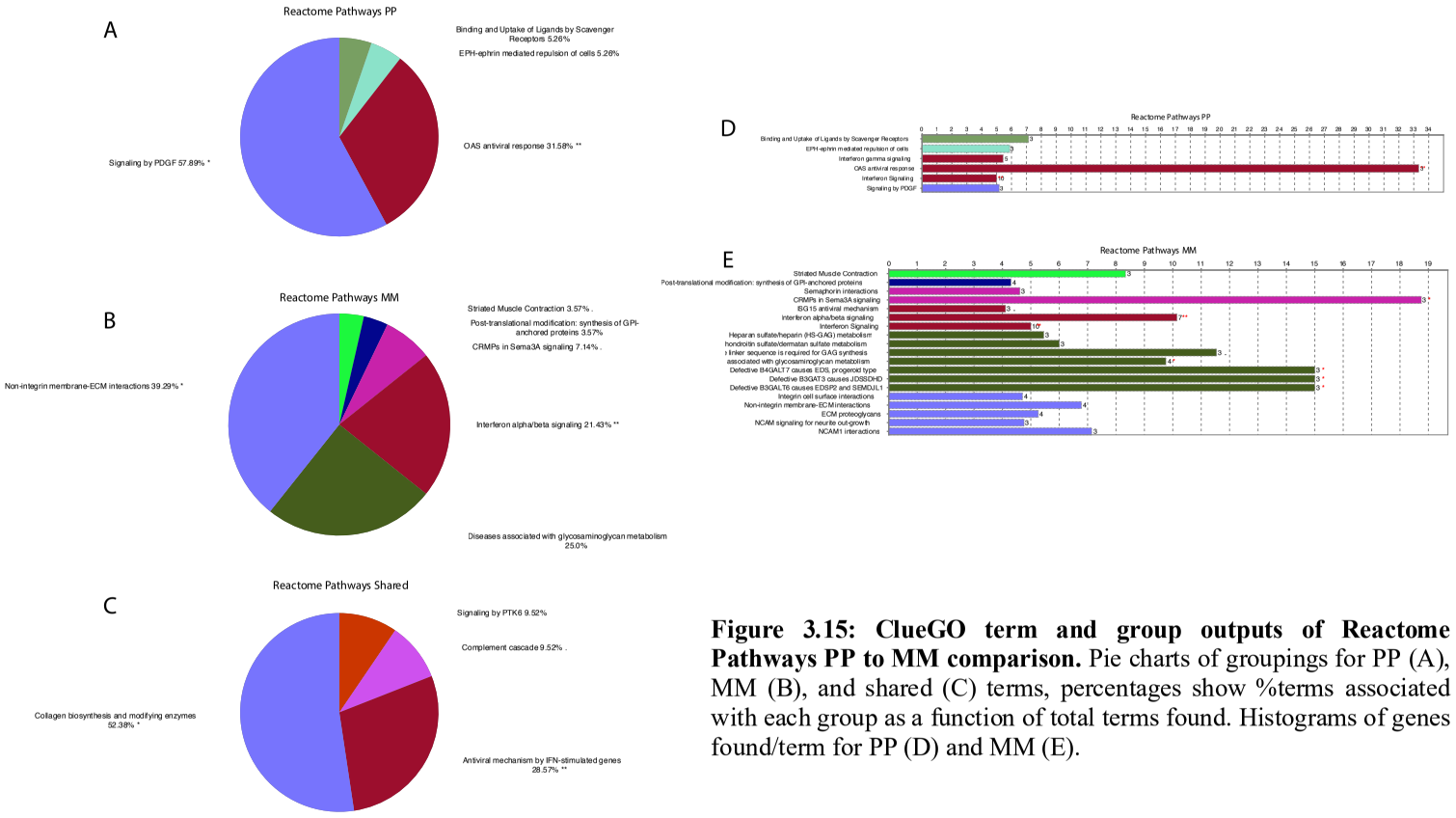


Figure 3.15: ClueGO term and group outputs of Reactome Pathways PP to MM comparison. Pie charts of groupings for PP (A), MM (B), and shared (C) terms, percentages show %terms associated with each group as a function of total terms found. Histograms of genes found/term for PP (D) and MM (E).

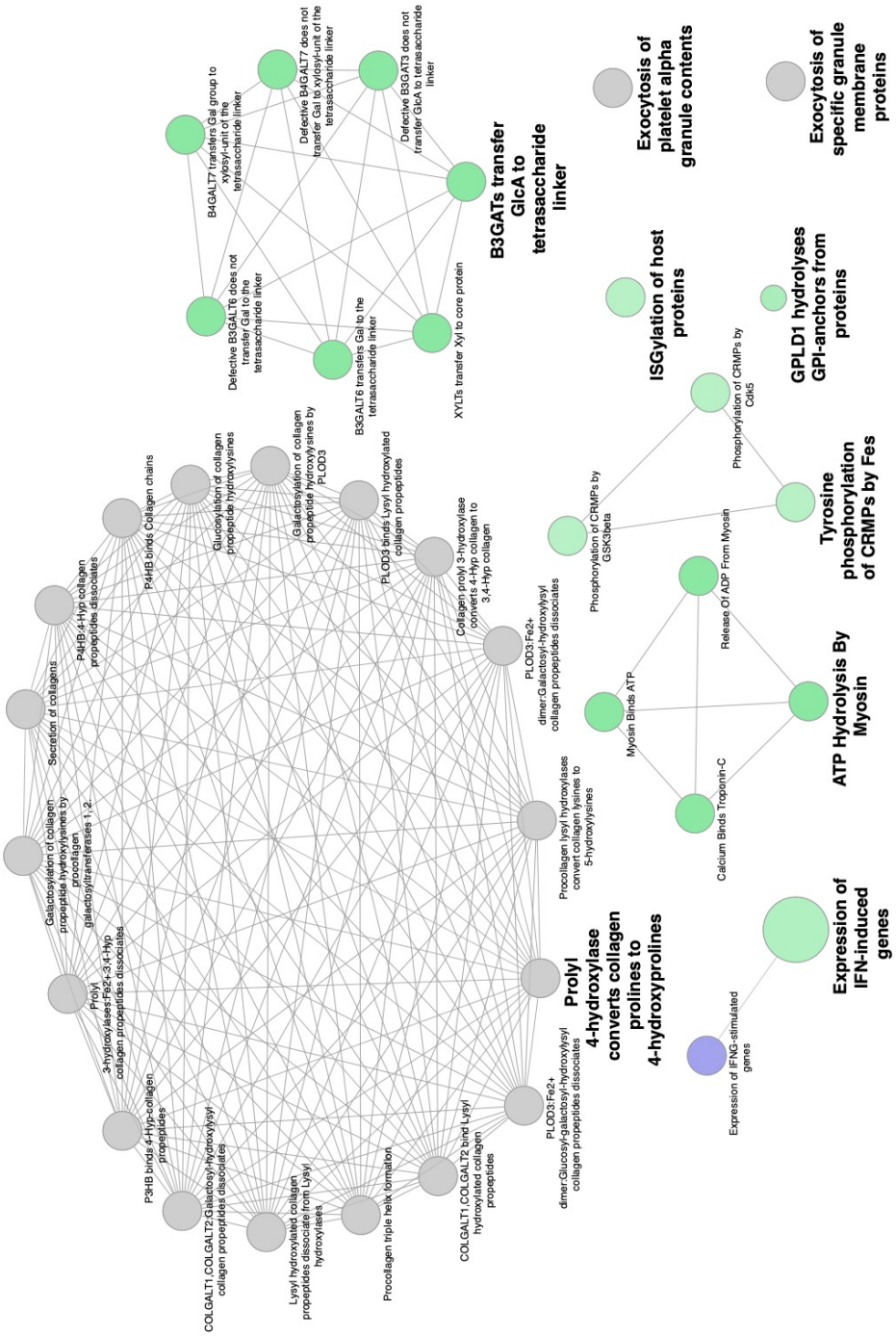


Figure 3.16: ClueGO Reactome Reactions network map. Grey nodes denote shared terms while blue nodes denote enriched terms in PP comparison while green nodes denote enriched terms in MM comparison

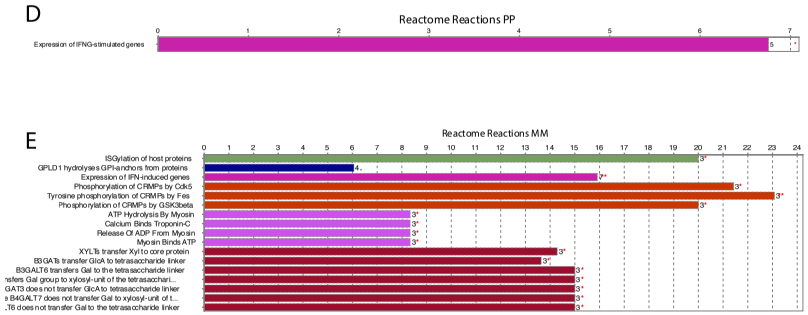


Figure 3.17: ClueGO term and group outputs of Reactome Reactions PP to MM comparison. Pie charts of groupings for PP (A), MM (B), and shared (C) terms, percentages show %terms associated with each group as a function of total terms found. Histograms of genes found/term for PP (D) and MM (E).

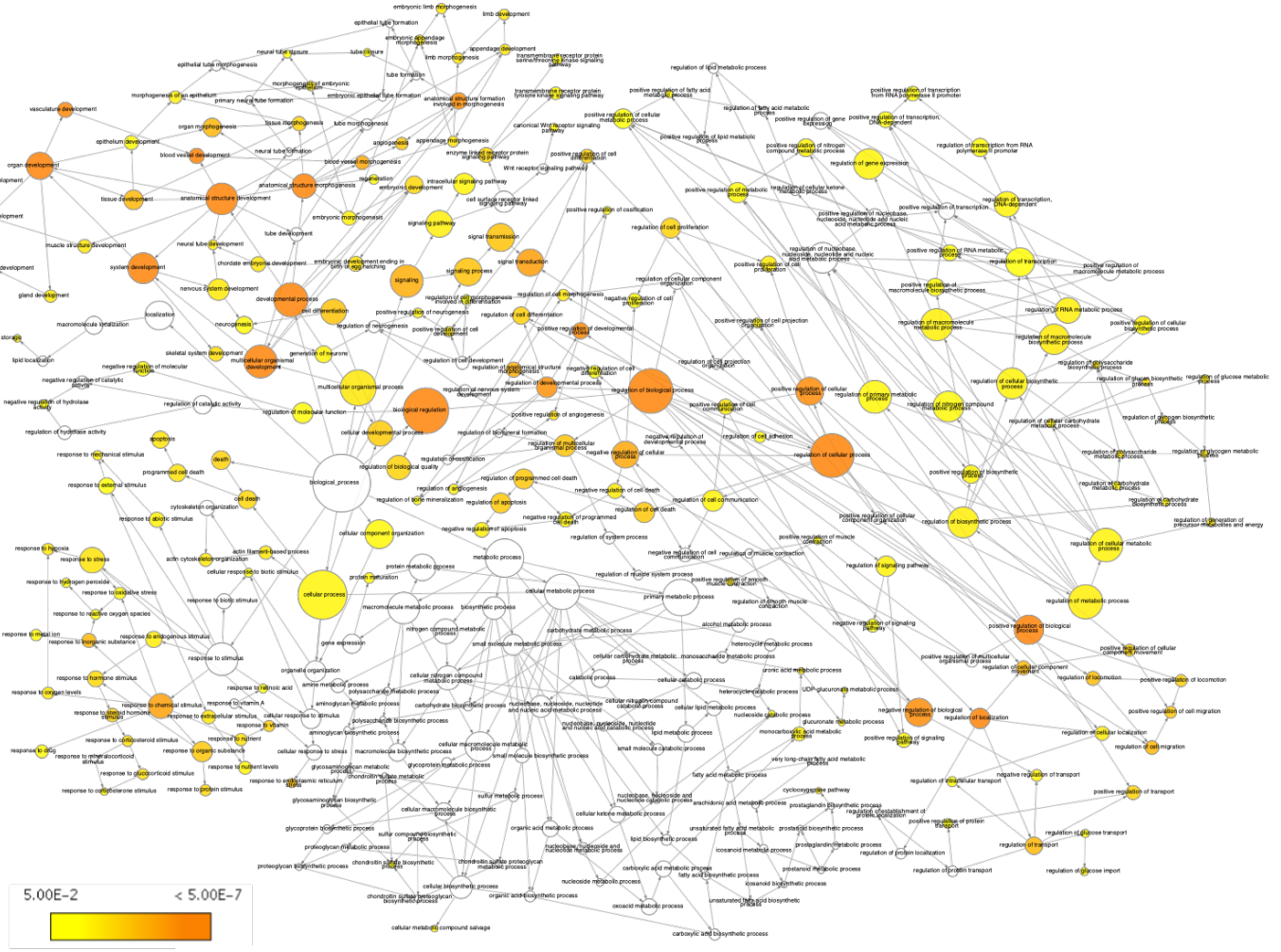


Figure 3.18: BiNGO network map PP Biological Process. Nodes are colored according to their term P value. White nodes are intermediate terms and are not significant.

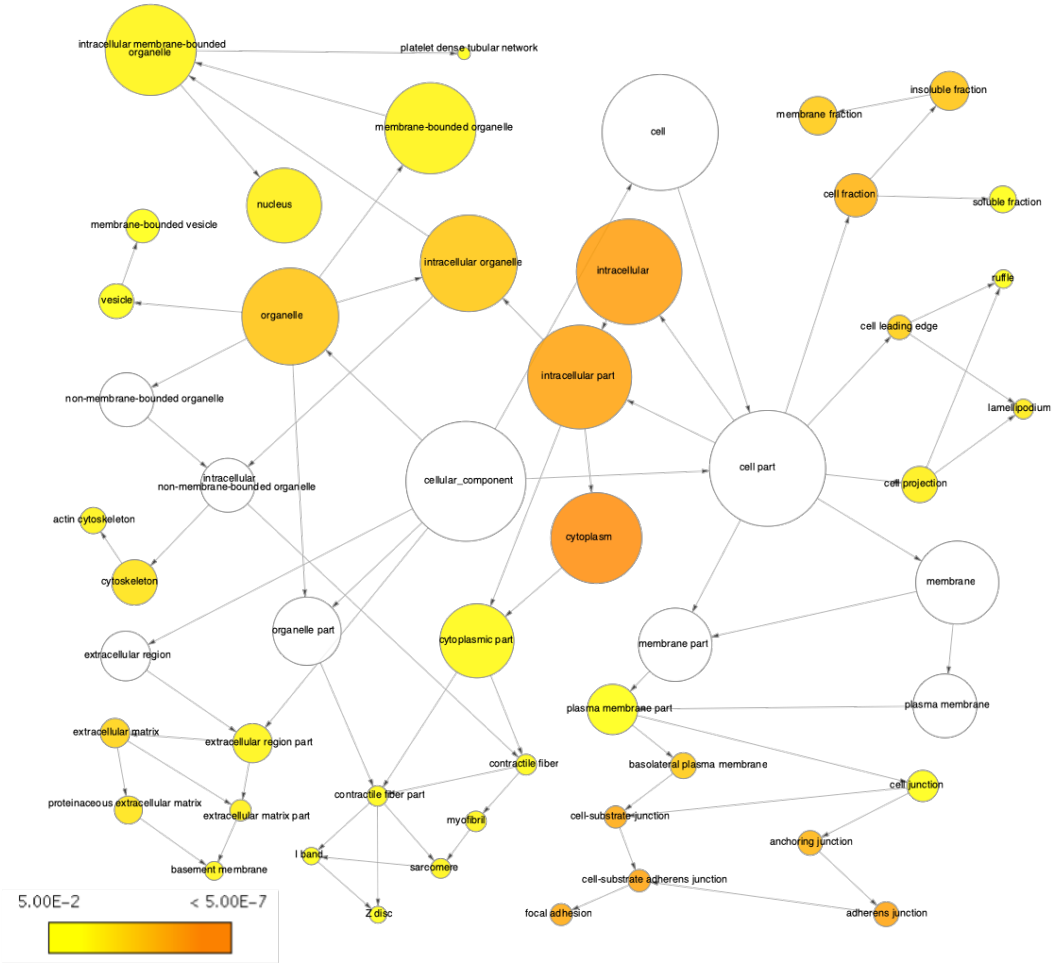


Figure 3.19: BiNGO network map PP Cellular Component. Nodes are colored according to their term P value. White nodes are intermediate terms and are not significant.

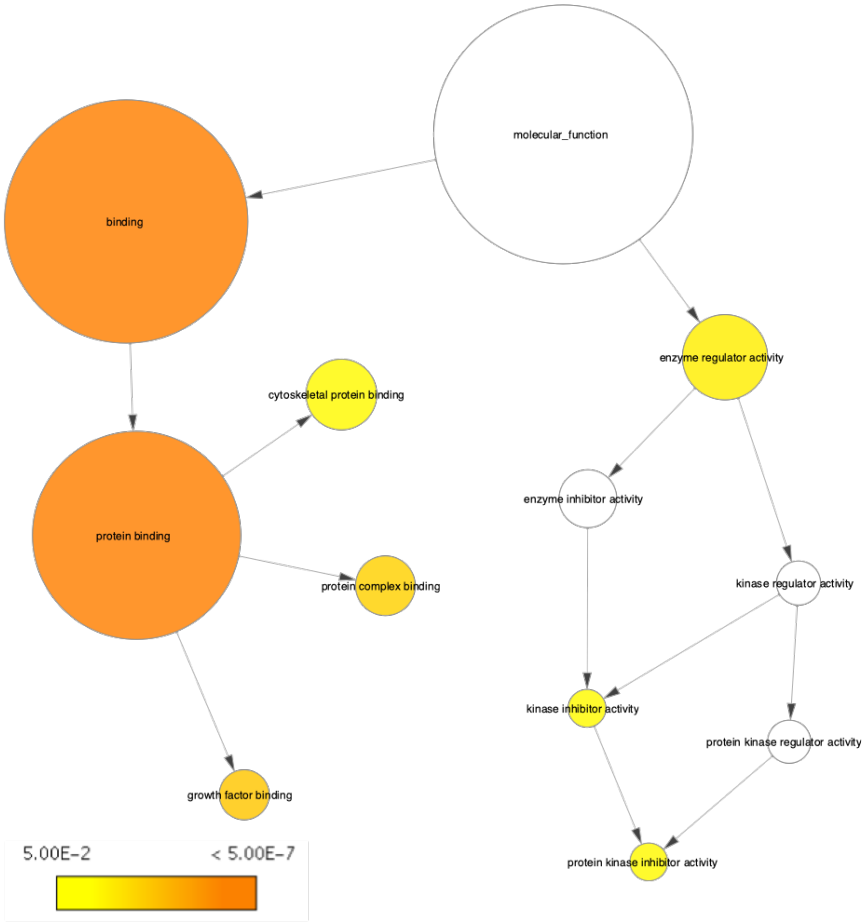


Figure 3.20: BiNGO network map PP Molecular Function. Nodes are colored according to their term P value. White nodes are intermediate terms and are not significant.

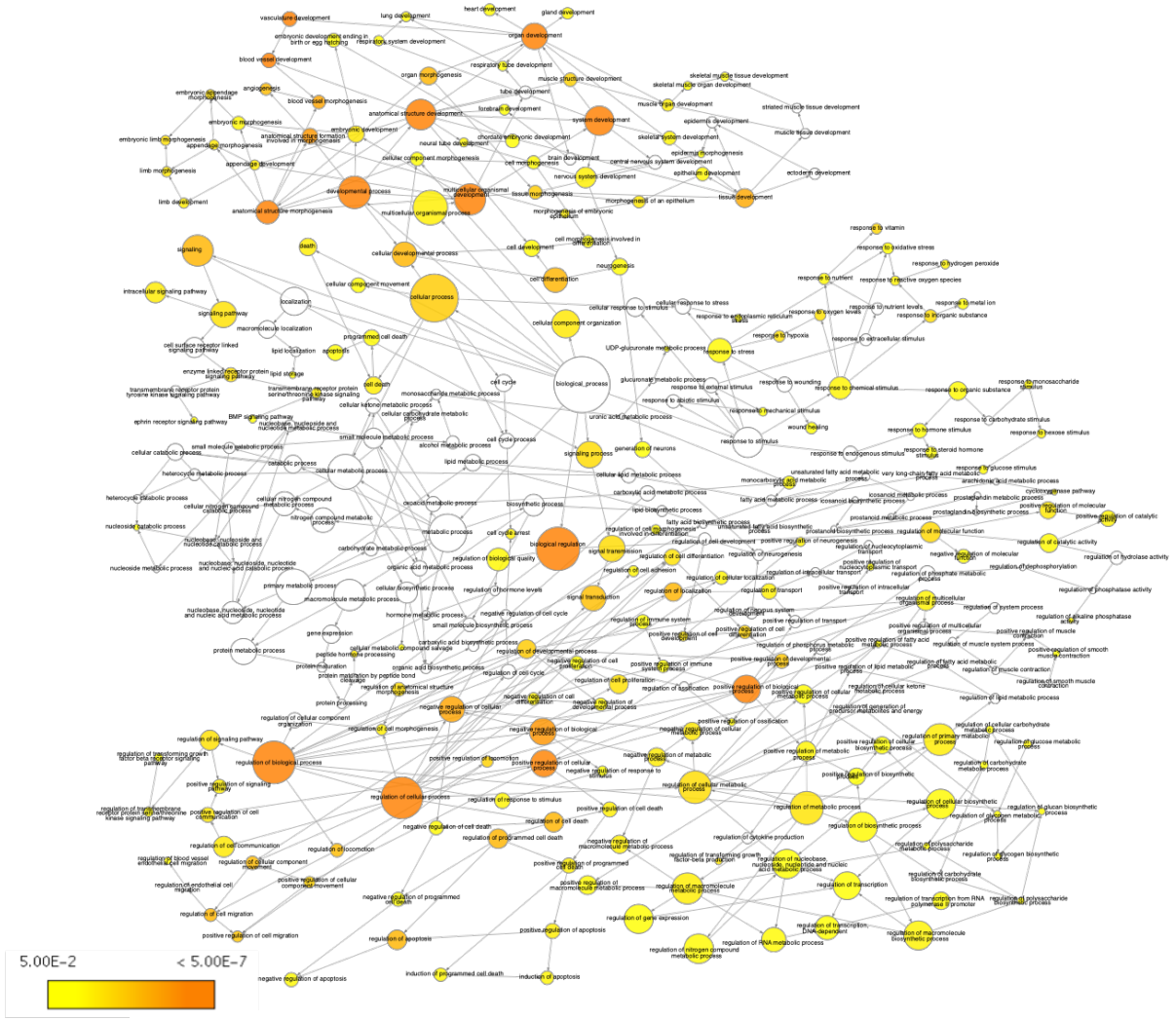


Figure 3.21: BiNGO network map MM Biological Process. Nodes are colored according to their term P value. White nodes are intermediate terms and are not significant.

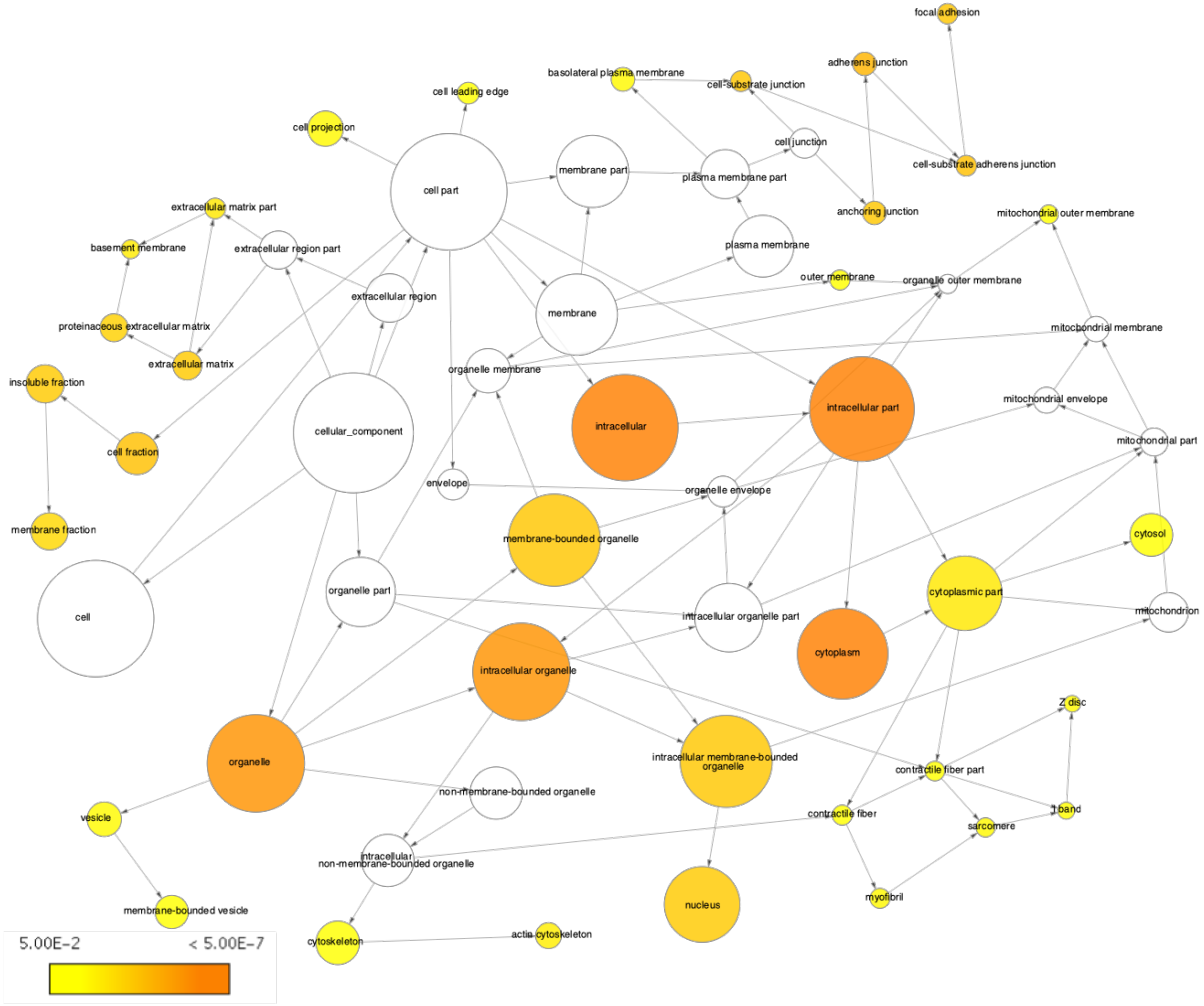


Figure 3.22: BiNGO network map MM Cellular Component. Nodes are colored according to their term P value. White nodes are intermediate terms and are not significant.

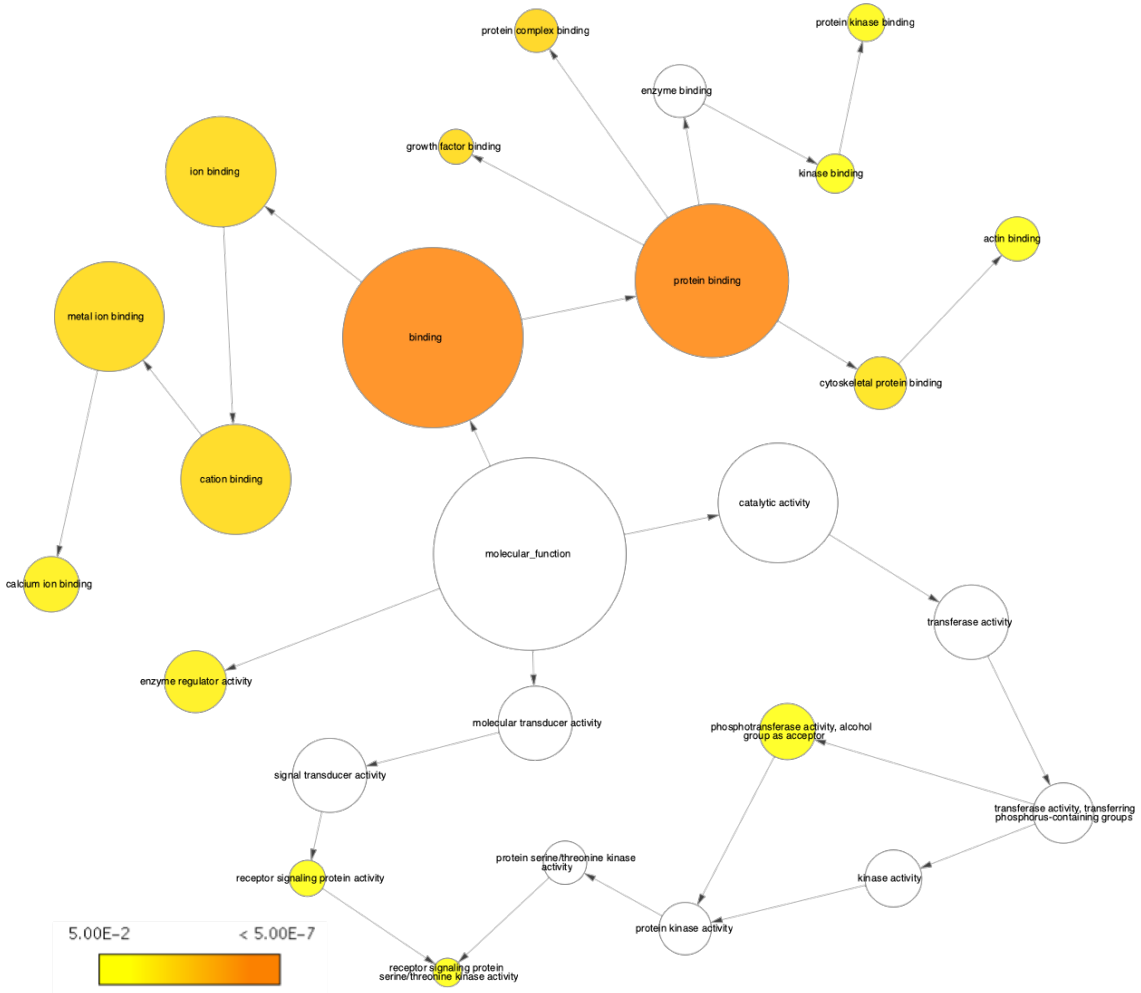


Figure 3.23: BiNGO network map MM Molecular Function. Nodes are colored according to their term P value. White nodes are intermediate terms and are not significant.

Chapter Four: Transcriptomic Characterization of crATIC

Introduction

De novo purine synthesis (DNPS) is essential for cellular function. Purines are critical as 1) components of DNA and RNA: the information carrying molecules of cells, 2) intra and intercellular signaling molecules, for example G (guanine) protein coupled receptors, 3) as the major source of energy currency in the form of ATP and 4) substrates and co-enzymes for many cellular functions. In humans and other animals DNPS is accomplished via ten sequential enzymatic reactions resulting in conversion of phosphoribosyl pyrophosphate (PRPP) to inosine monophosphate (IMP) mediated by six enzymes (seven of the ten reactions are catalyzed by three multifunctional enzymes). IMP can then be converted through two reactions to either AMP or GMP (Figure 4.1).

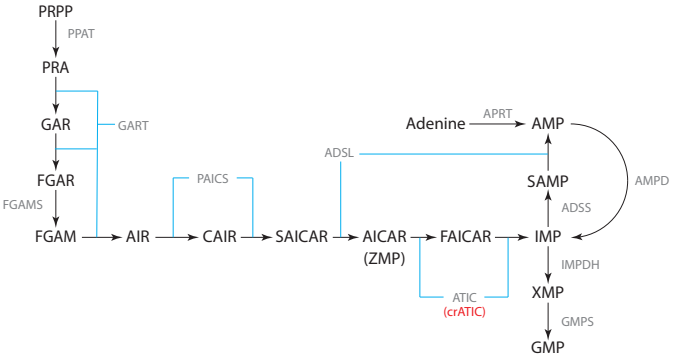


Figure 4.1: De novo purine synthesis pathway. DNPS is accomplished via the conversion of PRPP to IMP which is subsequently converted to AMP or GMP. The HeLa ATIC KO, crATIC, is indicated. Figure generated by Dr. Guido Vacano.

AMP-activated protein kinase (AMPK) is a major regulator of cellular metabolism. AMPK regulates the mammalian Target of Rapamycin (mTOR) pathway. AMPK activation shuts down mTOR signaling directly via phosphorylation of Raptor

(Saxton and Sabatini 2017) and indirectly via activation and phosphorylation of Tuberous Sclerosis Complex 1 and 2 (TSC1 and TSC2). mTOR regulates vital cellular anabolic and catabolic processes involved in lipid synthesis, glycolysis, mitochondrial and lysosomal biosynthesis, apoptosis, glucose metabolism, cytoskeletal rearrangement, and cell migration (Saxton and Sabatini 2017). Recently, AMPK activation has been implicated in different cellular processes such as inflammation suppression (Jeon 2016), and suppression of several IFN-γ-induced cytokines and chemokines in primary astrocytes and microglia via its restriction of IFN-γ signaling (Meares et al. 2013).

AMP is an allosteric effector of AMPK, and AMPK responds to increases in the AMP:ATP ratio by inhibiting ATP catabolism and promoting ATP anabolism (Garcia and Shaw 2017; Hardie 2011). AMPK can activate these processes in response to ATP depletion produced via fasting, exercise (Cantó et al. 2010) or other means. Generally speaking, AMP has three roles in AMPK control: promotion of AMPK phosphorylation (Hawley et al. 1995), inhibition of AMPK dephosphorylation (Davies et al. 1995), and allosteric activation of phosphorylated AMPK (Corton et al. 1995).

ZMP is an AMP mimetic and a potent AMPK agonist. While AMPK has garnered the most attention as the best characterized effector of ZMP accumulation, several studies have shown that ZMP has multiple cellular targets (Hawley et al. 1995; Douillet et al. 2019; Kirchner, Brüne, and Namgaladze 2018). This supports the hypothesis that ZMP can allosterically regulate enzymes ordinarily regulated by AMP. In multiple studies analyzing ZMP function, cells were fed AICA riboside (AICAr, the dephosphorylated form of ZMP) which is converted to ZMP within the cell. This approach involves adenosine transporters and adenosine kinases to ensure ZMP production and

accumulation in the cell (Garcia and Shaw 2017), however, once ZMP is formed, cells with active ATIC enzyme will catalyze it via DNPS. Recent work indicates that AICAr itself affects cell metabolism independently of its role as substrate for ZMP synthesis. For example, AICAr administration induces a potentially deleterious intracellular ATP depletion in hepatocytes (Guigas et al. 2006). Drug intervention inhibiting ATIC dimer formation has been shown to be efficacious in reducing tumor cell proliferation (Spurr et al. 2012), but can produce off target effects. Therefore, cell models that accumulate ZMP without exogenous AICAr should be advantageous for investigating optimal ZMP dosage levels in cells to produce beneficial effects.

The bifunctional enzyme 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (2.1.2.3) / inosine monophosphate cyclohydrolase (3.5.4.10) (ATIC) catalyzes the final two reactions of IMP synthesis, converting ZMP to FAICAR and then FAICAR to IMP (Figure 4.1). It is a homodimer, and dimerization is required for activity (Asby et al. 2015). Under some conditions ATIC may be a rate limiting step of the DNPS pathway (Marie et al. 2004; Baresova et al. 2016). So far, a single human with ATIC deficiency (AICA-ribosiduria) (Marie et al. 2004) has been identified and the mutations characterized: a mis-sense mutation in one allele resulting in K426R (transformylase region) and a frameshift in the other allele. This individual presented with profound developmental delay. It is likely that most mutations in ATIC are embryonic lethals, consistent with its critical role in DNPS and metabolism. Since ZMP is the substrate for ATIC, ATIC deficiency is likely to activate AMPK and have major consequences for cellular metabolism. DNPS nulls were recently generated in HeLa cells via CRISPR-Cas9 (Baresova et al. 2016). The crATIC cell line (Baresova et al. 2016), which has no

ATIC activity and accumulates ZMP, will allow detailed study of the consequences of mutations in ATIC. For example, given that adenine supplementation effectively shuts down DNPS (Holmes et al. 1973; Tu and Patterson 1978), analysis of crATIC in adenine depleted growth media will allow detailed assessment of the effect of ZMP accumulation on transcription, translation, and metabolism. Transfection of crATIC with ATIC clones containing various mutations will allow cellular analysis of specific amino acid substitutions on ATIC structure and function. As an initial approach to these studies we present an analysis of the crATIC and HeLa cells in the presence or absence of adenine.

Materials and Methods

Cell Culture

crATIC cells were described previously (Baresova et al. 2016). HeLa cells were purchased from ATCC (Manassas VA USA). Cells were grown on 60 mm TPP (Techno Plastic Products, AG, Switzerland) plates using DMEM with 10% fetal calf serum (FCS), 30 μM adenine, and Normocin (InvivoGen). For purine depletion experiments (starvation conditions) cells were incubated in similar medium but using fetal calf macroserum (FCM: serum dialyzed against a 3.5 kDa barrier) with or without 100 μM adenine. Complete media was regularly refreshed, and ten to twelve hours before experiments, was replaced with adenine-depleted or 100 μM adenine-supplemented media. Cells were subjected to starvation conditions for ten hours. For histological staining, 10,000 cells were plated overnight in complete growth media (DMEM 10%FCS with 30 μM adenine and Normocin), media was replaced with starvation conditions (DMEM 10% FCM, Normocin, with or without 100 μM adenine) and after six days were fixed using 10%

ethanol / 3.5% acetic acid solution and stained using 0.1% crystal violet. Entire culture areas were imaged.

HPLC analysis of ZMP metabolite accumulation

At two-hour intervals for ten hours, cells were washed once with cold 1x PBS and extracted with 500 µl ice cold 80% ethanol. Plates were scraped and the extract was centrifuged twice at 14,000 x g. Supernatant was collected and dried using a Speedvac then resuspended in 300 µl freshly prepared mobile phase. Samples were cleared twice by centrifugation at 14,000 x g and stored at -20 °C until analysis. HPLC-EC separation of ZMP was as previously described (Mazzarino et al. 2019). Analytes were detected using a CoulArray HPLC system (model 5600A, ESA) with EC channel potentials set from 0 to 900 mV in 100 mV increments, then 1200 mV, and 0 mV to oxidize and detect ZMP. The autosampler was kept at 10 °C over the course of the runs. Sum of primary peak area was used to assess analyte accumulation.

RNA-seq

Cells were cultured as described above. RNA-seq was performed as previously described (Mazzarino et al. 2019). Four replicates (a single culture was split into four plates) of crATIC and HeLa were cultured in adenine-supplemented or adenine-depleted (starvation) media for 10 hours. Total RNA was extracted using Tri-reagent (Sigma) according to the manufacturer's protocol, followed by spin columns (Machery Nagel) and elution with 50 µl DEPC treated water (Sigma). RNA purity and concentration were assessed using a NanoDrop One (Thermo Scientific). RNA was stored at -80 °C. RNA quality assessment and RNA-seq was performed by The Genomics and Microarray Core Facility at the University of Colorado, Denver. mRNA libraries were constructed using

the Nugen Universal Plus mRNA-Seq + UDI kit (cat # 9144–96), and 50 bp single read sequencing was performed employing the Illumina HiSEQ4000. The sequencing quality Q score was >38 for all reads. Conversion of .bcl to FASTQ files was done using CASAVA 2.0.

Data Processing

Computation was performed as previously described (Mazzarino et al. 2019). For each comparison group, the gene_exp.diff file (Cufflinks output) was filtered for significant entries where FPKM values ≥ 1 and log2 fold change values were log2 ≥ 1 or log2 ≤ -1. In each comparison group the 100 differentially expressed genes (DEGs) with the highest absolute log2 values (both 100 most positive and 100 most negative) were combined for ClueGO analysis. Total gene lists from our cutoffs were used for BiNGO analysis. Comparisons of crATIC to HeLa in adenine-depleted conditions are labeled as MM (minus to minus) or in adenine-supplemented conditions labeled as PP (plus to plus).

Gene Ontology analysis

Cytoscape (version 3.7.0) apps ClueGO (version 2.5.2) and BiNGO (version 3.0.3) were used to provide representative biological information from the lists of differentially expressed genes (DEGs). ClueGO using the top 100 most positive and 100 most negative DEGs was run using pairwise comparisons: HeLa plus adenine vs crATIC plus adenine (PP) and HeLa minus adenine vs crATIC minus adenine (MM). BiNGO is capable of utilizing our complete DEG lists but cannot run pairwise comparisons. The BiNGO analysis was limited to running one list of DEGs as a supplement to ClueGO results. The ClueGO analysis included biological process, cellular component, and molecular function gene ontologies as well as Reactome pathway and reactions, while

BiNGO was only run with biological process, cellular component, and molecular function gene ontologies.

qPCR validation of DEGs

qPCR was performed to validate the RNA-seq analysis. Total RNA was isolated as described above with cDNA prepared using the iScript cDNA synthesis kit (Bio-Rad #1708890) and qPCR conditions previously described (Mazzarino et al. 2019). Candidate gene primers were purchased from IDT PrimeTime service: TGFβI (Hs.PT.58.40018323), ALPP (Hs.PT.56a.38602874.g), Twist1 (Hs.PT.58.18940950), IQGAP2 (Hs.PT.58.28018594), GATA3 (Hs.PT.58.19431110), β-Actin (Hs.PT.39a.22214847), OASL (Hs.PT.58.50426392), TUSC3 (Hs.PT.58.3740957), and DPYSL3 (Hs.PT.58.39796068). C_t values were normalized to β-Actin.

Results

crATIC requires adenine for proliferative growth

HeLa and crATIC cells were cultured in complete media then subsequently cultured in DMEM supplemented with dialyzed fetal calf macroserum with or without adenine. HeLa cells showed proliferative growth in both media conditions while crATIC cells only showed proliferative growth in adenine- supplemented conditions (Figure 4.2).

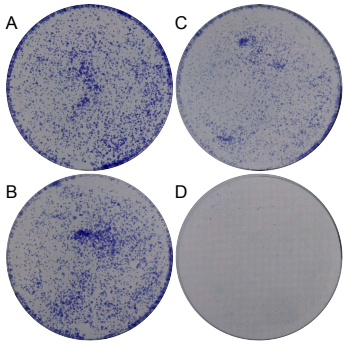


Figure 4.2: crATIC requires adenine for proliferative growth. HeLa (A,B) and crATIC (C,D) cells were plated and cultured in adenine-supplemented (A,C) or adenine-depleted (B,D) media. Plates were stained with crystal violet.

crATIC cells accumulate ZMP in adenine-depleted but not adenine- supplemented growth conditions

crATIC cells were cultured in adenine- depleted and adenine-supplemented media. Metabolites were ethanol extracted from cells in culture every two hours for ten hours. In samples from adenine-depleted (starved) cells, a ZMP peak was observed at the first time point in HPLC-EC traces (24.0 mins) and this peak increased linearly until the last time point measured (Figures 4.3, 4.4). A ZMP peak was not observed in samples from adenine- supplemented cells, which is consistent with previous results (Mazzarino et al. 2019).

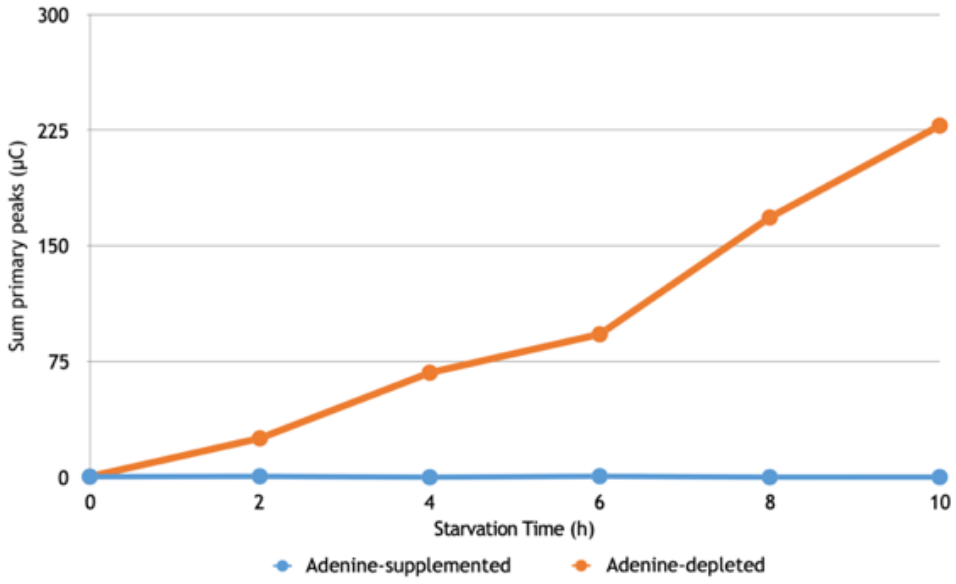


Figure 4.3: ZMP accumulates in crATIC. crATIC was grown in media with or without supplemental adenine. Accumulation of ZMP was measured by HPLC-EC and was observed only in cells grown in adenine-depleted media.

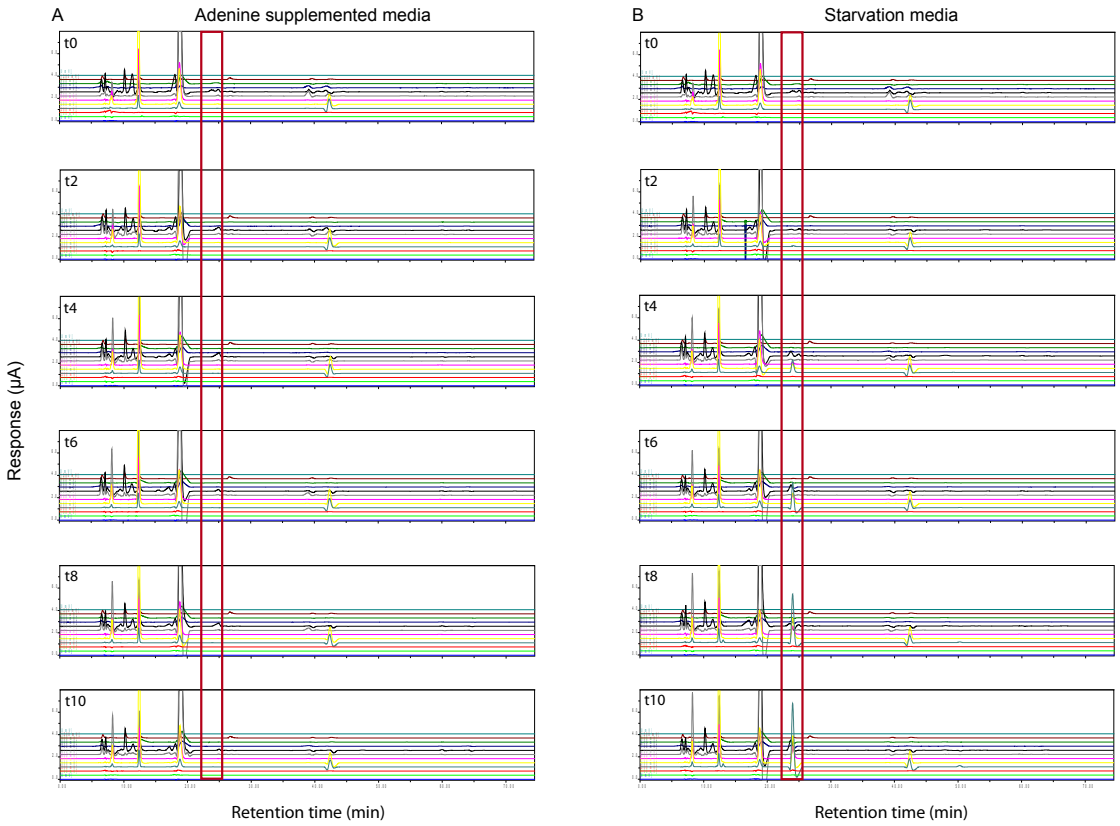


Figure 4.4: HPLC-EC traces of metabolites from crATIC cells. Cells were cultured in media supplemented FCM with (A) or without (B) adenine and metabolites extracted in two-hour increments. The red rectangle indicates elution time of ZMP with primary peak in channel 4 (300mV). Aligned and cropped two-hour chromatogram, purine supplemented (plus), purine deficient (minus), and purine deficient with ZMP spike. Dashed line indicates primary channel peak height in deficient and deficient with spike sample (C).

crATIC shows differentially expressed genes compared to HeLa in adenine- depleted and adenine-supplemented conditions

The crATIC and HeLa cell transcriptomes were compared after culture in adenine supplemented (PP) and adenine-depleted (MM) conditions. In both comparison groups, DEGs were limited by cutoffs (see Materials and Methods). In the PP comparison, we obtained 1311 DEGs while in the MM comparison, we obtained 1662 DEGs. This suggests that adenine supplementation corrects for many of the differences in gene transcription in the two cell types. The DEGs with the top 100 (positive) and bottom 100 (negative) log2 values were selected for further analysis. In the PP comparison, these represent log(2)fold ranges of -8.60573 to -2.31272 and 2.26702 to 8.20331. In the MM comparison, the DEGs with the top 100 positive and bottom 100 negative log2 values represent log(2)fold ranges of 8.39229 to 2.31793 and -8.87985 to -2.41031. These 200 gene lists were used for ClueGO analysis (Figure 4.5).

Table 4.1: Shared and unique DEGs between comparison groups. Total gene counts that satisfied previously defined cutoffs in the total gene list in the top 200 genes that were used for ClueGO analysis parsed into the PP and MM comparison or shared between the two comparison groups.

	Total DEGs	Top 200 DEGs
Shared	1083	156
Unique PP	228	44
Unique MM	579	44

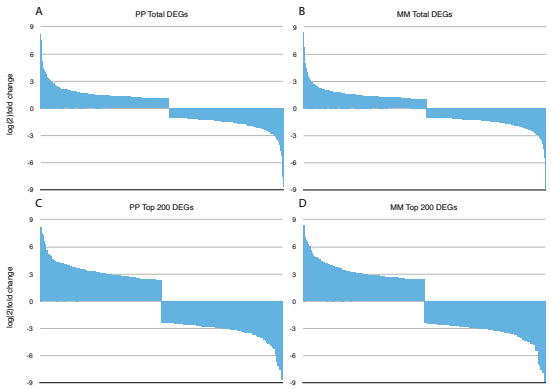


Figure 4.5: log(2) fold change of DEGs in cell lines under experimental conditions. A and B: DEGs in PP (A) and MM (B) conditions. C and D: 100 most positively and 100 most negatively changed DEGs in PP (C) and MM (D) conditions.

Comparing the MM and PP groups, 228 genes were unique to the PP group while 579 genes were unique to the MM group. 1083 genes were common to both groups. When comparing DEGs with the

100 most positive and 100 most negative log2 values (200 total), 44 genes were found unique to each group, and 156 genes were shared between the two groups (Table 4.1). Principle component analysis showed tight clustering by cell type and media supplementation (Figure 4.6).

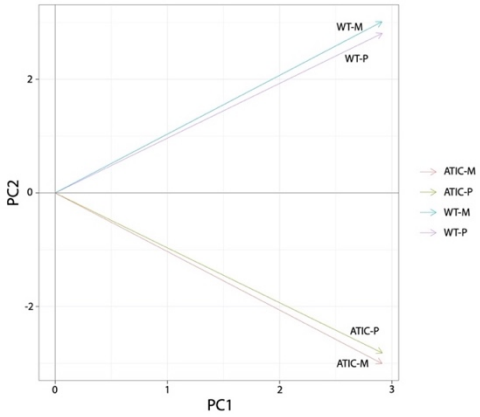


Figure 4.6: Principle component analysis of crATIC and HeLa experimental groups. PCA shows robust differences by cell type. Figure generated by Dr. Guido Vacano.

DEGs showed enrichment in ClueGO Gene Ontology and Reactome database analysis

RNA-seq experiments can provide important data bearing on global cellular changes in response to stressors and changes in environment.

The lists of 200 DEGs described above were used as input for ClueGO, a Cytoscape application that extracts representative functional biological information for large lists of genes or proteins (Mlecnik, Galon, and Bindea 2018; Shannon et al. 2003). We queried Gene Ontology (GO, biological process, cellular component, and molecular function) (Ashburner et al. 2000) and the Reactome Knowledgebase (pathways and reactions) (Fabregat et al. 2018) to identify significant terms from comparison of the crADSL and HeLa transcriptomes. Although we identified many GO and Reactome terms, we focused on a subset of specific terms implicated in Alzheimer's disease and

cellular aging. All ontology network maps, term, and grouping visualization figures are located after the text of this chapter. Prominent findings are highlighted (Figure 4.7).

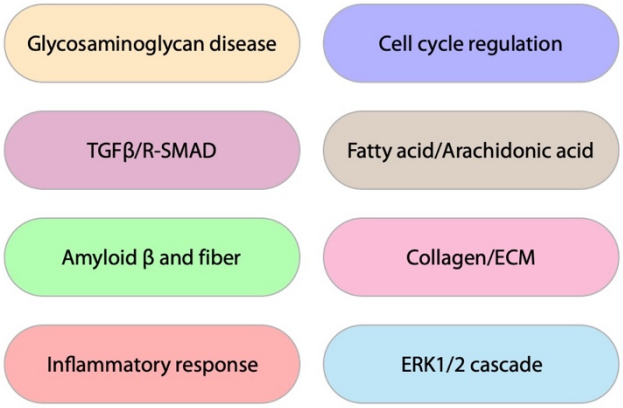


Figure 4.7: Notable GO groups from ClueGO analysis.

For Biological Process GO ontologies, we obtained 55 groups: 34 associated with shared terms, 33 associated with the PP comparison, and 23 associated with the MM comparison. Notable shared terms included inflammation / immune response terms such as IL-1β secretion, t-cell activation involved in immune response, eicosanoid metabolic process, prostanoid metabolic process, prostaglandin metabolic process, negative regulation of TNF product, response to amyloid beta, glutamine family biosynthetic process, and long chain fatty acid biosynthetic process and transport. Regulation of pathway-restricted SMAD protein phosphorylation and transforming growth factor beta terms were also in the list. For the PP comparison group there was enrichment of fatty acid terms, including arachidonic acid metabolic process, and the cyclooxygenase pathway. Other terms included neuron cellular homeostasis and neuron migration, midbrain development, and neurotransmitter metabolic process. Regulation of cellular response to TGFβ stimulus, response to hyperoxia, negative regulation of embryonic

development, and negative regulation of G1/S transition of mitotic cell cycle were also enriched terms. For the MM comparison, neuroinflammatory response, regulation of epithelial to mesenchymal transition, negative regulation of erk1/2 cascade, cellular response to fatty acid, and cellular response to prostaglandin stimulus were obtained in the analysis (Figures 4.9, 4.10).

For Cellular Component ontologies, we obtained 10 groups. Five groups were from shared comparisons, 2 from PP and 5 from MM. Shared terms included golgi lumen, melanosome, and complex of collagen trimers. Terms enriched in the PP comparison included dystrophin-associated glycoprotein complex and brush border. Terms associated with the MM comparison included extracellular matrix component, cell division site, and actomyosin (Figures 4.11, 4.12).

In the Molecular Function ontologies, 14 groups of terms were identified with 5 groups from shared, 6 groups from the PP comparison, and 3 from the MM comparison. Terms found within the shared groupings were collagen binding, proteoglycan binding, neuropeptide receptor binding, and amino acid binding. In the PP comparison, terms of note include steroid hormone receptor activity, adenylyl transferase activity, cell-cell adhesion mediator activity, and lysophospholipase activity. In the MM comparison, terms such as Aβ binding and l-amino acid transmembrane transporter activity were found (Figures 4.13, 4.14).

In the results from querying the Reactome Knowledgebase, we obtained 10 groups in Pathways, 5 shared, 6 from the PP comparison, and 2 from the MM comparison. Within the shared groupings, we obtained terms such as signaling by retinoic acid, amyloid fiber formation, signaling by NOTCH1, arachidonic acid metabolism, and

collagen formation. In the PP comparison we obtained nuclear receptor transcription pathway, TP53 regulates transcription of cell cycle genes, transcriptional regulation of the AP-2 (TFAP2) family of transcription factors, IL-4 and IL-13 signaling, and synthesis of prostaglandins (PG) and thromboxanes (TX). In the MM comparison we obtained IL-10 signaling, ECM proteoglycans, and diseases associated with glycosaminoglycan metabolism (Figures 4.15, 4.16).

In Reactome Reactions, we obtained 7 groups of terms with 4 groups shared, 3 groups from the PP comparison, and no groups from the MM comparison. Shared groups included expression of IFNγ-stimulated genes, exocytosis of specific granule membrane proteins, and terms associated with collagen. In the PP comparison we obtained formation of NR-MED1 coactivator complex, keratin filaments bind cell-cell adhesion complexes, and binding of AP1 transcriptional activator complexes to CCND1 promoter (Figures 4.17, 4.18).

Corroboration of ClueGO results via BiNGO analysis

To validate our ClueGO results, we performed enrichment analysis using the BiNGO application in Cytoscape (Maere, Heymans, and Kuiper 2005) and the complete significant DEG lists. While we obtained some new terms, on the whole this analysis produced term enrichment closely similar to that from our ClueGO analysis (Figures 4.19-4.24).

Validation of RNA-seq by qPCR of candidate genes

To assess the validity of our RNA-seq results, we employed qPCR to confirm transcript levels for select genes. The results for these genes showed similar transcript

levels and trends based on Ct value comparison with the log(2)fold values from our RNA-seq results (Figure 4.8).

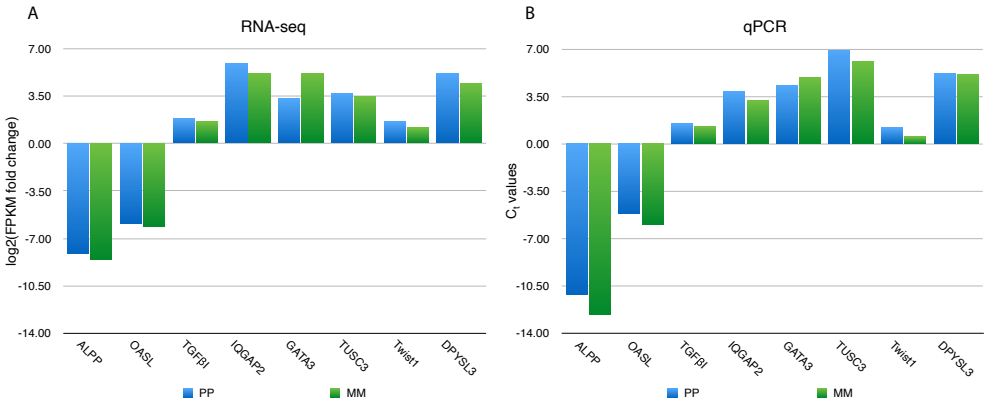


Figure 4.8: Candidate gene verification by qPCR. Gene transcription in PP and MM conditions measured by log(2)fold change of FPKM values from RNA-seq (A) and Ct values from qPCR (B). Values were normalized to β-Actin.

Discussion

In the present study, we evaluated dietary requirements for crATIC and metabolite accumulation during purine starvation.

In addition to its role in rescuing the DNPS KO phenotype, adenine supplementation shuts down DNPS (Holmes et al. 1973; Tu and Patterson 1978) and prevents ZMP accumulation in crATIC. Adenine phosphoribosyltransferase (APRT) mediates conversion of adenine and phosphoribosyl pyrophosphate (PRPP) to AMP and pyrophosphate (PPi) and is present in all mammalian tissues. APRT is uniquely responsible for metabolic adenine salvage from dietary sources (Silva et al. 2008). Adenine supplementation also provides a source for synthesis of GMP. AMP can be converted to IMP by AMP deaminase, and then converted to GMP by IMP

dehydrogenase [IMPDH, converts IMP into xanthine monophosphate (XMP)] and GMP synthase (converts XMP into GMP) (Watts 1974).

We employed RNA-seq to compare the crATIC and HeLa transcriptomes in adenine supplemented and adenine-depleted conditions, and we performed qPCR to verify these results. We established a 10-hour starvation (cell culture in adenine-depleted media). At the end of this time course, ZMP levels were still increasing in crATIC cells, and we have not yet established a time point at which ZMP levels plateau. It is possible ZMP accumulation during this period may not have been adequate for full AMPK activation. As discussed above, ZMP is an AMP mimetic and likely has multiple enzymatic targets. The crATIC HeLa system can be employed to identify these targets.

Our results demonstrate that crATIC requires purine (adenine) supplementation for proliferative growth, and that ZMP accumulates linearly over a time course of ten hours during purine starvation. After analysis of the RNA-seq data, we obtained many DEGs both by cell type and adenine supplementation.

Although DEGs mapped to numerous gene ontologies, we focused our discussion on terms of specific interest. AMPK sensitivity is decreased in aged models (Reznick et al. 2007), however in Alzheimer's disease (AD), an aging related disorder, pAMPK is abnormally elevated in tangle and pre-tangle bearing neurons (Vingtdeux et al. 2011). Data also suggest that insulin resistance seen in AD reduces the astrocytic energy supply via inhibition of AMPK, contributing significantly to the neurodegeneration observed in AD (Erol 2008). AMPK has a major role in Tau protein phosphorylation, as well as mTOR and autophagy pathways, which are dysfunctional in AD (Cai et al. 2012). Energy homeostasis in Alzheimer's disease is also dysregulated possibly via an AMPK related

mechanism (Caberlotto et al. 2013), illustrating the need to understand energetic processes in dementia. Our results suggest a role for ZMP or ATIC in AD and possibly other amyloid or tau-based dementias. AD is a progressive, age related, dementia characterized by amyloid-β plaques, tau fibril formation, and extensive neuronal degradation in the brain. One feature of AD is chronic neural inflammation (Kinney et al. 2018). Typically, inflammation is caused by a cellular response to stimuli and is normally terminated by a process called resolution. Arachidonic acid (AA) may play an important role in the inflammatory response in AD (Olivier 2016), especially in initiation of resolution (Wang et al. 2015; Whittington, Planel, and Terrando 2017; Serhan et al. 2015). Chronic inflammation in AD is marked by a dysregulation of resolution in which the inflammatory response persists (Wang et al. 2015) .

Normally, AA is a constituent of phospholipids. The circulating form is rare, as AA is rapidly scavenged and bound to albumin. Free AA can be cleaved from phospholipids by phospholipase (PLA) and is typically produced in response to injury or other stimuli. Free AA is metabolized rapidly through enzymatic pathways based on PLA protein- protein interactions with cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450 (CYP), fatty acid amide hydrolase (FAAH), or non-enzymatic lipid peroxidation and oxidative stress. FAAH is a concentration-dependent reversible reaction, producing anandamide (a neurotransmitter) from AA and ethanolamine, and AA release can be stimulated by anandamide (Scala et al. 2018; Ritter 2016). AA metabolic products have roles in processes such as platelet aggregation, vasoconstriction and dilation, toxic shock based organ dysfunction, inflammatory response, female fertility, fever mediation, Alzheimer's and Parkinson's disease neurodegeneration, mediation of

cAMP, suppression of excitatory neuronal signals via endocannabinoids, and smooth muscle response (Hanna and Hafez 2018).

Cytosolic phospholipase A2 (cPLA2) is expressed in higher levels in brain tissue from AD patients (Stephenson et al. 1996) and eicosanoids, a product of AA COX and LOX based metabolism, act as mediators of neuroinflammation. In AD, COX2 is overexpressed, and this overexpression has been correlated with AD progression (Ho et al. 2001). In addition, the COX1 splice variant, COX3, has been found in AD brain (J.-G. Cui et al. 2004). The prostaglandin metabolite, PGD2, is overproduced in glial cells surrounding amyloid plaques (Mohri et al. 2007). However, NSAIDs which act as COX2 inhibitors have so far been ineffective at mitigating the AD- associated inflammation (McGeer and McGeer 2007). Interestingly, LXA4, a metabolite produced via the LOX pathways from AA, has been implicated in reduction of reactive oxygen species (Y. Wu et al. 2012), inhibition of interleukin expression (Decker, McBean, and Godson 2009) and reduction of Aβ levels (Medeiros et al. 2013). LXA4 is also implicated in resolution of inflammation (Zhu et al. 2016). In cerebrospinal fluid and brain tissue from AD patients, LXA4 levels are lower than from control groups, while 15-LOX-2, the enzyme that catalyzes production of LXA4, was elevated in glial cells from AD patients (Wang et al. 2015). These results suggest that AA metabolism is differentially regulated in AD. Our results suggest that ZMP, ATIC, and DNPS may have important roles in AA metabolism and may be attractive targets for further investigation and development of new therapies.

Microglial cells respond to diverse cues from injured neurons by becoming activated and inducing phagocytosis to initiate clearance of apoptotic cells or extruded

proteins (e.g., Aβ). Activation is accompanied by release of cytokines such as IL-1β and TNFα (Kettenmann et al. 2011) and regulatory cytokines (Carniglia et al. 2017). There are inherent differences between aged, chronically activated microglia compared to young microglia (Koellhoffer, McCullough, and Ritzel 2017). In young microglia, cells are sensitive to TGFβ signaling which leads to a reduction in cytokine release (Paglinawan et al. 2003), neuroprotection (Lieb 2003), and promotion of phagocytosis (Wyss-Coray et al. 2001). However, in chronically activated microglia, there is decreased phagocytosis (Ritzel et al. 2015), and a reduced response to TGFβ signaling, resulting in increased neurotoxicity and reduced uptake of Aβ (Ramírez, Rey, and von Bernhardi 2008; von Bernhardi et al. 2007). This diminished response is largely attributed to reduced TGFβ-Smad coupling, the canonical pathway of TGFβ signaling, that occurs in AD (Colangelo et al. 2002) and aging (Tichauer et al. 2014). In non-canonical pathways, TGFβ activates MAPK, PI3K, and JNK: each are linked under certain situations to a pro-inflammatory response (Derynck and Zhang 2003). In canonical TGFβ signaling, activation of the TGFβ-Smad pathway induces glial cells to transcribe MAPK phosphatase (MKP), a serine-threonine phosphatase responsible for negative regulation of inflammation that results in decreased TNFα production and reduced Aβ– mediated activation of MAPK and NFκB signaling (Flores and von Bernhardi 2012). However, in cases of chronic microglial inflammation observed in aging and AD, the TGFβ-Smad pathway is inhibited, resulting in reduced amelioration of inflammation. Our identification of ATIC and DNPS involvement in TGFβ signaling coupled with FA based inflammatory regulation provides a novel avenue for continued AD research.

During embryogenesis and fetal development, purines have an important role as signaling molecules (Massé and Dale 2012), particularly in neuronal development (Zimmermann 2011; Fumagalli et al. 2017; Rodrigues, Marques, and Cunha 2019). DNPS has been found upregulated at the G1/S cell cycle transition (Chan et al. 2015; Zhao et al. 2015). So far, mutations that cause reduction in PAICS, ADSL, or ATIC activity in humans all lead to significant developmental delay. These findings indicate the importance of the pathway for producing purines for DNA and RNA synthesis in embryogenesis and development. Consistent with these roles, in our comparison of the crATIC and HeLa transcriptomes, we identified many DEGs that mapped to ontology terms concerned with development and neuronal function as well as ontology terms associated with rapid cellular proliferation, such as tumorigenesis and cancer. Multiple terms also mapped to cell cycle checkpoints and terms associated with the G1/S phase interface.

Previously, we characterized the crADSL cell line transcriptome (Mazzarino et al. 2019). Common ontology terms from the crADSL and crATIC transcriptome analyses are likely related to general DNPS function rather than the specific mutated DNPS enzyme or accumulated intermediates. Common terms include transforming growth factor beta, collagen/ECM, glycosaminoglycan metabolism, interferons, and embryonic development. Terms specific to the crATIC analysis include G1/S checkpoint transition, dystrophin associated glycoprotein complex, retinoic acid response, arachidonic acid metabolism, and prostaglandin and thromboxane. Of note, phospholipase activity was common to both crADSL and crATIC while lysophospholipase was found only in the crATIC analysis.

As discussed above, the crATIC cell line is advantageous in that ZMP accumulation is achieved via cellular metabolism, not conversion of exogenous AICAr in the media. The crATIC cells and crATIC cells transfected with identified patient allele and other mutant forms of ATIC should provide important cellular models of DNPS, AICA-ribosiduria, and ZMP accumulation. The results reported here are an important first step in establishing this model, and for further investigation of ZMP's role as an AMP mimetic.

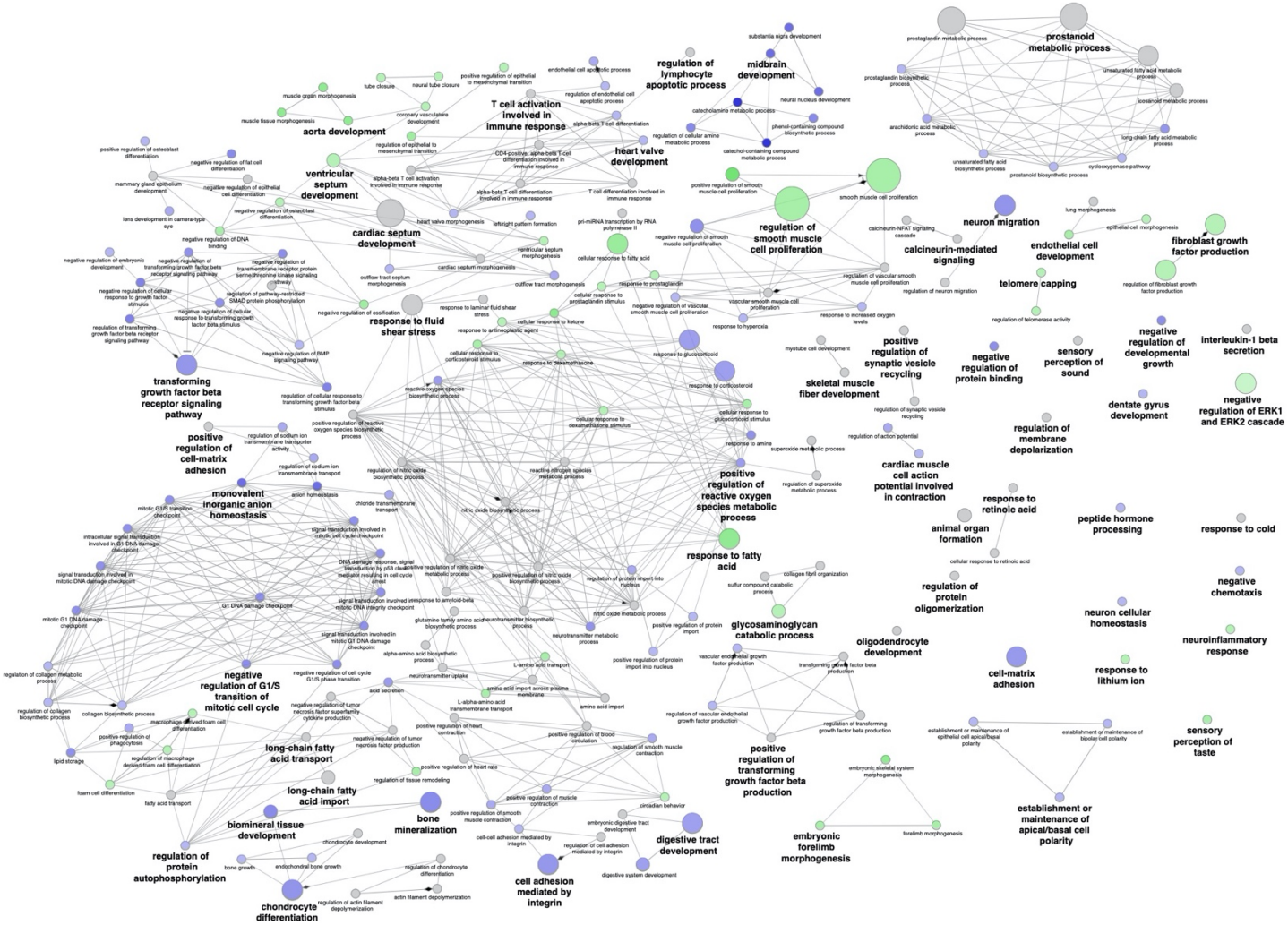


Figure 4.9: ClueGO Biological process network map. Grey nodes indicated shared terms, blue nodes indicate terms enriched in PP comparison, and green nodes indicate terms enriched in MM comparison.

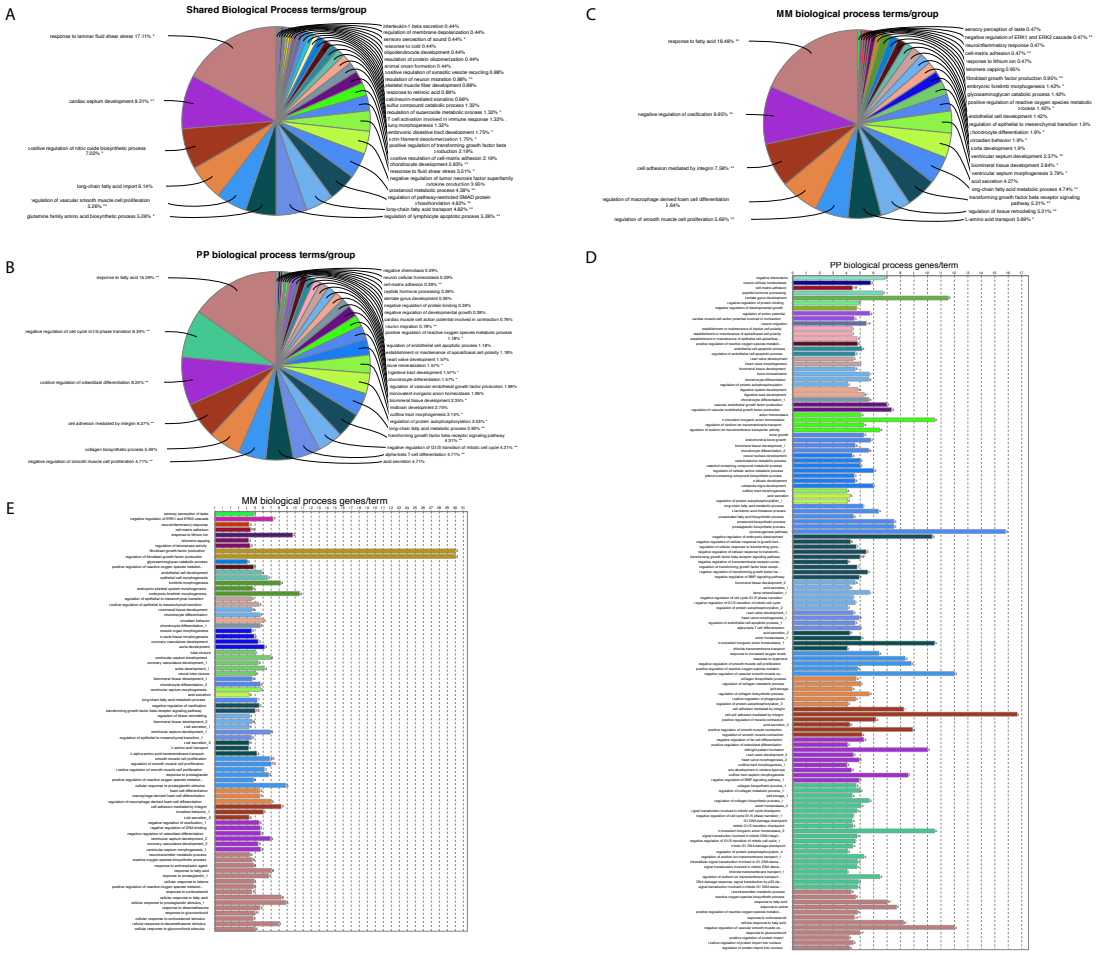


Figure 4.10: ClueGO Biological process terms and groups. Pie charts show the proportion of the terms associated with the groups for Shared (A), PP (B), and MM (C) comparison groups. Histograms indicated number of genes found per term for PP (D), and MM (E) comparison groups.

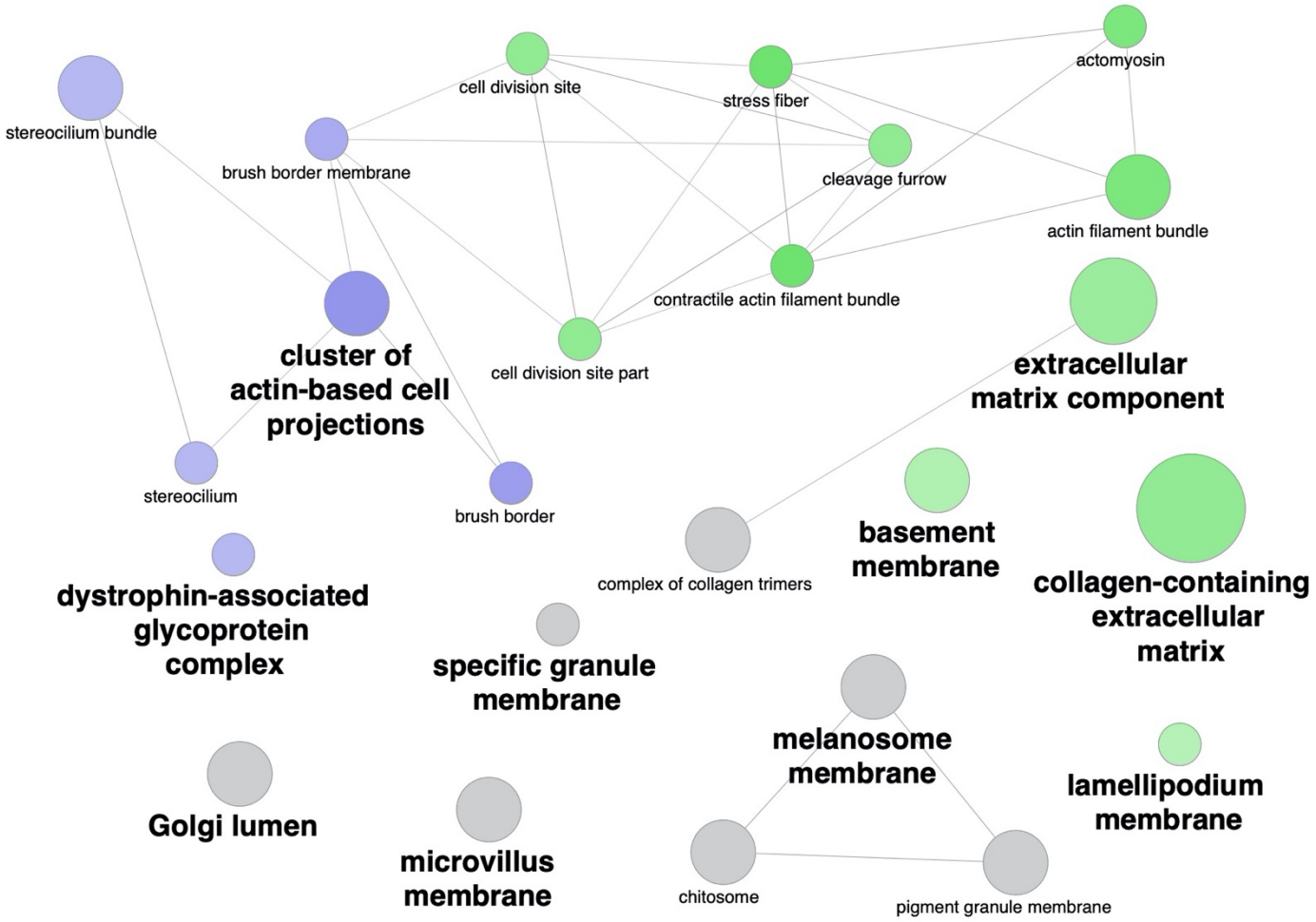


Figure 4.11: ClueGO Cellular component network map. Grey nodes indicated shared terms, blue nodes indicate terms enriched in PP comparison, and green nodes indicate terms enriched in MM comparison.

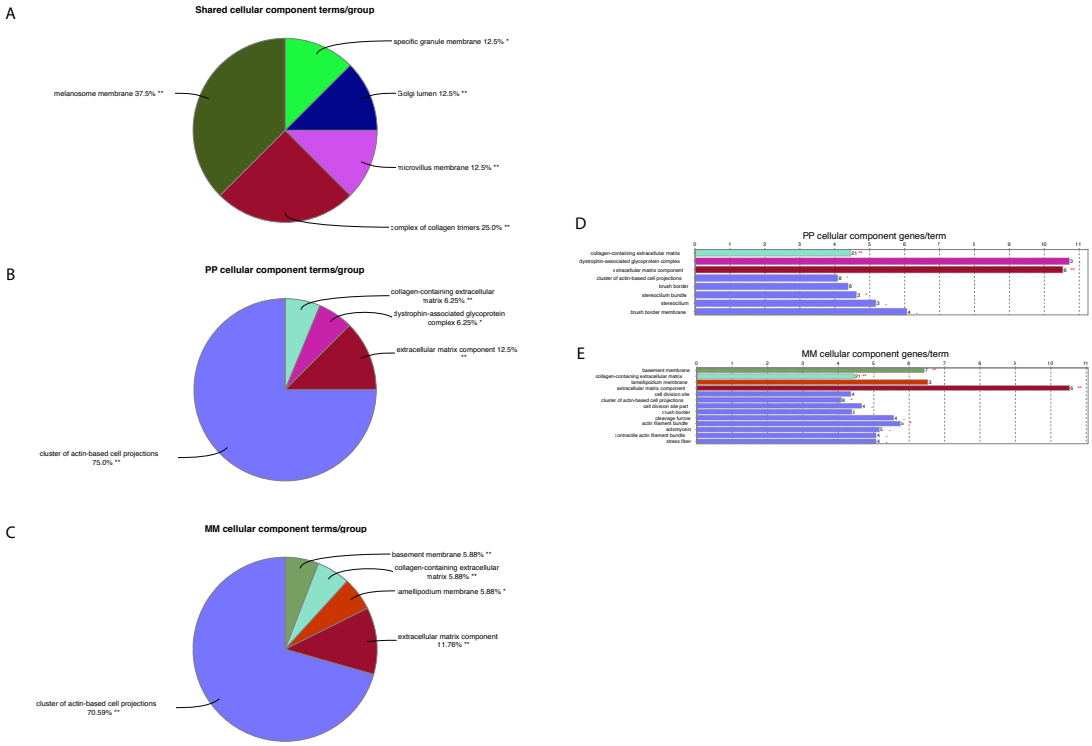


Figure 4.12: ClueGO Cellular component terms and groups. Pie charts show the proportion of the terms associated with the groups for Shared (A), PP (B), and MM (C) comparison groups. Histograms indicated number of genes found per term for PP (D), and MM (E) comparison groups.

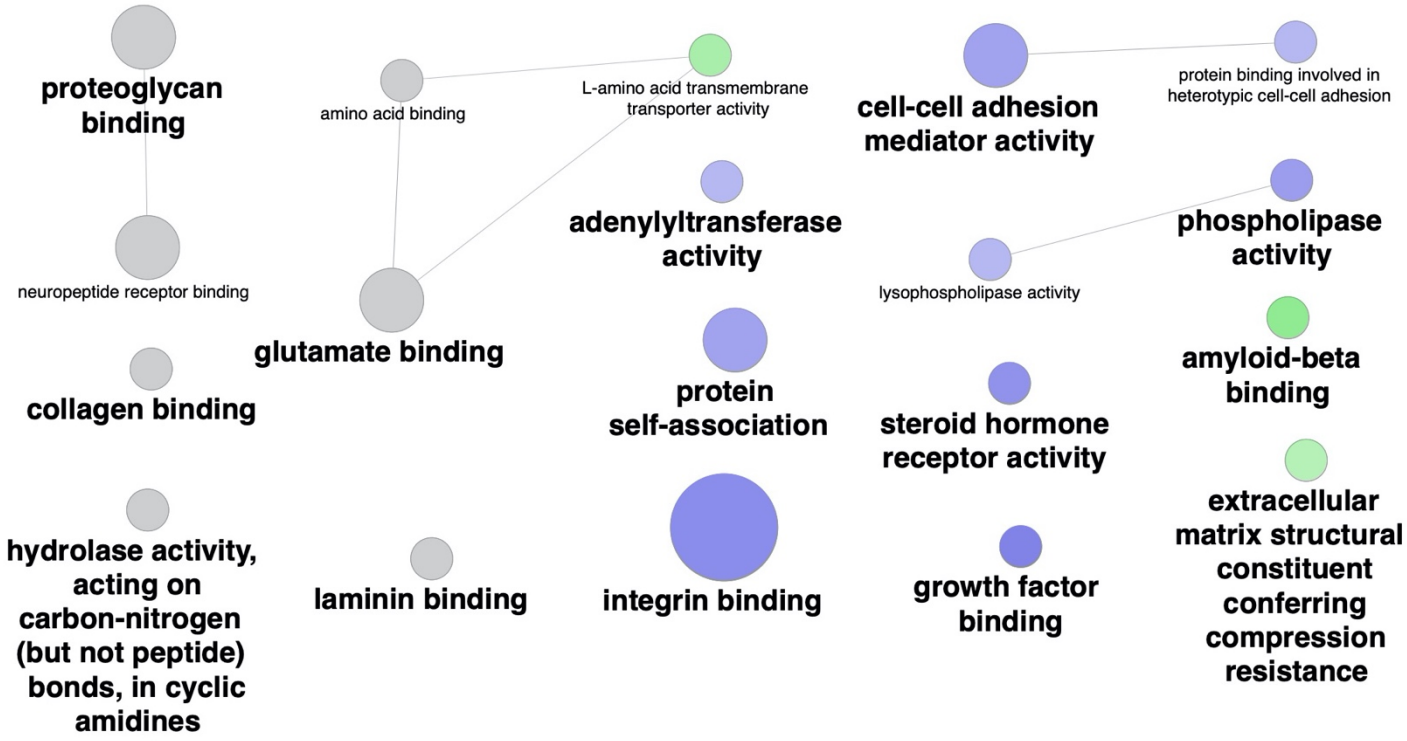


Figure 4.13: ClueGO Molecular function network map. Grey nodes indicated shared terms, blue nodes indicate terms enriched in PP comparison, and green nodes indicate terms enriched in MM comparison.

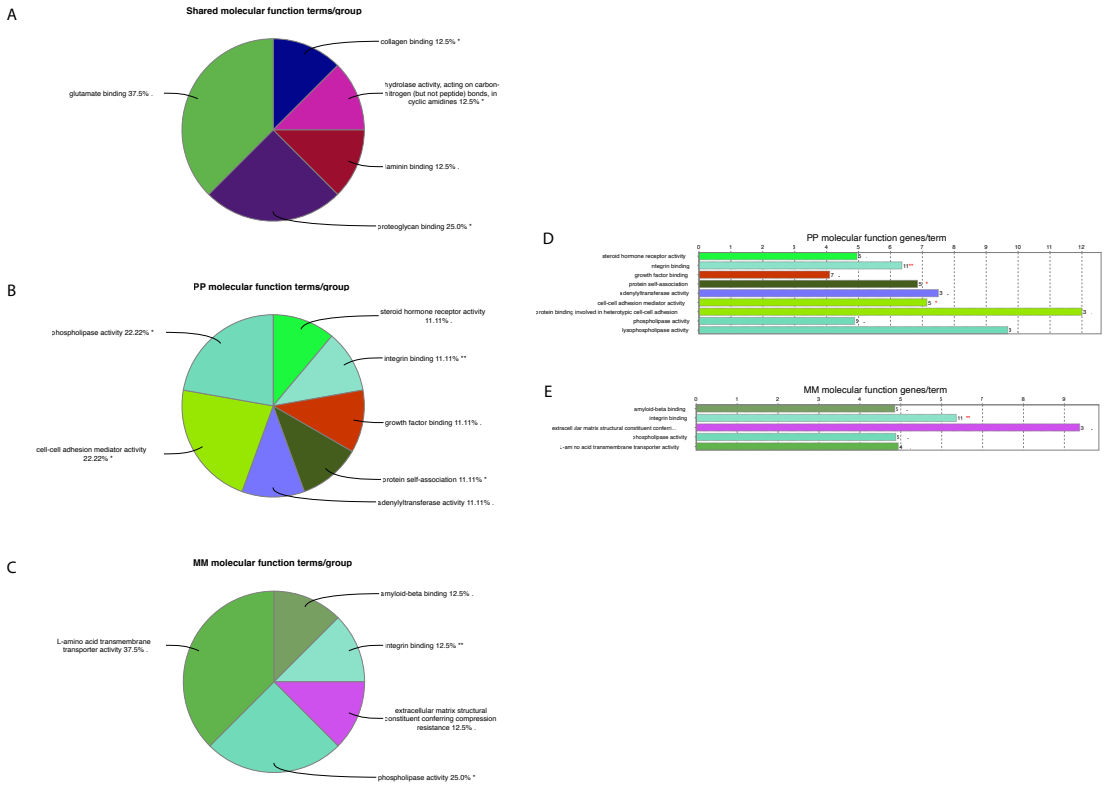


Figure 4.14: ClueGO Molecular function terms and groups. Pie charts show the proportion of the terms associated with the groups for Shared (A), PP (B), and MM (C) comparison groups. Histograms indicated number of genes found per term for PP (D), and MM (E) comparison groups.

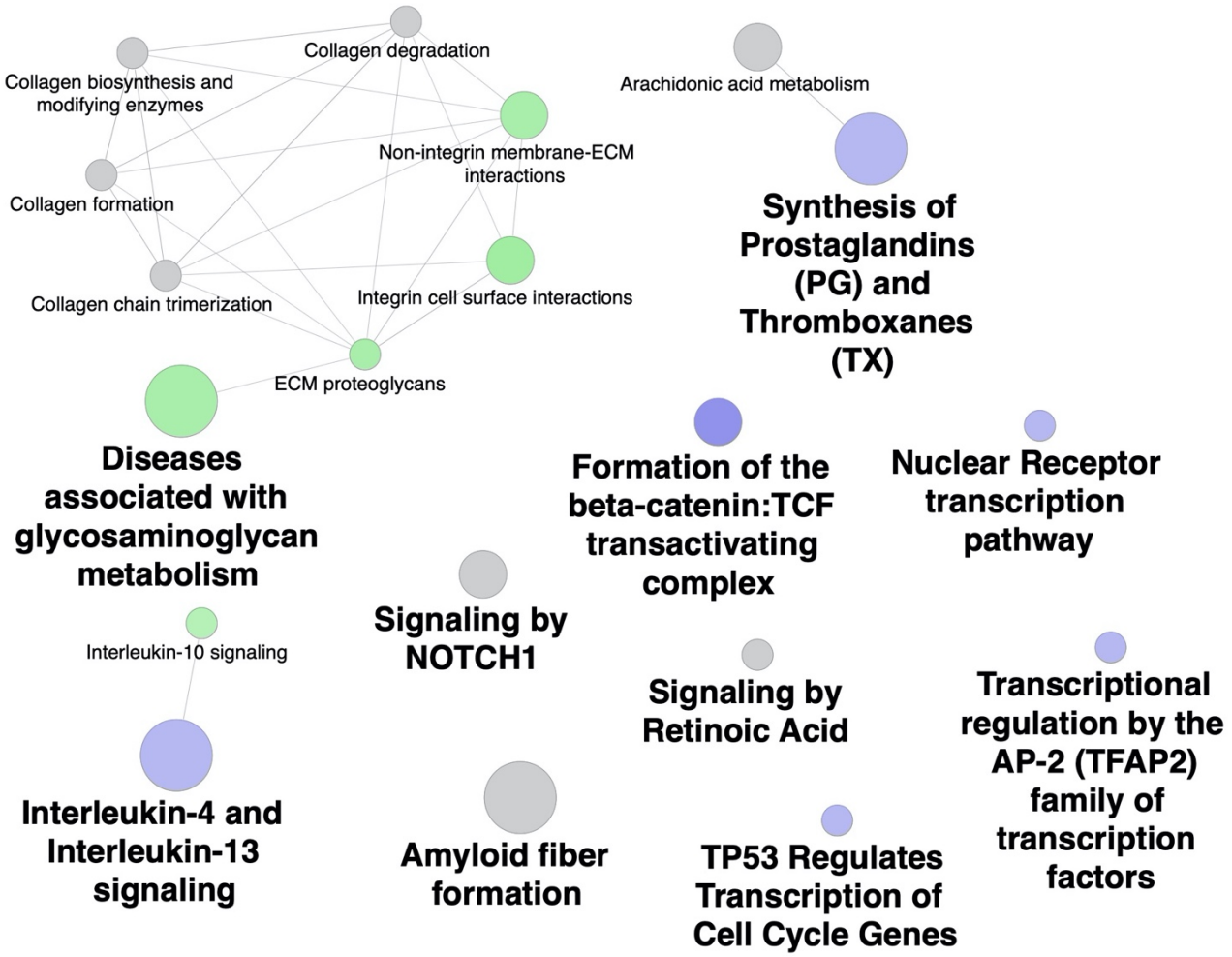


Figure 4.15: ClueGO Reactome pathways network map. Grey nodes indicated shared terms, blue nodes indicate terms enriched in PP comparison, and green nodes indicate terms enriched in MM comparison.

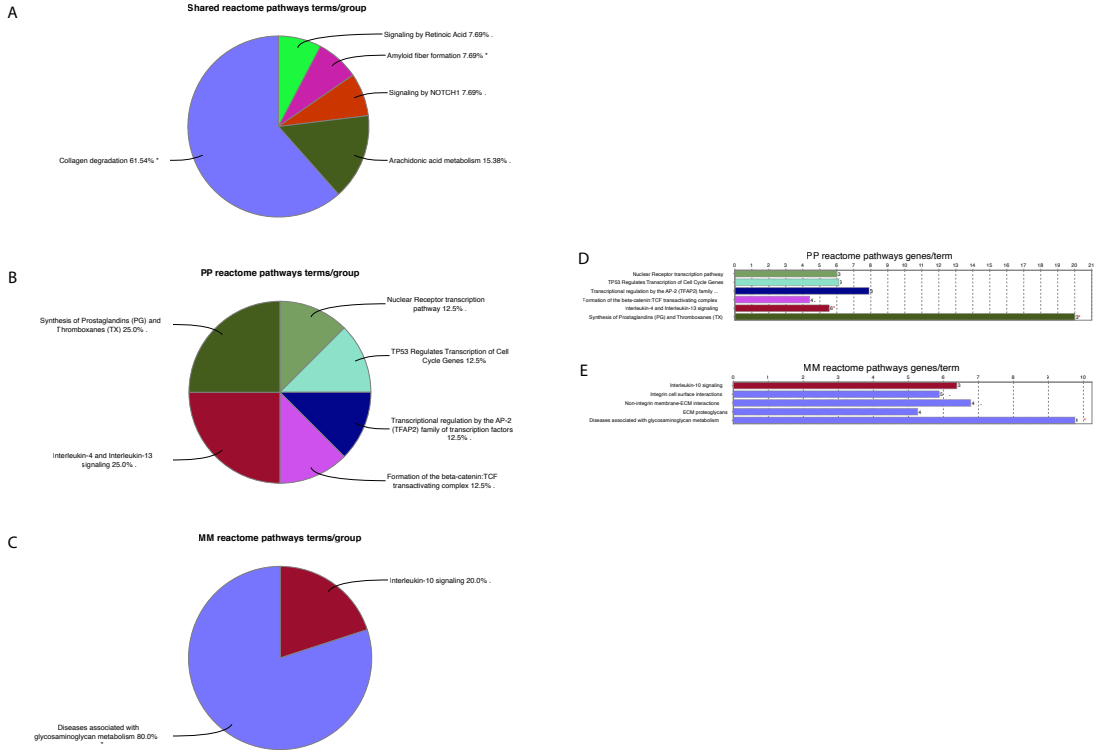


Figure 4.16: ClueGO Reactome pathways terms and groups. Pie charts show the proportion of the terms associated with the groups for Shared (A), PP (B), and MM (C) comparison groups. Histograms indicated number of genes found per term for PP (D), and MM (E) comparison groups.

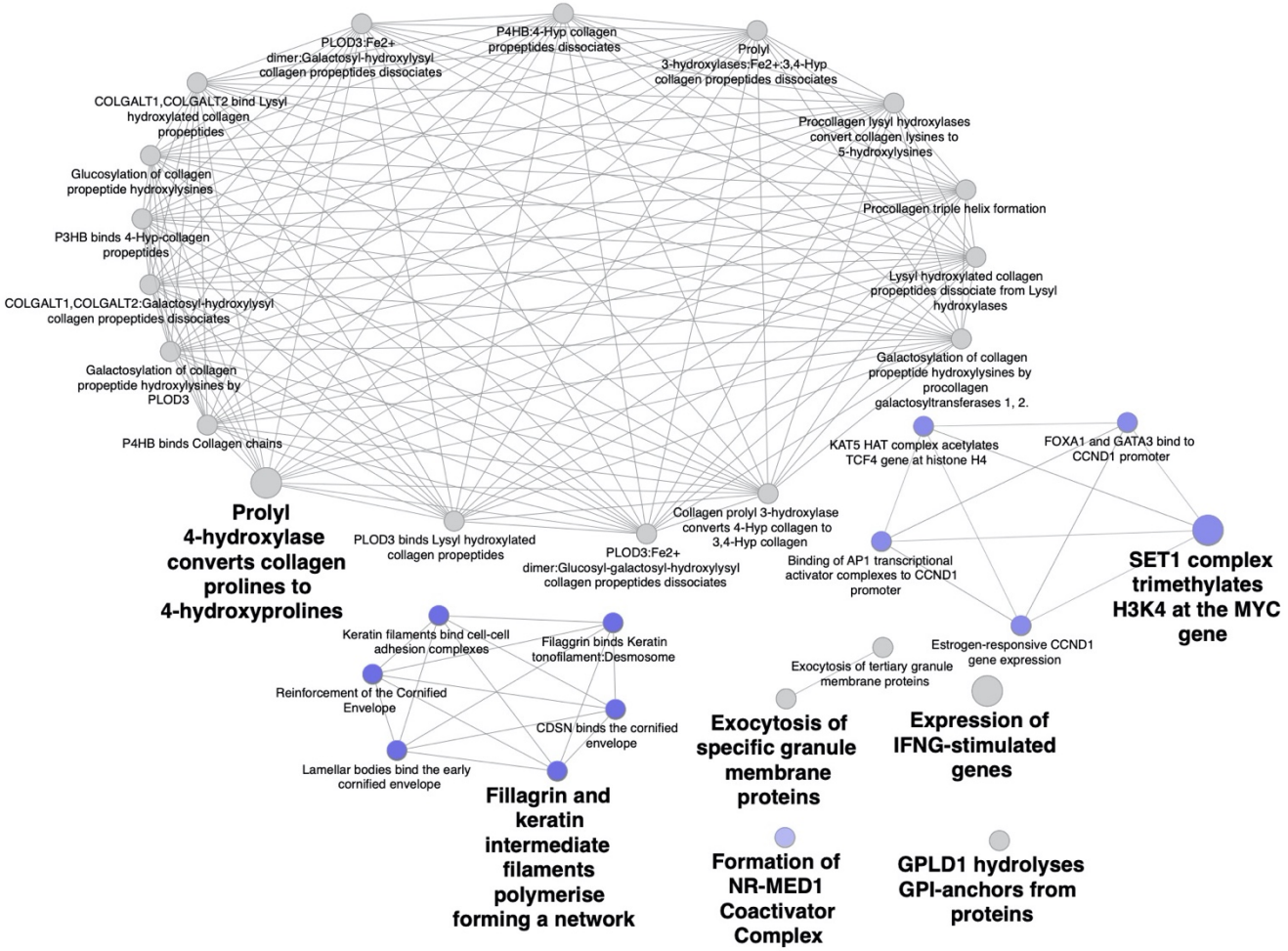


Figure 4.17: ClueGO Reactome reactions network map. Grey nodes indicated shared terms, blue nodes indicate terms enriched in PP comparison, and green nodes indicate terms enriched in MM comparison.

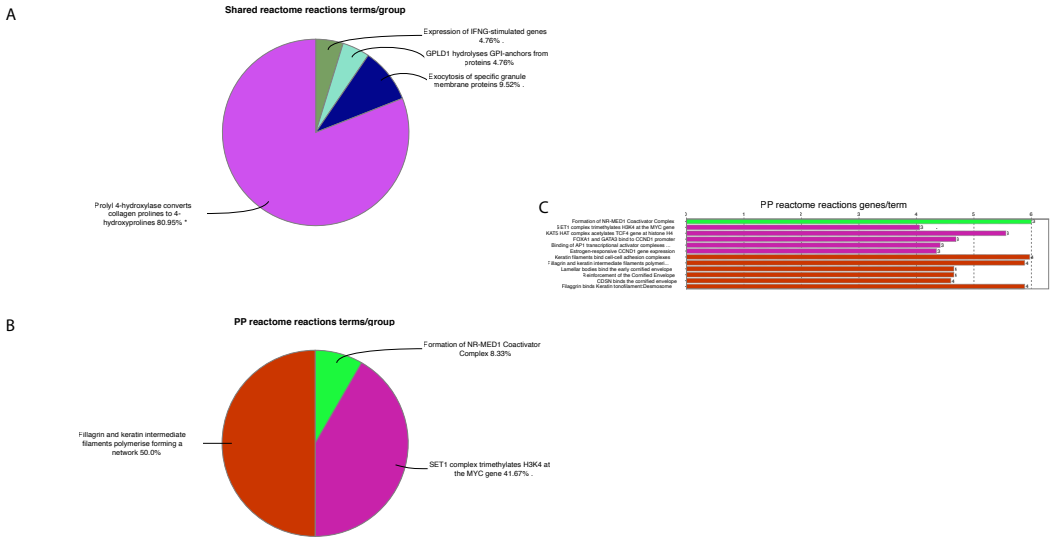


Figure 4.18: ClueGO Reactome reactions terms and groups. Pie charts show the proportion of the terms associated with the groups for Shared (A) and PP (B) comparison groups. Histogram indicated number of genes found per term unique to PP (C). No terms were found selectively enriched for the MM comparison group

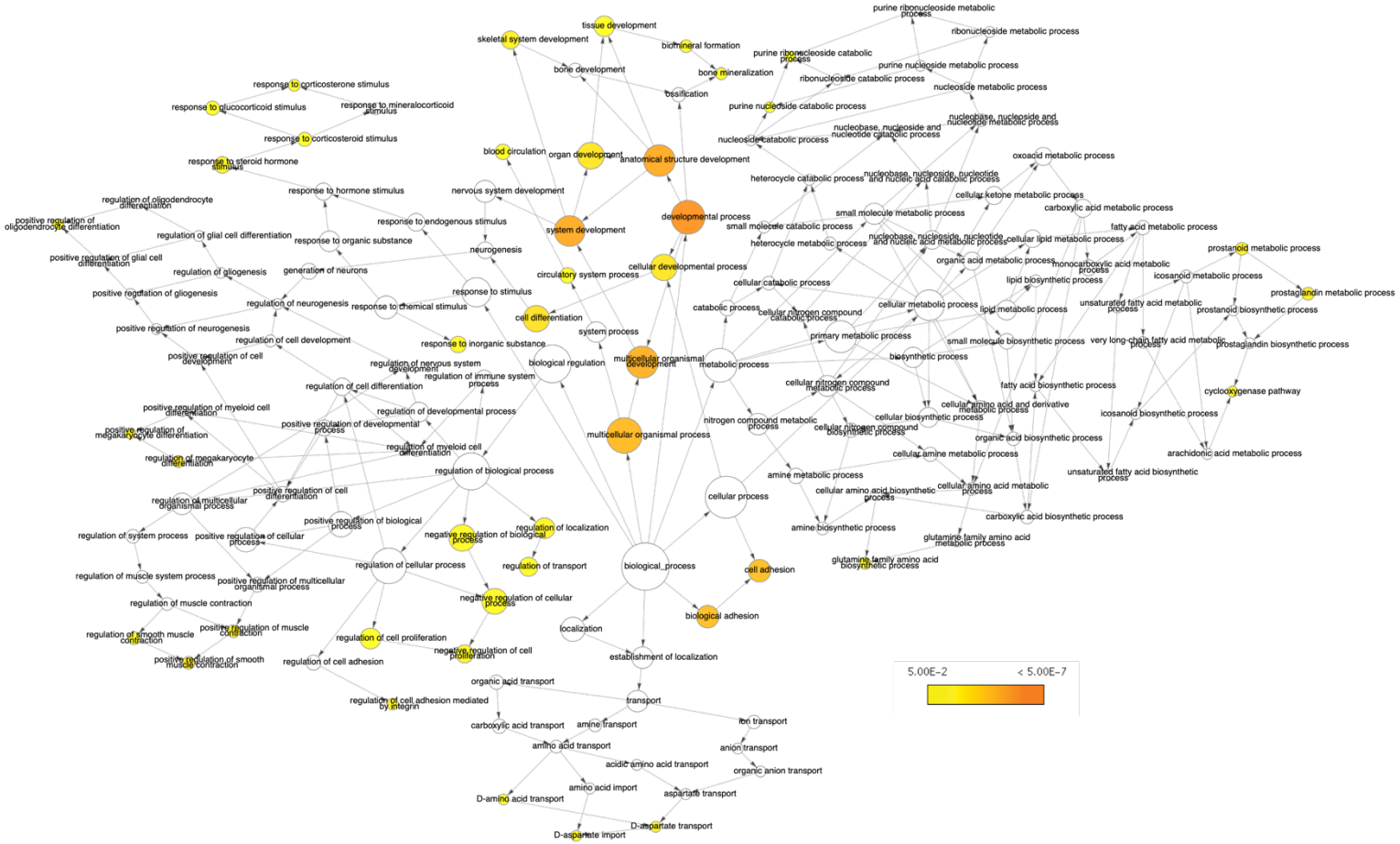


Figure 4.19: BiNGO network map of Biological process for PP comparison. Node color indicates the term P value. White nodes are not significant by P value but are included by hierarchy to link significant terms.

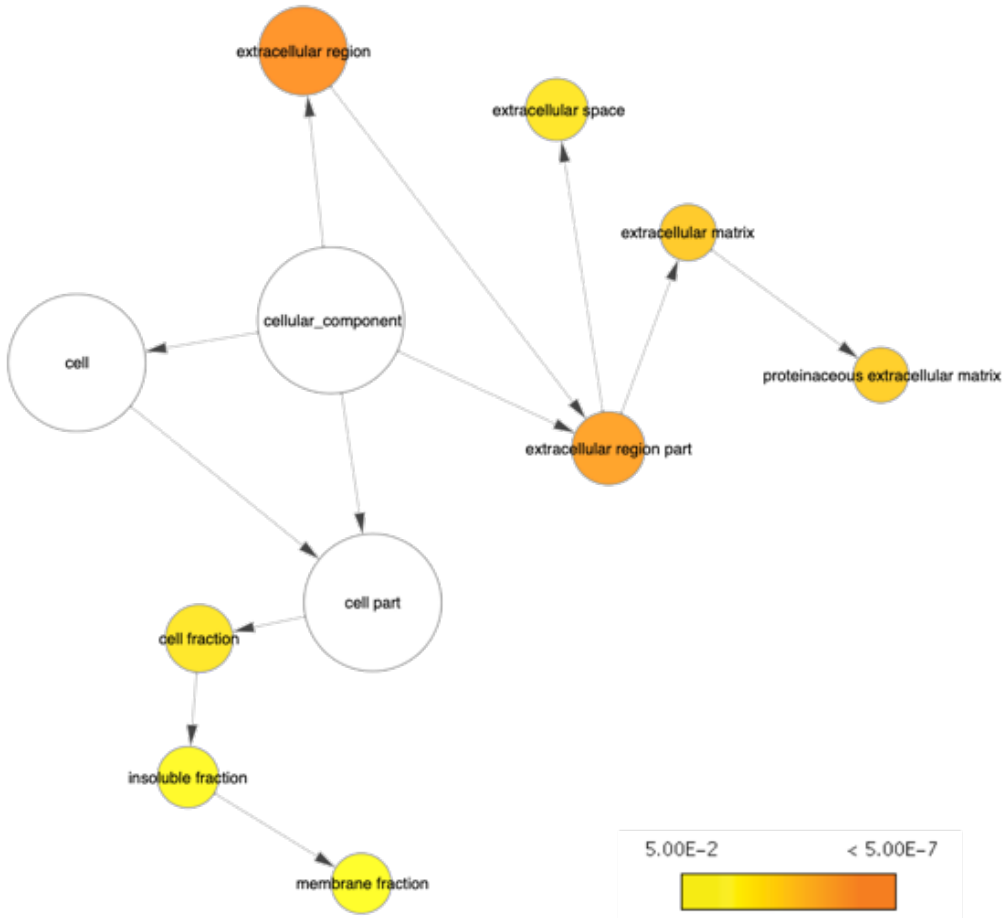


Figure 4.20: BiNGO network map of Cellular component for PP comparison. Node color indicates the term P value. White nodes are not significant by P value but are included by hierarchy to link significant terms.

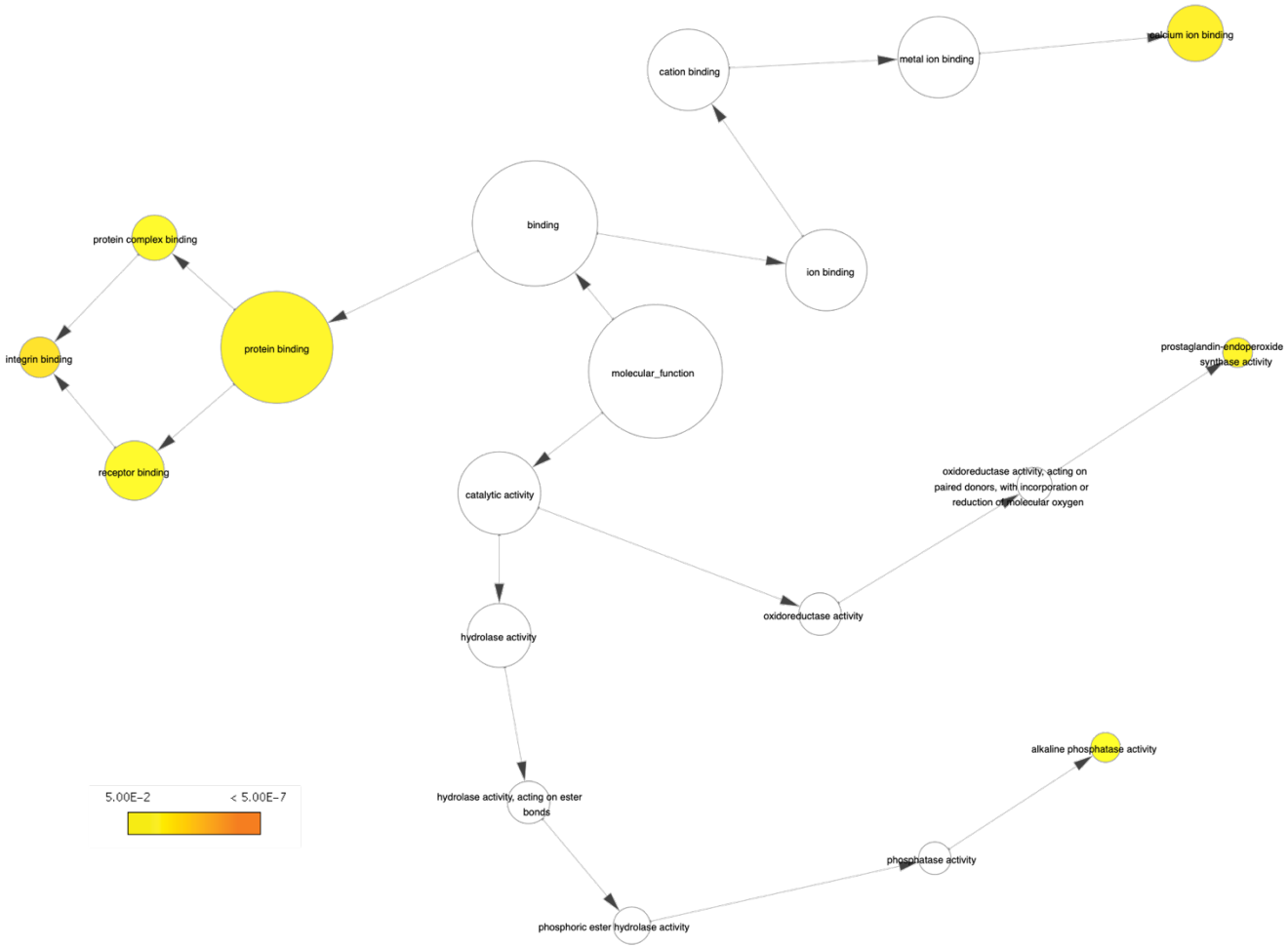


Figure 4.21: BiNGO network map of Molecular function for PP comparison. Node color indicates the term P value. White nodes are not significant by P value but are included by hierarchy to link significant terms.

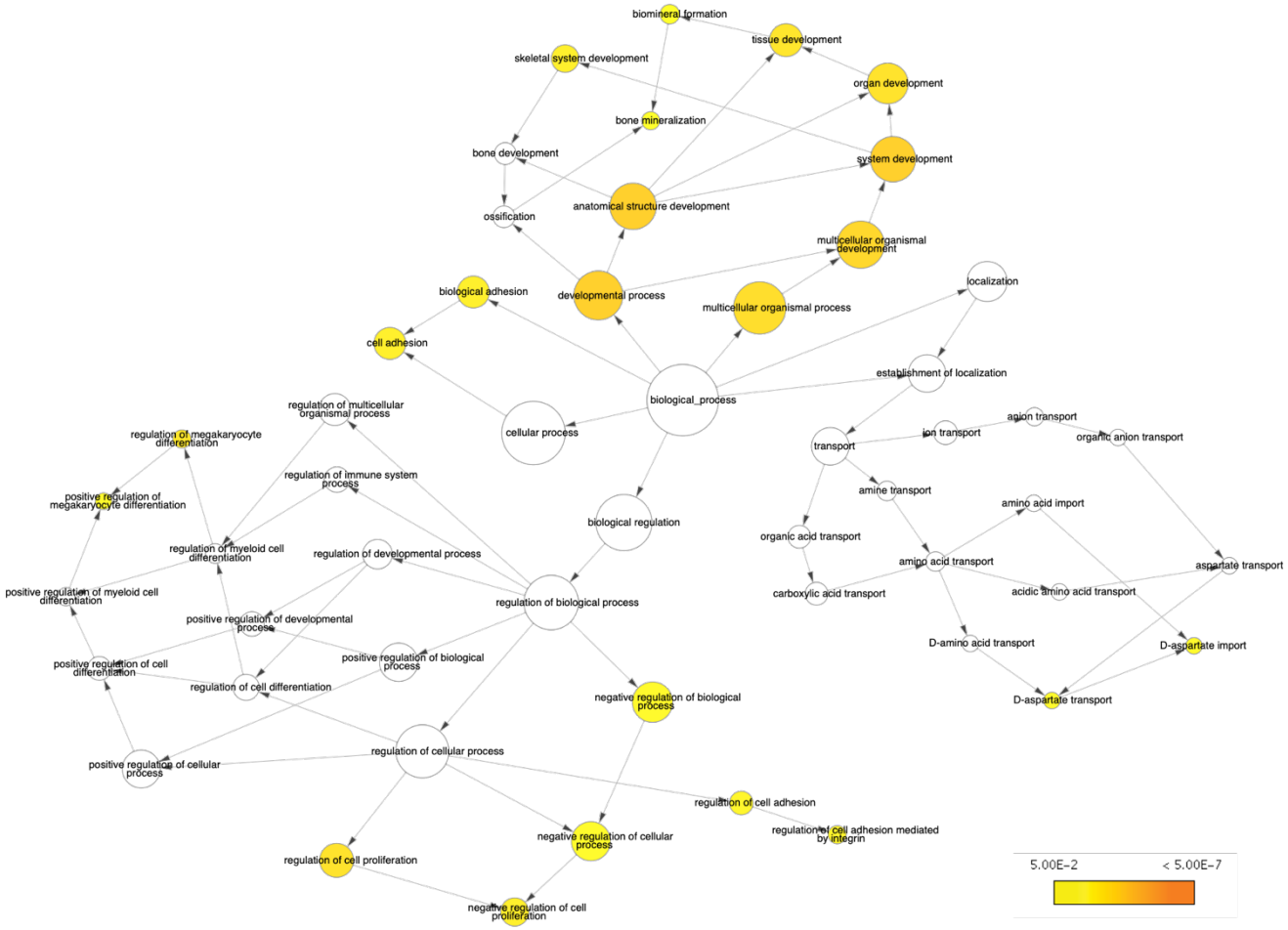


Figure 4.22: BiNGO network map of Biological process for MM comparison. Node color indicates the term P value. White nodes are not significant by P value but are included by hierarchy to link significant terms.

Figure 4.23: BiNGO network map of Cellular component for MM comparison. Node color indicates the term P value. White nodes are not significant by P value but are included by hierarchy to link significant terms.

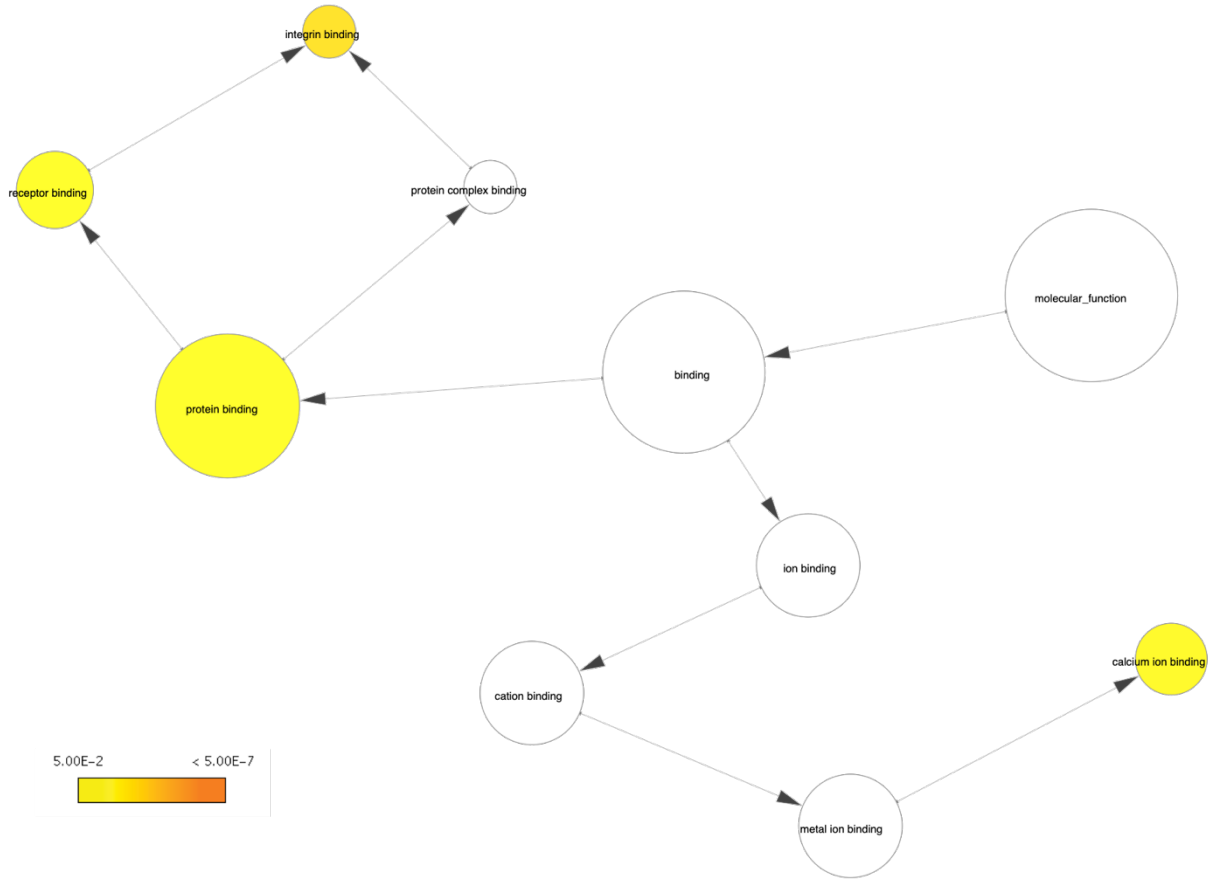


Figure 4.24: BiNGO network map of Molecular function for MM comparison. Node color indicates the term P value. White nodes are not significant by P value but are included by hierarchy to link significant terms.

Chapter Five: Metabolomic Characterization of crGART

Introduction

Purines are essential small molecules for cellular and organismal survival and function. Purines function as genomic bases in RNA and DNA, intra and intercellular signaling molecules, energy currency, substrates, and co-enzymes. *De novo* purine synthesis (DNPS) is among the first evolved and most fundamental biochemical pathways (Caetano-Anollés et al. 2009). In mammals, inosine monophosphate (IMP) is produced via a six-enzyme, ten-reaction pathway starting with phosphoribosyl pyrophosphate (PRPP) (Figure 5.1). The first step in the pathway, conversion of PRPP to 5-PRA catalyzed by PRAT, is the first irreversible step in DNPS. In humans, the enzyme GART is trifunctional, catalyzing steps 2, 3, and 5 in DNPS and is encoded by the GART gene, located on Hsa21. 5-PRA is the first substrate for GART and is rapidly broken down to ribose-5-phosphate (Rudolph and Stubbe 1995). Adenine and PRPP are converted to AMP and PPi by adenine phosphoribosyl transferase (APRT), and AMP can then be converted to IMP + NH_4 via AMP-deaminase (AMPD) (Jinnah, Sabina, and Van Den Berghe 2013). IMP can subsequently be used to produce GMP, hence, adenine supplementation successfully rescues deficits in DNPS (Figure 5.1).

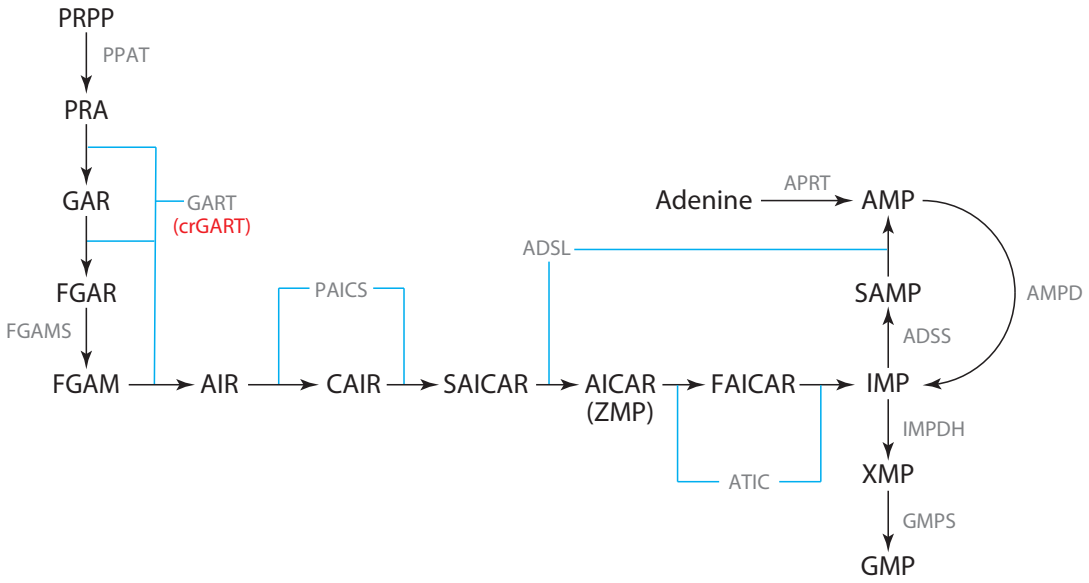


Figure 5.1: De novo purine synthesis pathway. DNPS mediates the conversion of PRPP to IMP. IMP is subsequently converted to AMP or GMP. The HeLa GART KO, crGART, is indicated. Figure generated by Dr. Guido Vacano.

Metabolomic profiles reflect a combination of genetic and environmental influences and can therefore be critical for diagnosis and for understanding biochemical pathway alterations associated with disease and environmental insults (Alonso, Marsal, and JuliÃ 2015). Deficiencies in DNPS have been identified and are rare. Most involve ADSL deficiency and fewer than 100 individuals exhibiting ADSL deficiency have been identified (Jurecka et al. 2015). Two individuals (siblings) have been identified with PAICS deficiency (Pelet et al. 2019), and only one patient has been identified with AICAribosiduria (ATIC deficiency) (Marie et al. 2004). The vast majority of these cases have genomic mutations which manifest as point mutations in the amino acid sequence, resulting in decreased enzymatic activity. AICAribosiduria and ADSL deficiency can be rapidly screened using the inexpensive Bratton-Marshall assay of body fluids to detect, respectively, AICAriboside (the dephosphorylated form of ATIC's substrate, ZMP) or SAICAR. More recently, genomic sequencing has been used for definitive diagnosis.

PAICS deficiency was diagnosed via exome sequencing (Pelet et al. 2019). To date, no cases of GART deficiency have been identified.

DNPS nulls for ADSL, ATIC, GART, PAICS and PFAS were recently produced in HeLa cells via CRISPR-Cas9 induced mutagenesis (Baresova et al. 2016) and characterized. The GART null, crGART, has no measurable GART protein or activity (Baresova et al. 2016; Mádrová et al. 2018).

In purine free media, the crADSL (ADSL null) and crATIC (ATIC null) cell lines accumulate the intermediates ZMP and SAICAR, respectively (Baresova et al. 2016). Adenine supplementation inhibits DNPS (and therefore intermediate accumulation). However, RNA-seq analysis of crADSL, crATIC, and crGART versus HeLa revealed robust transcriptome differences by cell type, many of which were not affected by adenine supplementation [versus 10 hours of purine starvation (Mazzarino et al. 2019; 2020). These results suggest that adenine supplementation, which is necessary for proliferative growth of the KO cell lines, does not completely correct the transcriptome phenotype of these cell lines.

CRISPR based techniques are more specific than their previous iterations of genomic manipulation however off-target Cas9 activity has been noted (Newton et al. 2019; X. Wu, Kriz, and Sharp 2014). To ensure that differences seen in metabolic profiles are solely due to the GART-KO instead of CRISPR artefacts, we compared crGART-KO (designated KO) with crGART stably transfected with pCMV-GART-K1 (designated pGART). Our results show that pGART has a notably different metabolomic profile versus crGART in purine-supplemented and purine starved media.

Methods

Cell culture

Cells were grown and starved for purines as previously described (Mazzarino et al. 2019). Cells were prepared for metabolite profiling as per Metabolon instructions. Briefly, cells were lifted using Detachin (Genlantis) and pelleted and washed in 1xPBS then aspirated and stored at -80°C. For purine auxotrophy assessment, 1,000 cells were seeded into 6-well plates (TechnoPlasticProducts AG Switzerland) and allowed to grow for 1-2 days. Media was exchanged for DMEM high glucose with l-glutamine, 10% FCM, 100 μg/mL normocin (Invivogen), with or without 30 μM adenine and allowed to grow for 7-10 days. FCM is fetal calf serum dialyzed against a 3.5kDa barrier to remove endogenous purines. 1,000 cells were plated, starved, fixed, and stained using crystal violet as previously described (Mazzarino et al. 2019).

Cloning K1-GART CHO-K1 into pTarget

The CHO-K1 GART cDNA (NCBI accession number EU622913.1) was cloned into the CMV promoted pTarget vector (Promega) (Knox et al. 2009). pTarget CHO-K1-GART was amplified in Stellar Competent Cells (Clontech) *E.coli* then prepared using NucleoBond Maxi kit as per manufacturers protocol (Machery Nagel). Cloned inserts in pTarget vectors were sequenced using T7, pTarget-seq, or SP6 primers and gene-specific primers (GART_K1_711_F and GART_K1_957_F) by premixed DNA/primer samples (Eurofins Genomics).

crGART transfections with K1-GART-pTarget plasmid

crGART HeLa cells were transfected with K1-GART-pTarget. Cells were grown continuously in high glucose DMEM (Thermo Fisher Scientific), 10% FCS (Hyclone

FetalClone II Serum (US), Thermo Fisher Scientific), supplemented with Normocin (InvivoGen), Geneticin (Thermo Fisher Scientific), and Adenine (30 μM) (Millipore Sigma). Transfections were performed using Lipofectamine 2000 as per manufacturer protocol (Invitrogen). Stable transfections were selected by Geneticin (Invivogen) and adenine starvation.

Metabolomic profiling

Cell pellets were sent to Metabolon for metabolic profiling using ultra high-perfomance liquid chromatography/tandem accurate mass spectrometry (UHPLC/MS/MS). Peak detection, determination of relative concentrations, and database mapping was performed. Statistical analysis and interpretation were provided by Metabolon. DRM significance was determined by Welsh's two-sample t-test for p values and False Discovery Rate for q-values (Storey and Tibshirani 2003), while effect groupings were determined by Two-Way ANOVA.

Results

Transfection of crGART with pCMV-GART-K1

We will be referring to the pCMV-GART-K1 GART transfected cell line as pGART and the non-transfected cell line as KO.

The pGART and KO cell lines were first assessed for purine auxotrophy. We previously demonstrated that pCMV-GART-K1 rescued the purine deficit the CHO GART-KO cell line AdeC (Knox et al. 2009). Given that human and CHO GART protein sequences exhibit 86% homology and 93% similarity (Knox et al. 2009), we expected that transfection of this plasmid into the crGART HeLa cell line would likely rescue the purine deficient phenotype. Crystal violet staining shows that pGART are capable of

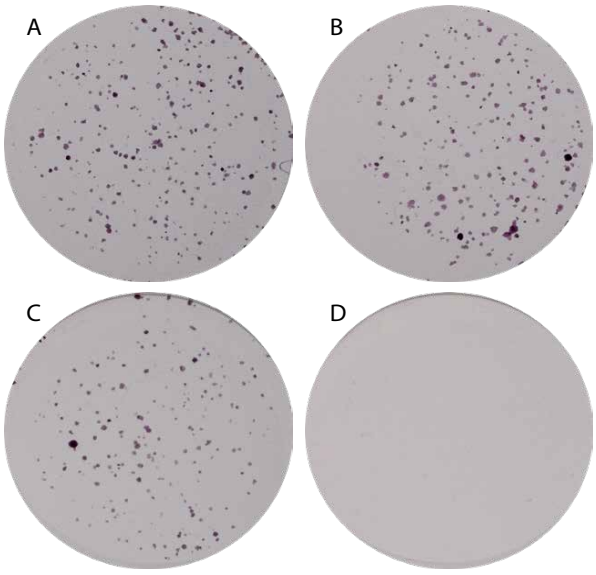


Figure 5.2: Adenine is required for proliferative growth of KO but not pGART. Transfected pGART cells (A, B) and KO HeLa (C, D) were cultured in DMEM supplemented with 10% FCM with (A, C) or without (B, D) 100 μM adenine then fixed and stained with crystal violet. Entire cell growth area was imaged. Transfection, growth, and staining performed by Terry Wilkinson II.

proliferative growth in adenine supplemented and unsupplemented media while KO cells are purine auxotrophs and require supplemental adenine for proliferative growth (Figure 5.2).

MS analysis reveals differentially regulated metabolites

Fold change analysis use comparison groups to produce ratios of differentially regulated metabolites (DRMs), significance is then calculated between experimental conditions and DRMs are sorted into genotype and treatment effects. For our analysis, the term "genotype effect" refers to DRMs that change significantly by cell type (KO versus pGART) while "treatment effect" refers to DRMs that change significantly by adenine supplementation. Significance and assignment of each effect per individual metabolite are calculated by Metabolon. A total of 505 metabolites were identified. 377 metabolites were found attributed to genotype effect while 157 metabolites were found attributed with treatment effect.

Pathway enrichment groups individual metabolites by functional association. Metabolon provides a calculated pathway enrichment value based upon their subpathway groupings each DRMs assigned to are (such as TCA cycle, Glutathione metabolism, etc.) and a subpathway enrichment value is calculated (Figure 5.3). Enrichment value is a

calculated number based solely on the number of DRMs found between experimental comparison groups and does not represent a directional change of metabolites or their respective pathways. This primary analysis aides in identification of subpathways that show large groupings of DRMs. Full output of subpathway enrichment analysis is given (Table 5.1). Heat maps of DRMs in prominent subpathways are given (Figures 5.4).

$$Enrichment\ Value = \frac{\frac{k}{m}}{\frac{n-k}{N-m}}$$

Figure 5.3: Enrichment Value calculation. *m* is number of metabolites in the subpathway identified, *k* is number of significant metabolites identified in the subpathway, *n* is the total number of significant metabolites per effect, and *N* is total number of metabolites identified.

Table 5.1: Subpathway Enrichment Value. Metabolites are grouped into respective subpathway category and enrichment value is calculated based on significant changes metabolites in genotype or treatment effect groupings. Highlighted subpathways have a calculated enrichment value above 1 and are therefore of interest for further investigation.

Genotype Effect						Treatment Effect					
Category	**k**	**m**	**n**	**N**	**Enrichment**	**Category**	**k**	**m**	**n**	**N**	**Enrichment**
Alanine and Aspartate Metabolism	6	6	377	505	1.35	Alanine and Aspartate Metabolism	0	6	157	505	-0.00
Aminosugar Metabolism	6	7	377	505	1.15	Aminosugar Metabolism	4	7	157	505	1.86
Ascorbate and Aldarate Metabolism	1	1	377	505	1.34	Ascorbate and Aldarate Metabolism	0	1	157	505	-0.00
Bacterial/Fungal	0	1	377	505	-0.00	Bacterial/Fungal	0	1	157	505	-0.00
Benzoate Metabolism	1	3	377	505	0.45	Benzoate Metabolism	1	3	157	505	1.07
Carnitine Metabolism	2	2	377	505	1.34	Carnitine Metabolism	1	2	157	505	1.61
Ceramide PEs	1	1	377	505	1.34	Ceramide PEs	0	1	157	505	-0.00
Ceramides	5	7	377	505	0.96	Ceramides	2	7	157	505	0.92
Chemical	4	5	377	505	1.07	Chemical	2	5	157	505	1.29
Creatine Metabolism	2	2	377	505	1.34	Creatine Metabolism	0	2	157	505	-0.00
Diacylglycerol	6	6	377	505	1.35	Diacylglycerol	0	6	157	505	-0.00
Dihydroceramides	1	1	377	505	1.34	Dihydroceramides	1	1	157	505	3.23
Dihydrosphingomyelins	5	5	377	505	1.34	Dihydrosphingomyelins	0	5	157	505	-0.00
Dipeptide	4	6	377	505	0.89	Dipeptide	2	6	157	505	1.07
Drug - Antibiotic	0	1	377	505	-0.00	Drug - Antibiotic	0	1	157	505	-0.00
Endocannabinoid	4	7	377	505	0.76	Endocannabinoid	1	7	157	505	0.46
Fatty Acid Metabolism (Acyl Carnitine, Long Chain Saturated)	4	5	377	505	1.07	Fatty Acid Metabolism (Acyl Carnitine, Long Chain Saturated)	3	5	157	505	1.95
Fatty Acid Metabolism (Acyl Carnitine, Medium Chain)	0	2	377	505	-0.00	Fatty Acid Metabolism (Acyl Carnitine, Medium Chain)	0	2	157	505	-0.00
Fatty Acid Metabolism (Acyl Carnitine, Monounsaturated)	1	3	377	505	0.45	Fatty Acid Metabolism (Acyl Carnitine, Monounsaturated)	1	3	157	505	1.07
Fatty Acid Metabolism (Acyl Carnitine, Polyunsaturated)	0	1	377	505	-0.00	Fatty Acid Metabolism (Acyl Carnitine, Polyunsaturated)	0	1	157	505	-0.00
Fatty Acid Metabolism (Acyl Carnitine, Short Chain)	1	1	377	505	1.34	Fatty Acid Metabolism (Acyl Carnitine, Short Chain)	1	1	157	505	3.23
Fatty Acid Metabolism (Acyl Choline)	5	5	377	505	1.34	Fatty Acid Metabolism (Acyl Choline)	5	5	157	505	3.29
Fatty Acid Metabolism (also BCAA Metabolism)	3	3	377	505	1.34	Fatty Acid Metabolism (also BCAA Metabolism)	2	3	157	505	2.16
Fatty Acid Synthesis	0	1	377	505	-0.00	Fatty Acid Synthesis	0	1	157	505	-0.00
Fatty Acid, Branched	1	3	377	505	0.45	Fatty Acid, Branched	0	3	157	505	-0.00
Fatty Acid, Dicarboxylate	1	4	377	505	0.33	Fatty Acid, Dicarboxylate	0	4	157	505	-0.00
Fatty Acid, Dihydroxy	1	1	377	505	1.34	Fatty Acid, Dihydroxy	1	1	157	505	3.23
Fatty Acid, Monohydroxy	2	4	377	505	0.67	Fatty Acid, Monohydroxy	0	4	157	505	-0.00
Folate Metabolism	2	2	377	505	1.34	Folate Metabolism	1	2	157	505	1.61
Food Component/Plant	4	6	377	505	0.89	Food Component/Plant	1	6	157	505	0.53
Fructose, Mannose and Galactose Metabolism	5	5	377	505	1.34	Fructose, Mannose and Galactose Metabolism	3	5	157	505	1.95
Galactosyl Glycerolipids	1	1	377	505	1.34	Galactosyl Glycerolipids	0	1	157	505	-0.00
Gamma-glutamyl Amino Acid	13	14	377	505	1.25	Gamma-glutamyl Amino Acid	5	14	157	505	1.15
Glutamate Metabolism	7	10	377	505	0.94	Glutamate Metabolism	5	10	157	505	1.63
Glutathione Metabolism	11	11	377	505	1.35	Glutathione Metabolism	4	11	157	505	1.17
Glycerolipid Metabolism	3	3	377	505	1.34	Glycerolipid Metabolism	2	3	157	505	2.16
Glycine, Serine and Threonine Metabolism	4	8	377	505	0.67	Glycine, Serine and Threonine Metabolism	5	8	157	505	2.04
Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	7	8	377	505	1.18	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	7	8	157	505	2.90
Guanidino and Acetamido Metabolism	1	1	377	505	1.34	Guanidino and Acetamido Metabolism	0	1	157	505	-0.00
Hexosylceramides (HCER)	4	4	377	505	1.34	Hexosylceramides (HCER)	0	4	157	505	-0.00
Histidine Metabolism	2	4	377	505	0.67	Histidine Metabolism	1	4	157	505	0.80
Inositol Metabolism	1	1	377	505	1.34	Inositol Metabolism	0	1	157	505	-0.00
Lactosylceramides (LCER)	3	3	377	505	1.34	Lactosylceramides (LCER)	0	3	157	505	-0.00
Leucine, Isoleucine and Valine Metabolism	11	19	377	505	0.77	Leucine, Isoleucine and Valine Metabolism	4	19	157	505	0.67
Long Chain Monounsaturated Fatty Acid	6	7	377	505	1.15	Long Chain Monounsaturated Fatty Acid	0	7	157	505	-0.00
Long Chain Polyunsaturated Fatty Acid (n3 and n6)	4	9	377	505	0.59	Long Chain Polyunsaturated Fatty Acid (n3 and n6)	1	9	157	505	0.35
Long Chain Saturated Fatty Acid	2	6	377	505	0.44	Long Chain Saturated Fatty Acid	0	6	157	505	-0.00
Lysine Metabolism	8	11	377	505	0.97	Lysine Metabolism	6	11	157	505	1.78
Lysophospholipid	19	22	377	505	1.17	Lysophospholipid	3	22	157	505	0.43
Lysoplasmalogen	3	4	377	505	1.00	Lysoplasmalogen	0	4	157	505	-0.00
Medium Chain Fatty Acid	1	4	377	505	0.33	Medium Chain Fatty Acid	0	4	157	505	-0.00
Methionine, Cysteine, SAM and Taurine Metabolism	14	17	377	505	1.11	Methionine, Cysteine, SAM and Taurine Metabolism	9	17	157	505	1.75
Mevalonate Metabolism	0	1	377	505	-0.00	Mevalonate Metabolism	1	1	157	505	3.23
Monoacylglycerol	6	6	377	505	1.35	Monoacylglycerol	0	6	157	505	-0.00
Neurotransmitter	0	1	377	505	-0.00	Neurotransmitter	1	1	157	505	3.23
Nicotinate and Nicotinamide Metabolism	5	6	377	505	1.12	Nicotinate and Nicotinamide Metabolism	4	6	157	505	2.17
Nucleotide Sugar	3	6	377	505	0.67	Nucleotide Sugar	4	6	157	505	2.17
Oxidative Phosphorylation	2	2	377	505	1.34	Oxidative Phosphorylation	1	2	157	505	1.61
Pantothenate and CoA Metabolism	5	5	377	505	1.34	Pantothenate and CoA Metabolism	4	5	157	505	2.61
Partially Characterized Molecules	0	2	377	505	-0.00	Partially Characterized Molecules	0	2	157	505	-0.00
Pentose Metabolism	7	8	377	505	1.18	Pentose Metabolism	4	8	157	505	1.62
Pentose Phosphate Pathway	1	1	377	505	1.34	Pentose Phosphate Pathway	1	1	157	505	3.23
Phenylalanine Metabolism	1	4	377	505	0.33	Phenylalanine Metabolism	1	4	157	505	0.80
Phosphatidylcholine (PC)	15	20	377	505	1.00	Phosphatidylcholine (PC)	0	20	157	505	-0.00
Phosphatidylethanolamine (PE)	10	11	377	505	1.22	Phosphatidylethanolamine (PE)	0	11	157	505	-0.00
Phosphatidylglycerol (PG)	2	2	377	505	1.34	Phosphatidylglycerol (PG)	0	2	157	505	-0.00
Phosphatidylinositol (PI)	6	7	377	505	1.15	Phosphatidylinositol (PI)	0	7	157	505	-0.00
Phosphatidylserine (PS)	1	1	377	505	1.34	Phosphatidylserine (PS)	0	1	157	505	-0.00
Phospholipid Metabolism	6	8	377	505	1.00	Phospholipid Metabolism	1	8	157	505	0.40
Plasmalogen	8	10	377	505	1.07	Plasmalogen	0	10	157	505	-0.00
Polyamine Metabolism	6	7	377	505	1.15	Polyamine Metabolism	2	7	157	505	0.92
Primary Bile Acid Metabolism	2	2	377	505	1.34	Primary Bile Acid Metabolism	0	2	157	505	-0.00
Pterin Metabolism	0	1	377	505	-0.00	Pterin Metabolism	1	1	157	505	3.23
Purine and Pyrimidine Metabolism	1	1	377	505	1.34	Purine and Pyrimidine Metabolism	1	1	157	505	3.23
Purine Metabolism, (Hypo)Xanthine/Inosine containing	5	7	377	505	0.96	Purine Metabolism, (Hypo)Xanthine/Inosine containing	5	7	157	505	2.34
Purine Metabolism, Adenine containing	9	10	377	505	1.21	Purine Metabolism, Adenine containing	5	10	157	505	1.63
Purine Metabolism, Guanine containing	6	7	377	505	1.15	Purine Metabolism, Guanine containing	5	7	157	505	2.34
Pyrimidine Metabolism, Cytidine containing	2	5	377	505	0.53	Pyrimidine Metabolism, Cytidine containing	2	5	157	505	1.29
Pyrimidine Metabolism, Orotate containing	2	2	377	505	1.34	Pyrimidine Metabolism, Orotate containing	2	2	157	505	3.25
Pyrimidine Metabolism, Thymine containing	2	3	377	505	0.89	Pyrimidine Metabolism, Thymine containing	2	3	157	505	2.16
Pyrimidine Metabolism, Uracil containing	9	11	377	505	1.10	Pyrimidine Metabolism, Uracil containing	7	11	157	505	2.10
Riboflavin Metabolism	2	3	377	505	0.89	Riboflavin Metabolism	1	3	157	505	1.07
Secondary Bile Acid Metabolism	1	2	377	505	0.67	Secondary Bile Acid Metabolism	1	2	157	505	1.61
Sphingolipid Synthesis	1	3	377	505	0.45	Sphingolipid Synthesis	1	3	157	505	1.07
Sphingomyelins	18	25	377	505	0.96	Sphingomyelins	2	25	157	505	0.25
Sphingosines	1	2	377	505	0.67	Sphingosines	2	2	157	505	3.25
Sterol	2	3	377	505	0.89	Sterol	1	3	157	505	1.07
TCA Cycle	7	8	377	505	1.18	TCA Cycle	2	8	157	505	0.80
Thiamine Metabolism	1	1	377	505	1.34	Thiamine Metabolism	0	1	157	505	-0.00
Tocopherol Metabolism	0	1	377	505	-0.00	Tocopherol Metabolism	0	1	157	505	-0.00
Tryptophan Metabolism	1	3	377	505	0.45	Tryptophan Metabolism	0	3	157	505	-0.00
Tyrosine Metabolism	4	6	377	505	0.89	Tyrosine Metabolism	3	6	157	505	1.62
Urea cycle; Arginine and Proline Metabolism	10	11	377	505	1.22	Urea cycle; Arginine and Proline Metabolism	3	11	157	505	0.87
Vitamin A Metabolism	0	1	377	505	-0.00	Vitamin A Metabolism	1	1	157	505	3.23
Vitamin B6 Metabolism	4	5	377	505	1.07	Vitamin B6 Metabolism	2	5	157	505	1.29

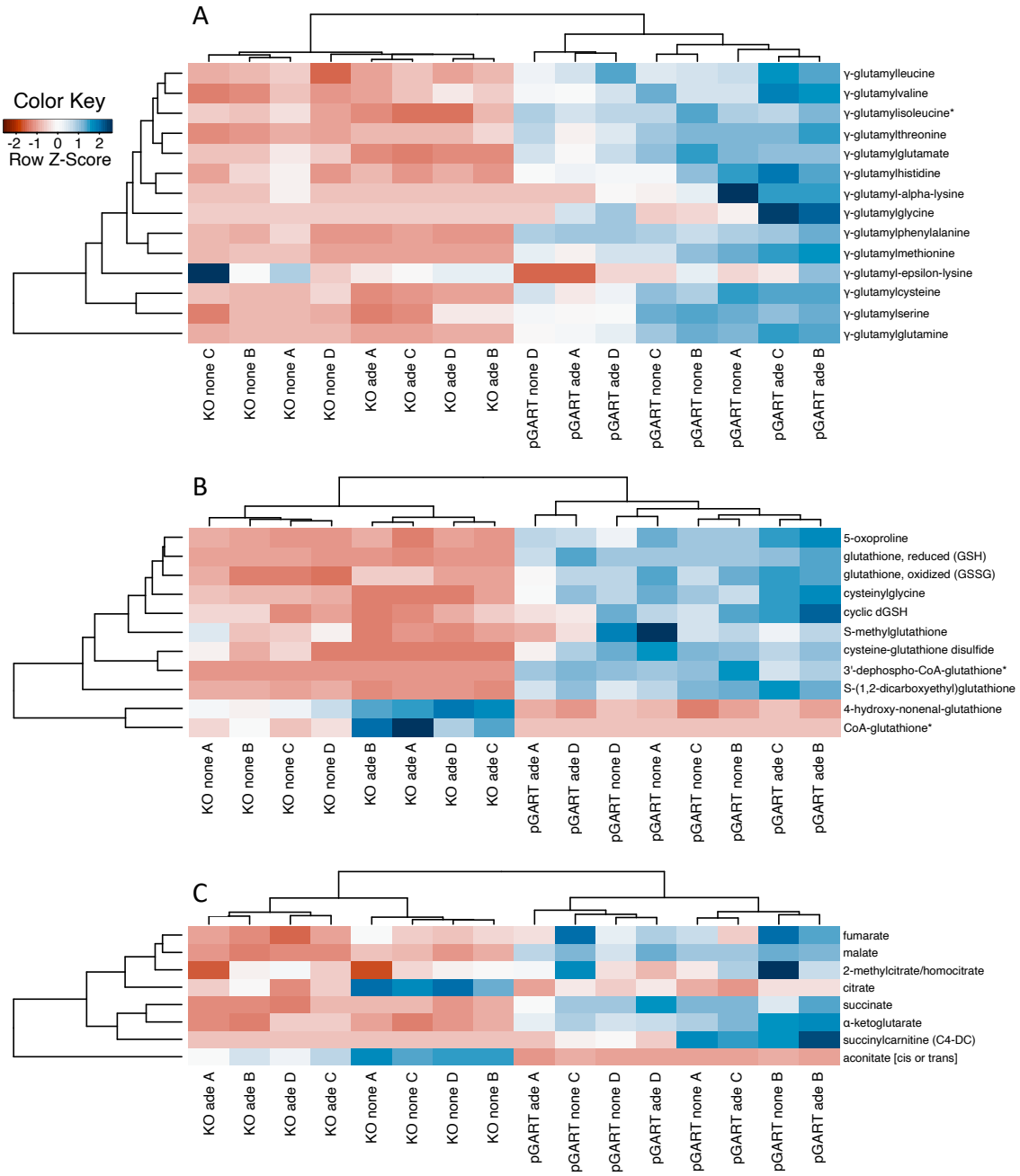


Figure 5.4: Heatmaps of select subpathway metabolites. The γ-glutamyl amino acid (A), glutathione metabolism (B), and TCA cycle (C) subpathways showed enrichment by genotype comparison but not by treatment. This is visualized by upper dendrogram. KO and pGART showed groupings. Metabolites were preferentially enriched in pGART samples. Sample labels are coded "ade" for adenine supplemented conditions and "none" for adenine deficient conditions. "A-D" denotes sample condition replicate.

Genotype analysis shows enrichment in subpathways

Enrichment analysis of genotype effect reveals DRMs are clustered within multiple subpathways (Table 5.1). Of particular interest to our laboratory were gamma-glutamyl amino acid, TCA cycle, glycolysis gluconeogenesis and pyruvate metabolism, glutathione, fatty acid metabolism, phosphatidylethanolamine and inositol, panothenate and CoA metabolism, and urea cycle; arginine and proline metabolism.

Selective subpathways associated with purine and pyrimidine metabolism were enriched. Curiously, (hypo)xanthine/inosine containing was below the enrichment analysis equation threshold for highlighting a pathway of interest. These terms are directly related to purine catabolism where nucleotides are broken down through inosine, hypoxanthine, xanthine, and then to uric acid in humans. However, hypoxanthine is utilized as a salvage input, directly converted to IMP in a single step (Figure 5.1). It was expected that salvage synthesis is upregulated in DNPS-KOs and would result in decreased catabolic products, however these data may contradict this expectation. These data suggest that purine catabolism is unaffected by DNPS dysregulation. Subpathways are focused around energy, and energy production/maintenance type pathways. These involve glycolysis, gluconeogenesis, pyruvate, creatine, and the TCA cycle (Table 5.1). This is perhaps to be expected as adenine containing nucleobase production, the fundamental base of the major cellular energy currency ATP, is compromised in DNPS defects. Enrichment analysis unveiled urea cycle in genotype comparison. The urea cycle is closely tied to nucleotide synthesis and TCA cycle. Gamma-glutamyl amino acid terms were almost all selectively enriched in genotype analysis.

Treatment analysis shows enrichment in DRM subpathways

Enrichment analysis of treatment effect reveals DRMs are clustered within multiple subpathways. Of interest, are aminosugar metabolism, dipeptide, fatty acid metabolism, gamma-glutamyl amino acid, glutamate metabolism, glutathione metabolism, pantothenate and CoA metabolism, and vitamin B6 metabolism (Table 5.1). Unsurprisingly, adenine supplementation showed robust enrichment in all terms identified associated with purine and pyrimidine metabolic subpathways such as purine metabolism, pyrimidine metabolism, nucleotide sugar, nicotinate and nicotinamide metabolism, and pentose metabolism. The (hypo)xanthine/inosine containing subpathway showed selective enrichment in the KO treatment effect group, with the large majority of metabolites elevated in purine supplemented cells. This is unsurprising as these metabolites are involved in salvage synthesis of purines. These data suggest that catabolic output of purine synthesis in DNPS dysregulation is reliant on salvage input products. Treatment effect also showed term enrichment in amino acid metabolism, specifically through lysine, glycine, serine, threonine, glutamate, and tyrosine metabolism. Amino acids are used as functional building blocks of proteins as well as cofactors in various metabolic processes. Amino acids are used as substrates in DNPS pathway, specifically, glutamine is used in the conversion of PRPP to 5-PRA and FGAR to FGAM, glycine is used in the conversion of 5-PRA to GAR, and aspartate is used in the conversion of CAIR to SAICAR. Amino acid metabolism may be differentially regulated to account for the reliance on DNPS synthesis or other metabolic processes influenced by DNPS dysregulation.

DNPS requires glycine, glutamine, and aspartate for synthesis of a singular IMP molecule. AMP is an inhibitor of PRAT, the first enzyme of the DNPS pathway. Adenine is directly converted into AMP and it stands to reason that adenine treatment will inhibit DNPS and therefore pathways associated with amino acid synthesis.

Discussion

In this study, we evaluated the purine dietary requirement for the crGART KO and generated pGART cell lines. We generated metabolic profiles of each cell line in adenine-supplemented and adenine depleted conditions and analyzed the metabolite profiles to functional pathways.

Adenine supplementation inhibits DNPS via formation of AMP through salvage synthesis as AMP inhibits PRAT, the first enzyme of DNPS. AMP can be converted into IMP via AMP-deaminase and subsequently synthesized into GMP (Figure 5.1) (Jinnah, Sabina, and Van Den Berghe 2013). We have previously reported that adenine supplementation is effective at inhibiting DNPS (Mazzarino et al. 2019; 2020).

GART catalyzes three non-sequential reactions in DNPS, the first of which is the conversion of 5-PRA to GAR. 5-PRA is highly unstable, breaking down with approximately 5 seconds under cellular conditions (Rudolph and Stubbe 1995). This makes GART attractive as a model of generalized DNPS deficiency, as other DNPS-KOs accumulate substrates that alter cellular processes outside of DNPS (Corton et al. 1995; Keller, Tan, and Lee 2012). Here, we report on metabolic alterations as a function of adenine starvation and supplementation as a function of the DNPS enzyme, GART. Our results have uncovered DRMs as a function of adenine treatment as well as cell type.

During our analysis, we noted many subpathways and connections to the Down's syndrome (DS) phenotype. GART is located on Hsa21 and is therefore triplicated in DS. Expression of GART as well as purine levels in DS are dysregulated (Patterson 2009). Our system was only evaluated for purine auxotrophy and therefore no conclusions can be directly drawn about gene dosage effects. However, our analysis highlights pathways that GART or DNPS influence, and therefore is likely to inform the involvement of GART in the DS phenotype. I have compiled these notable findings together with transcriptomic data to highlight potential cellular processes and pathways of interest and how they relate (Figure 5.5).

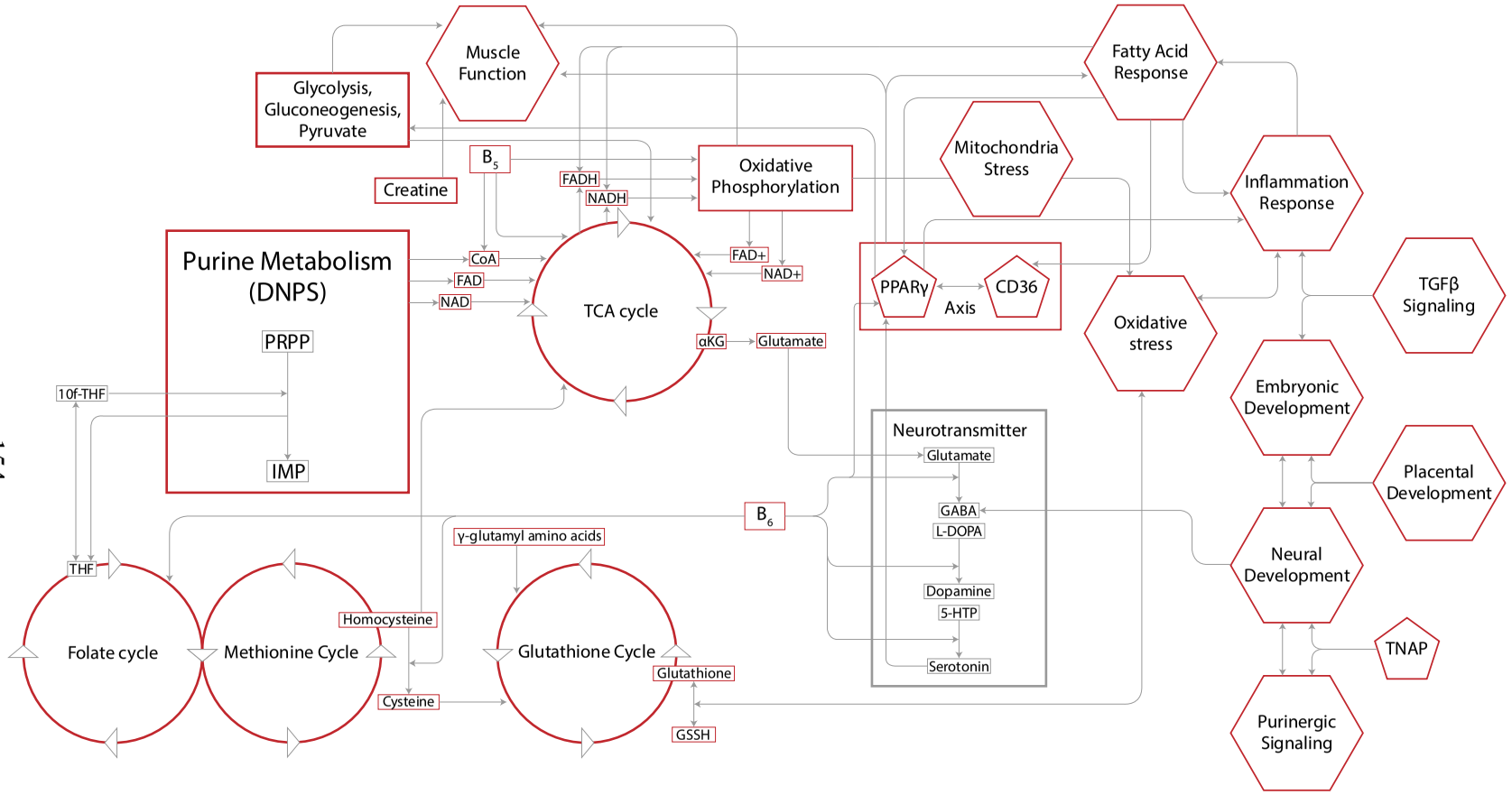


Figure 5.5: Grand themes of transcriptomic and metabolomic data, proposed. Figure has been generated through literature search and compilation of themes found within metabolomic and transcriptomic analysis of altered cellular processes in DNPS-KO cell lines. Within the figure, red outlines are differentially regulated while grey outlines are connectors. Arrows indicate how themes and findings are interrelated. Large boxes are processes, circles are cycles, small boxes are metabolites, pentagons are transcriptomic themes, and pentagons are genes. Our analysis has uncovered linkages and provided new pathways to guide research in the future.

Increased oxidative damage and reactive oxygen species load is a well-documented phenomenon in DS (Lott 2012b; Arbuzova, Hutchin, and Cuckle 2002), aging (H. Cui, Kong, and Zhang 2012), and Alzheimer's disease (Lott 2012b). In DS, multiple gene families involved in regulating cellular oxidative stress are found altered, including SOD, APP, PPAR, among others (Izzo et al. 2017; 2018). Superoxide Dismutase 1 (SOD1) is a major protein antioxidant responsible for conversion of free radical oxygen to hydrogen peroxide (Perluigi and Butterfield 2012). Glutathione (γ-glutamyl cysteinylglycine) is another primary redox buffer in mammalian cells, directly scavenge oxidizing species as well as through glutathione peroxidase activity and hydrogen peroxide generated by SOD (G. Wu et al. 2004). Glutathione levels are decreased in DS (Pogribna et al. 2001), as well as an increased in the activity ratio of SOD:Glutathione peroxidase (Pastore et al. 2003) all leading to increased hydrogen peroxide levels and oxidative stress. γ-glutamyl amino acids and glutathione are linked in mammalian cells by either the γ-glutamyl amino acid cycle or glutathione cycle(Orlowski and Meister 1970; Bachhawat and Yadav 2018). In either model, γ-glutamyl amino acids are used as a reservoir for γ-glutamyl groups for glutathione synthesis in mammals. In a recent metabolomic study, an abundance of γ-glutamyl amino acids are differentially regulated in the amniotic fluid of second trimester DS fetuses (X. Liu et al. 2020). DNPS and GART are directly involved in folate and one-carbon donor metabolism (Perluigi and Butterfield 2012). DS is characterized by hyperuricemia, or an excess uric acid build up which a product of purine catabolism. Hyperuricemia can be caused by an imbalance in the native antioxidant defense mechanisms (Perluigi and Butterfield 2012; Zana, Janka, and Kálmán 2007). An overwhelming majority of γ-glutamyl amino acids and glutathione

metabolites identified are selectively enriched in the genotype comparison, specifically enriched in the crGART-CDK line, with adenine treatment showing a minimal effect. This observation bolsters our position that correct dosage of the DNPS enzyme GART is necessary in antioxidant response.

Hypotonia is displayed in nearly all children diagnosed with DS and is among the most common phenotypes (Dey et al. 2013). This phenotype is not well understood. Hypotonia is characterized by excessive joint flexibility and inadequate muscle tone and strength, resulting in hyper flexibility, poor motor ability, and control (Lauteslager, Vermeer, and Helders 1998) and can be improved with physical therapy intervention (Maïano et al. 2019). As hypotonia in DS can be rescued to a degree, a portion of the hypotonia phenotype may be overcome by increasing muscle mass. Children with DS exhibit hypotonic phenotypes at a young age, with inability to crawl and standing behavior delayed compared to disomic counterparts (Lauteslager, Vermeer, and Helders 1998). These behaviors could be seen as strength training at a young age allowing muscle building for processive developmental milestones. Decreased muscle function could contribute to this delay in muscle development seen in DS. Mitochondrial dysfunction is a phenotype in DS (Arbuzova, Hutchin, and Cuckle 2002) including decreased ATP production (Izzo et al. 2017) and altered gene expression patterns, among other mitochondrial phenotypes (Izzo et al. 2018). There is evidence that links these altered mitochondrial states to the peroxisome proliferator activated receptor gamma (PPARγ) (Izzo et al. 2018). Mitochondrial function and proper ATP production are critical for muscle growth via strength training. Here, the enrichment of metabolic subpathway terms also associated with energy production, such as TCA cycle which generates high energy

electrons in the form of the adenine base FADH and NADH, creatine metabolism which acts as an ATP storage for muscle, as well as glycolysis and gluconeogenesis enriched purely along genotype comparison group lines suggests that GART is linked to these processes. The previously reported transcriptomic profiling of the crGART-KO cell line showed differences enriched in muscle structure and function terms as well as locomotor behavior (Chapter 2). This data, taken in tandem with our current metabolic profiling of the crGART-KO cell line, indicates a potential link between GART or DNPS and muscle structure and function relating to the hypotonia phenotype.

B class vitamins are essential micronutrients with a variety of cellular functions and must be taken in via diet. Our data show an enrichment of DRMs assigned to B5 (found in data as pantothenate) and B6 metabolism in genotype comparison. B5 metabolites also showed DRM enrichment in adenine treated and untreated KO comparison. Vitamin B5 is used as a cofactor in the TCA cycle, the electron transport chain, as well as in the synthesis of coenzyme A, and is therefore critical for cellular energy homeostasis (Kennedy 2016). Vitamin B6 is involved as a cofactor in the synthesis of folates, has been found essential for nervous system (Kennedy 2016), mitochondrial function (Janssen et al. 2019), glutathione metabolism, and amino acid metabolism (Dalto and Matte 2017). B6 has been shown to activate PPARγ via weak agonism (Yanaka et al. 2011). The folate cycle produces one carbon donors, necessary small molecules for pathways such as purine (one of three GART reactions requires a folate derivative) and pyrimidine synthesis (Moffatt and Ashihara 2002). Transsulfuration of homocysteine to cysteine is B6 dependent and accounts for approximately half of the cysteine used in glutathione synthesis for intracellular pools (Mosharov, Cranford, and

Banerjee 2000). B6 in the nervous system acts as a cofactor for synthesis of multiple neurotransmitters such as γ-amino-butyric acid (GABA), serotonin, and dopamine (Calderón-Ospina and Nava-Mesa 2020). B6 administration has shown efficacious in mitigating chronic seizure activity (Spinneker et al. 2007; Tong 2014) as well as attenuating excitotoxic effects of neurotoxin exposure (Dakshinamurti, Sharma, and Geiger 2003), mediated possibly through its involvement in GABA synthesis. Regarding DS, differences in GABA (Contestabile, Magara, and Cancedda 2017) as well as an increased rate of seizures have been noted (Lott and Dierssen 2010). Studies have also found B6 metabolic abnormalities in DS children (McCoy, Colombini, and Ebadi 1969) and amniotic fluid (Baggot et al. 2008), although supplementation has shown ineffective (Kleijnen and Knipschild 1991). We have previously reported that the transcript levels of the ALPL gene is drastically reduced in GART-KO compared to WT HeLa (Chapter 2). ALPL encodes the tissue-non-specific alkaline phosphatase enzyme, an ectoenzyme capable of dephosphorylating circulatory, extracellular B6 (Whyte et al. 1985) thus allowing membrane permeability and its function to be exerted on the cell. Unsurprisingly, GABA ontological terms were enriched in our analysis (Chapter 2).

Our analysis has uncovered only a small subset of differentially regulated lipid metabolites and owing to the difficulties associated with lipidomics we will be analyzing lipid metabolites with broad strokes. Lysophospholipids are single tailed lipid chains found typically as a byproduct of phospholipase activity. This lipid category is involved in PPARγ regulation (Tsukahara, Matsuda, and Haniu 2017) and has implications in immune response and inflammatory signaling mediated through GPCR coupling (Gräler 2002), lipid droplet protein expression and formation (Guilherme et al. 2008), and

response to low-density lipoprotein (McIntyre et al. 2003). Our data presented here indicates that GART or DNPS deficiency decreases lysophospholipids and is not attenuated by adenine supplementation, suggesting a possible relation to PPARγ regulatory element or immune response. Diacylglycerols act as building blocks for glycerophospholipids and as lipid second messengers (Eichmann and Lass 2015). Ceramides, including lactosyl- and hexosylceramide, are involved in sphingolipid synthesis, and with regulation potential in cellular events including signal transduction, cell growth, differentiation, apoptosis, as well as protein kinase C activity, immune response, and insulin sensitivity (Young et al. 2012; Sofi et al. 2017; Alarcon-Barrera et al. 2020; Nakamura et al. 2013; Pralhada Rao et al. 2013). The data presented suggests that GART or DNPS deficiency increases lactosyl- and hexosylceramide metabolites and is not rescued by adenine supplementation. Sphingomyelins account for approximately 10% of mammalian cellular lipids and is a part of the phosphosphingolipid family and is critical to myelination of neurons, insulin response, cholesterol association, cellular signaling (Slotte 2013), and but also are found as constituent in atherosclerotic plaques (Boini et al. 2018).

Interestingly, PPARγ activity is found linked within myriad metabolic pathways examined in this study, and acts as a transcription factor, with myriad genes under its control (Kroker and Bruning 2015). PPARγ controls genes related to lipid metabolism, glucose and energy metabolism, inflammation, apoptosis, cell proliferation, and insulin sensitivity (Kroker and Bruning 2015; Janani and Ranjitha Kumari 2015). Additionally, PPARγ transcript levels are enriched in the crGART cell line over WT HeLa of log2(fold change) 4.48. The scavenger receptor CD36 is excessively expressed in the crGART line,

with a log2(fold change) 8.52. Both PPARγ and CD36 transcript levels are near zero for WT HeLa cells. A feedback loop exists between CD36 and PPARg, where activation of CD36 induces PPARγ transcription factor activity, where transcript identity synthesized is CD36-ligand specific (Maréchal et al. 2018). For example, oxidized low density lipoprotein will activate CD36 and in turn induces PPARγ transcription activity causing CD36 transcript synthesis, however the synthetic growth hormone releasing peptide hexarelin can activate CD36 and subsequently PPARγ transcription activity but the CD36 gene is not transcribed (Maréchal et al. 2018). Indeed, PPARγ is sensitive to activation by phosphorylation or agonist outside of CD36, with different subsets of genes transcribed based upon activation signal type (Kroker and Bruning 2015). PPARγ in adipocytes was found activated by the B6 vitamer, pyroxidal-5'-phosphate, via a probable agonist mechanism, although partial or weak agonism is suspected (Yanaka et al. 2011). The neurotransmitter serotonin, in which B6 is directly involved in synthesis of as a cofactor, was also found to be a potent agonist of PPARγ (Waku et al. 2010). The involvement of PPARγ activity with influence in metabolic pathways uncovered here, as well as the heavily elevated CD36 and PPARγ transcript levels found with in DNPS-KOs, suggests that PPARγ or the CD36-PPARγ axis is in some way under the influence of DNPS.

Chapter Six: Summary, Discussion, and Future Directions

Summary and Discussion

The work presented here is not without the vital input from others. The Genomic sequencing core was responsible for the processing of RNA and RNA-seq FASTQ file generation. Dr. Guido Vacano performed the FASTQ to DEG lists and was instrumental in assisting me with the start-up of the ontological analysis. Dr. Vacano also generated heatmaps found in Chapter 2 as well as Chapter 5. Terry Wilkinson II performed the transfection and generation of the stable CMV promoted CHO-K1 GART crGART cell line as well as plating and staining the cells for purine auxotroph analysis. Metabolon processed and initially analyzed the metabolomic data for DRM significance.

Purines are essential molecules for sustaining life in the form of cellular signaling events, genetic information, and energy production/regulation. DNPS is critical in embryonic development as DNPS is upregulated in replicating cells. Previous work robustly indicates the need for tight regulation of DNPS. DNPS deficits in patients, although rare, manifest as congenital defects, with phenotypes ranging from mild to severe mental retardation, physical dysmorphic features, seizures, and shortened life expectancy or embryonic death. DNPS metabolic intermediates have known effects outside of purine synthesis that include alteration of activity of PKM2, a kinase thought to be the root of the metabolic shift seen in tumors, as well as AMPK, a master regulator

of cellular metabolism. DNPS dysregulation is therefore critical to study for understanding widespread effects related to cellular and organismal function.

The work described here is a characterization of the crADSL, crATIC, and crGART cell lines, probing the many cellular and organismal processes which are affected by DNPS dysregulation and outlining future research queries. I have shown that the cell lines used require exogenous purine supplementation in the form of adenine for proliferative growth, and that adenine supplementation inhibits DNPS, as there is no detectable SAICAR or ZMP found within the crADSL and crATIC cell lines respectively. I have generated a library of processes attributed to DNPS dysfunction. The novel CRISPR generated DNPS-KO cell lines represent a new cellular model to understand the wide-spread effects seen in patients as well as DNPS contribution into processes such as TGFβ/SMAD signaling, placentation, vitamin B6 metabolism, embryonic development, muscle function, tumorigenesis, and inflammation as uncovered in the work presented here.

Replicate considerations for RNA-seq and other -omic experiments are critical. Two forms of replicates are used, biological and technical. Technical replicates have the biological material used from the same source where biological replicates consist of biological samples from different sources. The questions we sought to answer were between differences in the transcriptome seen between two distinct biological samples. Therefore, technical replicates of each biological sample were necessary to ensure accuracy of measurements between the distinct cell line populations.

Purine starvation in DNPS-KOs cell lines investigated results in eventual cell death (Figures 2.2, 3.2, 4.2, 5.2), although the kinetics have not been explored

extensively. crATIC and HeLa cells were evenly plated in normal growth media then exchanged for adenine supplemented or deficient media and allowed to proliferate for 6 days. In 24 hour time intervals, plates were fixed and stained using crystal violet, a general histological stain for adherent cells. HeLa cells showed proliferative growth for the full 6 days in both adenine supplemented and deficient conditions. As the HeLa cells have a functional DNPS pathway, this result is unsurprising. crATIC only showed proliferative growth in adenine supplemented conditions for the full 6 days. crATIC in adenine deficient conditions appear to arrest proliferation within 1-2 days, with a notable unhealthy morphology by day 3 progressively worsening through the sixth day (data not shown). It is unknown if these cells undergo a quiescent or senescent phase, nor the temporal scale for induction and the time a vegetative state would persist before eventual cell death. Ten hours was chosen as a time point for these experiments to balance the accumulation of intermediates and potential transcriptomic alterations for vegetative or death signaling cascades. Ten hours also represents approximately 40% of the average replicative cycle for WT HeLa cells of 24-25hours. Previous transcriptomic experimentation on Chinese Hamster Ovary cell based DNPS-KOs were purine starved for six hours, with an average WT-CHO replicative cycle of 14 hours, representing 40%. As DNPS is upregulated for cellular replication, this 40% of replicative cycle is likely ideal to minimize off target effects related to inability to replicate the genome during the S phase of the cell cycle.

Purine supplementation supporting proliferative growth in these cell lines were achieved through the use of adenine and has been shown to be efficacious for all cell lines used for these studies (Figure 2.2, 3.2, 4.2, 5.2). Adenine is a salvage input and is

converted by adenine phosphoribosyltransferase directly into AMP, which is capable of being metabolized into IMP via AMP deaminase and subsequently made into GMP. Hypoxanthine is a salvage input capable of making IMP directly through hypoxanthine phosphoribosyl transferase and into GMP or AMP (Figure 6.1). Therefore, crATIC and crGART should proliferate using hypoxanthine as a purine source but not crADSL, as the ADSL enzyme is required to in the conversion of IMP to AMP.

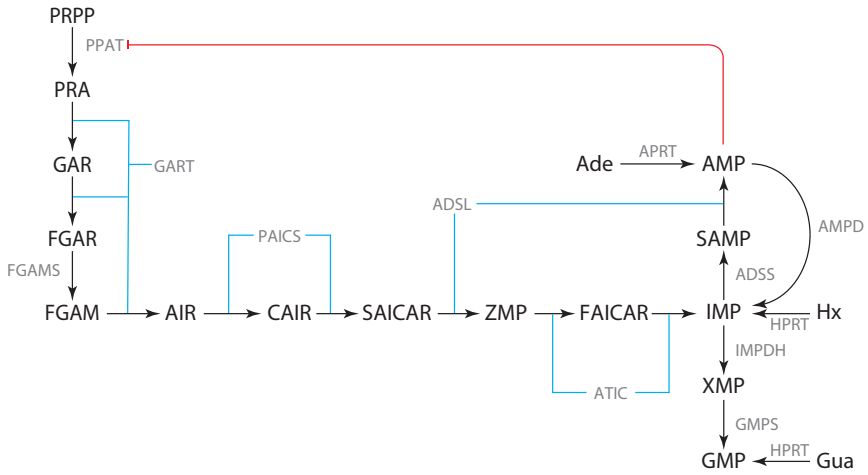


Figure 6.1: DNPS pathway with salvage inputs. DNPS converts PRPP to IMP via ten sequential steps using six enzymes. Salvage synthesis utilizes adenine (ade), hypoxanthine (Hx), or Guanine (Gua) and through one step will synthesize AMP, IMP, or GMP respectively. AMP can be converted into IMP through AMP deaminase (AMPD) and subsequently synthesized into GMP. The enzyme ADSL catalyzes SAICAR to ZMP and SAMP to AMP. AMP inhibits the first step of DNPS. Figure base generated by Dr. Guido Vacano.

Consistent with previously reported CHO cell line based DNPS-KOs, we have shown that Hela crADSL and crATIC accumulate their substrates, SAICAR and ZMP respectively, upon removal of an exogenous purine source. I used HPLC-electrochemical detection methods for substrate identification (Duval et al. 2013), which functions by the redox potential of the metabolites analyzed and is considered an extremely sensitive technique with detection limits in the 1-10 picogram range dependent on analyte (Kristal et al. 2007). SAICAR and ZMP are detected in this system by oxidation with

electrochemical channels set at 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1200mV. Primary peaks were seen in the 300mV channel for SAICAR and ZMP (Figures 3.4, 4.4). Cells were supplied exogenous adenine and can therefore synthesize purine nucleotides necessary for sustaining proliferative growth. It was expected that exogenous adenine supplementation would halt the DNPS pathway as AMP is an inhibitor of the enzyme PRAT, the first step in DNPS. This expectation was met, as SAICAR and ZMP were not detected in the crADSL and crATIC cell lines (respectively) when subjected to adenine supplementation (Figures 3.3, 3.4, 4.3, 4.4). It was hypothesized that upon removal of exogenous adenine and inducing a starvation state, DNPS would begin and metabolic intermediates would accumulate linearly. Indeed, this is the case with crATIC as the ZMP accumulation curve is relatively linear (Figure 4.3). However, crADSL exhibited a lag phase, with no detectable SAICAR from zero to four hours in purine starvation until measurable accumulation began (Figure 3.3). This result has been rather puzzling, although understanding this phenomenon was not within the scope of the experiments presented here. SAICAR can be dephosphorylated and exist as SAICAriboside which is excreted by the cell. It is not known if SAICAriboside is electrochemically active and therefore detectable in our HPLC-EC generated spectra. It is possible that the lag phase is due to an equilibrium that exists between SAICAR to SAICAriboside conversion or SAICAriboside excretion. SAICAriboside may also elute at retention times not covered in our methodologies. Alternatively, metabolite extraction methodology was performed as previously published (Duval et al. 2013) and may explain the lag phase. In short, cells were washed with phosphate buffered saline and then metabolites were extracted with ice-cold 80% ethanol solution. Alcohol based solutions

promote entropic collapse of proteins and as PKM2 is a binding partner of SAICAR, it is possible that SAICAR remains bound to PKM2 upon alcohol exposure and therefore unmeasurable due to protein sequestration. Neither of these hypotheses are particularly attractive. ZMP also exists in equilibrium with its dephosphorylated form AICAriboside (Vincent, Bontemps, and den Berghe 1996) which can be similarly excreted. ZMP is also an allosteric effector of AMPK and has been shown to have myriad binding partners (Douillet et al. 2019), although not much has been explored for binding affinities and sites of these other protein partners. However, the AMPK-ZMP interaction has been characterized; ZMP induces phosphorylation of Thr172 as well as direct allosteric activation of phosphorylated AMPK (Hardie 2011). The mechanism of action for the SAICAR-PKM2 interaction is still being elucidated. However, evidence suggests that it binds dimeric PKM2 and may induce tetramerization (Dayton, Jacks, and Vander Heiden 2016) or produce activity directly in the dimeric form (Ming Yan et al. 2016) and it is plausible that SAICAR only influences PKM2 activity when bound (i.e. binding does not induce a post translational modification). This would give credence to the protein-metabolite sequestration hypothesis posited. Debris pellet after ethanol extraction could be solubilized with ammonium and metabolites released to assess this possibility. SAICAriboside excretion can be assessed via measuring media concentration of SAICAriboside by the cheap, sensitive, and simple Bratton Marshall assay (Jurecka et al. 2015).

ADSL was found to have a single nucleotide polymorphism that resulted in a single amino acid point mutation in Neanderthals compared to modern day humans. This amino acid change from Neanderthals to modern day humans is found across the species

and is not attributed to genetic variation within the species. The mutation in ADSL from Neanderthal to modern human manifests as decreased stability of the ADSL protein structure in humans, as monitored by circular dichroism spectra (Stepanova et al. 2020). This result is incorporated into a manuscript that is currently submitted to eLife. This work is an insight into DNPS evolution and refinement of protein sequences for optimal purine synthesis.

Immortalized cell lines, especially the HeLa line, are known to drift and have variability over time and passage count. The cells used within this study are variable from the start. Within chapters 2, 3, and 4, the CRISPR DNPS-KO cells were gifts from our collaborators, Drs. Veronika Barešová and Marie Zikánová from The Charles University in Prague, Czech Republic. These cells were made from an ATCC sourced, CCL2 HeLa line. The WT HeLa cell line were purchased from ATCC by our own lab. The comparisons drawn, from a HeLa cell line in culture in the Czech Republic to our own HeLa cell line could likely produce artefacts in the analysis. In 2019, a manuscript was published comparing 14 HeLa strains in culture from 13 independent labs across the globe. They employed multi omics analysis to understand the differences seen across all of the lines. There are three common HeLa cell lines, CCL2 (original HeLa), S3 (third clone isolated from the CCL2 line), and Kyoto; the study mainly centered around the CCL2 and Kyoto lines. PCA analysis of copy number variation, mRNA, protein, and k_{loss} (a measure of protein turn-over rate) reveals two distinct clusters, Kyoto and CCL2 lines. The plots show that while intra cell line variation was notable, there is indeed clustering (Y. Liu et al. 2019). These results indicate that our experimental design, while not ideal, is with merit. Indeed, CD36 gene transcript was found robustly expressed in all of our

DNPS-KO cell lines, was also highly expressed in an analogous experiment performed in the CHO cell lines. qPCR was also performed almost a year later with freshly harvested RNA. These results bolster my position of the validity of the experimental parameters.

In the crADSL and crATIC analyses, transcriptomic terms were returned that are consistent with the phenotype, such as neural development and function, muscle function, embryonic development, and energy functions. The consistency of these terms to the phenotype indicates that we are indeed on the correct path to identify the underlying issues resulting from DNPS deficiency and processes likely influenced by DNPS.

Future Directions

Commonalities within DNPS HeLa-KOs: The CD36-PPARγ axis

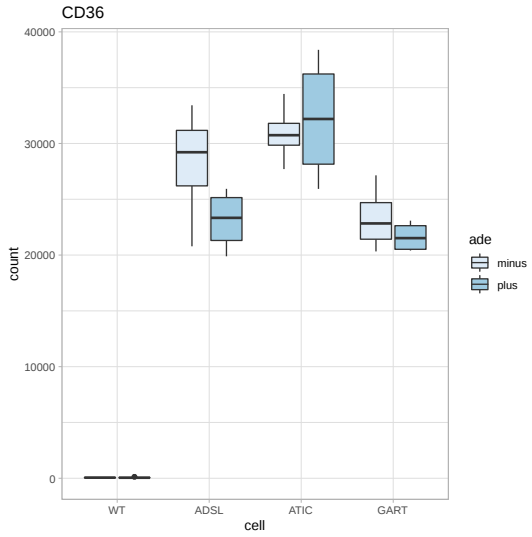


Figure 6.2: CD36 gene count in HeLa and DNPS-KO mutant cell lines. Box plot of CD36 transcript counts of replicates by cell type and purine supplementation. WT HeLa cells, with fully functioning DNPS pathway, show negligible levels of CD36 transcript while all DNPS-KO mutants show elevated transcript levels, purine supplementation in the form of adenine shows negligible effect on transcript levels. Figure generated by Dr. Guido Vacano.

The data presented in this dissertation show similarities and differences in transcriptomic expression. These similarities are likely due to generalized DNPS deficiency and can provide key insight to processes likely affected by DNPS dysregulation.

A common theme amongst the transcriptomics data set is the vast increase within the transcript count for the scavenger receptor gene Cluster of Differentiation 36 (CD36), also known as fatty acid translocase seen in all genetic knockouts (Figure 6.2);

CD36 was one of the most differentially regulated genes, transcript level does not appear to be augmented by adenine supplementation (Figure 6.2). CD36 has been found linked to a wide range of seemingly unrelated cellular processes and pathologies. CD36 is involved in fatty acid uptake and metabolism (Pepino et al. 2014), inflammation cascade (Kuda et al. 2011), angiogenesis, apoptosis, thrombosis, atherosclerosis, Alzheimer's disease, and insulin resistance (Silverstein and Febbraio 2009). Unsaturated fatty acids such as ω3 and ω6 class were found to alter CD36 mRNA expression with increase or decrease dependent on cell type (Vallvé et al. 2002; Pietsch et al. 1995).

Peroxisome proliferator activated receptor gamma (PPARγ) transcript was also found elevated within all DNPS-KO cell lines, however gene expression does appear to decrease slightly with adenine supplementation (Figure 6.3). 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 (Kroker and Bruning 2015). PPARγ and CD36 are interrelated, where CD36 incorporates a signal and activates PPARγ transcription activity. PPARγ transcription

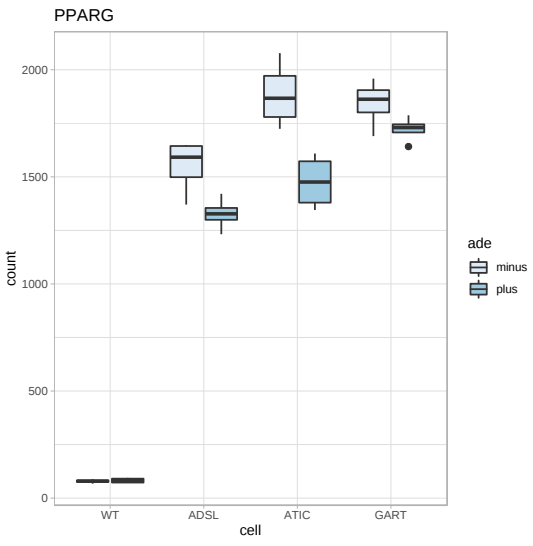


Figure 6.3: PPARγ gene count in HeLa and DNPS-KO mutant cell lines. Box plot of PPARγ transcript counts of replicates by cell type and purine supplementation. WT HeLa cells, with fully functioning DNPS pathway, show negligible levels of PPARγ transcript while all DNPS-KO mutants show elevated transcript levels, purine supplementation in the form of adenine slightly decreases mean transcript levels in DNPS-KOs negligible effect on transcript levels. Figure generated by Dr. Guido Vacano.

activity appears to be signal source specific, as agonist binding and ligand induced phosphorylation cause different subsets of PPARγ regulated genes to be expressed (Kroker and Bruning 2015). Curiously, CD36 is a target gene of agonist based PPARγ transcriptional activity (Kroker and Bruning 2015). The CD36- PPARγ axis has shown involvement in modulating fatty acid storage, triglyceride synthesis, glucose uptake, regulating fatty acid metabolism (Maréchal et al. 2018). It is unclear as to why DNPS-KOs would be robustly expressing transcript for these genes.

The ontologies elucidated suggest that inflammatory signaling and fatty acid processing are systems of interest, due to the control of CD36-PPARγ within these, we hypothesize that DNPS plays a role in mediating a chronic inflammatory cascade, as a complete rewiring of cells not native to expressing both PPARγ and CD36 are not likely to generate a new process for acute inflammation, and would instead rely on other means. Another plausible explanation is a defect in energy homeostasis. This idea is supported by the metabolomics data set, due to the large enrichment of differentially regulated metabolites as a function of GART enzyme KO suggesting an altered cellular energy landscape. DNPS-KOs may be more reliant on fatty acid hydrolysis, a process that can be regulated by CD36-PPARγ axis, for the generation of high energy elections for oxidative phosphorylation. Work has begun to i) identify if the increased transcript correlates to protein levels (Figure 6.4) and ii) to understand the DNPS-CD36-PPARγ

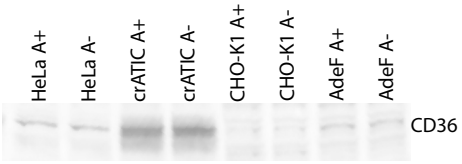


Figure 6.4: Preliminary Western Blot of cellular lysate, CD36. HeLa, crATIC, CHO-K1, and AdeF cells were grown and purine starved as previously described, lysed in RIPA buffer, and 30μg total protein was separated on 10% TG gel. Gel was transferred to a NC membrane and processed using 1:1000 αCD36. crATIC shows elevated levels of CD36 protein over HeLa. A+/- denotes with or without supplemental adenine

phenomenon through probing fatty acid oxidation and glycolytic flux as well as altering pro- and anti-inflammatory fatty acid signaling.

These transcript regulation findings are likely not an artefact of our methodology. Indeed, our laboratory performed RNA-seq on analogous DNPS-KO mutants to those presented here in the Chinese Hamster Ovary (CHO) cell line and showed increases of CD36 and PPARγ transcript over their WT CHO-K1 counterpart (Data not shown). These CHO mutants were made over three decades ago with vastly different techniques than CRISPR (Patterson 1975; 1976; Oates and Patterson 1977).

crADSL may provide insight for epithelial to mesenchymal transition

Transcriptomic analysis of the crADSL cell line uncovered potential research targets in epithelial to mesenchymal transition (EMT). EMT is a process by which polar, adherent epithelial cells undergo changes and become unattached and freely motile mesenchymal cells. EMT is strongly associated development, however the processes able to reactivate after development in certain instances like wound healing and cancer. Mesenchymal cells have increased resistance to apoptosis and are highly migratory and invasive (Kalluri 2009; Kalluri and Weinberg 2009). Loss of proper EMT control can result in deleterious processes such as tumor metastasis (Thiery 2002) and is therefore extensively studied in cancer biology. The ADSL substrate, SAICAR, is a known PKM2 activator, an enzyme directly related to cancer metabolism as well as metastasis (Fan et al. 2014; Hamabe et al. 2014).

The crADSL line presents a novel approach to understanding EMT. DNPS is critical for proper embryonic development and its substrate, SAICAR, is an effector of PKM2, an enzyme thought to be the center of the Warburg effect, altering most tumor's

energy production pathways. EMT can be initially assessed via addition of TGFβ or with EMT Induction Supplement and screened for biomarker proteins such as e-cadherin and fibronectin (Tang et al. 2013). Migration and infiltration experiments can also be assessed using similar methodology in a three-dimensional cell culture. The crADSL cell line can be used for understanding the role of DNPS as well as SAICAR in the EMT process, showing benefit for developmental and tumor biology.

ADSL deficiency has clinical relevance, manifest as a developmental disorder. Deficiency is typically diagnosed via identification of the dephosphorylated substrates of ADSL, SAICAR and SAMP (Figure 6.1) (dephosphorylated forms known as SAICAriboside and SAdo) in biofluids, although with the recent advent of the affordable and rapid Next Gen whole genome sequencing (Metzker 2010; Dunn et al. 2018) we expect more patients to be identified by genomic means. Generally speaking, severity of phenotype is correlated to the SAdo:SAICAriboside ratio, with values above 2 are mild with values closer to 1 present as severe (Jurecka et al. 2015). The variability in the phenotype is striking, ranging from fatal neonatal encephalopathy with hypokinesia, to developmental delay compatible with life at least to the teenage years (Gitiaux et al. 2009; Mouchegh et al. 2007). A heterozygous patient with two missense mutations in or near the catalytic active site, E80D and D87E, presented with a mild phenotype, although no reference data on substrate accumulation is known. It is not known if the patient is expressing both transcripts or if both mutant forms of ADSL proteins are present. Most studies of ADSL mutations have been performed using cell free extracts or purified proteins. The ADSL mutations, E80D and D87E, were identified in a single patient. Our lab were collaborators on the intital biochemical characterization of these mutants

(Sivendran et al. 2004). Previous work on the E80D and D87E ADSL mutants reveal increased stability and large amounts of aggregate formation of the D87E mutant while E80D show slightly decreased levels stability and slightly elevated aggregate formation over WT (Ray 2013). Currently, transfection of WT, E80D, and D87E mutant enzymes into the crADSL cell line show proliferative growth in adenine with WT and E80D ADSL, but not the D87E mutant. The aggregation characteristics of D87E may lead to sequestration or increased proteolytic clearance of this isoform in cells. We therefore hypothesize that only E80D ADSL protein is made in the cell, and will use this system to gain insight into isoform selection of this heterozygous patient. These two phenomena would likely explain the crADSL mutant isoform purine requirement differences.

crATIC and ZMP accumulation provides insight into energy production and inflammatory effects

The ZMP metabolite that accumulates in the crATIC cell line is a known AMP mimetic and activator of AMPK, a master regulator of cellular metabolism. AMPK sensitivity is diminished with age (Reznick et al. 2007) and shows aberrant hyperactivation in Alzheimer's disease neurons (Vingtdeux et al. 2011). Alzheimer's disease is characterized by neural extracellular amyloid β plaques and tau fibrils, as well as low grade chronic peripheral and neural inflammation (Kinney et al. 2018). Within the brain, mitochondrial function is diminished, tissue becomes insensitive to insulin signaling, and there are general aberrations in cellular energy homeostasis (Yin et al. 2016). AMPK is not only an effector of cellular energy metabolism, but also has been implicated in regulating inflammation (X. Chen et al. 2018). In an animal model of induced rheumatoid arthritis (a known model of chronic inflammation), pro-inflammation

signaling induced by complete Freund's adjuvant was mitigated by administration with AICAriboside (Xiang et al. 2019). The crATIC HeLa cell line represents an endogenous source of ZMP, non-reliant on drug interactions, gene silencing RNA, or exogenous AICAriboside. Therefore, the utilization of crATIC represents a novel means of understanding energy homeostasis and fatty acid processing in inflammatory disorders. Fatty acids in inflammatory cascades are processed via enzymatic activity, with the resultant molecule producing a pro- or anti-inflammatory effect. Arachidonic acid is an ω-6 fatty acid, with potential to be processed into the largely pro-inflammatory molecules prostaglandins and thromboxanes (Hanna and Hafez 2018). The ω-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid are typically processed into potent anti-inflammatory specialized proresolving mediators such as maresins and resolvins (Calder 2012). Given the energetic dysregulation and chronic inflammation characterized by Alzheimer's disease, a cellular model capable of accumulating ZMP to regulate AMPK activation and therefore temper inflammation as well as energy metabolic processes is attractive. In this model, I would achieve insight into energy signaling roles seen in chronic inflammatory disorders such as AD. Within these experiments, I propose to induce an inflammatory response with lipopolysaccharide or Amyloid β (Zhu et al. 2016) at various time points of ZMP accumulation. Mass spectrometry is a beneficial tool capable of identifying and, in certain cases, quantifying metabolite profiles. I would employ lipidomic MS techniques to identify free fatty acid composition and concentration ratios to understand pro- or anti-inflammatory signaling cascades as a function of AMPK activation and energy homeostasis derived fatty acid processing.

crGART reveals neural and placentation embryonic development defects

A theory for the root cause of Down syndrome (DS) is the Gene Dosage hypothesis. This postulates that the phenotype of DS is the result of the cumulative effect related to the triplication and overexpression of the genes located on chromosome 21 (Gardiner 2004). Here, I have outlined possible cellular processes influenced by the Hsa21 chromosome GART.

Alkaline phosphates are a class of four membrane bound exoenzymes, in humans, responsible for the hydrolysis of phosphate esters (Štefková, Procházková, and Pacherník 2015). Our results have shown that, in the crGART cell line, three alkaline phosphatase enzymes had elevated transcript levels whereas tissue non-specific alkaline phosphatase (TNAP) showed heavily decreased transcript levels compared to WT HeLa. Curiously, the three elevated genes are ALPP, ALPI, and ALPG are located together on chromosome 2, while the decreased gene, ALPL, is located on chromosome 1 (NCBI, GeneCards). TNAP is encoded by the alkaline phosphatase liver/bone/kidney (ALPL) gene and is abundant in these tissues although expression has been shown fairly ubiquitous (Sebastián-Serrano et al. 2015; Štefková, Procházková, and Pacherník 2015). TNAP activity is observed during neural developement (Langer et al. 2007), with expression promoting axonal growth (Díez-Zaera et al. 2011). The altered regulation of alkaline phosphatases, especially TNAP, may provide novel insight to mechanisms associated with DNPS dysfunction, axonal growth/synapse formation, and associated cognitive defects.

Our transcriptomic analysis of crGART revealed perturbations in gene expression related to placental development, with emphasis related to the labyrinthine layer. As

DNPS is heavily relied upon for development, this finding is unsurprising. The placenta is the first and largest embryonic organ, responsible for hormone signaling as well as gas and nutrient exchange between the mother and embryo (Turco and Moffett 2019; Gude et al. 2004). The trophoectoderm forms in a pre-implantation embryo with interaction between the trophoectoderm and the uterine wall driving successful embryonic implantation (Pillai et al. 2019; Knöfler et al. 2019) and begins to gain complexity and structure upon implantation deriving the trophoblast lineage and proper placentation begins (Knöfler et al. 2019). Analysis of morphology of over 100 embryonic lethal mouse models have revealed that a majority of these lines exhibit placental malformations, with fetal phenotype commonalities of abnormalities in cardiac, neural, and vascular systems (Perez-Garcia et al. 2018). The labyrinthine layer is a heavily vascularized embryonic derived structure. Mouse embryonic stem cells lacking or triplicated GART would be beneficial for understanding the early phases of pre-implantation trophoectoderm differentiation. While a true living mouse model lacking GART would be ideal for understanding placentation as a function of DNPS dysregulation, this is not feasible as DNPS is necessary for embryonic development and the embryo would likely not survive, in fact so far it has not been possible to isolate GART null mice. Transgenic mice expressing human GART under its own regulatory elements have been made in two laboratories, Dr. David Patterson's laboratory and the laboratory of John Gearhardt at Johns Hopkins. In both cases there was minimal if any alteration in phenotype. Nonetheless, the crGART mutant could be used to make transfected cell lines expressing different levels of GART activity. Comparison of the

transcriptomes of these lines should allow precise definition of which transcript changes are actually due to alterations in GART levels.

Metabolic effects for Vitamin B6 and DNPS dysregulation

Clinical manifestation of DNPS dysregulation as well as DS are characterized by seizure activity (Marie et al. 2004; Jurecka et al. 2015; Lott and Dierssen 2010). Seizure activity was not identified in PAICS deficient patients, however the only two patients identified died within three days postpartum (Pelet et al. 2019) making observation unlikely. Metabolomic analysis of the crGART line showed differential regulation of small molecules related to myriad processes, among them was Vitamin B6 metabolism. B6 is a vitamer involved as a cofactor for folate synthesis (Kennedy 2016), neurotransmitter synthesis (Calderón-Ospina and Nava-Mesa 2020), mitochondrial function (Janssen et al. 2019), and amino acid and glutathione metabolism (Dalto and Matte 2017). In neurotransmitter synthesis, B6 acts as a cofactor in the formation of serotonin, dopamine, as well as GABA (Kennedy 2016; Calderón-Ospina and Nava-Mesa 2020). GABA, largely known as the main inhibitor neurotransmitter (Contestabile, Magara, and Cancedda 2017) and is synthesized from the precursor glutamate, an excitatory neurotransmitter (Deidda, Bozarth, and Cancedda 2014). Dysregulation of B6 can lead to GABA deficiency and excess glutamate, creating an imbalance between synaptic excitation and inhibition potential manifesting as seizures (Wilson et al. 2019). Indeed, B6 supplementation has been shown beneficial in mitigating chronic seizure activity (Spinneker et al. 2007; Ohtahara, Yamatogi, and Ohtsuka 2011) as well as efficacious in mitigating neurotoxin induced seizures (Dakshinamurti, Sharma, and Geiger 2003).

Seizure activity in individuals with DNPS deficiencies may stem from a B6 metabolic deficits or improper dosage of TNAP, a regulator of B6. B6 exists as an active, but non-cell permeable phosphorylated form and a cell permeable dephosphorylated inactive form. B6 is ingested in its phosphorylated form and able to circulate freely. TNAP dephosphorylates B6, crossing cellular membranes or the blood brain barrier, and subsequently rephosphorylated, trapping it inside the cell (Calderón-Ospina and Nava-Mesa 2020). To probe this, DNPS-KO or knock-down neuronal cells would be generated, targeting GART first, and assessed for TNAP transcript and protein activity in culture (Díez-Zaera et al. 2011) as well as modulating vitamin B6 media levels. Understanding excitatory and inhibitor neurotransmitter load as a function of DNPS will yield insights into neurotransmitter synthesis and processing. Quantification of neurotransmitter load will also be assessed under purine starved and rich conditions, as well as by GART activity level via MS techniques to understand if conversion from glutamate to GABA is occurring.

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