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MODELING THE REPRODUCTIVE PHENOTYPES OF ADENYLOSUCCINATE LYASE DEFICIENCY IN *C. ELEGANS*

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ABSTRACT

Mutations in enzymes that function in purine metabolism result in human syndromes with a wide variety of symptoms. Adenylosuccinate lyase deficiency (ASLD) is characterized by the decrease in function of adenylosuccinate lyase (ADSL), a bi-functional enzyme within *de novo* purine biosynthesis. In humans, this syndrome is characterized by neuronal, developmental, and metabolic defects which include symptoms of seizures, encephalopathy, psychomotor retardation, and autistic features. The molecular mechanisms driving this disease are currently unknown. I report on the reproductive phenotypes associated with a knockdown of ADSL in *Caenorhabditis elegans*. I identify sterility in animals with significant knockdown of *adsl-1* expression, as well as embryonic lethality and oogenesis defects associated with a partial knockdown of *adsl-1* expression. Using supplementation, I correlate these phenotypes with a decreased flux through *de novo* purine biosynthesis. Although reproductive phenotypes are not directly correlated with human symptoms, these findings still have impact on the role of purines in the development of patients with ASLD.

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LIST OF ABBREVIATIONS

ADSL	adenylosuccinate lyase
ASLD	adenylosuccinate lyase deficiency
SAICAR	succinylaminoimidazole carboxamide ribotide
SAICAr	succinylaminoimidazole carboxamide riboside
S-AMP	succinyladenosine monophosphate
S-Ado	succinyladenosine
R5P	ribose-5-phosphate
AICAR	aminoimidazole carboxamide ribotide
FAICAR	formamidoimidazole carboxamide ribotide
IMP	inosine monophosphate
AMP	adenosine monophosphate
XMP	xanthine monophosphate
GMP	guanosine monophosphate
MTX	methotrexate
Ad	adenosine
Gu	guanosine

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Chapter 1. Introduction

Inborn Errors of Purine Metabolism

Rare syndromes associated with inborn errors of purine metabolism are a result of mutations in enzymes that function within the purine biosynthetic pathway. Due to low prevalence, limited awareness and phenotypic variability, many of these disorders are either undiagnosed or misdiagnosed.[1] Severe clinical manifestations, including death, can be associated with several of these syndromes; due to the similarity of many symptoms with other disorders, abnormal accumulation of purine metabolites is typically the only way to diagnose them.[1] Often times, if the disorder is treated early the most severe symptoms can be avoided; however, a number of these conditions have no known effective treatments.[1] Using a simple, yet effective system to model defects in purine metabolism provides further insight into the mechanistic nature of these crippling syndromes for future therapeutic development.

Purines nucleotides are considered basic building blocks of life along with pyrimidine nucleotides. Together they form DNA and RNA, but purines alone also have roles in cellular metabolism, energy conservation and transport, and formation of coenzymes.[2] There are two main pathways by which purines are synthesized: *de novo* and salvage. The *de novo* pathway consists of eleven steps starting with ribose-5-phosphate (R5P) and ending with inosine monophosphate (IMP), which can subsequently be converted into purine nucleotide monomers (Fig 1). The salvage pathway uses purines from the diet or elsewhere in the body to synthesize new purine products.

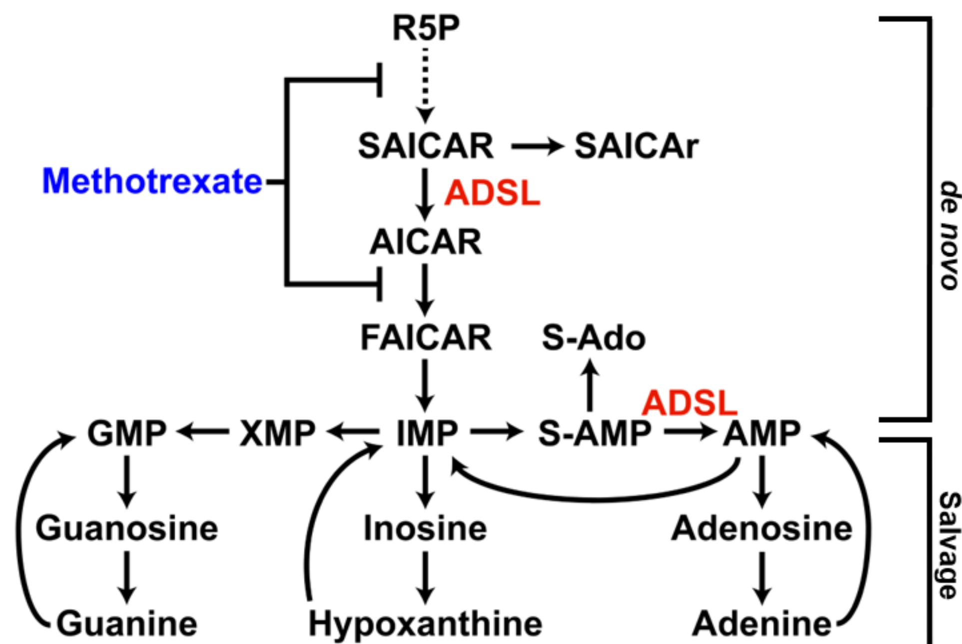

Figure 1. Purine biosynthesis pathways.

The *de* novo and parts of the salvage purine biosynthesis pathways are conserved across most eukaryotes.[3] ADSL is a bifunctional enzyme in *de novo* purine biosynthesis. Methotrexate is a small molecule that inhibits amidophosphoribosyltransferase, the second enzyme in *de novo* synthesis, and indirectly inhibits AICAR transformylase, another enzyme in *de novo* synthesis.[4,5] Abbreviations: Ribose-5-phosphate (R5P); succinylaminoimidazole carboxamide ribotide (SAICAR); succinylaminoimidazole carboxamide riboside (SAICAr); aminoimidazole carboxamide ribotide (AICAR); formaminoimidazole carboxamide ribotide (FAICAR); inosine monophosphate (IMP); succinyladenosine monophosphate (S-AMP); succinyladenosine (S-Ado); adenosine monophosphate (AMP); xanthine monophosphate (XMP); guanosine monophosphate (GMP). *Figure adapted from Fenton, Janowitz, McReynolds, Wang, Hanna-Rose submitted July 2017.

Adenylosuccinate Lyase Deficiency

Adenylosuccinate lyase (ADSL) is a bifunctional enzyme within the *de novo* purine synthesis pathway. It catalyzes the conversion of succinylaminoimidazole carboxamide ribotide (SAICAR) to aminoimidazole carboxamide ribotide (AICAR), and succinyladenosine monophosphate (S-AMP) to adenosine monophosphate (AMP) through the cleavage of a fumarate (Fig 1). Adenylosuccinate lyase deficiency (ASLD) is a human disorder that results from inadequate enzymatic activity. Clinical manifestations include any combination of seizures, ataxia, encephalopathy, psychomotor retardation, and autistic-like features – nonverbal, repetitive behaviors, failure to maintain eye-contact, irritability, and aggressiveness to name a few.[6,7] Accumulation of the dephosphorylated versions of SAICAR and S-AMP, succinyl-aminoimidazole carboxamide riboside (SAICAr) and succinyladenosine (S-Ado), in urine and cerebrospinal fluid is the only method of diagnosis.[6] To date, more than 80 patients have been reported with over 40 different mutations within ADSL.[7] The prognosis for patients is variable: the most critical cases, classified as fatal neonatal, resulting in premature death.[7]

There are several hypotheses regarding the pathology of ASLD. Patients have varying residual functionality of ADSL, which is negatively correlated with the severity of their symptoms.[7,8] This is contrary to the initial thought that the ratio of S-Ado to SAICAr was responsible for the severity, with higher ratios corresponding to less severe, leading to the hypothesis that S-Ado was protective while SAICAr was toxic.[7–9] Although in many cases, lower ratios of S-Ado to SAICAr are correlated to more severe symptoms, it is now hypothesized that the differences have to do with the age of the patient during analysis.[7,8] Neurotoxicity of

succinylpurines in humans has yet to be demonstrated, thus their role in ASLD pathology is still to be determined.[7]

An alternate hypothesis concerns the blockage in *de novo* synthesis of purines contributing to disease etiology. A decrease in ADSL activity suggests inhibited flux through the *de novo* pathway, thus causing a larger reliance on salvage synthesis of purines. A decreased concentration of purine products may be expected, especially those containing adenine due to the enzyme's multifunction in the production of AMP. However, this is not the case in ASLD patients as there is no significant difference in liver, kidney, and muscle purine levels.[10] ADSL activity is not fully eradicated by the genetic mutations – the largest reduction in patients being 3% of normal function – thus the residual activity may be sufficient to support overall purine levels in conjunction with the salvage pathway.[7,11] It is also possible that tissue types aside from liver, kidney, and muscle are more severely affected by ADSL loss of function resulting in a specific tissue deficiency in purines that leads to ASLD symptoms; however, there is no current data to support this theory.

Currently, there are no effective treatments for patients with ASLD.[12] Several therapies have been tested including, supplementation of D-ribose – a precursor to ribose-5-phosphate – or S-adenosyl-l-methionine – a proposed alternative to adenosine, which have had no significant results.[7,12,13] Additionally, patients that present with severe seizures have been treated using traditional methods for epilepsy, such as anticonvulsants and a ketogenic diet, to reduce seizure activity.[7] Despite ASLD's recognizable and diagnosable etiology, the molecular mechanisms have yet to be clarified, thus targeted and efficient therapies cannot be developed.

Using *C. elegans* as a Model System

Studying human syndromes in invertebrate model systems is a cheap and effective way to grasp basic understanding of the mechanistic nature of a disease. *Caenorhabditis elegans* are nematodes that are commonly used as a model organism due to the accessibility of their genome and well-studied development.[14] *C. elegans* are self-fertilizing hermaphrodites or males which allows their genetics to be easily manipulated and maintained.[14] Moreover, their transparent cuticles allow cellular processes to be directly visualized.[14] In addition to the structural characteristics and basic genetics of *C. elegans*, the *de novo* purine biosynthetic pathway is conserved (Fig. 2), just as it is in most other eukaryotes, making *C. elegans* a functional model to study ASLD.[3]

I developed a *C. elegans* model to probe the molecular mechanisms linking phenotypes of ASLD to distinct disruptions in purine biosynthesis in order to develop therapeutic approaches. The homology of purine biosynthesis in *C. elegans* and humans in conjunction with various genetic tools allow for specific targeting of genes for analysis. Specifically, RNA interference (RNAi) involves feeding *C. elegans* an *E. coli* strain expressing double stranded RNA in order to obtain gene-specific knockdown.[15] This genetic tool allowed me to establish an efficient working model of ASLD in *C. elegans.*

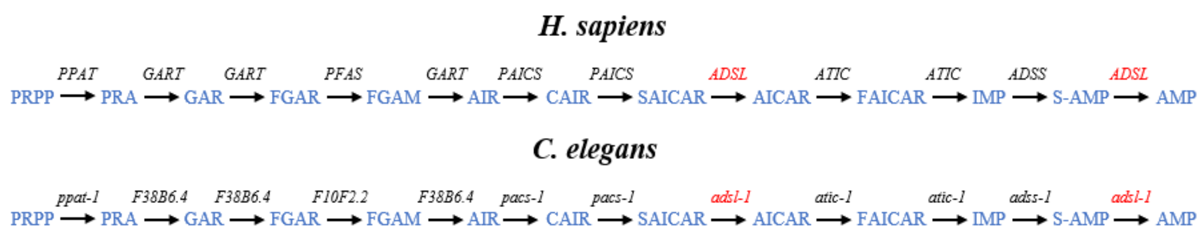

Figure 2. A comparison of *de novo* purine biosynthesis in *H. sapiens* and *C. elegans*.

The homologous genes encoding each enzyme are listed for the *de novo* purine biosynthesis pathways in humans and *C. elegans.* The metabolites in the pathway are indicated in blue and the genes encoding adenylosuccinate lyase are indicated in red.

Chapter 2. Results

*The following data can also be found within a manuscript by Fenton, A., Janowitz, H., McReynolds, M., Wang, W., Hanna-Rose, W., which is currently under review, titled: "A *Caenorhabditis elegans* model of adenylosuccinate lyase deficiency reveals neuromuscular and reproductive phenotypes of distinct etiology."

A deficiency in ADSL results in reproductive defects

I used *adsl-1*(RNAi) and *adsl-1(tm3328)* null mutants to look for phenotypes associated with a deficiency in ADSL in *C. elegans*. Anytime I knocked down *adsl-1* via RNAi, I used *eri-1 (mg366)*, which is a strain of *C. elegans* that has increased sensitivity to RNAi due to the loss of an exonuclease that targets siRNAs.[16] I exposed animals in their fourth larval stage to RNAi of *adsl-1* and progeny were produced, but with 18% embryonic lethality (Fig. 3). Continued growth of the progeny with RNAi of *adsl-1* resulted in 100% sterility of this generation (n>40). *adsl-1 (tm3328)* mutants also displayed sterility so they were maintained as balanced heterozygotes with the balancer containing GFP allowing for animals homozygous for *tm3328* to be selected. A typical *C. elegans* hermaphrodite generates approximately 300 progeny over their entire reproductive period.[14] A disruption of ADSL function results in the inability of these animals to produce healthy offspring that will mature into healthy adults. I conclude that ADSL is needed for normal reproduction.

In order to further characterize the reproductive defects, I compared the gonad arms of adult homozygous *adsl-1(tm3328),* a loss of function null allele for *adsl-1,* to the standard N2

control strain (Fig. 4A) and they appeared shrunken and deformed, without any indication of normal germ cell production or oogenesis (Fig. 4B). I also compared gonad arms of *adsl-1*(RNAi) to an empty vector(RNAi) and saw a similar phenotype, though less severe. Due to decreased severity, I verified the knockdown efficiency of *adsl-1*(RNAi) using qRT-PCR to measure relative mRNA levels. Compared to the empty vector (EV) RNAi, *adsl-1* expression was decreased by 82% in *adsl-1* RNAi (Fig. 5). Because there is a lack of formation of a proper gonad in either *adsl-1(tm3328)* or *adsl-1*(RNAi), I conclude that ADSL function is necessary for gonadogenesis.

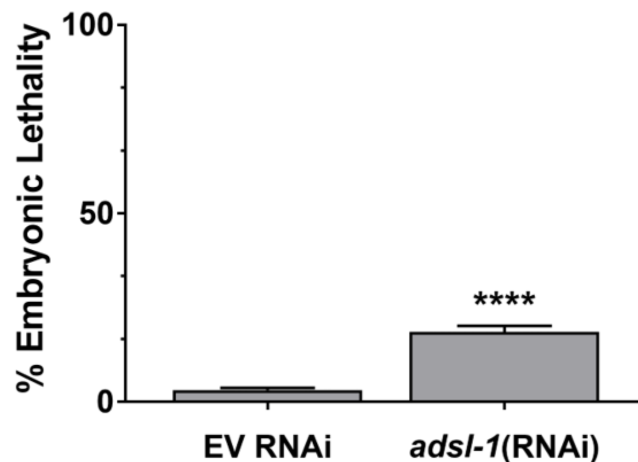

Figure 3. Disruption of ADSL function leads to embryonic lethality

Offspring of animals exposed to *adsl-1*(RNAi) during their fourth larval stage display 18% embryonic lethality compared to the 3% of the empty vector control, EV(RNAi). n>450 for each condition. Error bars are 95% confidence intervals. ****, p<0.0001 using a student's two-tailed t test.

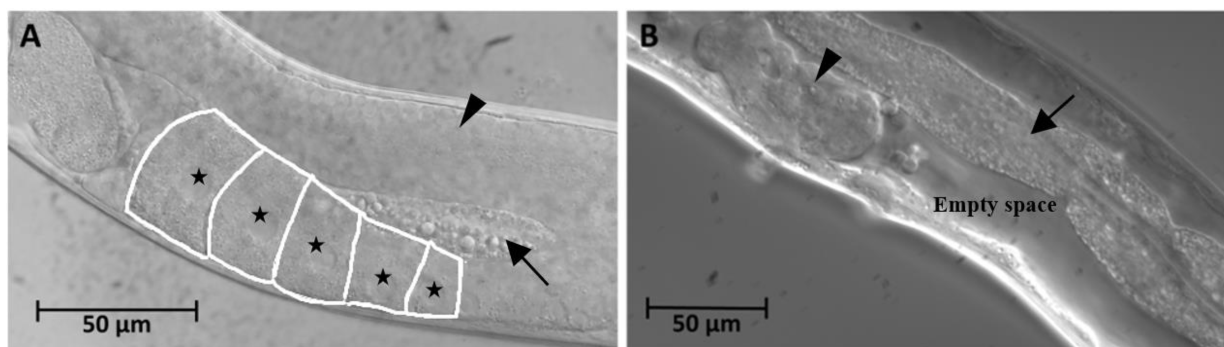

Figure 4. Gonad morphology of *adsl-1(tm3328)*.

(A) N2 gonad displaying normal oogenesis. **(B)** *adsl-1(tm3328)* gonad displaying shrunken and deformed phenotype. Stars indicate oocyte nuclei, arrow heads indicate germ cells in gonad, and arrows indicate intestinal tissue.

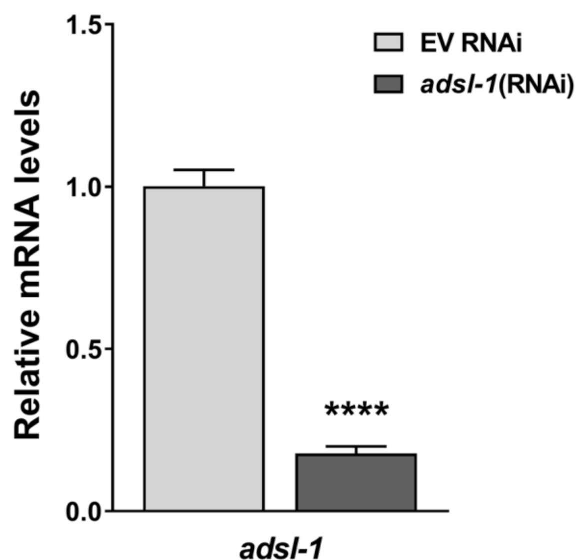

Figure 5. Expression of *adsl-1* is decreased in *adsl-1*(RNAi)

Relative mRNA levels of *adsl-1* were measured in empty vector (EV) RNAi and *adsl-1*(RNAi) using qRT-PCR. Values are averages of two biological replicates, done in duplicate. Error bars represent standard deviation. **** represents $p<0.0001$ using a student's two-tailed t test.

ADSL function is needed acutely for oogenesis

Because embryonic lethality could be induced in the progeny of animals exposed to RNAi of *adsl-1* during their fourth larval stage, I decided to analyze the gonad structure and progression of oogenesis in these animals for defects that may contribute to lethality. After 24 hours exposure, I saw an array of phenotypes not present in the N2 control (Fig. 6A), including distal gonad deterioration (Fig 6B.), double oocytes in the proximal gonad (Fig. 6C), and germ cells in the proximal gonad (Fig. 6D). In normal N2 worms, germ cells can be seen progressing from mitosis through the stages of meiosis I as they migrate proximally within the gonad arm.[17] Double oocytes and germ cells in the proximal gonad indicate that ADSL function is needed acutely for the maintenance of oogenesis; however, its specific role in oogenesis is still unknown.

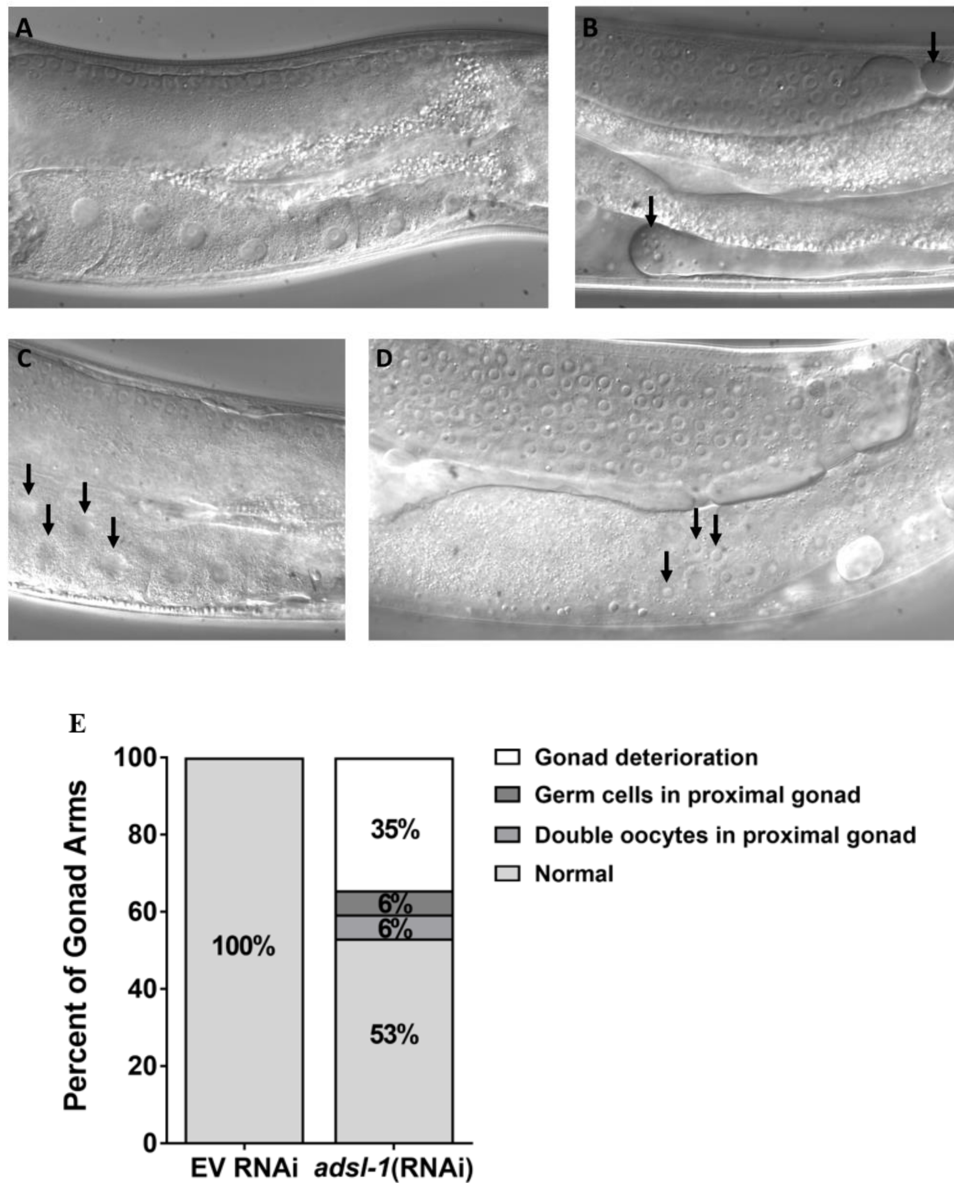

Figure 6. Disruption of ADSL function causes reproductive defects

Gonad morphology and oogenesis are disrupted in animals exposed to RNAi of *adsl-1* during their fourth larval stage. **(A)** Normal N2 gonad, **(B)** deterioration in the distal gonad, **(C)** double oocytes in the proximal gonad, **(D)** germ cells in the proximal gonad, and **(E)** quantification of *adsl-1*(RNAi) reproductive defects compared to N2 control. N=32 animals per condition, two gonad arms per animal.

Reproductive defects correlate with a blockage of *de novo* purine biosynthesis

The literature proposed mechanism for which I have developed two competing hypotheses for symptoms associated with ASLD – a build-up of the potentially toxic intermediate SAICAr, or a decrease in the production of purine products.[6,7] In order to address these hypotheses, I used pharmacological supplementation. The first hypothesis predicted that the accumulation of SAICAr, the riboside form of SAICAR, was toxic.[7–9] In order to test this hypothesis, I supplemented *adsl-1*(RNAi) with methotrexate (MTX), an antimetabolite inhibitor upstream of SAICAR production (Fig. 1).[4,5] MTX supplementation should continue to block *de novo* purine biosynthesis, but there should not be any accompanying accumulation of SAICAR. If SAICAr is toxic and correlating to reproductive dysregulation, then I would expect a restoration of fertility in *adsl-1*(RNAi) animals. When *adsl-1*(RNAi) were exposed to 22 µM MTX, fertility to was not restored (Fig. 6). This negative result is supported by the ability of MTX to alleviate movement defects also seen in both *adsl-1(tm3328)* and *adsl-1*(RNAi).[18] From these results, I conclude that the accumulation of SAICAR is not correlated to the reproductive defects in animals deficient in ADSL.

The alternate hypothesis suggests that a decrease in the production of purine products contributes to ASLD symptoms.[7] To test this hypothesis, I supplemented *adsl-1*(RNAi) with the riboside forms of purines – adenosine (Ad) and guanosine (Gu). By providing additional purine products, I hypothesized that I could replenish any purine deficit that might exist from a blockage in *de novo* synthesis. When I supplemented *adsl-1*(RNAi) with 10 mM Ad, I saw a restoration of fertility in 90% of the animals, n = 20 (Fig. 7A). Additionally, when I supplemented with 10 mM Gu, I saw a restoration of fertility in 80%, n = 20 (Fig. 7A). The overall fecundity of the animals with restored fertility was decreased in comparison to the empty

vector(RNAi) control, with a mean of 48 eggs for Ad supplementation and 30 eggs for Gu supplementation (Fig. 6B). Each purine product had the ability to restore fertility, and fecundity to some degree, in *adsl-1*(RNAi), with Ad being the most effective. Adenosine was tested for its ability to restore fertility in *adsl-1(tm3328)*, but was found to be unsuccessful. From these results, I conclude that the blockage of *de novo* purine biosynthesis contributes to the reproductive defects seen in *adsl-1*(RNAi); however, the more severe defects seen in *adsl-1 (tm3328)* seem to be a result of more than just the blockage of *de novo* purine biosynthesis.

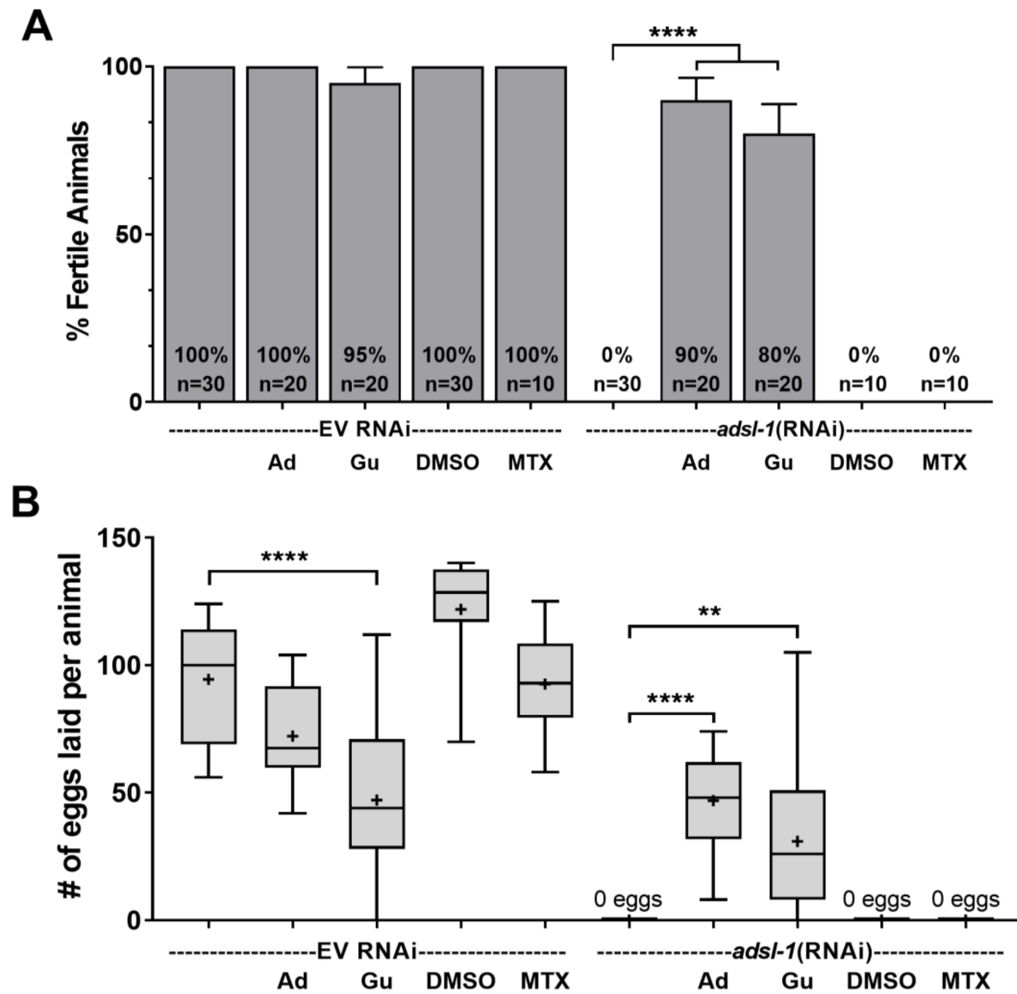

Figure 7. Purine supplementation restores fertility and fecundity in *adsl-1*(RNAi)

(A) Supplementation with 10 mM adenosine (Ad) or 10 mM guanosine (Gu) restores fertility in *adsl-1* (RNAi). Supplementation with 22 µM methotrexate (MTX) does not restore fertility. Error bars are 95% confidence intervals. **(B)** Supplementation with 10 mM adenosine or 10 mM guanosine restores fecundity in *adsl-1*(RNAi). Supplementation with 22 µM methotrexate does not restore fecundity. Boxes indicate the upper and lower quartiles, + indicates the mean, and the line indicates the median. Error bars indicate the maximum and minimum within the population. *, **, and **** represent $p<0.05$, $p<0.01$, and $p<0.0001$, respectively. Significance was calculated using ANOVA.

Global purines levels are unaffected by *adsl-1* knockdown

Another member of the lab conducted a metabolomic analysis of *adsl-1*(RNAi) using liquid chromatography – mass spectroscopy to quantify the metabolic levels of purines. The levels of four purine monophosphates – AMP, GMP, IMP, and XMP – were measured in *adsl-1*(RNAi) compared to an empty vector control(RNAi). There were no detectable differences between the *adsl-1* knockdown and the control, although AMP did show a consistent downward trend (Fig. 8A). IMP also showed a slight downward trend, but there was more variance between each biological replicate than AMP (Fig. 8B). Both GMP and XMP had a lot of variance between each biological replicate (Fig. 8C-D). From this data, it was concluded that purine levels were unaffected by a knockdown of *adsl-1*.[18] Although global purine levels are unaffected, my findings still indicate that decreased flux through the *de novo* purine biosynthesis pathway is correlated to the reproductive defects.

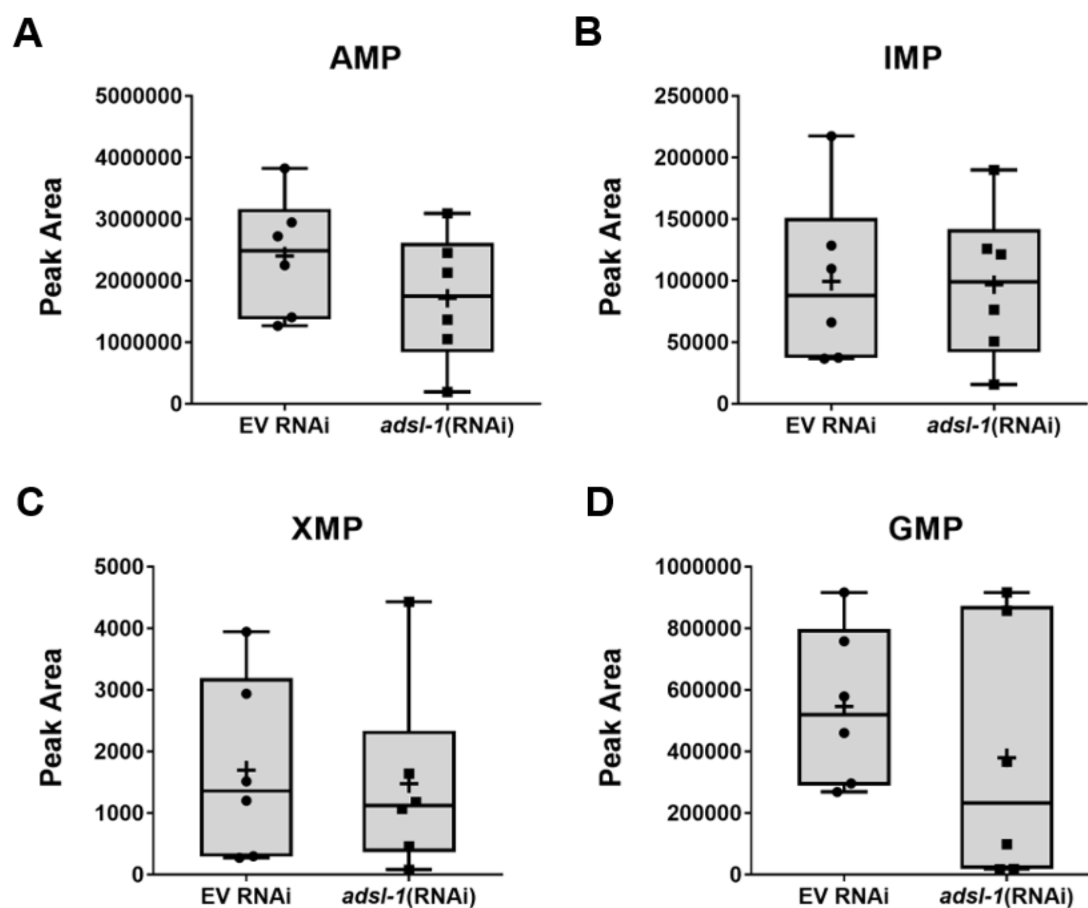

Figure 8. Purine monophosphate levels are unaffected by *adsl-1* knockdown

(**A**) AMP levels trend downward in *adsl-1* knockdowns. (**B**) IMP levels slightly trend downward in *adsl-1* knockdowns. (**C**) XMP levels are variable, but remain unchanged in *adsl-1* knockdowns. (**D**) GMP levels remain unchanged in *adsl-1* knockdowns. None of the changes in metabolite levels are statistically significant. Box plots are described in Figure 5.

Chapter 3. Discussion

In this study, I developed a model of ASLD in *C. elegans* and discovered a correlation between the decreased flux through *de novo* purine biosynthesis and reproductive defects. Previous research suggested that phenotypes of the human syndrome resulted from either a toxic buildup of the dephosphorylated substrate of ADSL, SAICAr, or decreased production of purine products via the *de novo* pathway.[7–9] My data emphasizes that symptoms of ASLD in *C. elegans* are correlated to the decreased production of purine products through *de novo* biosynthesis. Furthermore, this is interesting due to the fact that in both humans and *C. elegans,* global purine monophosphate levels are not depressed.[10,18] Thus, this is a detail that can now be taken into consideration when designing therapies.

In order to investigate the link between ASLD symptoms and distinct molecular perturbations of purine metabolism, first I used RNAi and a mutant allele of *adsl-1* to establish a model of ALSD in *C. elegans.* I identified a host of reproductive defects associated with a knockdown of *adsl-1*, including sterility, embryonic lethality, oogenesis defects, and reproductive development dysregulation. After characterizing the infertility of *adsl-1*(RNAi), I used pharmacological supplementation to probe the associated molecular mechanism and test the proposed hypotheses of the literature. I found that supplementation with either 10 mM adenosine or guanosine restored fertility in 90% and 80% of animals, respectively. It also partially restored fecundity to an average of 48 and 30 eggs per animal, respectively, compared to the 90 eggs per animal for the control. The addition of purines to control animals did not increase fecundity, so I concluded that purines are necessary for normal reproduction in animals with ASLD and are not just beneficial for fertility in general. Additionally, supplementation of guanosine to control

animals actually decreased fertility and fecundity suggesting some level of toxicity. On the contrary, supplementation with 22 μM of methotrexate had no effect on either fertility or fecundity. This data suggests that a toxic buildup of SAICAr does not contribute to reproductive defects in *adsl-1*(RNAi).

Fertility could not be restored in *adsl-1(tm3328)* via adenosine supplementation; however, the gonad deterioration is much more severe in the mutant than in *adsl-1*(RNAi). The *adsl-1(tm3328)* allele contain a 792 bp deletion (55% of the gene) that includes the active site, making it a null allele. Animals homozygous for the allele have a complete loss of ADSL function, whereas the animals exposed to RNAi of *adsl-1* contain only a partial knockdown (Fig. 4). The penetrance of RNAi is variable, but there still remains some residual ADSL activity. The severity of the human syndrome is correlated with residual activity, so it makes sense that this too is the case in the *C. elegans* model.[7,8] It is possible that adenosine supplementation contributed to a partial rescue of fertility, meaning that there could have been increased formation of gonad structures. Animals were only characterized by their ability to lay eggs, so a future analysis of gonad structure during supplementation will be necessary to verify this hypothesis.

Aside from sterility, I also noticed a high degree of embryonic lethality (18%) in the progeny of animals exposed to RNAi of *adsl-1* during their fourth larval stage. The knockdown efficiency increases during the initial exposure to RNAi, so it appeared that as *adsl-1* expression decreased in the embryos, their viability also decreased. This lethality indicates that ADSL function is likely necessary for embryonic development, whether to prevent the buildup of SAICAr or to provide *de novo* purines. Further investigation is necessary to determine which stage the embryos are dying at and if all of them are dying at the same stage. The majority of cell

divisions occur during the first half of embryogenesis, while the larger morphological changes occur primarily during the latter half.[19] Purines are likely in high demand during the period of rapid cell division so a decrease in *de novo* purines may be detrimental for embryogenesis leading to lethality. Alternatively, a specific stage later in embryogenesis may be sensitive to a loss of *de novo* purines, or a buildup of SAICAr.

In order to further investigate the reproductive defects of *adsl-1*(RNAi), I exposed *eri-1* animals to RNAi of *adsl-1* during their fourth larval stage and analyzed their reproductive structures 24 hours later. The development of a *C. elegans* takes approximately 3 days from egg to egg-laying adult.[20] They progress through four larval stages to a young adult and then finally a mature adult. Each stage of development takes approximately 12 hours, except the L1 stage, which is around 16 hours.[20] Gonadogenesis begins during the late L1 stage and continues through the L3 stage, as the germ line begins to develop.[17] Germ cell amplification occurs during the L4 stage, along with spermatogenesis, while oogenesis begins during the young adult stage and continues throughout the reproductive lifetime.[17] Inducing a knockdown of *adsl-1* after the majority of gonadogenesis, but prior to oogenesis allowed me to determine if oogenesis defects were present. After 24 hours, *adsl-1*(RNAi) exhibited distal gonad deterioration (35% of arms), germ cells in the proximal gonad (6% of arms), and double oocytes in the proximal gonad (6% of arms). Each of these defects could be explained through mitotic or meiotic errors. The distal gonad contains a region of replicating germ cells, while the proximal gonad contains oocytes in meiosis I.[21] Additionally, during the maturation of the oocyte, the gonad sheath cells play a role in oocyte progression through the gonad as well as ovulation.[22] Since oogenesis defects could be induced in these previously healthy animals, I concluded that purines synthesized through the *de novo* pathway were needed acutely for normal reproduction.

Because a lack of purines seemed to be correlated to infertility, another member of the lab performed metabolomic analysis on a mixed stage population of *adsl-1*(RNAi) to determine the relative levels of purine monophosphates. No significant difference was found between the empty vector control(RNAi) and the *adsl-1*(RNAi), suggesting that global purine levels are unaffected by the a blockage in *de novo* synthesis.[18] This result was not surprising considering the fact that ASLD patients also show normal levels of purine monophosphates in liver, kidney, and muscle tissue; however, it raises the question of why purine supplementation can restore fertility and fecundity in *adsl-1* knockdowns.[10] Since the metabolite levels were measured in a mixed stage, whole animal population, it remains possible that a specific stage, or perhaps only the reproductive organs are deficient in purines. Deficits resembling these would be masked in a metabolite analysis such as the one we conducted.

Purines via the *de novo* pathway have been previously suggested to play a role in reproduction. Mice that are deficient in 10-formyltetrahydrofolate synthetase activity, an enzyme required for formation of a coenzyme of *de novo* purine biosynthesis, are not viable due to embryonic lethality.[23] Essentially, an indirect blockage in *de novo* purine synthesis prevents normal embryonic development. Both *de novo* and salvage purine pathways have also been implicated in hormone-induced maturation of meiotically arrested oocytes in mice.[24] Even though a deficit in purines is not apparent in *adsl-1*(RNAi), the purines that are synthesized specifically via the *de novo* pathway seem to be important for normal reproduction.

Although reproductive defects have not been investigated or reported in the human syndrome, my findings still have implications for future mechanistic studies. Because there is such a wide array of symptoms associated with ASLD, it remains possible that other symptoms are a result of decreased *de novo* synthesis of purines. This is important to keep in mind as

therapeutic options are developed. Supplementation with purines may contribute to the alleviation of some of the symptoms of ASLD.

Alternatively, some symptoms could be alleviated by blocking the buildup of SAICAr. Another phenotype associated with both *adsl-1*(RNAi) and *adsl-1(tm3328)* are neuromuscular defects resulting in sluggish, irregular movement patterns which can be rescued via supplementation with 22 μM methotrexate, but not purine supplementation suggesting that SAICAr contributes to the phenotype.[18] *adsl-1*(RNAi) animals also have measurable levels of the normally undetectable metabolite SAICAR, which are also decreased upon supplementation of 22 μM methotrexate, further supporting the toxicity of either SAICAr or its phosphorylated version, SAICAR.[18] Furthermore, ASLD patients have detectable levels of SAICAR in their cerebrospinal fluid and present with neuromuscular symptoms like ataxia and psychomotor retardation which could potentially have the same underlying mechanism allowing a similar treatment.[6,7] Importantly, two distinct phenotypes in the ASLD *C. elegans* model appear to have distinct molecular mechanisms, which has significant implications in developing treatments for patients with ASLD.

As a whole, I have developed a model for studying certain characteristics of ASLD in *C. elegans*. My data describes the reproductive defects found in animals deficient in *adsl-1* expression and connects them to a deficiency in purines synthesized *de novo*; however, the exact mechanism associated with these defects requires further analysis. I showed that there are a combination of oogenesis defects and gonadogenesis dysregulation, which may contribute to embryonic defects and infertility. Supplementation with purine products restored fertility and fecundity in *adsl-1*(RNAi), despite apparent normal levels of purines measured via metabolomic

analysis. Overall purine levels may be maintained by salvage synthesis, but my results suggest that purines synthesized explicitly through the *de novo* pathway are important for reproduction.

Chapter 4. Materials and Methods

Nematode strains and maintenance

C. elegans strains were maintained at 20°C on *E. coli* OP50, according to standard methods. The following strains were used: N2, control; *eri-1*; and *adsl-1(tm3328)*. Both the N2 and *eri-1* strains were obtained from the *Caenorhabditis* Genetics Center (CGC). *adsl-1(tm3328)* was obtained from the National BioResource Project in Tokyo, Japan and outcrossed with N2 three times. This allele is homozygous sterile so it was balance with the *C. elegans* 2nd and 3rd chromosome balancer, hT2, which causes pharyngeal expression of GFP. Animals homozygous for *adsl-1(tm3328)* were used for phenotypic analysis.

RNA interference

The *adsl-1* RNAi clone was from the *C. elegans* RNAi Library (Source Bioscience, Nottingham, UK). RNAi was induced through feeding of *E. coli* that contained aforementioned clone.[15] The *E. coli* strain HT115 containing an empty RNAi vector L4440 was used as a control.

Embryonic Lethality Analysis

Embryonic lethality assays were conducted at 20°C on RNAi agar plates (NGM agar with 1mM IPTG, 25 µg carbenicillin) spotted with 200 µL of RNAi culture grown overnight in LB with 100 mg/ml carbenicillin and 12 µg/ml of tetracycline. 2 L4 animals (*eri-1)* were placed on each plate and allowed to lay eggs for 24 hours before moving to a new plate. Every 24 hours the animals were moved to a new plate for a total of five days. Each day, the number of eggs laid were counted. Two days after the eggs were laid, the total number of animals hatched were counted. At the end of the assay, embryonic lethality was assessed by comparing the total

number of hatched animals to the total number of eggs laid. Results represent an average of three trials.

Fertility Analysis

For supplemented fertility assays, 117 mM adenosine (Sigma) in 10% 1M NaOH, 150 mM guanosine (Sigma) in 20% 1 M NaOH, or 22 mM methotrexate (Sigma) in DMSO was added to RNAi agar plates spotted with 200 µL of RNAi culture for a final concentration of 10 mM for purines and 22 µM for methotrexate. 2 L4 animals (*eri-1)* were placed on RNAi plates and allowed to propagate. 10 L4 animals were selected from the RNAi plate and placed on their own individual RNAi plates. Fertility was assessed by counting the number of eggs laid for five days.

Quantitative RT-PCR

RNA was isolated from mixed stage animals grown on RNAi medium for 3 – 5 days using the TRIzol reagent (Invitrogen). 2 µg of RNA was used to synthesize cDNA using the Applied Biosystems High Capacity cDNA kit (Thermo Fisher). cDNA was diluted 1:10 and used for quantitative PCR on Applied Biosciences RT-PCR machine. SYBR Green was used as the fluorescent probe. A combination of three primer sets (*cdc-42, tba-1,* and *pmp-3*) were used as a control. Results represent two biological replicates, done in duplicate.

Appendix A

Additional Methods

Metabolomics of Embryos

The Metabolomics Core Facility at Penn State assisted with the LC-MS metabolomic analysis. 100 mL of OP50 *E. coli* culture was concentrated to 15 mL and 400 µL was spotted on 90 mm plates of NGM. 3 plates of gravid N2 adults were lysed and their eggs were synchronized at the L1 stage. The L1 animals were then platted on the spotted NGM plates and allowed to mature and reproduce. The adults were then washed away with ddH_2O and ~20 µL of embryos were collected in ddH_2O using a metal pick. The samples were flash frozen in liquid nitrogen and stored at -80°C. 15 µL samples were extracted in 1 mL of 3:3:2 acetonitrile:isopropanol:H_2O with 1 µM chloropropamide as an internal standard. A Precellys™ 24 homogenizer was used to homogenize samples. The extracts were dried under a vacuum and resuspended in HPLC Optima Water (Thermo Scientific). The samples were divided into two fractions, one for LC-MS and the other for BCA protein analysis. A modified version of an ion-pairing reversed phase negative ion electrospray ionization method was used to analyze the samples via LC-MS.[25] A Supelco (Bellefonte, PA) Titan C18 column (100 x 2.1 mm, 1.9 µm particle size) and a water-methanol gradient with tributylamine was used to separate samples. The LC-MS platform consisted of Dionex Ultimate 3000 quaternary HPLC pump, 3000 column compartment, 3000 autosampler, and an Exactive plus orbitrap mass spectrometer controlled by Xcalibur 2.2 software (all from ThermoFisher Scientific, San Jose, CA). The HPLC column was maintained at 30°C and a flow rate of 200 µL/min. Solvent A was 3% aqueous methanol with 10 mM tributylamine and 15 mM

acetic acid and solvent B was methanol. The gradient was as follows: 0 min., 0% B; 5 min., 20% B; 7.5 min., 20% B; 13 min., 55% B; 15.5 min., 95% B; 18.5 min., 95% B; 19 min., 0% B; 25 min., 0% B. The orbitrap was operated in the negative ion mode at the maximum resolution (140,000) and scanned from m/z 85 to m/z 1000. Protein concentrations determined by the BCA assay (Thermo Fisher) were used to correct metabolite levels.

Appendix B

Additional Figures

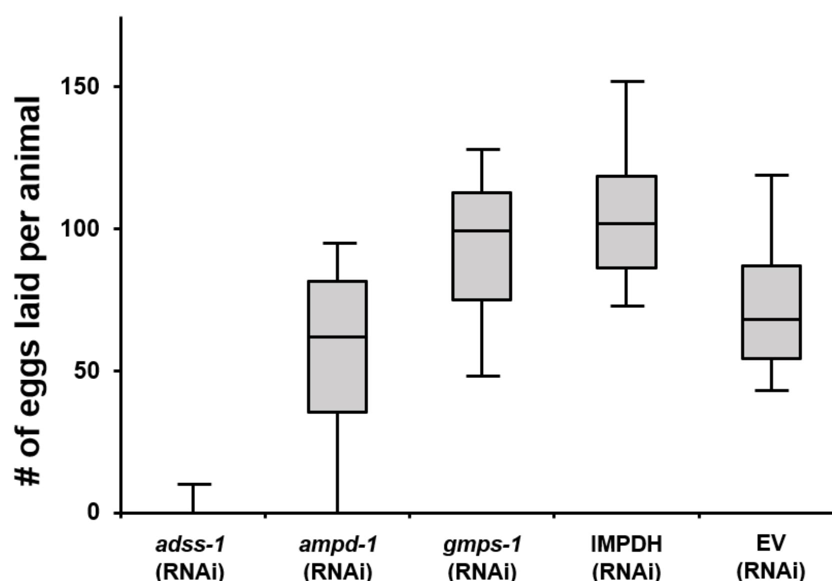

Figure 9. RNAi of other genes in purine biosynthesis have varying effects on fecundity *adss-1* codes for the enzyme that converts IMP to S-AMP in the step prior to *adsl-1* function. *ampd-1* codes for the enzyme that converts AMP back into IMP. Together, *adss-1, adsl-1,* and *ampd-1* form the purine nucleotide cycle which provides fumarate that can be incorporated into the TCA cycle for energy production.[26] IMPDH codes for the enzyme that converts IMP to XMP, and *gmps-1* codes for the enzyme that converts XMP to GMP.

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heterogeneity of adenylosuccinate lyase deficiency. *Hum. Mutat. 31*, 445–455.

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Haley Janowitz

EDUCATION

Bachelor of Science with Honors in Biochemistry and Molecular Biology — December 2017
Minor in Psychology
Schreyer Honors College, The Pennsylvania State University, University Park, PA

Study Abroad in Biochemistry and Molecular Biology — Summer 2015
Fudan University, Shanghai, China

RESEARCH EXPERIENCE

Undergraduate Researcher, The Department of Biochemistry and Molecular Biology
The Pennsylvania State University — 2015 - Present
Thesis: Modelling Reproductive Defects of Adenylosuccinate lyase deficiency in C. elegans

- Modelled a purine metabolic disorder in *C. elegans*, nematodes, to investigate how *de novo* purine biosynthesis is involved in reproductive development and oogenesis. Analyzed fertility, or the lack thereof, in the absence of the gene *adsl-1* and demonstrated the acute need for purines during reproduction.
  - Maintained *C. elegans* cultures
  - Isolated RNA and synthesized cDNA for qPCR and carried out qPCR to analyze expression levels of various genes
  - Knocked down expression of specific genes involved in purine biosynthesis using RNA interference
  - Analyzed structure and development of *C. elegans* reproductive structures in both wildtype and our purine metabolic disease model at various stages
  - Cloned the *adsl-1* gene and promoter to make a translational fusion to GFP in order to look at *adsl-1* expression patterns in *C. elegans*
  - Prepared samples of *C. elegans* for metabolite analysis using LC/MS
- Attended weekly lab meetings to discuss and present results and troubleshoot
- Assisted in daily lab duties such as maintaining reagents and making plates for *C. elegans* cultures, broth and plates for bacterial cultures, keeping supplies up to date and glassware clean, autoclaving waste, and filling pipette tip boxes
- Practiced writing proposals, presenting data, giving chalk talks, and reading primary literature
- Participated in ethical discussions regarding responsible conduct of research
- Assisted in managing lab finances: updated purchasing card record sheet, collected receipts from orders within the lab, corresponded with Administrative Assistant of lab PI

Undergraduate Researcher, The Department of Pharmacology and Chemical Biology
University of Pittsburgh — Summer 2017

- Developed therapeutics for neurodegenerative diseases using NS5AA1b, protein derived from a Hepatitis C virus
  - Cloned versions of NS5A1b with a metal sensitive promoter (MRE) combined with an additional basal promoter region derived from a heat shock protein
  - Cloned truncated versions of NS5A1b translational fusions to GFP to isolate region of interest
  - Maintained *Drosophila* and carried out cross schemes to analyze the effects of NS5A1b expression *in vivo.*
- Secondary project: Assisted in developing a synapse targeted redox sensitive GFP sensor and cross into oxidative stress disease models to verify localization and efficacy
  - Carried out crosses between various redox sensitive GFP lines and neural promoter UAS lines for UAS-GAL4 expression system
  - Dissected larva and performed immunohistochemistry for neuromuscular junction imaging
  - Carried out a five-step cross to develop a fly line that contained the synapse targeted redox sensitive GFP and an ATP 6 mutant allele.
- Attended bi-weekly graduate seminars

PUBLICATIONS AND PRESENTATIONS

- Adam Fenton, Haley Janowitz, Melanie McReynolds, Wenqing Wang, Wendy Hanna-Rose. *A C. elegans model of adenylosuccinate lyase deficiency reveals neuromuscular and reproductive phenotypes of distinct etiology.* The Pennsylvania State University. June 2017. (Submitted)
- Haley Janowitz. *Possible Therapeutic effects of a Hepatitis C Virus Protein on Neurodegenerative Diseases.* **Summer Undergraduate Research Fellowship Pharmacology Symposium**, University of Pittsburgh. July 2017. (Oral)
- Adam Fenton, Haley Janowitz, Wendy Hanna-Rose. *Modelling locomotive and developmental defects of adenylosuccinate lyase deficiency in Caenorhabditis elegans.* **Undergraduate Research Exposition**, The Pennsylvania State University. April 2017. (Poster)
- Adam Fenton, Haley Janowitz, Wendy Hanna-Rose. *Elucidating the mechanism of reproductive and locomotive defects in an inborn error of purine biosynthesis.* **Mid-Atlantic Society for Developmental Biology Meeting**, Howard University. May 2016. (Poster)
- Adam Fenton, Haley Janowitz, Wendy Hanna-Rose. *Elucidating the mechanism of reproductive and locomotive defects in an inborn error of purine biosynthesis.* **Undergraduate Research Exposition**, The Pennsylvania State University. April 2016. (Poster)

RELEVANT SKILLS

- Familiarity with *C. elegans* and *Drosophila* model systems
- Knowledge of cloning procedure: designing constructs, PCR, designing primers, restriction digests, DNA purification techniques, gel electrophoresis, ligations, transformations, sequencing, plasmid preps
- Experience with qRT-PCR: isolating RNA, synthesizing cDNA, running qRT-PCR, analyzing data
- Basic understanding of statistics and how to analyze various types of data
- Intermediate proficiency in Microsoft Excel and GraphPad Prism
- Practiced in presenting research project to audiences with and without a scientific background
- Well versed in the components of grant writing and research proposals
- Skilled in critically reading and presenting primary literature
- Informed about responsible conduct of research and the associated ethics

GRANTS/AWARDS

April 2017

- Second place at the Undergraduate Research Exhibition at University Park in Health and Life Sciences

Summer 2016

- Erickson Discovery Grant, The Pennsylvania State University

Summer 2016

- Summer Undergraduate Research Fund Award, The Department of Biochemistry and Molecular Biology, The Pennsylvania State University (declined)

2014 - 2015

- Women in Science and Engineering Research (WISER) Grant, NASA

ADDITIONAL EXPERIENCE

Organic Chemistry Instrument Room Teaching Assistant, The Department of Chemistry
The Pennsylvania State University 2017 - Present

- Assisted students with using analytical machinery (^{1}H NMR, IR, GC, UV-Vis) and analyzing chemical spectra

Orientation Team Leader, Schreyer Honors College
The Pennsylvania State University Fall 2017

- Organized and carried out the logistics for the Schreyer Honors Orientation for around 300 new students
- Communicated with faculty and staff to book rooms for the events and meals
- Collaborated with other 13 other Team Leaders and staff to plan the orientation weekend
- Guided 70 orientation mentors on their role for incoming students
- Attended a leadership workshop at the career center to evaluate and develop leadership skills

Orientation Mentor, Schreyer Honors College
The Pennsylvania State University Fall 2016

- Communicated with, welcomed, and guided new honors students before and during the Schreyer Honors Orientation
- Encouraged new students to become involved in the academic, as well as social opportunities that both Schreyer Honors College and Penn State has to offer
- Led a group of 15 new honors students during the three-day orientation

Honors General Chemistry Grader, The Department of Chemistry
The Pennsylvania State University Fall 2015

- Reviewed and graded homework (12), quizzes (12), and exams (4) for around 70 students

ACTIVITIES

Science LionPride, The Pennsylvania State University
Treasurer 2016 - 2017

- Managed club account (approximately $3,000) and Alumni endowment ($5,000)
- Designed, ordered, and distributed club merchandise (T-shirts, stickers, water bottles, hats, quarter zips, polo shirts, name tags, windbreakers)
- Collaborated with other executive board members about general club agendas

Member 2015 - Present

- Provided tours for prospective students
- Represented Eberly College of Science
- Raised over $8,000 for Penn State Dance Marathon (THON)
- Participated in science outreach events in the community

Springfield, The Pennsylvania State University
Member 2014 - 2015

- Raised over $120,000 for THON