Canonical and Noncanonical Mechanisms of Resistance to Arginine Starvation in Cancer

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Canonical and Noncanonical Mechanisms of Resistance to Arginine Starvation in Cancer

by

Leonard Rogers

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
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# Table of Contents

List of Figures ......................................................................................................................... v
List of Tables ............................................................................................................................ vi
Acknowledgments .................................................................................................................... vii
Abstract ................................................................................................................................... x

Chapter 1: Introduction: Innate and Adaptive Resistance Mechanisms to Arginine Deprivation Therapies in Sarcoma and Other Cancers ........................................................................... 1

1.1 Abstract ............................................................................................................................... 1

1.2 Introduction .......................................................................................................................... 2

1.2.1 Urea Cycle ....................................................................................................................... 3

1.2.2 Functional Consequences of ASS1 Silencing .................................................................. 4

1.3 Resistance ............................................................................................................................ 6

1.3.1 Adaptive Resistance .......................................................................................................... 6

1.3.2 Immunogenicity of ADI .................................................................................................. 10

1.3.3 Other Mechanisms of Resistance .................................................................................. 11

1.4 Current Clinical Trials ........................................................................................................ 13

1.5 Conclusions ......................................................................................................................... 14

1.6 References .......................................................................................................................... 18

Chapter 2: Intracellular Arginine-Dependent Translation Sensor Reveals the Dynamics of Arginine Starvation Response and Resistance in ASS1-Negative Cells ........................................... 24

2.1 Abstract ................................................................................................................................ 24

2.1.1 Background ...................................................................................................................... 24

2.1.2 Methods .......................................................................................................................... 25

2.1.3 Results ............................................................................................................................. 25

2.1.4 Conclusions ...................................................................................................................... 25

2.2 Background .......................................................................................................................... 25

2.3 Methods ............................................................................................................................... 28

2.3.1 Cell Culture .................................................................................................................... 28

2.3.2 Automated Cell Imaging ................................................................................................. 28

2.3.3 Individual Cell Tracking ............................................................................................... 30

2.3.4 Capillary Electrophoresis ............................................................................................... 30
Chapter 3: Macropinocytosis from the Microenvironment Enables Growth of Arginine Auxotrophic Tumors in the Absence of Extracellular Arginine

3.1 Abstract ................................................................. 72
3.2 Introduction ............................................................ 73
3.3 Results ................................................................. 75
  3.3.1 Arginine starvation fails to inhibit protein translation in vivo ........................................... 75
  3.3.2 ASS1 deficiency is not advantageous for spontaneous murine sarcomas.......................... 76
  3.3.3 Cells without ASS1 grow robustly through arginine deprivation in vivo but die in vitro........ 77
  3.3.4 Ass1 KO tumor cell growth is enabled by macropinocytosis of EVs from ASS1-competent MEFs during arginine deprivation................................................................. 78
  3.3.5 Autophagy/lysosomal degradation is required for cells receiving but not cells supplying growth support ................................................................................................................ 80
3.4 Discussion ................................................................. 82
3.5 Methods .................................................................. 85
List of Figures

Figure 1.1: Urea and Citrulline-N0 Cycles with Connections to Arginine Deprivation by ADI-PEG20 .................................................................15
Figure 1.2: Common Pathway of Resistance to ADI-PEG20.........................................................16
Figure 2.1: Response to arginine deprivation in ASS1-deficient cells ....................................50
Figure 2.2: Design and demonstration of arginine sensor .........................................................52
Figure 2.3: Validating degradation of sensor ...........................................................................53
Figure 2.4: Individual cell responses to arginine deprivation ....................................................55
Figure 2.5: Effects of intracellular arginine concentrations on translation ...............................57
Supplemental Figure S2.1 ........................................................................................................63
Supplemental Figure S2.2 ........................................................................................................64
Supplemental Figure S2.3 ........................................................................................................65
Supplemental Figure S2.4 ........................................................................................................67
Figure 3.1: Arginine starvation fails to inhibit protein translation in vivo .................................94
Figure 3.2: ASS1 deficiency is not advantageous for spontaneous murine sarcomas ..............95
Figure 3.3: Cells without ASS1 grow robustly through arginine deprivation in vivo but die in vitro .........................................................................................96
Figure 3.4: Ass1 KO tumor cell growth is enabled by macropinocytosis of EVs from ASS1-competent MEFs during arginine deprivation .................................................97
Figure 3.5: Autophagy/lysosomal degradation is required for cells receiving but not cells supplying growth support .................................................................99
Figure 3.6: Overview of growth support ...................................................................................101
Supplemental Figure S3.1: In vivo imaging ..............................................................................102
Supplemental Figure S3.2: ASS1 expression in spontaneous murine sarcoma cell lines and growth of Ass1 KO tumors in vivo .........................................................106
Supplemental Figure S3.3: Fibroblasts support growth of Ass1 WT murine and human sarcoma cell lines .........................................................................................107
Supplemental Figure S3.4: Validation of gene knockouts and ASS1 expression in MEFs .......108
Supplemental Figure S3.5: Evidence against other possible growth support mechanisms and MEF toxicity .........................................................................................110
Supplemental Figure S3.6: RNA sequencing of tumors ..............................................................112
List of Tables

Table 1.1: List of All Clinical Trials of ADI-PEG20 ................................................................. 17
Supplemental Table S2.1 ................................................................. 59
Supplemental Table S2.2 ................................................................. 60
Supplemental Table S2.3 ................................................................. 61
Supplemental Table S3.1: Tumor histology classifications and derived cell lines .............. 104
Supplemental Table S3.2: Antibodies and primers ................................................................. 113
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The enzyme argininosuccinate synthetase 1 (ASS1) catalyzes the condensation of citrulline and aspartate into argininosuccinate as part of the urea cycle and citrulline-nitric oxide cycle. This reaction is essential for mammals to synthesize the amino acid arginine, which is required for all cells. Nearly all human tissues express at least some ASS1, but they import most of their arginine from the extracellular space after it is produced and released by the kidneys. Most solid tumors lack a functional level of ASS1, including over 85% of sarcomas, which are cancers of connective tissues. Published evidence suggests that this provides a proliferation advantage by reducing the consumption of aspartate by ASS1, resulting in a larger supply of aspartate to be used in the production of pyrimidines for nucleic acid synthesis. However, ASS1 deficiency causes these cancers to rely on extracellular arginine for survival and growth, which can be targeted through arginine deprivation therapy. PEGylated arginine deiminase (ADI-PEG20), an enzymatic drug that degrades extracellular arginine to citrulline, is the most widely used arginine deprivation therapy, currently being tested in many clinical trials.

While often effective at slowing growth, many cancers, especially sarcomas, gain resistance to ADI-PEG20 in the long term by upregulating their expression of ASS1 and gaining the ability to
synthesize arginine from the now abundant citrulline. This is so common because the ASS1 gene is almost always transcriptionally repressed rather than being deleted or mutated. However, it takes time to increase ASS1 expression sufficiently. In the short term, cells also upregulate autophagy to provide a temporary source of arginine for protein translation and survival. These canonical mechanisms have been studied extensively, and ASS1 re-expression is the only published pathway of long-term resistance to arginine deprivation therapy. Many other pathways could theoretically provide long-term resistance to ADI-PEG20, but none have yet been shown to do so.

To determine whether ASS1 deficiency truly provides an advantage to sarcomas in vivo, a murine model of spontaneous sarcomas was developed with Ass1 knocked out (KO). Conditional Ass1 KO mice did not develop tumors sooner than control mice, nor did their tumors grow faster. In fact, tumors that expressed high levels of ASS1 initiated earlier and grew faster. These data cast doubt on the importance of previous findings explaining the advantages of ASS1 silencing; they suggest that the main reason for a lack of ASS1 in sarcomas may be inheritance from their tissues of origin. The apparent advantage conferred by ASS1 overexpression in these tumors remains unexplained but is a good target for future study.

To characterize the kinetics and heterogeneity of the development of resistance to ADI-PEG20 in ASS1-deficient cancers, a sensor system was developed to monitor the availability of intracellular arginine for protein translation. The sensor consists principally of a genomically integrated gene encoding a reporter protein downstream of an arginine-rich region. Sensor expression is thereby regulated at the translational level, as ribosomes stall or move more slowly at the arginine-rich region, causing reporter protein expression to decrease when arginine supplies are low. Nuclear localization of the reporter and automated imaging allowed tracking of
resistance to arginine deprivation in individual live cells. It was found that all ASS1-deficient cancer cells reduced their expression of the sensor when treated with ADI-PEG20 in vitro, followed by a period of heterogeneous recovery of expression. The timing and magnitude of resistance varied widely among individual cells. However, the sensor expression profile was quite different in vivo, as ADI-PEG20 unexpectedly had no impact on the expression of the arginine sensor even while tumor growth was slowed.

Ass1 KO tumor cell lines generated from the mouse model described above also did not decrease their expression of the arginine sensor when grafted into syngeneic mice and treated with ADI-PEG20. Unexpectedly, these tumors grew robustly through arginine deprivation therapy in vivo, where they were expected to die as they do in vitro. This suggested that the tumor microenvironment lent strong growth support to the tumors by supplying arginine. This hypothesis was further supported with in vitro experiments showing that ASS1-competent fibroblasts could support growth of Ass1 KO tumor cells during ADI-PEG20 treatment. This growth support effect was found to likely be mediated through the uptake of fibroblast-derived extracellular vesicles (EVs) by macropinocytosis into cancer cells, followed by degradation and recycling of the EV components by autophagy/lysosomal degradation to yield free arginine for the cancer cells to use. Inhibition of this growth support phenomenon was shown to be possible by targeting either EV production and macropinocytosis or autophagy both in vitro and in vivo.

These experiments uncovered a novel mechanism of resistance to arginine deprivation therapy, completely independent of intrinsic ASS1 expression, which was previously thought necessary. Further, these results highlight the importance and previously unknown magnitude of the ability of the tumor microenvironment to metabolically support tumors. Finally, this work has opened multiple promising avenues for future research that deserve to be explored.
Chapter 1: Introduction: Innate and Adaptive Resistance Mechanisms to Arginine Deprivation Therapies in Sarcoma and Other Cancers

This chapter is based on the publication listed below and has been reformatted to adhere to dissertation guidelines.


Contributing authors are Leonard Rogers and Brian Van Tine.

1.1 Abstract

Many cancers lack functional expression of the enzyme argininosuccinate synthetase 1 (ASS1) that is necessary for synthesis of L-arginine. These cancers must import arginine for survival and growth, and this reliance can be targeted by arginine-degrading extracellular enzymatic drugs, most commonly PEGylated arginine deiminase. These enzymes can become targets of the immune system, reducing their effectiveness, but PEGylation improves the in vivo stability. Arginine deprivation causes cell death in some cancers, but others gain resistance by expressing ASS1 after a starvation response is induced. Other resistance mechanisms are possible and explored, but these have not been observed specifically in response to arginine deprivation. Future studies, especially focusing on the mechanisms of ASS1 upregulation and metabolic
adaptations, may yield insights into preventing or taking advantage of resistance adaptations to make arginine deprivation therapy more effective.

1.2 Introduction

Our understanding of metabolic therapies and how to use them clinically is rapidly evolving. Current therapies based on tumor metabolism are focused on glutaminase inhibitors, isocitrate dehydrogenase (IDH) inhibitors, pyruvate transport via monocarboxylate transporter 1 (MCT1), and amino acid-degrading enzymes that target asparagine or arginine. It is the ability to systematically degrade arginine and how to apply this clinically that is the focus of this review. Currently, arginine starvation results in an adaptive metabolic reprogramming that renders cells resistant to arginine starvation\textsuperscript{1-3}. By better understanding the mechanisms of this adaptation, successful therapeutic strategies based on arginine starvation and its related biomarker ASS1 will result.

Amongst the more commonly arginine auxotrophic cancers are all the subtypes of sarcoma. Regardless of histology, about 88% lack significant expression of argininosuccinate synthetase 1 (ASS1)\textsuperscript{1}. This finding is not unique to sarcoma, as most solid tumors, including many melanomas, bladder cancers, prostate cancers, small cell lung cancers, and hepatocellular carcinomas, are deficient in this enzyme\textsuperscript{4-9}.

Sarcomas often originate in muscle and bone, and these two tissues, along with the heart and lungs, are among the lowest ASS1 mRNA-expressing tissues in the body\textsuperscript{10-12}. The high incidence of ASS1 silencing in sarcoma may be a consequence of its tissues of origin, but this is a subject of ongoing research. This explanation is also supported by the fact that the ASS1 gene is rarely mutated or deleted in cancer. Rather, its transcription is regulated by epigenetic modifications
and transcription factors. A common mechanism of ASS1 silencing is hypermethylation of its promoter region, causing decreased transcription. Some cancers have also shown competition between the repressive HIF-1α and activating c-Myc transcription factors binding to the E-box of the ASS1 promoter. These are all reversible regulation mechanisms that can dynamically respond when the cell is stressed by a lack of arginine. Further work to fully understand the nature of ASS1 silencing in sarcomas is ongoing.

1.2.1 Urea Cycle
The urea cycle in humans, shown in full in Figure 1.1, takes place mainly in the liver as a way to convert waste ammonia to urea. Ammonia is first converted to carbamoyl phosphate by condensation with carbonic acid and a phosphate group, a reaction catalyzed by carbamoyl phosphate synthetase I (CPS1). The carbamoyl phosphate is then condensed with ornithine with the loss of inorganic phosphate, catalyzed by ornithine transcarbamoylase (OTC), forming citrulline. ASS1 then catalyzes the condensation of aspartate with citrulline to form argininosuccinate. Argininosuccinate lyase (ASL) then cleaves argininosuccinate into fumarate and arginine. The arginine is subsequently hydrolyzed into ornithine and urea by arginase 1 (ARG1).

Although urea production is its main purpose, the cycle is not isolated and exchanges intermediates with other pathways in the cell. In fact, many cells outside the liver do not perform the full cycle but use portions for other functions, namely to produce nitric oxide (NO) and synthesize arginine, as shown in Figure 1.1. In adult humans, the kidneys import extracellular citrulline to synthesize arginine for the rest of the body. Because of this, most cells in the body have no need to produce arginine, but many still employ part of the urea cycle to produce NO. This is achieved by the enzyme NO synthase (NOS), using arginine and oxygen to produce NO.
and citrulline\textsuperscript{19}. The citrulline can then be recycled to arginine by ASS1 and ASL, in what is sometimes called the citrulline-NO cycle\textsuperscript{19}.

1.2.2 Functional Consequences of ASS1 Silencing

There are many possible benefits of suppressing ASS1. The leading hypothesis is that silencing this gene may accelerate cellular growth by causing an increase in available aspartate\textsuperscript{20}. Aspartate is consumed in the essentially irreversible reaction catalyzed by ASS1 to ligate citrulline and aspartate (Figure 1.1)\textsuperscript{17}. ASS1 deficiency therefore increases levels of aspartate, which is needed to make pyrimidines for nucleotide synthesis, allowing cell proliferation. In fact, the lethal metabolic disorder citrullinemia type I, caused by ASS1 deficiency or mutation, results in increased pyrimidine synthesis and proliferation\textsuperscript{20}. Supporting this hypothesis are multiple studies that have shown aspartate to be a key limiting metabolite for the growth of cancer cells\textsuperscript{21,22}.

Another possibility is that ASS1 downregulation helps maintain a higher intracellular pH under acidic stress in the tumor microenvironment\textsuperscript{23}. This happens when the urea cycle cannot function due to ASS1 loss, leaving basic ammonia free in the cells to scavenge protons. The net consequence of this is increased intracellular pH. Recently, another beneficial role has been proposed for ammonia in cancer, as its nitrogen may be directly incorporated into amino acids to maximize growth efficiency\textsuperscript{24}. There are likely other beneficial reasons that are yet to be elucidated.

ASS1 deficiency is also predicted to decrease cellular levels of fumarate, a product of the urea cycle downstream of ASS1. Although this metabolite seems to be unexplored in the context of ASS1 deficiency, any decrease is unlikely to be beneficial, considering that excess fumarate has been found to induce epithelial-to-mesenchymal-transition (EMT) and promote cancer
progression. ASS1 is also essential in the citrulline-NO cycle that produces nitric oxide (NO) for signaling. However, the citrulline-NO cycle is not necessary, as the substrate needed for NO synthesis is arginine, which can normally be imported from extracellular sources. Regardless of the advantages conferred by ASS1 deficiency, this state renders the cells sensitive to arginine deprivation in a targetable way.

Multiple enzymes are capable of targeting ASS1-deficient cells by degrading arginine in the bloodstream. Arginine decarboxylase (ADC) can be found in bacteria, plants, and mammals. ADC converts arginine to agmatine, which is toxic to normal cells and cannot be converted back to arginine, thus limiting the therapeutic potential of this enzyme. Nitric oxide synthase (NOS) could also theoretically be used to degrade arginine in the blood, but the increased NO levels may have unintended signaling effects, and NOS has not been used in this fashion to our knowledge. Recombinant human arginase I has been adapted as a drug to degrade extracellular arginine. The effectiveness has been greatly improved over time by several modifications, but arginase is less commonly used as a therapy than the following enzyme because of its lower affinity for arginine. The most widely used of the arginine-degrading enzymes is arginine deiminase, an enzyme found in many microbial organisms that hydrolyzes arginine into citrulline and ammonia. Arginine deiminase has been conjugated to polyethylene glycol of 20 kDa average size (ADI-PEG20), helping to increase its in vivo half-life drastically.

This drug is being tested in multiple clinical trials and is very promising for multiple reasons. First, it deprives the entire body of arginine regardless of whether the site is accessible, as demonstrated by its efficacy against intracranial glioblastoma, which is protected by the blood brain barrier. Importantly, ADI-PEG20 has strong effects on ASS1-deficient tumors while having only benign side effects of injection site rash and increased uric acid levels. These
differing effects on normal tissues versus tumors can be at least partially explained by the fact that most cells in the body express ASS1. The liver and kidneys have extremely high levels of the enzyme, as the liver performs the urea cycle for the body, and the kidneys produce excess arginine to export to the blood\textsuperscript{18}. Most other tissues express varying levels of ASS1 for less clear reasons, but it may be to carry out the citrulline-NO cycle\textsuperscript{19}. As a result, most tissues can import extracellular citrulline (a product of the ADI-PEG20 reaction) and synthesize arginine for survival. Further explaining the more severe response to ADI-PEG20 in cancer specifically is the increased rate of growth in tumors. While heart, lung, muscle, and bone express very low levels of ASS1, these tissues are relatively static in their growth and therefore unaffected compared to rapidly proliferating cancer cells. When starved of arginine, ASS1-deficient cancers are forced into cytostasis and have been shown to rely partially on autophagy for survival\textsuperscript{1}. Other methods of arginine acquisition likely also play a part and will be discussed later.

1.3 Resistance

1.3.1 Adaptive Resistance

Some ASS1-deficient cancers undergo cell death when starved of arginine by ADI-PEG20, but many others gain resistance to arginine deprivation\textsuperscript{1-3}. This is especially common in sarcomas, as they usually do not die as a consequence of arginine starvation\textsuperscript{1,2}. The most obvious pathway to resistance is upregulation of ASS1 expression. Indeed, this is the only cellular long-term resistance mechanism that has been confirmed to occur in response to ADI-PEG20\textsuperscript{1,6,15,30}. This path is open to nearly every ASS1-deficient cancer, as mutation or deletion of \textit{ASS1} is rare compared to transcriptional silencing. Virtually all regulation of \textit{ASS1} has been found at the transcriptional level, and the mechanisms by which \textit{ASS1} is upregulated in response to arginine deprivation are the subject of much research.
Figure 1.2 illustrates the fully repressed and fully active states of the ASS1 promoter and shows how arginine deprivation can lead to increased ASS1 expression and resistance to ADI-PEG20. In ASS1-deficient lymphoid malignancies, demethylation of the ASS1 promoter with a demethylating agent has been shown to rescue cells from ADI-PEG20 treatment\textsuperscript{13}. More importantly, ASS1-deficient mesothelioma cells were shown to autonomously demethylate their ASS1 promoter to gain resistance\textsuperscript{31}. However, more research has focused on the HIF-1α/c-Myc axis. Some cancers display a competition between the repressive HIF-1α and activating c-Myc transcription factors at the E-box of the promoter\textsuperscript{15}. These two transcription factors seem to be regulated through a multitude of pathways that are affected by arginine deprivation.

Mammalian target of rapamycin (mTOR) activity normally upregulates HIF-1α, repressing ASS1, as shown in Figure 1.2\textsuperscript{32}. Because mTOR is a cellular nutrient sensor, its activity is inhibited by the absence of certain amino acids, including arginine. This results in decreased HIF-1α as ADI-PEG20 inhibits mTOR in ASS1-deficient cells (Figure 1.2). As HIF-1α levels decrease, the Ras/ERK pathway and phosphoinositide 3-kinase (PI3K)/AKT/GSK-3β kinase cascades are activated, resulting in an increase in c-Myc levels\textsuperscript{15,30}. Specifically, ERK phosphorylates c-Myc at S62, which is stabilizing\textsuperscript{33}. Separately, AKT inhibits GSK-3β from phosphorylating c-Myc at T58, a site that promotes degradation\textsuperscript{33}. The two pathways thereby work together to stabilize c-Myc as it replaces HIF-1α on the ASS1 promoter. Our understanding of how arginine starvation can be utilized with mTOR pathway modulation needs further exploration.

Another possible mechanism of resistance can result from downregulation of differentiated embryonic chondrocyte 1 (DEC1), another E-box-binding transcription factor, an upstream regulator that promotes an increase in HIF-1α and decrease in c-Myc\textsuperscript{34}. Silencing
DEC1 increased ASS1, lending support to the hypothesis that HIF-1α/c-Myc balance is the dominant mechanism controlling ASS1 expression in many cells. However, this study used cells that lacked significant ASS1 promoter methylation in the untreated state, leaving open the possibility that an unmethylated promoter is required for expression. Performing a similar study in cells that have been shown to silence ASS1 by promoter methylation would be elucidating.

Arginine starvation can also lead to degradation of HIF-1α by way of the p300-HDAC2-Sin3A chromatin remodeling system. The histone acetyltransferase p300 normally maintains specific histone acetylations that help to stabilize HIF-1α on the ASS1 promoter. p300 dissociates upon arginine starvation, and HDAC2 deacetylates these histones, allowing the formation of a HIF-1α-proteasomal complex that leads to degradation. Although other transcription factors such as Sp4 and p53 are known to affect ASS1 transcription, there is no evidence that these factors differentially affect ASS1 expression under arginine starvation.

Upregulation of ASS1 takes time, and cells must sustain themselves in the meantime utilizing a starvation pro-survival response. For this, many ASS1-deficient cancers induce autophagy in response to ADI-PEG20. This is caused largely by arginine deprivation inhibiting mTORC1, which allows for increased autophagy. Autophagy, the process by which cells consume parts of themselves, enables the recycling of intracellular arginine, which is incorporated into proteins. This is a short-term response. By definition, autophagy is not sustainable indefinitely as a sole source of nutrients. Cells must obtain outside resources to grow. Therefore, autophagy can serve as a sort of bridge to ASS1 upregulation and long-term resistance. However, a reliance on autophagy for any period of time presents a targetable weakness in these cells that can be exploited by combining an autophagy inhibitor with ADI-
PEG20 in an attempt to kill cells before they can gain resistance. Indeed, multiple studies have shown that the autophagy inhibitor chloroquine enhances the apoptotic effect of ADI-PEG20\textsuperscript{1,13,36}. This combination may be more effective in an immune-competent system, as autophagy-defective T cells show enhanced anti-tumor activity\textsuperscript{37}.

Arginine deprivation also has major metabolic effects on arginine-auxotrophic cells which may contribute to resistance. In one study, melanoma cell lines were found to increase their reliance on both glycolysis and glutamine metabolism after becoming resistant to ADI-PEG20\textsuperscript{3}. Supporting this conclusion about glutamine is a paper showing that ASS1-depleted cells rely less on extracellular glutamine than ASS1-positive cells, which parallels the difference between cells that are sensitive and resistant to arginine deprivation\textsuperscript{23}. In addition to the increased reliance on glycolysis in melanoma cells, another study shows a shift toward oxidative phosphorylation in both melanoma and sarcoma cell lines\textsuperscript{2}. This study once again finds an increased dependence on glutamine metabolism in resistant cells\textsuperscript{2}. Multiple melanoma and breast cancer cell lines have been shown to become more resistant to glutamine deprivation as ASS1 expression increased in the absence of arginine starvation, whereas loss of ASS1 sensitizes cells to combined arginine and glutamine starvation\textsuperscript{38}.

Many adaptive changes may be a consequence of increased c-Myc activity, which is necessary for ASS1 upregulation, rather than adaptations to help the cell survive arginine starvation\textsuperscript{3}. At least one study has demonstrated significant cell death when glutaminase inhibition is combined with ADI-PEG20, signifying that these cells may need glutamine in order to make more glutamate and feed the tricarboxylic acid cycle\textsuperscript{2}. The upregulation of c-Myc may also allow therapeutic opportunities. Active, nuclear c-Myc is greatly increased by the combination of ADI-PEG20 and docetaxel\textsuperscript{39}. This in turn leads to increased human equilibrative nucleoside
transporter 1 (hENT1) expression, which imports the nucleoside analog gemcitabine, significantly enhancing its efficacy\textsuperscript{39}.

1.3.2 Immunogenicity of ADI

Another important aspect of the response to arginine deprivation therapy occurs only \textit{in vivo}.

Humans and other mammals mount a strong immune response to pure, recombinant ADI, as it is a foreign bacterial enzyme. This results in a short circulating half-life, about 4 hours in mice, and severely limits its effectiveness as a drug\textsuperscript{40}. This is the main reason that the PEGylated version of the drug was created. The polyethylene glycol cloaks the enzyme from recognition by the immune system and greatly enhances its effectiveness \textit{in vivo}\textsuperscript{28,41}. However, many patients still have immune responses to ADI-PEG20 over time\textsuperscript{42,43}. Immune reactions are much slower to occur than with pure ADI, but they can limit the time window in which ADI-PEG20 is effective. Blood arginine levels commonly show a gradual increase after roughly 8 weeks of treatment, which has been correlated to worse patient outcomes when used as a single agent\textsuperscript{42-44}. However, this window of effectiveness can be extended to at least 18 weeks by some combination therapies, as has been shown recently in multiple clinical trials utilizing triple drug regimens that include ADI-PEG20\textsuperscript{45,46}. Correlated with this was a delayed buildup of anti-ADI-PEG20 antibodies\textsuperscript{42,44-46}. These promising results should encourage further research to develop combination therapies that kill quickly. Additionally, the immune adaptation period can likely be harnessed for therapeutic purposes as tumors metabolically evolve in that time \textit{in vivo}.

While the immune system can neutralize ADI-PEG20, the drug may also have a negative effect on the immune system by depleting arginine. Low arginine levels are known to inhibit T cell proliferation, as myeloid-derived suppressor cells do this naturally through the activity of arginase I\textsuperscript{47}. An arginase inhibitor therefore has potential to be effective in combination with
ADI-PEG20 if anti-tumor T cells express ASS1 themselves and can make arginine. However, this may inadvertently also provide tumor cells with arginine, possibly negating the immune benefit. Arginine depletion has another detrimental effect on the anti-tumor activity of the immune system, as ASS1 upregulation causes increased programmed death-ligand 1 (PD-L1) expression, which is a negative regulator of T cells\textsuperscript{48,49}. This suggests PD-1/PD-L1 immune checkpoint blockade to be used in conjunction with ADI-PEG20\textsuperscript{48,49}. There is even evidence that ADI-PEG20 can enhance infiltration of T cells into tumors in mice, giving further support to the potential of this combination\textsuperscript{49}.

ASS1-deficient cells have also been found to have higher levels of cationic amino acid transporter 1 (CAT-1), which is the most important arginine importer and a potential drug target\textsuperscript{50,51}. Furthermore, ASS1-deficient cells have been shown to increase import of arginine through CAT-1 in response to ADI-PEG20 treatment\textsuperscript{51}. The extent of the contribution of this response to resistance was not determined, but it is likely mild and not enough to sustain growth, since extracellular arginine levels remain extremely low.

### 1.3.3 Other Mechanisms of Resistance

ASS1 re-expression and autophagy have been discussed, but there are a plethora of other ways for cells to obtain arginine, many of them unexplored as pathways for arginine deprivation resistance. Here we summarize what is known about such pathways and their relevance to ADI-PEG20 resistance. Macropinocytosis can be utilized by cancer cells to import and break down extracellular proteins for any amino acid, including arginine\textsuperscript{52,53}. A reliance on this pathway is common in Ras-transformed cells and pancreatic cancers, but can also be used by other cancers\textsuperscript{52,53}. It is therefore possible that macropinocytosis may be an important resistance pathway to arginine deprivation, as it has been shown to increase when mTORC1 is inhibited\textsuperscript{54}. 


Similarly, many breast cancers can import necrotic cell debris in a process called necrocytosis. After three days of amino acid starvation, 35-71% of cellular protein biomass was found to originate from necrocytosed peptides, and this process also provides carbohydrates, lipids, and nucleotides. Macropinocytosis and necrocytosis can also contribute significant amounts of amino acids under normal growth conditions, suggesting that these pathways could immediately provide relief from arginine starvation even without ASS1 upregulation. There is also some evidence that extracellular proteins, particularly albumin, can be taken up by receptor-mediated endocytosis, although this process has not been shown to promote cell growth to the same extent as macropinocytosis.

Phagocytosis is another possible pathway to resistance, albeit unlikely, as only a few cell types perform this activity. With this method, the cell would gain both cytoplasmic arginine and protein-incorporated arginine from consumed cells, exosomes, or other material, along with all other nutrients necessary for survival in the case of whole-cell phagocytosis. This phenomenon has rarely been demonstrated in nonhematopoietic cells, but some breast cancer cell lines are able to phagocytize yeast and extracellular matrix. Some cancers can also perform a process called entosis, whereby one cell invades another. The internal cell can then either be released or degraded by lysosomal enzymes, providing nutrients to the engulfing cell. The end result would be almost identical to phagocytosis with regards to nutrients. It is also conceivable that slower-adapting cells are engulfed more often, which would hasten the onset of resistance in the tumor overall.

Direct sharing of arginine from other cells through gap junctions (for example, immune cells and fibroblasts in the stroma) also has not been ruled out. Heterologous gap junctions are presumably rare but have been found between some cancer and non-cancer cells. However, gap junctions
are generally downregulated in cancer and therefore less likely to play a role in resistance.\(^6^1\)

Alternatively, other cells in the body may supply arginine to ASS1-deficient cancer cells indirectly. This mechanism has been shown with another amino acid, as mesenchymal cells in the microenvironment where leukemic cells grow can produce and secrete asparagine, conferring resistance to asparaginase treatment in some cases of acute lymphoblastic leukemia.\(^6^2\)

The most effective form of resistance to arginine depletion is innate. Many cancers do not downregulate ASS1 and are therefore immune to arginine deprivation therapy. Some apparently ASS1-deficient cancers may also be heterogeneous in their expression. It would take only a small number of highly expressing cells to eliminate the possibility of eradication through arginine deprivation treatment. Even if the other cells all died, ASS1-high cells would be clonally selected and grow out. This heterogeneity has not been a focus of research, but the field would benefit from its investigation.

### 1.4 Current Clinical Trials

There have been many completed clinical trials involving ADI-PEG20, while others are currently active or planned. These trials are summarized in Table 1.1. Numerous cancer types are included across the trials, and many involve ADI-PEG20 monotherapy. However, some test ADI-PEG20 in combination with other drugs, which is likely to be more effective for many cancers that are not killed by monotherapy. There is great potential for similar combination trials in the future, as ADI-PEG20 causes many potentially targetable adaptations in ASS1-deficient cancers while having few side effects.
1.5 Conclusions

Arginine auxotrophic cancers can be targeted by enzymes that degrade arginine in the blood. However, many cancers gain resistance to the most widely used of these enzymes, ADI-PEG20, by upregulating ASS1 and converting the degradation product citrulline back into arginine. Before ASS1 levels are sufficiently increased for resistance, many cancers rely on autophagy to temporarily recycle arginine and sustain themselves. There are also other possible mechanisms by which cells could gain resistance. The most studied of these is macropinocytosis, with which cells can ingest extracellular proteins to use as amino acids sources. Additionally, some parts of a heterogeneous tumor may express higher levels of ASS1 than the rest and be resistant from the start of treatment. Cells that are sensitive to arginine deprivation respond with numerous changes in cellular metabolism and transcription factor activity, amongst other things. Many of these changes can be targeted by other drugs in combination with ADI-PEG20. Exploration of the multitude of metabolic adaptations to arginine deprivation has only just begun, and the future of therapy development is bright.
Figure 1.1. Urea and Citrulline-NO Cycles with Connections to Arginine Deprivation by ADI-PEG20

Within the mitochondria, carbamoyl phosphate synthetase I (CPS1) incorporates ammonia into carbamoyl phosphate. Ornithine transcarbamoylase (OTC) then condenses carbamoyl phosphate with ornithine, forming citrulline. In the cytoplasm, argininosuccinate synthetase 1 (ASS1) catalyzes the condensation of aspartate with citrulline to form argininosuccinate. Argininosuccinate lyase (ASL) cleaves argininosuccinate into fumarate and arginine. Arginine is hydrolyzed into ornithine and urea by arginase 1 (ARG1), completing the cycle. Alternatively, nitric oxide synthase (NOS) can hydrolyze arginine to regenerate citrulline while producing nitric oxide (NO). ADI-PEG20 degrades arginine to citrulline and ammonia extracellularly. Citrulline can then be imported and converted to arginine by ASS1 and ASL.
Figure 1.2. Common Pathway of Resistance to ADI-PEG20

Arginine deprivation caused by ADI-PEG20 inhibits mTORC1, resulting in a decrease of repressive HIF-1α activity. By separate pathways, the positive transcription factor c-Myc is upregulated and causes increased ASS1 transcription and translation. ADI-PEG20 treatment also increases citrulline levels, which the cell can convert to arginine after ASS1 re-expression, resulting in resistance and the ability to proliferate.
<table>
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Table 1.1. List of All Clinical Trials of ADI-PEG20
1.6 References


35 Tsai, W.-B. *et al.* Chromatin remodeling system p300-HDAC2-Sin3A is involved in Arginine Starvation-Induced HIF-1α Degradation at the ASS1 promoter for ASS1 Derepression. *Scientific Reports* **7**, doi:10.1038/s41598-017-11445-0 (2017).


Chapter 2: Intracellular Arginine-Dependent Translation Sensor Reveals the Dynamics of Arginine Starvation Response and Resistance in ASS1-Negative Cells

This chapter is based on the publication listed below and has been reformatted to adhere to dissertation guidelines.


Contributing authors are Leonard Rogers, Jing Zhou, Adriana Baker, Charles Schutt, Prashanta Panda, and Brian Van Tine.

2.1 Abstract

2.1.1 Background
Many cancers silence the metabolic enzyme argininosuccinate synthetase 1 (ASS1), the rate-limiting enzyme for arginine biosynthesis within the urea cycle. Consequently, ASS1-negative cells are susceptible to depletion of extracellular arginine by PEGylated arginine deiminase (ADI-PEG20), an agent currently being developed in clinical trials. As the primary mechanism of resistance to arginine depletion is re-expression of ASS1, we sought a tool to understand the temporal emergence of the resistance phenotype at the single cell level.
2.1.2 Methods
A real-time, single-cell florescence biosensor was developed to monitor arginine-dependent protein translation. The versatile, protein-based sensor provides temporal information about the metabolic adaptation of cells, as it is able to quantify and track individual cells over time.

2.1.3 Results
Every ASS1-deficient cell analyzed was found to respond to arginine deprivation by decreased expression of the sensor, indicating an absence of resistance in the naïve cell population. However, the temporal recovery and emergence of resistance varied widely amongst cells, suggesting a heterogeneous metabolic response. The sensor also enabled determination of a minimal arginine concentration required for its optimal translation.

2.1.4 Conclusions
The translation-dependent sensor developed here is able to accurately track the development of resistance in ASS1-deficient cells treated with ADI-PEG20. Its ability to track single cells over time allowed the determination that resistance is not present in the naïve population, as well as elucidating the heterogeneity of the timing and extent of resistance. This tool represents a useful advance in the study of arginine deprivation, while its design has potential to be adapted to other amino acids.

2.2 Background
Arginine is acquired either extracellularly from the blood stream or synthesized intracellularly by enzymes from the urea cycle. The rate limiting step in arginine biosynthesis is catalyzed by argininosuccinate synthetase 1 (ASS1) (EC 6.3.4.5). As most arginine is made and exported from kidney cells for utilization by other cell types, many cancers take advantage of this extracellular supply by silencing ASS1 expression without compromising their arginine supply.
Cancer is thought to silence ASS1 to free aspartate pools for the biosynthesis that is associated with rapid cell division. ASS1-deficient cells must import extracellular arginine to survive and grow, making arginine depletion an attractive therapeutic strategy. Therefore, multiple arginine-depleting enzymes have been developed. The most clinically tested of these is arginine deiminase conjugated to polyethylene glycol, ADI-PEG20. This enzyme functions extracellularly to break down arginine into citrulline and ammonia. Most cells in the body are not greatly affected, as their ASS1 expression levels are sufficient for arginine production and/or their metabolic requirements for arginine are much lower than rapidly proliferating cells.

There are multiple possible mechanisms of resistance to arginine starvation, but the most common mechanism is the re-expression of ASS1. This is because the gene remains unaltered at the sequence level in most ASS1-deficient cells, as it is silenced by methylation. Mechanistically, when arginine is depleted by ADI-PEG20, c-Myc is activated and translocates to the nucleus, where it binds to the ASS1 promoter and upregulates transcription, leading to intracellular arginine production. The timing and heterogeneity of the emergence of resistance are important to understand for clinical translation, but until now, tools have been lacking to effectively study arginine metabolism at the single-cell level.

Many methods have been developed to measure biological arginine concentrations. While these assays can be useful, they are often expensive and difficult to use, and most are aimed toward food and medical systems. Very few of the available methods measure intracellular concentrations in live cells, and most of these do not allow for real-time measurements. The only published dynamic intracellular sensor of arginine in mammalian cells relies on an arginine-
binding protein from *Chlamydia pneumoniae*\(^\text{18}\). Unfortunately, this system is not suitable for studying biologically relevant arginine concentrations, as it responds only to arginine concentrations that are higher than physiologic arginine levels\(^\text{18}\).

Most organisms also have mechanisms for sensing amino acid concentrations. In higher organisms, the mTOR pathway senses nutrients, including arginine\(^\text{19-21}\), and is tied to cell proliferation\(^\text{22}\). In bacteria, *trp* operon attenuation utilizes a potentially useful mechanism. As the operon is transcribed, a ribosome translates the nascent mRNA, following closely behind the RNA polymerase. When tryptophan is abundant, the ribosome proceeds along the mRNA, allowing a transcription termination signal to form in the mRNA and halt transcription\(^\text{23}\). When tryptophan is scarce, the ribosome stalls, an alternate mRNA structure forms, and transcription continues\(^\text{23}\). Such ribosomal stalling during amino acid scarcity has been demonstrated in human cells specifically in response to arginine deprivation\(^\text{24}\).

This study characterizes and describes the development of an arginine translation sensor (ArgSen) based on the principle of ribosomal stalling. Several different structural components proved useful in studying cellular responses to arginine deprivation, as they were combined to create a novel chimeric protein that would need to satisfy multiple criteria. The ArgSen monitors cellular arginine-dependent translation in individual cells and entire populations over time without adversely affecting growth or response to stimuli. We demonstrate that when ASS1-deficient cells are treated with ADI-PEG20, there is a homogeneous decrease in translational capacity, but a heterogeneous pattern of resistance. Finally, we calculate the minimum concentration of arginine needed for optimal sensor translation.
2.3 Methods

2.3.1 Cell Culture
SKLMS1, SKUT1, and SKMEL2 cell lines were obtained from American Type Culture Collection (Manassas, VA) and are listed in Supplemental Table S2.1. WT cancer cell lines were grown in MEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Bio-Techne, Minneapolis, MN), 1.3% 100X penicillin-streptomycin (10,000U/mL) (Thermo Fisher Scientific), and 2.5 µg/mL Plasmocin (InvivoGen, San Diego, CA). LTAT cells were grown in this medium with 1 µg/mL ADI-PEG20 (Polaris, San Diego, CA) added. All ADI-PEG20 treatments were performed by replacing media with media that had been pre-treated with 1 µg/mL ADI-PEG20 for at least 8 hours at 37° C.

Mouse embryonic fibroblasts (MEFs) were generated as detailed later. MEFs were grown in IMDM (Thermo Fisher Scientific) supplemented with 20% FBS, 1% 100X MEM non-essential amino acids (Thermo Fisher Scientific), 0.0007% 2-mercaptoethanol (MilliporeSigma, Burlington, MA), 1% 100X penicillin-streptomycin (10,000U/mL), and 2.5 µg/mL Plasmocin.

2.3.2 Automated Cell Imaging
Cells were transduced with Incucyte® Nuclight Red Lentivirus Reagent (Essen BioScience, Ann Arbor, MI) and selected with puromycin. SKLMS1, SKUT1, and SKMEL2 cells were plated in 96-well plates at 3X10³, 7.5X10³, and 5X10³ cells per well, respectively. The next day, fresh phenol red-free medium was added to start treatment along with 50 nM YOYO™-1 Iodide (Thermo Fisher Scientific), which fluoresces green within the nuclei of dead cells. Images were taken with Incucyte® ZOOM or Incucyte® S3 (Essen BioScience) automated fluorescent microscopes at a rate of 0.5-1 per hour. Red and green cell numbers were quantified automatically with an analysis algorithm within the Incucyte software (Essen BioScience).
SKLMS1, SKUT1, and SKMEL2 cells expressing the indicated GFP arginine sensor variants were plated in 96-well plates at 3X10³, 7.5X10³, and 5X10³ cells per well, respectively. The next day, fresh phenol red-free medium was added to start treatment. Glutamine deprivation experiments used media supplemented with One Shot™ dialyzed FBS (Thermo Fisher Scientific) in place of standard FBS, and 2 mM L-Glutamine (Corning, Corning, NY) was either added or omitted. Images were taken with Incucyte® ZOOM or Incucyte® S3 automated fluorescent microscopes at a rate of 0.5-1 per hour. Sensor fluorescence was quantified automatically with an analysis algorithm within the Incucyte software, and average integrated intensities of green fluorescence in individual cells were taken.

SILAC RPMI 1640 Flex Media, with no glucose and no phenol red (Thermo Fisher Scientific) was used in experiments to vary arginine concentrations. Glucose (Agilent Technologies, Santa Clara, CA), L-glutamine, and L-lysine hydrochloride (MilliporeSigma) were added to the same concentrations as in RPMI 1640 (Thermo Fisher Scientific), along with 10% dialyzed FBS and 1.3% pen/strep. L-arginine (MilliporeSigma) was added to the indicated concentrations. Cells were grown and passaged in RPMI 1640 for at least one week before arginine concentration experiments. Fluorescence of the arginine sensor was measured with the Incucyte S3 at 0, 1, and 2 hours of the indicated treatments. The areas under the curve for this time period were then plotted against arginine concentration, with 0 represented as 0.01 µM. Data were fit to the following equation:

\[
Y = Bottom + \frac{(X^{\text{HillSlope}}) \times (\text{Top} - \text{Bottom})}{(X^{\text{HillSlope}} + EC_{50}^{\text{HillSlope}})}
\]  

(2.1)

Effective concentration (EC) of any other value (F) was then calculated with the following equation:
ECF = \left( \frac{F}{100-F} \right)^{\text{HillSlope}} \times EC50 \quad (2.2)

Where indicated, 100 µM cycloheximide (CHX) (MilliporeSigma) or 1 µM bortezomib (BTZ) (Millennium Pharmaceuticals, Cambridge, MA) was added to media. Fluorescence data in the presence of CHX were fit to the following exponential one-phase decay equation:

\[ Y = (Y_0 - \text{Plateau}) \times e^{-K \times X} + \text{Plateau} \quad (2.3) \]

Half-lives were calculated as (ln(2)/K).

### 2.3.3 Individual Cell Tracking

Arginine sensor fluorescence of individual cells was tracked over time by hand within the Incucyte S3 software by recording the integrated intensity of green objects (nuclei) detected by the analysis algorithm. 100 cells were initially targeted for tracking in each experimental condition. Results include data from less than 100 cells, as not all cells were able to be tracked with certainty. At timepoints where fluorescence in a cell was too low to be detected by the algorithm, the integrated intensity value was recorded as 0.

### 2.3.4 Capillary Electrophoresis

For obtaining lysates after a time course of ADI-PEG20, SKLMS1 cells were plated in 60-mm dishes, and treatments were administered so that all ended simultaneously. 7.5X10^4 LTAT cells were plated for every timepoint. 7.5X10^4 WT cells were plated for samples with 12 or fewer hours of treatment, and the number was doubled for each full day of treatment, with 6X10^5 cells plated for 72 hours. Treatment was started by the addition of fresh media the next day. All samples received fresh media 2 hours before harvesting. After lysis, immunoblots were performed with a Wes automated immunoblot machine (Bio-Techne) according to the manufacturer’s protocol. Each sample was normalized to its own total protein. Two ASS1
primary antibodies were used: a non-commercial mouse monoclonal from Polaris and a rabbit polyclonal ab175607 (Abcam, Cambridge, MA). Mouse monoclonal GFP antibody sc-9996 (Santa Cruz Biotechnology, Dallas, TX) was used to detect the arginine sensor. Two bands were detected at approximately the expected molecular size of the GFP sensor, and both were quantified and added together before normalization to total protein. Although a single band was expected, the identity of the two separate bands was not investigated. Antibodies are listed in Supplemental Table S2.2.

2.3.5 Cloning
A gBlocks® Gene Fragment (Integrated DNA Technologies, Coralville, IA) of the first arginine sensor variant (ArgSen (-) NLS), containing a P2A site and Fast-FT without a nuclear localization sequence (NLS), was cloned using AscI and NotI restriction sites. All gBlocks® in this study were ordered with extra nucleotides on each end to allow for direct restriction digestion and ligation into the vector. All arginine sensor variations were first cloned into plasmid pKLV2-EF1a-BsdCas9-W (replacing the original insert), then subcloned into plasmid pLV-EF1a-IRES-Puro. pKLV2-EF1a-BsdCas9-W was a gift from Kosuke Yusa (Addgene plasmid #67978; http://n2t.net/addgene:67978; RRID:Addgene_67978)26. pLV-EF1a-IRES-Puro was a gift from Tobias Meyer (Addgene plasmid #85132; http://n2t.net/addgene:85132; RRID:Addgene_85132)27. Successful cloning was confirmed by Sanger sequencing with primers EF1a fwd, pKLV2 seq rev, pLV seq rev, and Rad23b end fwd. All oligonucleotides in this study were ordered from Integrated DNA Technologies and are listed in Supplemental Table S2.3.

After cloning ArgSen (-) NLS, multiple modifications were made. A C-terminal SV40 Large T-antigen NLS was added by amplification of Fast-FT with SalI BamHI FastFT fwd and NotI NLS FastFT rev primers, followed by replacement of Fast-FT with Fast-FT-NLS using BamHI and
NotI restriction sites (ArgSen). Separately, the P2A site was removed by SalI restriction digestion followed by ligation (Argsen (-)P2A (-)NLS). Fast-FT in ArgSen (-)P2A (-)NLS was then replaced by enhanced green fluorescent protein with a C-terminal NLS (GFP-NLS) that had been PCR amplified with SalI BamHI GFP fwd and NotI NLS GFP rev primers, using BamHI and NotI restriction sites (GFP ArgSen). The polyarginine region in this variant was then replaced with a region of identical length encoding random non-arginine amino acids, obtained as a gBlock® and cloned with restriction sites XbaI and EcoRI (GFP RanSen).

Variations of the sensor with regions deleted to test degradation were derived from GFP ArgSen. DNA oligonucleotide pairs designed to replace targeted regions with short linkers were phosphorylated on their 5’ ends by T4 Polynucleotide Kinase (New England Biolabs, Ipswich, MA), then annealed. After annealing, each double-stranded oligonucleotide possessed complementary overhangs with the correct sequences to bind to the digested restriction sites on either side of its target region. The target region was cut out of the vector at these sites, and the newly annealed oligonucleotide was ligated in. NucGFP was made with Reporter only oligonucleotides using Ascl and BamHI restriction sites. Δ Degradation Domain was made with Proteasomal del oligonucleotides using Ascl and BstBI restriction sites. Δ Disordered Region was made with Disordered del oligonucleotides using Ascl and NsiI restriction sites. Δ Rad23b UbL was made with Rad23b del oligonucleotides using NsiI and XbaI restriction sites. After publication, all constructs will be available from Addgene (Watertown, MA).

Lentiviral particles were made with Lenti-X™ 293T cells (Takara Bio USA, Mountain View, CA). Experimental cells were transduced with virus and selected with puromycin.
2.3.6 Flow Cytometry
SKLMS1 ArgSen cells were plated in 6-well plates for a time course of ADI-PEG20, along with control WT cells for each timepoint. To enable simultaneous harvesting at similar levels of confluency, the following numbers of cells were plated per well according to the length of ADI-PEG20 treatment: 1X10^5 for 72 hours, 6.5X10^4 for 48 hours, 4X10^4 for 24 and 18 hours, and 2.5X10^4 for 12 or fewer hours. All samples received fresh media 2 hours before harvesting. Cells were washed with PBS, harvested with trypsin, and measured for fluorescence of blue Fast-FT by flow cytometry with a 407 nm laser and 50 nm wide bandpass filter centered at 450 nm. Fluorescence values of paired WT samples were subtracted from ArgSen samples, and values were then normalized to untreated fluorescence levels.

2.3.7 RT-qPCR
For isolating RNA after a time course of ADI-PEG20 treatment, SKLMS1 cells were plated in 60-mm dishes, and treatments were administered so that all ended simultaneously. 7.5X10^4 cells were plated for samples with 12 or fewer hours of treatment, and double for each full day of treatment, with 6X10^5 cells plated for 72 hours. All samples received fresh media 2 hours before harvesting. Cells were washed in ice-cold PBS, scraped, and centrifuged. RNA was isolated by Direct-zol™ RNA Miniprep Plus kit (Zymo Research, Irvine, CA) according to the manufacturer’s protocol. The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to produce cDNA according to the manufacturer’s protocol. The Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) was then used to quantify arginine sensor and GAPDH mRNA according to the manufacturer’s protocol. qrtPCR ArgSen fwd, qrtPCR ArgSen rev, qrtPCR GAPDH fwd, and qrtPCR GAPDH rev primers were used in the RT-qPCR reactions.
2.3.8 Protein Translation Assay
SKLMS1 WT cells were plated at 6X10^3 cells per well in a 96-well plate. The next day, media was replaced and indicated treatments were given at indicated times so that the assay began simultaneously for all samples. The Click-&-Go Plus 647 OPP Protein Synthesis Assay Kit (Click Chemistry Tools, Scottsdale, AZ) was used according to manufacturer’s instructions. Red fluorescence was measured on the Incucyte S3, and cell-by-cell analysis was performed to quantify the mean intensity of fluorescence in each cell. The average fluorescences of CHX controls for each timepoint were subtracted from each sample before normalization to the untreated 0 timepoint.

2.3.9 Mouse Embryonic Fibroblast Generation
HEPD0731_5_F08 mutant embryonic stem cell clones were ordered from the European Conditional Mouse Mutagenesis Program (EUCOMM). These cells contain an allele of ASS1 harboring FRT-flanked lacZ and neomycin resistance genes, followed by a loxP site upstream of critical exon 4 and another loxP site downstream of exon 4. Embryos were grown from these stem cells, and the resulting mice were crossed with mice expressing FLP recombinase. This cross removed the cassette containing lacZ and neomycin resistance genes, allowing the ASS1 allele to encode functional ASS1 protein. These mice were then backcrossed to C57BL/6 mice with confirmed FLP-negative genotypes three times to remove FLP and possess ASS1^{F/+} alleles. ASS1^{F/+} mice were crossed to each other, and a colony of ASS1^{F/F} mice was established.

MEFs were generated from embryos harvested from an ASS1^{F/F} X ASS1^{F/F} mating. MEFs were allowed to grow normally and became spontaneously immortalized. MEFs were infected with either Ad5CMVCre or Ad5CMVcytoLacZ adenoviral particles (University of Iowa, Iowa City,
IA) to knock out ASS1 or serve as a negative control, respectively. Complete knockout of ASS1 was confirmed by both genotyping and immunoblot.

### 2.3.10 Metabolomics
For metabolomic analysis, SKLMS1 WT and LTAT cells expressing GFP ArgSen were plated in replicates of four on 100 mm dishes at 2X10^6 cells per dish for every condition except LTAT with 48 hours of ADI-PEG20, which had 1X10^6 cells. The next day, cells were treated with ADI-PEG20 for the indicated time periods, with each sample getting fresh media one hour before harvesting. Viable cells were counted for one sample from each condition, to be used for normalization. Metabolites were extracted from the remaining three samples with methanol using the protocol for extraction of metabolites from adherent cells from Human Metabolome Technologies America (HMT) (Boston, MA). Purified metabolites and viable cell counts were then sent to HMT for metabolomic analysis. Data from HMT were then normalized to ratios of individual sample total metabolites to average total metabolite levels per 1X10^6 cells. The concentrations of arginine in ADI-PEG20-treated MEM and untreated MEM were also determined by HMT.

### 2.3.11 Cell Volume Determination
For determining cell volume after 48 hours of ADI-PEG20 treatment, cells were plated and treated identically to the metabolomics experiment, then harvested by trypsin. Samples were mixed well, and 20 µL of each were taken, mixed with an equal volume of trypan blue (Thermo Fisher Scientific), pipetted into Countess™ Cell Counting Chamber Slides (Thermo Fisher Scientific), and analyzed by a Countess™ II Automated Cell Counter (Thermo Fisher Scientific). Average cell diameter was calculated from this data. The remaining cells were analyzed by flow
cytometry, measuring forward scatter intensity to determine relative cell diameters. Individual and average cell volumes were then calculated with FlowJo software (FlowJo, Ashland, OR).

2.3.12 Statistics
Data were analyzed using GraphPad Prism 8 Software (GraphPad Software, San Diego, CA). mRNA and GFP immunoblot error bars represent standard error of the mean. All other error bars represent standard deviation. ASS1 protein expression differences were analyzed by unpaired t test. Growth rate differences were analyzed by one-way ANOVA. Metabolite differences at 48 hours were analyzed by Welch’s t test. “ns” denotes non-significant difference between groups. * denotes p < .05, ** denotes p < .01, *** denotes p < .001, and **** denotes p < .0001.

2.4 Results

2.4.1 Resistance to ADI-PEG20
The established working model of resistance to arginine starvation is demonstrated in Figure 2.1a. In ASS1-deficient cells, arginine is imported from the extracellular space. Upon the addition of ADI-PEG20, extracellular arginine is converted to citrulline, which causes a cellular starvation state that induces a c-Myc-dependent re-expression of ASS1. This is the primary mechanism of arginine starvation resistance, as re-expression of ASS1 allows the intracellular production of arginine through urea cycle enzymes. As more ASS1 is expressed, the cell can synthesize more arginine from citrulline, thus allowing cells to grow in the absence of extracellular arginine. ASS1-deficient cells moderately increase expression of ASS1 in the short term in response to arginine deprivation, but in the long term, ASS1 is greatly upregulated (Figure 2.1b).

To model the response of cells to the arginine starvation state, two well-characterized human sarcoma cell lines, SKLMS1 and SKUT1, and one human melanoma cell line, SKMEL2, were
used\textsuperscript{12,16,28}. As these lines express very little ASS1 at baseline, long term ADI-PEG20 treatment (LTAT) resistant cell lines were derived as previously described by Kremer et al.\textsuperscript{12}. The longer the cell lines were cultured with ADI-PEG20, the more ASS1 protein was expressed (Figure 2.1b). Next, we confirmed that arginine starvation in ASS1-negative cell lines leads to a cytostatic response, as all three WT cell lines demonstrated significantly decreased cellular proliferation when monitored by Incucyte Nulight Red counts over 72 hours, whereas LTAT cells grew at a similar rate as untreated WT cells (Figure 2.1c). Finally, we demonstrated that cell death is not meaningfully increased in the arginine starvation state (Figure 2.1d).

\textbf{2.4.2 Arginine Sensor Design}
To gain a better understanding of the temporal emergence of resistance to ADI-PEG20 in real time, a fluorescence-based protein sensor was designed to measure arginine-dependent protein translation. A lentiviral delivery system is utilized to integrate the sensor gene into the host genome. The EF1\textalpha promoter was chosen to mimic endogenous arginine-dependent translation, as this drives homogeneous and stable transcription in human cells\textsuperscript{29}. In addition, seven components were incorporated into the sensor (Figure 2.2a). A fluorescent timer protein was used to optimize the first-generation reporter (ArgSen), as classical fluorescent proteins do not degrade fast enough for their fluorescence to noticeably decrease within a few hours of stopping translation. Fast-FT was chosen, as it matures from blue to red over a few hours in the cell, allowing only recently translated proteins to be measured by blue fluorescence\textsuperscript{30}. However, most experiments were performed with an enhanced green fluorescent protein version of the sensor (GFP ArgSen), made possible by attaching a domain that rapidly degrades the GFP. Finally, a nuclear localization signal was added to enable better quantification via microscopy.

The core part of the arginine sensor is a polyarginine region that enables a more specific
response to intracellular arginine levels (Figure 2.2a). When the cell is starved of arginine with ADI-PEG20, this region should be translated more slowly, causing ribosomal pausing and/or stalling\textsuperscript{24}, resulting in slower translation of the downstream reporter protein. 20 arginine residues are included in this region.

Upstream of and attached to the polyarginine motif is a strong proteasomal degradation signal (Figure 2.2a), allowing the arginines to be recycled quickly, as the incorporated arginine residues might deplete cytoplasmic levels of the amino acid in the cell. The degradation domain also causes rapid depletion of the reporter when translation rates decrease, which is critical for the temporal sensitivity of the sensor. The degradation domain consists of three parts, Rad23b ubiquitin-like domain (UbL), the N terminus of human thymidylate synthase (hTS), and a disordered region. To promote ubiquitin-independent proteasomal degradation, a human Rad23b UbL domain was added. This domain has been shown to localize attached proteins to the proteasome and facilitate their rapid degradation\textsuperscript{31}. hTS is degraded in a manner totally independent of ubiquitin\textsuperscript{32}. The 30 amino acids from the N terminus of hTS are essential for its degradation and were therefore chosen as the N terminus of the sensor\textsuperscript{32}. The hTS N terminus is short, but studies have shown that longer disordered regions generally lead to faster proteasomal degradation\textsuperscript{33,34}. For this reason, a long, disordered region was included. The measles virus phosphoprotein C-terminal domain flexible disordered linker was chosen as the long disordered region from a data set used to validate the Multilayered Fusion-based Disorder predictor v. 2.00 (MFDp2)\textsuperscript{35-37}. Additional groups have confirmed that this region is a natural long, disordered linker\textsuperscript{38-40}.

Due to the intrinsic ability of Fast-FT to signal only recently translated proteins by blue fluorescence, a P2A site was included after the polyarginine motif and before the Fast-FT in this
first version of the sensor. The P2A essentially acts as a self-cleavage site, and its inclusion is an effective way to translate two polypeptides from the same mRNA in the same quantity\textsuperscript{41}, which frees Fast-FT to function independently while recycling the upstream arginine (Figure 2.2a). The GFP ArgSen, standardly used for most of the studies, does not include the P2A site, causing the GFP to be degraded quickly.

2.4.3 Sensor Response to ADI-PEG20
Flow cytometric analysis was performed on SKLMS1 cells expressing the Fast-FT arginine sensor (ArgSen) to characterize its fluorescence over time when cells were treated with ADI-PEG20 to deplete extracellular arginine. Flow cytometric analysis shows a decrease of ArgSen fluorescence with a minimum signal seen at 12 hours, followed by a recovery of fluorescence back to untreated levels by 72 hours (Figure 2.2b), which is consistent with re-expression of ASS1 (Figure 2.1b) and the reinitiation of proliferation (Figure 2.1c). After transducing cells with GFP ArgSen, the response to ADI-PEG20 was compared to a variant in which the arginine residues of the polyarginine motif were replaced with random non-arginine amino acid residues (GFP RanSen) (Figure 2.2c). This decreased the overall arginine content in the protein from 37 in GFP ArgSen to 17 residues in GFP RanSen, and the response to ADI-PEG20 was reduced correspondingly (Figure 2.2c). GFP ArgSen mRNA levels do not decrease with ADI-PEG20 treatment, staying relatively steady for 48 hours, with some increase but no decrease in transcription seen after that time, suggesting that this is a translational and not transcriptional probe for arginine starvation response (Figure 2.2d).

Without treatment, sensor expression gradually decreases over time as cells grow, deplete nutrients, and become more confluent (Figure 2.2e). In WT cells treated with ADI-PEG20, sensor fluorescence is reduced initially, then recovers over time, whereas untreated and LTAT
cells maintain relatively steady fluorescence (Figure 2.2e). Some cell lines, such as SKLMS1, are dependent on glutamine and therefore grow slowly in response to glutamine deprivation\textsuperscript{28}. In response to glutamine withdrawal, SKLMS1 cells reduce GFP ArgSen signal overtime, but with slower kinetics than ADI-PEG20 treatment, and SKUT1 and SKMEL2 respond similarly (Figure 2.2e). This is consistent with the sensor also responding to general protein translation. Glutamine deprivation has less effect on global translation rates than ADI-PEG20 in SKLMS1 (shown in Supplemental Figure S2.1), but Figures 2.2c and 2.2e taken together indicate that GFP ArgSen expression is preferentially affected by arginine availability over other amino acids.

Cells were then treated with ADI-PEG20 for 12 hours, near the minimum point of GFP ArgSen fluorescence, and then placed in normal growth media (Figure 2.2f). GFP ArgSen fluorescence increased rapidly over the few hours following addition of complete media, stabilizing at roughly the baseline level, then slowly decreasing at a similar rate as untreated cells.

To test whether ASS1 expression is required for ADI-PEG20 resistance, GFP ArgSen was expressed in MEFs with ASS1 either knocked out (ASS1\textsuperscript{−/−}) or expressed at normal levels (ASS1\textsuperscript{F/F}). When treated with ADI-PEG20, ASS1\textsuperscript{F/F} MEFs experienced a moderate decrease in fluorescence followed by recovery, whereas fluorescence decreased more rapidly and did not recover in ASS1\textsuperscript{−/−} MEFs (shown in Supplemental Figure S2.2). ADI-PEG20 also completely stopped the growth of ASS1\textsuperscript{−/−} MEFs but not ASS1\textsuperscript{F/F} MEFs (shown in Supplemental Figure S2.2).

2.4.4 Sensor Component Characterization
Degradation and recycling of the sensor probe’s arginines are important for temporal resolution and to avoid unwanted arginine depletion. The GFP ArgSen is in a constant state of balance between rapid synthesis and rapid degradation, resulting in a relatively low steady state level of GFP due to the incorporation of its rapid degradation domain. Any disturbance of either
translation or degradation, such as ADI-PEG20 treatment or translation or proteasome inhibitors, should affect the levels of GFP in the cell. With this design, faster degradation gives better temporal resolution to the sensor. For this reason, we characterized the importance of each upstream (of reporter) sensor component to the efficiency of degradation. GFP ArgSen degradation was measured in the presence of the translation inhibitor cycloheximide (CHX), and this was compared to variants with deletions of various regions upstream of the GFP (Figure 2.3a). The complete sensor (GFP ArgSen) was found to degrade the fastest, as measured by its half-life of approximately 2.7 hours (Figure 2.3b). However, deletion of either the designed disordered region or the ubiquitin-like domain led to only a slight increase in half-life of less than 1 hour (Figure 2.3b). All variants that included the polyarginine motif degraded much faster than nuclear GFP alone (labeled NucGFP; >100 hours half-life), including the variant with no degradation domain (Figure 2.3b).

ADI-PEG20 causes a similar but slower decline in sensor fluorescence when compared to CHX (Figure 2.3c). Images show that translation inhibitor CHX rapidly depletes sensor fluorescence in cells, while proteasomal inhibitor bortezomib (BTZ) temporarily increases fluorescence before cell death (Figure 2.3c). To test whether translation and proteasomal degradation of the sensor continue even after arginine depletion, SKLMS1 WT cells were treated with CHX at multiple time points after starting ADI-PEG20 treatment. Results demonstrated the expected pattern of a rapid decrease in fluorescence, indicating that proteasomal degradation is not significantly affected by ADI-PEG20 (Figure 2.3d). Next, cells were treated with BTZ at 0 or 48 hours after ADI-PEG20 treatment. BTZ treatment at either time point resulted in higher sensor fluorescence in the following hours compared to control (Figure 2.3e).
To correlate sensor fluorescence with protein expression by an independent method, immunoblots by capillary electrophoresis for arginine sensor reporter protein were performed over a time course of ADI-PEG20 in SKLMS1 WT, showing a similar kinetic pattern as flow cytometry and microscopy measurements (Figures 2.3f and 2.3g). The pattern of sensor expression is also relatively unchanged when normalized to mRNA levels, with the exception of a moderate decrease at 72 hours due to increased mRNA at that time (Figure 2.3g).

2.4.5 Single Cell Tracking
To determine the dynamics of cellular responses to arginine deprivation, integrated green fluorescence intensities of individual WT cells were tracked over time with and without arginine depletion by ADI-PEG20. In the absence of arginine depletion, the majority of cells increased or maintained relatively steady sensor fluorescence before undergoing mitosis (Figure 2.4a). With ADI-PEG20 treatment, every measured cell decreased expression of the sensor before some cells recovered expression over time (Figure 2.4a). The timing of recovery varied among cells, and the magnitude varied even more, indicating heterogeneous resistance (Figure 2.4a). Higher proportions of cells in Figure 2.4a die with ADI-PEG20 treatment than are shown in Figure 2.1d. This is because Figure 2.4a does not include daughter cells from divisions, which increase the total live cell numbers by 72 hours.

SKLMS1, SKUT1, and SKMEL2 WT cells with and without ADI-PEG20 over time were imaged with the Incucyte S3 (Figure 2.4b). Representative images at 4, 24, and 72 hours are shown. At 4 and 24 hours, most treated cells have significantly decreased fluorescence compared to their untreated controls. This fluorescence is recovered in some treated cells at 72 hours, while untreated cells have become confluent, causing downregulation of translation, especially in SKLMS1 and SKMEL2.
We then determined the percentage of cells that had divided, died or neither (labeled as “analyzed”) at both 4 and 24 hours, shown in Figure 2.4c. In each cell line at both time points, a significantly lower percentage of cells treated with ADI-PEG20 had divided, supporting the growth data in Figure 2.1c. The cells which neither divided nor died through each time point were then categorized according to their change in arginine sensor fluorescence. At 4 hours, no treated cells had increased expression of the sensor, while over 83% of untreated cells in each cell line had increased expression or had not changed. At 24 hours, fluorescence remained low in 90-96% of treated cells, while the remaining subset had already started to recover (Figures 2.4a and 2.4c). Less than 7% of untreated cells in any cell line neither divided nor died by 24 hours, limiting the usefulness of categorizing. A subset of cells that divided and their descendants were able to be tracked, but most descendants were lost due to confluent crowding and imaging limitations of the Incucyte S3. Those descendants that could be tracked with confidence showed a very similar pattern of sensor fluorescence as cells that did not divide within 72 hours (shown in Supplemental Figure S2.3).

2.4.6 Metabolic Analysis
Metabolomic analysis by mass spectrometry of SKLMS1 WT and LTAT cells treated with ADI-PEG20 over time demonstrates a decrease in intracellular arginine concentration that occurs within 1 hour (Figure 2.5a). Arginine levels do not recover within 48 hours (Figure 2.5a). However, the steady state intracellular arginine levels are roughly five times higher in LTAT cells than in WT cells when treated with ADI-PEG20 (Figure 2.5a). As expected, intracellular citrulline levels start low and increase greatly over time (Figure 2.5b). Maximum citrulline levels are not reached by 12 hours, and citrulline levels stay significantly lower in LTAT cells than in WT cells (Figure 2.5b), as expected in cells with a functional amount of ASS1.
2.4.7 Minimum Arginine Concentration Needed for Optimal Sensor Translation

In order to determine concentrations of arginine needed for optimal sensor translation, WT and LTATs of each cell line were treated with media containing varying concentrations of arginine, and the fluorescence of GFP ArgSen was quantified by microscopy (Figure 2.5c). These conditions limit the arginine concentrations of both WT and LTAT cells, as the media contains no citrulline that could be used by ASS1 to synthesize arginine, and no cells outside of the small intestine express all the necessary genes to synthesize citrulline de novo\textsuperscript{42}. EC50s were found to range from 2.59-8.10 µM (Figure 2.5d). To compare arginine sensor translation at various arginine media concentrations to actual intracellular arginine concentrations with ADI-PEG20, a combination of microscopy and flow cytometry was used to measure the diameters and calculate the volumes of individual cells. Then intracellular concentrations of arginine were calculated using metabolomics data and average cell volumes at 48 hours (Figures 2.5e and 2.5f). The resulting figures were 13.9 µM of intracellular arginine for WT cells and 81.7 µM for LTAT cells.

2.5 Discussion

The ArgSen is a new single-cell sensor to monitor real-time arginine-dependent protein translation. Arginine starvation strategies are being developed clinically, but single agent use of therapeutics like ADI-PEG20 has failed to translate to patients\textsuperscript{43}. Therefore, an understanding of the cellular adaption to arginine deprivation is an unmet need. By understanding the intracellular dynamics of arginine starvation, a better understanding of when to add additional agents may lead to better therapeutic strategies. Therefore, the development of the ArgSen will allow not only for an understanding of the temporal dynamics of arginine starvation, but also the stromal...
response when multiple colored sensors are fully implemented \textit{in vitro} and \textit{in vivo}, which is the subject of ongoing work.

The specificity of the ArgSen to arginine is supported by two experiments. First, the initial response to arginine deprivation is greatly blunted when arginine residues in the polyarginine motif are replaced by random non-arginine amino acids (RanSen). Second, glutamine deprivation, while decreasing the maximal rate of translation, slows synthesis much more gradually than arginine deprivation. These results indicate a preference and specificity of the sensor for monitoring arginine-rich translation and support the idea that the dominant mechanism of regulation is a decrease in translation. For use in other systems where arginine deficiency may or may not be occurring, the RanSen may be used as a control for ArgSen to determine whether the observed response is preferential for arginine availability.

The ArgSen demonstrates a rapid decrease in expression followed by a gradual recovery over time in ASS1-deficient cells. The Fast-FT ArgSen and GFP ArgSen show some differences in the magnitude of response to ADI-PEG20. This is likely attributable to the different mechanisms by which their signals are degraded, as well as different methods of measurement, as average GFP ArgSen fluorescence is susceptible to overestimation, as some cells fall below the detection threshold.

In contrast to WT cells, resistant LTAT cells demonstrate no change in expression in response to ADI-PEG20, as expected. This long-term resistance is conferred mostly by increased ASS1 expression. WT cells also show a short-term increase in ASS1 corresponding to ArgSen recovery from ADI-PEG20 treatment. However, this increase in ASS1 expression likely does not totally account for the increased sensor expression, as autophagy is known to help these cells cope with
arginine starvation. Post-translational ASS1 activity regulation also cannot be ruled out. The sensor is a more dynamic measure of resistance, as it increases significantly by 24 hours, whereas ASS1 increases are small and difficult to measure at this early time point. Both continue to increase thereafter.

The ArgSen model depends on constant sensor mRNA levels in the cell for reporter protein levels to accurately reflect the ability of the cell to use arginine for translation. When measured over a time course of ADI-PEG20 treatment, sensor mRNA levels do not significantly decrease at any point, and the overall pattern of sensor fluorescence is very similar regardless of whether it is normalized to mRNA, confirming that sensor protein expression levels are driven by translation rather than transcription.

Next, while the full-length sensor possesses the shortest half-life and therefore the best temporal resolution, deletion of some parts of the degradation domain still resulted in only marginally longer half-lives, specifically when the polyarginine motif remained. We speculate that this is because the polyarginine motif is likely disordered, and the presence of a disordered region is critical and often sufficient for proteasomal degradation\textsuperscript{32,44,45}.

Finally, CHX and BTZ worked as expected in sensor-expressing cells, rapidly depleting or causing a buildup of the reporter protein, respectively. This supports the proposed mechanism of the sensor that relies on both rapid synthesis and rapid degradation, leading to a relatively low steady state level of reporter protein. Immunoblots further confirmed that the reporter protein is indeed being degraded as designed.

Tracking of arginine sensor expression in individual ASS1-deficient cells demonstrated that ADI-PEG20 treatment initially depleted the reporter protein in every cell analyzed, without
exception. This is strong evidence that resistant cells are not present in naïve populations. Subsequent recovery of expression indicates a metabolic adaptation in response to arginine deprivation, but the magnitude of recovery varies widely. While population averages show a slow recovery of expression back to roughly untreated levels by 72 hours, depending on the cell line, single cell tracking reveals a diverse range of responses. After three days, many cells still express very little arginine sensor, while some have roughly normal levels, and a few even increased well past initial levels. The timing of recovery also varies among cells, with a small subset gaining resistance earlier than most. These cells are present in all three cell lines, but they are most prominent in SKUT1. Throughout the study, SKUT1 seemed to recover expression more quickly than the other cell lines, which agrees with the fact that it has a higher basal level of ASS1 expression. These results overall show a fairly homogeneous initial response to ADI-PEG20 followed by a heterogeneous recovery period wherein some cells increase expression while others remain low.

When EC50s of arginine for sensor translation were measured, no difference between WT and LTAT of the same cell line exceeded 2 µM, as LTAT cells are able to increase their supply of arginine during ADI-PEG20 treatment rather than using it more efficiently. Measurements of actual arginine concentrations in cells with ADI-PEG20 treatment suggest that LTATs are able to maintain their concentrations of intracellular arginine at roughly five times the level of WT cells. While SKLMS1 LTAT arginine levels are still far below those of untreated media (about 340 µM, shown in Supplemental Figure S2.4), the cells maintain a concentration that is nearly twice their EC90, explaining why translation in LTATs is unaffected by ADI-PEG20. However, the intracellular concentration of arginine in WT cells is substantially below this level when treated with ADI-PEG20, which greatly inhibits protein translation.
Interestingly, after the start of treatment with ADI-PEG20, citrulline takes many hours to reach a steady intracellular concentration. This is likely because these cells do not readily transport citrulline across the plasma membrane. WT cells maintain higher citrulline levels than LTATs, likely as a result of faster conversion to arginine by ASS1 in LTATs. More importantly, and unexpectedly, arginine levels did not significantly recover within 48 hours of ADI-PEG20 treatment. Pairing this with the previous data, arginine sensor expression is seen to increase in WT cells while intracellular arginine concentrations remain constant. This seems contradictory, but the most likely mechanism is that, after initial arginine depletion, synthesis of arginine increases over time, and utilization of arginine increases to match, thereby maintaining a steady concentration of arginine while increasing translation. We performed experiments to test this hypothesis by inhibiting both translation and proteasomal degradation separately up to 48 hours after the initiation of ADI-PEG20 treatment. The results showed that robust translation and degradation continue throughout the course of arginine deprivation and that the sensor we have designed measures adaptive resistance more accurately than actual intracellular arginine concentrations by directly monitoring the ability of cells to use arginine for translation.

2.6 Conclusions

The ability to track responses in individual cells makes the sensor useful for the study of arginine deprivation for clinical development. Using the sensor, multiple ASS1-deficient cell lines were shown to completely lack resistance to ADI-PEG20 at the single cell level in naïve populations but develop a heterogeneous pattern of resistance. This is promising for the potential effectiveness of treatment with ADI-PEG20 and combination therapies with it. The finding that resistance is heterogeneous suggests that temporally targeting the early response to ADI-PEG20 with additional drugs may yield a more effective, homogeneous response. Finally, the concept of
the sensor could be applied to other amino acids through modification, having potential to be used in the study of many other biological systems.
Figure 2.1. Response to arginine deprivation in ASS1-deficient cells

Figure 2.2. Design and demonstration of arginine sensor

a. Diagram of the main components of the arginine sensor. b. ArgSen fluorescence in SKLMS1 cells measured by flow cytometry. Error bars represent standard deviation. c. GFP ArgSen and GFP RanSen fluorescence in SKLMS1 cells measured by microscopy. Error bars represent standard deviation. d. Arginine sensor mRNA in SKLMS1 GFP ArgSen cells. Error bars represent standard error of the mean. e. GFP ArgSen fluorescence with no treatment, glutamine deprivation, ADI-PEG20 treatment, and ADI-PEG20 resistance in three cell lines. Error bars represent standard deviation. f. GFP ArgSen fluorescence with ADI-PEG20 treatment for 12 hours, followed by normal media or continuation of treatment, in three cell lines. Error bars represent standard deviation.
Figure 2.3. Validating degradation of sensor

a. Degradation of GFP ArgSen and deletion variants in SKLMS1 in the presence of a translation inhibitor (CHX). Error bars represent standard deviation. b. Best approximations of half-lives with 95% confidence intervals calculated from data in a. ND = not determined. c. Representative images of SKLMS1 GFP ArgSen fluorescence during treatment with ADI-PEG20, CHX, or a proteasome inhibitor (BTZ). d. GFP ArgSen fluorescence in SKLMS1 cells with ADI-PEG20, adding CHX at various time points. Error bars represent standard deviation. e. GFP ArgSen fluorescence in SKLMS1 cells with ADI-PEG20, adding BTZ at various time points. Error bars represent standard deviation. f. Immunoblots of GFP ArgSen protein in SKLMS1 over ADI-PEG20 time course. g. Quantification of f, along with same data normalized to corresponding mRNA levels from 2.2d. Error bars represent standard error of the mean.
Figure 2.4. Individual cell responses to arginine deprivation

a. GFP ArgSen fluorescence in individual cells over time in three cell lines. b. Representative images of growth and GFP ArgSen fluorescence over three days with or without ADI-PEG20 in three cell lines. c. Counts of cell fates and tiered changes in GFP ArgSen fluorescence at 4 and 24 hours with and without ADI-PEG20 in three cell lines.
**Figure 2.5. Effects of intracellular arginine concentrations on translation**

**a.** Arginine concentrations in SKLMS1 WT and LTAT cells expressing GFP ArgSen over time with ADI-PEG20 treatment as measured by mass spectrometry. **b.** Citrulline concentrations in SKLMS1 WT and LTAT cells expressing GFP ArgSen over time with ADI-PEG20 treatment. **c.** Average GFP ArgSen fluorescence over two hours with various concentrations of arginine in media in three cell lines. Horizontal dotted lines indicate the values with ADI-PEG20 treatment. Vertical dotted lines indicate calculated intracellular arginine concentrations after 48 hours of ADI-PEG20 treatment. **d.** Effective concentration (EC) values for arginine driving translation of GFP ArgSen in three cell lines, calculated from **c.** **e.** Histograms of cell volumes after 48 hours of ADI-PEG20 treatment. **f.** Intracellular arginine concentrations after 48 hours of ADI-PEG20 treatment, along with values used to calculate concentrations. All error bars represent standard deviation.
## Supplemental Table S2.1

List of cell lines and sources.

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**Supplemental Table S2.2**

List of antibodies and sources.
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Supplemental Table S2.3

List and full sequences of DNA oligonucleotides used in this study.
Supplemental Figure S2.1

Global protein translation rates in SKLMS1 WT over 12 hours with no treatment, ADI-PEG20, glutamine deprivation, or ADI-PEG20 plus glutamine deprivation. Error bars represent standard deviation.
Supplemental Figure S2.2

GFP ArgSen fluorescence and growth over 72 hours with ADI-PEG20 treatment in ASS1\(^{+/+}\) and ASS1\(^{-/-}\) MEFs. Error bars represent standard deviation.
A  SKLMS1

Average Green Fluorescence (% of Initial)

Hours

B  SKUT1

Average Green Fluorescence (% of Initial)

Hours

C  SKMEL2

Average Green Fluorescence (% of Initial)

Hours
Supplemental Figure S2.3

Analysis of GFP ArgSen fluorescence in subsets of dividing cells from Figure 2.4. Five untreated cells and roughly 20 ADI-PEG20-treated cells from each cell type were tracked, along with all their descendants, for 24 and 72 hours respectively, excluding cells that could not be tracked with confidence. Data points represent the average green fluorescence of cells at the indicated timepoint, calculated from the averages of each initial cell or its trackable descendants at that time. Error bars represent standard error of the mean.
Supplemental Figure S2.4

Concentrations of arginine in MEM after 72 hours with SKLMS1 WT and LTAT cells with and without ADI-PEG20 treatment. Bars for ADI-PEG20-treated media are not visible because values are too low. Error bars represent standard deviation.
2.7 References


Lee, F. & Yanofsky, C. Transcription termination at the trp operon attenuators of Escherichia coli and Salmonella typhimurium: RNA secondary structure and regulation


Chapter 3: Macropinocytosis from the Microenvironment Enables Growth of Arginine Auxotrophic Tumors in the Absence of Extracellular Arginine

This chapter is based on a manuscript of the same name that is in preparation for publication at the time of writing.

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3.1 Abstract

A common metabolic abnormality in cancer is arginine auxotrophy caused by silencing of the rate limiting enzyme argininosuccinate synthetase 1 (ASS1) in the urea cycle. Here, conditional Ass1 knockout (KO) in a spontaneous murine sarcoma model affected neither tumor initiation nor growth rates, contradicting the prevalent idea that ASS1 silencing confers a proliferative advantage in vivo. Use of extracellular arginine deprivation therapy has encouraged studies of the metabolic adaptations that cause resistance. Classically, resistance occurs by upregulating ASS1, allowing tumors to synthesize arginine directly. Here, Ass1 KO cells grew robustly through arginine starvation in vivo, but died in vitro. Co-culture with Ass1-competent fibroblasts rescued growth through macropinocytosis of vesicles and/or cell fragments, followed by recycling of protein-bound arginine. This process was inhibited in vivo by imipramine or chloroquine. This identifies a new ASS1-independent resistance mechanism to arginine deprivation and
demonstrates complete microenvironmental compensation for amino acid deprivation therapy in vivo.

3.2 Introduction

The enzyme argininosuccinate synthetase 1 (ASS1) is essential for both humans and mice to synthesize arginine, an amino acid that is required for protein synthesis and survival. However, most solid tumors, including many hepatocellular carcinomas, prostate cancers, bladder cancers, small cell lung cancers, and melanomas, amongst others, lack a functional level of ASS1, making it one of the most commonly silenced genes in cancers\(^1\)\(^-\)\(^6\). This is also true of sarcomas, with over 85% being deficient in this enzyme\(^7\). Lack of ASS1 causes these tumors to be auxotrophic for arginine, relying on the naturally plentiful extracellular supply for growth and survival.

Studies have shown that ASS1 downregulation can confer proliferation advantages, particularly by reducing aspartate consumption by ASS1 and diverting it towards pyrimidine synthesis\(^8\). However, this has not been established as the reason for frequent silencing of ASS1 in tumors, nor has it been shown to affect initiation or growth in a spontaneous tumor model. In the case of sarcomas, where this phenotype is frequent, the cells of origin should be considered. For example, ASS1 is essentially undetectable in muscle, from which many sarcomas originate, and is also low in adipose tissue\(^9\). This study directly investigates the effects of a conditional knockout of Ass1 in a spontaneous murine sarcoma model.

Despite the advantage that ASS1 deficiency might confer to tumors in a typical environment, this characteristic makes tumors metabolically vulnerable to arginine deprivation. PEGylated arginine deiminase (ADI-PEG20) is an enzymatic drug that degrades extracellular arginine to citrulline. Treatment with ADI-PEG20 has little effect on most normal cells because they express
a functional level of ASS1 to convert citrulline back into arginine as part of the urea cycle. However, ASS1-deficient cancer cells, with their high demand for arginine and inability to synthesize it, enter a starvation state and become cytostatic\textsuperscript{4,6,7,10–12}. They must adapt to survive or die.

Upregulation of autophagy in response to ADI-PEG20 has been shown to compensate in tumor cells without an extracellular source of arginine\textsuperscript{4,6,7,13,14}. This adaptation is driven by low levels of intracellular arginine inhibiting mTORC1 that in turn causes autophagy to increase. Autophagy can recycle arginine within the cell and provide enough nutrients to sustain the cell in the short term.

In order to gain long term resistance to arginine deprivation therapy, studies have shown that these cancers simply increase their expression of ASS1, often through a c-Myc-dependent mechanism, which is possible because the gene is typically epigenetically repressed rather than being mutated or deleted\textsuperscript{3,7,11,15}. In fact, ASS1 upregulation is the only published mechanism of long-term resistance in this system.

The ultimate required metabolic adaptation in this system is that cancer cells acquire an adequate supply of arginine to grow and proliferate. This may not necessarily be achieved solely by upregulating ASS1 and synthesizing more arginine. There are a multitude of other mechanisms by which cancer cells could theoretically acquire arginine\textsuperscript{16}. Among others, macropinocytosis, necrocytosis, receptor-mediated endocytosis, phagocytosis, entosis, extracellular vesicle transfer, nutrient sharing through gap junctions, and arginine secretion by nearby cells provide plentiful possibilities\textsuperscript{16–28}. It is unclear which, if any, of these mechanisms have the capacity to supply a sufficient amount of arginine for tumors to progress in the absence of plasma arginine. Which
mechanism is best able to provide cancer cells with nutrients under starvation conditions has not been fully elucidated, but a recent report suggests that macropinocytosis helps to overcome similar aspartate limitations. Most import mechanisms would also require the newly acquired materials to be broken down by autophagy/lysosomal degradation to produce free arginine to incorporate into new proteins.

This study defines the first alternative mechanism for resistance to arginine starvation whereby the microenvironment supports tumor growth in the absence of ASS1 expression.

### 3.3 Results

#### 3.3.1 Arginine starvation fails to inhibit protein translation in vivo

As has been demonstrated previously in ASS1 deficient sarcoma cell lines, the proliferation of human sarcoma cell line SKLMS1 is significantly perturbed when treated with ADI-PEG20 (Figure 3.1a) because of very low to no ASS1 expression (Figure 3.1c). Although it slows growth, ADI-PEG20 does not cause meaningful death in these cells (Figure 3.1b). SKLMS1 can become resistant to ADI-PEG20 with long-term exposure by upregulating the expression of ASS1, and resistant cells are called LTAT (long-term ADI-PEG20-treated) (Figure 3.1c).

To test the kinetics of resistance, we tested intracellular arginine-dependent translational capacity of SKLMS1 under arginine starvation conditions. mApple, chosen for its ability to serve as a reporter protein for both in vitro and in vivo experiments, was cloned into a previously published lentiviral construct for reporting the availability of arginine for translation within cells. Matching previously published data using other reporters, ADI-PEG20 caused a rapid decrease in expression of the sensor mApple reporter in vitro compared to no treatment (Figure 3.1d). In contrast, despite the fact that ADI-PEG20 slowed growth similarly in vitro and in vivo (Figures
3.1a and 3.1e), no decrease in sensor expression was found in vivo (Figure 3.1f). Representative sensor fluorescence images are shown in Supplemental Figure S3.1a.

Metabolite measurements revealed that ADI-PEG20 was indeed very effective in vivo, decreasing arginine levels in serum to near zero within two days and suppressing them for the duration of treatment (Figure 3.1g). Correspondingly, citrulline levels increased and reached a steady state within 7 days (Figure 3.1h). Metabolite measurements in the tumors showed a similar but less dramatic pattern, with the tumors losing 81% of their arginine by two days and 90% by 15 days (Figure 3.1i). Citrulline levels likewise increased in the tumors as in the serum (Figure 3.1j).

These decreased arginine concentrations resulting from ADI-PEG20 treatment caused metabolic changes in the tumors. To confirm that a metabolic shift to glutamine biology was still occurring in vivo as shown previously in vitro, positron emission tomography (PET) was used to estimate utilization of glutamine by the tumors by measuring the uptake of analog molecule $^{18}$F-$(2S,4R)$4-fluoroglutamine ({$^{18}$F-$(2S,4R)$4-FGln}). ADI-PEG20 indeed caused increased uptake of glutamine in SKLMS1 tumors (Supplemental Figures S3.2b and S3.2c). This suggests that the metabolic shifts caused by ADI-PEG20 hold in this system, but that cells in the intratumoral environment rapidly compensate for the loss of arginine and are able to maintain arginine-dependent translation.

### 3.3.2 ASS1 deficiency is not advantageous for spontaneous murine sarcomas

In order to better understand the effects of the loss of ASS1 on tumor biology, a genetic model was made with $\text{Myf5}$-driven Cre expression causing recombination of floxed $p53$ genes in both groups and floxed $\text{Ass1}$ genes in only one group of a spontaneous murine sarcoma experiment, resulting in nonfunctional protein products (Figure 3.2a). Most resulting tumors were identified
morphologically as rhabdomyosarcomas, and a significant proportion were osteosarcomas (Supplemental Table S3.1a), with images shown in Figures 3.2b and 3.2c. Knocking out Assl did not significantly change the rate of tumor initiation in this sarcoma model (Figure 3.2d). Likewise, the difference in growth rates between the two groups was not significant (Figure 3.2e).

When ASS1 protein was measured by immunoblot of frozen tumors, all Assl null tumors had low ASS1 (Figure 3.2f). A small amount of ASS1 was detected in these tumors, indicating the presence of stromal cells that did not express Myf5-Cre and therefore had an intact Assl gene. 18 of 26 (69%) Assl WT tumors also expressed ASS1 at a level lower than the highest-expressing Assl null tumor (Figure 3.2f). This group of tumors was then segregated along this dividing line for further analysis. There was a trend toward sooner initiation in tumors with high ASS1, and when compared to Assl null tumors, the ASS1 high tumors appeared significantly sooner (Figure 3.2g). ASS1 high tumors also had significantly higher growth rates than ASS1 low tumors (Figure 3.2h).

3.3.3 Cells without ASS1 grow robustly through arginine deprivation in vivo but die in vitro

Cell lines were made from many of the spontaneous sarcomas from Figure 3.2 (Supplemental Table S3.1b). Those cell lines with low ASS1 from both Assl null and WT tumors were shown to be highly sensitive to ADI-PEG20 and unable to proliferate after several hours, while many cells died within the first few days of treatment (Figures 3.3a and 3.3b). Not all Assl WT tumor cell lines died with ADI-PEG20 treatment, as BVM03O showed only slowed proliferation (Figure 3.3a), similar to SKLMS1, likely due to its higher level of ASS1 (Supplemental Figure S3.2a). ASS1 low cell lines differed in their responses to long-term ADI-PEG20 treatment
depending on their genotype. Ass1 null cell lines invariably died completely when treated with ADI-PEG20, while all Ass1 WT cell lines developed resistance over weeks to months of treatment.

To determine the effects of ADI-PEG20 on the proliferation of Ass1 null cells in vivo, multiple Ass1 null murine sarcoma cell lines were injected subcutaneously into either syngeneic C57BL/6J mice or NU/J mice. There, very little effect of ADI-PEG20 was found in NU/J mice in two of the three cell lines tested, whereas ADI-PEG20 seemed to slow but not stop growth in immune-competent syngeneic C57BL/6J mice (Figures 3.3c and 3.3d and Supplemental Figure S3.2b). Crucially, Ass1 null tumors from all tested cell lines were able to grow robustly through ADI-PEG20 treatment in vivo while the drug remained 100% lethal to their parental cell lines in vitro, illustrating a stark contrast in the effectiveness of ADI-PEG20 between the different environments and explaining the in vivo arginine sensor data (Figure 3.1f).

3.3.4 Ass1 KO tumor cell growth is enabled by macropinocytosis of EVs from ASS1-competent MEFs during arginine deprivation

To further investigate this phenomenon, BVMA01R Ass1 null tumors that had grown through ADI-PEG20 were taken ex vivo and made into cell lines. These cells remained sensitive to ADI-PEG20 in vitro (Figure 3.4a) and could not survive long-term treatment. When cultured on a feeder layer of mouse embryonic fibroblasts (MEFs), these cells were more resistant to death, and the shown cell line from mouse 1037 (1037 tumor cells) accepted support from the MEFs well enough to proliferate through ADI-PEG20 treatment (Figure 3.4a). All tested ASS1 low cell lines showed a similar ability to accept growth support from fibroblasts during ADI-PEG20 treatment, including Ass1 WT murine sarcoma lines (Supplemental Figures S3.3a and S3.3b). The human sarcoma cell lines SKLMS1 and SKUT1, which expresses slightly more ASS1¹⁰,
also accepted support from human fibroblasts (Supplemental Figures S3.3c and S3.3d). While the observed growth support was far less robust than what tumors received in vivo, this model provided an opportunity to investigate the mechanisms by which arginine auxotrophic cell proliferation could be supported by other cells in the absence of free extracellular arginine.

Co-culturing 1037 tumor cells with MEFs rather than growing them on a MEF feeder layer provided a similar growth support effect (Figure 3.4b). This effect could not be sustained unless the MEFs expressed ASS1 (Figure 3.4b). Knockout of Ass1 in MEFs was validated in Supplemental Figures S3.4a-S3.4d. Multiple possible mechanisms of growth support were investigated, and it was found that neither MEF-conditioned media nor inhibition of gap junctions or clathrin-mediated endocytosis could recreate or prevent the growth support phenomenon, respectively (Supplemental Figures S3.5a-S3.5c). When MEFs were stained with a green fluorescent membrane dye, large fragments of MEF membranes were seen disconnected from their cells of origin, making contact with the tumor cells, showing that cell fragments and extracellular vesicles (EVs) could likely be transferred from MEFs to tumor cells (Figure 3.4c).

To test whether this was a major mechanism by which MEFs supported tumor cell growth, EVs were isolated from MEFs and added to 1037 tumor cells in the presence of ADI-PEG20. These EVs protected from death and supported growth similarly to co-cultured MEFs (Figure 3.4d). Further, the EV production inhibitor EST completely abrogated the growth supportive effect of MEFs (Figure 3.4e) while not affecting the growth of the MEFs themselves in the presence of ADI-PEG20 (Supplemental Figure S3.5d), indicating that the growth support was not diminished because of decreased MEF viability. Another EV production inhibitor, imipramine, had a similar effect to EST (Figure 3.4f), even though the two inhibitors target different pathways of EV production30,31.
This may be due to the fact that imipramine can also inhibit macropinocytosis\textsuperscript{32}. Therefore, we tested the effect of imipramine on the uptake of fluorescently labeled membranes from MEFs into 1037 cells. Indeed, we found that imipramine significantly decreased the amount of membrane that 1037 cells took from the media over 24 hours when treated with ADI-PEG20 (Figure 3.4g). Because of its apparent inhibition of macropinocytosis in this model, directly targeting the tumor cells, along with its proven track record of safe use in both mice and humans, imipramine was tested for its ability to slow growth of BVMA01R tumors \textit{in vivo} in combination with ADI-PEG20. The combination of ADI-PEG20 and imipramine significantly slowed tumor growth, while either single agent did not (Figure 3.4h). This was not due to lower ASS1 expression, as no treatment group differed significantly in tumor ASS1 levels compared to untreated (Figure 3.4i).

\textbf{3.3.5 Autophagy/lysosomal degradation is required for cells receiving but not cells supplying growth support}

As ingested proteins must be degraded by tumor cells to obtain useful arginine, the autophagy/lysosomal degradation inhibitor chloroquine was tested with the co-culture of 1037 cells and MEFs to determine whether autophagy was essential to the growth support process. Chloroquine completely inhibited growth of the tumor cells in co-culture with MEFs and ADI-PEG20 (Figure 3.5a).

In order to determine whether autophagy was essential for the growth support process in the tumor cells, MEFs, or both, two MEF cell lines were generated with either \textit{Atg7} or \textit{Fip200} floxed. These genes were then knocked out with Cre to inhibit autophagy specifically in the MEFs (Figures 3.5b and 3.5c and Supplemental Figures S3.4e-S3.4g). The effects on co-culture growth support were tested, and both floxed cell lines were able to support proliferation of 1037
cells in the presence of ADI-PEG20. *Atg7* KO MEFs supported growth to a lesser extent than their floxed counterparts, while *Fip200* KO MEFs were completely unable to support growth (Figures 3.5d and 3.5e). These results mirrored the effects of the gene knockouts on the MEFs themselves, as the *Atg7* KO MEFs stayed relatively healthy in the presence of ADI-PEG20, while the *Fip200* KO MEFs died rapidly due in part to extremely low ASS1 (Supplemental Figures S3.4e and S3.4h). To separate the effects of MEF viability from the effects of MEF autophagy, GFP-ASS1 was exogenously expressed in these same cell lines, giving them ample resistance to ADI-PEG20 (Supplemental Figures S3.4e and S3.4i). The ASS1-expressing floxed MEFs provided slightly more growth support than their *Ass1* WT counterparts (Figures 3.5d and 3.5e), and ASS1 expression rescued the ability of *Atg7* and *Fip200* KO MEFs to provide growth support (Figures 3.5d and 3.5e).

When BVMA01R tumors were treated *in vivo* with both ADI-PEG20 and chloroquine, only the combination significantly slowed tumor growth (Figure 3.6f). Again, this was shown to not be due to a difference in tumor ASS1 levels among the groups, as none significantly differed from untreated (Figure 3.6g). RNA sequencing of these tumors revealed many differentially regulated genes (Supplemental Figure S3.6a) and pathways. Relevant pathways are shown in Supplemental Figure S3.6b. Notably, ADI-PEG20 greatly increased expression of genes related to cell adhesion, supporting the observation that ADI-PEG20 promoted cell-cell contacts and clustering of these cells *in vitro*, an effect that was more prominent in the presence of MEFs (Supplemental Figure S3.6c). Phagosome and lysosome pathways were also upregulated, supporting the hypothesis that the tumor cells uptake and digest bulk nutrients from outside the cell. Strikingly, 18 of the 20 most differentially regulated pathways with ADI-PEG20 treatment are upregulated, with the cell cycle pathway being a notable exception (Supplemental Figure S3.6d).
Additionally, 13 of the 20 pathways are related to the immune system, which may indicate increased immune cell infiltration, a possibility that is under current investigation.

### 3.4 Discussion
Based on the published literature describing the lack of ASS1 in many cancers and the potential advantages this condition confers, knocking out Ass1 in addition to p53 in a murine sarcoma model was expected to result in faster initiation and/or faster growth of tumors. This was not the case, largely because of the naturally low levels of ASS1 in most of the Ass1 WT tumors. In retrospect, the fact that the spontaneous murine sarcoma model roughly recapitulated the finding of naturally low ASS1 in most human sarcomas should have been expected. Muscle, the main tissue of tumor origin in this model, expresses as little ASS1 any tissue in the body. This fact combined with the results of this study suggest that the main reason for a lack of ASS1 in many sarcomas is simply inheritance of suppressed ASS1 expression from their cells of origin. This interpretation conflicts with studies showing advantages of ASS1 suppression in cancer cells.

In fact, ASS1 upregulation gives an advantage to tumors in this model, which opposes prevailing thought on the topic, and is the focus of further investigation.

When using arginine deprivation therapy to target ASS1-deficient cancers, it was surprising that SKLMS1 tumors did not decrease their translational capacity when treated with ADI-PEG20 in vivo. This was the first bit of evidence indicating that the in vitro and in vivo environments may prove to be crucially different in the context of arginine deprivation therapy. When all tested Ass1 KO tumors were able to grow rapidly through ADI-PEG20 treatment in multiple mouse strains, it was clear that a novel mechanism of resistance to arginine deprivation therapy must be responsible. The evidence pointed strongly toward microenvironmental metabolic support of tumor growth.
This metabolic adaptation to ADI-PEG20 was then confirmed to not be intrinsic to the cancer cells, but could be recapitulated to a lesser extent \textit{in vitro} by co-culturing tumor cells with ASS1-competent MEFs, further supporting the hypothesis of microenvironmental growth support. While most major routes of possible nutrient transfer were tested, experiments showed that the most important path in this system \textit{in vitro} is likely the excretion of EVs and/or cell fragments from MEFs and subsequent uptake of these membrane-bound particles into tumor cells by macropinocytosis, followed by recycling of the proteins within to supply the tumor cells with arginine (Figure 3.6). This recycling seems to be achieved largely through autophagy/lysosomal degradation, as shown by the results of chloroquine treatment. It was further determined that the mechanism of growth support does not require autophagy in the cells providing the support but rather the cells receiving the nutrients.

These findings result in new clinical opportunities for ADI-PEG20 combination therapies and clinical trials. Autophagy proved critical enough in this system that chloroquine had pronounced synergy in slowing tumor growth in combination with ADI-PEG20 \textit{in vivo}. Imipramine, an inhibitor that theoretically targets the tumors more specifically than chloroquine in this system, largely by inhibiting macropinocytosis, also showed clear synergy with ADI-PEG20 \textit{in vivo}. Additionally, RNA sequencing strongly suggests that ADI-PEG20 recruits immune cells to tumors, which makes combination with immune therapy a very promising avenue for future research.

This study also elucidates the magnitude of the ability of the microenvironment to metabolically support tumor growth. The system featured totally arginine-auxotrophic cancer cells, in conditions that would otherwise be lethal, growing through ADI-PEG20-induced arginine starvation robustly with the help of host cells that were able to synthesize arginine and provide it...
to the tumor. Some experiments even showed no apparent effect of ADI-PEG20 on the growth of the Ass1 KO tumors, indicating the magnitude to which this phenomenon can assist tumors. To our knowledge, there have been no reports of microenvironmental tumor growth support to this extent.

Overall, we have identified a new noncanonical mechanism for ADI-PEG20 resistance and a first step in metabolic adaptation to ADI-PEG20. While re-expression of ASS1 via c-Myc expression is the canonical adaptation mechanism, macropinocytosis alleviates tumors acutely as the first step in overcoming arginine starvation in arginine-auxotrophic tumors. These findings explain the negative phase 3 clinical trials in some cancers such as hepatocellular carcinoma (HCC), where ADI-PEG20 was found to lower circulating arginine levels but not to affect tumor progression-free survival or overall survival. Additionally, this work suggests that new clinical trials using chloroquine, imipramine, or a combination of both with ADI-PEG20 in HCC may overcome the ability of the microenvironment to compensate for ADI-PEG20-induced starvation.

As has become clear from multiple clinical trials, amino acid starvation in arginine-auxotrophic tumors requires multiple agents. Currently, ADI-PEG20 is being tested in combination with pemetrexed and cisplatin in mesothelioma (NCT02709512)\textsuperscript{34} and with docetaxel and gemcitabine in sarcoma (NCT pending)\textsuperscript{35}. The observations in this study suggest that the addition of chloroquine or imipramine to these regiments would prevent macropinocytosis and increase the efficacy of arginine starvation as a therapeutic. The complexity of cancer metabolic redundancies is demonstrating that development of agents such as ADI-PEG20 will require not only a full understanding of metabolic adaptations, but also that we must target the urea cycle and the adaptive pathways simultaneously.
3.5 Methods

3.5.1 Spontaneous Murine Sarcoma Model

Ass1^{F/F} mice were bred as previously described\textsuperscript{12}. Strain #008462 p53^{F/F} mice were obtained from The Jackson Laboratory (Bar Harbor, ME)\textsuperscript{36}. Strain #010529 Myf5-Cre mice were also obtained from The Jackson Laboratory\textsuperscript{37}. Mice were bred so that all experimental mice were females that were heterozygous for Myf5-Cre and homozygous p53^{F/F}. The indicated mice were bred to also be homozygous Ass1^{F/F}.

Experimental mice were carefully checked for tumors two times per week. All discovered tumors were measured with calipers at least every other day, and volume was calculated as half of length X width\textsuperscript{2}. Growth of each tumor was fitted in GraphPad Prism (GraphPad Software, San Diego, CA) to the following exponential growth equation:

\[ Y = Y_0 \times e^{k \times X} \]  

(3.1)

where \( Y_0 \) is the Y value when X (time) is 0, and \( k \) is the rate constant of growth. Graphs of tumor growth with a fitted curve used this same method and constrained \( Y_0 \) to the mean of the Y values on Day 0.

All small animal experiments were approved by the Animal Studies Committee at Washington University School of Medicine, and all protocol guidelines were followed. Tumors in this study were harvested only when a protocol endpoint was reached while the mouse was alive. A portion of each harvested tumor was fixed in 10% neutral buffered formalin and embedded in paraffin. 5 \( \mu \text{m} \) thick slides were cut for hematoxylin and eosin (H&E) staining where shown. All tumor histology classifications were done blindly by a trained pathologist. Another portion of each harvested tumor was flash frozen in liquid nitrogen and stored at -80\(^\circ\) C.
3.5.2 Cell Line Generation
Ass1\textsuperscript{F/F} MEFs were generated as previously described\textsuperscript{12}. Cell lines were generated from tumors as follows. Tissue was taken from the interior of the tumor, minced, washed, and incubated in 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA) at 4° C overnight. Trypsin was then aspirated, and tissue pieces were incubated with residual trypsin at 37° C for 30 min. Tissue pieces were then dispersed by pipetting with warm, complete I-20 media (described below), filtered through sterile 100 µm nylon mesh, and plated in I-20 media. Cells were then grown and passaged before being used for experiments.

3.5.3 Cell Culture
SKLMS1, SKUT1, and HDF\textalpha{} cell lines were obtained from the American Type Culture Collection (Manassas, VA). Atg7\textsuperscript{F/F} MEFs were generated from Atg7\textsuperscript{F/F} mice, which were made and provided by Dr. M. Komatsu (Tokyo Metropolitan Institute of Medical Science)\textsuperscript{38,39}. Fip200\textsuperscript{F/F} MEFs were generated from Atg200\textsuperscript{F/F} mice\textsuperscript{40,41}. All MEFs used in experiments were first spontaneously immortalized as previously described\textsuperscript{12}. All LTAT cells were developed by passaging cells normally with 1 µg/mL ADI-PEG20 (Polaris Pharmaceuticals, San Diego, CA) until growth rate stabilized. SKLMS1 and SKUT1 cells were grown in media consisting of MEM (Thermo Fisher Scientific) with 10% fetal bovine serum (FBS) (Bio-Technne, Minneapolis, MN), 1% 100X penicillin/streptomycin (Thermo Fisher Scientific), and 2.5 µg/mL Plasmocin (InvivoGen, San Diego, CA). All other cell lines were grown in I-20 media, consisting of IMDM (Thermo Fisher Scientific) with 20% FBS, 1% 100X penicillin/streptomycin, 1% 100X MEM nonessential amino acids (Corning, Corning, NY), 0.0007% 2-mercaptoethanol (MilliporeSigma, St. Louis, MO), and 2.5 µg/mL Plasmocin. Phenol red-free versions of these media were used for any experiment requiring quantification of green fluorescence. Cells were grown in a cell culture incubator with 5% CO\textsubscript{2} at 37° C.
Cell line experiments were conducted in 96-well plates and started the day after plating except where indicated. For these plates, cells were plated at a density per well of 3,000 for SKLMS1, HDFa, and MEFs; 5,000 for murine tumor cell lines; and 7,500 for SKUT1. For co-culture experiments, cells were plated and allowed to settle and attach together. The growth media of the cell line being counted was used when cell lines in the same well typically used different media. Feeder layers of cells were plated two days before the start of the experiment and treated with mitomycin C (MMC) (MilliporeSigma) the next day, immediately before the tumor cells were seeded on top.

MMC treatment was performed by adding 10 µg/mL MMC to cells for 2 hours at 37° C, then washing three times with DPBS (Thermo Fisher Scientific). DiOC$_{18}(3)$ (3,3’-Dioctadecyloxacarbocyanine Perchlorate) (Thermo Fisher Scientific) staining was performed according to the manufacturer’s instructions. All in vitro ADI-PEG20 treatment was done with media that had been pre-treated with 1 µg/mL ADI-PEG20 overnight at 37° C. EST (aloxistatin) (MedChemExpress, Monmouth Junction, NJ), imipramine (MilliporeSigma), chloroquine (MilliporeSigma), tonabersat (Cayman Chemical Company, Ann Arbor, MI), carbenoxolone (Apexbio Technology, Houston, TX), and Pitstop 2 (MilliporeSigma) were administered at the indicated concentrations, and controls were treated with the same volume of vehicle.

### 3.5.4 Vesicle Uptake Assay

3X10$^6$ DiOC$_{18}(3)$-stained or unstained MEFs were plated in a 10-cm dish, and 10 mL phenol red-free media was added the next day and left on for 24 hours. Media was then taken off MEFs and centrifuged for 5 min at 200 X g, and the supernatant was collected. Conditioned media for the supplemental growth support experiment was obtained in the same way with unstained
MEFs, plating them at the same density per cm\(^2\) and adding the same depth of media as in the 96-well plates, and treating with MMC before conditioning media.

MMC-treated BVMA01R cells were plated in a 96-well plate at 15,000 cells per well, and media collected from MEFs was pre-treated with ADI-PEG20 and added to the BVMA01R cells with or without imipramine. This media was collected again after 24 hours, and DiOC\(_{18}(3)\) fluorescence was measured on a Tecan Infinite M200 plate reader (Tecan, Männedorf, Switzerland). Data was normalized so that 0% represented the average fluorescence of conditioned media from unstained MEFs and 100% represented the average fluorescence of media from stained MEFs, both without being added to BVMA01R cells.

### 3.5.5 Extracellular Vesicle Isolation
MEFs were plated on five 10-cm dishes of 8X10\(^5\) cells each. The next day, 5 mL of media was added to each plate for 24 hours, then harvested with Total Exosome Isolation Reagent (from cell culture media) (Thermo Fisher Scientific) according to the manufacturer’s instructions. Isolated EVs were resuspended in ADI-PEG20 pre-treated media and added to 1037 cells in a 96-well plate at the indicated concentrations.

### 3.5.6 Automated Cell Imaging
All automated cell imaging was done on cells in 96-well plates with either an Incucyte ZOOM (Sartorius, Göttingen, Germany) or Incucyte S3 (Sartorius) automated cell imaging system. Cells that were counted were first transduced with either Incucyte® Nuclight Red Lentivirus (EF1a, Puro) (Sartorius) or Incucyte® Nuclight Green Lentivirus (EF1a, Puro) (Sartorius) and selected with puromycin for stable expression. Incucyte software was used to count the red or green nuclei of transduced cells to measure the number of live cells at each timepoint. The cell impermeable DNA-binding dye YOYO™-1 Iodide (Thermo Fisher Scientific) was used to stain
dead cells and was quantified in the same way. mApple ArgSen expression was measured as the average integrated intensity of the nuclei.

3.5.7 Immunoblotting
Cells were plated at 1X10^5 cells per well of a 6-well plate for all immunoblot experiments. Fresh media was added the next day, and the cells were harvested 24 hours later. Cell pellets were lysed in 1X Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) with 1X Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) added. Mouse tissues were lysed in 2X Cell Lysis Buffer with 1X Halt™ Protease and Phosphatase Inhibitor Cocktail. Tissues were mashed with Biomasher II® Disposable Micro Tissue Homogenizers (DWK Life Sciences, Millville, NJ). After resuspension or homogenization in lysis buffer, samples were incubated on ice for 20 min with periodic vortexing, then sonicated. Samples were centrifuged at 21,130 X g at 4° C for 10 min, and the supernatant was collected. Samples were run on a Wes automated immunoblot machine (Bio-Techne) according to the manufacturer’s instructions, using the primary antibodies indicated in Supplemental Table S3.2a. Protein amounts were quantified with Compass software for Simple Western (Bio-Techne) and normalized to total protein.

3.5.8 Cloning
All primer sequences are listed in Supplemental Table S3.2b. Primers were ordered from Integrated DNA Technologies (Coralville, IA). The mApple gene was PCR amplified from plasmid mApple-N1 (Addgene, Watertown, MA) with SalI BamHI mApple fwd and NotI NLS mApple rev primers to add restriction sites and a C-terminal nuclear localization signal. mApple-N1 was a gift from Michael Davidson (Addgene plasmid #54567; http://n2t.net/addgene:54567; RRID: Addgene_54567)^42. The amplicon was then cloned into the pKLV2-EF1a-GFP ArgSen
plasmid (Addgene plasmid #184695)\textsuperscript{12} with restriction sites BamHI and NotI, replacing EGFP with mApple. The sensor gene with mApple reporter was then subcloned into the pLV-EF1a-GFP ArgSen plasmid (Addgene plasmid #184704)\textsuperscript{12}, completely replacing the original insert. This pLV-EF1a-mApple ArgSen plasmid was used for experiments.

The EGFP gene was PCR amplified with BamHI EGFP fwd and BglII EGFP rev primers, then cloned into the MSCV puro plasmid (Plasmid #68469)\textsuperscript{43} by digesting BglII on the vector and C terminus of the insert, and BamHI (compatible with BglII) on the N terminus of the insert, followed by ligation. Cloning destroyed the N terminal restriction site and preserved the C terminal BglII site. MSCV Puro was a gift from Tyler Jacks (Addgene plasmid #68469; http://n2t.net/addgene:68469; RRID: Addgene_68469). ASS1 cDNA was then PCR amplified from a HEK 293T cDNA library with ASS1 fwd and ASS1 rev primers. This PCR product was further amplified while adding restriction sites with BglII ASS1 fwd and MfeI ASS1 rev primers. This was then cloned into the MSCV EGFP puro plasmid with BglII on the N terminus of the insert, linking the ASS1 and EGFP genes, and leaving the C terminus of the insert undigested and ligating with the blunt end left by HpaI digestion of the vector. The resulting GFP-ASS1 fusion sequence was then PCR amplified with AscI EGFP fwd and NotI ASS1 rev primers and cloned into the pLV vector with AscI and NotI restriction sites.

All cloned constructs were integrated into lentiviruses and used to transduce the indicated cells, which were selected with puromycin.

**3.5.9 In Vivo Arginine Sensor**

1.5 million SKLMS1 cells expressing mApple ArgSen were grafted subcutaneously into female NU/J mice (Strain #002019, The Jackson Laboratory) 4-6 weeks of age. All subcutaneous grafts in this study were done by suspending cells in media, mixing 1:1 with Matrigel (Corning), and
injecting 100 µL under the skin on the flank. mApple ArgSen mice were fed an alfalfa-free, low fluorescence diet, the Teklad global 18% protein diet (Inotiv, West Lafayette, IN). Treatment and imaging were started after tumors reached 200 mm³. Images of mApple fluorescence were acquired by a Bruker Multispectral FX Pro system (Bruker, Billerica, MA), and images were analyzed with Fiji software (fiji.sc).

3.5.10 Tumor Growth Experiments
5 million of the indicated cells were grafted subcutaneously into female C57BL/6J (Strain #000664, The Jackson Laboratory) or NU/J mice 4-6 weeks of age. Treatments began after tumors reached 200 mm³. ADI-PEG20 treatments were administered by intramuscular injection as 13 µL of 11 mg/mL ADI-PEG20 every three days. Imipramine was dissolved in 138 mM NaCl at 30 mg/mL and administered intraperitoneally (IP) at 30 mg/kg/day. Chloroquine was dissolved in DPBS at 20 mg/mL and administered IP at 60 mg/kg/day. Mice were euthanized after 30 days of treatment or when tumors reached 1,600 mm³ for the imipramine experiment or 2,000 mm³ for the chloroquine experiment.

3.5.11 Gene Knockout
Ass1+/− MEFs and Ass1F/F controls were generated as previously described¹². Atg7F/F and Fip200F/F MEFs were infected with either Ad5CMVCre or Ad5CMVempty adenoviral particles (University of Iowa, Iowa City, IA) to recombine and knock out the floxed genes or serve as negative controls, respectively. Cells were lysed for genotyping with DirectPCR Lysis Reagent (Cell) (Viagen Biotech, Los Angeles, CA) following the manufacturer’s instructions. Ass1 genotyping used Ass1 del fwd, Ass1 FF fwd, and Ass1 geno rev primers. Atg7 genotyping used Atg7 geno fwd, Atg7 FF rev, and Atg7 del rev primers. Fip200 genotyping used Fip200 del fwd,
Fip200 FF fwd, and Fip200 geno rev primers. All gene knockouts were also validated by immunoblot.

3.5.12 Metabolomics
Blood and tumors were harvested from NU/J mice harboring SKLMS1 tumors at the indicated timepoints. Blood was allowed to clot at room temperature for 30 min, then centrifuged at 1,500 X g for 10 min at 4° C. The supernatant serum was collected and immediately stored at -80° C. Tumors were flash frozen upon harvesting and stored at -80° C. Samples were shipped on dry ice for metabolomic analysis at Human Metabolome Technologies America (Boston, MA).

3.5.13 RNA Sequencing
RNA was isolated from frozen tumor samples with the Direct-zol RNA Miniprep Plus kit (Zymo Research, Irvine, CA). RNA samples were submitted to the Genome Technology Access Center at the McDonnell Genome Institute (GTAC@MGI) of Washington University in St. Louis. GTAC@MGI prepared the RNA using the KAPA RiboErase method, sequenced with an Illumina NovaSeq sequencer (Illumina, San Diego, CA), and analyzed with their standard RNA-seq analytical pipeline.

3.5.14 Positron Emission Tomography
Female NU/J mice harboring SKLMS1 tumors (n=4) underwent small animal positron emission tomography (PET) imaging one day prior to ADI-PEG20 administration (NT) and 13 days after starting treatment. The mice were anesthetized with 1% isoflurane/oxygen followed by dynamic PET acquisition at 0-60 min after intravenous tail injection of 8-12 MBq of $^{18}$F-(2 S,4 R)-4-FGln using the INVEON imaging system. The animals were deprived of food for four hours prior to the $^{18}$F-(2 S,4 R)-4-FGln studies. The animals were maintained at 37° C during the study using a
warming lamp. Computed tomography (CT) images were also acquired with the INVEON system.

The PET data were analyzed by manually drawing 3-dimensional regions of interest (ROIs) over the tumor identified on the PET studies with correlation to CT to confirm the tumor location. The uptake data were expressed as mean standardized uptake values (SUVs) for each ROI at 50-60 min after injection of $^{18}$F-(2 S,4 R)4-FGln.

### 3.5.15 Quantification and Statistical Analysis
Statistical tests were performed in GraphPad Prism 9 software. Differences between time series were analyzed by 2-way ANOVA, using a mixed model when necessary. Grouped data were analyzed by Mann-Whitney test, Kolmogorov-Smirnov test, paired t test, or unpaired t test as appropriate. Time-to-event data were analyzed by Log-rank (Mantel-Cox) test. All error bars show standard deviation, except tumor growth error bars show standard error of the mean. All statistical details of experiments can be found in the figure legends. P-values are denoted in the following way: ns: $p > 0.05$; *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$. 
Figure 3.1. Arginine starvation fails to inhibit protein translation in vivo

a. Proliferation of SKLMS1 cells with and without ADI-PEG20 treatment in vitro. b. Death of SKLMS1 cells with and without ADI-PEG20 treatment in vitro. c. ASS1 expression in WT and LTAT SKLMS1 cells. d. Expression of mApple ArgSen in SKLMS1 cells with and without ADI-PEG20 treatment in vitro. e. Growth of SKLMS1 tumors with and without ADI-PEG20 treatment in NU/J mice. f. Expression of mApple ArgSen in SKLMS1 tumors with and without ADI-PEG20 treatment in NU/J mice. See also Supplemental Figure S3.1a. g. Serum arginine concentrations of NU/J mice treated with ADI-PEG20. h. Serum citrulline concentrations of NU/J mice treated with ADI-PEG20. i. SKLMS1 tumor arginine concentrations from NU/J mice treated with ADI-PEG20. j. SKLMS1 tumor citrulline concentrations from NU/J mice treated with ADI-PEG20. Data are mean ± s.d. except in e (mean ± s.e.m.) (n = 12 in a, b; n = 3 in c, g-j; n = 8-12 in d; n = 12-24 in e; n = 6 in f). Two-way ANOVA tests except in c (two-tailed unpaired t-test).
Figure 3.2. ASS1 deficiency is not advantageous for spontaneous murine sarcomas

a. Ass1 knockout genetic schema and example genotyping result. Gel image is cropped for clarity. b. Example H&E stainings of spontaneous murine rhabdomyosarcomas from both control and Ass1 KO groups. Numbers indicate mouse IDs. Scale bar = 100 µm. c. Example H&E stainings of spontaneous murine osteosarcomas from both control and Ass1 KO groups. Numbers indicate mouse IDs. Scale bar = 100 µm. d. Tumor-free survival plot of both control and Ass1 KO groups. e. Growth rates of tumors from both control and Ass1 KO groups. f. ASS1 expression of tumors from both control and Ass1 KO groups. g. Tumor-free survival plot of Ass1 KO group along with control mice segregated (along dotted line in f) by ASS1 expression in their tumors. h. Growth rates of tumors of control group mice segregated by ASS1 expression in the tumors. Data are mean ± s.d. (replicates as shown). Log-rank tests for d, g. Mann-Whitney tests for e, f, h.
Figure 3.3. Cells without ASS1 grow robustly through arginine deprivation *in vivo* but die *in vitro*

**a.** Proliferation *in vitro* with and without ADI-PEG20 of three *Myf5-Cre;p53<sup>-/-</sup>;Ass1<sup>+/+</sup>* cell lines isolated from spontaneous murine sarcomas. **b.** Proliferation *in vitro* with and without ADI-PEG20 of three *Myf5-Cre;p53<sup>-/-</sup>;Ass1<sup>-/-</sup>* cell lines isolated from spontaneous murine sarcomas. **c.** Tumor growth of Ass1<sup>-/-</sup> BVMA01R grafts in both C57BL/6J and NU/J mice with and without ADI-PEG20 treatment. **d.** Tumor growth of Ass1<sup>-/-</sup> BVMA02R grafts in both C57BL/6J and NU/J mice with and without ADI-PEG20 treatment. Data are mean ± s.d. in **a** (n = 4), **b** (n = 12), mean ± s.e.m. in **c, d** (n = 3-4). Two-way ANOVA tests. See also Supplemental Figure S3.2.
Figure 3.4. Ass1 KO tumor cell growth is enabled by macropinocytosis of EVs from ASS1-competent MEFs during arginine deprivation

a. Proliferation of Ass1 KO murine sarcoma cell line (1037) taken ex vivo after growing through ADI-PEG20 treatment in vivo. 1037 cells die when treated with ADI-PEG20, but they grow through ADI-PEG20 when cultured on feeder layers of various numbers of MMC-treated MEFs.
b. Proliferation of 1037 cells when treated with ADI-PEG20 and co-cultured with MEFs that have Ass1 either floxed or knocked out. See also Supplemental Figures S3.4a-S3.4d.
c. Images of 1037 cells with red nuclei co-cultured with MEFs with green-stained membranes. Yellow arrows indicate examples of large fragments of MEFs contacting 1037 cells. Images are cropped for clarity.
d. Proliferation of 1037 cells when treated with ADI-PEG20 with or without the addition of EVs isolated from MEFs.
e. Fold change in number of live 1037 cells after 96 hours of treatment with ADI-PEG20 with and without both co-cultured MEFs and 25 µM EST. See also Supplemental Figure S3.5d.
f. Fold change in number of live 1037 cells after 96 hours of treatment with ADI-PEG20 with and without both co-cultured MEFs and 25 µM imipramine.
g. Uptake of MEF EVs by 1037 cells with or without 20 µM imipramine during ADI-PEG20 treatment. More fluorescence remaining in media indicates less uptake.
h. Growth of BVMA01R tumors grafted into syngeneic C57BL/6J mice with and without both ADI-PEG20 and imipramine treatments.
i. Quantification of ASS1 levels in harvested tumors from h. Data are mean ± s.d. except in h (mean ± s.e.m.) (n = 2 in a; n = 3 in b, e, f, g; n = 4 in d; n = 5-12 in h; n = 3-6 in i). Two-way ANOVA tests for a, b, d, h. Two-tailed paired t-tests for e, f, g. Two-tailed unpaired t-test for i.
Figure 3.5. Autophagy/lysosomal degradation is required for cells receiving but not cells supplying growth support

a. Proliferation of 1037 cells when treated with ADI-PEG20 with and without both co-cultured MEFs and 20 μM chloroquine. b. Genotyping of $Atg7^{FF}$ MEFs infected with adCre to trigger recombination of the floxed gene or adEmpty as a control. c. Genotyping of $Fip200^{FF}$ MEFs infected with adCre to trigger recombination of the floxed gene or adEmpty as a control. d. Fold change in number of live 1037 cells after 72 hours of treatment with ADI-PEG20 with and without co-cultured MEFs having $Atg7$ either floxed or knocked out, along with each of these MEF cell lines overexpressing GFP-ASS1. e. Fold change in number of live 1037 cells after 72 hours of treatment with ADI-PEG20 with and without co-cultured MEFs having $Fip200$ either floxed or knocked out, along with each of these MEF cell lines overexpressing GFP-ASS1. d and e were performed simultaneously and used the same controls. f. Growth of BVMA01R tumors grafted into syngeneic C57BL/6J mice with and without both ADI-PEG20 and chloroquine treatments. g. Quantification of ASS1 levels in harvested tumors from f. Data are mean ± s.d. except in f (mean ± s.e.m.) (n = 3 in a; n = 4-8 in d, e; n = 9-10 in f; n = 8-10 in g). Two-way ANOVA tests for a, f. Two-tailed unpaired t-tests for d, e, g. Images are cropped for clarity. See also Supplemental Figures S3.4e-S3.4i.
Figure 3.6. Overview of growth support

Tumor stromal cells produce extracellular vesicles that contain proteins and other nutrients. During arginine deprivation therapy, ASS1-deficient cancer cells take up these EVs and use autophagy/lysosomal degradation to recycle the ingested proteins and produce sufficient free arginine for survival and growth. Created with BioRender.com.
a.

![Images showing changes over time with ADI-PEG20 treatment.](image)

0 Days 1 Day 3 Days
ADI-PEG20 - + - + - +
7 Days 10 Days 14 Days
ADI-PEG20 - + - + - +

b.

![Graph showing standard uptake value with ADI-PEG20 treatment.](image)

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c.

![Images comparing NT and ADI-PEG20 treated groups.](image)

NT (Day -1) ADI-PEG20 (Day 13)
Supplemental Figure S3.1. *In vivo* imaging

**a.** Representative fluorescence images of SKLMS1 tumors expressing mApple ArgSen in NU/J mice with and without ADI-PEG20 treatment. **b.** Quantified uptake of $^{18}$F-(2 S,4 R)4-FGln radioactive glutamine tracer measured by PET before and after 13 days of ADI-PEG20 treatment in SKLMS1 tumors grafted into NU/J mice. **c.** Computed tomography/PET combination images of mice from **b** with tumors outlined in red. Data are mean ± s.d. (n = 4). Two-tailed paired t-test. Images are cropped for clarity.
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Supplemental Table S3.1. Tumor histology classifications and derived cell lines

a. All harvested spontaneous murine sarcomas were stained with hematoxylin and eosin, then classified according to morphology by a trained pathologist. Counts and percentages are shown.

b. Cell lines generated from spontaneous murine sarcomas. Cell line name, mouse of origin, genotype, morphological classification, and other notes are listed.
Supplemental Figure S3.2. ASS1 expression in spontaneous murine sarcoma cell lines and growth of Ass1 KO tumors in vivo

a. Quantification of ASS1 immunoblots for six spontaneous murine sarcoma cell lines featured in Figures 3.3a and 3.3b. b. Tumor growth of Ass1 KO BVMA03R grafts in NU/J mice with and without ADI-PEG20 treatment. Data are n = 1 in a, mean ± s.e.m. in b (n = 3-4).
Supplemental Figure S3.3. Fibroblasts support growth of Ass1 WT murine and human sarcoma cell lines

a. Proliferation of Myf5-Cre;p53\(^{-/-}\);Ass1\(^{+/+}\) BVM01R cell line when treated with ADI-PEG20 with or without a feeder layer of MEFs. 
b. Proliferation of Myf5-Cre;p53\(^{-/-}\);Ass1\(^{+/+}\) BVM02R cell line when treated with ADI-PEG20 with or without a feeder layer of MEFs. 
c. Proliferation of human sarcoma cell line SKLMS1 when treated with ADI-PEG20 with or without a feeder layer of adult human dermal fibroblasts (HDFa). 
d. Proliferation of human sarcoma cell line SKUT1 when treated with ADI-PEG20 with or without a feeder layer of HDFa cells. Data are mean ± s.d. (n = 4). Two-way ANOVA tests.
Supplemental Figure S3.4. Validation of gene knockouts and ASS1 expression in MEFs

**a.** Genotyping of Ass1<sup>F/F</sup> MEFs infected with adCre to trigger recombination of the floxed gene or adLacZ as a control. **b.** ASS1 immunoblots of Ass1<sup>F/F</sup>, Ass1<sup>−/−</sup>, and Ass1<sup>F/F</sup> LTAT MEFs. **c.** Quantification of **b.** **d.** Proliferation of Ass1<sup>F/F</sup> and Ass1<sup>−/−</sup> MEFs when treated with ADI-PEG20. **e.** Immunoblots of ATG7, FIP200, exogenous GFP-ASS1, and endogenous ASS1 in MEFs expressing GFP-ASS1 with either Atg7 or Fip200 floxed or knocked out. **f.** Quantification of ATG7 in MEFs with Atg7 floxed or knocked out. **g.** Quantification of FIP200 in MEFs with Fip200 floxed or knocked out. **h.** Quantification of endogenous ASS1 in all four MEF lines from e. **i.** Quantification of GFP-ASS1 in all four MEF lines from e. Data are mean ± s.d. (n = 3 in c, f-g; n = 15 in d). Two-tailed Welch’s t-test for c. Two-way ANOVA test for d. Two-tailed ratio paired t-tests for f, g. Images are cropped for clarity.
Supplemental Figure S3.5. Evidence against other possible growth support mechanisms and MEF toxicity

a. Proliferation of 1037 cells when treated with ADI-PEG20 in either normal media or media conditioned by MEFs. b. Proliferation of 1037 cells when treated with ADI-PEG20 with and without co-cultured MEFs and the gap junction inhibitors carbenoxolone (50 μM) and tonabersat (50 μM). c. Proliferation of 1037 cells when treated with ADI-PEG20 with and without co-cultured MEFs and the clathrin-mediated endocytosis inhibitor Pitstop 2 (80 μM). d. Proliferation of MEFs co-cultured with 1037 cells with ADI-PEG20 treatment and with or without 25 μM EST. Data are mean ± s.d. (n = 3 in a, d; n = 4 in b, c). Two-way ANOVA tests.
a. BVMA01R tumors from C57BL/6J mice were harvested, and their RNA was sequenced. Shown is a volcano plot of gene expression when tumors were treated with ADI-PEG20 versus no treatment.
b. Changes in expression of RNAs belonging to three pathways when tumors were treated with ADI-PEG20, chloroquine, or both.
c. Images showing the effects of ADI-PEG20 on 1037 cells over 24 hours alone or in co-culture with MEFs.
d. Top 20 most differentially regulated pathways from RNA sequencing data with ADI-PEG20 treatment. Red coloring indicates pathways that are related to the immune system. (n = 3 in a, b, d.)
### Antibodies and primers

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#### b. Primers used for polymerase chain reactions

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<tr>
<td>BglII EGFP rev</td>
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<td>TGTATTGACGGCTTGGTTCTGG</td>
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<tr>
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<td>GGAATTAAGATATGTCCAGCAAAGGCTCCGTGGTTCTGG</td>
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<tr>
<td>Mfle1 ASS1 rev</td>
<td>GGTCTAAGTCTGATCAATTGGCAGTGACCTTG</td>
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<tr>
<td>AscI EGFP fwd</td>
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<tr>
<td>NotI ASS1 rev</td>
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</tr>
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</tr>
<tr>
<td>Ass1 geno rev</td>
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<tr>
<td>Fip200 geno rev</td>
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</table>

**Supplemental Table S3.2. Antibodies and primers**

a. Antibodies used for immunoblots. Antibody target, vendor, and catalog number are listed. b. Primers used for polymerase chain reactions. Primer names and 5’→3’ sequences are listed.
# 3.6 References


Chapter 4: Future Directions

The work detailed in this dissertation has elucidated the mechanisms, heterogeneity, and kinetics of both canonical and noncanonical mechanisms of resistance to arginine deprivation therapy in cancer. Just as it has answered many questions, it has spurred an even larger number of new ones. Many of these new questions are quite interesting and deserve to be explored further. To that end, some promising future directions for research building on the work presented in this dissertation are outlined here.

4.1 Arginine Sensor

The arginine sensor described in Chapter 2 effectively tracked the availability of arginine for translation within cells. However, the design leaves room for improvement in multiple ways.

Firstly, improvements to the degradation domain are likely possible to allow more rapid degradation of the reporter and thereby grant greater temporal resolution to the sensor. An improved degradation domain may also be simpler and/or smaller, which should improve the performance of the sensor. At least one design has been shown to degrade GFP with a half-life of less than 15 minutes\(^1\). Therefore, it may be possible to implement a similar design to increase the efficiency of degradation of the arginine sensor.

In order to control for changes in global translation rates, two reporter proteins may be incorporated into the sensor, with only one reporter downstream of the polyarginine region. This could take the form of one reporter downstream and one upstream of the polyarginine region, so that the upstream reporter is affected by global translation rates, and the downstream reporter is affected in roughly the same way by global translation, with the additional effect of slowed translation when arginine specifically is scarce. These two reporters would likely be most
effective if separated by a P2A site, each with their own degradation domain, but could still function if connected.

Alternatively, the arginine sensor could be expressed under the control of a specific internal ribosome entry site (IRES) that changes expression very little in response to global signals such as mTOR inhibition. Cricket paralysis virus IRES requires no initiation factors to initiate translation of its mRNA and would therefore be nearly ideal for this purpose\(^2\). This design would insulate sensor expression from changes in global protein expression. Combining the previous two ideas of using an IRES and a second fluorescent protein as a control may yield even better performance than using either alone.

It is also tempting to expand on the concept of the arginine sensor design to create sensors for the availability of other amino acids in cells. This would theoretically be easy to achieve by swapping out the polyarginine region for a region encoding a different amino acid or even a combination of amino acids. Asparagine and glutamine may be targets of interest for these efforts, but other amino acids could be used as well.

However, there may be a limited number of amino acids for which this sensor design is useful under physiologically relevant conditions. For example, some amino acids may become limiting for other cellular processes and cause cell death at higher concentrations than would cause a decrease in translation rate. Situations such as this would require a more complicated system to isolate the effects of amino acid concentration on translation and are unlikely to yield results that give useful physiological insight.
4.2 Microenvironmental Growth Support

The arginine sensor led to the discovery of a new mechanism of resistance to arginine deprivation. Chapter 3 describes the basic mechanisms underlying this phenomenon, namely that Ass1 KO cancer cells overcome arginine starvation by endocytosing extracellular vesicles through macropinocytosis, then degrading the proteins contained within those vesicles through autophagy/lysosomal degradation and recycling the resulting arginine. However, much elucidation remains to be done, including the determination of which particular host cells provide most of the growth support in vivo.

To answer this question, conditional knockout of Ass1 could be performed in specific subsets of murine host cells that typically make up a significant portion of tumors, such as fibroblasts, monocytes, and endothelial cells. The effects of ADI-PEG20 on growth of Ass1 KO tumors in these mice could then be tested. Alternatively, subsets of cells could be depleted completely, such as using a mouse line that lacks monocytes. After identifying the most important cell type(s) for in vivo growth support, co-culture experiments could be performed with that cell type in vitro to validate the findings.

Further, the role of macropinocytosis in the growth support mechanism should be elucidated more rigorously. Macropinocytosis can be measured by adding fluorescently labeled high molecular weight dextran, a marker of macropinocytosis, to cells in culture and imaging macropinosomes. First, the effects of imipramine on macropinocytosis in Ass1 KO cancer cells should be measured directly with this method to further confirm that imipramine is acting mainly through macropinocytosis inhibition.
There are no known proteins that function only in macropinocytosis, which would be ideal targets to knock out in order to inhibit macropinocytosis specifically. Therefore, a double point mutant of KR987/989AA in CARMIL1, an actin capping protein regulator, could be made with CRISPR. This double mutation inhibits macropinocytosis while leaving most other functions of CARMIL1 unaffected. The effects of these mutations on co-culture growth support and macropinocytosis could then be tested.

The importance of macropinocytosis in vivo must also be confirmed more specifically. To do this, the effects of ADI-PEG20 on the growth of CARMIL1 mutant tumors could be tested. In addition, it would be useful to inject fluorescent dextran into these tumors and harvest them to visualize macropinocytosis in vivo. This should be done with both WT and CARMIL1 mutant tumors and after treatment with ADI-PEG20 and/or imipramine.

Additionally, the importance of EVs must be shown in vivo. After identifying important cell types for tumor growth support in vivo, one could proceed to determine the dominant pathway of EV production for that cell type in vitro. This could be done by separately treating the cells with inhibitors that target all three major pathways of EV production, then measuring the effects on EV production by isolating vesicles and measuring abundance with nanoparticle tracking analysis. If an effective inhibitor is found, it could be used in vivo in combination with ADI-PEG20 while the effects on Ass1 KO tumor growth are measured. For a more targeted approach, one could generate a mouse line with a critical gene in the important EV production pathway knocked out in the relevant cell type, then test tumor growth support with ADI-PEG20 in those mice.
It would also be beneficial to identify the specific components of EVs that are critical for growth support. The most likely factor is the bulk protein content contained within the vesicles, a rich source of arginine. Synthetic liposomes can help test the importance of these proteins as well as other factors, as they can be loaded with different components of EVs individually, then added to cancer cells to test their ability to rescue growth. To test whether the protein or lipid content of the EVs can support growth, liposomes could be loaded with high concentrations of protein such as bovine serum albumin or fatty acids or other lipids. To further separate the effects of proteins from the membranes of the liposomes, the cell culture media could simply contain a large concentration of soluble protein. This may be more difficult with lipids if they are poorly soluble. Liposomes could also be used to test the effects of other molecules, such as purified ASS1 enzyme.

Albeit bulk protein is likely the main factor, Ass1 mRNA and enzyme are also plausible contributors to growth support that may be transferred from ASS1-competent cells to Ass1 KO cells through EVs. To test this hypothesis, one could isolate vesicles from MEFs with intact or knocked out Ass1 genes and test their ability to support Ass1 KO cancer cell growth with ADI-PEG20. If no difference is found, then likely neither Ass1 mRNA nor enzyme is a contributing factor in vivo. Optionally, EVs could be isolated from Ass1 KO MEFs expressing large amounts of exogenous WT or mutant ASS1. If EVs from MEFs with WT ASS1 support growth better, then mRNA and/or enzyme would be shown to theoretically be able to help in large enough amounts. One could then attempt to distinguish between the effects of mRNA and enzyme by expressing Ass1 shRNA in the recipient cancer cells.

Finally, it would be useful to knock out ASS1 in multiple human cell lines and determine whether the same growth support phenomenon occurs in a wide variety of cancers. This would give
insight into how clinically impactful this mechanism is, as most cancers have intact ASS1 genes and are able to upregulate it to achieve long-term resistance to ADI-PEG20. If most cancer lines are shown to be able to overcome arginine deprivation therapy by scavenging arginine from extracellular vesicles \textit{in vivo}, it will increase the urgency to find therapies that effectively synergize with ADI-PEG20 to kill.

### 4.3 Impact of Arginine Starvation on the Immune Microenvironment

To that end, the effects of ADI-PEG20 on the immune system and potential for synergy with immunotherapies also deserve to be explored. After RNA sequencing of tumors indicated a large increase in immune-related gene signatures during ADI-PEG20 treatment, analysis using advanced computational models developed in the laboratory of Dr. Everett Moding confirmed that ADI-PEG20 caused an increase in immune cell infiltration of tumors. This preliminary data shows increases in multiple immune cell types, including both pro-tumor and antitumor subsets. This suggests that immune checkpoint inhibitors may be effective in combination with ADI-PEG20.

To further elucidate the changes caused by ADI-PEG20 in the immune component of tumors, immunohistochemistry (IHC) could be performed to identify changes in immune markers and immune evasion markers, especially PD-1 and PD-L1. In addition, the tumor cell lines isolated from the spontaneous murine sarcoma model detailed in Chapter 3 of this dissertation could be screened for immune evasion markers. The effect of ADI-PEG20 on growth of selected cell lines with various levels of immune evasion markers (likely PD-L1) could then be measured \textit{in vivo} in both immune-competent syngeneic mice and immune-deficient mice. Sensitivity to ADI-PEG20
in immune-competent mice but not immune-deficient mice would indicate that ADI-PEG20 may be slowing growth by stimulating the immune antitumor response. This seems to be the case with at least one cell line from Chapter 3. Cell lines with low expression of immune evasion markers would be expected to be more sensitive to immune attack in this model.

Selected tumor cell lines that are susceptible to ADI-PEG20-induced immune attack in vivo could then be co-cultured with multiple types of immune cells in vitro while measuring the effects of ADI-PEG20 on immune cell responses to the tumor cells. This would help elucidate which immune cells are mediating the antitumor response in vivo. Cells could then be grown in syngeneic mice with different immune components knocked out (e.g. B cells, T cells, monocytes). If the effect of ADI-PEG20 is diminished, that would indicate that the knocked out immune component in that particular mouse model is important for mediating the antitumor response.

While cells that are sensitive to ADI-PEG20-induced immune attack can help elucidate the mechanisms of the immune response, cells that are less responsive, likely with higher expression of immune evasion markers, are likely to be more responsive to combination therapies of ADI-PEG20 with immune checkpoint inhibitors. The sensitivity of these cells to combination therapy should therefore be tested both in co-culture with relevant immune cells and in immune-competent mice. A suitable combination therapy may be ADI-PEG20 with an anti-PD-1 antibody. Therapies targeting other immune signals may also be effective, and should be explored depending on the results of earlier experiments. In theory, immune checkpoint inhibition combined with ADI-PEG20 may also be effective against tumors that are already susceptible to immune attack, although the addition of immunotherapy may make a smaller difference in this case.
4.4 Conclusions

As detailed here, the research done for this dissertation has opened many promising avenues for future research. The paths to be taken are clear and vary widely. There are opportunities to expand on the concept of the arginine sensor to develop better methods for monitoring cellular responses to amino acid deprivation. The discovery of a new mechanism of long-term resistance to arginine deprivation would benefit from further elucidation of both the *in vivo* mechanism and the prevalence and relative contribution of the mechanism in other cancers and during clinical arginine deprivation therapy. This new resistance mechanism also underscores the importance of finding effective therapies to use in combination with ADI-PEG20 to eradicate tumors rather than just slowing their growth. One promising direction, suggested by the observed increased immune infiltration caused by ADI-PEG20, is to explore the combination of arginine deprivation with immunotherapy, as described in this chapter. Overall, the work presented in this dissertation has contributed greatly to the understanding of resistance to arginine deprivation therapies while providing a basis for many more exciting advances to be built upon.
4.5 References

