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Rom Keshet[1,2*], Joo Sang Lee[3-5*], Lital Adler[1*], Muhammed Iraqi[6], Yarden Ariav[1], Lisha Qin Jin Lim[1], Shaul Lerner[1], Shiran Rabinovich[1], Roni Oren[7], Rotem Katzir[3], Hila Tishler[1], Noa Stettner[1,7], Omer Goldman[1], Hadas Landesman[1], Sivan Galai[1], Yael Kuperman[7], Yuri Kuznetsov[7], Alexander Brandis[8], Tevi Mehlman[8], Sergey Malitsky[8], Maxim Itkin[8], S. Eleonore Koehler[9], Yongmei Zhao[10], Keyur Talsania[10], Tsai-wei Shen[10], Nir Peled[11], Igor Ulitsky[1], Angel Progador[6], Eytan Ruppin[3,*,**] and Ayelet Erez[1,*,**]

[1]Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel
[2]The Bert W. Strassburger Lipid Center, Sheba Medical Center, Tel Hashomer, Israel
[3]Cancer Data Science Lab, National Cancer Institutes of Health, Bethesda, MD 20892, USA
[4]Department of Health Sciences and Technology, Samsung Advanced Institute for Health Sciences & Technology, Samsung Medical Center, Sungkyunkwan University, Seoul 06351, Republic of Korea
[5]Department of Precision Medicine, School of Medicine, Sungkyunkwan University, Suwon 16419, Republic of Korea.
[6]Faculty of Health Sciences, The Shraga Segal Department of Microbiology, Immunology and Genetics, Ben-Gurion University of the Negev, Beer Sheva, 84105, Israel.
[7]Department of Veterinary Resources, Weizmann Institute of Science, Rehovot, Israel
[8]Department of Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot, Israel
[9]Department Anatomy & Embryology, Maastricht University, Maastricht, Netherlands
[10]Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA
[11]The Legacy Heritage Oncology Center and Dr. Larry Norton Institute, Soroka Medical Center and Ben-Gurion University, Beer-Sheva, Israel

[*]These authors contributed equally to this work
[**]Correspondence to: Ayelet Erez MD, PhD (Lead Contact); Weizmann Institute of Science, Rehovot, Israel; Phone- 972-8-934-3714; FAX- 972-8-934-3739; email: ayelet.erez@weizmann.ac.il.
Eytan Ruppin, MD, PhD; National Cancer Institute, National Institutes of Health, Bethesda MD; Phone-1-240-858-3169; FAX-1-301-496-2905; email:eyruppin@gmail.com

Abstract

ASS1 downregulation in different tumors has been shown to support cell proliferation and yet, in several common cancer subsets ASS1 expression associates with a poor patients' prognosis. Here we demonstrate that ASS1 expression under glucose deprivation is induced by c-MYC, providing survival benefit by increasing NO synthesis and activating the gluconeogenic enzymes PC and PCK2 by S-nitrosylation. The resulting increased flux through gluconeogenesis enhances serine, glycine and subsequently purine synthesis. Notably, high ASS1-expressing breast cancer mice do not respond to immune checkpoint inhibitors and breast cancer patients with high ASS1 have more metastasis. We further find that inhibiting purine synthesis increases pyrimidine to purine ratio, elevates the expression of the immunoproteasome and significantly enhances the response of autologous primary CD8+ T cells to anti-PD1. These results suggest that treating patients with high ASS1 cancers with purine synthesis inhibition is beneficial and may also sensitize them to immune checkpoint inhibition therapy.

Introduction

Argininosuccinate synthase (ASS1) catalyzes the formation of argininosuccinate from citrulline and aspartate and is a key component in the liver urea cycle (UC). Outside the liver, ASS1 and its subsequent UC enzyme argininosuccinate lyase (ASL) are expressed to form the citrulline-arginine cycle. This cycle supplies the cellular needs for arginine and its downstream essential metabolites, including polyamines, nitric oxide (NO) and proline in a cell specific manner[1,2]. Accordingly, ASS1 expression is differentially regulated in different cancers. In many cancer types, ASS1 expression is epigenetically downregulated and is associated with poor patients' prognosis[3-6]. In these cancers, we previously reported that ASS1 downregulation increases the availability of its substrate aspartate for pyrimidine synthesis, supporting cell proliferation[7]. Furthermore, in many cancers, we and others have demonstrated a more general tumor metabolic urea cycle dysregulation (UCD), which further enhances pyrimidine synthesis[8,9]. The increase in pyrimidine levels generates a mutational bias favoring pyrimidine that correlates with higher levels of hydrophobic-immunogenic antigens and hence with increased response to immunotherapy[9]. Nevertheless, in several common types of cancer, ASS1 is overexpressed for a yet unknown survival benefit[10-11]. Interestingly, anecdotal studies have reported that the expression of ASS1 in cancer is induced together with gluconeogenic genes without a known functional interaction[12,13]. We were therefore intrigued to decipher the metabolic gains that high ASS1 expression may provide to such cancers, surmising that such understanding may further advance our ability to treat these cancers.

Results

Analysis of The Cancer Genome Atlas (TCGA) revealed ten types of tumors where ASS1 expression was significantly upregulated compared to the corresponding noncancerous tissues (**Figure 1A**). In contrast to low ASS1 tumors which we previously found to associate with a general dysregulation in expression of other UC genes and quantified as having high UCD scores[9], high ASS1 expressing cancers have low UCD scores, testifying to a different kind of metabolic rewiring (**Extended Data Figure 1A**). Interestingly, we found a significant positive correlation between high ASS1 expression and the expression

of the key unidirectional gluconeogenic enzymes pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PCK2) and fructose-1,6-bisphosphatase 1 (FBP1). This was in contrast to the expression of the unidirectional glycolysis enzyme hexokinase 1 (HK1), which showed no significant correlation with ASS1 expression (**Figure 1B and Extended Data Figure 1B**). We thus hypothesized that high levels of ASS1 in tumors, in correlation with gluconeogenic enzymes, can support glucose homeostasis under limited glucose conditions.

To test our hypothesis, we focused on studying subtypes of prevalent cancers which have high ASS1 expression, thinking that they may demonstrate dependence on ASS1 for maintaining glucose levels. These cancers included human non-small cell lung (NSCLC, A549 cells) and colon cancer (SW620 cells). In addition, we found high ASS1 expression in the syngeneic mouse breast cancer cells (4T1) which enabled us to further test our findings *in vivo*. Indeed, following glucose deprivation, ASS1 RNA expression was significantly upregulated in all three-cancer types (**Figure 1C**). In comparison to high baseline expression of ASS1 in colon and breast cancer cells, lung cancer cells expressed relatively moderate ASS1 levels, enabling us to detect elevations in ASS1 levels following additional nutrient manipulations. Consequently, we were able to demonstrate in lung cancer cells an increase in ASS1 protein levels following glucose depletion and a further elevation when we additionally removed the serum[14] from the media (**Figure 1C and Extended Data Figure 1C, top panel**). Importantly, we found that even cancer cells known to express very low levels of ASS1 as MNNG human osteosarcoma[15] upregulate ASS1 expression when grown under glucose deprivation (**Extended Data Figure 1C bottom panel**). To confirm the functional significance of ASS1 upregulation, we knocked down its expression by using *shASS1* RNA in lung, colon and breast cancer cells and found that in contrast to growth under in regular medium, ASS1 depletion under glucose starvation decreases survival and increases cancer cell apoptosis, while its overexpression rescues the phenotype (**Figure 1D and Extended Data Figure 1D-F**). In corroboration with our findings, colon cancers have been previously shown to depend on ASS1 expression for maintaining glucose metabolism and survival[16]. We further found that decreasing ASS1 expression in 4T1 murine breast cancer cells significantly restricted their orthotopic growth in the mammary fat pads of female BALB/c mice in comparison to

control mice injected with ASS1-expressing breast cancers cells (**Figure 1E**). By further analysis of the TCGA data, we found that ASS1 expression strongly associated with decreased patients' survival- both in lung adenocarcinoma as well as in a high ASS1 expressing breast cancer subtype (**Figure 1A, Figure 1F and Extended Data Figure 1G**). Collectively, these *in vitro*, *in vivo* and human results suggest that tumors and cancer cells with high ASS1 expression contribute to cancer survival at least under low-nutrient stress conditions.

As cancer cells often hijack physiological mechanisms for their survival, we next tested whether ASS1 has a role in adaptation to glucose-deficient states in non-transformed cells. Interestingly, citrullinemia type I (CTLN I) and type II patients who lack a functional ASS1, have an abnormal liver morphology with increased glycogen deposits, and there are reports of hypoglycemic episodes in Type II, supporting irregular glucose homeostasis for an unclear cause[17-19]. Indeed, studying fibroblasts from CTLN I patients, we found that under acute glucose-deficient conditions the survival of ASS1-deficient cells is markedly reduced in comparison to control fibroblasts (**Figure 2A**). To determine if ASS1 plays a similar role in adaptation to no glucose states *in-vivo* outside the cancer context, we generated a site-specific knockout of ASS1 in hepatocytes by crossing *ASS1*$^{f/f}$ mice with transgenic mice overexpressing albumin (Alb) Cre[20,21] (**Figure 2B**). Since the liver is the main organ for gluconeogenesis[22], we fasted control and hepatic ASS1-deficient mice. While both groups showed a significant decrease in glucose levels following fasting, we found no significant difference in glucose levels between the groups either before or after fasting (**Figure 2C**). Next, we evaluated gluconeogenesis activation following fasting and pyruvate injection by measuring plasma glucose levels along the time course to recovery. Here, we found a profound reduction in fasting plasma glucose levels following pyruvate injection in mice with hepatic ASS1 knockout compared to controls (**Figure 2D**). Together, these results suggest that in non-cancerous human cells and in mice, ASS1 enables survival under glucose deprivation by supporting gluconeogenesis. In addition, chronic loss of functional ASS1 likely leads to other adaptive mechanisms for maintaining glucose levels.

To study whether these findings are relevant for gluconeogenesis in cancer, we grew control and ASS1-knocked-down human lung cancer cells in serum and glucose-free

media with or without the addition of pyruvate. While pyruvate significantly promoted survival of control cells in glucose-free medium, it failed to do so in ASS1-deficient cells (**Extended Data Figure 2A**). In the absence of glucose, pyruvate levels were significantly higher in ASS1-depleted lung cancer cells than in control cells, supporting the specific contribution of ASS1 for pyruvate utilization under such conditions (**Extended Data Figure 2B**). Thus, ASS1 expression contributes to gluconeogenic adaptation under low-nutrient conditions in non-cancerous and cancerous human cells as well as in wild type animals.

Several studies reported that glucose-starved cancer cells activate proximal gluconeogenesis for serine and glycine biosynthesis rather than glucose[12]. We thus supplemented lung cancer cells with ^{13}C-labeled glutamine and measured ^{13}C-labeled glycine and glucose using gas chromatography mass-spectrometry (GCMS). Indeed, while glucose and serum deprivation resulted in increased production of ^{13}C-labeled glycine, glucose levels dropped to an undetectable level (**Extended Data Figure 2C**). Interestingly, ASS1-deficient cancer cells generated a significantly lower ^{13}C-labeled serine and glycine levels under no glucose nutrient deprivation, in comparison to controls (**Figure 3A and Extended Data Figure 2D**). Notably, we found no significant changes in total serine and glycine levels under these conditions (**Extended Data Figure 2E**). Additionally, following no-glucose starvation ASS1 deficient breast cancer cells demonstrated decreased levels of the gluconeogenesis intermediate phosphoenolpyruvate (PEP) as well as of ^{13}C-glutamine labelling of the gluconeogenesis intermediate 3-phosphoglycericacid (3PG) and of glycine (**Extended Data Figure 2F**). Supplementing serine to ASS1-knocked down lung cancer cells grown in low serine medium, rescued lung cancer cells' survival (**Figure 3B**). These results collectively support that the induction of gluconeogenesis under glucose depletion in cancer cells depends on ASS1 to support serine and glycine synthesis rather than glucose.

ASS1 is expressed ubiquitously in extra-hepatic tissues as a key component of the citrulline-arginine cycle, a major source of endogenous arginine for the production of nitric oxide (NO) by NO synthase (NOS)[1]. NO levels regulate multiple metabolic pathways *via* cyclic GMP (cGMP) and by post-translational modification through S-nitrosylation[23,24].

Since NO has been established to induce survival mechanisms in glucose-starved cells[25], we hypothesized that ASS1 activity may activate gluconeogenesis through NO production. To test this hypothesis, we grew control and ASS1-knocked down lung cancer cells in the more extreme nutrient-deprived medium (i.e., without glucose and serum) with and without the addition of the NO donor S-nitroso-N-acetylpenicillamine (SNAP)[26]. Expectedly, cancer cells with low ASS1 levels had lower arginine levels (**Figure 3C**). SNAP supplementation indeed significantly increased serine and glycine levels, and the survival of ASS1-depleted cancer cells relative to controls (**Figure 3D and Extended Data Figure 3A**). In contrast, adding the NOS inhibitor- N(G)-nitroarginine methyl esther (L-NAME) or the ASS1 inhibitor α-methyl-DL-aspartic acid (MDLA)[27], significantly restricted serine production and cancer survival (**Figure 3E-F and Extended Data Figure 3B**). We found that in no-glucose nutrient-deprived conditions, ASS1 was necessary for S-nitrosylation of the unidirectional gluconeogenic enzymes PC and PCK2, since decreasing ASS1 levels reduced the S-nitrosylation of PC and PCK (**Figure 3G and Extended Data Figure 3C-D**). Importantly, we found that ASS1 contributes to *in vivo* S-nitosylation of PC in hepatocytes obtained from mice after fasting (**Figure 3H and Extended Data Figure 3E**). Finally, it was shown by others that NO synthesis can also promote cancer survival *via* cGMP - dependent induction of pro-survival genes[28]. Indeed, we found that cGMP levels were markedly elevated in glucose-deprived breast cancer cells, and diminished in cancer cells treated with ASS1 inhibitor (**Figure 3I**). Taken together, these results indicate that ASS1 supports NO-driven metabolic regulation of gluconeogenesis through both S-nitrosylation and cGMP production.

c-MYC induces adaptation to poor-nutrient states in cancer[29] and is one of the few transcription factors that have been found to activate ASS1[30]. Analysis of the TCGA dataset reveals that the c-MYC nutrient deprivation (ND) signature[29] highly correlates with high ASS1 expression in breast and lung, while in colon cancers the trend is similar but less significant (**Figure 4A and Extended Data Figure 4D**). Indeed, the detrimental effect of ASS1 expression on the prognosis of patients is accentuated among patients whose tumors express the c-MYC driven nutrient deprivation signature (**Figure 4B**). In cancer cells, we found that while the elevation of c-MYC protein expression following nutrient deprivation was prominent in lung and colon cancers and less so in breast cancer, inhibiting

c-MYC by the drug 10074-G5[31] caused extensive cell death in glucose-deprived conditions in cells of all three cancer types (**Figure 4C-D and Extended Data Figure 4B)** Notably, the reduced survival under nutrient deprivation and treatment with c-MYC inhibitor was associated with decreased NO production (**Extended Data Figure 4C**). Since ASL, the subsequent enzyme to ASS1 in the citrulline-arginine cycle, is essential for NO generation[32], we performed analysis of CHIP-Seq data[33,34] and found that indeed, c-MYC also binds to the promoter of *ASL* (**Figure 4E**). We found that following glucose deprivation, c-MYC inhibition prevents the increase in *ASS1* and *ASL* RNA expression levels, as well as the levels of two other genes known to be induced by MYC under nutrient starvation for serine synthesis[29] - phosphoglycerate dehydrogenase (PHGDH), and phosphoserine phosphatase (PSPH), (**Figure 4F and Extended Data Figure 4D**). Interestingly, at the protein levels following treatment with c-MYC inhibitor, we found reduction in ASS1 levels (**Extended Data Figure 4E**), and not in ASL (Data not shown). This is likely similar to other conditions requiring NO synthesis in which ASL protein levels tend to remain constant relative to ASS1[35]. Indeed, the combined expression of NO synthesis genes (*ASL+ASS1+NOS1)* strongly correlated with the expression of gluconeogenic genes (**Extended Data Figure 4F**). Together, these results suggest that under limited nutrient availability, c-MYC promotes survival by inducing the citrulline-arginine cycle for NO synthesis. We thus defined an integrated *nutrient-deprivation/NO (NDNO) signature* that combines the gene expression of *ASS1, ASL*, and *NOS1* and extends the c-MYC induced nutrient deprivation signature genes to include the NO-synthesis related genes (see Methods).

By increasing serine and glycine synthesis, gluconeogenesis flux has been reported to promote purine synthesis in cancer cells[12]. We hence hypothesized that ASS1-overexpressing tumors might have a reverse, *purine-rich mutational bias (PRB)*, characterized by a higher purine to pyrimidine ratio. Analyzing the TCGA data, we found that ASS1 levels correlate with a purine rich mutational bias in breast, lung and colorectal cancers collectively (**Figure 5A**). Indeed, we found that in murine breast and colon cancer cells deprived of glucose, purine levels increased, resulting in a significant elevation in the total purine to pyrimidine metabolites' ratio (**Figure 5B, Extended Data Figure 5A and Supplementary Table 1**). We next confirmed that purine levels decrease in murine breast

cancer cells with ASS1 depletion under glucose deprivation (**Figure 5C and Supplementary Table 2**). Furthermore, NO synthesis inhibition decreased the purine to pyrimidine ratio in the murine breast cancer cells with and without ASS1 knockdown (**Extended Data Figure 5B**). Exome sequencing of high ASS1-expressing cancer cells verified that the high purine to pyrimidine ratio leads to a PRB signature (**Figure 5D**). Notably, treatment of these cells with mizoribine, a purine synthesis inhibitor[36], reversed their nucleotide imbalance towards higher pyrimidines and resulted in the reversal of the PRB to a pyrimidine-rich mutational signature and in inhibition of' proliferation (**Figure 5E-F, Extended Data Figure 5C-D and Supplementary Table 3**). In support, inhibition of one-carbon metabolism by methotrexate treatment in high ASS1 expressing lung cancer cells also decreased the purine to pyrimidine ratio (**Figure 5G and Supplementary Table 4**).

We have previously demonstrated that the pyrimidine-rich transversion mutational bias observed in low ASS1 tumors enhances sensitivity to anti-PD1 therapy[9]. We thus expected that high ASS1 tumors and/or tumors with high PRB levels would be less responsive to immunotherapy. We examined this hypothesis by analyzing the TCGA gene expression data together with the overall response rates to anti-PD1/PDL1 therapy reported for different cancer types[37]. Though there are outliers, in general we find that tumors with high ASS1 expression and those with high PRB levels show decreased response for checkpoint therapy compared to other tumors (**Figure 6A-B**). Specifically in NSCLC cohort treated with anti-PD1, we find that ASS1 expression and NDNO-score are significantly associated with lack of response (**Figure 6C**). Taken together, these results testify that high ASS1 expression in nutrient deprived conditions contributes to high PRB levels and poor response to immunotherapy.

Given these findings, we turned to study their potential translational value regarding treatment of high ASS1 tumors. We have previously shown that high pyrimidine synthesis generates higher levels of hydrophobic peptides that are more immunogenic[9]. We now analyzed the protein amino acid composition of NSCLC tumor samples data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) compendium and found that indeed ASS1 protein expression levels inversely correlate with peptides' hydrophobicity

(**Figure 6D**). Since immunoproteasome activity has been associated with enhanced neoantigen presentation and with improved immune responses[38], we tested the association of its induced subunit LMP-7[39,40] with ASS1 levels and with mizoribine treatment. Both interventions are expected to increase immunogenicity by increasing the pyrimidine to purine ratio because mizoribine inhibits purine synthesis, and downregulating ASS1 expression levels with *shASS1* is expected to both promote pyrimidine synthesis[9] as well as decrease purine synthesis. Indeed, we find that treatment with mizoribine and low ASS1 levels are both associated with a higher immunoproteasome LMP-7 expression (**Figure 6E**). These results suggest a potential adjuvant benefit for mizoribine treatment of ASS1-high expressing tumors, by promoting the anti-cancer immune response *via* enhancing immune-proteasome activity. To test the relevance of these findings *in vivo*, we had to separate the putative purine inhibitory effects of mizoribine that act directly on the tumor cells from its potential immunosuppressive effects on the tumor microenvironment[41]. Thus, we treated 4T1 breast cancer cells with mizoribine *in vitro* before injecting them into the mammary pads of immunocompetent mice. We found that while mizoribine and ASS1 inhibition each restricted tumor growth, they generated a more significant response when given in combination with anti-PD1 in breast cancer mice unresponsive to anti-PD1 (**Figure 6F**).

To study the importance of our results for human tumors on a more mechanistic level, we first grew patient derived xenografts (PDX) in immunosuppressed mice. These PDXs were generated from NSCLC explants with high ASS1 expression, taken from cancer patients who were not responding to immune checkpoint therapy (**Extended Data Figure 6A**). Following *in vitro* treatment with anti-PD1 with or without mizoribine, we found a significant elevation in secretion of IFN-γ by the patients' autologous primary CD8+ T cells in response to the combined treatments, compared to IFN-γ levels secreted by these cells following treatment with Anti-PD1 alone (**Figure 6G and Extended Data Figure 6B**). These results together demonstrate that in ASS1 expressing tumors, the purine inhibitor mizoribine can both reverse the high purine to pyrimidine ratio, and enhance the response to immunotherapy.

Since cancer cells that can survive in a low nutrient environment and can evade the immune system have a higher chance to metastasize successfully[42], these findings naturally gave rise to the possibility that high ASS1 expression may contribute to tumor metastatic progression. In support of this notion, others have shown that in gastric cancer, decreasing ASS1 expression can repress metastasis formation[43]. To evaluate whether high ASS1 expression is associated with metastatic risk, we analyzed the TCGA data for ASS1 expression and metastasis incidence in 683 breast cancer patients (101 metastasis cases and 582 control) with local/regional recurrence after mastectomy. We found that ASS1 levels are significantly higher in primary tumors that developed metastases as compared to primary tumors that did not (**Figure 6H**).

Discussion

Activation of gluconeogenesis, a physiologically liver-bound process, is one strategy adopted by cancer cells to overcome glucose deficiency[12,44]. In glucose-starved cancer cells, UC genes were found to be upregulated together with gluconeogenic genes, but the functional role of these changes has remained unclear[12]. Here we describe a dynamic crosstalk between signaling and metabolism; under glucose deprivation c-MYC drives the expression of the UC genes ASL and ASS1 resulting in increased NO production that promotes gluconeogenesis by nitrosylation of the two unidirectional key gluconeogenic enzymes PCK2 and PC (**Extended Data Figure 6C**). The flux through gluconeogenesis leads to increased serine and glycine synthesis, which has been associated with poor prognoses in breast and lung cancer patients[45,46]. Intuitively, under glucose deprivation one would anticipate gluconeogenesis to support glucose production. Yet, we find that even in media without glucose, gluconeogenesis supports serine and glycine production, with no significant effect on glucose levels. These results complement the established data demonstrating that the main goal of enhanced glycolysis in cancer is to supply subsidiary pathways that enable proliferation by generating the necessary macromoelcules[47], showing similar functions occurring when gluconeogenesis is activated.

We found that the resulting increased serine synthesis leads to enhanced purine synthesis causing a nucleotide imbalance favoring purines. The consequent purine excess leads to a purine rich mutational signature and decreases the response to immunotherapy. Both manifestations are reversed by treating mouse and human cancer cells as well as

human tumors with mizoribine (**Extended Data Figure 6C**). Mizoribine exerts its activity through selective inhibition of inosine monophosphate synthetase and guanosine monophosphate synthetase, resulting in inhibition of purine nucleotide synthesis[36]. Our findings show high cancer killing following mizoribine treatment in high ASS1 breast tumors and suggest that these tumors might be sensitive to purine analogs commonly used in cancer such as mercaptopurine and thioguanine[48] . In addition, our study further supports our earlier findings that high pyrimidine to purine ratio promotes response to immune cell therapy (ICT). Beyond that, it shows that high ASS1 expressing tumors respond poorly to ICT and that treatment of these cells with mizoribine, a purine synthesis inhibitor, results in the reversal of their PRB signature to a pyrimidine-rich mutational signature, in induction of the immune-proteasome and overall improved response to ICT (**Extended Data Figure 6C**). Yet, current purine inhibitors have been reported to have immune-suppressive effects[36] and thus, there is a clear need to develop new ways to target purine synthesis that do not contribute to immunosuppression.

Taken together, we demonstrate here that elevated ASS1 expression in cancers provides survival benefits under glucose deficiency by increasing serine and glycine synthesis and by reducing cancer immunogenicity. As such, ASS1 expression may contribute to cancer metastatic progression. Our findings give rise to the possibility that ASS1 expression may change dynamically along tumor progression to provide unique metabolic benefits that are deemed at different cancer stages- from primary tumors where its low expression promotes proliferation *via* enhanced pyrimidine production to tumor metastases where its high expression supports cancer survival in nutrient poor and immune rich environment.

Acknowledgments:

We acknowledge and thank the Weizmann Institute for providing financial and infrastructural support. We thank Shawn Mcguirck, McGill University, Montreal, Canada, for assistance with GC-MS data analysis. The MC-38 cancer cell line was kindly given to us by Eran Elinav from the Weizmann Institute of Science. A. Erez is incumbent of the Leah Omenn Career Development Chair and is supported by research grants from the European research program (ERC818943), and from the Israel Science Foundation (860/18). AE received additional support from The Moross Integrated Cancer Center, Sagol Institute for Longevity Research, Adelis Foundation, Rising Tide Foundation, and from Manya and Adolph Zarovinsky. L. Adler receives support from Robin Lynn & Lawrence S. Blumberg. E. Ruppin gratefully acknowledges support from NCI/CCR intramural funding. JSL is partly supported by a grant of the National Research Foundation of Korea funded by the Korean Government (NRF-2020R1A2C2007652). AP is supported by the NRF-ISF grant 3127/19.

Author Contribution

R.Keshet, JSL and LA led the genomic analysis and molecular experiments; YA, LQJL, SL, SR, HT, HL, SG, helped with the molecular experiments; RO, NS, YK, Y. Kuznetsov, OG were responsible for the animal experiments; MT, SM, AB, M. Itkin, TM contributed to the metabolomic analysis; R.Katzir, YZ, KT, TWS, and IU helped with the genomic analysis. M.Iraqi and AP and NP performed the PDX experiments. SEK kindly provided the ASS1 flox mice, ER and AE initiated and led the study and wrote the manuscript. All authors were involved in discussions about study design, contributed to the writing and reviewed the manuscript.

Competing Interests Statement – We have no competing interest**.**

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Figure legends:

Figure 1. High ASS1 expression in cancer provides a metabolic survival benefit under glucose deprivation. A) ASS1 is overexpressed in 10 cancer types in TCGA as compared to the corresponding normal tissues (Genome Tissue Expression (GTEX) data) based on a two-sided Wilcoxon ranksum test with multiple hypothesis correction. Y-axis shows ASS1 expression levels in cancer (red) and in the corresponding normal tissues (blue), and the X-axis shows the cancer types (n=25 cancer types). ** denotes P<0.01, ****P<0.0001. **B**) The expression levels (Y-axis) of unidirectional gluconeogenesis enzymes (X-axis) are

significantly higher in ASS1 high tumors (top tertile, n=2916, 2897, 2925, and 2913, respectively; red) compared to ASS1 low tumors (bottom tertile, n=2932, 3127, 2921, and 2927, respectively; blue) based on one-sided Wilcoxon ranksum test (P=2.9E-21, 4.9E-26, 1.9E-04, and 9.4E-01, respectively). PC- pyruvate carboxylase, PCK1- Phosphoenolpyruvate Carboxykinase 1, FBP1- Fructose-Bisphosphatase 1, HK1- Hexokinase 1. **** denotes P<0.0005. **C**) Low nutrient states induce ASS1 expression in breast, colon and lung cancer cells. 4T1 and SW620 cells were grown either in normal medium or in medium without glucose for 48h; A549 cells were grown either in normal medium or in medium without glucose and serum for 72h. ASS1 levels were measured by real-time PCR and western blotting. n=3 biological replicates in each group. p value was calculated using two-tailed student's t-test. Left panel: p=0.0006; Middle panel: p= 0.034; Right panel: p= 0.0096 for control *vs.* no glucose and 0.0002 for control *vs.* no glucose and no serum. **D**) ASS1 supports glucose-independent survival in human lung cancer cells. A549 cells (expressing either *ASS1-shRNA* or control *GFP-shRNA*) were grown without glucose for 96h. Left panel: Gene expression was measured by real-time PCR. Middle panel - Survival was measured by crystal violet staining. Right panel: ASS1 overexpression (OE) rescues ASS1 inhibition-induced cell death. A549 cells including; shGFP (control), shASS1 or shASS1 that was infected with plasmid carrying ASS1 (shASS1+ASS1 OE) were incubated in Glucose free medium for 72hrs. Annexin V/7ADD assay was used to determine the percentage of apoptotic and dead cells (% Cell death). n=3 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. Left panel: p= 8.7E-05 for control *vs.* shASS1#1 and 8.45E-05 for control *vs.* shASS1#2. Middle panel: p=0.019 for control *vs.* shASS1#1 and 0.0014 for control *vs.* shASS1#2. Right panel: p=0.002. **E**) ASS1 is required for 4T1 breast cancer tumor growth in mice. 4T1 cells expressing two different clones of ASS1-shRNA or control GFP-shRNA were injected into mouse mammary fat pads. Tumors were resected and weighed on day 14. n=10 mice in each group. p value was calculated using Wilcoxon rank sum test. p=0.0008 for control *vs.* shASS1#1 and 0.05 for control *vs.* shASS1#2. **F**) Tumor samples with ASS1 overexpression (top tertile, n=165 and 358) associate with poor survival in TCGA lung adenocarcinoma (left) and breast cancer (right) patients compared to the rest of the patients (n=329 and 717) (two-sided logrank p-values are denoted in the figure, and median survival differences are 232 and 673 days, respectively). The pink and light blue area denote confidence intervals of the survival curves. The ASS1-high samples in breast cancer are those denoted in **A**.

Tumor type abbreviations: THCA - thyroid carcinoma, COAD - colon adenocarcinoma, PAAD - pancreatic adenocarcinoma, OV - ovarian serous cystadenocarcinoma, STAD- stomach adenocarcinoma, LUAD - lung adenocarcinoma, ESCA - esophageal carcinoma, LUSC- lung squamous cell carcinoma, DLBC - lymphoid neoplasm diffuse large B-cell lymphoma, CESC - cervical squamous cell carcinoma and endocervical adenocarcinoma, PRAD - prostate adenocarcinoma, UCEC - uterine corpus endometrial carcinoma, BLCA- bladder carcinoma, HNSC- head-neck squamous cell carcinoma, TGCT - testicular germ cell tumors, KIRC - kidney renal clear cell carcinoma, KIRP - kidney renal papillary cell carcinoma, LIHC - liver hepatocellular carcinoma, KICH - kidney chromophobe, GBM - glioblastoma multiforme, LAML - acute myeloid leukemia, LGG - brain lower grade glioma, BRCA- breast invasive carcinoma, SKCM - skin cutaneous melanoma, SARC – sarcoma. Boxplots in this figure follows the standard definition, where the box is drawn

from top quartile (75-percentile) to bottom quartile (25-percentile) of the data with a horizontal line drawn in the middle to denote the median value. The lowest point of the bottom whisker is the minimum of the data and the highest point of the top whisker is the maximum of the data.

Figure 2. ASS1 has a physiologic role in adaptation to no-glucose conditions. A) Left panel: A western blot showing ASS1 protein levels in normal fibroblasts and in fibroblasts from citrullinemia type 1 (CTLN1) patients. Right panel: Fibroblasts from CTLN1 patients have impaired glucose-independent survival. Control and CTLN1 fibroblasts were grown without glucose for 72h. Survival was measured by crystal violet staining. n=4 biological replicates for each cell line. p value was calculated using two-tailed student's t-test. p=0.015 for control *vs.* CTLN1#1 and 0.025 for control *vs.* CTLN1#2. **B**) A western blot showing ASS1 protein levels in livers of control mice and mice with hepatocyte-specific ASS1 conditional knockout (*Alb Cre CKO*). The experiment was conducted On three randomly-picked mice from each group. **C)** ASS1 inhibition does not significantly change baseline and fasting glucose levels in *ASS1$^{f/f}$ -Alb Cre C57BL/6* mice or control. *ASS1$^{f/f}$* mice were fasted for 16h. Glucose was measured before and after the 16h fast. n=12 mice for control groups and 10 for KO groups. p value was calculated using two-tailed student's t-test. p=4.6E-09 for glucose levels at baseline *vs.* fasting in control and 3.7E-04 for glucose levels at baseline *vs.* fasting in ASS1 hepatocytes CKO. **D**) ASS1 contributes to gluconeogenesis. *ASS1$^{f/f}$ -Alb Cre C57BL/6* mice or control *ASS1$^{f/f}$* mice were fasted for 16h. Pyruvate was injected IP 2gr/kg. Glucose was measured at the following time points: 0,15,30,45,60,75 minutes. Delta glucose levels between fed and fasted states were compared between knockout and control mice using repeated-measures ANOVA, with treatment and batch as between-subject factors. The analysis was done using Statistica, v. 12. The experiment was repeated twice, each time with n=10 in each group. Shown is the combines analysis with n=20 mice in each group].

Figure 3. **ASS1 supports serine synthesis under low nutrients *via* increasing NO signalling**. **A**) ASS1 supports serine and glycine production from glutamine under nutrient deprivation. A549 cells were grown for 48h and cultured with U-[^{13}C] glutamine during the last 6h of incubation. Shown is the proportion of the U-[^{13}C]-labelled serine and glycine mass pools/M+0 as quantified by GC/MS. n=4 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. p=0.0157 for serine and 0.0028 for glycine. **B)** Addition of 1mM serine Biological industries) to medium without glucose rescues the survival of shASS1 clones to the same survival level as the shGFP. A549 cells were grown in glucose and serine free medium or in the same medium supplemented with 1mM serine. Following 72h, the cells were stained with 0.2% crystal violet and the staining was quantified at 595nm as fold change from day 0. n=3 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. Left panel: p= 0.041 for control *vs.* shASS1#1 and 0.033 for control *vs.* shASS1#2. **C**) A549 cells were grown for 72h in full medium and arginine levels were measured with LC/MS. Arginine concentrations were normalized to protein levels of the respective samples. n=3 biological replicates in control group and 4 in shASS1 group**.** p value was calculated using two-tailed student's t-test. p=0.0066. **D**) NO donors support serine and glycine production in nutrient-starved lung cancer cells. A549 cells were grown in the absence of glucose and serum for 48h and with 10mM SNAP for the last 1h of incubation and analysed by GC/MS. n=3 biological

replicates in each group**.** p value was calculated using two-tailed student's t-test. p=0.0365 for serine and 0.0382 for glycine total levels at low nutrients with SNAP *vs.* low nutrient conditions. **E**) NO supports serine production through gluconeogenesis. A549 cells were grown with or without glucose and serum (Low Nutrient), and supplemented with either 10mM L-Name or 10mM MDLA for 48h. Cells were incubated with U-[13C] glutamine for the last 6h of culture without serine. Shown are the total levels quantified by GC/MS as the sum of areas under the curve (AUC) measurement for the different masses/ M+0. n=3 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. p=2.2E-05 for control *vs.* low nut.; 3.6E-05 for control *vs.* low nut.+MDLA; and 0.0035 for control *vs.* low nut.+L-Name. **F**) A549 control and shASS1 clones were incubated in Glucose free medium for 72hrs with and without supplementation of 10mM of L-Name at 24h and 48h. After 72hrs, the cells were stained with 0.2% crystal violet and staining was quantified at 595nm as fold change from day 0. n=4 biological replicates in no glucose-control group, 3 in L-Name-control and no glucose-shASS1 groups and 2 in L-Name-shASS1 groups.in each group as depicted in the graph**.** p value was calculated using two-tailed student's t-test. p=0.023 for control and 0.041 for shASS1#2. **G**) ASS1 contributes to starvation-induced S-nitrosylation of gluconeogenic enzymes in nutrient-deficient lung cancer cells. A549 cells were grown in the absence of glucose and serum for 48h. Detection of protein nitrosylation was performed using the SNO-RAC method followed by a western blot and quantification. **H**) ASS1 contributes to starvation-induced S-nitrosylation of gluconeogenic enzymes in livers of fasting mice. Alb/Cre and control mice were fed regularly or fasted for 16h. Detection of protein nitrosylation in the livers was performed using the SNO-RAC method followed by a western blot. Quantification is shown in Extended Data Figure 3F. **I**) ASS1 contributes to glucose- starvation induced cGMP production. 4T1 cells were grown for 48h with and without 10 mM ASS1 inhibitor (MDLA) and cGMP levels were measured using LC-MS. n=3 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. p=0.018 for control *vs.* no glucose; 0.013 for no glucose *vs.* MDLA; and 0.014 for no glucose *vs.* no glucose+MDLA.

Figure 4. **ASS1 expression in cancer is part of the transcriptional signature activated by c-MYC in response to nutrient deficiency**. **A**) ASS1 expression is significantly higher when c-MYC nutrient deprivation signature is high (top 20%, red, n=215, 99, 98, and 59, respectively) *vs.* low (bottom 20%, blue, n=215, 99, 98, and 59, respectively) in TCGA breast, lung and colon cancer using one-sided Wilcoxon ranksum test (P=4.0E-34, 1.3E-02 1.4E-02, and 7.4E-02). This trend is less significant but similar with a 33% cut-off as shown in Extended Data Figure 4A. The box is drawn from top quartile (75-percentile) to bottom quartile (25-percentile) of the data with a horizontal line drawn in the middle to denote the median value. The lowest point of the bottom whisker is the minimum of the data and the highest point of the top whisker is the maximum of the data. **B**) Among patients whose tumors express the c-MYC driven nutrient deprivation signature, patients with high expression of ASS1 (red; n=84, 268, 74) have a worse prognosis in lung, breast and colon cancers compared to controls (blue; n=163, 269, 74). Two-sided logrank p-values are denoted in the figure, and median survival differences are 354 and 995 days for lung and breast cancer, respectively; Median survival differences is not available for colon cancer because the survival probability of ASS1 low group does not reach 0.5. The pink and light blue area denote confidence intervals of the survival curves. **C**) c-MYC levels in lung and

colon cancer cells increase in glucose starvation. A549 cells were grown either in normal medium or in medium without glucose for 96h; SW620 cells were grown either in normal medium or in medium without glucose for 24h. **D**) Restriction of cancer cell growth by c-MYC inhibition is intensified by nutrient deprivation. A549 cells were grown either in normal medium or in medium without glucose and serum for 24h; 4T1 and SW620 cells were grown either in normal medium or in medium without glucose for 24h. Cells were exposed to either 30mM 10074-G5 (MYC inhibitor) or to DMSO as control. Cell viability was measured using crystal violet staining. n=3 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. In A549 p=0.004 for survival comparison in normal medium; p=0.02 for survival comparison in no glucose+no serum conditions of cells with and without MYC inhibitor. In 4T1 p=0.024 for normal medium; p=0.019 for no glucose. In SW620 p=0.018 for normal medium; p=0.012 for no glucose. **E**) Binding of c-MYC to the ASL promoter in ChIP-seq data from ENCODE project34 (gray bars, from K562, MCF-7, MCF10A, and NB4 cells) and U2OS cells35 (blue bars). Gray color intensity indicates peak scores as defined by the ENCODE project and presented in the UCSC Genome Browser. Green box indicates the position of a consensus Myc binding motif. **F**) c-MYC supports the no glucose-dependent ASS1 and ASL gene expression upregulation. A549 cells were grown and treated as described in C. Gene expression was measured by real-time PCR. n=3 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. p=0.0009 for ASS1 and 0.0006 for ASL elevation under no glucose conditions.

Figure 5. ASS1 supports purine synthesis in cancer. **A**) Purine mutational bias is significantly higher in high ASS1 samples (grey, top-tertile, n=923) of TCGA samples of breast, lung and colorectal cancers collectively compared to the low ASS1 samples (white, bottom-tertile, n=923) using one-sided Wilcoxon ranksum test (P=0.001). The box is drawn from top quartile (75-percentile) to bottom quartile (25-percentile) of the data with a horizontal line drawn in the middle to denote the median value. The lowest point of the bottom whisker is the minimum of the data and the highest point of the top whisker is the maximum of the data. **B**) The purine (R)/pyrimidine (Y) ratio is increased upon glucose deprivation in murine breast cancer cells. 4T1 cells were grown in the absence of glucose for 24h (baseline) and 48h and quantified by liquid chromatography–mass spectrometry (LC-MS). Nucleotide levels were measured in μg/ml and corrected for protein concentration. Purine/pyrimidine ratio was calculated by dividing (AMP+GMP) by (UMP+CMP) relative concentrations. n=6 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. p=0.035. **C**) ASS1 inhibition decreases purine/pyrimidine ratio under glucose deprivation. 4T1 cells were grown in the presence or absence of glucose for 72h and analysed as in 5B. n=3 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. p=0.022. **D**) DNA sequencing analysis reveals that glucose deprivation results in a purine mutational bias in 4T1 breast cancer cells. Cells were grown and treated as in 5B. p value was calculated using Fisher's exact test (p=0.003). **E**) Purine synthesis inhibition with mizoribine results in a decreased purine/pyrimidine ratio in murine breast cancer cells. 4T1 cells were grown either with or without 300μM mizoribine for 24h. Nucleotide levels were measured by LC-MS. The purine/pyrimidine ratio was calculated as in B. n=3 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. p=0.0002. **F**) DNA sequencing analysis reveals that purine synthesis inhibition by a 24h treatment with 300μM

mizoribine results in a pyrimidine mutational bias in 4T1 breast cancer cells. p value was calculated using Fisher's exact test (p=0.01). **G**) A549 cells were grown for 48hr in control medium or supplemented with 1.2μM Methotrexate (Holland-Moran). Nucleotide levels were measured as described below by LC-MS. Purine/pyrimidine ratio was calculated by dividing (AMP+GMP+ATP+GTP) concentration by (UMP+CMP+TMP+UTP+CTP+TTP) concentration before and after methotrexate. n=3 biological replicates in each group**.** p value was calculated using two-tailed student's t-test. p=0.002.

Figure 6. High ASS1 cancers are more sensitive to purine synthesis inhibition than low ASS1-expressing tumors. **A**) High ASS1 tumors (median ASS1 expression within top tertile; n=9) show significantly lower objective response rate to anti-PD1 therapy across cancer types compared to low ASS1 tumors (n=11) using one-sided t-test (P=0.05). **B**) High PRB tumors (median PRB levels within bottom tertile; n=8) show significantly lower objective response rate to anti-PD1 therapy across cancer types than low PRB tumors (n=10; see Methods) using one-sided Wilcoxon ranksum test (P=0.07). **C**) ASS1 mRNA expression levels and NDNO scores are significantly higher in non-responders (orange; n=14) compared to responders (green; n=7) to anti-PD1 therapy in non-small cell lung cancer (NSCLC) (one-sided Wilcoxon ranksum P=0.05 and 0.03, respectively). **D**) Proteomic-based NDNO scores associate with lower hydrophobicity of peptide repertoire (one-sided t-test P<0.05) in a (CPTAC3) lung adenocarcinoma cohort. NDNO scores were used to determine the top tertile (red; n=36) and bottom tertile (blue; n=36); Proteomic NDNO scores denote the ratio of NNDO scores of the tumor vs the matched normal sample (Methods). Hydrophobicity scores were determined by the metric from Janin et al[2], where the hydrophobicity of each sample's peptide repertoire denotes the mean hydrophobicity of all its peptides (Methods). **E**) 4T1 breast cancer cells were treated with 300μM mizoribine for 24 hours and analysed by western for LMP-7 immunoproteasome (Left panel). Quantification of the western is shown on the right panel. **F**) Purine inhibition promotes response to immunotherapy checkpoint inhibitors: 4T1 cells (either control shGFP cells or shASS1 cells) were treated in vitro with mizoribine before injection into female mice mammary fat pads. Mice (each group n=10) were treated with intraperitoneal injection of either PBS control or 250mg/mouse a-PD1on days 7,11,13. Mizoribine was given 300μM *in vitro*. Tumor size was calliper-measured on the designated days (upper panel) and at sacrifice on day 14 (lower panel, p = 0.0476 using student's t-test). The experiment was conducted with biological and technical replicates and repeated twice Control: n=30; α-PD1: n=26; shASS1: n=13; mizoribine (Mizo.): n=10; α-PD1+shASS1: n=19; α-PD1+Mizo.: n=8; Mizo+shASS1: n=4. p value was calculated using one-way t-test. p=0.008 for control *vs.* shASS1; 0.0004 for control *vs.* Mizo. 0.002 for control *vs.* α-PD1+shASS1; 3.1E-07 for control *vs.* α-PD1+Mizo.; 8.7E-08 for control *vs.* Mizo.+shASS1; 0.007 for α-PD1 *vs.* α-PD1+shASS1; 1.1E-06 for α-PD1 *vs.* o α-PD1+Mizo.; 0.032 for Mizo *vs.* α-PD1+Mizo.; 0.018 for Mizo *vs.* Mizo.+shASS1. **G**) Autologous CD8+T cells from ICT-Non-responsive patients respond to matching NSCLC PDXs explant following Mizo treatment. Human IFNγ was measured using ELISA in wells containing cultured CD8+T-cells and PDX explants from two patients (Pat#1, LEP 19 and Pat#2, LEP 20) with metastatic NSCLC non responsive to anti-PD1. In the supernatants of

the explant alone, either treated or not-treated with mizoribine, we did not detect human IFNγ. Similarly, adding autologous CD8+T cells to mizoribine-treated or non-treated explants did not induce detectable IFNγ levels. Treatment with anti-PD1, either nivolumab or pembrolizumab, induced a significant elevation in IFNγ secretion only when T cells were incubated with mizoribine-treated explants. n=3 biologically independent counts in each group. One-way t-test was performed among the groups; comparison was done between each line-connected two groups. Pat#1: p=0.00013 for 5^{th} *vs.* 6^{th} group; p=0.00024 for 7^{th} *vs.* 8^{th} group. Pat#2: p=0.00066 for 5^{th} *vs.* 6^{th} group; p=3.9E-05 for 7^{th} *vs.* 8^{th} group. **H**) A boxplot showing ASS1 expression levels in primary tumor samples that developed metastasis (red; n=101) *vs.* ASS1 expression in primary samples that did not develop metastasis (blue; n=582) (Wilcoxon ranksum P<0.05). Boxplots in this figure follow the standard definition, where the box is drawn from top quartile (75-percentile) to bottom quartile (25-percentile) of the data with a horizontal line drawn in the middle to denote the median value. The lowest point of the bottom whisker is the minimum of the data and the highest point of the top whisker is the maximum of the data.

Methods

Cell cultures: Cells were cultured using standard procedures in a 37°C humidified incubator with 5% CO2 in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) (for A549 cells and primary fibroblasts) or RPM1 1640 medium (Gibco) (for 4T1, SW620 and MC38 cells) supplemented with 10%–20% heat-inactivated fetal bovine serum, 10% pen-strep and 2 mM glutamine. The cancer cell-lines under glucose-deprivation were grown in a medium containing dialyzed serum and no glucose. Cell line sources are provided in the Reporting Summary.

Virus infection: Cancer cells were infected with either pLKO-based lentiviral vector with short hairpin RNA (shRNA) sequences against either GFP (as a non-target control) or human ASS1 (Dharmacon). Infected cells were selected with 2μg mL−1 puromycin. For ASS1 over expression rescue, cells were transduced with pLenti6.3/TO/V5-DEST-based lenti viral vector with the human ASS1 transcript. Transduced cells were selected with 12.5μg/ml Blasticidin.

Crystal violet staining: Cells were seeded in 12-well plates at 30,000-100,000 cells/well in a triplicate. Time 0 was calculated as the time the cells became adherent, which was about 20 hours after plating. Cell's staining with Crystal violet was done as described[9], and absorbance was measured at 595 nm 24-72 hours following time 0.

Flow Cytometry:

Annexin V 7-AAD cell viability assay: A549 cells were seeded in 6-well plates at 100,000-120,000 cells/well. Following 72hrs of incubation in glucose free medium with or without treatment with 10mM of L-Name for 48hrs, cells were detached from the surface with TrypLE solution (Biological Industries), washed with PBS, stained with Annexin V 7-AAD (Biolegend) according to the manufacture instruction. Data was acquired on Attune nxt flow cytometer and analyzed using FlowJo V10.0.6.

Flow cytometry for NO measurements: A549 cells were grown in glucose and serum free medium for 20hrs and treated with 10μM 10074-G5 (c-Myc inhibitor, Sigma). Data was acquired on Attune nxt flow cytometer and analyzed using FlowJo v10.0.6.

Intracellular NO was detected by incubation of cells for 15 min at 37C with 10 mM DAF-FM diacetate followed by extensive washing, according to the manufacturer's instructions (Molecular Probes).

Flow cytometry for PD-1 detection: CD8+ T cells were collected after incubation with PDX's, washed twice, and co-stained with anti-CD3 conjugated PE (Biolegend, clone UCHT1, dilution 1:100) and anti-PD-1 conjugated FITC (Biolegend, clone eh12.2h7, dilution 1:100) DAPI added and the cells analyzed on FACSCanto II (BD Biosciences) and analyzed using FlowJo V10.0.7.

Methotrexate treatment: A549 cells were grown for 48hr in control medium or supplemented with 1.2μM Methotrexate (Holland-Moran). Nucleotide levels were measured as described below by LC-MS. Purine/pyrimidine ratio was calculated by dividing (AMP+GMP+ATP+ADP+GTP) concentration by (UMP+CMP+TMP+UTP+CTP+TTP) concentration.

Nitrosylation assay: Detection of protein nitrosylation was performed using the SNO-RAC method [49] with minor adjustments. Briefly, cell lysates were prepared and a total of ~ 2 mg protein was used for each experimental condition. The blocking step was performed for 30 min at 50°C, in the presence of 40 mM NEM with frequent vortexing. After acetone precipitation, proteins were recovered by centrifugation at 2000g for 5 min at 4°C, and the pellets were resuspended in HENS buffer (100 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS, pH 7.5). This material was added to 100μl thiopropyl sepharose beads (GE Healthcare) in the presence of 50 mM sodium ascorbate. Following rotation in

darkness overnight at 4°C, beads were washed with 4×1 ml HENS buffer, then 2×1 ml HENS/10 buffer (HENS diluted 1:10). Captured proteins were eluted with 30µl HENS/10 containing 250 mM 2-mercaptoethanol for 20 min at room temperature, and analyzed by Western blotting.

Western blotting: Cells were lysed with RIPA (Sigma-Aldrich) and 1% protease inhibitor cocktail (Calbiochem). Following centrifugation, supernatant was collected and protein content was evaluated by Bradford assay or BCA Protein Assay Kit (ThermoFisher Scientific, cat # 23225). 80 µg from each sample under reducing conditions were loaded into each lane and separated by electrophoresis on a 10% SDS polyacrylamide gel. Following electrophoresis, proteins were transferred to Cellulose Nitrate membranes (Tamar, Jerusalem, Israel). Nonspecific binding was blocked by incubation with TBST (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk for 1h at room temperature. Membranes were subsequently incubated with antibodies as detailed in the Reporting Summary "Antibodies" section. Gels were quantified by Gel Doc XR+ (BioRad) and analyzed by ImageLab 5.1 software (BioRad).

RNA processing and quantitative PCR: RNA was extracted from cells by using RNeasy Mini Kit (QIAGENe # 74104). cDNAwas synthesized from 1 µg RNA using qScript cDNA Synthesis Kit (Quanta #95749). Detection was performed using either SYBR green PCR master mix (Thermo Fisher scientific #4385612) or FastStart Universal Probe Master (Roche #04914058001) with the required primers. Please see the **Supplementary table 5** of primers for the sequence of the oligonucleotides used.

***In vivo* animal studies:** Please refer to the "Animals and other organisms" section in the Reporting Summary.

LC-MS/MS Arginine measurement: A549 cells were seeded at 500× 105 and grown for 72hrs in full DMEM medium, washed with ice-cold saline, lysed with a mixture of 50% methanol in water and quickly scraped for three freeze-thaw cycles in liquid nitrogen. The insoluble material was pelleted in a cooled centrifuge (4°C) and was used to measure

protein levels in samples. The supernatant was collected for consequent LC-MS/MS analyses, as described[50]. Arginine levels were calculated using a standard curve of the Arginine concentration in each sample and normalized to protein levels.

LC-MS/MS Nucleotides measurement: A549 or 4T1 cells were grown as detailed for each experiment and nucleotides analysis was done as described[9].

The LC-MS/MS instrument consisting of an Acquity I-class UPLC system (Waters) and Xevo TQ-S triple quadrupole mass spectrometer (Waters), equipped with an electrospray ion source, and operated in positive ion mode was used for analysis of nucleoside monophosphates. MassLynx and TargetLynx software (version 4.1, Waters) were applied for the acquisition and analysis of data. Chromatographic separation was done on a 100 mm × 2.1 mm internal diameter, 1.8-µm UPLC HSS T3 column equipped with 50 mm × 2.1 mm internal diameter, 1.8-µm UPLC HSS T3 pre-column (both Waters Acquity) with mobile phases A (10 mM ammonium acetate and 5 mM ammonium hydrocarbonate buffer, pH 7.65 adjusted with 10% acetic acid) and B (acetonitrile) at a flow rate of 0.3 mL min−1 and column temperature 25°C. A gradient was used as follows: for 0-3 min the column was held at 0.2% B, then 3-3.5 min a linear increase to 100% B, 3.5-4.0 min held at 100% B, 4.0-4.5 min back to 0.2% B and equilibration at 0.2% B for 2.5 min. Samples kept at 8°C were automatically injected in a volume of 3 µl. For mass spectrometry, argon was used as the collision gas with a flow of 0.10 mL min−1. The capillary voltage was set to 2.50 kV, source temperature 150°C, desolvation temperature 400°C, cone gasflow 150 l hr−1, desolvation gas flow 800 l hr−1. Nucleotide concentration was calculated using a standard curve of the relevant nucleotide concentration in each sample. Standard curves included increasing concentration of all measured nucleotides ranging from 0-10ug/ml that were positioned at the beginning and at the end of each run. All the calculated values for the different nucleotides in each sample fell within the standard curve range. Analytes were detected in positive mode using multiple-reaction monitoring listed previosely[9].

For PEP and 3-PG quantitation the instrument configuration was used as for nucleotide analysis, whereas LC separation was done in conditions as described for LC-MS polar metabolites analysis[9]. ESI (+) was used for MS detection, with MRM transitions 169.0>81.0, 169.0>123.0, 169.0>151.0m/z and collision energies 15, 10, and 4eV,

respectively, for PEP, and 187.0>99.0, 187.0>141.0m/z and collision energies 12 and 9eV, respectively, for 3-PG. 13C10-ATP was used as internal standard.

LC-MS/MS labeled metabolite detection: A549 cells were seeded at 1.4-2 × 10^6 cells per 10 cm plate and were incubated in full DMEM or in glucose and serum free DMEM and treated with either 10mM L-NAME (Cayman Chemical) or 10mM MDLA (sigma) for 48hrs. In the last 6 hrs. of incubation the medium was replaced with medium containing 4mM of U-C13 Glutamine without Serine. Following the incubation with labeled Glutamine the cells were washed with ice-cold saline, washed with ice-cold saline, lysed with a mixture of 50% methanol in water \and quickly scraped followed by three freeze-thaw cycles in liquid nitrogen. The insoluble material was pelleted in a cooled centrifuge (4°C) and the supernatant was collected for consequent LC-MS/MS analysis as described[9].
GC-MS labeled metabolite detection: A549 cells were seeded at 0.9-2.5 × 106 cells per 10 cm plate and were incubated in full DMEM or in glucose and serum free DMEM and treated with either 10mM L-NAME or 10mM MDLA for 48hrs. In the last 6 hrs. of incubation the medium was replaced with medium containing 4mM of U-C13 Glutamine with or without Serine. Following the incubation with the labeled Glutamine the cells were washed with ice-cold saline, lysed with a mixture of 50% methanol in water added with 2 µg/mL ribitol as an internal standard and quickly scraped followed by three freeze-thaw cycles in liquid nitrogen. The insoluble material was pelleted in a cooled centrifuge (4°C) and the supernatant was collected for consequent GC-MS analysis as described[9]. Natural abundance of labeled metabolite was calculated using Metran analysis.

Sample Procurement and Generation of PDXs and patient's peripheral CD8+ T cells: Two patients bearing metastatic non-small cell lung cancer (NSCLC) were included in this study. Blood samples and fresh tumor tissue samples were procured just after their biopsy with patient consent and with Helsinki approval (number 0093-19-SOR). The samples were placed in serum free DMEM (Gibco) media then processed for implantation in NSG (NOD *scid* gamma) mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl, The Jackson Laboratory). Tumor tissue samples were implanted subcutaneously in dorsal flanks of the 7-9 weeks old male mice to form patient-derived xenografts (PDXs).

After isolation of peripheral blood mononuclear cells (PBMCs) from the patients according to standard protocol using Lymphocyte Separation Medium (LSM, MP Biomedicals, SKU 0850494-CF), $CD8^+$ T cells were isolated from PBMCs by employing human CD8 Microbeads (Miltenyi Biotec, 130-045-201), LS column (Miltenyi Biotec, 130-042-401) and MidiMACS Separator (130-042-302) following manufacturer's protocol. For culturing and maintenance of CD8 T cells, RPMI containing 10% human male AB plasma (Sigma, H4522), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 1% penicillin/streptomycin, 10 mM HEPES (Life Technologies), 200 IU/mL recombinant human IL-2 (PeproTech) and 50ng/mL anti-human CD3 Antibody (BioLegend, 317302) were used for the first 48 hrs followed by culturing and passaging in media containing 200 IU/mL of IL-2.

Co-culture of tumor tissue explants with autologous CD8+ T cells: $2x2x2mm^3$ tumor tissue explants were cut from PDXs with a specific tissue cutter and seeded singly in 48 well flat-bottom plates and incubated with either mizoribine (0.3mM) in 400µl of media or with only media for 10 hrs. Then, explants were washed once and transferred into new 48 well flat-bottom plates containing 2×10^5 CD8+T cells/well along with nivolumab (Opdivo, 20µg/mL), pembrolizumab (Keytruda, 20µg/mL) or mock solventin fresh 400µl RPMI containing 20IU/ml ofIL-2. After 18 hours of incubation, the supernatant was collected from the wells and then assayed for standard IFNγ ELISA assay (ELISA MAX, Biolegend) according to manufacturer's instructions.

Immunohistochemistry of PDX tumors: Immunostaining of PDX tumor sections was done as described[9], except sections were incubated with the ASS1 antibody (1:100 dilution, Abcam, ab124465, CA, USA).

TCGA whole-exome sequencing analysis: We downloaded the cancer genome atlas (TCGA) gene expression and phenotypic information of 9,273 patients (6,228 GTEx healthy tissue samples) encompassing 25 cancer types for which corresponding healthy control samples via UCSC Xena browser (http://xena.ucsc.edu)[51]. We compared the expression of ASS1 in these cancer *vs* healthy tissue samples using Wilcoxon rank-sum test.

Mutation analysis: We have downloaded the mutation data from cbioportal (N=11,072), as it integrates the mutation analysis from different TCGA centers to avoid center-specific bias in mutation calls. (For the analyses described in the Methods Section "*ASS1 expression in tumor vs healthy tissue samples*," we used the TCGA data from UCSC Xena browser (http://xena.ucsc.edu) because it includes TCGA RNAseq data normalized together with GTEx healthy tissue samples, which are absent in cbioportal.)

To study the *Purine-Rich mutational Bias (PRB)* we consider the fraction of transversions from puRines (R) to pYrimidines (Y); f(R->Y) denotes the ratio of R->Y point mutations to all point mutations on the DNA sense strand in a given sample. The fraction of transversions from pyrimidines to purines (Y->R), is defined in an analogous manner. PRB is defined as the difference between the two fractions, i.e.

$$\mathrm{PRB} = \frac{N(Y \to R) - N(R \to Y)}{mutational\ load} = f(Y \to R) - f(R \to Y),$$

where N(R->Y) and N(Y->R) denote the number of R->Y and Y->R single nucleotide polymorphisms (SNPs) on the DNA sense strand, respectively, and 'mutational load' is the total number of SNPs in a given sample. PRB was calculated using all single nucleotide variants (SNV). We compared PRB levels for ASS1-low (bottom tertile) vs ASS1-high (top tertile) samples using Wilcoxon ranksum test for lung, breast, and colorectal cancers collectively.

***In silico* gluconeogenesis:** We considered the expression levels of the key gluconeogenic (PC, PCK1, and FBP1) enzymes and the unidirectional glycolytic enzyme HK1. We evaluated the association between the expression values of these enzymes with ASS1 expression levels using Wilcoxon ranksum test in 11 cancer types (where ASS1 is either overexpressed or significantly associated with patient survival as shown in **Figure 1A, F**). We checked the association between ASS1+ASL+NOS1 expression (see the section '*In silico* S-nitrosylation' below) and the summed expression levels of all gluconeogenic enzymes (PC, PCK1, PCK2, FBP1 and FBP2) in lung (LUAD, LUSC), breast, and colon cancers. We rank-normalized the expression levels across samples in each cancer type, and compared the gluconeogenic enzymes expression levels in high (top tertile) vs low (bottom tertile) ASS1+ASL+NOS1 samples using Wilcoxon ranksum test.

***In silico* S-nitrosylation:** We considered the expression levels of ASS1, ASL, NOS1 to estimate the activity of S-nitrosylation. We evaluated the association between the estimated S-nitrosylation levels and gluconeogenesis levels estimated by the mean expression levels of gluconeogenic enzymes using Wilcoxon ranksum test.

Association of ASS1 expression with c-MYC-driven nutrient deprivation signature: The association between ASS1 expression levels and c-MYC driven nutrient deprivation (ND) signature [29] was evaluated for TCGA breast, lung and colon cancer samples using Wilcoxon ranksum test. For the ND score, we considered the summed expression of Phosphoglycerate dehydrogenase (PHGDH), Phosphoserine aminotransferase (PSAT1), Phosphoserine phosphatase (PSPH), Serine hydroxymethyltransferase 1 (SHMT1) and Serine hydroxymethyltransferase 2 (SHMT2) (Sun et al., 2015). The mean expression level of the ND signature genes was calculated in each sample and then compared to ASS1 expression.

NDNO signature: The nutrient deprivation signature was combined with the expression levels of enzymes involved in S-nitrosylation (ASS1, ASL, NOS1) to construct the nutrient deprivation/NO (NDNO) signature. The mean expression level of the ND signature genes

together with ASS1, ASL, and NOS1 was calculated in each sample and then compared to ASS1 expression.

Association of ASS1 expression and NDNO signature with patients' prognosis: Kaplan Meier analysis was performed to identify the association of ASS1 with patient survival. We compared the survival of patients with high ASS1 samples (top tertile) vs the remainder of the samples using a logrank test [52]. We performed an analogous analysis for the samples that suffer from nutrient deprivation, where c-MYC nutrient deprivation signature is high (>50-percentile) in lung, breast, and colon cancers; for lung cancer, the same top tertile was used, while for breast and colon cancers, top 50-percentile was used to select ASS1-high tumors. The effect size was quantified by median survival differences.

Urea cycle dysregulation (UCD) score: UCD-score quantifies the extent of dysregulation of urea cycle that enhance pyrimidine synthesis. It is defined in our previous study[9], as the weighted summation of gene expression of six urea cycle enzymes, i.e., UCD-score = -ASL-ASS1+CPS1-OTC+SLC25A13-SLC25A15, where the names of genes denote their gene expression levels.

Whole-exome sequencing analysis: 4T1 cells treated as outlined above were followed by lysis and DNA isolation using DNeasy blood and tissue kit (Quiagen). Dragen was used to carry out somatic variant calling for the set of samples. The following Dragen filter was used: "SNP filter:snp: QD<2.0 || FS>60.0; indel filter:indel: QD<2.0 || FS>200", and the resulting variant call was annotated using Ensembl VEP [53]. Purine to pyrimidine (R>Y) and pyrimidine to purine (Y>R) mutations on the sense strand were counted and pooled per condition to achieve sufficient values for statistical analysis. For low glucose condition, a Fisher's exact test was performed on R>Y versus Y>R with corresponding mutation rates with CCLE [54] data as background. For low glucose condition with mizoribine treatment, a Fisher's exact test was performed on R>Y versus Y>R with corresponding mutation rates in low glucose condition as background.

Association of NDNO protein signature and hydrophobicity of peptides in CPTAC lung cancer data: We analyzed recent CPTAC3 lung adenocarcinoma mass spectrometry data (https://cptac-data-portal.georgetown.edu/cptac/s/S046) to evaluate the association of ASS1 over-expression and hydrophobicity of peptides. The mass spectrometry data estimates the presence of peptides in the sample (n=109). Proteomic NDNO score was calculated by getting the regular NDNO score for tumor and matched normal samples and taking the ratio of the NDNO score of tumor over normal sample. The hydrophobicity of peptides was determined by R library 'Peptides' following Janin et al [55]. We evaluated the association between proteomic NDNO-signature and mean peptide hydrophobicity using Wilcoxon ranksum test.

Association of ASS1 expression and NDNO signature with immune checkpoint therapy response: We first analyzed the association of median ASS1 expression and the objective response rate (ORR) to anti-PD1/PDL1 therapy across cancer types obtained from Dr. Yarchoan[37] using Wilcoxon rank-sum test. We analyzed the association of median ASS1 expression and PRB levels in each cancer with cross-cancer ORR to anti-PD1/PDL1 therapy. In difference from the case of ASS1 (top-tertile vs the remainder), low PRB tumors were determined as the bottom tertile of PRB levels, while the high PRB tumors were the remainder of cancer types. This is because there are many cancer types whose median PRB levels are zero (or close to zero), and PRB levels are mostly negative. A total of 19 cancer types were considered where TCGA molecular profiles and anti-PD1 ORR data were available for both analyses. We next analyzed the anti-PD1 treatment cohort of lung cancer patients, where pre-treatment transcriptomic data and response information is available with 7 responders and 14 nonresponders. We compared ASS1 expression levels and NDNO signature between responders vs nonresponders using Wilcoxon ranksum test.

Metastasis analysis: We analysed gene expression and metastasis incidence data of 683 breast cancer patients (101 metastasis cases and 582 control) with local/regional recurrence after mastectomy in estimating distant metastasis risk (GSE102484)[56]. We compared ASS1 expression levels in metastasis vs control groups using Wilcoxon ranksum test.

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Statistics and Reproducibility: Unless otherwise specified, all statistical analyses were performed using one-way ANOVA, Tukey multiple comparisons of means, Student's t-test or Wilcoxon rank sum test of multiple or two groups, with Dunnett's correction when required. Log-transformed data were used where differences in variance were significant and variances were correlated with means. The sample size was chosen in advance based on common practice of the described experiment and is mentioned for each experiment. When samples were distributed non-normally, Mann-Whitney analysis was performed. Statistical tests were done using Statsoft's STATISTICA, ver. 10. No statistical method was used to predetermine sample size, no data were excluded from the analyses. The experiments were not randomized and the Investigators were not blinded to allocation during experiments and outcome assessment. All error bars represent SER. $p \leq 0.05$ was considered significant in all analyses (* denotes $p<0.05$, **$p<0.01$, ***$p<0.001$). Unless specified otherwise, each experiment was conducted with at least three biological replicates repeated at least three times, and with at least three technical replicates within each biological replicate. All error bars represent SER. NS – non-significant, * denotes $P \leq 0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$ unless noted otherwise.

Data Availability:

Whole exome-sequencing data that support the findings of this study have been deposited in the SRA repository under accession code PRJNA637021 (https://www.ncbi.nlm.nih.gov/sra/PRJNA637021). Transcriptomics, whole-exome sequencing, and clinical data for Figure 1, 4, 5 from the TCGA Research Network: http://cancergenome.nih.gov/. The transcriptomics and clinical data derived from this resource that supports the findings of this study is available in UCSC Xena browser (https://xena.ucsc.edu): https://toil.xenahubs.net/download/TcgaTargetGtex_RSEM_Hugo_norm_count.gz and https://toil.xenahubs.net/download/TcgaTargetGTEX_phenotype.txt.gz. Whole exome sequencing data was downloaded from cbioportal (https://www.cbioportal.org/). CPTAC

proteomics data (https://proteomics.cancer.gov/programs/cptac) that were re-analyzed here are available under accession code S046 (https://cptac-data-portal.georgetown.edu/cptac/s/S046). Source data for Fig. 1-6 and Extended Data Fig. 1-5 have been provided as Source Data files. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Code Availability: The custom code used for TCGA analysis in the revised manuscript is available via github repository at https://www.github.com/jooslee/ASS1.

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