Abstract: Methods of diagnosing and treating autism spectrum disorders are provided.
METHODS OF TREATING AUTISM SPECTRUM DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of priority of US Provisional Application Nos. 62/435,986, filed December 19, 2016; 62/559,765, filed September 18, 2017; and 62/582,472, filed November 7, 2017; each of which is incorporated by reference herein in its entirety for any purpose.

FIELD

[002] The present application relates to field of treatment of autism spectrum disorders.

BACKGROUND

[003] Autism spectrum disorders (ASDs) are one of a group of linked neurodevelopment disorders (NDDs). ASDs are characterized by abnormalities in social interaction and communication, restricted interests, and repetitive behaviors. Symptoms of autism typically appear in the first two years of life and affect brain function and development. Classification of ASDs in the Diagnosis and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) lists distinct forms including Asperger syndrome, Rett syndrome, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS). Many other NDDs exhibit behaviors and symptoms similar to autism.

[004] The US Centers for Disease Control and Prevention (CDC) estimate that 1 in 88 children in the US have an ASD, with a ten-fold increase in prevalence over the past 40 years that is only partially explained by improved diagnosis and awareness. Compared with girls, boys are approximately four to five times more likely to be diagnosed with an ASD.

[005] ASDs are highly heritable and exhibit a 2-3% recurrence rate in siblings and a 60%-90% concordance rate in siblings. However, known genetic causes (including chromosomal abnormalities or Fragile-X syndrome) account for only 10%-20% of ASD cases. The interaction of numerous genes, as well as environmental factors, is thought to confer susceptibility to ASDs. Cellular dysfunction, including neuroinflammation, oxidative stress, mitochondrial abnormalities, and abnormal synaptic plasticity, have been proposed as cellular mechanisms predisposing individuals to ASDs.

[006] There are currently no effective methods of treatment or prevention of ASDs. Treatments are needed that can improve core features of ASDs and affect the neurodevelopmental trajectory of ASDs.

SUMMARY

[007] In some embodiments, a method of treatment of an autism spectrum disorder (ASD) is provided comprising modulating the activity of the molecular pathway involved in the
conversion of IMP to AMP and/or downstream signaling through AMP-kinase (AMPK) in a subject in need thereof.

[008] In some embodiments, a method of treatment of an autism spectrum disorder is provided comprising modulating the amount or activity of one or more enzymes in the molecular pathways involved in the conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP) or downstream signaling through AMPK in a subject in need thereof.

[009] In some embodiments, a method of treatment of an ASD is provided comprising modulating the amount or activity of one or more metabolites of the molecular pathways involved in the conversion of IMP to AMP or downstream signaling through AMPK in a subject in need thereof.

[0010] In some embodiments, a method of treatment of an ASD is provided comprising administering to a subject in need thereof an adenylosuccinate (succinyl-adenosine monophosphate or S-AMP) modulator.

[0011] In some embodiments, the S-AMP modulator is an adenylosuccinate synthetase (ADSS) inhibitor. In some embodiments, the ADSS inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.

[0012] In some embodiments, the ADSS inhibitor is a small molecule. In some embodiments, the ADSS inhibitor is L-alanosine or D,L-alanosine.

[0013] In some embodiments, the ADSS inhibitor is selected from hydantocidin, hydantocidin phosphate, hydantocidin-hadacidin S hybrid inhibitor, and hydantocidin-hadacidin R hybrid inhibitor, shown below.

[0014] In some embodiments, the ADSS inhibitor is selected from GE-101, GE-109, and hadacidin, shown below.
In some embodiments, the ADSS inhibitor is selected from AdSS-1 and AdSS-2:

![AdSS-1](image1)

![AdSS-2](image2)

In some embodiments, the ADSS inhibitor is a compound having structure A:

![Structure A](image3)

wherein each of \( R_1 \) and \( R_2 \) is independently selected from the group consisting of \(-H\), a halogen, \(-NH_2\), \(-OH\), \(-NH-R_3\), and \(-O-R_3\);

each of \( G_1 \), \( G_2 \), and \( G_4 \), is independently selected from the group consisting of \( \text{CH} \), \( \text{N} \), \( \text{O} \), and \( \text{S} \), or \( G_4 \) is independently \( \text{C}=0 \) group;

\( G_3 \) is independently selected from the group consisting of \( \text{CH}_2 \), \( \text{NH} \), \( \text{O} \), \( \text{C}=0 \) group and \( \text{S} \);

\( G_5 \) is independently selected from the group consisting of \( \text{C} \) and \( \text{N} \);

\( L \) is absent or is selected from the group consisting of \( \text{O} \), \( \text{NH} \), and \( \text{S} \);

\( R_3 \) is selected from a group consisting of \(-H\), an \( \text{C}-\text{Cis alkyl} \), an \( \text{aryl}-\text{C}(0)-\text{H} \), and \( \text{C}(0)-\text{alkyl} \);

\( R_4 \) is selected from a group consisting of \(-H\), \( \text{-C}(0)0-\); and \( \text{-C}(0)-\text{R}_3 \);

\( R_5 \) is selected from a group consisting of \(-H\), an \( \text{C}-\text{Cis alkyl} \), and an \( \text{aryl} \);

\( M \) is absent or is selected from the group consisting of \(-\text{CH}_2-\); \(-\text{NH}-\); \(-\text{NH-C}(0)-\); \(-\text{O}-\); and \(-\text{S}-\);

and

\( n \) is an integer having the value between 1 and 6.

In some embodiments, \( G_1 \), \( G_2 \), and \( G_4 \) are \( \text{N} \), \( G_3 \) is \( \text{NH} \), and \( G_5 \) is \( \text{C} \).

In some embodiments, the ADSS inhibitor is an antisense oligonucleotide. In some embodiments, the antisense oligonucleotide is complementary to a portion of the ADSS mRNA.

In some embodiments, the ADSS inhibitor is an siRNA. In some embodiments, the siRNA targets a portion of the ADSS mRNA.

In some embodiments, the ADSS inhibitor is a peptide.

In some embodiments, the S-AMP modulator is an adenylosuccinate lyase activator. In some embodiments, the adenylosuccinate lyase activator increases the amount of adenylosuccinate lyase and/or increases the activity of adenylosuccinate lyase.

In some embodiments, the method comprises increasing the amount of adenylosuccinate lyase. In some embodiments, the amount of adenylosuccinate lyase is increased by administering a nucleic acid that encodes adenylosuccinate lyase. In some embodiments, the amount of adenylosuccinate lyase is increased by inhibiting its degradation.

In some embodiments, the method comprises increasing the activity of adenylosuccinate lyase. In some embodiments, the activity of adenylosuccinate lyase is increased by the addition of an activator. In some embodiments an activator of adenylosuccinate lyase activity is a small molecule. In some embodiments, an activator of adenylosuccinate lyase activity is a peptide.

In some embodiments, a method of treating an ASD is provided comprising reducing the amount of S-Ado in a subject in need thereof. In some embodiments, a method of treating an ASD is provided comprising administering to a subject in need thereof a succinyl-adenosine (S-Ado) reducing agent. In some embodiments, the S-Ado reducing agent is an antibody that binds S-Ado. In some embodiments, the antibody is an antibody fragment. In some embodiments, the antibody fragment is selected from an scFv, Fab, Fab', F(ab')2 fragment. In some embodiments, the S-Ado reducing agent is an abzyme.

In some embodiments, an S-Ado reducing agent is an agent that modulates the activity of one or more enzymes responsible for the synthesis or degradation of S-Ado. In some embodiments, an S-Ado reducing agent is an agent that inhibits the enzymatic synthesis of S-Ado. In some embodiments, an S-Ado reducing agent is an agent that activates the enzymatic degradation of S-Ado. In some embodiments, an S-Ado reducing agent is an agent that activates the conversion of S-Ado into a non-S-Ado form. In some embodiments, an S-Ado reducing agent is a peptide. In some embodiments, an S-Ado reducing agent is a small molecule.

In some embodiments, a method of treating an ASD is provided comprising reducing the amount or activity of AMPK. In some embodiments, the activity of AMPK is modulated by the administration of an AMPK inhibitor. In some embodiments, the amount of AMPK is modulated by decreasing the amount of AMP. In some embodiments, the AMPK inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.
In some embodiments, the AMPK inhibitor is a small molecule. In some such embodiments, the small molecule inhibits the activity of AMPK. In some embodiments, the AMPK inhibitor is dorsomorphin, such as dorsomorphin hydrochloride.

In some embodiments, the AMPK inhibitor is an antisense oligonucleotide. In some such embodiments, the antisense oligonucleotide reduces the amount of AMPK. In some embodiments, the antisense oligonucleotide is complementary to a portion of the AMPK mRNA.

In some embodiments, the AMPK inhibitor is an siRNA. In some such embodiments, the siRNA reduces the amount of AMPK. In some embodiments, the siRNA is complementary to a portion of the AMPK mRNA.

In some embodiments, the AMPK inhibitor is a peptide. In some such embodiments, the peptide inhibits the activity of AMPK.

In some embodiments, the subject has a 16pl1.2 deletion. In some embodiments, the subject has a mutation in the KCTD13 gene. In some embodiments, the mutation in the KCTD13 gene is a loss-of-function mutation. In some embodiments, the mutation in the KCTD13 gene is a partial or total deletion of the KCTD13 gene, or a missense mutation, or a nonsense mutation.

In some embodiments, the subject has a mutation in the CUL3 gene. In some embodiments, the mutation in the CUL3 gene is a loss-of-function mutation. In some embodiments, the mutation in the CUL3 gene is a partial or total deletion of the CUL3 gene, or a missense mutation, or a nonsense mutation.

In some embodiments, the subject has an elevated level of S-Ado. In some embodiments, the elevated level of S-Ado is determined in a blood, urine, or CSF sample from the subject.

In some embodiments, treating an autism spectrum disorder comprises alleviating at least one symptom of the autism spectrum disorder. In some embodiments, alleviating at least one symptom comprises reducing the number, severity, and/or frequency of seizures; preventing and/or slowing developmental delay; improving and/or slowing the decline in intellectual ability; reducing the incidence of obesity; reducing social interaction deficit; improving language; reducing repetitive behaviors; reducing sleep disorders; reducing mood disorders; reducing anxiety; reducing gastrointestinal symptoms; reducing hyperactivity; and/or reducing attention deficits.

In some embodiments, a method of identifying a subject who would benefit from treatment with an ADSS inhibitor is provided comprising determining the level of S-Ado in a sample from the subject, wherein an elevated level of S-Ado in the sample indicates the subject would benefit from treatment with an S-AMP modulator. In some embodiments, the level of S-Ado in the sample is compared to a reference level of S-Ado. In some embodiments, the method
further comprises determining whether the subject has a 16pl 1.2 deletion, wherein a 16pl 1.2 deletion indicates the subject would benefit from treatment with an S-AMP modulator.

[0036] In some embodiments, the method further comprises determining whether the subject has a mutation in the \textit{KCTD13} gene, wherein a mutation in the \textit{KCTD13} gene indicates the subject would benefit from treatment with an S-AMP modulator. In some embodiments, the mutation in the \textit{KCTD13} gene is a loss-of-function mutation. In some embodiments, the mutation in the \textit{KCTD13} gene is a partial or total deletion of the \textit{KCTD13} gene.

[0037] In some embodiments, the method further comprises determining whether the subject has a mutation in the \textit{CUL3} gene, wherein a mutation in the \textit{CUL3} gene indicates the subject would benefit from treatment with an S-AMP modulator. In some embodiments, the mutation in the \textit{CUL3} gene is a loss-of-function mutation. In some embodiments, the mutation in the \textit{CUL3} gene is a partial or total deletion of the \textit{CUL3} gene.

[0038] In some embodiments, a method of identifying a subject who would benefit from treatment with an ADSS inhibitor is provided comprising determining whether the subject has a 16pl 1.2 deletion, wherein a 16pl 1.2 deletion indicates the subject would benefit from treatment with an ADSS inhibitor.

[0039] In some embodiments, a method of identifying a subject who would benefit from treatment with an ADSS inhibitor is provided comprising determining whether the subject has a mutation in the \textit{KCTD13} gene, wherein a mutation in the \textit{KCTD13} gene indicates the subject would benefit from treatment with an ADSS inhibitor. In some embodiments, the mutation in the \textit{KCTD13} gene is a loss-of-function mutation. In some embodiments, the mutation in the \textit{KCTD13} gene is a partial or total deletion of the \textit{KCTD13} gene.

[0040] In some embodiments, a method of identifying a subject who would benefit from treatment with an ADSS inhibitor is provided comprising determining whether the subject has a mutation in the \textit{CUL3} gene, wherein a mutation in the \textit{CUL3} gene indicates the subject would benefit from treatment with an ADSS inhibitor. In some embodiments, the mutation in the \textit{CUL3} gene is a loss-of-function mutation. In some embodiments, the mutation in the \textit{CUL3} gene is a partial or total deletion of the \textit{CUL3} gene.

[0041] In some embodiments, the method further comprises determining the level of S-Ado in a sample from the subject, wherein an elevated level of S-Ado in the sample indicates the subject would benefit from treatment with an S-AMP modulator.

[0042] In some embodiments, the subject exhibits at least one symptom of an autism spectrum disorder. In some embodiments, at least one symptom of an autism spectrum disorder is selected from development delay, intellectual disability, seizures, increased risk of obesity; social interaction deficit; language impairment; repetitive behaviors; sleep disorder; mood disorder;
anxiety; gastrointestinal symptoms; hyperactivity; and attention deficits. In some embodiments, the subject has been previously diagnosed as having an autism spectrum disorder.

[0043] In some embodiments, the subject does not have an adenylosuccinate lyase deficiency.

[0044] In some embodiments, the S-AMP modulator is selected from an ADSS inhibitor, an adenylosuccinate lyase activator, and an S-Ado reducing agent.

[0045] In some embodiments, the method comprises administering to the subject an S-AMP modulator. In some embodiments, the S-AMP modulator is an ADSS inhibitor.

[0046] In some embodiments, the ADSS inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.

[0047] In some embodiments, the ADSS inhibitor is a small molecule. In some embodiments, the ADSS inhibitor is L-alanosine or D,L-alanosine.

[0048] In some embodiments, the ADSS inhibitor is selected from hydantocidin, hydantocidin phosphate, hydantocidin-hadacidin S hybrid inhibitor, and hydantocidin-hadacidin R hybrid inhibitor, shown below.

[0049] In some embodiments, the ADSS inhibitor is selected from GE-101, GE-109, and hadacidin, shown below.

[0050] In some embodiments, the ADSS inhibitor is selected from AdSS-1 and AdSS-2:
In some embodiments, the ADSS inhibitor is a compound having structure A:

\[
A
\]

wherein each of \( R_1 \) and \( R_2 \) is independently selected from the group consisting of -H, a halogen, -NH \(_2\), -OH, -NH-R\(_3\), and -O-R\(_3\);

each of \( G_i \), \( G_2 \), and \( G_4 \) is independently selected from the group consisting of CH, N, O, and S, or \( G_4 \) is independently C=0 group;

\( G_3 \) is independently selected from the group consisting of CH\(_2\), NH, O, C=0 group and S;

\( G_5 \) is independently selected from the group consisting of C and N;

L is absent or is selected from the group consisting of O, NH, and S;

\( R_3 \) is selected from a group consisting of -H, an \( \text{Ci-Cis alkyl} \), an aryl,-C(0)-H, and -C(0)-alkyl;

\( R_4 \) is selected from a group consisting of -H, -C(0)O-, and -C(0)-R3;

\( R_5 \) is selected from a group consisting of -H, an \( \text{Ci-Cis alkyl} \), and an aryl;

M is absent or is selected from the group consisting of -CH\(_2\), -NH-, -NH-C(O)-, -O-, and -S-; and

\( n \) is an integer having the value between 1 and 6.

In some embodiments, \( G_i \), \( G_2 \), and \( G_4 \) are N, \( G_3 \) is NH, and \( G_5 \) is C.


In some embodiments, the ADSS inhibitor is an antisense oligonucleotide. In some embodiments, the antisense oligonucleotide is complementary to a portion of the ADSS mRNA.

In some embodiments, the ADSS inhibitor is an siRNA. In some embodiments, the siRNA targets a portion of the ADSS mRNA.

In some embodiments, the ADSS inhibitor is a peptide.

In some embodiments, the S-AMP modulator is an adenylosuccinate lyase activator. In some embodiments, the adenylosuccinate lyase activator increases the level of adenylosuccinate lyase and/or increases the activity of adenylosuccinate lyase. In some embodiments, the method comprises administering a nucleic acid that encodes adenylosuccinate lyase.

In some embodiments, the method comprises administering to the subject a succinyl-adenosine (S-Ado) reducing agent. In some embodiments, the S-Ado reducing agent is an
antibody that binds S-Ado. In some embodiments, the antibody is an antibody fragment. In some embodiments, the antibody fragment is selected from an scFv, Fab, Fab', F(ab')2 fragment.

[0058] In some embodiments, a method of monitoring treatment of a subject with an S-AMP modulator or S-Ado reducing agent comprises determining the level of S-Ado in a sample from the subject. In some embodiments, the level of S-Ado is determined at least two time points. In some embodiments, the level of S-Ado is determined in a first sample from the subject and in a second sample from the subject, wherein the second sample from the subject is taken at a later point in time than the first sample from the subject. In some embodiments, the first sample from the subject is taken prior to treatment with an S-AMP modulator or S-Ado reducing agent and the second sample from the subject is taken after administration of at least one dose of an S-AMP modulator or S-Ado reducing agent.

[0059] In some embodiments, the first sample from the subject is taken at a first time point and the second sample from the subject is taken at a second time point, wherein at least one dose of an S-AMP modulator or S-Ado reducing agent is administered between the first time point and the second time point. In some embodiments, a decrease in the level of S-Ado in the second sample compared to the first sample indicates the treatment is effective.

[0060] In some embodiments, the method is a method of monitoring treatment of a subject with an S-AMP modulator. In some embodiments, the S-AMP modulator is an ADSS inhibitor.

[0061] In some embodiments, the ADSS inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule. In some embodiments, the ADSS inhibitor is a small molecule.

[0062] In some embodiments, the ADSS inhibitor is L-alanosine or D,L-alanosine.

[0063] In some embodiments, the ADSS inhibitor is an antisense oligonucleotide. In some embodiments, the antisense oligonucleotide is complementary to a portion of the ADSS mRNA.

In some embodiments, the ADSS inhibitor is an siRNA. In some embodiments, the siRNA is complementary to a portion of the ADSS mRNA. In some embodiments, the ADSS inhibitor is a peptide. In some embodiments, the S-AMP modulator is an adenylosuccinate lyase activator.

[0064] In some embodiments, the adenylosuccinate lyase activator increases the level of adenylosuccinate lyase and/or increases the activity of adenylosuccinate lyase.

[0065] In some embodiments, the method comprises administering a nucleic acid that encodes adenylosuccinate lyase. In some embodiments, the method is a method of monitoring treatment of a subject with an S-Ado reducing agent. In some embodiments, the S-Ado reducing agent is an antibody that binds S-Ado. In some embodiments, the antibody is an antibody fragment. In some embodiments, the antibody fragment is selected from an scFv, Fab, Fab', F(ab')2 fragment.
In some embodiments, the sample is selected from a blood sample, a urine sample, and a CSF sample.

In any of the embodiments described herein, a method of treating an ASD further comprises placing the subject on a low purine diet.

In some embodiments, a method of treating an autism spectrum disorder in a subject comprises placing the subject on a low purine diet. In some embodiments, the subject has a 16pl 1.2 deletion. In some embodiments, the subject has a mutation in the KCTD13 gene. In some embodiments, the mutation in the KCTD13 gene is a loss-of-function mutation. In some embodiments, the mutation in the KCTD13 gene is a partial or total deletion of the KCTD13 gene, a missense mutation, or a nonsense mutation. In some embodiments, the subject has a mutation in the CUL3 gene. In some embodiments, the mutation in the CUL3 gene is a loss-of-function mutation. In some embodiments, the mutation in the CUL3 gene is a partial or total deletion of the CUL3 gene, a missense mutation, or a nonsense mutation. In some embodiments, the subject has an elevated level of S-Ado. In some embodiments, the elevated level of S-Ado is determined in a blood, urine, or CSF sample from the subject. In some embodiments, treating an autism spectrum disorder comprises alleviating at least one symptom of the autism spectrum disorder. In some embodiments, alleviating at least one symptom comprises reducing the number, severity, and/or frequency of seizures; preventing and/or slowing developmental delay; improving and/or slowing the decline in intellectual ability; reducing the incidence of obesity; reducing social interaction deficit; improving language; reducing repetitive behaviors; reducing sleep disorders; reducing mood disorders; reducing anxiety; reducing gastrointestinal symptoms; reducing hyperactivity; and/or reducing attention deficits.

In some embodiments, a method of monitoring treatment of a subject having an autism spectrum disorder with low purine diet is provided, comprising determining the level of S-Ado in a sample from the subject. In some embodiments, the level of S-Ado is determined at at least two time points. In some embodiments, the level of S-Ado is determined in a first sample from the subject and in a second sample from the subject, wherein the second sample from the subject is taken at a later point in time than the first sample from the subject. In some embodiments, the first sample from the subject is taken prior to treatment with a low purine diet and the second sample from the subject is taken after treatment with the low purine diet. In some embodiments, the second sample from the subject is taken after at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 6 weeks, at least 2 months, at least 3 months, at least 6 months, or at least 1 year after the start of treatment with the low purine diet. In some embodiments, a decrease in the level of S-Ado in the second sample compared to the first sample indicates the
treatment is effective. In some embodiments, the method is a method of monitoring treatment of a subject with a low purine diet.

[0070] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the claims.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0071] Figure 1 provides western blot analysis of KCTD13 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels in Kctd13A47 mice compared to wildtype (WT).

[0072] Figure 2 shows an overview of the stable isotope labeling using amino acids in cell culture (SILAC) labeling and trypsin digestion procedures.

[0073] Figures 3A-3B show ubiquitination (A) and proteome (B) analysis of Kctd13A47 mice compared to WT.

[0074] Figure 4 describes the reaction mediated by ADSS.

[0075] Figures 5A-5B shows immunoblot (A) and quantification (B) of ADSS protein levels in neurons from Kctd13A47 mice versus wildtype controls (D = DMSO; B = bortezomib; M = MLN4924). Kctd13- refers to Kctd13A47 mice.

[0076] Figures 6A-6B show experimental protocol (A) and results (B) on the effect of KCTD13 transfection on ubiquitination of ADSS in a HEK model with exogenous expression of HisUb and RBX1/CUL3. Ni-NTA refers to magnetic beads. IB=immunoblot; IP=immunoprecipitation; Ub=ubiquitin.

[0077] Figures 7A-7B show experimental protocol (A) and results (B) on the effect of KCTD13 and HisUb cotransfection with ADSS in a HEK model without exogenous RBX/CUL3.

[0078] Figures 8A-8B show experimental protocol (A) and results (B) on the concentration-dependent effect of KCTD13 transfection on ubiquitination of ADSS in HEK cells.

[0079] Figures 9A-9C show experimental design (A), signaling effect (B), and experimental results (C) of a dominant-negative CUL3 (DNCUL3) on ADSS ubiquitination by KCTD13 in HEK cells.

[0080] Figures 10A-10B show the experimental conditions (A) and results (B) for the immunoprecipitation experiment to study the ADSS-KCTD13-CUL3 interaction. i=input; Ig=non-specific Ig control; K=KCTD13 antibody; m=myc antibody.

[0081] Figures 11A-I IB show experimental design (A) and results (B) on ubiquitination of ADSS following expression of the different adaptor proteins KCTD13, KCTD12, and TNFAIP1. The top panel in Figure 11B shows ubiquitination results, while the lower blots show western blots confirming expression of the different adaptor proteins.

[0082] Figure 12 shows KCTD13 mRNA levels in 16pl 1.2 deletion patient fibroblasts and unaffected (U) control fibroblasts.
Figure 13 shows metabolic results from LC/MS analysis of cell lysate samples from 16pl 1.2 deletion patient fibroblasts and control (con) fibroblasts.

Figure 14 shows metabolic results from LC/MS analysis of media samples from 16pl 1.2 deletion patient fibroblasts and control (con) fibroblasts.

Figure 15 shows purine metabolite levels in urine from WT or KO (Kctdl 3/147) mice.

Figure 16 shows purine metabolite levels in cell lysates of neurons at 21 days in vitro (DIV21) from WT or KO (Kctdl 3/147) mice.

Figure 17 shows purine metabolite levels in media supernatants of division 21 (DIV21) neurons from WT or KO (Kctdl 3Δ47) mice.

Figure 18A-D shows a summary of changes in purine metabolites in cell lysates and media supernatants of DIV21 neurons from WT or KO (Kctdl 3Δ47) mice.

Figure 19 shows purine metabolite levels in human fibroblast cell lysates following treatment with Hepes (H), L-alanosine (LA, an inhibitor of ADSS), or untreated (U).

Figures 20A-B show IMP levels in cell lysates of DIV21 neurons from WT mice following treatment with D, L-alanosine or L-alanosine, or untreated (U).

Figure 21 shows S-Ado levels in cultured neurons from wild-type and Kctdl 3/147 (KO) mice, and in neuronal media.

Figure 22 shows S-Ado levels in cultured neurons from wild-type and Kctdl 3/147 (KO) mice contacted with 0, 0.01, or 0.1 µM L-alanosine.

DESCRIPTION OF CERTAIN EMBODIMENTS

1. Definitions

As used herein, the term "about" refers to a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. The term "about" generally refers to a range of numerical values (e.g., +/-5-10% of the recited range) that one of ordinary skill in the art would consider equivalent to the recited value (e.g., having the same function or result). When terms such as at least and about precede a list of numerical values or ranges, the terms modify all of the values or ranges provided in the list. In some instances, the term about may include numerical values that are rounded to the nearest significant figure.

The term "antibody" is used herein in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity. In some embodiments, an antibody may be a chimeric antibody, a humanized antibody, or a human antibody.
The term antibody includes, but is not limited to, fragments that are capable of binding to an antigen, such as Fv, single-chain Fv (scFv), Fab, Fab', di-scFv, sdAb (single domain antibody) and (Fab')₂ (including a chemically linked F(ab')₂). The term antibody also includes, but is not limited to, chimeric antibodies, humanized antibodies, and antibodies of various species such as mouse, human, cynomolgus monkey, etc. Antibody fragments also include either orientation of single chain scFvs, tandem di-scFv, diabodies, tandem tri-sdcFv, minibodies, etc. Antibody fragments also include nanobodies (sdAb, an antibody having a single, monomeric domain, such as a pair of variable domains of heavy chains, without a light chain). An antibody fragment can be referred to as being a specific species in some embodiments (for example, human scFv or a mouse scFv).

An "abzyme" or "catalytic antibody" refers to a monoclonal antibody with catalytic activity.

The term "antisense oligonucleotide" refers to a single-stranded oligonucleotide comprising 8 to 50 monomeric units and having a nucleobase sequence that permits hybridization to a corresponding segment of a target nucleic acid. An antisense oligonucleotide may comprise natural, non-natural, and/or modified nucleosides and/or internucleoside linkages.

The term "siRNA" refers to a double-stranded oligonucleotide comprising a first strand comprising 10 to 30 monomeric units and a second strand comprising 10 to 30 monomeric units that is complementary to the first strand, wherein the first strand or second strand has a nucleobase sequence that permits hybridization to a corresponding segment of a target nucleic acid. The first strand and second strand may have 0, 1, 2, or 3 mismatches with respect to one another.

The term "monoclonal antibody" refers to an antibody of a substantially homogeneous population of antibodies, that is, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each antibody in a monoclonal antibody preparation is directed against a single determinant on the antigen. Thus, a sample of monoclonal antibodies can bind to the same epitope on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies may be made by the hybridoma method, by recombinant DNA methods, or be isolated from phage libraries.
The term "peptide" as used herein refers to a molecule formed by linking at least two, and up to 300, amino acids by amide bonds. The amino acids of a peptide may be natural, non-natural, and/or modified amino acids. In some embodiments, a peptide comprises 2-200 amino acids, or 2-100 amino acids, or 2-50 amino acids, or 2-30 amino acids, or 10-300 amino acids, or 10-200 amino acids, or 10-100 amino acids, or 10-50 amino acids.

The term "vector" is used to describe a polynucleotide that can be engineered to contain a cloned polynucleotide or polynucleotides that can be propagated in a host cell. A vector may include one or more of the following elements: an origin of replication, one or more regulatory sequences (such as, for example, promoters and/or enhancers) that regulate the expression of the polypeptide of interest, and/or one or more selectable marker genes (such as, for example, antibiotic resistance genes and genes that can be used in colorimetric assays, for example, β-galactosidase). The term "expression vector" refers to a vector that is used to express a polypeptide of interest in a host cell.

A "host cell" refers to a cell that may be or has been a recipient of a vector or isolated polynucleotide. Host cells may be prokaryotic cells or eukaryotic cells. Exemplary eukaryotic cells include mammalian cells, such as primate or non-primate animal cells; fungal cells, such as yeast; plant cells; and insect cells.

The term "isolated" as used herein refers to a molecule that has been separated from at least some of the components with which it is typically found in nature or produced. For example, a polypeptide is referred to as "isolated" when it is separated from at least some of the components of the cell in which it was produced. Where a polypeptide is secreted by a cell after expression, physically separating the supernatant containing the polypeptide from the cell that produced it is considered to be "isolating" the polypeptide. Similarly, a polynucleotide is referred to as "isolated" when it is not part of the larger polynucleotide (such as, for example, genomic DNA or mitochondrial DNA, in the case of a DNA polynucleotide) in which it is typically found in nature, or is separated from at least some of the components of the cell in which it was produced, for example, in the case of an RNA polynucleotide. Thus, a DNA polynucleotide that is contained in a vector inside a host cell may be referred to as "isolated".

The term "biological sample" means a quantity of a substance from a living thing or formerly living thing. Such substances include, but are not limited to, blood, (for example, whole blood), plasma, serum, urine, amniotic fluid, synovial fluid, endothelial cells, leukocytes, monocytes, cerebrospinal fluid, other cells, organs, and tissues.

A "reference" as used herein, refers to any sample, standard, or level that is used for comparison purposes. A reference may be obtained from a healthy and/or non-diseased sample. In some examples, a reference may be obtained from an untreated sample, or may be a
sample from the subject prior to treatment. In some examples, a reference is obtained from one or more healthy individuals who are not the subject or patient.

[00106] An "autism spectrum disorder" or an "ASD" refers to any one of a group of complex disorders of brain development. "Autism" may be used interchangeably with ASD. ASD includes, but is not limited to, autistic disorder, Rett syndrome, childhood disintegrative disorder, pervasive developmental disorder - not otherwise specified (PDD-NOS), and Asperger syndrome. ASD can be associated with intellectual disability, impairments in communication skills and social interactions, difficulties in motor coordination and attention, seizures, increased risk of obesity, and other symptoms such as sleep and gastrointestinal disturbances. ASD encompasses disorders with varying degrees of impairment, and symptoms may also include restricted, repetitive, and stereotyped patterns of behavior. ASD may have a single-gene or multi-gene etiology, but the etiology of an ASD in an individual subject may also be unknown.

[00107] "Adenylosuccinate synthetase" and "ADSS" as used herein refer to any native ADSS that results from expression and processing of ADSS in a cell. As used herein, "ADSS" also comprises related adenylosuccinate synthetase like (ADSSL) proteins. The term includes ADSS from any vertebrate source, including mammals such as primates (e.g., humans and cynomolgus monkeys) and rodents (e.g., mice and rats), unless otherwise indicated. The term also includes naturally occurring variants of ADSS, e.g., splice variants, isoforms, isozymes, or allelic variants. The amino acid sequence of an exemplary human ADSS protein is shown in SEQ ID NO: 12.

[00108] "Adenyl succinate lyase" and "ADSL" as used herein refer to any native ADSL that results from expression and processing of ADSL in a cell. The term includes ADSL from any vertebrate source, including mammals such as primates (e.g., humans and cynomolgus monkeys) and rodents (e.g., mice and rats), unless otherwise indicated. The term also includes naturally occurring variants of ADSL, e.g., splice variants, isoforms, isozymes, or allelic variants. The amino acid sequence of an exemplary human ADSL protein is shown in SEQ ID NO: 13.

[00109] "AMP kinase" and "AMPK" and "5'-AMP-activated protein kinase" as used herein refer to any native AMP kinase that results from expression and processing of AMP kinase in a cell. The term includes AMP kinase from any vertebrate source, including mammals such as primates (e.g., humans and cynomolgus monkeys) and rodents (e.g., mice and rats), unless otherwise indicated. The term also includes naturally occurring variants of AMP kinase, e.g., splice variants, isoforms, isozymes, or allelic variants. AMP kinase is a heterotrimeric protein comprising α, β, and γ subunits. The amino acid sequences of exemplary human AMP kinase subunits are shown in SEQ ID Nos: 17-19.
An "S-AMP modulator" or "adenylosuccinate modulator" or "succinyl-adenosine monophosphate modulator" refers to an agent that decreases the production or level of S-AMP. S-AMP modulators include, but are not limited to, adenylosuccinate synthetase (ADSS) inhibitors and adenylosuccinate lyase activators.

An "AMPK modulator" refers to an agent that decreases the amount or activity of AMPK. AMPK modulators include, but are not limited to, AMPK inhibitors and AMP reducing agents.

An "AMPK inhibitor" or refers to an agent that inhibits the expression or activity of AMPK, and/or reduces the level of AMPK.

An "AMP reducing agent" refers to an agent that decreases the amount of AMP.

An "adenylosuccinate synthetase inhibitor" or "ADSS inhibitor" refers to an agent that inhibits the expression or activity of ADSS, and/or reduces the level of ADSS.

An "adenylosuccinate lyase activator" refers to an agent that increases the expression, level, or activity of adenylosuccinate lyase.

An "succinyl-adenosine reducing agent" or "S-Ado reducing agent" refers to an agent that reduces the level of free S-Ado and/or its metabolites in a subject. In some embodiments, an S-Ado reducing agent reduces the level of extracellular free S-Ado and/or its metabolites in a subject. In some embodiments, an S-Ado reducing agent reduces the level of S-Ado and/or its metabolites in blood, urine, and/or cerebrospinal fluid. Reduction of free S-Ado and/or its metabolites includes degradation of S-Ado and/or its metabolites and/or binding of S-Ado and/or its metabolites such that its deleterious effects are substantially mitigated.

An "effective amount" of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

An "individual" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In various embodiments, the individual or subject is a human.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A
pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

[00121] A "pharmaceutically acceptable salt" means a physiologically and pharmaceutically acceptable salt of a compounds, *i.e.*, a salt that retains the desired biological activity of the compound and does not impart undesired toxicological effects thereto. Any of the compounds described herein, such as any of the small molecule inhibitors, includes pharmaceutically acceptable salts thereof.

[00122] As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of a disease or condition, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease or condition, decreasing the rate of disease progression, amelioration or palliation of the disease or condition, and remission or improved prognosis. In some embodiments, methods are provided that delay development of a condition or disease or one or more symptoms of the condition or disease, or slow the progression of a disease or condition.

**II. Exemplary Therapeutic Methods**


[00124] The present inventors have identified adenylsuccinate synthetase (ADSS) as a substrate of a ubiquitin ligase complex involving Kctdl3 and Cul3, and have demonstrated that deletion of **KCTD13** gene results in a statistically significant decrease in ADSS ubiquitination and a concomitant increase in ADSS protein levels. As discussed below, the increase in ADSS protein levels is expected to lead to the presence of extracellular S-Ado and its metabolites in individuals with loss-of-function **KCTD13** gene mutations.

[00125] ADSS catalyzes the conversion of IMP to adenylsuccinate (S-AMP), which is then converted to AMP by adenylsuccinate lyase. *See, e.g.*, Figure 4. A build-up of S-AMP leads to dephosphorylation of S-AMP and secretion of S-Ado from cells. S-Ado is not detectable in the blood, urine, or cerebrospinal fluid of healthy individuals, suggesting that
secretion of S-Ado is the result of a dysregulated AMP synthesis pathway. Intriguingly, mutations in the second enzyme in the pathway, adenylosuccinate lyase (ADSL), can result in a rare condition referred to as ADSL deficiency, in which S-Ado and another ADSL substrate, succinylaminoimidazole carboxamide ribotide (SAICAR), accumulate in urine, CSF, and plasma. Patients with ADSL deficiency exhibit neurological symptoms, including severe psychomotor retardation, microcephaly, early onset of seizures, and autistic features. See, e.g., Jurecka et al., 2015, *J. Inherit. Metab. Dis.*, 38: 231-242.

Increased S-AMP levels also result in increased AMP levels through the normal enzymatic conversion of S-AMP to AMP by ADSL. AMP is a key regulator of AMP Kinase (AMPK). In the presence of high levels of AMP, AMPK is upregulated and actives a number of different molecular pathways including those involved in glucose metabolism, lipid metabolism, cell growth/autophagy, polarity, and transcription. See, e.g., Mihaylova et al., 2012, *Nat. Cell Biol.*, 13: 1016-23. Thus, the loss-of-function *KCTD13* gene mutations may lead to aberrantly activated signaling through one or more of these pathways via aberrantly upregulated AMPK.

Prior the present disclosure, there was no known link between ADSS and the AMP synthesis pathway and the risk loci identified in autism.

Methods of treating an autism spectrum disorder are provided herein. In some embodiments, a method of treatment of an autism spectrum disorder is provided comprising administering to a subject in need thereof an adenylosuccinate (S-AMP) modulator. In some embodiments, a method of treating an autism spectrum disorder (ASD) is provided comprising administering to a subject in need thereof a succinyl-adenosine (S-Ado) reducing agent. In some embodiments, a method of treating an ASD is provided comprising reducing the amount or activity of AMPK. In some such embodiments, the method comprises administering to a subject with ASD an AMPK modulator, such as an AMPK inhibitor or an AMP reducing agent. In some embodiments, a method of treating an ASD in a subject comprises a low purine diet, alone or in combination with other treatments, including the treatments described herein. Low purine diets are well known, e.g., for the treatment and prevention of kidney stones and gout.

In various embodiments, the subject has a 16p1 1.2 deletion. The 16p1 1.2 deletion may include deletion of all or a portion of the *KCTD13* gene. In some embodiments, the subject has a mutation in the *KCTD13* gene, which may be a partial or full deletion, insertion, point mutation, and the like. In some embodiments, the mutation in the *KCTD13* gene is a loss-of-function mutation. In some embodiments, the loss-of-function mutation in the *KCTD13* gene is a partial or total deletion of the *KCTD13* gene.

In some embodiments, the subject has a mutation in the *CUL3* gene, which may be a partial or full deletion, insertion, point mutation, and the like. In some embodiments, the
mutation in the CUL3 gene is a loss-of-function mutation. In some embodiments, the mutation in the CUL3 gene is a partial or total deletion of the CUL3 gene.

[00131] In some embodiments, the subject has an elevated level of S-Ado. In some embodiments, the elevated level of S-Ado is determined in a blood, plasma, urine, or CSF sample from the subject.

[00132] In some embodiments, the subject has both a 16pl 1.2 deletion, which may include a partial or full deletion of the KCTD13 gene and an elevated level of S-Ado. In some embodiments, the subject has both a loss-of-function mutation in the KCTD13 gene and an elevated level of S-Ado. In some embodiments, the subject has both a loss-of-function mutation in the CUL3 gene and an elevated level of S-Ado. In some embodiments, the subject has an elevated level of S-Ado and exhibits one or more symptoms of an autism spectrum disorder. As discussed above, the elevated level of S-Ado may be determined in a blood, plasma, urine, or CSF sample from the subject.

[00133] In some embodiments, treating an autism spectrum disorder comprises alleviating at least one symptom of the autism spectrum disorder. In some such embodiments, alleviating at least one symptom comprises reducing the number, severity, and/or frequency of seizures; preventing and/or slowing developmental delay; improving and/or slowing the decline in intellectual ability; reducing the incidence of obesity; reducing social interaction deficit; improving language; reducing repetitive behaviors; reducing sleep disorders; reducing mood disorders; reducing anxiety; reducing gastrointestinal symptoms; reducing hyperactivity; and/or reducing attention deficits.

[00134] In various embodiments, methods comprise administering to a subject with an autism spectrum disorder, or a subject suspected of having an autism spectrum disorder, or a subject predicted to develop an autism spectrum disorder, or a subject at risk for developing an autism spectrum disorder, a modulator of adenylsuccinate (S-AMP modulator). In various embodiments, the subject has been identified as having an autism spectrum disorder, or suspected of having an autism spectrum disorder, or predicted to develop an autism spectrum disorder, or at risk for developing an autism disorder, using any diagnostic criteria in the art or described herein. In various embodiments, the S-AMP modulator reduces the production or level of S-AMP.

A. Exemplary S-AMP Modulators

[00135] In some embodiments, the S-AMP modulator is an adenylsuccinate synthetase (ADSS) inhibitor. An ADSS inhibitor refers to an agent that inhibits the expression or activity of ADSS, and/or reduces the level of ADSS. That is, in various embodiments, an ADSS inhibitor may inhibit the expression of the ADSS protein, e.g., by inhibiting translation of the
ADSS mRNA into the ADSS protein. In some embodiments, an ADSS inhibitor inhibits the activity of ADSS, such as by binding to ADSS and interfering with its enzymatic activity.

[00136] In some embodiments, the S-AMP modulator is an adenyllosuccinate lyase activator. An adenyllosuccinate lyase activator refers to an agent that increases the expression, level, or activity of adenyllosuccinate lyase. That is, in various embodiments, an adenyllosuccinate lyase activator may involve expressing adenyllosuccinate lyase in a cell, such as by administering a nucleic acid encoding adenyllosuccinate lyase. An adenyllosuccinate lyase activator may also be an inhibitor of a cellular factor that itself inhibits adenyllosuccinate lyase, such as a microRNA.

1. **Exemplary ADSS Inhibitors**

[00137] In various embodiments, an adenyllosuccinate synthetase (ADSS) inhibitor is an agent that inhibits the expression or activity, and/or reduces the level of ADSS. An ADSS inhibitor may, in various embodiments, be a small molecule, a peptide, an siRNA, or an antisense oligonucleotide.

[00138] In some embodiments, an ADSS inhibitor is a small molecule. A small molecule ADSS inhibitor may, in some embodiments, bind to the active site of ADSS and compete for binding of the natural substrates, such as IMP and/or L-aspartate. In some embodiments, a small molecule ADSS inhibitor is an IMP mimic. In some embodiments, a small molecule inhibitor is an L-aspartate mimic.

[00139] In some embodiments, an ADSS inhibitor is a peptide. A peptide is a polymeric compound of amino acids comprising up to 300 amino acid units linked by amide bonds. In some embodiments, a peptide inhibitor comprises fewer than 200, fewer than 100, fewer than 50, fewer than 40, fewer than 30, fewer than 20, or fewer than 10 amino acids. In some embodiments, a peptide inhibitor comprises 2-200 amino acids, or 2-100 amino acids, or 2-50 amino acids, or 2-30 amino acids, or 10-300 amino acids, or 10-200 amino acids, or 10-100 amino acids, or 10-50 amino acids. The amino acids of a peptide may be natural, non-natural, and/or modified. In some embodiments, a peptide ADSS inhibitor comprises an L-aspartate or L-aspartate mimic and competitively inhibits binding of ADSS substrate L-aspartate.

[00140] In some embodiments, an ADSS inhibitor is an antisense oligonucleotide. Antisense oligonucleotides are well known in the art. Antisense oligonucleotides are typically 8-50, 8-40, or 8-30 nucleosides long and, in some embodiments, comprise one or more modified nucleosides and/or modified base moieties and/or modified internucleoside linkages. In some embodiments, an antisense oligonucleotide mediates RNaseH activity, which causes degradation of the target mRNA. Antisense oligonucleotides are reviewed, for example, in Antisense Drug Technology, Ed. Stanley T. Corrke, CRC Press, 2007.
In some embodiments, an ADSS inhibitor is an siRNA. siRNAs are double-stranded oligonucleotides in which one strand has a nucleobase sequence that permits hybridization to a corresponding segment of a target nucleic acid. siRNAs may comprise various modifications. Such modifications, and siRNAs generally, are well known in the art. See, e.g., siRNA Design: Methods and Protocols, Ed. Debra J. Taxman, Springer-Verlag New York, LLC, 2013.

A nonlimiting exemplary small molecule ADSS inhibitor is L-alanosine [L-2-amino-3-(N-hydroxy-N-nitrosamino) propionic acid]. Another nonlimiting exemplary small molecule ADSS inhibitor is D,L-alanosine (3-(Hydroxynitrosoamino)-D,L-alanine). Further exemplary ADSS inhibitors include, but are not limited to, hydantocidin, hydantocidin phosphate, hydantocidin-hadacidin S hybrid inhibitor, and hydantocidin-hadacidin R hybrid inhibitor, shown below.

Further exemplary ADSS inhibitors include, but are not limited to, GE-101, GE-109, and hadacidin, shown below.

Further exemplary ADSS inhibitors include AdSS-1 and AdSS-2:

Further exemplary ADSS inhibitors include compounds having structure A:
wherein each of $R_i$ and $R_2$ is independently selected from the group consisting of $-H$, a halogen, $-NH_2$, $-OH$, $-NH-R_3$, and $-O-R_3$;

each of $G_i$, $G_2$, and $G_4$ is independently selected from the group consisting of $CH$, $N$, $O$, and $S$,
or $G_4$ is independently selected from the group consisting of $C=0$ group;

$G_3$ is independently selected from the group consisting of $CH_2$, $NH$, $O$, $C=0$ group and $S$;

$G_5$ is independently selected from the group consisting of $C$ and $N$;

$L$ is absent or is selected from the group consisting of $O$, $NH$, and $S$;

$R_3$ is selected from a group consisting of $-H$, an $Ci$-$Cis$ alkyl, an aryl,$-C(0)-H$, and $-C(0)-alkyl$;

$R_4$ is selected from a group consisting of $-H$, $-C(0)$-$O$; and $-C(0)$-$R_3$;

$R_5$ is selected from a group consisting of $-H$, an $Ci$-$Cis$ alkyl, and an aryl;

$M$ is absent or is selected from the group consisting of $-CH_2$; $-NH$; $-NH-C(O)$; $-O^-$, and $-S$; and

$n$ is an integer having the value between 1 and 6.

In some embodiments, $G_i$, $G_2$, and $G_4$ are $N$, $G_3$ is $NH$, and $G_5$ is $C$.


2. Exemplary Adenylosuccinate Lyase Activators

[00147] In various embodiments, an adenylosuccinate lyase activator is an agent that increases the expression, level and/or activity of adenylosuccinate lyase. In various embodiments, an adenylosuccinate lyase activator may involve expressing adenylosuccinate lyase in a cell, such as by administering a nucleic acid encoding adenylosuccinate lyase.

Administering a nucleic acid encoding adenylosuccinate lyase may comprise gene therapy, for example. Gene therapy strategies are reviewed, for example, in Naldini, 2015, Nature, 526: 351-360.

[00148] In some embodiments, an adenylosuccinate lyase activator may also be an inhibitor of a cellular factor that itself inhibits adenylosuccinate lyase, such as a microRNA. For example, an antisense oligonucleotide that targets a microRNA that inhibits adenylosuccinate lyase will result in an increase in adenylosuccinate lyase levels.
B. Exemplary S-Ado Reducing Agents

[00149] In various embodiments, methods comprise administering to a subject with an autism spectrum disorder, or a subject suspected of having an autism spectrum disorder, or a subject predicted to develop an autism spectrum disorder, or a subject at risk for developing an autism spectrum disorder, a succinyl-adenosine reducing agent (S-Ado reducing agent). In various embodiments, the subject has been identified as having an autism spectrum disorder, or suspected of having an autism spectrum disorder, or predicted to develop an autism spectrum disorder, or at risk for developing an autism disorder, using any diagnostic criteria in the art or described herein. In various embodiments, the S-Ado reducing agent reduces the level of S-Ado and/or its metabolites outside of cells. In various embodiments, the S-Ado reducing agent may sequester S-Ado and/or its metabolites and/or cause degradation of S-Ado and/or its metabolites.

[00150] In some embodiments, an S-Ado reducing agent is an antibody that binds S-Ado and/or one or more of its metabolites. In some embodiments, an S-Ado reducing agent is an antibody that binds S-Ado. In some such embodiments, by binding S-Ado and/or one or more of its metabolites, and antibody reduces or eliminates one or more negative effects of S-Ado and/or one or more of its metabolites. As noted herein, the term "antibody" includes various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity. In some embodiments, an S-Ado reducing agent is an abzyme.

C. Exemplary AMPK Modulators

[00151] In various embodiments, methods comprise administering to a subject with an autism spectrum disorder, or a subject suspected of having an autism spectrum disorder, or a subject predicted to develop an autism spectrum disorder, or a subject at risk for developing an autism spectrum disorder, an AMPK modulator. In various embodiments, the subject has been identified as having an autism spectrum disorder, or suspected of having an autism spectrum disorder, or predicted to develop an autism spectrum disorder, or at risk for developing an autism disorder, using any diagnostic criteria in the art or described herein. In various embodiments, the AMPK modulator reduces the amount or activity of AMPK. In some embodiments, the activity of AMPK is modulated by the administration of an AMPK inhibitor. In some embodiments, the amount of AMPK is modulated by decreasing the amount of AMP.

[00152] In some embodiments, the AMPK inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.

[00153] In some embodiments, an AMPK inhibitor is a small molecule. A small molecule AMPK inhibitor may, in some embodiments, bind to AMPK and compete for binding
of the natural ligand(s), such as AMP. In some embodiments, a small molecule AMPK inhibitor is an AMP mimic. Nonlimiting exemplary AMPK inhibitors include dorsomorphin, such as dorsomorphin hydrochloride.

In some embodiments, an AMPK inhibitor is a peptide. A peptide is a polymeric compound of amino acids comprising up to 300 amino acid units linked by amide bonds. In some embodiments, a peptide inhibitor comprises fewer than 200, fewer than 100, fewer than 50, fewer than 40, fewer than 30, fewer than 20, or fewer than 10 amino acids. In some embodiments, a peptide inhibitor comprises 2-200 amino acids, or 2-100 amino acids, or 2-50 amino acids, or 2-30 amino acids, or 10-300 amino acids, or 10-200 amino acids, or 10-100 amino acids, or 10-50 amino acids. The amino acids of a peptide may be natural, non-natural, and/or modified. In some embodiments, a peptide AMPK inhibitor competitively inhibits binding of AMPK to a ligand.

In some embodiments, an AMPK inhibitor is an antisense oligonucleotide. Antisense oligonucleotides are well known in the art. Antisense oligonucleotides are typically 8-50, 8-40, or 8-30 nucleosides long and, in some embodiments, comprise one or more modified nucleosides and/or modified base moieties and/or modified internucleoside linkages. In some embodiments, an antisense oligonucleotide mediates RNaseH activity, which causes degradation of the target mRNA. Antisense oligonucleotides are reviewed, for example, in Antisense Drug Technology, Ed. Stanley T. Corrke, CRC Press, 2007.

In some embodiments, an AMPK inhibitor is an siRNA. siRNAs are double-stranded oligonucleotides in which one strand has a nucleobase sequence that permits hybridization to a corresponding segment of a target nucleic acid. siRNAs may comprise various modifications. Such modifications, and siRNAs generally, are well known in the art. See, e.g., siRNA Design: Methods and Protocols, Ed. Debra J. Taxman, Springer-Verlag New York, LLC, 2013.

In some embodiments, an AMPK modulator inhibits AMPK amount and/or activity by reducing the level of AMP (an AMP reducing agent). In some embodiments, an AMP reducing agent is an antibody that binds AMP. In some embodiments, an AMP reducing agent is an abzyme. In some embodiments, an AMP reducing agent increases the activity or amount of an enzyme that drives conversion of AMP to another molecule. For example, in some embodiments, an AMP reducing agent increases the activity or amount of adenylate kinase (which converts AMP + ATP to 2 ADP), ATP synthase (which converts ADP to ATP), myoadenylate deaminase (which converts AMP to IMP), and/or nucleotidase (which converts AMP to adenosine).
D. Exemplary Pharmaceutical Compositions and Routes of Administration

[00158] In some embodiments, compositions comprising one or more of the therapeutic agents provided herein are provided in formulations with a wide variety of pharmaceutically acceptable carriers (see, for example, Gennaro, Remington: The Science and Practice of Pharmacy with Facts and Comparisons: Drugfacts Plus, 20th ed. (2003); Ansel et al., Pharmaceutical Dosage Forms and Drug Delivery Systems, 7th ed., Lippencott Williams and Wilkins (2004); Kibbe et al., Handbook of Pharmaceutical Excipients, 3rd ed., Pharmaceutical Press (2000)). Various pharmaceutically acceptable carriers, which include vehicles, adjuvants, and diluents, are available. Moreover, various pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are also available. Non-limiting exemplary carriers include saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof.

[00159] In some embodiments, pharmaceutical compositions are administered in an amount effective for treatment of (including prophylaxis of) an autism spectrum disorder. The therapeutically effective amount is typically dependent on the weight of the subject being treated, his or her physical or health condition, the extensiveness of the condition to be treated, or the age of the subject being treated.

[00160] A therapeutic agent provided herein may be administered in vivo by various routes, including, but not limited to, intravenous, intra-arterial, parenteral, intraperitoneal or subcutaneous. The appropriate formulation and route of administration may be selected according to the particular therapeutic agent and intended application.

[00161] A therapeutic agent provided herein may be administered in conjunction with a low purine diet.

III. Exemplary Diagnostic Methods

[00162] In some embodiments, a method of identifying a subject who would benefit from treatment with an S-AMP modulator and/or low purine diet is provided. In some such embodiments, the method comprises determining the level of S-Ado in a sample from the subject, wherein an elevated level of S-Ado in the sample indicates the subject would benefit from treatment with an S-AMP modulator and/or low purine diet. In some embodiments, the level of S-Ado in the sample is compared to a reference level of S-Ado. The level of S-Ado may be determined in a sample selected from a blood, plasma, urine, and/or CSF sample. In various embodiments, the subject may be selected for S-Ado testing because they have been identified as having an autism spectrum disorder, or suspected of having an autism spectrum disorder, or predicted to develop an autism spectrum disorder, or at risk for developing an autism spectrum disorder. Such identifications may be made on the basis of any criteria in the art for identifying...
such subjects, and may include, for example, neurological assessments, genetic assessments, cognitive testing and/or language testing. In some embodiments, a subject is identified using the Autism-Diagnosis Interview-Revised (ADI-R), the Autism Diagnostic Observation Schedule (ADOS-G), and/or the Childhood Autism Rating Scale (CARS).

[00163] In some embodiments, a method of identifying a subject who would benefit from treatment with an S-AMP modulator and/or a low purine diet comprises determining whether the subject has a 16pl 1.2 deletion, wherein a 16pl 1.2 deletion indicates the subject would benefit from treatment with an S-AMP modulator and/or a low purine diet.

[00164] In some embodiments, a method of identifying a subject who would benefit from treatment with an S-AMP modulator and/or a low purine diet comprises determining whether the subject has a mutation in the KCTD13 gene, wherein a mutation in the KCTD13 gene indicates the subject would benefit from treatment with an S-AMP modulator and/or a low purine diet. In some embodiments, the mutation in the KCTD13 gene is a loss-of-function mutation. In some embodiments, the mutation in the KCTD13 gene is a partial or total deletion of the KCTD13 gene.

[00165] In some embodiments, a method of identifying a subject who would benefit from treatment with an S-AMP modulator and/or a low purine diet comprises determining whether the subject has a mutation in the CUL3 gene, wherein a mutation in the CUL3 gene indicates the subject would benefit from treatment with an S-AMP modulator and/or a low purine diet. In some embodiments, the mutation in the CUL3 gene is a loss-of-function mutation. In some embodiments, the mutation in the CUL3 gene is a partial or total deletion of the CUL3 gene.

[00166] A method of identifying a subject who would benefit from treatment with an S-AMP modulator and/or a low purine diet may comprise any combination of determining the level of S-Ado in a sample from the subject, determining whether the subject has a 16pl 1.2 deletion, determining whether the subject has a mutation in the KCTD13 gene, determining whether the subject has a mutation in the CUL3 gene, and/or determining whether the subject exhibits autism spectrum disorder symptoms, or is otherwise predicted to develop an autism spectrum disorder or at risk of developing an autism spectrum disorder.

[00167] In some embodiments, the subject exhibits at least one symptom of an autism spectrum disorder. In some embodiments, at least one symptom of an autism spectrum disorder is selected from development delay, intellectual disability, seizures, and increased risk of obesity; social interaction deficit; language impairment; repetitive behaviors; sleep disorder; mood disorder; anxiety; gastrointestinal symptoms; hyperactivity; and attention deficits. In some embodiments, the subject has been previously diagnosed as having an autism spectrum disorder.
In some embodiments, the subject does not have an adenylosuccinate lyase (ADSL) deficiency.

A. Exemplary Methods of Detecting Nucleic Acid Variations

Methods of determining presence of genomic variations, such as 16pl 1.2 deletions, mutations in the KCTD13 gene, and/or mutations in the CUL3 gene in a sample from a subject are known in the art. For example, assays for detection of specific variations, using real-time PCR are known (available from, for example, Qiagen, Valencia, CA).

A nucleic acid, may be e.g., genomic DNA, RNA transcribed from genomic DNA, or cDNA generated from RNA. A nucleic acid may be derived from a vertebrate, e.g., a mammal. A nucleic acid is said to be "derived from" a particular source if it is obtained directly from that source or if it is a copy of a nucleic acid found in that source.

Variations in nucleic acids and amino acid sequences may be detected by certain methods known to those skilled in the art. Such methods include, but are not limited to, DNA sequencing; primer extension assays, including allele-specific nucleotide incorporation assays and allele-specific primer extension assays (e.g., allele-specific PCR, allele-specific ligation chain reaction (LCR), and gap-LCR); allele-specific oligonucleotide hybridization assays (e.g., oligonucleotide ligation assays); cleavage protection assays in which protection from cleavage agents is used to detect mismatched bases in nucleic acid duplexes; analysis of MutS protein binding; electrophoretic analysis comparing the mobility of variant and wild type nucleic acid molecules; denaturing-gradient gel electrophoresis (DGGE, as in, e.g., Myers et al. (1985) Nature 313:495); analysis of RNase cleavage at mismatched base pairs; analysis of chemical or enzymatic cleavage of heteroduplex DNA; mass spectrometry (e.g., MALDI-TOF); genetic bit analysis (GBA); 5' nuclease assays (e.g., TaqMan®); and assays employing molecular beacons. Certain of these methods are discussed in further detail below.

Detection of variations in target nucleic acids may be accomplished by molecular cloning and sequencing of the target nucleic acids using techniques known in the art. Alternatively, amplification techniques such as the polymerase chain reaction (PCR) can be used to amplify target nucleic acid sequences directly from a genomic DNA preparation from tissue. The nucleic acid sequence of the amplified sequences can then be determined and variations identified therefrom. Amplification techniques are known in the art, and include, for example, the polymerase chain reaction (PCR).

In various embodiments, the ligase chain reaction may be used to amplify target nucleic acid sequences. See, e.g., Wu et al., Genomics 4:560-569 (1989). In addition, allele-specific PCR may be used to detect variations (e.g., substitutions) in a nucleic acid sequence. See, e.g., Ruano and Kidd (1989) Nucleic Acids Research 17:8392; McClay et al. (2002)
Analytical Biochem. 301:200-206. In some embodiments of this technique, an allele-specific primer is used in which the 3’ terminal nucleotide of the primer is complementary to (i.e., capable of specifically base-pairing with) a particular variation in the target nucleic acid. If the particular variation is not present, an amplification product is not observed. In some embodiments, amplification Refractory Mutation System (ARMS) can also be used to detect variations (e.g., substitutions). ARMS is described, e.g., in European Patent Application Publication No. 0332435, and in Newton et al., Nucleic Acids Research, 17:7, 1989.

Other methods useful for detecting variations (such as substitutions or deletions) include, but are not limited to, (1) allele-specific nucleotide incorporation assays, such as single base extension assays (see, e.g., Chen et al. (2000) Genome Res. 10:549-557; Fan et al. (2000) Genome Res. 10:853-860; Pastinen et al. (1997) Genome Res. 7:606-614; and Ye et al. (2001) Hum. Mut. 17:305-316); (2) allele-specific primer extension assays (see, e.g., Ye et al. (2001) Hum. Mut. 17:305-316; and Shen et al. Genetic Engineering News, vol. 23, Mar. 15, 2003), including allele-specific PCR; (3) 5’nuclease assays (see, e.g., De La Vega et al. (2002) BioTechniques 32:S48-S54 (describing the TaqMan® assay); Ranade et al. (2001) Genome Res. 11:1262-1268; and Shi (2001) Clin. Chem. 47:164-172); (4) assays employing molecular beacons (see, e.g., Tyagi et al. (1998) Nature Biotech. 16:49-53; and Mhlanga et al. (2001) Methods 25:463-71); and (5) oligonucleotide ligation assays (see, e.g., Grossman et al. (1994) Nuc. Acids Res. 22:4527-4534; patent application Publication No. US 2003/01 19004 A1; PCT International Publication No. WO 01/92579 A2; and U.S. Pat. No. 6,027,889).

Variations may also be detected by mismatch detection methods. Mismatches are hybridized nucleic acid duplexes which are not 100% complementary. The lack of total complementarity may be due to deletions, insertions, inversions, or substitutions. One example of a mismatch detection method is the Mismatch Repair Detection (MRD) assay described, e.g., in Faham et al., Proc. Natl Acad. Sci. USA 102:14717-14722 (2005) and Faham et al., Hum. Mol. Genet. 10:1657-1664 (2001). Another example of a mismatch cleavage technique is the RNase protection method, which is described in Winter et al., Proc. Natl. Acad. Sci. USA, 82:7575, 1985, and Myers et al., Science 230:1242, 1985. For example, a method of the invention may involve the use of a labeled riboprobe which is complementary to the human wild-type target nucleic acid. The riboprobe and target nucleic acid derived from the tissue sample are annealed (hybridized) together and subsequently digested with the enzyme RNase A, which is able to detect some mismatches in a duplex RNA structure. If a mismatch is detected by RNase A, it cleaves at the site of the mismatch. Thus, when the annealed RNA preparation is separated on an electrophoretic gel matrix, if a mismatch has been detected and cleaved by RNase A, an RNA product will be seen which is smaller than the full-length duplex RNA for the
riboprobe and the mRNA or DNA. The riboprobe need not be the full length of the target nucleic acid, but can be a portion of the target nucleic acid, provided it encompasses the position suspected of having a variation.

[00176] In a similar manner, DNA probes can be used to detect mismatches, for example through enzymatic or chemical cleavage. See, e.g., Cotton et al., Proc. Natl. Acad. Sci. USA, 85:4397, 1988; and Shenk et al., Proc. Natl. Acad. Sci. USA, 72:989, 1975. Mismatches may also be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes. see, e.g., Cariello, Human Genetics, 42:726, 1988. With either riboprobes or DNA probes, the target nucleic acid suspected of comprising a variation may be amplified before hybridization. Changes in target nucleic acid can also be detected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

[00177] In some embodiments, restriction fragment length polymorphism (RFLP) probes for the target nucleic acid or surrounding marker genes can be used to detect variations, e.g., insertions or deletions. Insertions and deletions may also be detected by cloning, sequencing and amplification of a target nucleic acid. Single stranded conformation polymorphism (SSCP) analysis can also be used to detect base change variants of an allele. See, e.g., Orita et al., Proc. Natl. Acad. Sci. USA 86:2766-2770, 1989, and Genomics, 5:874-879, 1989.

[00178] In some embodiments, a genomic deletion may be determined using deletion analysis. In some embodiments, the deletion may be genotyped based on genomic testing that determines the copy number of sequences, such as chromosomal microarray (CMA) or fluorescence in situ hybridization (FISH). In some embodiments, a genomic deletion may be determined using multiplex ligation-dependent probe amplification (MLPA). In some embodiments, a commercially available service for determining a genomic deletion may be used (e.g., CGC Genetics).

[00179] In some embodiments, compositions suitable for use in performing the methods described herein are provided. For example, arrays are provided that can be used in such methods. In some embodiments, an array comprises individual or collections of nucleic acid molecules useful for detecting variations. For instance, an array may comprise a series of discretely placed individual allele-specific oligonucleotides or sets of allele-specific oligonucleotides. Several techniques are known in the art for attaching nucleic acids to a solid substrate such as a glass slide. One method is to incorporate modified bases or analogs that contain a reactive moiety that is capable of attachment to a solid substrate, such as an amine group, a derivative of an amine group, or another group with a positive charge, into nucleic acid molecules that are synthesized. The synthesized product is then contacted with a solid substrate,
such as a glass slide coated with an aldehyde or other reactive group. The aldehyde or other reactive group will form a covalent link with the reactive moiety on the amplified product, which will become covalently attached to the glass slide. Other methods, such as those using amino propyl silican surface chemistry are also known in the art.

[00180] The presence of 16p1.2 deletions, mutations in the KCTD13 gene, and/or mutations in the CUL3 gene according to any of the methods described herein may be determined using any suitable biological sample obtained from a subject. Biological samples may be obtained from vertebrate animals, and in particular, humans.

B. Exemplary Methods of Determining S-Ado Levels

[00181] In some embodiments, levels of succinyladenosine (S-Ado) and/or its metabolites are determined in extracellular fluids such as blood, plasma, cerebrospinal fluid and/or urine. Any method of determining S-Ado levels may be used. Nonlimiting exemplary methods of detecting S-Ado and/or its metabolites include, but are not limited to, HPLC with UV detection or HPLC-MS. See, e.g., Jurecka et al., 2015, J. Inherit. Metab. Dis. 38: 231-242). In some embodiments, a Bratton-Marshall assay (using N-1-naphthyl ethylene diamine dihydrochloride) and thin-layer chromatography (TLC) is used to identify S-Ado in urine, cerebrospinal fluid, blood and/or plasma. See, e.g., Jaeken, J. Inherit. Metab. Dis. 15: 416-418, 1992. In some embodiments, the Bratton-Marshall test and TLC with Pauly reagent detects the presence of urinary S-Ado. In some embodiments, high-performance liquid chromatography with photodiode array detection (HPLC-DAD) can resolve S-Ado from serum, urine, blood and/or CSF by reverse-phase high-pressure liquid chromatography (RP-HPLC) with detection by UV spectroscopy. In some embodiments, levels of S-Ado and/or its metabolites can be measured using HPLC combined with electrospray ionization tandem mass spectrometry.

EXAMPLES

[00182] The examples discussed below are intended to be purely exemplary of the invention and should not be considered to limit the invention in any way. The examples are not intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (for example, amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1. Generation of Kctdl3A47 mice

[00183] As the role of ubiquitin ligase pathways in schizophrenia and autism is unclear, a mouse model of alteration in expression of KCTD13 was developed. KCTD13 is a substrate-
specific adapter of a BTB-CUL3-RBX1 (BCR) E3 ubiquitin-protein ligase complex involved in regulation of cytoskeleton structure.

[00184] The CRISPR/Cas9 system guide RNAs targeting the first exon oIkctdl3 were used to create a deletion in exon 1 of the mouse Kctdl3 gene (NCBI Gene ID 233877). Out of frame deletion in exon 1 of Kctdl3 would be expected to result in edited sequences that do not produce any protein products.

A. **In vitro validation of single guide RNAs (sgRNAs)**

[00185] sgRNAs were designed using the CRISPRtool (crispr.mit.edu), the sequences of which were screened for Kctdl3 are (sgRNA1 is the one that made the mouse) SEQ ID Nos: 1-3 (Table 1). To validate sgRNA targeting of the Kctdl3 locus, U6-sgRNA PCR products were generated using Herculase II DNA polymerase (Agilent), purified using QIAquick PCR Purification Kit (Qiagen), and co-transfected with a Cas9 expression plasmid into mouse N2a cells (ATCC) using Lipofectamine 2000 (Life Technologies). Three days after transfection, genomic DNA was extracted with QuickExtract DNA Extraction Solution (Epicenter) and used as a template for PCR amplification with Herculase II DNA polymerase (Agilent). PCR amplicons were purified, and 200 ng was used as an input into the SURVEYOR assay (Transgenomic), run on a 2% E-gel (Life Technologies), and quantified using relative band intensities.

**Table 1: sgRNAs designed for Kctdl3**

<table>
<thead>
<tr>
<th>SEQ ID NO</th>
<th>Description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sgRNA1</td>
<td>gccggctgcggcgaagtct</td>
</tr>
<tr>
<td>2</td>
<td>sgRNA2</td>
<td>aggggctcagactgtacga</td>
</tr>
<tr>
<td>3</td>
<td>sgRNA3</td>
<td>cacacgctgccacccctca</td>
</tr>
</tbody>
</table>

B. **Preparation of Cas9 mRNA and sgRNA RNA for zygote injection**

[00186] Human codon optimized Cas9 (from Streptococcus pyogenes) capped and polyadenylated mRNA was prepared by *in vitro* transcription using mMessage mMachine T7 ULTRA Transcription Kit (Ambion). sgRNA RNA was prepared by *in vitro* transcription using Megashortscript T7 Transcription Kit (Ambion) with an annealed partially double stranded template (Table 2).

**Table 2: sgRNA sequences**

<table>
<thead>
<tr>
<th>SEQ ID NO</th>
<th>Description</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>8</td>
<td>Reverse sgRNA IVT primer 1</td>
<td>Aaaaaagcac cgcactcgggtg ccaccttttcc aagtgtgatat ccgactagcc ttatattaac ttgctatattc tagctcttaaa acagcattcg gcgcgcgcgc gccttatagt gatgcgtatt a</td>
</tr>
</tbody>
</table>
[00187] Both Cas9 mRNA and sgRNA RNA were purified by MEGAclear Transcription Clean-Up Kit (Ambion) and mixed to a final concentration of 200 ng/µl Cas9 mRNA and 50 ng/µl sgRNA RNA in H2O for injection.

C. Generation of germline mutant mice

[00188] Three-week old C57BL/6N (Taconic) female mice (superovulation and plugged 0.5 dpc) were used as zygote donors and CD-1(ICR) females were used as foster mothers. Three days prior to zygote injections, pregnant mare’s serum (PMS) 5 R7 was administered IP to each donor female. Forty-seven hours later hCG 5 IU was administered by IP injection and then females were paired with stud males. Donor females were sacrificed 0.5 pcd and oviducts were collected and placed into 0.1% hyaluronidase/flushing holding media (FHM) (Millipore). Using two pairs of forceps the swollen ampulla was torn open releasing the eggs/cumulus cell bunch. The zygotes were washed in drops of FHM and the cumulus cells were removed and put into KSOM-aa culture medium (Millipore) for an hour before injection. 5 µl of Cas9/sgRNA RNA mixture was loaded into a microinjection needle (prepared by Needle puller Sutter P-97) and attached to the microinjector (Eppendorf microinjector 5242). Sets of eggs were placed into 100 µl FHM drops covered with mineral oil at room temperature. The larger pronucleus was injected until an obvious expansion occurred. Eggs were placed back into the warm and equilibrated KSOM-aa culture medium and incubated overnight. Twelve hours later the two-cell stage embryos were surgically implanted bilaterally into the oviducts of 0.5 dpc CD-I recipients. A maximum of 26 two-cell embryos were transferred into one recipient and monitored for pregnancy. To identify progeny that contained indels in the targeted Kctdl3 exon, the genotyping primers Kctdl3 forward primer 1 (SEQ ID NO: 4) and Kctdl3 reverse primer 2 (SEQ ID No: 6) were used.

Table 3: Primers for identification of edited products

<table>
<thead>
<tr>
<th>SEQ ID NO</th>
<th>Description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Kctdl3 forward primer 1</td>
<td>cgagcgagtg ctggagaggtg g</td>
</tr>
<tr>
<td>5</td>
<td>Kctdl3 reverse primer 1</td>
<td>AAAAAAGCA CCGACTCGGT TCCACTTTTT CAAGTTGATA Acgccctgacct ttgctatttc tagctcttaa AACagcatttc ggccccgagcc ggccggtTTT CTGTCCTCTCC ACaag</td>
</tr>
</tbody>
</table>
D. Genotyping kctdl3A47 mice

During the course of working with the kctdl3A47 mice, an amplification bias was found that made genotyping heterozygote mice difficult. A primer pair and probe was developed suitable for droplet digital PCR that allowed accurately genotyping all allelic combinations of the kctdl3A47 allele. The genotyping primers were SEQ ID NOs: 14-16.

Table 4: kctdl3A47 genotyping probe set

<table>
<thead>
<tr>
<th>SEQ ID NO</th>
<th>Description</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>14</td>
<td>Kctd13 F 47_64nt</td>
<td>tccgtcactgccaagtgc</td>
</tr>
<tr>
<td>15</td>
<td>Kctd13 R 47_64nt</td>
<td>ccacactgagggctagg</td>
</tr>
<tr>
<td>16</td>
<td>Kctd13 probe 47_64nt</td>
<td>/56-FAM/tgaggagga/ZEN/atgctggagtc/31ABkFQ/</td>
</tr>
</tbody>
</table>

To genotype mice, genomic DNA from either ear clip or tail clips was obtained by adding 75uL of 25mM NaOH, 0.2mM EDTA to the tissue and incubating for 30 minutes at 95°C followed by neutralization with 75uL 40mM Tris-HCL pH5. Genotyping was carried out by mixing 1 µL of the genomic DNA mixture with the ddPCR assay and 1X ddPCR mix for Probes (Bio-Rad). Droplets were prepared according to the manufacturer's instructions in a Bio-Rad Droplet generator. Droplets were cycled according to the manufacturer's instructions. Following cycling droplets were counted in an X100 ddPCR instrument (Bio-Rad). Bio-Rad ddPCR Quantasoft software was used to determine the DNA content of each sample/genotype of each mouse.

E. Kctdl3 antibody generation and confirmation of Kctdl3 knockout

A peptide specific antibody was generated against the C-terminal peptide CVRRHITHDERPHGQQIVFKD-OH of KCTD13 (SEQ ID NO: 11). This peptide was injected into two New Zealand white rabbits (Dana-Farber Institute, 21st Century Biochemicals). The resulting serum was affinity purified against the same peptide and eluted into PBS. This polyclonal antibody, Pr2905, was used at a dilution of 1:1000 for western blots and 1:50 for immunoprecipitations.

Protein lysates were generated from mouse neurons prepared from wildtype or Kctdl3A47 mice. C57B6 timed pregnant females were euthanize following IACUC approved
E18 embryos were collected and rapidly decapitated. Embryos were then washed in dissection media (Hibernate-E (Hib-E, Gibco) supplemented with 100U/mL penicillin streptomycin (Pen/Strep, Gibco)). Brains were isolated, cortices were separated from the midbrain and meninges was removed from each cortex. Each cortex was cut into six pieces; five cortices were pooled for dissociation. To dissociate 5 cortices, one Papain kit (Worthington LK3 176) was reconstituted in 5mLs Hib-E and activated at 37°C for 10 minutes. Following activation, DNase I (Sigma) was added to a concentration of 1C^g/mL and filter sterilized using a 0.22µm filter. Cortices were washed twice with 5mLs of Hib-E and 5mLs of activated papain/DNAsel was added to the cortices and incubated for 8 minutes at 37°C. Following digestion, cortices were washed three times with 10mL Hib-E per wash. Cortices were then triturated 10-15 times with a P1000 pipette in 1 mL NBAActive4 supplemented with 10µg/mL DNAsel filter sterilized using a 0.22µm filter. The cell mixture was then allowed to settle for 1 minute at room temperature and the supernatant was transferred to a new tube containing 4mLs of NBAActive4 media and spun at 1K rpm for 5 minutes at room temperature. The supernatant was aspirated and the pellet was gently resuspended in 1mL NBAActive4 media. Cells were counted in a hemocytometer, diluted and plated at 1 million cells/well in a 6 well poly-D-lysine (Corning, Biocoat) or 6 million cells/10cm poly-D-lysine plate (Corning, Biocoat).

To inhibit proteasome function, neurons were treated for 5 hours with 2µM bortezomib in DMSO. To inhibit Cullin function, cells were treated for 5 hours with 2µM MLN4924. Following treatments, cells were collected in ubiquitin lysis buffer (IX CST lysis buffer (Cell Signaling Technology), Phosphatase Inhibitor Cocktail 2 (PIC2; Sigma), Phosphatase Inhibitor Cocktail 3 (PIC3; Sigma), 0.1 mM chloracetimide (Sigma), 10mM NaF (Sigma), 2mM PMSF (Aldrich), Roche protease inhibitor mini (Roche), 50µM PR-619 (Lifesensors), 2 mM 1,10 orthophenanthroline (Sigma)) by scraping. Lysates were sonicated with Diagenode bath sonicator for 5' and spun at 12k for 10 minutes and supernatants were transferred to a new tube. Laemli buffer was added to samples, boiled, run on an Bis-Tris SDS PAGE gel and western transferred to a nitrocellulose membrane.

Antibodies used from western blots and immunoprecipitations were Myc-tag Rabbit mAb (CST 71D10-2278S), KCTD13 Rabbit (Pr2905), HA-tag Rabbit mAb (CST C29F4-3724S), IgG Rabbit polyclonal ChIP Grade 0.2mg/ml (ab27478), V5-tag Rabbit mAb (CST D3H8Q-13202S), Myc-tag Mouse mAb (CST 9B1 1-2276S), KCTD13 (B-12) Mouse monoclonal IgG 200ug/ml (sc-393994), KCTD13 Rabbit polyclonal Ab (21st Century Biochemicals, Pr2905), HA-tag Mouse mAb (CST 6E2-2376S), Anti-V5 Antibody Mouse monoclonal 50uL at 1.Olmg/mL (Invitrogen P/N 46-0705), and B-actin Rabbit mAb (HRP Conjugate) (CST D6A8-12620S).
Protein lysates from DIV21 cultures were run on western blots and probed with the Pr2905 antibody or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control antibodies. Figure 1 shows knockdown of KCTD13 levels in *Kctdl3A47* mice compared to wildtype (WT), with no change in GAPDH levels.

**Example 2: Proteomic and mass spectrometry analysis of *Kctdl3A47* mice**

Experiments were designed to quantitatively compare ubiquitylomes and proteomes between wildtype and *Kctdl3A47* mice. An overview of the stable isotope labeling using amino acids in cell culture (SILAC) labeling and trypsin digestion procedures is presented in Figure 2.

**A. SILAC labeling of mouse primary neurons**

Forebrains (cortex, hippocampus and striatum) were dissected from either wildtype C57B6 or *Kctdl3A47* C57B6. E18 timed pregnant females were euthanized using IACUC approved methods. Neurons were dissected and dissociated using papain as described above. Neurons were plated on 10cm poly-D-lysine coated plates at a density of 6 million cells per plate in either heavy or light SILAC media. Neurons were fed every other day for 21 days in vitro. This feeding schedule resulted in greater than 95% labeling of cells. Neurons were then scraped into ubiquitin lysis buffer. Plates of a common genotype and metabolic labeling state were pooled for further processing. SILAC labels were flipped for each genotype. K-ε-GG and total proteome profiling were later performed for both replicates.

**B. Cell lysis and trypsin digestion for K-ε-GG and proteome profiling**

SILAC-labeled neurons were lysed on plates by washing once with 1OmLs ice cold PBS and then scraping into 350µL of ice cold urea lysis buffer (8 M urea, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 µg/ml aprotinin (Sigma-Aldrich), 10 µg/ml leupeptin (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 µM PR-619, and 1 mM chloroacetamide). Plates of a common genotype and label were pooled for processing. Following lysis, samples were centrifuged at 20,000 x g for 15 minutes at 4°C to remove insoluble material. Protein concentrations were determined using a bicinecinonic acid (BCA) protein assay (Pierce) and samples were mixed equitably per SILAC state. Proteins were reduced with 5 mM dithiothreitol for 45 minutes at room temperature (RT) and subsequently carbamidomethylated with 10 mM iodoacetamide for 30 min at RT in the dark. Samples were diluted to 2 M urea with 50 mM Tris-HCl, pH 7.5, and digested with sequencing grade trypsin (Promega) at 25°C overnight using an enzyme to substrate ratio of 1:50. Digested samples were acidified to 1% formic acid (FA) (Sigma-Aldrich). Tryptic peptides were desalted on 500-mg tC18 Sep-Pak SPE cartridges (Waters). Cartridges were conditioned with 5 ml of 100% acetonitrile (MeCN), 5 ml of 50%MeCN/0.1%FA, and four times with 5 ml of 0.1% formic acid.
trifluoroacetic acid (TFA). Up to 15 mg of sample was loaded onto a single cartridge, and subsequently washed 3X with 5 ml of 0.1% TFA. Samples were eluted from cartridges by washing 2X with 3 ml of 50%MeCN/0.1%FA. Desalted samples were dried overnight in a Savant SC210A SpeedVac concentrator (Thermo Scientific).

C. **Basic pH reverse phase (bRP) fractionation**

Offline bRP fractionation was completed using a custom-manufactured Zorbax 300 Extend-C18 column (9.4 x 250 mm, 300 Å, 5 μm, Agilent) on an Agilent 1100 series HPLC system. Approximately 15 mg of peptide sample was resuspended in 1.8 ml of basic RP solvent A (2% MeCN, 5 mM ammonium formate, pH 10), separated into 2 FIPLC vials and injected with Solvent A at flow rate of 3 ml/min. A 64-min method was used for fractionation. The gradient was composed of an initial increase to 8% Solvent B (1.1% B/min) (90% MeCN, 5 mM ammonium formate), followed by a 38-minute linear phase (0.5% B/min) where the amount of solvent B was increased from 8% to 27% and ramp phases where the Solvent B amount was increased from 31% (1% B/min) to 39% (0.5% B/min), and finally to 60% (3% B/min). A total of 96 2ml fractions were collected every 0.66 min at a flow rate of 3 ml/min. For the proteome profiling, 5% of each fraction was pooled into 22 fractions. For ubiquitination profiling, 95% of each fraction was pooled into 8 fractions using a concatenated pooling strategy. Pooled samples were dried using a SpeedVac concentrator.

D. **K-ε-GG enrichment**

Samples were then enriched for the ubiquitin remnant motif (K-ε-GG). The anti-K-ε-GG antibody was obtained from the PTMScan® ubiquitin remnant motif (K-ε-GG) kit (Cell Signaling Technology). Prior to enrichment, the antibody was covalently coupled to Protein A agarose beads by chemical cross-linking with DMP. For cross-linking, the antibody bound beads were first washed 3x with 1 ml of 100 mM sodium borate, pH 9 and then incubated in 1 ml of 20 mM dimethyl pimelimidate (DMP) for 30 minutes with rotation at RT. The reaction was stopped by washing beads 2X with 1 ml of 200 mM ethanolamine, pH 8 followed by incubation for 2 hours at 4°C with rotation. Antibody-bound beads were washed three times in 1.5 ml of ice cold immunoprecipitation (IAP) buffer (50 mM MOPS, pH 7.2, 10 mM sodium phosphate, 50 mM NaCl), resuspended in IAP buffer, and stored at 4°C. For K-ε-GG enrichment, bRP fractions were reconstituted in 1.5 ml of IAP buffer and each fraction was incubated with 32 μg of cross-linked anti-K-ε-GG antibody for 1 hour, at 4°C, while rotating. Following incubation, samples were spun down at 2000 x g and the supernatant was removed. Antibody-bound beads were washed 4X with 1.5 ml of ice cold PBS and peptides were then eluted from the beads with 2 x 50 μl of 0.15% TFA. Eluted peptides were desalted using C18 StageTips. Each 8 StageTip was packed with two plugs of C18 material (EmporeTM C18 Extraction Disk; 3M) and then
conditioned with 100 μl of MeOH, 100 μl of 50% MeCN/0.1% FA, and 2x with 100 μl of 0.1% FA. K-ε-GG peptides were loaded onto the conditioned StageTips, washed 2x with 100 μl of 0.1% FA, eluted with 50 μl of 50% MeCN/0.1% FA, and dried to completeness.

E. LC-MS/MS analysis

K-ε-GG and global proteome fractions were reconstituted in 8 μl and 20 μl of 3% MeCN/1% FA, respectively, and analyzed by nanoflow-UPLC-HCD-MS/MS using a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled on-line to a Proxeon Easy-nLC 1000 system. 4 μl and 1 μl of K-ε-GG and global proteome samples was injected, respectively, for each analysis. Samples were injected onto a microcapillary column (360 μm OD x 75 μm ID) packed with 24 cm of ReproSil-Pul C18-AQ 1.9 μm beads (Dr. Maisch GmbH) that was equipped with an integrated electrospray emitter tip (10 μm). For online analyses, the column was heated to 50°C using a 20 cm column heater (Phoenix S&T). For LC separation, solvent A was 0.1% FA/3% MeCN and solvent B was 90% MeCN/0.1% FA. Peptides were eluted into the mass spectrometer at a flow rate of 200 nL/min using a gradient consisting of a linear phase at 0.3% B/min, followed by a ramp to 60% B (10% B/min). The total analysis time for each sample was 150 minutes. The Q Exactive instrument was operated in the data-dependent mode acquiring HCD MS/MS scans (R=1 7,500) after each MSI scan (R=70,000) on the 12 top most abundant ions using an MSI ion target of 3x 106 ions and an MS2 target of 5x104 ions. The maximum ion time utilized for the MS/MS scans was 120 ms; the HCD-normalized collision energy was set to 25; the dynamic exclusion time was set to 20s, and the peptide match and isotope exclusion functions were enabled.

F. K-ε-GG and proteome MS data analysis

MS data was analyzed with the MaxQuant software version 1.3.0.5 and searched against the mouse Uniprot database that contained 248 common laboratory contaminants was provided by the MaxQuant software package. The search parameters were as follows: enzyme specificity was set to trypsin, maximum number of mixed cleavages set to 2, precursor mass tolerance was at 20 ppm for the first search, and set to 6 ppm for the main search. Oxidized methionines and N-terminal protein acetylation were searched as variable modifications, with carbamidomethylation of cysteines searched as a fixed modification. For searching K-ε-GG data files, Gly-Gly addition to lysines was also searched as a variable modification. The minimum peptide length was set to 6, and false discovery rate for peptide, protein, and site identification was set to 1%. The filter labeled amino acids and peptide quantification functions were enabled. For proteome data, proteins were considered in the dataset if they were identified by 2 or more razor/unique peptides and quantified by 3 or more ratio counts in both biological replicates. For
the K-ε-GG data, K-ε-GG sites were considered if they were confidently localized (>0.75) and quantified in both biological replicates.

G. Identification of adenylosuccinate synthetase (ADSS) as a target regulated by Kctd13

Results on proteome and ubiquitination analysis in Kctd13A47 mice versus wildtype controls are shown in Figures 3A and 3B. For both ubiquitination analysis (Figure 3A) and proteome analysis (Figure 3B), adenylosuccinate synthase (ADSS) was identified as significantly regulated in Kctd13A47 mice versus wildtype controls. Thus, ADSS may be a ubiquitin ligase and proteasome substrate. Figure 4 highlights the role of ADSS in catalyzing the first committed step in the de novo synthesis of adenosine monophosphate (AMP) from inosine monophosphate (FMP).

Western blot results in Figures 5A (immunoblot) and 5B (quantification) show that ADSS protein levels are significantly increased by approximately 3.4-fold in neurons from Kctd13A47 mice versus wildtype controls. As a control, neurons were contacted with DMSO (D), bortezomib (B), or MLN4924 (M). Bortezomib is a proteasome blocker and MLN4924 is a general CULLIN inhibitor, which blocks the neddylation of the CULLIN subunit required for the ubiquitination of substrate. Bortezomib and MLN4924 are predicted to cause an increase in proteasome substrates. In neurons from Kctd13A47 mice, there is no change in ADSS levels after treatment with bortezomib (B) or MLN4924 (M) because in the absence of Kctd13, ADSS is not being ubiquitinated.

Example 3: Evaluation of ADSS ubiquitination in HEK cells

A method was developed to study ADSS ubiquitylation in HEK cells. The following plasmids were used for experiments: pCMV6 hADSS-myc-flag (Origene: RC204256), V82 hKCTD13_IRESPuro, V17 hCUL3-HA-Flag_IRESPuro, V20 hrBXI_IRESPuro, His-ubiquitin (WB Kailin, Dana-Farber Institute, Harvard Medical School).

A day before transfection (day -1) HEK 293T (HEK) cells were plated in 10cm dishes at 3.8-4 million cells per dish. The next day (day 0), the plasmids containing His-Ub (Gift of Dr. WKGaelin, Harvard Medical School), KCTD13, CUL3 and ADSS-myc-flag constructs were transfected into HEK cells. Two days following transfection, bortezomib was added to the transfected cells to a final concentration of 2 µM. Five hours later cells were harvested into PBS by scraping. 1 mL of the cell suspension was transferred to a tube for use as transfection controls and spun at 2500 rpm at 4°C. The remaining 9 mis were transferred to a 15 mL Falcon tube and spun at 1K for 5 minutes at 4°C. The supernatant was removed. The cell pellets were then frozen in liquid N₂. Samples were stored at -80°C until ready to proceed with pulldown. Cell pellets were resuspended in 1 mL of chilled (4°C) Buffer C (6M Gnd-HCl, 0.1M...
NaP04, 10 mM Imidazole) then sonicated (Branson sonicator, microtip, power 30%, cycle 50, 10 to 15 pulses). Buffer C equilibrated Ni-NTA magnetic beads (Invitrogen) were added to the sonicated lysate. Beads were incubated 2 hours at 4°C with rocking to collect His-Ub conjugated proteins. Beads were then washed twice with Buffer C supplemented with 2mM PMSF, 1X Roche Protease inhibitors, twice with Buffer D (1:3 volume ratio Buffer C:Buffer E) /2mM PMSF/Protease inhibitors (Roche), and once with Buffer E (25 mM Tris. CL, pH 6.8/20 mM imidazole/ protease inhibitor (Roche) /2 mM PMSF). Bound proteins were then eluted by boiling in 300mM imidazole, 2x Laemli PAGE buffer, 500 mM β-mercaptoethanol. Eluted samples were loaded on a 4-12% Bolt Bis-Tris PAGE gel (Invitrogen) and run in MOPS buffer. A western transfer was performed and probed with the appropriate primary and secondary antibodies.

For transfection controls, cell pellets were resuspended in 100 µl of CST lysis buffer or RIPA buffer +PMSF+ 1X protease inhibitor tablet (Roche) and sonicated with a Diagenode water bath sonicator for 10 minutes. Samples were spun 10 min, 12K rpm and supernatant was transferred to a new tube. An equal volume of 4X gel loading dye +BME was added and the samples were boiled for 10 min. Samples were western blotted with the appropriate primary and secondary antibodies.

Figure 6A presents the experimental protocol to study the effect of KCTD13 on ubiquitination of ADSS in the HEK model with exogenous expression of HisUb and RBX1/CUL3. Results in Figure 6B show that ubiquitination of ADSS was seen when KCTD13, HisUb, and RBX/CUL3 were cotransfected with ADSS.

Figure 7A presents the experimental protocol to study the effect of KCTD13 on ubiquitination of ADSS in the HEK model with exogenous expression of His-Ub. Results in Figure 7B show that ubiquitination of ADSS was seen when KCTD13 and HisUb were cotransfected with ADSS without exogenous RBX/CUL3. Ubiquitination of ADSS was not seen in the absence of coexpression of KCTD13.

Figures 8A and 8B show the concentration-dependent effect of KCTD13 transfection on ubiquitination of ADSS. Ubiquitination of ADSS increased as the concentration of KCTD13 transfected was increased from 0 to 1 µg in the transfection.

Figures 9A shows the experimental design to study the effect of a dominant-negative CUL3 (DNCUL3) on ADSS ubiquitination by KCTD13 in HEK cells. This DNCUL3 construct would be expected to block any ubiquitin-mediated effects of a cul3-dependent ubiquitin ligase, as shown in Figure 9B. As shown in Figure 9C, expression of the DNCUL3 construct blocked the effect of KCTD13 to increase ubiquitination of ADSS.
Example 4: Co-immunoprecipitation experiments in HEK 293 cells

Experiments were performed in HEK 293 cells to study interactions between KCTD13 and ADSS in a heterologous system.

The co-immunoprecipitation protocol was as follows. For transfections, 3.8 million cells were plated in a 10cm tissue culture dish 24 hours in advance of transfection. Cells were transfected according to manufacturer’s instructions using Lipofectamine 2000 in 6 well plates. Cells were collected 72 hours post-transfection. Media was aspirated, washed 1X with chilled PBS, collected in 10 mL chilled PBS, centrifuged (1000 RPM, 5 min, 4°C). Supernatant was aspirated and pellets were snap frozen in LN2. Pellets were stored at -80°C until processed. Cell pellets were lysed with 1mL Ubiquitin lysis buffer (IX CST lysis buffer (Cell Signaling Technology), PIC2 (Sigma), PIC3 (Sigma), 0.1 mM chloracetimide (Sigma), 10mM NaF (Sigma), 2mM PMSF (Aldrich), Roche protease inhibitor mini (Roche), 50uM PR-619 (Lifesensors), 2 mM 1,10 orthophenanthroline (Sigma). Prior to collecting antibody protein complexes, protein A magnetic Dynabeads (Thermo-Fisher/Invitrogen) were blocked in 5% BSA for 1 hr with rocking at 4°C. Dynabeads were then loaded with 0.5 µg CHIP grade IgG (Abeam) with rocking at 4°C for 1 hr and then 50µL of beads were added to the protein lysates to preclear of any nonspecific IgG-Dynabead binding proteins. Whole cell lysates were prepared from frozen pellets by sonicating pellets resuspended in 1mL ubiquitin lysis buffer in a Diagenode water bath sonicator on HIGH for 5 minutes at 4°C. Lysates were spun at 14K RPM for 10 min at 4°C. Supernatant were transferred to a new tube and 50µL of a 50% slurry of pre-loaded, pre-blocked beads were added to the lysate for 1 hour at 4°C with rocking. Beads were collected using a magnet and supernatant was transferred to a clean tube. Lysate was divided among 8 aliquots (125µL) and primary antibody against myc epitope (CST, clone) (rb) or KCTD13 or rabbit IgG was added at a 1:50 (i.e., 2.5 µL) dilution. Lysates were rocked at 4°C for 1 hour. While antibody-lysate mixtures were rocking, a fresh 50% slurry of protein A magnetic Dynabeads was prepared in ubiquitin lysis buffer (50% bead slurry). Beads were washed and resuspended as described previously. At the end of 1 hour 50 µL of bead slurry was added to each protein antibody mixture to collect antibody protein immunocomplexes. Following collection of immunocomplexes, beads were washed 5 times with 0.5 mL RIPA wash Buffer (10mM Tris-Cl, pH 8.0, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140mM NaCl) supplemented with 2mM PMSF and IX Roche protease inhibitor cocktail (Roche). After each addition of RIPA wash buffer, beads were collected using a magnet. After the final wash step buffer was completely aspirated to ensure all wash buffer was completely removed. Proteins were eluted from the beads by resuspending in 75uL of 2X Laemli buffer (BioRad) containing 500 mM mM β-mercaptoethanol. Samples were then heated.
at 95°C for 6 minutes to denature proteins and either western blots were run as described below or samples were stored at -80°C. To measure the input levels of transfected proteins in whole cell lysates, 50 μL of starting lysate was mixed with 48 μL 4X Laemli buffer plus 2 μL BME and heated at 95°C for 6 minutes. When necessary, lysates were further diluted with 2X Laemli buffer. Western transfers and immunoblotting were carried out using the Thermo-Fisher/Invitrogen Bolt system. Briefly, 5 μL of each eluted IP sample was loaded on an 8% Bolt Bis-Tris-Plus Gel (15-well) and run in Bolt IX MOPS Running buffer (BOLT). Gels were run at 165V for 32 minutes and transferred using an iBLot2 to nitrocellulose for 7 minutes.

Following western transfer, membranes were blocked in 5% BSA in IX Tris Buffered Saline plus Tween (TBS-T, Sigma) for 1 hour at RT on a rocking platform. Following blocking, primary antibody (Myc or Kctdl3) was added at 1:1000 and incubated on a rocking platform at 4°C overnight. Subsequently, membranes were washed with 3 brief washes to remove the antibody mixture, followed by three washes with IX TBS-T for 5 min each. Secondary antibody consisting of Donkey and rabbit conjugated to horseradish peroxidase (HRP, GE Healthcare, NA934V) diluted 1:5000 in 5% blotting milk (Bio-Rad) in TBS-T was then added to membranes and incubated at RT for 1 hour on a rocking platform. Membranes were washed with 3 brief washes to remove the antibody mixture, followed by three washes with IX TBS-T for 5 min each. Just prior to imaging, the membranes were rinsed once with Tris-Buffered Saline without Tween. Membranes were imaged by incubating each membrane in Femto ECL (Pierce) for 1 min and then imaging on a Bio-Rad Chemidoc. For loading controls for input levels of proteins, membranes were incubated with an antibody to B-actin coupled to HRP (Cell Signaling Technology) diluted in 5% BSA in TBS-T for total inputs for 1 hour followed by washing as described for other primary and secondary antibodies.

Figure 10A shows the experimental conditions for the immunoprecipitation experiment. Figure 10B shows that immunoprecipitation (IP) with antibody against KCTD13 (K lanes) led to immunoprecipitation of ADSS, as signal was seen with immunoblotting (IB) for the myc antibody, and CUL3, as signal was seen with IB for the HA antibody. The input lanes (i) show lysate sample that was not subjected to immunoprecipitation.

KCTD13 is an adapter protein that binds to CUL3. It was next investigated whether other adaptor protein, such as KCTD12 or TNFAIP1, could also promote ubiquitination of ADSS. Figure 11A shows the experimental outline for the experiment, with the transfection of the adaptor proteins KCTD13, KCTD12, or TNFAIP1 in parallel experiments. Figure 11B shows that only KCTD13, and not KCTD12 or TNFAIP1, expression increased the ubiquitination of ADSS. These experiments confirm that not all adaptor proteins are equivalent and that KCTD13 has a unique role in regulating ubiquitination of ADSS.
Example 5. Metabolomic profiling of fibroblasts and neurons

[00216] 16pl 1.2 deletion patient fibroblast samples were acquired from the Simons Foundation VIP collection. For comparison, fibroblasts from unaffected individuals were obtained from Mclean Hospital collection of primary fibroblasts (Cohen, Ongur, McPhee). Fibroblasts were grown in DMEM supplemented with 10% serum (Gibco). For metabolic experiments, 160,000 cells/well were plated in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) in 12 well plates. For neurons, 1 million primary mouse neurons/well were plated in 6 well plates and were extracted similar to fibroblasts as described below except volumes were doubled to accommodate the extra surface area.

[00217] It was first determined whether KCTD13 mRNA levels were altered in 16pl 1.2 deletion patient fibroblasts compared with control unaffected (U) fibroblasts. Figure 12 shows that KCTD13 mRNA levels were significantly lower in 16pl 1.2 deletion patient fibroblasts, confirming an alteration in expression of KCTD13 mRNA with this deletion.

[00218] Next, metabolic changes were determined between 16pl 1.2 deletion patient fibroblasts compared with control fibroblasts. For metabolite extraction, samples were collected from either cells or the cell media. Cell samples were extracted from cells grown in 12 well plates. Cell samples were extracted by washing cells once with 1mL ice-cold PBS, transferring to dry ice, followed by adding 400µL of 80% methanol (VWR) containing the internal standards inosine-^15N4, thymine-d4, and glycocholate-d4 (Cambridge Isotope Laboratories). Cells were then incubated for 15 minutes at -80°C, followed by scraping and transfer of the methanol and cells to a 1.5mL tube. Cells were centrifuged (10 min, 9,000g, 4°C), and the supernatant was transferred to a new 1.5mL tube. The pellet was extracted again with 50 µL of 80% methanol containing internal standards and centrifuged. The supernatant was then pooled with the previously collected methanol sample. Media supernatant samples (30 µL) were extracted using 120 µL of 80% methanol (VWR) containing the internal standards inosine-^15N4, thymine-d4, and glycocholate-d4 (Cambridge Isotope Laboratories). The samples were centrifuged (10 min, 9,000g, 4°C). The resulting supernatant was transferred to a new tube. Samples were stored at -80°C until analysis.

[00219] A method using basic hydrophilic interaction chromatography (HILIC) separation and negative ionization mode MS detection was established on an LC-MS system consisting of an ACQUITY UPLC (Waters Inc.) coupled to a 5500 QTRAP triple quadrupole mass spectrometer (AB SCIEX). Supernatants were injected directly onto a Luna NH2 column (150 x 2.0 mm, 5 µm particle size; Phenomenex) that was eluted at a flow rate of 400 µL/min with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide (Sigma-Aldrich) in water (VWR)) and 90% mobile phase B (10 mM
ammonium hydroxide in 75:25 v/v acetonitrile/methanol (VWR)) followed by a 10-min linear gradient to 100% mobile phase A. The ion spray voltage was -4.5 kV and the source temperature was 500°C.

[00220] Raw data were processed using MultiQuant 1.2 software (AB SCIEX) for automated LC-MS peak integration. All chromatographic peaks were manually reviewed for quality of integration and compared against a known standard for each metabolite to confirm compound identities. Internal standard peak areas were monitored for quality control, to assess system performance over time, and to identify any outlier samples requiring re-analysis. A pooled plasma reference sample was also analyzed after sets of 20 study samples as an additional quality control measure of analytical performance and to serve as reference for scaling raw LC-MS peak areas across sample batches. Metabolites with a signal-to-noise ratio <10 were considered unquantifiable.

[00221] Purine metabolites were not altered in fibroblast cell lysates (Figure 13) or media (Figure 14) from 16pl 1.2 deletion patients compared to control patients.

[00222] Purine metabolites were also compared in urine from adult WT and kctdl3A47 mice. The kctdl3A47 mice did not have alterations in purine metabolites in urine compared to WT mice (Figure 15). Blank graphs in Figure 15 were not detectable.

[00223] Next, purine metabolites were assessed in cell lysates and supernatants of cultured neurons from wild-type and kctdl3A47 mice. Neurons were cultured until division 21, at which point lysates and supernatants were prepared using the same protocol as for the fibroblast samples.

[00224] Purine metabolites downstream of ADSS (including s-Ado, AMP, ADP, and ATP) were present at higher levels in cell lysates of cultured neurons from kctdl3A47 mice compared to WT (Figure 16). See also Figure 21. Similarly, levels of S-Ado and AMP were increased in neuronal media from kctdl3A47 mice compared to WT (Figure 17). See also Figure 21. Figure 18A-D shows the levels of various purine metabolites in cell lysates and media of WT and kctdl3A47 (KO) mice. Levels of adenylsuccinate, guanine, and SAICAR were not detectable.

[00225] Without intending to be bound by any particular theory, these data suggest that an increase in ADSS activity in kctdl3A47 mice leads to changes in purine metabolism in neurons, with higher levels of metabolites downstream of ADSS. Since changes in purine metabolites were seen in neurons from kctdl3A47 mice but not in fibroblasts from 16pl 1.2 deletion patients, these results may indicate that neurons are particularly impacted by changes in ADSS function.

[00226] AMP is known to be able to signal through AMP-kinase to regulate numerous intracellular processes, including energy homeostasis. Thus, ADSS may function to influence
energy homeostasis by increasing AMP [see Stenesen D, et al., Cell Metab 17(1): 101-12 (2013) and Jacquemont S et al., Nature 478(7367):97-102 (2011)], which is a positive regulator of AMP-kinase. Thus, inhibition of AMP kinase activity may be another means to mitigate dysfunction of purine metabolism in 16p1.2 deletion patients.

**Example 6: Alterations in purine metabolism with ADSS inhibition**

[00227] L-alanosine has been characterized as an active anabolite and inhibitor of ADSS (see Tyagi AK, et al., Cancer Res 40(12):4390-7 (1980)). Thus, L-alanosine may be used as an ADSS inhibitor in metabolic profiling experiments.

[00228] Human control fibroblasts in culture were treated for 12 hours with HEPES, 10 mg/mL L-alanosine (LA), or were left untreated. As shown in Figure 19, treatment with LA increased levels of IMP and decreased levels of AMP and ADP, showing the ability of LA to functionally decrease ADSS activity and alter purine metabolism. Levels of aminomimidazole carboxamide ribotide (AICAR), a metabolite upstream of ADSS, were not affected by LA treatment.

[00229] A dose-response was performed with D,L-alanosine and L-alanosine in DIV21 mouse neurons. As shown in Figure 20, both the D,L and the L forms of alanosine produced a dose-dependent increase in IMP levels. Thus, the D,L and L forms of alanosine can functionally inhibit ADSS and may be useful for regulating purine metabolism.

[00230] In addition, S-Ado levels decreased in cultured neurons from kctdl3A47 mice contacted with increasing concentrations of L-alanosine (Figure 22).

[00231] It can also be determined whether exogenous expression of KCTD13 by viral transduction can rescue purine levels in 16p1.2 deletion patient fibroblasts to levels more similar to control fibroblasts. Transduction of a virus encoding KCTD13 is compared to a vector virus for the ability to rescue the purine phenotype of 16p1.2 deletion patient fibroblasts and make the profile more similar to that of wildtype fibroblasts, these changes include a reduction in AMP levels, S-AMP levels and S-Ado levels. In addition, changes in the metabolic profile of kctdl3A47 neurons is evaluated following transduction with a virus encoding KCTD13 compared to a vector virus. These data confirm the specific role of KCTD13 in regulating purine levels.

[00232] The foregoing written specification should enable one skilled in the art to practice embodiments within the scope of the appended claims. The foregoing description and Examples detail certain embodiments and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the embodiment may be practiced in many ways and should be construed in accordance with the appended claims and any equivalents thereof.
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What is Claimed is:

1. A method of treating an autism spectrum disorder comprising administering to a subject in need thereof an adenylosuccinate (S-AMP) modulator.

2. The method of claim 1, wherein the S-AMP modulator is an adenylosuccinate synthetase (ADSS) inhibitor.

3. The method of claim 2, wherein the ADSS inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.

4. The method of claim 3, wherein the ADSS inhibitor is a small molecule.

5. The method of claim 4, wherein the ADSS inhibitor is selected from L-alanosine, D,L-alanosine, hydantocidin, hydantocidin phosphate, hydantocidin-hadacidin S hybrid inhibitor, hydantocidin-hadacidin R hybrid inhibitor, AdSS-1, AdSS-2, GE-101, GE-109, and hadacidin.

6. The method of claim 4, wherein the ADSS inhibitor is a compound having structure A:

\[
\begin{align*}
R_1 & \quad \text{independently selected from the group consisting of } -H, \text{ a halogen, } -\text{NH}_2, -\text{OH}, -\text{NH}-R_3 \text{, and } -O-R_3; \\
G_1, G_2, \\ & \quad \text{independently selected from the group consisting of } CH, N, O, \text{ and } S; \\
G_3 & \quad \text{independently selected from the group consisting of } -H, \text{ an } Ci-Cis\text{ alkyl, an aryl}, -C(0)-H, \text{ and } -C(0)-alkyl; \\
R_4 & \quad \text{independently selected from the group consisting of } -H, -C(0)0-, \text{ and } -C(0)-R_3; \\
G_5 & \quad \text{independently selected from the group consisting of } CH2, NH, O, C=O group and S; \\
L & \quad \text{absent or is selected from the group consisting of } O, \text{NH, and S; } \\
R_3 & \quad \text{is selected from a group consisting of } -H, \text{ an Ci-Cis alkyl, an aryl}, -C(0)-H, \text{ and } -C(0)-alkyl; \\
R_5 & \quad \text{is selected from a group consisting of } -H, \text{ an Ci-Cis alkyl, and an aryl; } \\
M & \quad \text{absent or is selected from the group consisting of } -CH2-, -NH-, -NH-C(0)-, -O-, \text{ and } -S-; \\
& \quad \text{and } n \text{ is an integer having the value between } 1 \text{ and } 6. \\
\end{align*}
\]

7. The method of claim 6, wherein Gi, G2, and G4 are N, G3 is NH, and G5 is C.
8. The method of claim 3, wherein the ADSS inhibitor is an antisense oligonucleotide or an siRNA.

9. The method of claim 8, wherein the antisense oligonucleotide is complementary to a portion of the ADSS mRNA.

10. The method of claim 3, wherein the ADSS inhibitor is a peptide.

11. The method of claim 1, wherein the S-AMP modulator is an adenylosuccinate lyase activator.

12. The method of claim 11, wherein the adenylosuccinate lyase activator increases the level of adenylosuccinate lyase and/or increases the activity of adenylosuccinate lyase.

13. The method of claim 12, wherein the method comprises administering a nucleic acid that encodes adenylosuccinate lyase.


15. The method of claim 14, wherein the S-Ado reducing agent is an antibody that binds S-Ado or an abzyme.

16. The method of claim 15, wherein the antibody is an antibody fragment.

17. The method of claim 16, wherein the antibody fragment is selected from an scFv, Fab, Fab', F(ab')_2 fragment.


19. The method of claim 18, wherein the AMPK modulator is an AMPK inhibitor.

20. The method of claim 19, wherein the AMPK inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.

21. The method of claim 20, wherein the AMPK inhibitor is a small molecule.

22. The method of claim 21, wherein the AMPK inhibitor is dorsomorphin.

23. The method of claim 21, wherein the AMPK inhibitor is an antisense oligonucleotide or an siRNA.

24. The method of claim 23, wherein the antisense oligonucleotide is complementary to a portion of the AMPK mRNA.

25. The method of claim 20, wherein the AMPK inhibitor is a peptide.

26. The method of claim 18, wherein the AMPK modulator is an AMP reducing agent.

27. The method of claim 26, wherein the AMP reducing agent is an antibody that binds AMP or an abzyme.

28. The method of claim 27, wherein the antibody is an antibody fragment.
29. The method of claim 28, wherein the antibody fragment is selected from an scFv, Fab, Fab', F(ab')₂ fragment.
30. The method of any one of the preceding claims, wherein the subject has a 16pl 1.2 deletion.
31. The method of any one of the preceding claims, wherein the subject has a mutation in the KCTD13 gene.
32. The method of claim 31, wherein the mutation in the KCTD13 gene is a loss-of-function mutation.
33. The method of claim 31 or claim 32, wherein the mutation in the KCTD13 gene is a partial or total deletion of the KCTD13 gene, a missense mutation, or a nonsense mutation.
34. The method of any one of the preceding claims, wherein the subject has a mutation in the CUL3 gene.
35. The method of claim 34, wherein the mutation in the CUL3 gene is a loss-of-function mutation.
36. The method of claim 35, wherein the mutation in the CUL3 gene is a partial or total deletion of the CUL3 gene, a missense mutation, or a nonsense mutation.
37. The method of any one of the preceding claims, wherein the subject has an elevated level of S-Ado.
38. The method of claim 37, wherein the elevated level of S-Ado is determined in a blood, urine, or CSF sample from the subject.
39. The method of any one of the preceding claims, wherein treating an autism spectrum disorder comprises alleviating at least one symptom of the autism spectrum disorder.
40. The method of claim 39, wherein alleviating at least one symptom comprises reducing the number, severity, and/or frequency of seizures; preventing and/or slowing developmental delay; improving and/or slowing the decline in intellectual ability; reducing the incidence of obesity; reducing social interaction deficit; improving language; reducing repetitive behaviors; reducing sleep disorders; reducing mood disorders; reducing anxiety; reducing gastrointestinal symptoms; reducing hyperactivity; and/or reducing attention deficits.
41. A method of identifying a subject who would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator comprising determining the level of S-Ado in a sample from the subject, wherein an elevated level of S-Ado in the sample indicates the subject would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator.
42. The method of claim 41, wherein the level of S-Ado in the sample is compared to a reference level of S-Ado.
43. The method of claim 41 or claim 42, wherein the method further comprises determining whether the subject has a 16pl 1.2 deletion, wherein a 16pl 1.2 deletion indicates the subject would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator.

44. The method of any one of claims 41 to 43, wherein the method further comprises determining whether the subject has a mutation in the KCTD13 gene, wherein a mutation in the KCTD13 gene indicates the subject would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator.

45. The method of claim 44, wherein the mutation in the KCTD13 gene is a loss-of-function mutation.

46. The method of claim 44 or claim 45, wherein the mutation in the KCTD13 gene is a partial or total deletion of the KCTD13 gene.

47. The method of any one of claims 41 to 46, wherein the method further comprises determining whether the subject has a mutation in the CUL3 gene, wherein a mutation in the CUL3 gene indicates the subject would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator.

48. The method of claim 47, wherein the mutation in the CUL3 gene is a loss-of-function mutation.

49. The method of claim 47 or claim 48, wherein the mutation in the CUL3 gene is a partial or total deletion of the CUL3 gene.

50. A method of identifying a subject who would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator, comprising determining whether the subject has a 16pl 1.2 deletion, wherein a 16pl 1.2 deletion indicates the subject would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator.

51. A method of identifying a subject who would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator, comprising determining whether the subject has a mutation in the KCTD13 gene, wherein a mutation in the KCTD13 gene indicates the subject would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator.

52. The method of claim 51, wherein the mutation in the KCTD13 gene is a loss-of-function mutation.

53. The method of claim 51 or claim 52, wherein the mutation in the KCTD13 gene is a partial or total deletion of the KCTD13 gene.

54. A method of identifying a subject who would benefit from treatment with an ADSS inhibitor, comprising determining whether the subject has a mutation in the CUL3 gene,
wherein a mutation in the CUL3 gene indicates the subject would benefit from treatment with an ADSS inhibitor.

55. The method of claim 54, wherein the mutation in the CUL3 gene is a loss-of-function mutation.

56. The method of claim 54 or claim 55, wherein the mutation in the CUL3 gene is a partial or total deletion of the CUL3 gene.

57. The method of any one of claims 50 to 56, wherein the method further comprises determining the level of S-Ado in a sample from the subject, wherein an elevated level of S-Ado in the sample indicates the subject would benefit from treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator.

58. The method of any one of claims 41 to 57, wherein the subject exhibits at least one symptom of an autism spectrum disorder.

59. The method of claim 58, wherein alleviating at least one symptom comprises reducing the number, severity, and/or frequency of seizures; preventing and/or slowing developmental delay; improving and/or slowing the decline in intellectual ability; reducing the incidence of obesity; reducing social interaction deficit; improving language; reducing repetitive behaviors; reducing sleep disorders; reducing mood disorders; reducing anxiety; reducing gastrointestinal symptoms; reducing hyperactivity; and/or reducing attention deficits.

60. The method of any one of claims 41 to 59, wherein the subject has been previously diagnosed as having an autism spectrum disorder.

61. The method of any one of claims 41 to 60, wherein the subject does not have an adenylosuccinate lyase deficiency.

62. The method of any one of claims 41 to 61, wherein the S-AMP modulator is selected from an ADSS inhibitor, and an adenylosuccinate lyase activator.

63. The method of any one of claims 41 to 62, comprising administering to the subject an S-AMP modulator.

64. The method of claim 63, wherein the S-AMP modulator is an ADSS inhibitor.

65. The method of claim 64, wherein the ADSS inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.

66. The method of claim 65, wherein the ADSS inhibitor is a small molecule.

67. The method of claim 66, wherein the ADSS inhibitor is selected from L-alanosine or D,L-alanosine, hydantocidin, hydantocidin phosphate, hydantocidin-hadacidin S hybrid inhibitor, hydantocidin-hadacidin R hybrid inhibitor, AdSS-1, AdSS-2, GE-101, GE-109, and hadacidin.
68. The method of claim 66, wherein the ADSS inhibitor is a compound having structure A:

![Chemical structure](image)

wherein each of Ri and R2 is independently selected from the group consisting of -H, a halogen, -NH2, -OH, -NH-R3, and -O-R3;
each of Gi, G2, and G4 is independently selected from the group consisting of CH, N, O, and S, or G4 is independently selected C=0 group;
G3 is independently selected from the group consisting of CH2, NH, O, C=0 group and S;
G5 is independently selected from the group consisting of C and N;
L is absent or is selected from the group consisting of O, NH, and S;
R3 is selected from a group consisting of -H, an Ci-Cis alkyl, an aryl, -C(0)-H, and -C(0)-alkyl;
R4 is selected from a group consisting of -H, -C(0)0-; and -C(0)-R3;
R5 is selected from a group consisting of -H, an Ci-Cis alkyl, and an aryl;
M is absent or is selected from the group consisting of -CH2--; -NH--; -NH-C(O)--; -O-, and -S--; and
n is an integer having the value between 1 and 6.

69. The method of claim 68, wherein Gi, G2, and G4 are N, G3 is NH, and G5 is C.

70. The method of claim 65, wherein the ADSS inhibitor is an antisense oligonucleotide or an siRNA.

71. The method of claim 70, wherein the antisense oligonucleotide is complementary to a portion of the ADSS mRNA.

72. The method of claim 65, wherein the ADSS inhibitor is a peptide.

73. The method of claim 63, wherein the S-AMP modulator is an adenylosuccinate lyase activator.

74. The method of claim 73, wherein the adenylosuccinate lyase activator increases the level of adenylosuccinate lyase and/or increases the activity of adenylosuccinate lyase.

75. The method of claim 74, wherein the method comprises administering a nucleic acid that encodes adenylosuccinate lyase.
76. The method of any one of claims 41 to 62, comprising administering to the subject a succinyl-adenosine (S-Ado) reducing agent.

77. The method of claim 76, wherein the S-Ado reducing agent is an antibody that binds S-Ado.

78. The method of claim 77, wherein the antibody is an antibody fragment.

79. The method of claim 78, wherein the antibody fragment is selected from an scFv, Fab, Fab', F(ab')₂ fragment.

80. The method of any one of claims 41 to 62, comprising administering to the subject an AMPK modulator.

81. The method of claim 80, wherein the AMPK modulator is an AMPK inhibitor.

82. The method of claim 81, wherein the AMPK inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.

83. The method of claim 82, wherein the AMPK inhibitor is a small molecule.

84. The method of claim 83, wherein the AMPK inhibitor is dorsomorphin.

85. The method of claim 82, wherein the AMPK inhibitor is an antisense oligonucleotide or an siRNA.

86. The method of claim 85, wherein the antisense oligonucleotide is complementary to a portion of the AMPK mRNA.

87. The method of claim 82, wherein the AMPK inhibitor is a peptide.

88. The method of claim 80, wherein the AMPK modulator is an AMP reducing agent.

89. The method of claim 88, wherein the AMP reducing agent is an antibody that binds AMP or an abzyme.

90. The method of claim 89, wherein the antibody is an antibody fragment.

91. The method of claim 90, wherein the antibody fragment is selected from an scFv, Fab, Fab', F(ab')₂ fragment.

92. A method of monitoring treatment of a subject with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator, comprising determining the level of S-Ado in a sample from the subject.

93. The method of claim 92, wherein the level of S-Ado is determined at at least two time points.

94. The method of claim 93, wherein the level of S-Ado is determined in a first sample from the subject and in a second sample from the subject, wherein the second sample from the subject is taken at a later point in time than the first sample from the subject.
95. The method of claim 94, wherein the first sample from the subject is taken prior to treatment with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator and the second sample from the subject is taken after administration of at least one dose of an S-AMP modulator, S-Ado reducing agent, or AMPK modulator.

96. The method of claim 94, wherein the first sample from the subject is taken at a first time point and the second sample from the subject is taken at a second time point, wherein at least one dose of an S-AMP modulator, S-Ado reducing agent, or AMPK modulator is administered between the first time point and the second time point.

97. The method of any one of claims 92 to 96, wherein a decrease in the level of S-Ado in the second sample compared to the first sample indicates the treatment is effective.

98. The method of any one of claims 92 to 97, wherein the method is a method of monitoring treatment of a subject with an S-AMP modulator, S-Ado reducing agent, or AMPK modulator.

99. The method of claim 98, wherein the S-AMP modulator is an ADSS inhibitor.

100. The method of claim 99, wherein the ADSS inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.

101. The method of claim 100, wherein the ADSS inhibitor is a small molecule.

102. The method of claim 101, wherein the ADSS inhibitor is selected from L-alanosine or D,L-alanosine, hydantocidin, hydantocidin phosphate, hydantocidin-hadacidin S hybrid inhibitor, hydantocidin-hadacidin R hybrid inhibitor, AdSS-1, AdSS-2, GE-101, GE-109, and hadacidin.

103. The method of claim 101, wherein the ADSS inhibitor is a compound having structure A:

![Diagram of compound A]

wherein each of R1 and R2 is independently selected from the group consisting of -H, a halogen, -NH2, -OH, -NH-R3, and -O-R3;
each of G1, G2, and G4 is independently selected from the group consisting of CH, N, O, and S, or G4 is independently selected C=O group;
G3 is independently selected from the group consisting of CH2, NH, O, C=0 group and S;
G is independently selected from the group consisting of C and N;
L is absent or is selected from the group consisting of O, NH, and S;
R is selected from a group consisting of -H, an Ci-Ci alkyl, an aryl,-C(0)-H, and -C(0)-alkyl;
R is selected from a group consisting of -H, -C(0)R; and -C(0)-R;
R is selected from a group consisting of -H, an Ci-Ci alkyl, and an aryl;
M is absent or is selected from the group consisting of -CH2; -NH-; -NH-C(O)H; -O-; and -S-; and
n is an integer having the value between 1 and 6.

104. The method of claim 103, wherein Gi, G2, and G4 are N, G3 is NH, and G5 is C.

105. The method of claim 100, wherein the ADSS inhibitor is an antisense oligonucleotide or an siRNA.

106. The method of claim 105, wherein the antisense oligonucleotide is complementary to a portion of the ADSS mRNA.

107. The method of claim 100, wherein the ADSS inhibitor is a peptide.

108. The method of claim 99, wherein the S-AMP modulator is an adenylsuccinate lyase activator.

109. The method of claim 108, wherein the adenylsuccinate lyase activator increases the level of adenylsuccinate lyase and/or increases the activity of adenylsuccinate lyase.

110. The method of claim 109, wherein the method comprises administering a nucleic acid that encodes adenylsuccinate lyase.

111. The method of any one of claims 92 to 98, wherein the method is a method of monitoring treatment of a subject with an S-Ado reducing agent.

112. The method of claim 111, wherein the S-Ado reducing agent is an antibody that binds S-Ado.

113. The method of claim 112, wherein the antibody is an antibody fragment.

114. The method of claim 113, wherein the antibody fragment is selected from an scFv, Fab, Fab’, F(ab’)2 fragment.

115. The method of any one of claims 92 to 98, wherein the method is a method of monitoring treatment of a subject with an AMPK modulator.

116. The method of claim 115, wherein the AMPK modulator is an AMPK inhibitor.

117. The method of claim 116, wherein the AMPK inhibitor is an antisense oligonucleotide, an siRNA, a peptide, or a small molecule.

118. The method of claim 117, wherein the AMPK inhibitor is a small molecule.

119. The method of claim 118, wherein the AMPK inhibitor is dorsomorphin.
120. The method of claim 117, wherein the AMPK inhibitor is an antisense oligonucleotide or an siRNA.

121. The method of claim 120, wherein the antisense oligonucleotide is complementary to a portion of the AMPK mRNA.

122. The method of claim 117, wherein the AMPK inhibitor is a peptide.

123. The method of claim 115, wherein the AMPK modulator is an AMP reducing agent.

124. The method of claim 123, wherein the AMP reducing agent is an antibody that binds AMP or an abzyme.

125. The method of claim 124, wherein the antibody is an antibody fragment.

126. The method of claim 125, wherein the antibody fragment is selected from an scFv, Fab, Fab', F(ab')2 fragment.

127. The method of any one of claims 57 to 126, wherein the sample is selected from a blood sample, a urine sample, and a CSF sample.

128. The method of any one of claims 1 to 91, wherein the method further comprises placing the subject on a low purine diet.

129. A method of treating an autism spectrum disorder in a subject comprising placing the subject on a low purine diet.

130. The method of claim 129, wherein the subject has a 16pl 1.2 deletion.

131. The method of claim 129 or claim 130, wherein the subject has a mutation in the KCTD13 gene.

132. The method of claim 131, wherein the mutation in the KCTD13 gene is a loss-of-function mutation.

133. The method of claim 131 or claim 132, wherein the mutation in the KCTD13 gene is a partial or total deletion of the KCTD13 gene, a missense mutation, or a nonsense mutation.

134. The method of any one of claims 129 to 133, wherein the subject has a mutation in the CUL3 gene.

135. The method of claim 134, wherein the mutation in the CUL3 gene is a loss-of-function mutation.

136. The method of claim 135, wherein the mutation in the CUL3 gene is a partial or total deletion of the CUL3 gene, a missense mutation, or a nonsense mutation.

137. The method of any one of claims 129 to 136, wherein the subject has an elevated level of S-Ado.
138. The method of claim 137, wherein the elevated level of S-Ado is determined in a
blood, urine, or CSF sample from the subject.

139. The method of any one of claims 129 to 138, wherein treating an autism spectrum
disorder comprises alleviating at least one symptom of the autism spectrum disorder.

140. The method of claim 139, wherein alleviating at least one symptom comprises
reducing the number, severity, and/or frequency of seizures; preventing and/or slowing
developmental delay; improving and/or slowing the decline in intellectual ability; reducing the
incidence of obesity; reducing social interaction deficit; improving language; reducing repetitive
behaviors; reducing sleep disorders; reducing mood disorders; reducing anxiety; reducing
gastrointestinal symptoms; reducing hyperactivity; and/or reducing attention deficits.

141. A method of monitoring treatment of a subject having an autism spectrum
disorder with low purine diet, comprising determining the level of S-Ado in a sample from the
subject.

142. The method of claim 141, wherein the level of S-Ado is determined at at least
two time points.

143. The method of claim 142, wherein the level of S-Ado is determined in a first
sample from the subject and in a second sample from the subject, wherein the second sample
from the subject is taken at a later point in time than the first sample from the subject.

144. The method of claim 143, wherein the first sample from the subject is taken prior
to treatment with a low purine diet and the second sample from the subject is taken after
treatment with the low purine diet.

145. The method of claim 144, wherein the second sample from the subject is taken
after at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 6 weeks, at least
2 months, at least 3 months, at least 6 months, or at least 1 year after the start of treatment with
the low purine diet.

146. The method of any one of claims 141 to 145, wherein a decrease in the level of S-
Ado in the second sample compared to the first sample indicates the treatment is effective.

147. The method of any one of claims 141 to 146, wherein the method is a method of
monitoring treatment of a subject with a low purine diet.
Fig. 2
Fig. 4

GDP + Pi

GTP + aspartate

ADSS

adenylosuccinate

adenylosuccinate synthetase

fumarate

NH₃

AMP deaminase

AMP

IMP
Fig. 13

Metabolite Levels

AICAR
IMP
S-Ado

ADP
ATP

S-AMP
AMP
SAICAR

16p11.2 con
15 27 47 528 533 567

16p11.2 con
15 27 47 528 533 567

16p11.2 con
15 27 47 528 533 567
**Fig. 17**

The diagram illustrates metabolic pathways involving AICAR, SAICAR, ADSL, IMP, S-AMP, AMP, ADP, ATP, and S-Ado. The graph shows metabolite levels for WT and KO conditions, with statistical significance indicated by p-values: 0.0007, 0.09, and 0.5.
Fig. 22
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K 31/52 A61P 25/00 A61P 25/28

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEMABS Data, COMPENDEX, EMBASE, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>Y</td>
<td></td>
<td>4-7 8-40, 129-147</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

D. SPECIAL CATEGORIES OF CITED DOCUMENTS:

- **A** - document defining the general state of the art which is not considered to be of particular relevance
- **E** - earlier application or patent but published on or after the international filing date
- **L** - document which may throw doubts on priority claim(s) on which is cited to establish the publication date of another citation or other special reason (as specified)
- **O** - document referring to an oral disclosure, use, exhibition or other means
- **P** - document published prior to the international filing date but later than the priority date claimed
- **T** - later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- **X** - document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- **Y** - document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- **A** - document member of the same patent family

Date of the actual completion of the international search 12 March 2018

Date of mailing of the international search report 15/05/2018

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Authorized officer

Bareyt, Sebastiaan
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<td>A</td>
<td>compounds L-alanosine</td>
<td>8-40, 129-147</td>
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<td>A</td>
<td>paragraph [02.1]</td>
<td>1-3, 8-40, 129-147</td>
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<td>Category</td>
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<td>Relevant to claim No.</td>
</tr>
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# INTERNATIONAL SEARCH REPORT

## Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **☐** Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. **☐** Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. **☐** Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

> see additional sheet

1. **☐** All required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. **☐** As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. **☐** As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. **☐** No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   1–40, 129–147

### Remark on Protest

- **☐** The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- **☐** The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- **☐** No protest accompanied the payment of additional search fees.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-40, 129-147
   related to the treatment of autism spectrum disorder (ASD)

2. claims: 41-128
   related to the identification of subjects, and to the monitoring of treatments