ClinGen Rett/Angelman-like Syndromes Working Group

Specified ACMG/AMP guidelines for interpretation of variants in *CDKL5* (NM_001323289.2)*, FOXG1* (NM_005249.4), *MECP2* (NM_004992.3), *SLC9A6* (NM_006359.2), *TCF4* (NM_001083962.1), *UBE3A* (NM_130838.2)

Scope of work: All disorders associated with pathogenic variants in the specified genes

Criteria will be combined as described in Richards et al, 2015, with one modification: 1 Very Strong and 1 Supporting = Likely Pathogenic

EVIDENCE OF PATHOGENICITY

PVS1: *"Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function is a known mechanism of disease."*

***EP notes*:**

For truncating/frameshift variants:

- *FOXG1:* PVS1 is applicable up to p.S468 which corresponds to the distal most de novo truncating variant in an affected patient reported to date (PMID 30525188). Any truncating/frameshift variant distal of p.S468 should be downgraded to Strong (PVS1_Strong). The distal most de novo non-truncating variant in an affected patient reported to date is at p.Q480 (PMID 29655203). Any truncating/frameshift variant distal of p.Q480 should be downgraded to Moderate (PVS1_Moderate).

- *MECP2*: PVS1 is applicable up to p.E472 which corresponds to the distal most de novo truncating variant in an affected patient reported to date (PMID: 12081720). Any truncating/frameshift variant distal of p.E472 should be downgraded to Moderate (PVS1_Moderate). PVS1 can be applied to any frameshift variant that results in a read-through of the stop codon, as several such read-through variants have been described in individuals with Rett syndrome (PMID 16473305, 20108430).

- *UBE3A*: PVS1 is applicable up to p.K841 which corresponds to the distal most de novo truncating variant in an affected patient reported to date (PMID 9887341). Any truncating variant distal of p.K841 should be downgraded to Strong (PVS1_Strong). The distal most de novo non-truncating variant in an affected patient reported to date is at p.G850 (PMID 27620904). Any truncating variant distal of p.G850 should be downgraded to Moderate (PVS1_Moderate). PVS1 can be applied to any frameshift variant that results in a read-through of the stop codon, as several such read-through variants have been described in individuals with Angelman syndrome (PMID 9887341, 25212744).

- *TCF4*: PVS1 is applicable up to p.E643 which corresponds to the distal most de novo truncating variant in an affected patient reported to date (PMID 29695756). Any truncating variant distal of p.E643 should be downgraded to Moderate (PVS1_Moderate). PVS1 can be applied to any frameshift variant that results in a read-through of the stop codon, as several such read-through

variants have been described in individuals with Pitt-Hopkins syndrome (PMID 22045651, 18728071).

- *SLC9A6:* PVS1 is applicable up to p.A563 which corresponds to the boundary for predicted nonsense mediated decay (NMD). PVS1_Strong can be applied to any truncating variant between p.C564-p.T601 (affects NMD and >10% of protein is lost) and PVS1_Moderate can be applied to any truncating variant between p.Y602-p.A669 (affects NMD and <10% of protein is lost). PVS1_Moderate can be applied to any frameshift variant that results in a read-through of the stop codon.

- *CDKL5*: When using the major brain isoform (NM_001323289.2) PVS1 is applicable up to p.R948 which corresponds to the distal most de novo truncating variant in an affected patient reported to date (ClinVar variation ID: 489299). Any truncating variant distal of p.R948 should be downgraded to Moderate (PVS1_Moderate).

  When using NM_003159.2 (the historically used transcript) do not use for LOF in CDKL5 C-terminus (exons 19-21, or after p.P904) as the major brain isoform has an alternative C-terminus (PMID: 21748340, 23756444).

For initiation codon variants:

- *UBE3A* use PVS1 (initiation codon variant in this gene has been described in affected patients, PMID 29737008)

- *CDKL5*, *FOXG1*, *SLC9A6* and *TCF4* use PVS1_Supporting (initiation codon variants in these genes have not been described to date in affected patients and a downstream putative in-frame methionine start codon is present in each gene with no pathogenic variants described upstream of the putative in-frame methionine)

- *MECP2*: not applicable due to the MECP2E1 alternative isoform that includes exon 1 with an alternate start codon

For canonical splice site variants:

- Variants predicted to result in an out-of-frame product - use PVS1, except for variants in *CDKL5* C-terminus (exons 19-21, or after p.904) (when using the NM_003159.2 transcript).

- Variants predicted to preserve reading frame (also see flow chart):

  - Use PVS1 for variants that flank the exons listed below for which de novo pathogenic variants in the splice site region have been described to date:

    - *CDKL5* exons 7, 10, 13
    - *MECP2* exon 3
    - *SLC9A6* exon 10
    - *TCF4* exon 15

  - Downgrade to PVS1_strong for variants that flank the exons listed below for which de novo pathogenic variants in the canonical splice site region have not been described to date but where the transcript has been extensively studied and no normal variants in the splice junctions have been reported:

        - *UBE3A* exon 7

        (multiple pathogenic variants in this exon have been reported in affected individuals, in-frame loss of 55 amino acids is 6.4% of protein)

        - *UBE3A* exon 8

        (pathogenic variants in this exon have been reported in affected individuals, in-frame loss of 52 amino acids is 6.1% of protein. At least two different variants in the canonical splice sites have been described in affected patients but de novo status not determined – PMID 25212744, ClinVar ID 421239)

        - *SLC9A6* exon 3

        (non-canonical splice site variants have been described in affected patients and found to segregate in some families and aberrant mRNA splicing has been demonstrated – PMID 27256868, 19377476, 18342287)

    - Downgrade to PVS1_moderate for variants that flank the exons listed below for which pathogenic variants in the splice site region have not been described to date:

        - *CDKL5* exon 17

        (To date one pathogenic variant in this exon has been reported in an affected individual, in-frame loss of 40 amino acids is 3.9% of protein)

For intragenic deletions/duplications:

- Predicted to result in an out-of-frame product use PVS1
- Predicted to result in a product that preserves reading frame (see flow chart for gene specific details):
    - For single exon in-frame deletions assign the same strength (PVS1, PVS1_strong, or PVS1_moderate) as for splice site variants that preserve reading frame indicated above
    - For multiple exon in-frame deletions PVS1 can be assigned to deletions that include single in-frame exons in the PVS1 category listed in the splice site section above OR if the exon contains a functionally important domain as specified in PM1
    - Given the extensive data available for *CDKL5, MECP2, TCF4* and *UBE3A*, classifications for single or multi-exon in-frame deletions are assigned as PVS1 or PVS1_strong. Exceptions are *CDKL5* exon 17 (as described above) and *SLC9A6* due to a limited number of pathogenic variants reported to date.
- CDKL5 (exon 1) and TCF4 (exon 20) are non-coding exons. There is evidence that loss of just non-coding CDKL5 exon 1 is pathogenic given previous de novo finding in patients affected with CDKL5-disease (GeneDx internal data), therefore, for losses involving just CDKL5 exon 1, PVS1 can be applied. For single exon deletions that involve just TCF4 exon 20, as this does not affect the reading frame, PVS1_moderate can be applied.

PS1: *"Same amino acid change as a previously established pathogenic variant regardless of nucleotide change. Caveats: Beware of changes that impact splicing rather than at the amino acid/protein level."*

EP notes:

- Applicable to all genes as written

PS2: *"De novo (both maternity and paternity confirmed) in a patient with the disease and no family history. Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, and so on, can contribute to non-maternity."*

EP notes:

- Applicable to all genes in affected individuals identified as mosaic for the variant (as the presence of a variant in the mosaic state is confirmatory of the variant being de novo)
- Follow point system as recommended by ClinGen SVI committee with specifications as indicated below

  https://clinicalgenome.org/site/assets/files/3461/svi_proposal_for_de_novo_criteria_v1_0.pdf

- Because of the very high de novo rate of pathogenic variants in *MECP2*, *CDKL5*, *FOXG1*, *TCF4*, *UBE3A* and *SLC9A6*, de novo observation can be attributed the highest value points per proband (2 points for confirmed de novo and 1 point for assumed de novo) if the patient is known to be affected with a neurodevelopmental phenotype consistent with the gene.

Table 1. Points awarded per de novo occurrence

Phenotypic consistency	Points per Proband	
	Confirmed de novo	Assumed de novo
Phenotype highly specific for gene	2	1
Phenotype consistent with gene but not highly specific	1	0.5
Phenotype consistent with gene but not highly specific and high genetic heterogeneity*	0.5	0.25
Phenotype not consistent with gene	0	0

*Maximum allowable value of 1 may contribute to overall score

Table 2. Recommendation for determining the appropriate ACMG/AMP evidence strength level for de novo occurrence(s)

Supporting (PS2_Supporting or PM6_Supporting)	Moderate (PS2_Moderate or PM6)	Strong (PS2 or PM6_Strong)	Very Strong (PS2_VeryStrong or PM6_VeryStrong)
0.5	1	2	4

PS3: *"Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product. Note: Functional studies that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well-established."*

EP notes:

- PS3_Strong: RNA studies that demonstrate abnormal splicing and an out-of-frame transcript (not to be used for canonical splice site variants and when PVS1 is used)
- PS3_Supporting: RNA studies that demonstrate abnormal splicing and an in-frame product (unless it affects an in-frame exon specified in the PVS1 section)
- PS3_Supporting: *FOXG1*, *MECP2*, *CDKL5*, *TCF4*, *UBE3A* (see tables for accepted functional studies). Not to be used for *SLC9A6*

PS4: *"The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls."*

EP notes:

- Following rational of Rasopathy publication (PMID: 29493581): Can use PS4_supporting for 2nd independent occurrence. Bump up criteria for more independent observations, PS4_moderate for 3-4, PS4_strong for 5+.

- Detailed phenotype not needed. Need to confirm patient is 'affected with a neurodevelopmental phenotype consistent with the gene' at a minimum.
- Patient can be published OR an internal case OR observed at an outside lab (i.e. via ClinVar) OR described in the reputable databases [LOVD (*UBE3A)*, RettBASE (*MECP2, CDKL5, FOXG1)].* However independent case has to be confirmed to be a different patient than yours (compare gender/age).
- Do not use this criterion for variants where BS1 is applied or where PM2 does not apply.

PM1: *"Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation."*

EP notes:

- *FOXG1* (Forkhead: aa 181-275; PMID 18571142, 28661489)
- *TCF4* (basic Helix-Loop-Helix domain (bHLH): aa 564-617; PMID 17436254, 22045651)
- *CDKL5* (ATP binding region: aa 19-43; TEY phosphorylation site: aa 169-171; PMID: 28544139, 17993579, 23064044, 29264392)
- *MECP2* (Methyl-DNA binding [MDB]: aa 90-162; Transcriptional repression domain [TRD]: aa 302-306; PMID 21326358, 23770565)
- *UBE3A* (3' cysteine binding site: aa 820) (PMID 9887341)
- Not to be used for *SLC9A6*

PM2: *"Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium AND not observed in the homozygous state."*

EP notes:

- PM2_Supporting: Use if absent, zero observations in public databases.

This downgrade is in line with the recommendation by the ClinGen SVI committee for all VCEPs (https://clinicalgenome.org/working-groups/sequence-variant-interpretation/)

PM3: *"For recessive disorders, detected in trans with a pathogenic variant."*

EP notes:

- Not applicable for these genes

PM4: *"Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants."*

EP notes:

- *CDKL5*: Do not use for in-frame deletions/insertions in CDKL5 C-terminus (exons 19-21, or after p.904). (when using the NM_003159.2 transcript).
- *MECP2*: Do not use PM4 for in-frame deletions/insertions in the Proline-rich region of the gene (p.381-p.405).
- *FOXG1*: Do not use PM4 for in-frame deletions/insertions in the Histidine-rich region (p.37-p.57), Proline and Glutamine-rich region (p.58-p.86) and Proline-rich region (p.105-p.112).
- PM4_supporting: Smaller in-frame events (< 3 amino acid residues) unless they occur in a functionally important region (see PM1 for functionally important domains for each gene).
- *MECP2* and *UBE3A*: stop-loss variants can be upgraded to PM4_Strong as several stop loss variants in these genes have been described in affected individuals (PMIDs: 10814719, 11469283, 25212744).

PM5: *"Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before. Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level."*

EP notes:

- Applicable to all genes as written
- A Grantham or BLOSUM score comparison can be used to determine if the variant is predicted to be as or more damaging than the established pathogenic variant.

PM5_Strong:

≥2 different missense changes affecting the amino acid residue. Do not apply PM1 in these situations.

PM6: *"Assumed de novo but without confirmation of paternity and maternity."*

EP notes:

See PS2 for updated usage of de novo criteria

PP1: *"Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease."*

EP notes:

- PP1: 2 informative meiosis
- PP1_Moderate: 3-4 informative meiosis
- PP1_Strong: ≥5 informative meiosis

Note: individuals must have disease consistent with reported phenotype (even if on the mild end of spectrum of the disease)

PP2: *"Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease."*

EP notes:

- Not applicable for these genes

PP3: ***"****Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)"*

EP notes:

- For missense variants use REVEL with a score ≥ 0.75
- For splice site variants use MaxEntScan, NNSPLICE and HumanSplicingFinder when the majority of the predictions program support splicing alteration

(The above are in line with other ClinGen variant curation expert panels)

PP4: *"Patient's phenotype or family history is highly specific for a disease with a single genetic etiology."*

EP notes:

- See gene specific clinical phenotype guidelines

PP5: *"Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation."*

EP notes:

- Do not use

EVIDENCE OF BENIGN IMPACT

BA1: *"Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium."*

EP notes:

- Use large population databases (i.e. gnomAD)
- Use if variant is present at ≥0.0003 (0.03%) in any sub-population – cutoff is based on summation of prevalence of genes covered in the Rett/Angelman-like working group. The prevalence values were determined using the most conservative numbers found in the literature.
- Use if allele frequency is met in any general continental population dataset of at least 2,000 observed alleles

	MECP2	***UBE3A***	***CDKL5***	***FOXG1***	***TCF4***	***SLC9A6***	***BA1***	***BS1***
Disease allele frequency (DAF) = calculated from Hardy-Weinberg	*0.00008*	*0.0000167*	*0.000015*	*0.00001*	*0.000015*	*0.000125*	*0.0003* *(0.000262)*	*0.00008*
Reasoning for DAF	*DAF= 0.00008 (general population) based on 1 in 8,500 females (GeneReviews) / 1.5 (assumes 50/50 male/female ratio)*	*DAF = 0.0000167 Prevalence of 1 /12000-1/20000 (reported by NORD); 10-20 percent of individuals with Angelman syndrome have mutations in UBE3A. Conservatively, using 1/12000 and 20% attributable risk and*	*DAF = 0.000015 Based on a prevalence of atypical RTT of 1/45000 (orphanet) and adjusted for X-linked dominant*	*DAF = 0.00001, Based on a prevalence of atypical RTT of 1/45000 (orphanet)*	*Approximately 500 affected individuals have been reported worldwide. PTHS prevalence estimates: 1:200,000-300,000 or 1:34,000 - 41,000. DAF conservative PTHS: 0.000015 (1/34,000)*	*DAF: 0.000125 based on 1:12,000 prevalence and use 1.5 times your prevalence (since x-linked recessive), since gnomAD is a population of males and females; even if it isn't exactly 1/2 of*		

		multiplied times 2, bc approx 1/2 variant carriers will not be affected due to pat inheritance.				*each. CHRISTIANSON SYNDROME, OVERLAPS WITH ANGELMAN. Prevalence 1 in 12,000 for AS*		
Inheritance	*X-Linked Dominant*	*Autosomal (Imprinted)*	*X-Linked Dominant*	*Autosomal Dominant*	*Autosomal Dominant*	*X-Linked Recessive*		

BS1: *"Allele frequency is greater than expected for disorder."*

EP notes:

- Use large population databases (i.e. gnomAD)
- Use if variant is present at ≥0.00008 (0.008%) and <0.0003 (0.03%) in any sub-population – based on MECP2 expected disease allele frequency (1 in 8500 females /1.5 alleles (assumes 50/50 male/female ratio)
- Use if allele frequency is met in any general continental population dataset of at least 2,000 observed alleles

BS2: *"Observed in a healthy adult individual for a recessive (homozygous), or dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance at an early age."*

EP notes:

- Should be applied in cases where the healthy adult is devoid of neurodevelopmental phenotypes.
- Best to use with internal curated data that includes clinical information or published patients that have been phenotyped.
- 2 unaffected (related or unrelated) Het (*FOXG1, TCF4*), Hemi (*SLC9A6*), Het or Hemi (*CDKL5*, *MECP2*)
- 4 unaffected (related and maternally inherited or unrelated) Het (*UBE3A*)
- BS2_supporting: 1 unaffected (related or unrelated) Het (*FOXG1, TCF4*), Hemi (*SLC9A6*), Het or Hemi (*CDKL5, MECP2*)
- BS2_supporting: 2 unaffected (related and maternally inherited or unrelated) Het (*UBE3A*)

BS3: *"Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing."*

EP notes:

- BS3_Strong: RNA functional studies that demonstrate no impact on splicing and transcript composition. It can be downgraded based on quality of data
- Not applicable for these genes for other functional studies (see tables for other accepted functional studies)

BS4: *"Lack of segregation in a family. Caveat: The presence of phenocopies for common phenotypes."*

EP notes:

- Absent in a similarly affected family member, when seen in two or more families
- BS4_supporting: absent in a similarly affected family member

Note: Need to confirm that the family member is 'affected with a neurodevelopmental phenotype consistent with the gene' at a minimum.

BP1: *"Missense variant in a gene for which primarily truncating variants are known to cause disease."*

EP notes:

- Not applicable for these genes

BP2: *"Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern."*

EP notes:

- Applicable for *MECP2*, *TCF4*, *FOXG1* for *in trans* state (knock out of these genes results in embryonic lethality/drastic phenotype, PMID 11242117, 8649400, 17878293, 14704420)
- Not applicable for *SLC9A6*, *UBE3A* and *CDKL5* for *in trans* state (knock out of these genes result in disease but viable phenotype, PMID 21964919, 9808466, 23236174)

BP3: *"In-frame deletions/insertions in a repetitive region without a known function."*

EP notes:

- Inframe expansions or deletions in *FOXG1* repetitive regions: poly His (p.His47-p.His57), poly Gln (p.Gln70-p.Gln73) and poly Pro (p.Pro58-p.Pro61; p.Pro65-p.Pro69; p.Pro74-p.Pro80)

BP4: *"Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.). Caveat: Because many in silico algorithms use the same or very similar input for their predictions, each algorithm cannot be counted as an independent criterion. BP4 can be used only once in any evaluation of a variant."*

EP notes:

- For missense variants use REVEL with a score ≤ 0.15
- For splice site variants use MaxEntScan, NNSPLICE and HumanSplicingFinder when the majority of the predictions program support no splicing alteration

  (The above are in line with other ClinGen variant curation expert panels)

BP5: *"Variant found in a case with an alternate molecular basis for disease."*

EP notes:

- For example if a variant in one of the genes is identified in a patient with lissencephaly in whom a pathogenic variant is identified in the *PAFAH1B1* gene.
- *UBE3A*: variant should also be maternally inherited in the case with an alternate molecular basis for disease for this criteria to be used.
- *SLC9A6*: the variant should be in the hemizygous state in the case with an alternate molecular basis for disease for this criteria to be used.
- Do not apply for any gene if variant is de novo
- BP5_strong: ≥3 cases with alternate molecular basis for disease

BP6: *"Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation."*

EP notes:

- Do not use

BP7: *"A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved."*

EP notes:

- Applicable to all genes as written

- We have defined “not highly conserved" regions in BP7 as those with PhastCons score <1 and/or PhyloP score <0.1 and/or the variant is the reference nucleotide in one primate and/or three mammal species. This definition is in line with other VCEPs.

Supplement Tables:

MECP2 Functional Assays

Name of Assay	**MECP2 chromatin binding assay**	**MECP2 in vitro binding assay**	**In vitro transcription repression assay**
Measured Parameter	Localization of MECP2 to highly methylated heterochromatic loci by quantitative immunofluorescence assay (MECP2 and DAPI co-localization)	Association of MECP2 with NCoR/SMRT co-repressors	Luciferase activity in cell lysates co-expressing target reporters and wt or mutant MECP2 effector proteins
Expected Deleterious Result Range (PS3_Supporting)	MECP2 is distributed diffusely (no clustering pattern)	Abolished interaction by co-immunoprecipitation assay	Abolished transcription repression activity in cells transfected with the effector construct expressing mutant MECP2 compared to constructs expressing wild type MECP2
Expected Benign Result Range (BS3)	Not recommended	Not recommended	Not recommended
References	PMID: 27929079, 23770565, 29718204	PMID:23770565, 29718204	PMID 23452848

FOXG1 Functional Assays

Name of assay	Subcellular localization	CDKN1A expression	Chromatin localization	Stability of chromatin binding
Measured parameter	Immunofluorescence staining pattern	*CDKN1A* mRNA level quantitation	Chromocenter/nucleoplasmic ratios of fluorescence intensity	Strip-FRAP (fluorescence recovery after photobleaching)
Expected deleterious result range (PS3_Supporting)	Abnormal staining pattern such as nuclear speckles or nuclear and cytoplasmic localization instead of homogenous distribution throughout the nucleus	Increase of *CDKN1A* expression by ~30%	Ratio greater than 0.52 indicating more dispersed within chromatin compared to wild type (ratio of 0.45)	Decrease in chromatin affinity, t_2 of <2 seconds compared to 3 seconds or greater (wild type)
Expected benign result range (BS3)	Not recommended	Not recommended	Not recommended	Not recommended
References	PMID 21280142 22091895	PMID 21280142	PMID 22091895	PMID 22091895

CDKL5 Functional Assays

Name of Assay	***in vitro autophosphorylation assays***	***in vitro phosphorylation-TEY assay***	***sub cellular localization assay***	***in vitro kinase assay***
Measured Parameter	Auto-phosphorylation of CDKL5	phosphorylation of TEY motif	subcellular distribution	enzymatic activity of CDKL5
Expected Deleterious Result Range (PS3_Supporting)	Absence of auto-phosphorylation	Absence of phosphorylation	unidentifiable with Hoechst staining and localizes partially within the cytoplasm	Absence of phosphorylation of CDKL5 substrates (MeCP2 and Dnmt1)
Expected benign result range (BS3)	Not recommended	Not recommended	Not recommended	Not recommended
References	PMID: 16935860	PMID: 16935860	PMID: 16935860	PMID: 27265524 16935860

TCF4 Functional Assays

Name of assay	Subcellular localization assay	Homogenous time-resolved fluorescence assay for measurement of protein-protein interaction	Luciferase assay for measurement of transcriptional activity	Electrophoretic mobility shift assay (EMSA)	Western blot	Co-fractionation
Measured parameter	Subcellular distribution	Homodimer formation (with itself) and heterodimer formation (with other bHLH transcription factors)	Transcriptional activation of E-box containing promoter reporter constructs	DNA binding activity of homo- and heterodimers	Protein expression and stability	Localization to the chromatin
Expected deleterious result range (PS3_Supporting)	Localization different compared to wild type TCF4 (e.g. accumulated in nuclear dots, no nuclear accumulation)	<25% homodimer and heterodimer formation compared to wild type	p-value <0.05 compared to wild type luciferase activity	Comparison to wild type possible however no robust threshold available	Comparison to wild type possible however no robust threshold available	p-value < 0.05 compared to wild type TCF4. Localization to the soluble fraction
Expected benign result range (BS3)	Not recommended	Not recommended	Not recommended	Not recommended	Not recommended	Not recommended
References	PMID 22460224, 22777675	PMID 22777675	PMID 17436255, 19235238, 22460224, 22777675	PMID 22460224	PMID 22460224	PMID 22460224

UBE3A Functional Assays

Name of assay	E3 ubiquitin ligase activity	UBE3A protein expression
Measured parameter	E3 ubiquitin ligase activity	Protein levels monitored to reflect either protein stability or levels of self degradation.
Expected deleterious result range (PS3_Supporting)	Loss of substrate ubiquitination	Comparison to WT possible however no robust thresholds available.
Expected benign result range (BS3)	Not recommended	Not recommended
References	PMID: 15263005; 26255772	PMID: 26255772

***MECP2* clinical phenotype guidelines:**

Core phenotype (need to be met for PP4)

Regression of developmental progress and loss of at least 2 of 4 of following

Loss, partial or complete of fine motor skills (hand use)

Loss, partial or complete of spoken communication

Abnormal (dyspraxic) or absent gait

Stereotypies

Supportive Criteria (do not need to be met for PP4, however in the absence of one core phenotype, two or more supportive phenotypes can be used in its place)

Periodic breathing (breath-holding/hyperventilation) when awake

Bruxism when awake

Impaired sleep pattern

Abnormal muscle tone

Peripheral vasomotor disturbances

Scoliosis/kyphosis

Growth retardation (small stature)

Small, cold hands and feet

Inappropriate laughing/screaming spells

Diminished response to pain

Intense eye communication (“eye pointing”)

Additional notes: If information is provided such that a phenotype of Rett syndrome is suspected, with specific minimal features used for the diagnosis, then this can be used for PP4 in lieu of the specific clinical features listed.

***FOXG1* clinical phenotype guideline:**

Core phenotype (need to be met for PP4)

Microcephaly

Severe intellectual disability

Dyskinesia

No period of normal development

Neonatal hypotonia

Additional features (do not need to be met for PP4, however in the absence of one core phenotype, two or more supportive phenotypes can be used in its place)

Abnormal brain imaging (e.g. partial agenesis of the corpus callosum, simplified gyral pattern, reduced white matter volume)

Delayed motor development

Impairment of postnatal growth

Stereotypies

Generalized seizures

GE reflux

Poor sleep pattern

Unexplained episodes of crying

Recurrent aspiration

***CDKL5* clinical phenotype guidelines:**

Core phenotype (need to be met for PP4)

Seizures, including infantile spasms, beginning in infancy

Global developmental delay

Intellectual disability

Hypotonia

Severely impaired gross motor function

Cortical visual impairment in the first 12 months

Supportive criteria (do not need to be met for PP4, however in the absence of one core phenotype, two or more supportive phenotypes can be used in its place)

Sleep disturbances

Gastrointestinal dysfunction

Subtle dysmorphic features (broad forehead, large, deep-set eyes, tapered fingers, full lips, anteverted nostrils in males)

Bruxism

Hand stereotypies

Periodic breathing

Laughing, screaming spells

Cold hands and feet

Peripheral vasomotor dysfunction

TCF4 clinical phenotype guidelines:

Core phenotype (need to be met for PP4)

Global developmental delay

Intellectual disability

Behavioral problems (anxiety)

Hand flapping

Characteristic Facial Features (become more apparent with age)

Deeply set eyes with prominent supraorbital ridges

Mildly up-slanted palpebral fissures

Broad nasal root, wide nasal ridge, and wide nasal base with enlarged nostrils

Overhanging or depressed nasal tip, which may be pointed

Short philtrum

Thick vermilion of the lower lip, which is often everted

Widely spaced teeth

Supportive criteria (do not need to be met for PP4, however in the absence of one core phenotype, two or more supportive phenotypes can be used in its place)

Prominence of the lower face with a well-developed chin, with age the lower face becomes more prominent and facial features may coarsen

Mildly cupped ears with over folded helices

In some individuals, wide mouth with downturned corners and exaggerated Cupid's bow or tented vermilion of the upper lip

Happy, excitable, frequent smiling, laughter

Episodic periodic breathing

Additional notes: If information is provided such that a phenotype of Pitt Hopkins syndrome is suspected, with specific minimal features used for the diagnosis, then this can be used for PP4 in lieu of the specific clinical features listed.

***UBE3A* clinical phenotype guidelines:**

Mandatory criterion:

Severe ID (if 5 years of age or older) or global developmental delay (if <5 years of age)

In addition, the patient has to satisfy at least 4/5 of the following:

Ataxia/jerky movements

Characteristic EEG

Seizures

Absent speech or less than 5 words (if at least 4 years of age)

Frequent smiling

Additional notes: If information is provided such that a phenotype of Angelman syndrome is suspected, with specific minimal features used for the diagnosis, then this can be used for PP4 in lieu of the specific clinical features listed.

SLC9A6 **clinical phenotype guidelines:**

Core phenotype (need to be met for PP4)

Global developmental delay

Intellectual disability

Epilepsy

Autistic spectrum disorder

Ataxia

Craniofacial dysmorphism

Supportive criteria (do not need to be met for PP4, however in the absence of one core phenotype, two or more supportive phenotypes can be used in its place)

Happy, excitable, frequent smiling, laughter

Angelman-like features

Microcephaly