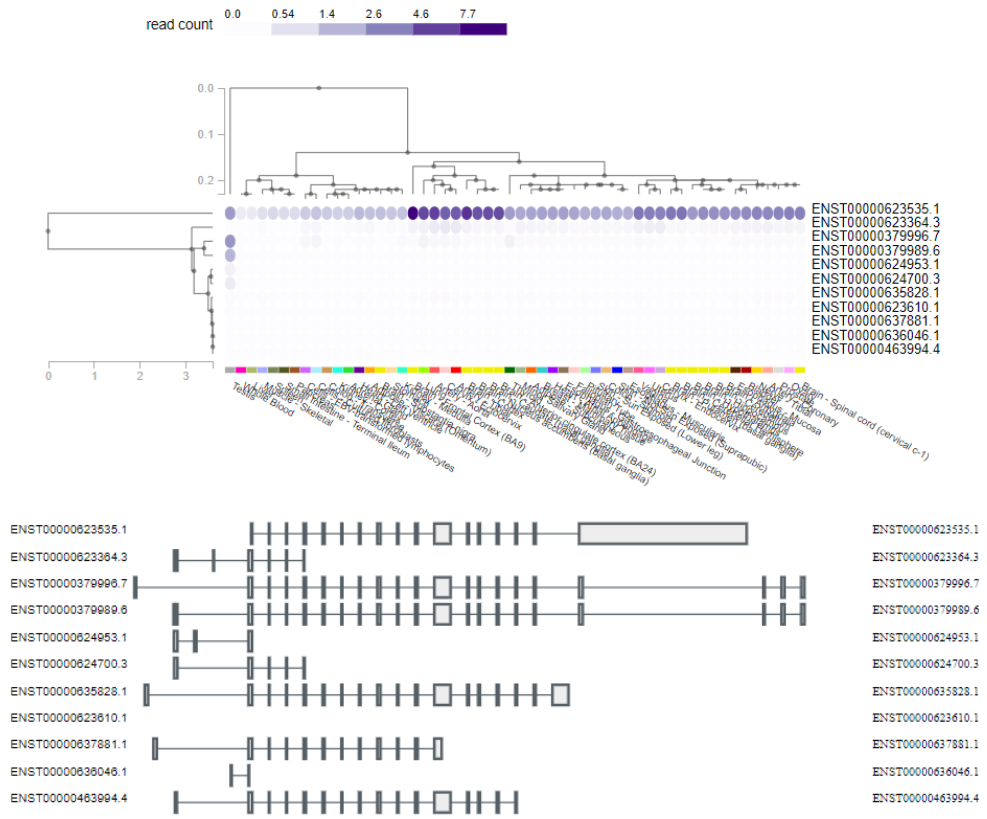
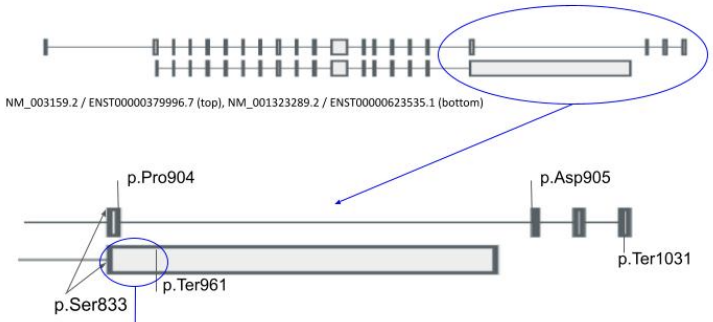
Supp. Figure S1. Rett / Angelman-like gene_CDKL5 v2

A. *CDKL5* isoform expression pattern. *NM_003159.2 is the commonly used transcript, **NM_001323289.2 is the most highly expressed transcript; the red node marks brain-specific expression. B. *CDKL5* isoform expression pattern. NM_003159.2 is the commonly used transcript; NM_001323289.2 is the most highly expressed transcript.

Supp. Figure S1a



Supp. Figure S1b



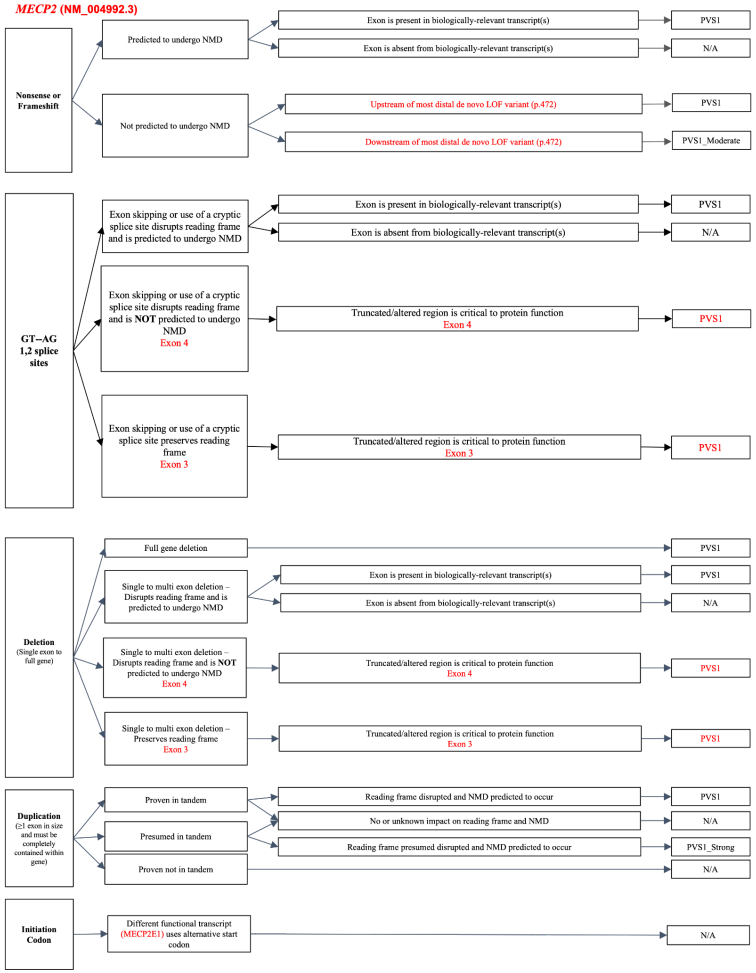
ClinVar ID	NM_003159.2 Unique Variant	Alleles in gnomAD
153543	c.2854C>T (p.Arg952Ter)	36
153544	c.2908C>T (p.Arg970Ter)	2
649912	c.3037_3046del (p.Val1013fs)	0

ClinVar ID	NM_001323289.2 Unique Variants	Origin
214547	c.2716C>T (p.Gln906Ter)	
508102	c.2733G>A (p.Trp911Ter)	Unknown
858746	c.2760_2761CA (p.Thr921fs)	Cfrm *de novo*
649910	c.2785C>T (p.Gln929Ter)	Unknown
649911	c.2821del (p.Tyr941fs)	Cfrm *de novo*
435196	c.2828_2829delGA (p.Arg943fs)	Cfrm *de novo*
482247	c.2842C>T (p.Arg948Ter)	Cfrm *de novo*

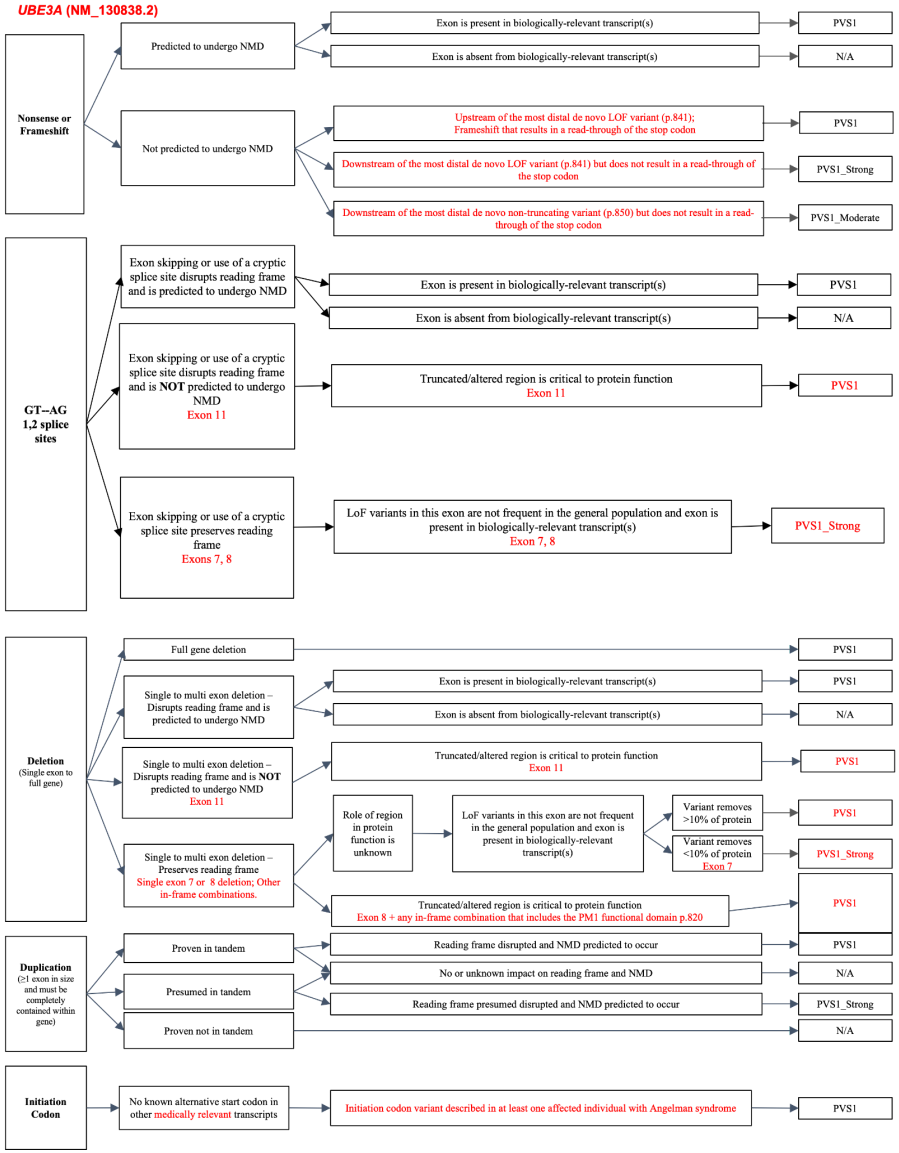
Supp Figure S2: Rett / Angelman-like genes PVS1 flowcharts

Rett Syndrome/Angelman-like genes PVS1 flowcharts. A. *MECP2*, B. *UBE3A,* C. *CDKL5,* D. *FOXG1*, E. *SLC9A6*, F. *TCF4*

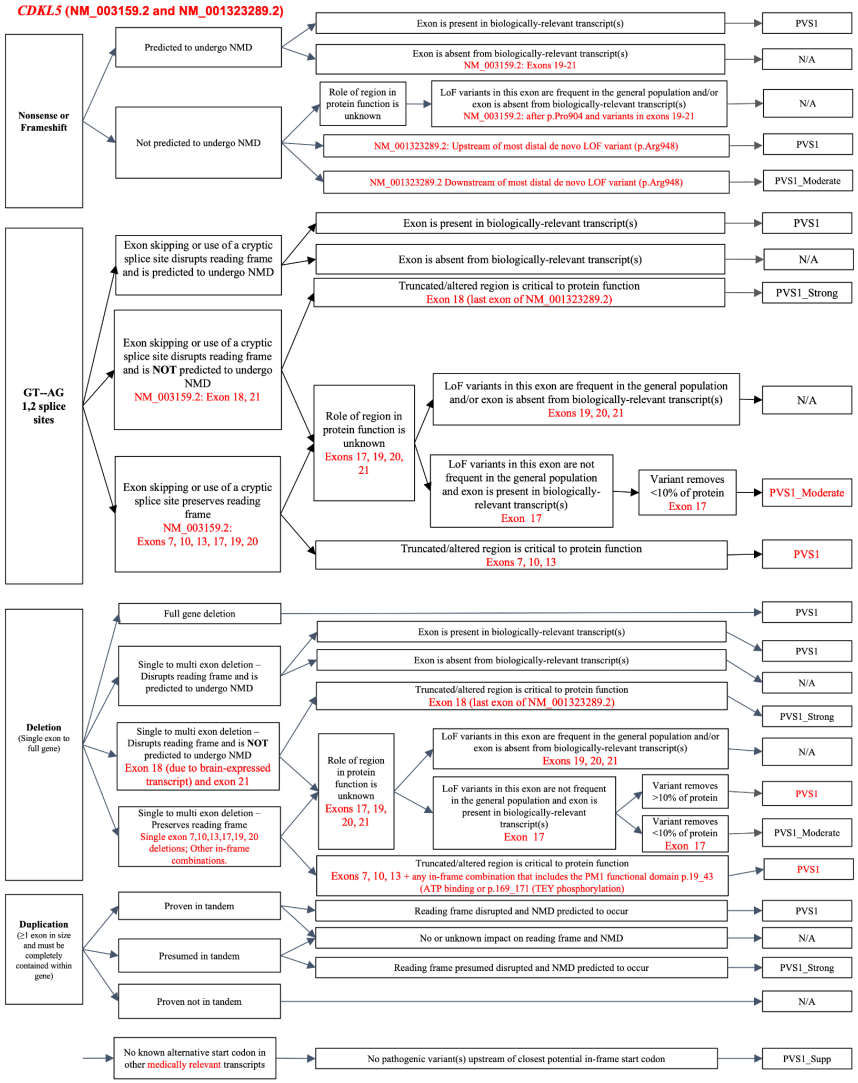
Supp. Figure S2a: *MECP2*



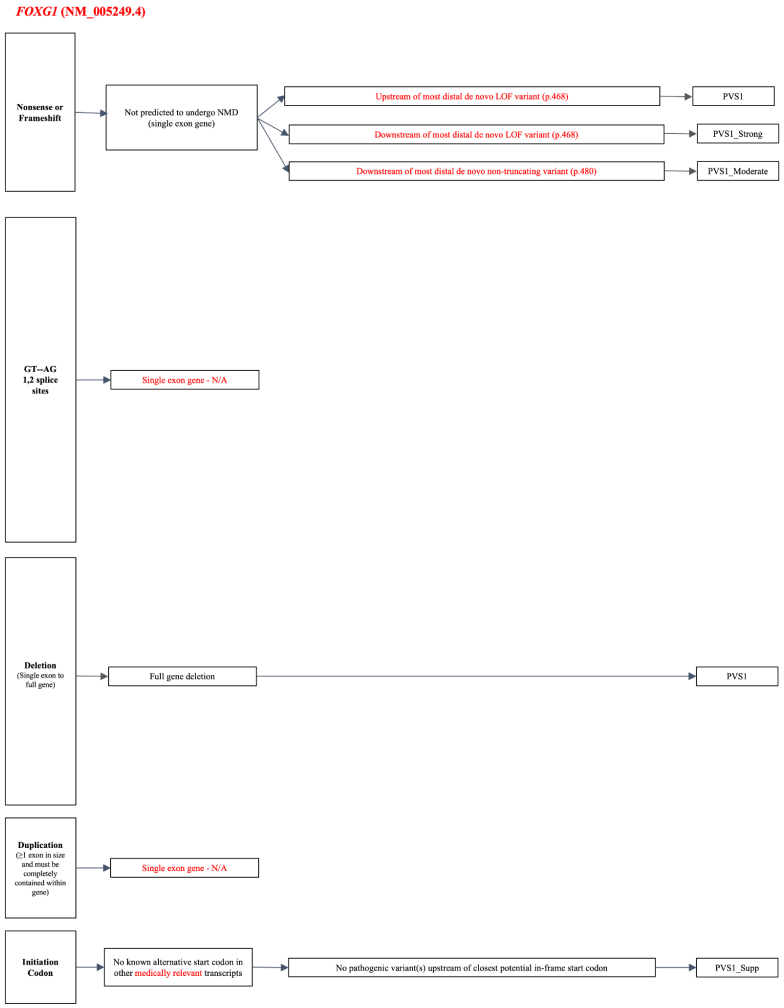
Supp. Figure S2b: *UBE3A*



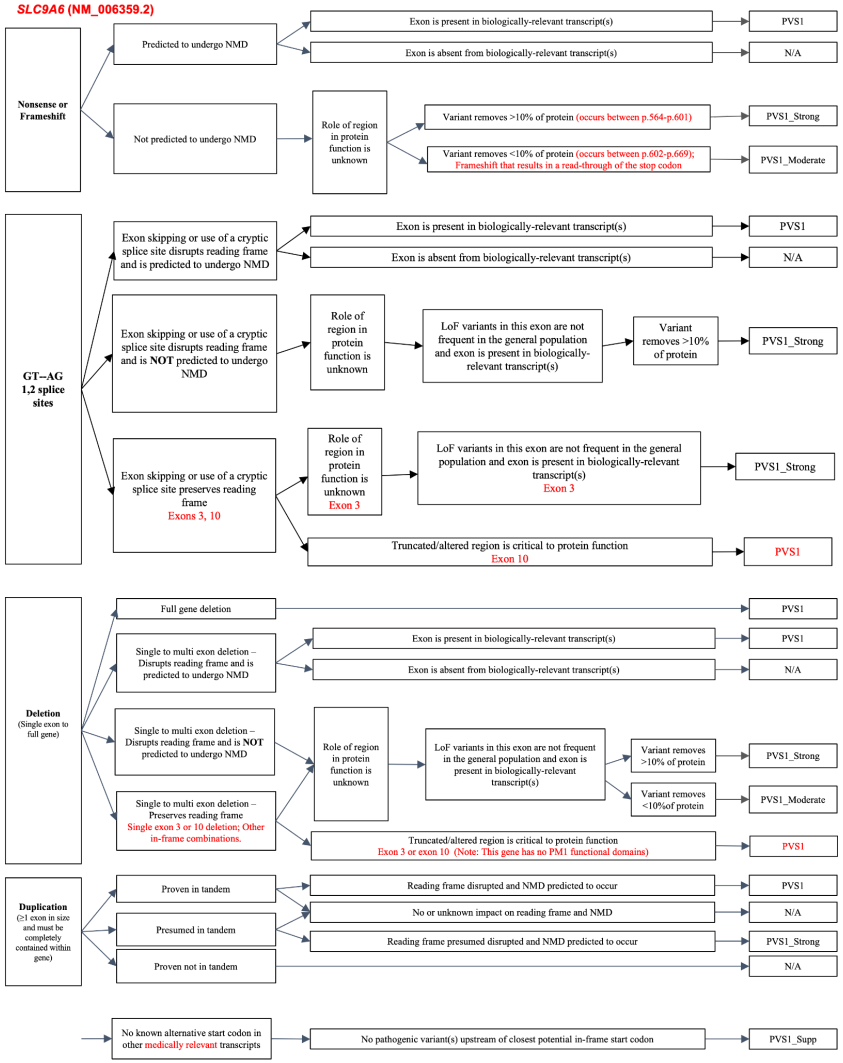
Supp. Figure S2c: *CDKL5*



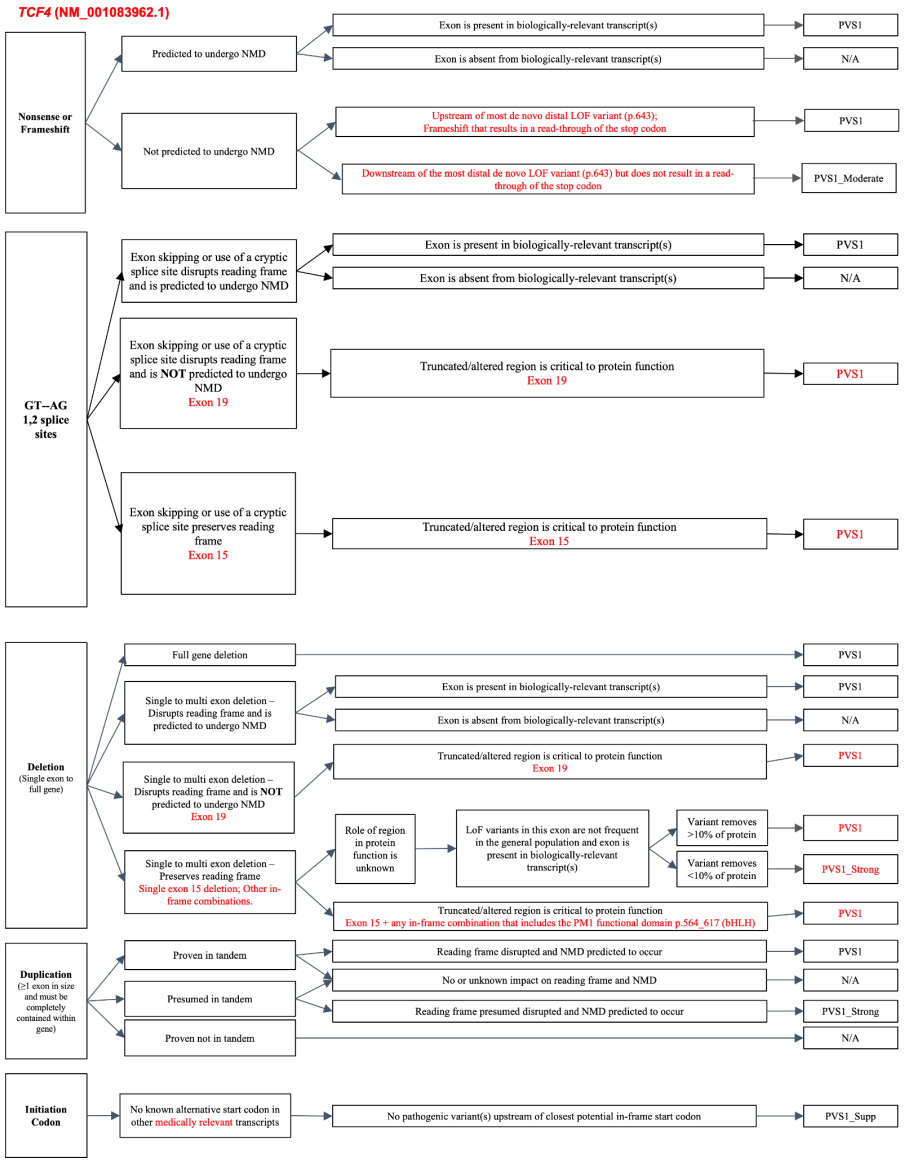
Supp. Figure S2d: *FOXG1*



Supp. Figure S2e: *SLC9A6*



Supp. Figure S2f: *TCF4*



Supp. Table S1: Population Frequencies

	MECP2	***UBE3A***	***CDKL5***	***FOXG1***	***TCF4***	***SLC9A6***	***BA1***	***BS1***
Disease allele frequency (DAF) = calculated from Hardy-Weinberg	*0.00008*	*0.00002*	*0.00002*	*0.00001*	*0.00002*	*0.0001*	*0.0003*	*0.00008*

Reasoning for DAF	*DAF= 0.00008 (general population).* Prevalence is estimated at 1:10,000-1:23,000 female births. *Using 1 in 10.000 females (adjust by 1.5 for XLD (assumes 50/50 male/female ratio) and very conservatively accounting for 20% due X inactivation patterns.*	*DAF = 0.00002 Prevalence of 1 /12000-1/24000; 10-20 percent (range from rarediseases. org at time of publication) of individuals with Angelman syndrome have mutations in UBE3A. Conservatively, using 1/12,000 (adjusted to allele frequency) and 20% attributable risk and multiplied times 2 as ~ 1/2 variant carriers will not be affected due to paternal inheritance.*	*DAF = 0.00002 Prevalence is* 1:40,000-60,000. Conservatively using *1/40000 and adjusting for X-linked dominant (as described for MECP2).*	*DAF = 0.00001 Based on a prevalence of atypical RTT of 1/45000 (adjusted to allele frequency) (orphanet at time of publication) .*	*DAF = 0.00002 Approximately 500 affected individuals have been reported worldwide. PTHS prevalence estimates: 1:34,000 - 41,000. DAF based on 1:34,000 (adjusted to allele frequency).*	*DAF: 0.0001 Prevalence is estimated as* 1:16,000 and 1:100,000. *Using 1:16,000 prevalence and use 1.5 times your prevalence (x-linked recessive),*		

Inheritance	*X-Linked Dominant*	*Autosomal (Imprinted)*	*X-Linked Dominant*	*Autosomal Dominant*	*Autosomal Dominant*	*X-Linked Recessive*		

*DAF numbers are conservatively rounded up to the most significant digit

Supp. Table S2: Functional Assays

A. *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

B. *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

C. *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

D. *TCF4* Functional Assays

Name of assay	Subcellular localization assay	Homogenous time-resolved fluorescence				Co-fractionation

		assay for measurement of protein-protein interaction	Luciferase assay for measurement of transcriptional activity	Electrophoretic mobility shift assay (EMSA)	Western blot	
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	PMI: 17436255, 19235238, 22460224, 22777675	PMID: 22460224	PMID: 22460224	PMID: 22460224

E. *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

Supp. Table S3: Application of PP4 Criteria

MECP2	***FOXG1***	***CDKL5***	***TCF4***	***SLC9A6***	***UBE3A***
Core Features	**Core Features**	**Core Features**	**Core Features**	**Core Features**	**Core Features**
Regression of developmental progress and loss of at least 2 of 4 of following	Microcephaly	Seizures, including infantile spasms, beginning in infancy	Global developmental delay	Global developmental delay	Delayed attainment of developmental milestones
Loss, partial or complete of fine motor skills (hand use)	Severe intellectual disability	Global developmental delay	Intellectual disability	Intellectual disability	Movement or balance disorder, usually ataxia of gait and/or tremulous movement of the limbs
Loss, partial or complete of spoken communication	Dyskinesia	Intellectual disability	Behavioral problems (anxiety)	Epilepsy	Behavioral uniqueness (increased frequent laughter/smiling, hand flapping)

Abnormal (dyspraxic) or absent gait	No period of normal development	Hypotonia	Hand flapping	Autistic spectrum disorder	Seizures
Stereotypies	Neonatal hypotonia	Severely impaired gross motor function	Characteristic Facial Features (become more apparent with age)	Ataxia	Abnormal EEG, with a characteristic pattern of large-amplitude slow spike waves
Supportive features	**Supportive features**	Cortical visual impairment in the first 12 months	Deeply set eyes with prominent supraorbital ridges	Craniofacial dysmorphism	Speech impairment with minimal use of words (if over 12 months)
Periodic breathing (breath-holding/hyper ventilation) when awake	Abnormal brain imaging (e.g. partial agenesis of the corpus callosum, simplified gyral pattern, reduced white matter volume)	**Supportive features**	Mildly up-slanted palpebral fissures	**Supportive features**	**Additional note**
Bruxism when awake	Delayed motor development	Sleep disturbances	Broad nasal root, wide nasal ridge, and	Happy, excitable, frequent smiling, laughter	“Angelman syndrome”

			wide nasal base with enlarged nostrils		
Impaired sleep pattern	Impairment of postnatal growth	Gastrointestinal dysfunction	Overhanging or depressed nasal tip, which may be pointed	Angelman-like features	
Abnormal muscle tone	Stereotypies	Subtle dysmorphic features (broad forehead, large, deep-set eyes, tapered fingers, full lips, anteverted nostrils in males)	Short philtrum	Microcephaly	
Peripheral vasomotor disturbances	Generalized seizures	Bruxism	Thick vermilion of the lower lip, which is often everted		
Scoliosis/kyphosis	GE reflux	Hand stereotypies	Widely spaced teeth		
Growth retardation (small stature)	Poor sleep pattern	Periodic breathing	**Supportive features**		

Small, cold hands and feet	Unexplained episodes of crying	Laughing, screaming spells	Prominence of the lower face with a well-developed chin, with age the lower face becomes more prominent and facial features may coarsen		
Inappropriate laughing/screaming spells	Recurrent aspiration	Cold hands and feet	Mildly cupped ears with over folded helices		
Diminished response to pain		Peripheral vasomotor dysfunction	In some individuals, wide mouth with downturned corners and exaggerated Cupid's bow or tented vermilion of the upper lip		
Intense eye communication ("eye pointing")			Happy, excitable, frequent smiling, laughter		
Additional note			Episodic periodic breathing		

“Rett syndrome”			**Additional note**		
			“Pitt Hopkins syndrome”		

PP4 may be applied when the core features are met or in the absence of a single core features, two or more supportive features can be used in it’s place. For *UBE3A*, the mandatory feature must be met in addition to round of 4 of the 5 additional features to apply PP4. For *MECP2*, *TCF4*, and *UBE3A*, PP4 may be used when an additional note (a specific clinical diagnosis) is used to describe the patient’s clinical presentation.

Gene	***MECP2***	***FOXG1***	***CDKL5***	***TCF4***	***SLC9A6***	***UBE3A***
Core Features	Regression of developmental progress and loss of at least 2 of 4 of following	Microcephaly	Seizures, including infantile spasms, beginning in infancy	Global developmental delay	Global developmental delay	**Mandatory Feature**
	Loss, partial or complete of fine motor skills (hand use)	Severe intellectual disability	Global developmental delay	Intellectual disability	Intellectual disability	Severe ID (if 5 years of age or older) or global developmental

						delay (if <5 years of age)
	Loss, partial or complete of spoken communication	Dyskinesia	Intellectual disability	Behavioral problems (anxiety)	Epilepsy	**Additional features**
	Abnormal (dyspraxic) or absent gait	No period of normal development	Hypotonia	Hand flapping	Autistic spectrum disorder	Ataxia/jerky movements
	Stereotypies	Neonatal hypotonia	Severely impaired gross motor function	Characteristic Facial Features (become more apparent with age)	Ataxia	Characteristic EEG
			Cortical visual impairment in the first 12 months	Deeply set eyes with prominent supraorbital ridges	Craniofacial dysmorphism	Seizures
				Mildly up-slanted palpebral fissures		Absent speech or less than 5 words

						(if at least 4 years of age)
				Broad nasal root, wide nasal ridge, and wide nasal base with enlarged nostrils		Frequent smiling
				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 features	Periodic breathing (breath-holding/hyperventilation) when awake	Abnormal brain imaging (e.g. partial agenesis of the corpus callosum, simplified gyral pattern, reduced white matter volume)	Sleep disturbances	Prominence of the lower face with a well-developed chin, with age the lower face becomes more prominent and facial features may coarsen	Happy, excitable, frequent smiling, laughter	
	Bruxism when awake	Delayed motor development	Gastrointestinal dysfunction	Mildly cupped ears with over folded helices	Angelman-like features	
	Impaired sleep pattern	Impairment of postnatal growth	Subtle dysmorphic features (broad forehead, large, deep-set eyes, tapered fingers, full lips, anteverted nostrils in males)	In some individuals, wide mouth with downturned corners and exaggerated Cupid's bow or tented vermilion of the upper lip	Microcephaly	
	Abnormal muscle tone	Stereotypies	Bruxism	Happy, excitable, frequent smiling, laughter		

	Peripheral vasomotor disturbances	Generalized seizures	Hand stereotypies	Episodic periodic breathing		
	Scoliosis/kyphosis	GE reflux	Periodic breathing			
	Growth retardation (small stature)	Poor sleep pattern	Laughing, screaming spells			
	Small, cold hands and feet	Unexplained episodes of crying	Cold hands and feet			
	Inappropriate laughing/screaming spells	Recurrent aspiration	Peripheral vasomotor dysfunction			
	Diminished response to pain					

	Intense eye communication (“eye pointing”)					
Additional note	“Rett syndrome”			“Pitt Hopkins syndrome”		“Angelman syndrome”