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Analysis of X-inactivation status in a Rett syndrome natural history study cohort

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Abstract

Background: Rett syndrome (RTT) is a rare neurodevelopmental disorder associated with pathogenic *MECP2* variants. Because the *MECP2* gene is subject to X-chromosome inactivation (XCI), factors including *MECP2* genotypic variation, tissue differences in XCI, and skewing of XCI all likely contribute to the clinical severity of individuals with RTT.

Methods: We analyzed the XCI patterns from blood samples of 320 individuals and their mothers. It includes individuals with RTT ($n = 287$) and other syndromes sharing overlapping phenotypes with RTT (such as *CDKL5* Deficiency Disorder [CDD, $n = 16$]). XCI status in each proband/mother duo and the parental origin of the preferentially inactivated X chromosome were analyzed.

†This work is dedicated to the memory of Dr. Mary Jones, who passed away recently.

Results: The average XCI ratio in probands was slightly increased compared to their unaffected mothers (73% vs. 69%, $p = .0006$). Among the duos with informative XCI data, the majority of individuals with classic RTT had their paternal allele preferentially inactivated ($n = 180/220$, 82%). In sharp contrast, individuals with CDD had their maternal allele preferentially inactivated ($n = 10/12$, 83%). Our data indicate a weak positive correlation between XCI skewing ratio and clinical severity scale (CSS) scores in classic RTT patients with maternal allele preferentially inactivated XCI ($r_s = 0.35$, $n = 40$), but not in those with paternal allele preferentially inactivated XCI ($r_s = -0.06$, $n = 180$). The most frequent *MECP2* pathogenic variants were enriched in individuals with highly/moderately skewed XCI patterns, suggesting an association with higher levels of XCI skewing.

Conclusion: These results extend our understanding of the pathogenesis of RTT and other syndromes with overlapping clinical features by providing insight into the both XCI and the preferential XCI of parental alleles.

KEYWORDS

CDKL5 deficiency disorder, *MECP2*, preferential inactivation of parental alleles, Rett syndrome, X-chromosome inactivation

1 | INTRODUCTION

Rett syndrome (RTT) [MIM: 312750] is a rare, X-linked neurodevelopmental disorder. Individuals with RTT have apparently normal development for the first 6 to 18 months of life followed by a period of developmental stagnation and neurological regression (Jeffrey L. Neul et al., 2010; Percy et al., 2010). Characteristic repetitive, stereotypic hand movements, abnormal or absent speech, and abnormal or absent fine motor skills and ambulation are required in the diagnosis of classic RTT and may be accompanied by microcephaly, intellectual disability, breath-holding, and hyperventilation while awake, scoliosis, and seizures. RTT almost exclusively affects females, with a prevalence of about 1 in 10,000 females by 12 years of age (Laurvick et al., 2006). Pathogenic, loss-of-function variants in the X-linked *MECP2* [MIM: 300005] gene, which encodes the methyl-CpG binding protein 2, are present in 95%–97% of individuals meeting the criteria for classic or typical RTT (Lyst & Bird, 2015; Neul et al., 2010). The majority of pathogenic variants in the *MECP2* gene occur de novo (Hampson et al., 2000; Huppke et al., 2000; Yamashita et al., 2001), with parental studies indicating that up to 96% of these alterations occur on paternal allele (Girard et al., 2001; Trappe et al., 2001; Zhu et al., 2010). Rare males with *MECP2* pathogenic variants present with variable features, ranging from developmental delay to early infantile epileptic encephalopathy (Budden et al., 2005; Chahil et al., 2018; Neul et al., 2019; Reichow et al., 2015).

Variant or atypical RTT, representing about 13% of individuals with RTT, must meet two of four necessary criteria and five of 11 supportive criteria for a clinical diagnosis (Neul et al., 2010; Percy et al., 2007). Whole gene duplication of *MECP2* causes a severe neurodevelopmental phenotype characterized by developmental delay, infantile hypotonia, seizures, feeding difficulty, poor or absent speech, and history of recurrent infections (del Gaudio et al., 2006; Peters et al., 2019). This disorder is separately classified as *MECP2* duplication syndrome and occurs mainly in males. Other syndromes, such as developmental and epileptic encephalopathy type 2 [MIM: 300672], may display overlapping phenotypes with classic RTT symptoms but are caused by pathogenic variants in genes other than *MECP2*. Pathogenic variants in cyclin-dependent kinase-like 5 (*CDKL5*) [MIM: 300203], also X-linked, and forkhead box G1 (*FOXG1)*[MIM: 164874], an autosomal gene, result in the neurodevelopmental disorders *CDKL5* deficiency disorder (CDD) (Weaving et al., 2004) and *FOXG1* disorder (FD) [MIM: 613454] (Ariani et al., 2008; Kortüm et al., 2011; Mitter et al., 2018; Olson et al., 2019), respectively. Although these disorders were originally considered with Rett-like phenotypes, they are now regarded as unique disorders based on their clinical features and molecular etiologies.

X-chromosome inactivation (XCI) is a DNA methylation-mediated gene-silencing process unique to the X chromosome, which ensures appropriate dosage compensation between the sexes in mammals (Augui et al., 2011; Duncan et al., 2018; Patrat et al., 2020). In females, inactivation of one or the other X chromosome occurs early in development and is, in general, a random process (Cohen et al., 2003; Wu et al., 2014). As a result, females inherently have a mosaic pattern of active and inactive X chromosomes in cells throughout the body (Migeon, 2007). XCI skewing occurs when one X chromosome is preferentially inactivated over the other in a nonrandom manner. XCI skewing may be caused by the presence of a mutation in an X-linked gene, usually resulting in preferential inactivation of the X chromosome containing the mutant allele, and/or the presence of X-linked mutations that confer a selective advantage or disadvantage to the cells (Brown & Robinson, 2000; Migeon et al., 1981; Plenge et al., 2002). Previous reports have found ~8.8%–10% of healthy females with an XCI skewing ratio > 80%, and ~ 2% with an XCI skewing ratio > 90% (Amos-Landgraf et al., 2006; Shvetsova et al., 2019).

Since the *MECP2* gene is subject to XCI, phenotypic variability may be influenced by skewed XCI when a pathogenic variant exists on one of its alleles. While most RTT cases are sporadic, unaffected mothers that carry *MECP2* pathogenic variants have been reported, likely protected by highly skewed inactivation of the X chromosome carrying the mutant allele (Wan et al., 1999). Previous studies investigating the parental origin of the inactivated X chromosome in RTT individuals identified the paternally inherited X chromosome as preferentially inactivated in skewed cases (Knudsen et al., 2006). As the majority of de novo *MECP2* alterations occur on the paternal allele (Girard et al., 2001; Trappe et al., 2001; Zhu et al., 2010), this could indicate that a relatively small proportion of cells expressing the mutated copy of *MECP2* can result in RTT in affected individuals with highly skewed XCI patterns. Conversely, inherited *MECP2* mutations are mostly on the maternally inherited X chromosome, which results from either skewing of XCI in the asymptomatic mother or germline mosaicism (Venâncio et al., 2007).

In this study, we analyzed XCI patterns in 320 participants with RTT or RTT-related syndromes and their unaffected mothers from the Natural History of Rett Syndrome & Related Disorders study (NCT02738281). We sought to identify parent-of-origin skewing patterns in the probands and to determine if any relationship exists between the levels of XCI skewing and known mutational status in either *MECP2* or *CDKL5* gene. These results are intended to provide further insight into the pathogenesis of RTT and RTT-related syndromes

2 | MATERIALS AND METHODS

2.1 | Subjects

Peripheral blood samples from 320 duos of RTT or RTT-related syndromes participants and their mothers were collected from the Natural History of Rett Syndrome & Related Disorders (NCT02738281) and Biobanking of Rett Syndrome and Related Disorders (NCT02705677) studies. The *MECP2* (NM_004992.4) or *CDKL5* (NM_003159.3) variants in each patient are listed in Table S1. The biologic samples were collected under the biobanking protocol approved by the relevant Institutional Review Boards of the Rett Syndrome and Related Disorders consortium.

2.2 | DNA preparation and XCI analysis

Genomic DNA from each individual was extracted from peripheral blood using conventional DNA isolation methodology. The XCI pattern was determined by PCR analysis of a polymorphic trinucleotide repeat in the first exon of the androgen receptor (*AR*) gene with or without digestion with the methylation-sensitive enzyme *Hpa*II (Pegoraro et al., 1994). All samples were analyzed as proband-mother pairs. Degree of XCI skewing was calculated as the fractional peak area ratio (expressed as %) for the more strongly amplified allele. Degree of skewing thus varies between 50% and 100%, where 50% reflects a random pattern and 100% a completely skewed pattern. Samples with skewing ratio below 80% were classified as "Randomly inactivated", 80%–90% as "Moderately skewed", and 91–100% as "Highly skewed". The cutoff was set based on the XCI skewing distribution in healthy individuals (Amos-Landgraf et al., 2006; Shvetsova et al., 2019). If an individual was homozygous for their *AR* allele sizes, the XCI status was interpreted as "Uninformative". If results could not be determined due to poor sample quality, the XCI status is interpreted as "No result". Samples with borderline ratios (78%–82% and 89%–93%) were repeated in duplicate and the final ratio was generated based on the average from all three analyses.

AR repeat allele sizes were analyzed and the specific allele with XCI rate over 50% was considered preferentially inactivated in the individual. Parental origin of the inactivated X chromosome in the probands was assessed by comparison of *AR* repeat allele sizes in probands and their mothers. If the two alleles had the same XCI rate (i.e., 50%), the parental origin was interpreted as "Uninformative". If the preferentially inactivated allele was found in the mother, while the other allele (active allele) of the proband was not found, the preferentially inactivated allele was interpreted as "Maternal". If the preferentially inactivated

allele was not found in the mother, while the other allele (active allele) of the proband was found, it was extrapolated and interpreted to be "Paternal". If both proband and mother shared the same two alleles (of either same or different *AR* repeat sizes), the parental origin was interpreted as "Uninformative".

2.3 | Statistical data analysis

Statistical analysis was performed by student *t*-test (two-tail, paired comparison) for duos (mother and proband). For correlation analysis of clinical severity and parental ages, Pearson and Spearman correlation coefficients were measured and *p* value was generated based on standard procedure. Normal probability distribution was generated by Microsoft Excel. The variant analysis in probands was performed by student *t*-test (two-tail, unequal variance).

3 | RESULTS

3.1 | Clinical characteristics of participants

The RTT Natural History cohort used in this study consisted of individuals with classic RTT (261/320 individuals), atypical RTT (26/320 individuals), *MECP2* duplication disorder (2/320 individuals), CDD (16/320), and individuals with a *MEPC2* mutation but not meeting criteria for either classic or atypical RTT (non-RTT *MECP2*) (15/320). Of 320 probands, we were able to obtain XCI results for 263 with informative parental allele inactivation status (Figure 1 and Table 1).

3.2 | Differences in parental origin of XCI skewing in individuals with classic RTT versus CDD

The maternal XCI pattern was studied along with each proband's XCI pattern for individuals with informative results with classic RTT ($n = 220$), and the parental origin of the inactivated allele was determined as described in Materials and Methods section. For the individuals with classic RTT, the majority (82%, 180/220) had their paternal allele preferentially inactivated (Figure 2a, Table 2). In contrast, among individuals with CDD, most probands (83%, 10/12) had their maternal allele preferentially inactivated (Figure 2a, Table 2). Individuals with atypical RTT (58%, 11/19) or non-RTT MECP2 (54%, 6/11) showed intermediate ratio of paternal allele XCI.

In the probands with highly/moderately skewed XCI, the same preferential XCI patterns were observed (for classic RTT, 95% (77/81) paternally skewed; for CDD, 0% (0/3) paternally skewed; for atypical RTT, 63% (5/8) paternally skewed; for non-RTT MECP2, 0% (0/2) paternally skewed) (Figure 2b, Table 2).

3.3 | Identification of XCI patterns in the RTT natural history cohort

For probands with classic RTT, 20 were highly skewed, 69 were moderately skewed, and 151 individuals had random XCI (Table 3). For the CDD participants, none of those with informative results displayed highly skewed inactivation, while three individuals had moderately skewed inactivation and 12 had random inactivation. The average XCI skewing ratio was 73.1 ± 1.3% (Mean ± *SEM*,

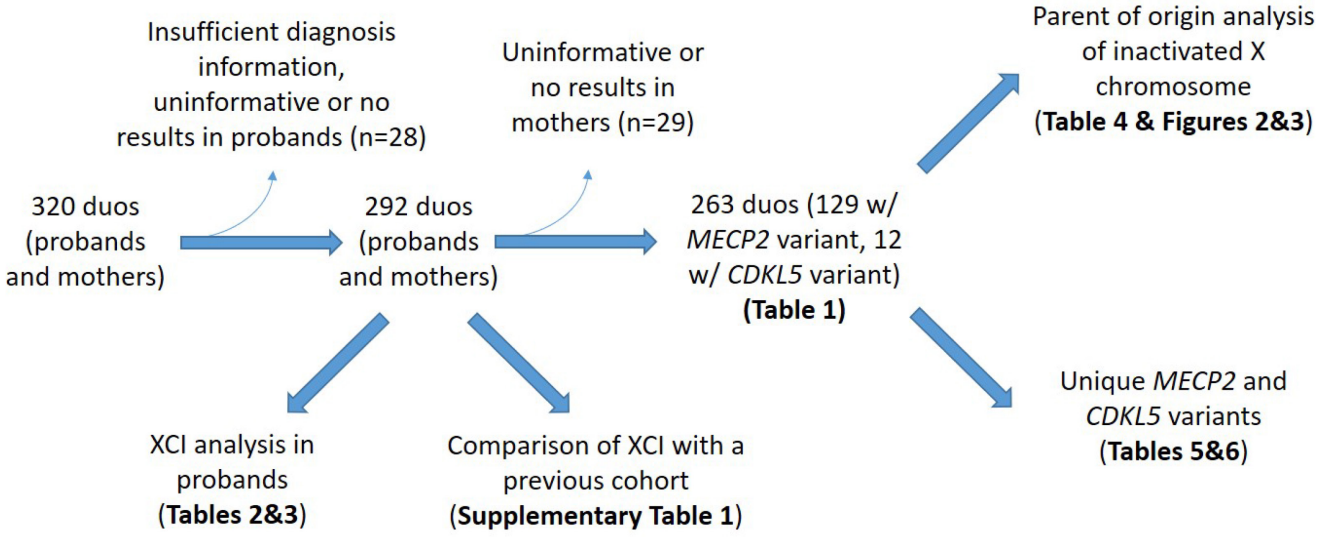

FIGURE 1 Flowchart of the study. XCI, X-chromosome inactivation

TABLE 1 Summary of the total number of probands and the resulting number available for X-chromosome inactivation (XCI) analysis

	XCI informative	Proband XCI uninformative	Proband no XCI results	Insufficient diagnosis information	Mother XCI uninformative
Total	263	24	4	5	29
Classic RTT	220	19	2	n/a	n/a
Atypical RTT	19	2	1	n/a	n/a
MECP2 duplication	1	1	0	n/a	n/a
CDD	12	1	0	n/a	n/a
Other *MECP2*-positive variant but non RTT	11	1	1	n/a	n/a

Abbreviations: CDD, *CDKL5* deficiency disorder; RTT, Rett syndrome.

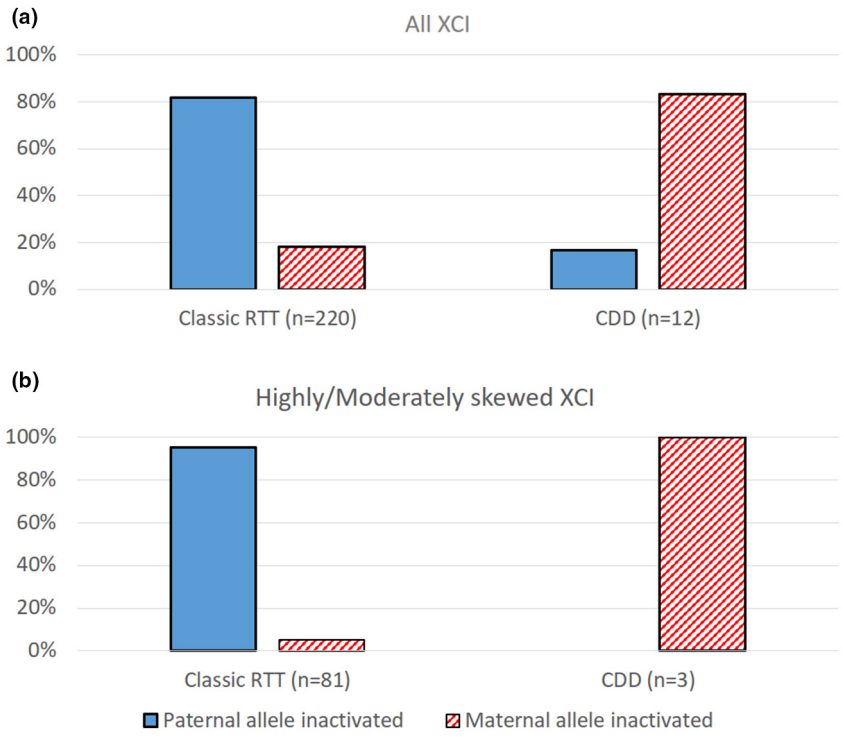

FIGURE 2 Parental origin analysis of preferentially inactivated X-chromosome allele in individuals with classic RTT and CDD. (a) XCI ratio (randomly, moderately, and highly skewed) of individuals with informative parental origin status of preferentially inactivated parental allele. (b) Individuals with highly or moderately skewed XCI and preferentially inactivated parental allele. XCI, X-chromosome inactivation; RTT, Rett syndrome; CDD, *CDKL5* deficiency disorder

TABLE 2 Parent of origin of preferentially inactivated X-chromosome

Probands	Total[a]	Paternal allele inactivated	Maternal allele inactivated
Classic RTT	220	180	40
Atypical RTT	19	11	8
MECP2 duplication	1	0	1
Other *MECP2*-positive variant but non RTT	11	6	5
With *CDKL5* variant	12	2	10

Abbreviation: RTT, Rett syndrome.

[a]Total number of probands with informative XCI results for both proband and mother.

$n = 297$) in the probands, and 68.8 ± 1.3% (Mean ± *SEM*, $n = 292$) in their unaffected mothers, which was determined to be significant ($p = .0006$, student *t*-test, paired comparison). The percentage of mothers with highly/moderately skewed XCI, regardless of their daughter's XCI status, was 22% (64/292).

3.4 | Association between XCI skewing patterns and clinical severity

To confirm whether there is any association between clinical severity and XCI skewing ratios, we performed a correlation analysis using clinical severity scale (CSS) and motor behavior analysis (MBA) (Lane et al., 2011) in patients with classic RTT. Overall, there is no correlation between XCI ratio and CSS (coefficient = −0.02, $n = 240$) or MBA (coefficient = 0.05, $n = 116$). However, in the probands with maternally inactive alleles, there is a weak positive correlation between XCI ratio and CSS (coefficient = 0.35, $n = 40$), and similar correlation between XCI ratio and MBA (coefficient = 0.30, $n = 17$) (Figure 3, Tables S1 and S2), which indicates that increased XCI might be correlated with stronger phenotypes in those individuals. This correlation was not found in probands with paternal allele preferentially inactivated (Figure 3). No definite correlation between XCI ratio and clinical severity was observed in probands with highly/moderately skewed XCI. Other factors, such as potential modifier gene variants, might also influence the XCI-clinical severity associations and are not taken into account in the current analysis.

TABLE 3 The skewing ratio distribution of individuals with classic RTT or CDD disorders

Skewing ratio range (%)	Classic RTT ($n = 240$)	CDD ($n = 15$)	Inactivation ratio interpretation
91–100	20 (8%)	0 (0%)	Highly skewed
80–90	69 (29%)	3 (20%)	Moderately skewed
70–79	63 (26%)	3 (20%)	Randomly inactivated
60–69	46 (19%)	6 (40%)	
50–59	42 (18%)	3 (20%)	

Abbreviations: CDD, *CDKL5* deficiency disorder; RTT, Rett syndrome.

3.5 | Molecular genotype and XCI

MECP2 pathogenic variants and their genotype–phenotype association with RTT have been recorded by multiple databases and summarized by different groups (Ehrhart et al., 2021). The mutational status of this cohort was known prior to current study, with 58 unique *MECP2* alterations (large deletions were grouped together as one variant type) and 16 unique *CDKL5* alterations (Table S3). The spectrum of pathogenic variants in the probands with highly/moderately skewed XCI consisted of 22 unique *MECP2* and three *CDKL5* alterations (Table 4). Eight of the 22 *MECP2* alterations were recurrent, while all *CDKL5* changes were only observed once (Table 4). The eight recurrent *MECP2* pathogenic variants are among the most commonly reported in individuals with RTT (Williamson & Christodoulou, 2006)

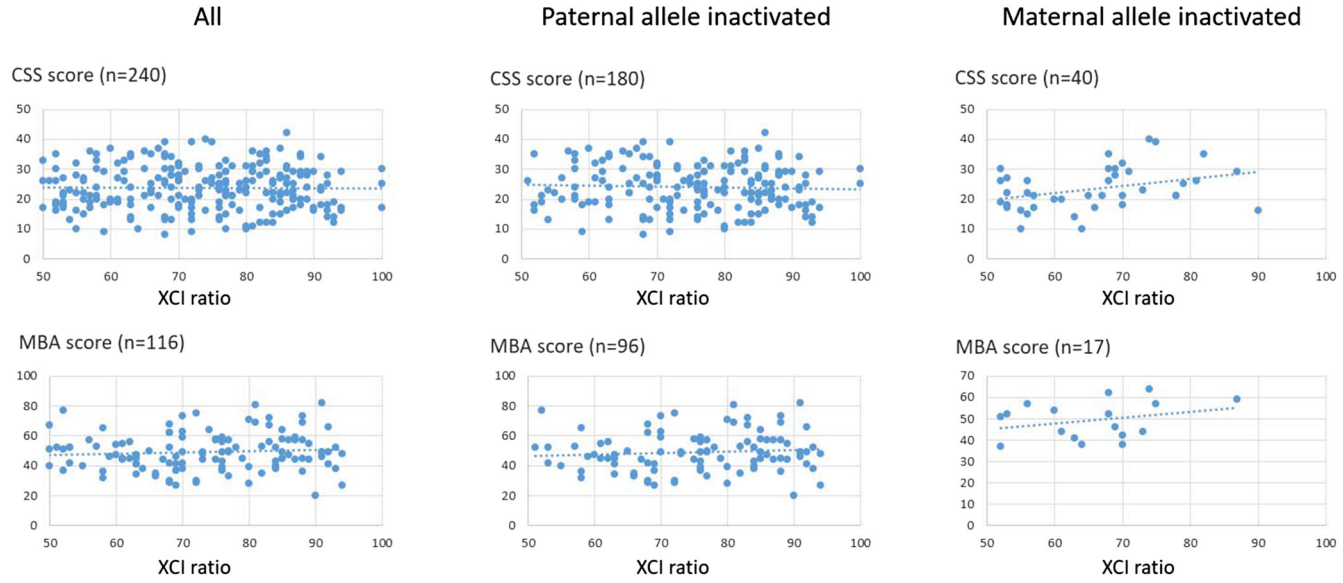

FIGURE 3 Clinical severity scale (CSS) and motor behavior analysis (MBA) scores were used to evaluate the clinical severity in probands with classic RTT. Correlation analysis between clinical severity and XCI in all probands combined, probands with paternal allele preferentially activated, and probands with maternal allele preferentially inactivated revealed weak positive correlation between XCI skewing ratio and CSS scores in individuals with maternal allele preferentially inactivated XCI ($r_s = 0.35$) but not in those with paternal allele preferentially inactivated XCI ($r_s = -0.06$). Dotted trendline is shown for each plot. XCI, X-chromosome inactivation; RTT, Rett syndrome

TABLE 4 *MECP2* and *CDKL5* variants in individuals with highly/moderate skewing XCI

***MECP2* variants**	**Number of probands with the specific variant**	**Percentage of pathogenic mutations reported (Williamson & Christodoulou, 2006)**
MECP2 c.502C > T (p.Arg168Ter)	7 (13.5%)	11.90%
MECP2 c.763C > T (p.Arg255Ter)	7 (13.5%)	10.70%
MECP2 c.808C > T (p.Arg270Ter)	6 (11.5%)	9.60%
[a]*MECP2* LargeDel	5 (9.6%)	N/A
MECP2 c.880C > T (p.Arg294Ter)	4 (7.7%)	8.20%
MECP2 c.316C > T (p.Arg106Trp)	3 (5.8%)	4.80%
MECP2 c.916C > T (p.Arg306Cys)	3 (5.8%)	6.40%
*MECP2*_c.473C > T (p.Thr158Met)	3 (5.8%)	12.20%
MECP2 c.1164_1207del (p.Pro389Ter)	1 (2%)	
MECP2 c.1308_1309del (p.Gln437AlafsTer49)	1 (2%)	
MECP2 c.430A > T (p.Lys144Ter)	1 (2%)	
MECP2 c.57_58ins17	1 (2%)	
MECP2 c.806del (p.Gly269AlafsTer20)	1 (2%)	
MECP2 c.856_859del (p.Lys286ProfsTer2)	1 (2%)	
MECP2 c.1081_1310del (p.Pro361AlafsTer49)	1 (2%)	
MECP2 c.1163_1188del (p.Pro388ArgfsTer8)	1 (2%)	
MECP2 c.454C > G (p.Pro152Ala)	1 (2%)	
MECP2 c.514_515insA (p.Pro172HisfsTer3)	1 (2%)	
MECP2 c.455C > G (p.Pro152Arg)	1 (2%)	
MECP2 c.622C > T (p.Gln208Ter)	1 (2%)	
MECP2 c.917G > A (p.Arg306His)	1 (2%)	
MECP2 c.423C > G (p.Tyr141Ter)	1 (2%)	
***CDKL5* variants**		**Frequency**
CDKL5 c.626C > G (p.Pro209Arg)		1
CDKL5 c.784 T > C (p.Tyr262His)		1
CDKL5 Deletion Xp22.13(18,401,075-18,455,975)		1
Non-RTT with *MECP2* alteration		**Present in highly/ moderately skewed cases**
MECP2 c.1164_1207del (p.Pro389Ter)		Yes
MECP2 c.1328C > T(p.Ala443Val)		No
MECP2 c.808C > T (p.Arg270Ter)		Yes
MECP2 c.923C > T (p.Thr308Ile)		No
MECP2 c.1151_1195del45 (p.Pro385_Pro399del)		No
MECP2 c.1135_1142del (p.Pro379ThrfsTer11)		No
MECP2 c.487G > T (p.Gly163Trp)		No
MECP2 c.397C > T (p.Arg133Cys)		No
MECP2 c.433C > T (p.Arg145Cys)		No

Notes: *MECP2* (NM_004992.4) and *CDKL5* (NM_003159.3) were used.

Abbreviations: RTT, Rett syndrome; XCI, X-chromosome inactivation.

[a]Large intragenic deletions are grouped as one variant type.

(Table 4). The percentage of individuals with those eight variants was increased in the highly/moderately skewed XCI group compared to randomly inactivated XCI group (mean 9.1% vs. 6.7%, $p = .03$) (Table 5). This suggests a potential enrichment of the recurrent *MECP2* variants in individuals with highly/moderately skewed XCI

TABLE 5 Recurrent *MECP2* variants found more often in individuals with skewed XCI patterns (high/moderate vs. random, $p = .03$)

	% of highly/moderately skewed XCI group ($n = 52$)	% of random inactivation group ($n = 92$)
MECP2 p.Arg168Ter	13.5% (7)	8.7% (8)
MECP2 p.Arg255Ter	13.5% (7)	9.8% (9)
MECP2 p.Arg270Ter	11.5% (6)	7.6% (7)
MECP2 LargeDel	9.6% (5)	5.4% (5)
MECP2 p.Arg294Ter	7.7% (4)	3.3% (3)
MECP2 p.Arg106Trp	5.8% (3)	2.2% (2)
MECP2 p.Arg306Cys	5.8% (3)	7.6% (7)
MECP2 p.Thr158Met	5.8% (3)	8.7% (8)
Average	9.1% (4.7)	6.7% (6.2)

Note: *MECP2* (NM_004992.4) was used.

Abbreviation: XCI, X-chromosome inactivation.

patterns, indicating that those variants might be associated with higher levels of XCI skewing. We performed a similar analysis for the probands with both *MECP2* variant and X-inactivation results in RettBASE database (Krishnaraj et al., 2017). Among all the qualified probands ($n = 265$), 91 were with highly/moderately skewed XCI and 174 with random XCI (Table S4). In addition to the eight recurrent variants reported in our cohort, there were three more variants (R133C, P389X, and R106W) which were reported for multiple times in both random XCI group and highly/moderately skewed XCI group. These 11 recurrent variants comprised 74.7% of all the variants found in probands with highly/moderately skewed XCI, while comprising 55.2% in those with random XCI. Thus, the increased prevalence of recurrent *MECP2* variants with highly/moderately skewed XCI in our study is consistent with the larger RettBASE cohort. However, some caution is warranted in making direct comparisons from our XCI data to data generated in different labs, considering that different methods and cutoff values might introduce potential bias in patients tested in the RettBASE database.

Next, we wanted to determine if probands with *MECP2* pathogenic variants and highly/moderately skewed XCI also had mothers that display highly/moderately XCI skewing. Overall, we found 52 probands with highly/moderately skewed XCI patterns and *MECP2* variants (Table S1). Notably, 12 of these probands have mothers who also exhibited highly/moderately skewed XCI patterns, a ratio that is close to previously published data (23% (12/52) in this study, 21% (3/14) by Knudsen et al.) (Knudsen et al., 2006).

To further analyze the pathogenic *MECP2* variants based on their clinical severity, we classified the variants into two groups, Severe and Mild (Table S5). The Severe group includes severe mutations, such as R016W, R168X, R255X, R270X, early truncations (before R270), and large deletions. The Mild group includes less severe mutations such as R133C, R294X, R306C, and C-terminal truncation. In individuals with classic RTT, XCI ratio was slightly increased in Severe group ($n = 64$) compared to Mild group ($n = 29$), yet the difference was not significant (student *t*-test, 75.8 vs. 72.5 [Severe vs. Mild], $p = .23$, Table S5). CSS and MBA scores were both increased in Severe group (CSS, 26.2 vs. 21.9, $p = .01$; MBA, 51.7 vs. 46.5, $p = .06$), which is expected (Table S5). More probands with highly/moderately skewed XCI pattern were observed in Severe group compared to Mild group (42.19% vs. 31.03%). There is no significant difference among probands with highly/moderately skewed XCI and those with random XCI based on their CSS or MBA scores when they are from the same severity group (Table S5).

3.6 | Association between XCI skewing patterns and specific *MECP2* and *CDKL5* alterations

We compared the XCI patterns between individuals with *MECP2* and *CDKL5* pathogenic variants. The average XCI ratio of those with *MECP2* variants was 73.2%, while those with *CDKL5* variants was 68.0%. We then evaluated the possible correlation between genetic etiology and degree of XCI skewing. Out of all the probands with highly/moderately skewed XCI, 52 had variants in *MECP2* (45 classic RTT, six atypical RTT, and one non-RTT *MECP2*), and three had variants in *CDKL5* (Table 6). Among those with *MECP2* pathogenic variants and highly/moderately skewed XCI patterns, 44 exhibited inactivation of the paternally inherited allele (12 highly skewed, 32 moderately skewed) and four

TABLE 6 Clinical distribution of individuals with highly or moderately skewed XCI

	Highly/moderately skewed probands/Total probands[a] (%)
With *MECP2* variant	52/129 (40%)
With *CDKL5* variant	3/12 (25%)
Classic RTT	45/113 (40%)
Atypical RTT	6/8 (75%)
MECP2 duplication	0/1 (0%)
Other *MECP2*-positive variant but non RTT	1/7 (14%)

Abbreviations: RTT, Rett syndrome; XCI, X-chromosome inactivation.

[a]Total number of probands with informative XCI results for both proband and mother.

exhibited inactivation of the maternally inherited allele (all moderately skewed). The three individuals with *CDKL5* pathogenic variants showed preferential inactivation of the maternal allele (Tables 6 and S1).

Among those with pathogenic *MECP2* variants yet no features of RTT (non-RTT *MECP2*), two had preferential inactivation of the maternal allele and five of the paternal allele. Only one participant with *MECP2* duplication syndrome had an XCI result. This individual and her mother both had a random XCI pattern, and the proband had inactivation of the maternal allele (Table S1, Family 156). The same allele was inactivated in the mother. This patient has *MECP2* triplication and her mother is confirmed to be normal (no duplication) by chromosomal microarray analysis.

4 | DISCUSSION

In this study, we describe the XCI skewing patterns of a large cohort of individuals with RTT and RTT-related syndromes from the Natural History of Rett Syndrome & Related Disorders study. The percentage of probands with classic RTT and highly/moderately skewed XCI (>80:20 XCI ratio) was 37.1% (89/240 individuals). This result is in line with data from a previously studied yet unpublished cohort from the same natural history study, in which 44% of probands (no sample from mothers available) with classic RTT were found to have highly/moderately skewed XCI (59/133 individuals, Table S1). We also determined the percentage of mothers with highly/moderately skewed XCI, regardless of their daughter's XCI status, to be 22%. These two percentages are increased from the observed highly/moderately skewed XCI ratio percentages of 5% and 14% in phenotypically unaffected newborns and adults, respectively (Amos-Landgraf et al., 2006), which represents a statistical difference. The increased percentage of mothers with highly/moderately skewed XCI observed in this study is consistent with a previous report of mothers of individuals with RTT (Knudsen et al., 2006); however, the mechanism and significance of this finding remain unclear.

4.1 | Differences in parental origin of preferentially inactivated X chromosomes between classic RTT and CDD

One advantage of this study is that maternal samples were available to determine the parent of origin of the preferentially inactivated X chromosome for probands with RTT or RTT-related syndromes (particularly CDD). Our finding that classic RTT individuals have preferential skewing of their paternal allele is concordant with previous studies (Knudsen et al., 2006; Nielsen et al., 2001). Although the number of individuals in this study with CDD was considerably smaller than those with classic RTT, a sharp contrast was observed for the parental origin of the preferentially inactivated X chromosome, with the majority of CDD probands showing preferential inactivation of the maternal allele (Figure 2).

The finding that the paternal X chromosome is preferentially inactivated in probands with classic RTT is consistent with the expected finding that up to 96% of de novo *MECP2* alterations occur on the paternal allele (Girard et al., 2001; Trappe et al., 2001; Zhu et al., 2010), and suggests that only a small portion of cells expressing the variant-carrying allele is necessary to produce clinical RTT symptoms. One potential explanation for preferential inactivation of the paternal X chromosome in RTT could be due to a survival advantage in blood cells that inactivate X chromosomes with *MECP2* pathogenic variants. Blood samples, by default, serve as a proxy for XCI in brain tissue, which is the critical tissue for individuals with RTT. A few studies, with smaller sample sizes, have performed XCI studies on brain tissue from individuals with Rett syndrome (Shahbazian et al., 2002; Zoghbi et al., 1990). In these studies, only random patterns of XCI were observed in the brain tissue of these individuals ($n = 10$ and $n = 3$). Zoghbi et al. examined the XCI pattern of both neuronal and nonneuronal tissue of the same individuals, and, interestingly, varying degrees of skewing were observed in the nonneuronal tissue, while XCI in brain tissue was random for all three (Zoghbi et al., 1990).

Currently, little is known about the parental origin of the inactivated X chromosome in individuals with CDD,

and, to our knowledge, this is the first study that examines parental origin of XCI in CDD probands. While our initial results suggest a difference in the XCI patterns between individuals with *MECP2* and *CDKL5* pathogenic variants, further studies are required to examine a larger cohort with *CDKL5* pathogenic variants and confirm this observation and its potential relationship to pathogenicity.

4.2 | Association between *MECP2* genotype and XCI skewing

Since the probands in this RTT Natural History study had previous genetic testing results, we investigated the relationship between genotype and X-inactivation pattern. While certain *MECP2* pathogenic variants were seen in multiple individuals with highly/moderately skewed XCI patterns, these alterations are among the most common changes observed in individuals with RTT (Table 4). Additionally, the common p.R270X pathogenic variant was also identified in probands with a moderate pattern of XCI and without RTT features (Williamson & Christodoulou, 2006) (Table 4). The percentage of individuals with the eight common variants was increased in the highly/moderately skewed XCI group compared to the random XCI group. These results suggest the common/recurrent *MECP2* variants might be associated with higher levels of XCI skewing. Interestingly, based on the weak positive correlation between skewing ratio of XCI and clinical severity in the probands with maternal allele inactivation, it is possible that in those individuals, the cells with higher ratio of XCI skewing would cause a more severe phenotype. Given that the maternal allele is inactivated and the paternal allele is active, the de novo pathogenic variants in each individual (located presumptively on paternal allele) would be expressed more than the wild-type allele, consequently causing a more destructive effect on the cell growth and survival. Conversely, in probands with paternal allele inactivated, the maternal allele is active, and the skewing ratio has less of an effect, since wild-type *MECP2* expression is increased over mutant *MECP2* expression. Nevertheless, based on XCI and clinical data presented in this study, the amount of mutant *MECP2* expression still has an effect on clinical outcome of these individuals, which suggests that a small amount of cells with defective *MECP2* can be enough to cause severe clinical phenotype. The timing and location of the cells expressing defective MECP2 might also contribute to the clinical severity.

4.3 | Effects of XCI status on clinical severity in RTT and CDD based on proportion of cells expressing active mutated allele

Given the literature that most of the *MECP2* variants in RTT are de novo (Hampson et al., 2000; Huppke et al., 2000; Yamashita et al., 2001), and most of the de novo variants are on the paternal allele (Girard et al., 2001; Trappe et al., 2001; Zhu et al., 2010), the paternal allele is therefore assumed to carry the *MECP2* pathogenic variants in the majority of RTT cases. The observed preferential inactivation of the paternal allele seems to be the result of a protective mechanism against the mutated allele, by natural selection of the cells with skewed XCI of the paternal allele. However, it is interesting that an individual who is highly skewed toward inactivating the paternal (presumably mutant) chromosome still displays the clinical features of RTT, which is suggested by our data.

One possible explanation is that the effect of specific pathogenic variants might be much larger than XCI, and only a small portion of cells expressing the active mutant allele would be required for clinical manifestation, although it might be difficult to detect the specific effect of XCI with various *MECP2* variants. By stratifying specific mutations, Archer et al. analyzed the correlation between clinical severity in individuals with RTT with two *MECP2* pathogenic variants, and found a statistically significant increase in clinical severity with increase in the proportion of active mutated allele (Archer et al., 2007).

Another possibility is that there might be other genetic modifiers that modulate the phenotypic expression. In the group of non-RTT *MECP2*, we analyzed individuals with pathogenic *MECP2* variants, who do not have RTT and demonstrate a milder phenotype without regression. The typical expectation is that these people would be highly skewed favoring the maternal/wild-type allele and skewing level might also depend on variants. Yet, our results showed that the percentage of inactivated paternal allele (55%, $n = 6/11$) is smaller than that of the individuals with classic RTT (82%, $n = 180/220$). In individuals with highly/moderately XCI skewing, the results were even more striking, with neither of the non-RTT *MECP2* individuals (0/2) showing skewed XCI of paternal allele, versus 95% in classic RTT (77/81). Some of the variants in the non-RTT *MECP2* group are not common and may have milder functional consequences (e.g., R145C, G163W, A443V), but other more severe variants, such as R133C and R270X, could disrupt all of the function of the protein (Ballestar et al., 2000; Yusufzai & Wolffe, 2000). It is surprising that the mutant alleles are not markedly skewed in the non-RTT *MECP2* individuals and that these individuals do not display the RTT phenotypes.

Previous work failed to find significant clinical severity differences between males and females with *CDKL5* pathogenic variants (Demarest et al., 2019; Fehr et al., 2016; MacKay et al., 2021), which was counterintuitive to researchers' expectation, as random XCI could have provided more variability and possibly milder clinical phenotypes in females. The finding that individuals with CDD tend to have maternal allele preferentially inactivated might provide insight into that unusual observation. That is, to manifest CDD, individuals need to have a majority of the cells in the body/brain expressing the pathogenic variant of *CDKL5*, and when there is random XCI in females with *CDKL5* pathogenic variants, they do not manifest the entire CDD phenotype, and appear to be unaffected or asymptomatic. This could explain why a marked severity difference is not noted between males and females with CDD, as only the females with a high proportion of active mutated alleles would show a clinical phenotype, similar to the males with all the cells expressing the mutated allele of *CDKL5*. This finding also has implications for the potential of an unrecognized pool of females with *CDKL5* pathogenic variants that are not being identified because of the observational bias in conducting genetic testing.

4.4 | Limitations, pitfalls, and future perspectives

An important caveat to this study, and most XCI studies, is that the assays were performed in DNA from peripheral blood, which is not typically the affected tissue(s) in X-linked disorders, such as RTT. Recently, Tukiainen et al. assessed XCI across human tissues based on RNA-seq, and demonstrated that the X-inactivation patterns are usually consistent across tissues, despite a few variable escapee behaviors (Tukiainen et al., 2017). These data suggest that blood samples could serve as a reliable nonneuronal tissue to determine XCI. However, it does not rule out the probability that a certain level of discordant XCI skewing exists in different tissues in individuals. Further studies with larger sample sizes involving both neural and nonneural tissues would be helpful to overcome the limitation of interpretation based on blood XCI status reflecting that of brain or other tissues, and explain the possible discordance of XCI status among different tissue types.

Another limitation in this study is the small cohort size of individuals with CDD. CDD has an incidence of 1 in 40,000 to 60,000 newborns, which makes it rarer than RTT and leads to an overall smaller patient population compared to RTT. As more patients with CDD are identified and their XCI status examined, a more thorough statistical analysis could be completed for this group. RettBASE provides a list of 578 individuals with *CDKL5* variants, and only 308 of the probands are considered with pathogenic ($n = 224$), likely pathogenic ($n = 41$), or variant of unknown significance ($n = 43$) alterations. The limited sample size largely restrains the effectiveness of statistical analysis, and the results might be affected by data distribution and dispersion as well (Figure S1). Appropriate parametric or nonparametric methods need to be selected for adequate analysis.

5 | CONCLUSION

The results from our study extend the understanding of the pathogenesis of RTT and RTT-related syndromes by providing insight into the preferential XCI of parental alleles in this patient cohort (paternal preferential inactivation in classic RTT and maternal preferential inactivation in CDD). Our results suggest that in classic RTT the paternal X chromosome frequently carries de novo *MECP2* pathogenic alterations while also being preferentially inactivated in XCI assays, and in CDD the maternal X chromosome is preferentially inactivated. We also identified that recurrent pathogenic *MECP2* variants are more commonly associated with XCI skewing in individuals with classic RTT. Further studies are needed to explore potential clinical implications of these findings.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

ETHICAL STATEMENT
This study has been approved by the Institutional Review Boards of the Rett Syndrome and Related Disorders Consortium.

AUTHOR CONTRIBUTIONS
XF, KMB, FA, JG, and RCC analyzed XI data; AB, TF, RCR, SS, PH, MJ, RH, DL, EDM, TAB, SS, JLN, and AKP part Rett Syndrome and Related Disorders Consortium that see and follow patients; RCC, MJF and AKP conceived of experiments; FA and RCC wrote paper. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are available in the supplementary material of this article

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REFERENCES

Amos-Landgraf, J. M., Cottle, A., Plenge, R. M., Friez, M., Schwartz, C. E., Longshore, J., & Willard, H. F. (2006). X chromosome-inactivation patterns of 1,005 phenotypically unaffected females. *American Journal of Human Genetics*, *79*(3), 493–499. https://doi.org/10.1086/507565

Archer, H., Evans, J., Leonard, H., Colvin, L., Ravine, D., Christodoulou, J., Williamson, S., Charman, T., Bailey, M. E., Sampson, J., de Klerk, N., & Clarke, A. (2007). Correlation between clinical severity in patients with Rett syndrome with a p.R168X or p.T158M MECP2 mutation, and the direction and degree of skewing of X-chromosome inactivation. *Journal of Medical Genetics*, *44*(2), 148–152. https://doi.org/10.1136/jmg.2006.045260

Ariani, F., Hayek, G., Rondinella, D., Artuso, R., Mencarelli, M. A., Spanhol-Rosseto, A., Pollazzon, M., Buoni, S., Spiga, O., Ricciardi, S., Meloni, I., Longo, I., Mari, F., Broccoli, V., Zappella, M., & Renieri, A. (2008). FOXG1 is responsible for the congenital variant of Rett syndrome. *American Journal of Human Genetics*, *83*(1), 89–93. https://doi.org/10.1016/j.ajhg.2008.05.015

Augui, S., Nora, E. P., & Heard, E. (2011). Regulation of X-chromosome inactivation by the X-inactivation Centre. *Nature Reviews Genetics*, *12*(6), 429–442. https://doi.org/10.1038/nrg2987

Ballestar, E., Yusufzai, T. M., & Wolffe, A. P. (2000). Effects of Rett syndrome mutations of the methyl-CpG binding domain of the transcriptional repressor MeCP2 on selectivity for association with methylated DNA. *Biochemistry*, *39*(24), 7100–7106. https://doi.org/10.1021/bi0001271

Brown, C. J., & Robinson, W. P. (2000). The causes and consequences of random and non-random X chromosome inactivation in humans. *Clinical Genetics*, *58*(5), 353–363. https://doi.org/10.1034/j.1399-0004.2000.580504.x

Budden, S. S., Dorsey, H. C., & Steiner, R. D. (2005). Clinical profile of a male with Rett syndrome. *Brain and Development*, *27*, S69–S71. https://doi.org/10.1016/j.braindev.2005.03.018

Chahil, G., Yelam, A., & Bollu, P. C. (2018). Rett syndrome in males: A case report and review of literature. *Cureus*, *10*(10), e3414. https://doi.org/10.7759/cureus.3414

Cohen, D. R. S., Matarazzo, V., Palmer, A. M., Tu, Y., Jeon, O. k.-H., Pevsner, J., & Ronnett, G. V. (2003). Expression of MeCP2 in olfactory receptor neurons is developmentally regulated and occurs before synaptogenesis. *Molecular and Cellular Neuroscience*, *22*(4), 417–429. https://doi.org/10.1016/S1044-7431(03)00026-5

del Gaudio, D., Fang, P., Scaglia, F., Ward, P. A., Craigen, W. J., Glaze, D. G., Neul, J. L., Patel, A., Lee, J. A., Irons, M., Berry, S. A., Pursley, A. A., Grebe, T. A., Freedenberg, D., Martin, R. A., Hsich, G. E., Khera, J. R., Friedman, N. R., Zoghbi, H. Y., … Roa, B. B. (2006). Increased MECP2 gene copy number as the result of genomic duplication in neurodevelopmentally delayed males. *Genetics in Medicine*, *8*(12), 784–792. https://doi.org/10.1097/01.gim.0000250502.28516.3c

Demarest, S. T., Olson, H. E., Moss, A., Pestana-Knight, E., Zhang, X., Parikh, S., Swanson, L. C., Riley, K. D., Bazin, G. A., Angione, K., Niestroj, L. M., Lal, D., Juarez-Colunga, E., & Benke, T. A. (2019). CDKL5 deficiency disorder: Relationship between genotype, epilepsy, cortical visual impairment, and development. *Epilepsia*, *60*(8), 1733–1742. https://doi.org/10.1111/epi.16285

Duncan, C. G., Grimm, S. A., Morgan, D. L., Bushel, P. R., Bennett, B. D., NISC Comparative Sequencing Program, Roberts, J. D., Tyson, F. L., Merrick, B. A., & Wade, P. A. (2018). Dosage compensation and DNA methylation landscape of the X chromosome in mouse liver. *Scientific Reports*, *8*(1), 10138. https://doi.org/10.1038/s41598-018-28356-3

Ehrhart, F., Jacobsen, A., Rigau, M., Bosio, M., Kaliyaperumal, R., Laros, J. F. J., Willighagen, E. L., Valencia, A., Roos, M., Capella-Gutierrez, S., Curfs, L. M. G., & Evelo, C. T. (2021). A catalogue of 863 Rett-syndrome-causing MECP2 mutations and lessons learned from data integration. *Scientific Data*, *8*(1), 10. https://doi.org/10.1038/s41597-020-00794-7

Fehr, S., Downs, J., Ho, G., de Klerk, N., Forbes, D., Christodoulou, J., Williams, S., & Leonard, H. (2016). Functional abilities in children and adults with the CDKL5 disorder. *American Journal of Medical Genetics. Part A*, *170*(11), 2860–2869. https://doi.org/10.1002/ajmg.a.37851

Girard, M., Couvert, P., Carrie, A., Tardieu, M., Chelly, J., Beldjord, C., & Bienvenu, T. (2001). Parental origin of de novo MECP2 mutations in Rett syndrome. *European Journal of Human Genetics*, *9*(3), 231–236. https://doi.org/10.1038/sj.ejhg.5200618

Hampson, K., Woods, C. G., Latif, F., & Webb, T. (2000). Mutations in the MECP2 gene in a cohort of girls with Rett syndrome. *Journal of Medical Genetics*, *37*(8), 610–612. https://doi.org/10.1136/jmg.37.8.610

Huppke, P., Laccone, F., Krämer, N., Engel, W., & Hanefeld, F. (2000). Rett syndrome: Analysis of MECP2 and clinical characterization of 31 patients. *Human Molecular Genetics*, *9*(9), 1369–1375. https://doi.org/10.1093/hmg/9.9.1369

Knudsen, G. P. S., Neilson, T. C. S., Pedersen, J., Kerr, A., Schwartz, M., Hulten, M., Bailey, M. E., & Orstavik, K. H. (2006). Increased skewing of X chromosome inactivation in Rett syndrome patients and their mothers. *European Journal of Human Genetics*, *14*(11), 1189–1194. https://doi.org/10.1038/sj.ejhg.5201682

Kortüm, F., Das, S., Flindt, M., Morris-Rosendahl, D. J., Stefanova, I., Goldstein, A., Horn, D., Klopocki, E., Kluger, G., Martin, P., Rauch, A., Roumer, A., Saitta, S., Walsh, L. E., Wieczorek, D., Uyanik, G., Kutsche, K., & Dobyns, W. B. (2011). The core FOXG1 syndrome phenotype consists of postnatal microcephaly, severe mental retardation, absent language, dyskinesia, and corpus callosum hypogenesis. *Journal of Medical Genetics*, *48*(6), 396–406. https://doi.org/10.1136/jmg.2010.087528

Krishnaraj, R., Ho, G., & Christodoulou, J. (2017). RettBASE: Rett syndrome database update. *Human Mutation*, *38*(8), 922–931. https://doi.org/10.1002/humu.23263

Lane, J. B., Lee, H. S., Smith, L. W., Cheng, P., Percy, A. K., Glaze, D. G., Neul, J. L., Motil, K. J., Barrish, J. O., Skinner, S. A., Annese, F., McNair, L., Graham, J., Khwaja, O., Barnes, K., & Krischer, J. P. (2011). Clinical severity and quality of life in children and adolescents with Rett syndrome. *Neurology*, *77*(20), 1812–1818. https://doi.org/10.1212/WNL.0b013e3182377dd2

Laurvick, C. L., de Klerk, N., Bower, C., Christodoulou, J., Ravine, D., Ellaway, C., Williamson, S., & Leonard, H. (2006). Rett syndrome in Australia: A review of the epidemiology. *The Journal of Pediatrics*, *148*(3), 347–352. https://doi.org/10.1016/j.jpeds.2005.10.037

Lyst, M. J., & Bird, A. (2015). Rett syndrome: A complex disorder with simple roots. *Nature Reviews Genetics*, *16*(5), 261–275. https://doi.org/10.1038/nrg3897

MacKay, C. I., Wong, K., Demarest, S. T., Benke, T. A., Downs, J., & Leonard, H. (2021). Exploring genotype-phenotype relationships in the CDKL5 deficiency disorder using an international dataset. *Clinical Genetics*, *99*(1), 157–165. https://doi.org/10.1111/cge.13862

Migeon, B. R. (2007). Why females are mosaics, X-chromosome inactivation, and sex differences in disease. *Gender Medicine*, *4*(2), 97–105. https://doi.org/10.1016/s1550-8579(07)80024-6

Migeon, B. R., Moser, H. W., Moser, A. B., Axelman, J., Sillence, D., & Norum, R. A. (1981). Adrenoleukodystrophy: Evidence for X linkage, inactivation, and selection favoring the mutant allele in heterozygous cells. *Proceedings of the National Academy of Sciences of the United States of America*, *78*(8), 5066–5070. https://doi.org/10.1073/pnas.78.8.5066

Mitter, D., Pringsheim, M., Kaulisch, M., Plümacher, K. S., Schröder, S., Warthemann, R., Abou Jamra, R., Baethmann, M., Bast, T., Büttel, H. M., Cohen, J. S., Conover, E., Courage, C., Eger, A., Fatemi, A., Grebe, T. A., Hauser, N. S., Heinritz, W., Helbig, K. L., … Brockmann, K. (2018). FOXG1 syndrome: Genotype–phenotype association in 83 patients with FOXG1 variants. *Genetics in Medicine*, *20*(1), 98–108. https://doi.org/10.1038/gim.2017.75

Neul, J. L., Benke, T. A., Marsh, E. D., Skinner, S. A., Merritt, J., Lieberman, D. N., Standridge, S., Feyma, T., Heydemann, P., Peters, S., Ryther, R., Jones, M., Suter, B., Kaufmann, W. E., Glaze, D. G., & Percy, A. K. (2019). The array of clinical phenotypes of males with mutations in methyl-CpG binding protein 2. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics*, *180*(1), 55–67. https://doi.org/10.1002/ajmg.b.32707

Neul, J. L., Kaufmann, W. E., Glaze, D. G., Christodoulou, J., Clarke, A. J., Bahi-Buisson, N., Leonard, H., Bailey, M. E., Schanen, N. C., Zappella, M., Renieri, A., Huppke, P., Percy, A. K., & RettSearch Consortium. (2010). Rett syndrome: Revised diagnostic criteria and nomenclature. *Annals of Neurology*, *68*(6), 944–950. https://doi.org/10.1002/ana.22124

Nielsen, J. B., Henriksen, K. F., Hansen, C., Silahtaroglu, A., Schwartz, M., & Tommerup, N. (2001). MECP2 mutations in Danish patients with Rett syndrome: High frequency of mutations but no consistent correlations with clinical severity or with the X chromosome inactivation pattern. *European Journal of Human Genetics*, *9*(3), 178–184. https://doi.org/10.1038/sj.ejhg.5200600

Olson, H. E., Demarest, S. T., Pestana-Knight, E. M., Swanson, L. C., Iqbal, S., Lal, D., Leonard, H., Cross, J. H., Devinsky, O., & Benke, T. A. (2019). Cyclin-dependent kinase-like 5 deficiency disorder: Clinical review. *Pediatric Neurology*, *97*, 18–25. https://doi.org/10.1016/j.pediatrneurol.2019.02.015

Patrat, C., Ouimette, J.-F., & Rougeulle, C. (2020). X chromosome inactivation in human development. *Development*, *147*(1), dev183095. https://doi.org/10.1242/dev.183095

Pegoraro, E., Schimke, R. N., Arahata, K., Hayashi, Y., Stern, H., Marks, H., Glasberg, M. R., Carroll, J. E., Taber, J. W., & Wessel, H. B. (1994). Detection of new paternal dystrophin gene mutations in isolated cases of dystrophinopathy in females. *American Journal of Human Genetics*, *54*(6), 989–1003.

Percy, A. K., Lane, J. B., Childers, J., Skinner, S., Annese, F., Barrish, J., Caeg, E., Glaze, D. G., & MacLeod, P. (2007). Rett syndrome: North American database. *Journal of Child Neurology*, *22*(12), 1338–1341. https://doi.org/10.1177/0883073807308715

Percy, A. K., Neul, J. L., Glaze, D. G., Motil, K. J., Skinner, S. A., Khwaja, O., Lee, H. S., Lane, J. B., Barrish, J. O., Annese, F., McNair, L., Graham, J., & Barnes, K. (2010). Rett syndrome diagnostic criteria: Lessons from the natural history study. *Annals of Neurology*, *68*(6), 951–955. https://doi.org/10.1002/ana.22154

Peters, S. U., Fu, C., Suter, B., Marsh, E., Benke, T. A., Skinner, S. A., Lieberman, D. N., Standridge, S., Jones, M., Beisang, A., Feyma, T., Heydeman, P., Ryther, R., Kaufmann, W. E., Glaze, D. G., Neul, J. L., & Percy, A. K. (2019). Characterizing the phenotypic effect of Xq28 duplication size in MECP2 duplication syndrome. *Clinical Genetics*, *95*(5), 575–581. https://doi.org/10.1111/cge.13521

Plenge, R. M., Stevenson, R. A., Lubs, H. A., Schwartz, C. E., & Willard, H. F. (2002). Skewed X-chromosome inactivation is a common feature of X-linked mental retardation disorders. *American Journal of Human Genetics*, *71*(1), 168–173. https://doi.org/10.1086/341123

Reichow, B., George-Puskar, A., Lutz, T., Smith, I. C., & Volkmar, F. R. (2015). Brief report: Systematic review of Rett syndrome in males. *Journal of Autism and Developmental Disorders*, *45*(10), 3377–3383. https://doi.org/10.1007/s10803-015-2519-1

Shahbazian, M. D., Sun, Y., & Zoghbi, H. Y. (2002). Balanced X chromosome inactivation patterns in the Rett syndrome brain. *American Journal of Medical Genetics*, *111*(2), 164–168. https://doi.org/10.1002/ajmg.10557

Shvetsova, E., Sofronova, A., Monajemi, R., Gagalova, K., Draisma, H. H. M., White, S. J., Santen, G. W. E., Chuva de Sousa Lopes, S. M., Heijmans, B. T., van Meurs, J., Jansen, R., Franke, L., Kiełbasa, S. M., den Dunnen, J., 't Hoen, P. A. C., BIOS consortium, & GoNL consortium. (2019). Skewed X-inactivation is common in the general female population. *European Journal of Human Genetics*, *27*(3), 455–465. https://doi.org/10.1038/s41431-018-0291-3

Trappe, R., Laccone, F., Cobilanschi, J., Meins, M., Huppke, P., Hanefeld, F., & Engel, W. (2001). MECP2 mutations in sporadic cases of Rett syndrome are almost exclusively of paternal origin. *American Journal of Human Genetics*, *68*(5), 1093–1101. https://doi.org/10.1086/320109

Tukiainen, T., Villani, A.-C., Yen, A., Rivas, M. A., Marshall, J. L., Satija, R., Aguirre, M., Gauthier, L., Fleharty, M., Kirby, A., Cummings, B. B., Castel, S. E., Karczewski, K. J., Aguet, F., Byrnes, A., GTEx Consortium, Lappalainen, T., Regev, A., Ardlie, K. G., … MacArthur, D. (2017). Landscape of X chromosome inactivation across human tissues. *Nature*, *550*(7675), 244–248. https://doi.org/10.1038/nature24265

Venâncio, M., Santos, M., Pereira, S. A., Maciel, P., & Saraiva, J. M. (2007). An explanation for another familial case of Rett syndrome: Maternal germline mosaicism. *European Journal of Human Genetics*, *15*(8), 902–904. https://doi.org/10.1038/sj.ejhg.5201835

Wan, M., Lee, S. S., Zhang, X., Houwink-Manville, I., Song, H. R., Amir, R. E., Budden, S., Naidu, S., Pereira, J. L., Lo, I. F., Zoghbi, H. Y., Schanen, N. C., & Francke, U. (1999). Rett syndrome and beyond: Recurrent spontaneous and familial MECP2 mutations at CpG hotspots. *American Journal of Human Genetics*, *65*(6), 1520–1529. https://doi.org/10.1086/302690

Weaving, L. S., Christodoulou, J., Williamson, S. L., Friend, K. L., McKenzie, O. L., Archer, H., Evans, J., Clarke, A., Pelka, G. J.,

Tam, P. P., Watson, C., Lahooti, H., Ellaway, C. J., Bennetts, B., Leonard, H., & Gécz, J. (2004). Mutations of CDKL5 cause a severe neurodevelopmental disorder with infantile spasms and mental retardation. *American Journal of Human Genetics*, *75*(6), 1079–1093. https://doi.org/10.1086/426462

Williamson, S. L., & Christodoulou, J. (2006). Rett syndrome: New clinical and molecular insights. *European Journal of Human Genetics*, *14*(8), 896–903. https://doi.org/10.1038/sj.ejhg.5201580

Wu, H., Luo, J., Yu, H., Rattner, A., Mo, A., Wang, Y., Smallwood, P. M., Erlanger, B., Wheelan, S. J., & Nathans, J. (2014). Cellular resolution maps of X chromosome inactivation: Implications for neural development, function, and disease. *Neuron*, *81*(1), 103–119. https://doi.org/10.1016/j.neuron.2013.10.051

Yamashita, Y., Kondo, I., Fukuda, T., Morishima, R., Kusaga, A., Iwanaga, R., & Matsuishi, T. (2001). Mutation analysis of the methyl-CpG-binding protein 2 gene (MECP2) in Rett patients with preserved speech. *Brain Dev*, *23*(Suppl 1), S157–S160. https://doi.org/10.1016/s0387-7604(01)00378-3

Yusufzai, T. M., & Wolffe, A. P. (2000). Functional consequences of Rett syndrome mutations on human MeCP2. *Nucleic Acids Research*, *28*(21), 4172–4179. https://doi.org/10.1093/nar/28.21.4172

Zhu, X., Li, M., Pan, H., Bao, X., Zhang, J., & Wu, X. (2010). Analysis of the parental origin of de novo MECP2 mutations and X chromosome inactivation in 24 sporadic patients with Rett syndrome in China. *Journal of Child Neurology*, *25*(7), 842–848. https://doi.org/10.1177/0883073809350722

Zoghbi, H. Y., Percy, A. K., Schultz, R. J., & Fill, C. (1990). Patterns of X chromosome inactivation in the Rett syndrome. *Brain and Development*, *12*(1), 131–135. https://doi.org/10.1016/s0387-7604(12)80194-x

SUPPORTING INFORMATION

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