

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**Skewed X-chromosome inactivation in unsolved neurodevelopmental disease cases can guide re-evaluation For X-linked genes**

**This is a pre print version of the following article:**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1903052> since 2023-12-07T08:03:19Z

*Published version:*

DOI:10.1038/s41431-023-01324-w

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 **SKEWED X-CHROMOSOME INACTIVATION IN UNSOLVED**  
2 **NEURODEVELOPMENTAL DISEASE CASES CAN GUIDE RE-EVALUATION FOR X-**  
3 **LINKED GENES**  
4

5 Chiara Giovenino<sup>1</sup>, Slavica Trajkova<sup>1</sup>, Lisa Pavinato<sup>1</sup>, Simona Cardaropoli<sup>2</sup>, Verdiana Pullano<sup>1</sup>, Enza  
6 Ferrero<sup>1</sup>, Elena Sukarova-Angelovska<sup>3</sup>, Silvia Carestiatto<sup>1</sup>, Paola Salmin<sup>4</sup>, Antonina Rinninella<sup>1,5</sup>,  
7 Anthony Battaglia<sup>1</sup>, Luca Bertoli<sup>6</sup>, Antonio Fadda<sup>6</sup>, Flavia Palermo<sup>1</sup>, Diana Carli<sup>2</sup>, Alessandro  
8 Mussa<sup>2</sup>, Paola Dimartino<sup>7</sup>, Alessandro Bruselles<sup>8</sup>, Tawfiq Froukh<sup>9</sup>, Giorgia Mandrile<sup>10</sup>, Barbara  
9 Pasini<sup>1,4</sup>, Silvia De Rubeis<sup>11-13</sup>, Joseph D. Buxbaum<sup>11-16</sup>, Tommaso Pippucci<sup>17</sup>, Marco Tartaglia<sup>8</sup>,  
10 Marzia Rossato<sup>6</sup>, Massimo Delledonne<sup>6</sup>, Giovanni Battista Ferrero<sup>2</sup>, Alfredo Brusco<sup>1,4</sup>  
11

- 12 1. Department of Medical Sciences, University of Turin, 10126, Turin, Italy  
13 2. Department of Public Health and Pediatrics, University of Turin, 10126, Turin, Italy  
14 3. Department of Endocrinology and Genetics, University Clinic for Pediatric  
15 Diseases, Faculty of Medicine, Ss. Cyril and Methodius University in Skopje, 1000 Skopje,  
16 Republic of North Macedonia  
17 4. Medical Genetics Unit, Città della Salute e della Scienza University Hospital, 10126, Turin,  
18 Italy  
19 5. Department of Biomedical and Biotechnological Sciences, Medical Genetics, University of  
20 Catania, 94124, Catania, Italy  
21 6. Functional Genomics Lab, Department of Biotechnology, University of Verona, 37134,  
22 Verona, Italy  
23 7. Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy  
24 8. Genetics and Rare Diseases Research Division, Ospedale Pediatrico Bambino Gesù, IRCCS,  
25 00146, Rome, Italy.  
26 9. Department of Biotechnology and Genetic Engineering, Philadelphia University, Amman,  
27 Jordan

10. Medical Genetics Unit and Thalassemia Center, San Luigi University Hospital, University of Torino, Orbassano, TO, Italy.
11. Seaver Autism Center for Research and Treatment, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
12. Department of Psychiatry, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
13. The Mindich Child Health and Development Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
14. Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
15. Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
16. Department of Neuroscience, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
17. U.O. Genetica Medica, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italia

Corresponding Author: Professor Alfredo Brusco, University of Torino, Department of Medical Sciences, via Santena 19, 10126, Torino, Italy. Fax: +39 011 236 5926. Email: [alfredo.brusco@unito.it](mailto:alfredo.brusco@unito.it)

Running title: XCI in unsolved NDD

Keywords: chromosome X inactivation; X-linked gene; neurodevelopmental disorders; exome sequencing reanalysis; breakpoint definition; Xdrop technology.

54 **Abstract**

55 Despite major advances in genome technology and analysis, >50% of patients with a  
56 neurodevelopmental disorder (NDD) remain undiagnosed after extensive evaluation. A point in case  
57 is our clinically heterogeneous cohort of NDD patients that remained undiagnosed after FRAXA  
58 testing, chromosomal microarray analysis and trio exome sequencing (ES). In this study, we explored  
59 the frequency of non-random X chromosome inactivation (XCI) in the mothers of male patients and  
60 affected females, the rationale being that skewed XCI might be masking previously discarded genetic  
61 variants found on the X chromosome. A multiplex fluorescent PCR-based assay was used to analyse  
62 the pattern of XCI after digestion with *HhaI* methylation-sensitive restriction enzyme. In families  
63 with skewed XCI, we re-evaluated trio-based ES and identified pathogenic variants and a deletion on  
64 the X chromosome. Linkage analysis and RT-PCR were used to further study the inactive X  
65 chromosome allele, and Xdrop long-DNA technology was used to define chromosome deletion  
66 boundaries. We found skewed XCI (>90%) in 16/186 (8.6%) mothers of NDD males and in 12/90  
67 (13.3%) NDD females, far beyond the expected rate of XCI in the normal population (3.6%,  
68 OR=4.10; OR=2.51). By re-analyzing ES and clinical data, we solved 7/28 cases (25%) with skewed  
69 XCI, identifying variants in *KDM5C*, *PDZD4*, *PHF6*, *TAF1*, *OTUD5* and *ZMYM3*, and a deletion in  
70 *ATRX*. We conclude that XCI profiling is a simple assay that targets a subgroup of patients that can  
71 benefit from re-evaluation of X-linked variants, thus improving the diagnostic yield in NDD patients  
72 and identifying new X-linked disorders.

73

## 74 INTRODUCTION

75 Major advances in exome sequencing (ES) technologies and data analysis, along with the continuing  
76 identification of new disease genes, have greatly contributed to increasing the diagnostic rate of  
77 neurodevelopmental disorders (NDDs). However, despite these advances, from 50-70% of NDD  
78 cases remain unsolved.(1-3) Among the reasons that make molecular diagnostics challenging are: (i)  
79 mutations that create unspecific phenotypes; (ii) difficulties in interpreting variants of uncertain  
80 significance (VUS)(4); (iii) newly-defined diseases that describe few patients, making it difficult to  
81 draw conclusions about phenotypic expansion (4); and (iv) technical limitations of the diagnostic  
82 tools used.(5) Several complementary approaches can be attempted to increase the diagnostic yield  
83 of unresolved NDDs, such as transcriptome analysis and whole genome sequencing. Re-analysis of  
84 ES data has proven to be the most effective, increasing the diagnostic yield by 10-15%.(6)

85 Approximately 6% of NDDs ( 6% in males; 6.9% females) are estimated to be X-linked  
86 (7)where the pathogenic genetic lesions are can often lead to non-random X-chromosome inactivation  
87 (XCI) or skewing. Although this process was identified many decades ago, the actual mechanics and  
88 fine details of XCI have not yet been completely characterized. (8) Physiologically, XCI is random  
89 and results in an approximate equal ratio of cells expressing either maternal or paternal X  
90 chromosome genes. (9) XCI skewing is defined as ‘preferential’ (80:20%) or ‘extreme’ (90:10%),  
91 and is a frequent indication of the presence of an X-linked pathogenetic variant, be it in affected  
92 females (10) or in the mother of male patients. (11)

93 Females heterozygous for an X-linked pathogenetic variant are usually healthy as skewed XCI  
94 favors expression of the wild-type allele, thus protecting females from the deleterious effects of the  
95 variant.(11,12

96 More recently, skewed XCI has also been observed in affected females, (10) likely caused  
97 again by skewing, but this time favouring expression of a deleterious allele that reaches a  
98 pathogenicity threshold. Such female patients are susceptible to X-linked recessive conditions with a  
99 phenotype similar to that observed in male patients. (10) For X-linked dominant conditions, which

100 may be lethal in males, skewing that selects against the deleterious allele has also been observed, thus  
101 decreasing lethality. (13)

102 Of the 281 undiagnosed NNDs, 276 were informative by XCI skewing (90 females + 186  
103 mothers of males). We show that XCI skewing analysis combined with targeted re-evaluation of ES  
104 data and functional analyses can increase the diagnostic yield and identify novel X-linked disease  
105 genes.

106

107 We exploited the analysis of XCI skewing in 276 deeply phenotyped but undiagnosed NDD  
108 patients (90 females + 186 mothers of males). We show that XCI skewing analysis combined with  
109 targeted re-evaluation of ES data and functional analyses can increase the diagnostic yield and  
110 identify novel X-linked disease genes.

## 111 **MATERIAL AND METHODS**

### 112 *Study cohort*

113 From the patient cohort that is part of a large international collaborative study aimed at identifying  
114 the genetic bases of NDDs, we selected 91 affected females, and 189 mothers of affected males with  
115 negative results after trio-ES, CMA (50K Agilent) and FRAXA (see Supplemental materials and  
116 methods).

117

### 118 *X-chromosome inactivation analysis*

119 XCI was tested using DNA extracted from whole blood using an in-house developed protocol. The  
120 XCI pattern was calculated using three independent microsatellite polymorphic markers on the X  
121 chromosome : (i) the CA repeat in the promoter region of the SLIT and NTRK Like Family Member  
122 4 (*SLITRK4*) gene; (ii) the CAG repeat located in exon 1 of the androgen receptor (*AR*) gene(14); (iii)  
123 the CA and AG tandem repeats in the first intron of Proprotein Convertase Subtilisin/Kexin Type 1  
124 Inhibitor (*PCSKIN*) gene (Supplemental Figure 1; supplemental materials and methods).

125

126 *Characterization of ATRX breakpoints by indirect sequence capture coupled with Illumina*  
127 *sequencing*

128 Xdrop-based enrichment and subsequent amplification of enriched DNA was conducted at the  
129 Samplix facility as previously described(15) and subsequently sequenced in 150PE on a  
130 NovaSeq6000 (Illumina) (Supplemental materials and methods).

131

132 *RNA extraction and RT-PCR*

133 To determine which X-chromosome-derived allele (wt or variant) was expressed , we generated and  
134 amplified cDNA from total RNA extracted from patients' fresh blood (Supplemental materials and  
135 methods).

136

137 *X-chromosome inactivation phasing by linkage analysis*

138 Families with variants inherited from the mother and segregated in different subjects were analyzed  
139 by a set of markers to phase the identified variant with the active or inactive X chromosome and  
140 exclude recombination events. The following genetic markers: DXS993, DXS991, DXS986,  
141 DXS1068, DXS990, were amplified using AmpliTaq Gold DNA Polymerase (Thermo Fisher  
142 Scientific) (see Supplemental Material and Methods for details), separated by capillary  
143 electrophoresis on an ABI 3130xl DNA analyzer (Thermo Fisher Scientific) with the GeneScan 500  
144 LYZ size standard (Thermo Fisher Scientific) and analyzed using the GeneMapper software v.4  
145 (Thermo Fisher Scientific).

146

## 147 **RESULTS**

148 *X chromosome inactivation assay*

149 The HUMARA test, based on the analysis of a CAG repeat in the *AR* locus, is currently the gold  
150 standard method for XCI evaluation.(14) As HUMARA is not informative in ~21% of females (due  
151 to homozygosity or alleles of difficult interpretation), (16) we set up a fluorescent multiplex

152 methylation-sensitive PCR assay that simultaneously amplifies the *AR* and two additional  
153 independent polymorphic microsatellites within *SLITRK4* and *PCSKIN* (Supplemental figure 1).  
154 Firstly, we evaluated our assay in a female patient with a balanced Xq25;q24 translocation and  
155 complete XCI skewing (100:0), previously assessed by HUMARA.(17) Complete XCI skewing was  
156 confirmed using the two additional informative loci (Supplemental table 2), demonstrating the  
157 validity of the test.

158 To further test the assay, we evaluated the XCI pattern in four females with an NDD and four healthy  
159 mothers of NDD males: all had received a previous clinical and molecular diagnosis of an X-linked  
160 condition with potential skewed XCI (Supplemental Table 3). XCI skewing (>80%) was documented  
161 in three affected females and three healthy mothers. Our findings were consistent with the literature  
162 which describes the occurrence of XCI skewing in patients with pathogenetic variants in *NAA10*,  
163 *PQBP1*, *MECP2*, and *ACSL4*. (18) Similarly, we found random XCI in a healthy mother  
164 heterozygous for a pathogenic *IDS* variant, which is in line with previous observations indicating  
165 occurrence of skewing in one affected female only. (18) Finally, our female patient with *DDX3X* had  
166 a random XCI as reported for half of the patients affected by MRXSSB (MIM #300958). (18) No or  
167 limited information was available in the literature for *HNRNPH2*, *RBM10*.

#### 168 *XCI screening in unsolved NDD cases*

169 XCI screening was carried out on 281 individuals: 92 females with NDD and 189 healthy mothers of  
170 males with NDD. Probands had been previously studied in depth by CMA/trio-based ES without  
171 finding a genetic lesion. The phenotype of the patients varied: 35% (98/281) had intellectual disability  
172 (ID), 41% (115/281) had autism spectrum disorder (ASD) and 24% (68/281) were complex  
173 syndromic cases with facial dysmorphism (Supplemental Tables 4-5).

174 The XCI assay was informative for at least one marker in 276/281 cases (98.2%; 90/92 female  
175 patients, 186/189 mothers) (Supplemental Tables 6-7).



176 We observed 28 cases with extreme skewing (>90%) i: 12/90 female patients (13.3% vs.  
177 3.6%; OR=4.10; IC 95% 1.85-9.10) and 16/186 mothers (8.6% vs. 3.6%; OR=2.51; IC 95% 1.21-  
178 5.19) (Table1; Supplemental Figure 2-3, Supplemental Table 2).

179

#### 180 *Genetic analyses in XCI-skewed cases*

181 To exclude the possibility that there were genetic causes underlying the observed skewing of XCI,  
182 we sequenced the *XIST* minimal promoter in the above described 28 cases. The rationale of this  
183 method was to uncover rare variants which might cause epigenetic and functional differences between  
184 X chromosomes in females as described by Plenge *et al.*(19). No such variants were found. Thus, we  
185 decided to re-evaluate the ES data, focusing on X-chromosome variants and assessing their relevance  
186 also in the light of newly available clinical data. We identified an X-linked variant consistent with  
187 the phenotype in three females and five males (8/28; 28.6%) (Table 2), as detailed below.

188

#### 189 *A novel TAF1 variant in multiple affected members of family #113*

190 The proband was a 2-year-old girl with global developmental delay and delayed psychomotor  
191 development, and almost completely skewed XCI (95:5; Fig. 1A, Supplemental Table 2). She had a  
192 14-year-old brother with ID, delayed speech and language development, feeding difficulties and  
193 behavioral abnormalities (II.1); a second 11-year-old brother was healthy (II.2). Their mother (I.2)  
194 reported she had had teaching support at school.

195 We found a c.745G>A p.(Gly249Arg) variant in Transcription initiation factor TFIID subunit  
196 1 (*TAF1*), a gene associated with X-linked syndromic intellectual developmental disorder-33  
197 (MRX33; MIM# 300966). MRX33 is characterized by delayed psychomotor development, ID and  
198 typical facial dysmorphisms (Supplemental Table 8).20) The variant was inherited from the mother,  
199 who also showed skewed XCI (90:10). The variant segregated with the affected brother but not with  
200 the healthy brother (Fig.1A, B). The p.(Gly249Arg) variant was absent in the GnomAD database  
201 (ver.2.1.1), and changes a highly conserved nucleotide (PhyloP= 9.37; PhastCons= 1) and amino acid

202 residue, which is maintained from vertebrates to *Drosophila melanogaster* (Fig. 1C). The change was  
203 predicted to be intolerant by MetaDome(21) (Fig 1D) and deleterious by CADD (Phred: 24.7)(22).  
204 Most of the reported likely pathogenic/pathogenic *TAFI* variants are missense substitutions that  
205 cluster between exons 16-30, whereas p.(Gly249Arg) is located in exon 6 (Fig. 1E). However, using  
206 MutScore (which takes into consideration positional clustering of variants already detected in disease-  
207 associated genes and variants found in the population), we noted that the variant reached a predicted  
208 pathogenicity score of 0.96 (maximum 1).(23)  
209 The pathogenicity of p.(Gly249Arg) was also supported by the predicted structural damage triggered  
210 by disallowed phi/psi alert in Missense 3D (24) (Supplemental Material and Methods, Supplemental  
211 figure 4A). Using a series of microsatellite markers on the X chromosome (DXS993, DXS991,  
212 DXS986), we analysed the segregation of the haplotype containing the c.745G>A *TAFI* variant in  
213 the family. Because the haplotype also spanned the AR microsatellite, we could determine the  
214 c.745G>A was located on the inactive X chromosome (X<sub>i</sub>) in both the mother (I.2) and her daughter  
215 (II.3)(Fig. 1A).

216

#### 217 *A rare de novo PHF6 variant affecting the female proband of family NWM24*

218 The proband of family NWM24 was a 7-year-old girl, the second child of healthy parents. At birth,  
219 she was small for gestational age (SGA), and presented global developmental delay, autistic behavior  
220 several dysmorphic features, divergent strabismus and brachy/syndactyly. XCI was completely  
221 skewed (100:0)(Fig. 1F, Supplemental Table 2, Supplemental figure 6A). We identified a *de novo*  
222 c.890G>T p.(Cys297Phe) variant in PHD finger protein 6 (*PHF6*; Fig. 1G), a gene associated with  
223 X-linked recessive Borjeson-Forssman-Lehmann syndrome (BFLS; MIM# 301900). We  
224 reconsidered this previously missed variant because, in the meantime, *de novo* heterozygous variants  
225 have been described in affected females with an overlapping but distinct phenotype including  
226 characteristic facial dysmorphism, dental, finger and toe abnormalities, and linear skin pigmentation  
227 (Supplemental Table 9).( 25, 26) These features are present in our patient. The variant is absent in

228 GnomAD (ver 2.1.1), and changes a very conserved nucleotide (PhyloP= 9.36; PhastCons= 1) and  
229 amino acid (Fig. 1H). Cys297 is located within the PHD-like zinc-binding domain where most *PHF6*  
230 pathogenic/likely pathogenic variants reported in ClinVar map (MutScore= 0.949) map. Cys297 is  
231 considered intolerant to change by MetaDome (Fig. 1I; PF13771; a.a. 239-330; UniProt: Q8IWS0).  
232 Bioinformatic analyses predict the change to be deleterious (CADD Phred= 29.5; REVEL= 0.97;  
233 Table 2). Pathogenicity of p.(Cys297Phe) was also supported by the predicted structural damage, the  
234 amino acid substitution triggering a clash alert(24) (local clash score: wild type=10.47; mutant=35.67;  
235 Supplemental Figure 4B).

236

#### 237 *A KDM5C variant with variable expressivity in family #237*

238 In Family 237, we found a 10-year-old girl with moderate ID and skewed XCI (Fig. 1J). She was the  
239 second of four siblings that included one affected brother (III.1) and two healthy sisters (III.3 and  
240 III.4). The parents were healthy, but several male maternal relatives were reported to have ID. We  
241 found a maternally inherited c.1204G>A p.(Asp402Asn) missense variant in Lysine-specific  
242 demethylase 5C (*KDM5C*), a gene associated with intellectual developmental disorder, X-linked,  
243 syndromic, Claes-Jensen type (MRXSCJ; MIM# 300534; fig. 1K). The variant was shared by the  
244 proband's affected brother (III.1), and one of her healthy sisters (III.3). The variant was predicted to  
245 be deleterious by bioinformatic analysis (CADD Phred: 29.7; REVEL: 0.866), and the affected  
246 residue mapped to a region that was considered intolerant to variation by MetaDome (Fig. 1L), and  
247 conserved from vertebrates to drosophila (Fig. 1M). Another variant affecting this amino acid residue  
248 [c.1204G>T p.(Asp402Tyr)] was previously demonstrated to compromise *KDM5C* stability and  
249 enzymatic activity.(27)

250 MRXSCJ is an X-linked recessive disorder, characterized by DD/ID with clinical  
251 heterogeneity in affected males.(28) Recurrent features include short stature, microcephaly,  
252 hyperreflexia and aggressive behavior, which were present both in the proband and her brother (III.1).  
253 Females with variants in *KDM5C*, as in case III.2, have only recently been found to be associated

254 with incomplete penetrance and a variable phenotype ranging from mild to severe ID (Supplemental  
255 Table 10).(28) The presence of both a male and female in this family initially led us to discard X-  
256 linked genes.

257 By determining the phase of AR alleles and the *KDM5C* alleles by linkage analysis, we  
258 demonstrated that the affected sister (III.2) had a preferentially active mutant allele (90%);  
259 conversely, the unaffected sister (III.3) and her mother had a preferentially inactive mutant allele (Fig.  
260 1J).

261

#### 262 *A genomic ATRX deletion characterized by the Xdrop method in family #236*

263 In family 236, the proband was a 13-year-old boy with a long diagnostic odyssey (Fig. 2A). At 3  
264 years of age, he presented with hypotonia, DD/ID and dysmorphisms. The phenotype was compatible  
265 with mental retardation-hypotonic facies syndrome (MRXFH1, MIM# 309580); however, ES was  
266 negative for an intragenic *ATRX* pathogenic variant (MIM\* 300032). We found complete XCI  
267 skewing (100:0) in the mother, prompting us to re-evaluate the genetic data. By visually inspecting  
268 the ES reads using IGV(29), we noticed no coverage of exons 3 and 4 of *ATRX* (Fig. 2B upper panel),  
269 suggesting the presence of an intragenic deletion. According to linkage analysis, the  $X_i$  chromosome  
270 in the mother carried the haplotype with the deletion (Fig.2A).

271 For in-depth characterization of the deletion, we used the Indirect Sequence Capture (Xdrop  
272 technology)( 30,31), a powerful method for characterizing specific genomic regions. We enriched for  
273 a region of ~100 kb within the *ATRX* gene, spanning the deletion. This region was subsequently  
274 sequenced at high coverage using the Illumina NGS platform. The analysis identified the breakpoints  
275 of the deletion (Supplemental Figure 5), with an uncertainty of 3 bp, identical on both sides of the  
276 interrupted region (hg38; chrX:77,697,545-77,703,516; chrX:77,697,542-77,703,513) (Fig. 2B).  
277 Remapping of the Illumina reads on the reconstructed sequence demonstrated perfect alignment,  
278 without mismatches, thus confirming the correctness of the breakpoints (Figure 2B, lower panel). The

279 deletion of 5,971 bp was confirmed by Sanger sequencing using flanking PCR primers and shown to  
280 be inherited from the mother (Fig. 2C, D).

281

#### 282 *PDZD4: a possible novel NDD gene in family NWM25*

283 In family NWM25, we identified a mother of an affected boy with 90:10 XCI (Fig 2E, Supplemental  
284 Table 2). Since the age of two, the son presented symptoms of DD, followed by the development of  
285 kyphoscoliosis with pectus excavatum, hyperelastic skin and joints, persistent hand tremors, facial  
286 dysmorphisms and polymicrogyria by brain MRI. Two maternal uncles were reported to be affected  
287 by undefined ID.

288 Re-analysis of the X-chromosome variants led to the identification of a c.2190G>C  
289 p.(Lys736Asn) missense variant in the PDZ domain-containing 4 gene (*PDZD4*; MIM\* 300634) (Fig.  
290 2F), which was inherited from the healthy mother. Lys736 is conserved in vertebrates (Fig 2G). Using  
291 linkage analysis, we showed that the haplotype with p.(Lys736Asn) was located on the inactive X-  
292 chromosome (Fig 2E).

293 By exploiting GeneMatcher (<https://genematcher.org/>), we identified a second affected 12-  
294 year-old female (II.1; family TF110, Figure 2H) with a *de novo* frameshift c.10\_16del  
295 p.(Asn4Alafs\*12) variant in *PDZD4*. She presented with an overlapping phenotype, including DD,  
296 microcephaly, ID and dysmorphisms. Also in this family we observed almost complete XCI (95:5)  
297 in the proband, although we could not determine if it the variant was located on the inactive X  
298 chromosome.

299

#### 300 *OTUD5 a novel recently identified gene in family #234*

301 The probands of family 234 were two brothers, aged 16 and 26 years, with mild ID. Their healthy  
302 mother showed complete skewing of XCI (100:0) (Fig. 2I). We identified a missense c.1526C>T  
303 p.(Pro509Leu) variant in OTU DOMAIN-CONTAINING PROTEIN 5 (*OTUD5*), a gene that has recently  
304 been associated with Multiple Congenital Anomalies-Neurodevelopmental syndrome (MCAND;

305 MIM# 301056).(31) MCAND is an X-linked recessive congenital multisystemic disorder  
306 characterized by poor growth, global developmental delay with impaired intellectual development  
307 together with variable abnormalities of the cardiac, skeletal, and genitourinary systems. Disease  
308 severity is highly variable, ranging from death in early infancy to survival into the second or third  
309 decade, suggesting the variant is hypomorphic.(32)

310 We first confirmed that the c.1526C>T allele was indeed expressed in the patient's blood (II.1,  
311 fig. 2J). Next, we compared the cDNA sequence of *OTUD5* from the patient's blood with the *OTUD5*  
312 genomic DNA sequence (gDNA) from the mother and showed that the c.1526C>T allele was not  
313 detectable, suggesting that the skewed X inactivation preferentially silenced the chromosome with  
314 the variant (I.2, fig. 2J). Bioinformatic analyses predicted the variant to be likely pathogenic (Table  
315 2). The substitution of leucine with proline triggers a structural damage with a local clash score of  
316 33.58 versus a score of 15.21 calculated for the wild type protein (Supplemental Figure 4C).(24)

317

318 *ZMYM3: a possible novel NDD gene in family NWM127*

319 **In family NWM127, subject II.1 (Fig. 2K) is a 13-year-old male with DD, moderate ID,**  
320 **cryptorchidism, porosis of bonesosteoporosisand dysmorphic features. He was the fourth child**  
321 **in a family of European ancestry and had an affected sister (II.2) presenting with severe ID due**  
322 **de novo tetrasomy for 15q11.2-q13.1 (MIM \*608636). He was severely hypotonic in early**  
323 **infancy and showed relevant delay in his gross motor milestones (head control at one year and**  
324 **sitting position at five years). He never developed fine motor skills nor acquired toilet training.**  
325 **Dysmorphic features included long face, tall forehead, thick eyebrows, deeply set eyes, broad**  
326 **nasal tip, and low-set flashy ears with cupped formed ear lobes. Upon re-analysis of the ES data,**  
327 **we found a c.1322G>A p.(Arg441Gln) variant in the Zinc Finger, MYM-type 3 (ZMYM3) gene.**  
328 **The mother showed completely skewed XCI (100:0), and similar XCI skewing was found in**  
329 **both the II.2 (90:10) and II.3 unaffected sister (85:15), with the mutant allele preferentially**  
330 **inactive. Furthermore, a p.(Arg441Trp) variant was described by Philips et al. in 2014 in three**

331 male probands with ID and several dysmorphic features shared with our proband II.1, and was  
332 recently confirmed as a recurrent variant in a novel ZMYM3-associated NDD.(33,34) Other  
333 potentially causative ES-detected variants were excluded by functional analysis [e.g., de novo  
334 OSBPL8: c.1535T>C; p.(Val512Ala)] that did not show altered protein activity (Prof. T. Balla,  
335 Bethesda, MD, personal communication)SION

336 Among the mechanisms that cause deviation from random X chromosome inactivation is  
337 selection against cells expressing X chromosomes carrying a pathogenic genetic lesion. We reasoned  
338 that we could take advantage of unbalanced XCI and use it as a guide for re-evaluating clinical and  
339 molecular data in NDD patients in which previous genetic testing failed to make a diagnosis. To test  
340 for XCI, we set up a multiplex fluorescent PCR that simultaneously analyzed the methylation status  
341 of three independent polymorphic markers on the X-chromosome. This assay allowed us to increase  
342 informativeness to >98%, compared to 80% using standard HUMARA.(14)

343 Analysis of 91 female NDD patients and 186 mothers of male NDD patients, previously  
344 undiagnosed by CMA and trio-ES, showed a significant enrichment of subjects with extremely  
345 unbalanced XCI, defined as a >90:10 XCI ratio (28/277, 10%) in line with the results of a similar  
346 study.(10) The extreme skewing of XCI suggested that some of our undiagnosed cases might be  
347 attributable to a gene located on the X chromosome. Proof of principle came from the re-evaluation  
348 of available trio-ES data: by focusing on X-linked coding regions, we identified likely pathogenic  
349 variants in 7/28 cases, solving 25% of NDDs with skewed XCI.

350 In our original survey of 575 NDD cases, we had 28 patients with skewed XCI: nine with X-  
351 linked variants classified as class 4 or 5, and 12 with class 3 variants. Taking into consideration these  
352 28 cases, we estimate that X-linked genes account for 6.4-8.5% (9+28/575; 21+28/757) of the patients  
353 in our survey. Our figures are in agreement with the data from a recent evaluation of the burden of  
354 X-linked coding variation based on 11,044 Developmental Disorder patients, which estimated X-  
355 linked causes in 6.0% of males and 6.9% of females.(7)

356 We previously missed seven variants on the X chromosome for one of three reasons, namely:

357 (i) the gene was not associated with disease at the time of the analysis (*OTUD5*, *PDZD4*, and

358 *ZMYM3*); (ii) the variant was a structural rearrangement missed by ES (*ATRX*) or (iii) the variant was

359 overlooked because it was apparently inconsistent with X-linked segregation, since both males and

360 females were affected (*TAF1*, *PHF6*, and *KDM5C*). In the first category, LINKage-specific-

361 deubiquitylation-deficiency-induced embryonic defects (LINKED) syndrome was first associated

362 with pathogenic *OTUD5* variants only in 2021,(32) whereas *PDZD4* and *ZMYM3* have been at present

363 only proposed to be disease-causing genes. Variants predicted to damage protein structure or function

364 in *ZMYM3* have been identified in patients with NDD in December 2022, (33) whereas *PDZD4* has

365 been at present only proposed to be disease-causing gene. (35)the family with a microdeletion in

366 *ATRX* highlights the importance of searching for genomic rearrangements, exploiting exome data, or

367 performing genome sequencing. In this case the deletion was missed by CMA due to lack of array

368 probes in the deleted tract. The strong clinical suspicion of ID-hypotonic facies syndrome (MIM#

369 309580) prompted us to analyze the coverage of all *ATRX* exons on ES data and to finally identify

370 the deletion of exons 3-4. We also chose to locate the precise breakpoints using a novel method based

371 on the enrichment for targeted resequencing by the Xdrop technology, which combines high-

372 resolution droplet PCR (dPCR) with droplet sorting and Multiple Displacement Amplification in

373 droplets (dMDA). This approach proved to be successful in fine-mapping the deletion breakpoints,

374 narrowing them down from a large putative region of ~20 kb between exons 2 and 5. Given the

375 flexibility of this technology, we expect it to be useful when analysing other similar cases where the

376 large size of the involved region hampers the efficient use of traditional assays for the characterization

377 of structural variations at the single-base resolution. Alternatively, achieving the same results would

378 have required either genome sequencing (more expensive) or a very large sets of PCR-based assays

379 and labor/time intensive work to map the whole 20 kb region, also because the deletion maps within

380 a region rich in repeated sequences. Availability of the deletion boundaries allowed us to set up a

381 simple PCR test to follow segregation of the variant in the family.



382           The X chromosome is often underestimated in the diagnosis of female NDD patients because  
383 of the common misconception that females are less susceptible to X-linked conditions.(18) Although  
384 many X-linked conditions show a profound sex-linked bias, given the specific mechanism of  
385 inheritance, an increasing number of X-linked diseases have been described that occur similarly in  
386 both female and male patients.(18) For example, in families 113 and NWM24, we identified a  
387 missense variant in *TAF1* and *PHF6* in a female; we overlooked/ignored these variants at the first ES  
388 reading because inconsistent with an X-linked recessive disease. However, the literature reported  
389 females with phenotypes consistent with variants in those genes. In the case of *TAF1*, completely  
390 skewed XCI is consistent with other recently described cases where the phenotype, which differs in  
391 females and males, is uniform within each sex.(36) XCI unbalance favors the wild-type allele in both  
392 the mother (mild phenotype) and the affected daughter, leaving the pathogenic mechanism unclear.  
393 We can speculate that: (i) **expression of** 5% of the pathogenic allele is sufficient to cause the  
394 phenotype or (ii) the XCI pattern is different in affected tissues such as brain, where the pathogenic  
395 allele is for some reason more expressed than in blood. In family NWM24, the phenotype associated  
396 with *PHF6* is consistent with the literature that reports two females carrying the *de novo*  
397 p.Cys305Phe, just a few amino acids distant from our proband's variant.(25, 37)

398           Family 237 is another example of X-linked gene complexity: three females carried a missense  
399 variant in *KDM5C* but we detected skewed XCI towards the deleterious allele only in the individual  
400 with the disease phenotype. Segregation analysis showed skewing towards the deleterious allele.  
401 *KDM5C* is known to escape XCI and thus the role of skewing in the phenotype is not clear.(38)

402           Among the various causes of female susceptibility to X-linked conditions, XCI certainly plays  
403 a key role at the penetrance level. Although the mechanism of XCI has been known for a very long  
404 time, evaluating XCI's influence on phenotype remains challenging. In some cases, the presence of  
405 skewed XCI is more easily explained by the selection of cells that inactivate the mutated allele,  
406 expressing only the wild-type allele and gaining a selective advantage during the early stages of  
407 development.(11) Typical examples are mothers heterozygous for *OTUD5*, *ATRX*, *ZMYM3* and

408 *PDZD4* variants who are protected against the deleterious effect of an X-linked pathogenetic variant  
409 by skewed XCI. In females with X-linked conditions, XCI can modulate expression of the  
410 phenotype;(39) it is likely that there are several mechanisms that underlie disease and skewing that  
411 currently escape our understanding and are not always easily identifiable. Finally, in 20 XCI-skewed  
412 cases, we could not identify any potentially causative variant. We hypothesize that the phenotype  
413 might be explained by variants in coding regions not covered by exome sequencing? or by noncoding  
414 variation, such as deep intronic variants that affect splicing or regulatory regions.

415 Taken together, our data conclude that XCI testing is a simple, inexpensive and productive  
416 means for re-evaluating exome data from the X chromosome.

417

#### 418 **DATA AVAILABILITY**

419 All variants have been deposited into ClinVar: SCV002583290, SCV002583291, SCV002583292,  
420 SCV002583293, SCV002583294, SCV002583295, SCV002583296, SCV002583297,  
421 SCV002583298, SCV002583299, SCV002583300, SCV002583301.

422

## 423 REFERENCES

- 424 1. Miles JH. Autism spectrum disorders--a genetics review. *Genet Med*. 2011;13(4):278-94.
- 425 2. Betancur C. Etiological heterogeneity in autism spectrum disorders: more than 100 genetic and  
426 genomic disorders and still counting. *Brain Res*. 2011;1380:42-77.
- 427 3. Satterstrom FK, Kosmicki JA, Wang J, Breen MS, De Rubeis S, An JY, et al. Large-Scale Exome  
428 Sequencing Study Implicates Both Developmental and Functional Changes in the Neurobiology of Autism.  
429 *Cell*. 2020;180(3):568-84.e23.
- 430 4. Hartley T, Lemire G, Kernohan KD, Howley HE, Adams DR, Boycott KM. New Diagnostic  
431 Approaches for Undiagnosed Rare Genetic Diseases. *Annu Rev Genomics Hum Genet*. 2020;21:351-72.
- 432 5. Boycott KM, Hartley T, Biesecker LG, Gibbs RA, Innes AM, Riess O, et al. A Diagnosis for All Rare  
433 Genetic Diseases: The Horizon and the Next Frontiers. *Cell*. 2019;177(1):32-7.
- 434 6. Basel-Salmon L, Orenstein N, Markus-Bustani K, Ruhrman-Shahar N, Kilim Y, Magal N, et al.  
435 Improved diagnostics by exome sequencing following raw data reevaluation by clinical geneticists involved  
436 in the medical care of the individuals tested. *Genet Med*. 2019;21(6):1443-51.
- 437 7. Martin HC, Gardner EJ, Samocha KE, Kaplanis J, Akawi N, Sifrim A, et al. The contribution of X-  
438 linked coding variation to severe developmental disorders. *Nat Commun*. 2021;12(1):627.
- 439 8. Gjaltema RAF, Schwämmle T, Kautz P, Robson M, Schöpflin R, Ravid Lustig L, et al. Distal and  
440 proximal cis-regulatory elements sense X chromosome dosage and developmental state at the Xist locus. *Mol*  
441 *Cell*. 2022;82(1):190-208.e17.
- 442 9. Harper PS. Mary Lyon and the hypothesis of random X chromosome inactivation. *Hum Genet*.  
443 2011;130(2):169-74.
- 444 10. Fieremans N, Van Esch H, Holvoet M, Van Goethem G, Devriendt K, Rosello M, et al. Identification  
445 of Intellectual Disability Genes in Female Patients with a Skewed X-Inactivation Pattern. *Hum Mutat*.  
446 2016;37(8):804-11.
- 447 11. Giorgio E, Brussino A, Biamino E, Belligni EF, Bruselles A, Ciolfi A, et al. Exome sequencing in  
448 children of women with skewed X-inactivation identifies atypical cases and complex phenotypes. *Eur J*  
449 *Paediatr Neurol*. 2017;21(3):475-84.

- 450 12. Plenge RM, Stevenson RA, Lubs HA, Schwartz CE, Willard HF. Skewed X-chromosome inactivation  
451 is a common feature of X-linked mental retardation disorders. *Am J Hum Genet.* 2002;71(1):168-73.
- 452 13. Li D, Strong A, Shen KM, Cassiman D, Van Dyck M, Linhares ND, et al. De novo loss-of-function  
453 variants in X-linked MED12 are associated with Hardikar syndrome in females. *Genet Med.* 2021;23(4):637-  
454 44.
- 455 14. Amos-Landgraf JM, Cottle A, Plenge RM, Friez M, Schwartz CE, Longshore J, et al. X chromosome-  
456 inactivation patterns of 1,005 phenotypically unaffected females. *Am J Hum Genet.* 2006;79(3):493-9.
- 457 15. Blondal T, Gamba C, Møller Jagd L, Su L, Demirov D, Guo S, et al. Verification of CRISPR editing  
458 and finding transgenic inserts by Xdrop indirect sequence capture followed by short- and long-read  
459 sequencing. *Methods.* 2021;191:68-77.
- 460 16. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI  
461 sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome  
462 inactivation. *Am J Hum Genet.* 1992;51(6):1229-39.
- 463 17. Di Gregorio E, Bianchi FT, Schiavi A, Chiotto AM, Rolando M, Verdun di Cantogno L, et al. A de  
464 novo X;8 translocation creates a PTK2-THOC2 gene fusion with THOC2 expression knockdown in a patient  
465 with psychomotor retardation and congenital cerebellar hypoplasia. *J Med Genet.* 2013;50(8):543-51.
- 466 18. Migeon BR. X-linked diseases: susceptible females. *Genet Med.* 2020;22(7):1156-74.
- 467 19. Plenge RM, Hendrich BD, Schwartz C, Arena JF, Naumova A, Sapienza C, et al. A promoter mutation  
468 in the XIST gene in two unrelated families with skewed X-chromosome inactivation. *Nat Genet.*  
469 1997;17(3):353-6.
- 470 20. O'Rawe JA, Wu Y, Dörfel MJ, Rope AF, Au PY, Parboosingh JS, et al. TAF1 Variants Are Associated  
471 with Dysmorphic Features, Intellectual Disability, and Neurological Manifestations. *Am J Hum Genet.*  
472 2015;97(6):922-32.
- 473 21. Wiel L, Baakman C, Gilissen D, Veltman JA, Vriend G, Gilissen C. MetaDome: Pathogenicity  
474 analysis of genetic variants through aggregation of homologous human protein domains. *Hum Mutat.*  
475 2019;40(8):1030-8.
- 476 22. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for  
477 estimating the relative pathogenicity of human genetic variants. *Nat Genet.* 2014;46(3):310-5.

- 478 23. Quinodoz M, Peter VG, Cisarova K, Royer-Bertrand B, Stenson PD, Cooper DN, et al. Analysis of  
479 missense variants in the human genome reveals widespread gene-specific clustering and improves prediction  
480 of pathogenicity. *Am J Hum Genet.* 2022;109(3):457-70.
- 481 24. Ittisoponpisan S, Islam SA, Khanna T, Alhuzimi E, David A, Sternberg MJE. Can Predicted Protein  
482 3D Structures Provide Reliable Insights into whether Missense Variants Are Disease Associated? *J Mol Biol.*  
483 2019;431(11):2197-212.
- 484 25. Zweier C, Kraus C, Brueton L, Cole T, Degenhardt F, Engels H, et al. A new face of Borjeson-  
485 Forssman-Lehmann syndrome? De novo mutations in PHF6 in seven females with a distinct phenotype. *J Med*  
486 *Genet.* 2013;50(12):838-47.
- 487 26. Gerber CB, Fliedner A, Bartsch O, Berland S, Dewenter M, Haug M, et al. Further characterization of  
488 Borjeson-Forssman-Lehmann syndrome in females due to de novo variants in PHF6. *Clin Genet.*  
489 2022;102(3):182-90.
- 490 27. Brookes E, Laurent B, Öunap K, Carroll R, Moeschler JB, Field M, et al. Mutations in the intellectual  
491 disability gene KDM5C reduce protein stability and demethylase activity. *Hum Mol Genet.* 2015;24(10):2861-  
492 72.
- 493 28. Carmignac V, Nambot S, Lehalle D, Callier P, Moortgat S, Benoit V, et al. Further delineation of the  
494 female phenotype with KDM5C disease causing variants: 19 new individuals and review of the literature. *Clin*  
495 *Genet.* 2020;98(1):43-55.
- 496 29. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative  
497 genomics viewer. *Nat Biotechnol.* 2011;29(1):24-6.
- 498 30. Grosso V, Marcolungo L, Maestri S, Alfano M, Lavezzari D, Iadarola B, et al. Characterization of.  
499 *Front Genet.* 2021;12:743230.
- 500 31. Madsen EB, Höijer I, Kvist T, Ameer A, Mikkelsen MJ. Xdrop: Targeted sequencing of long DNA  
501 molecules from low input samples using droplet sorting. *Hum Mutat.* 2020;41(9):1671-9.
- 502 32. Beck DB, Basar MA, Asmar AJ, Thompson JJ, Oda H, Uehara DT, et al. Linkage-specific  
503 deubiquitylation by OTUD5 defines an embryonic pathway intolerant to genomic variation. *Sci Adv.*  
504 2021;7(4).

33. Hiatt SM, Trajkova S, Sebastiano MR, Partridge EC, Abidi FE, Anderson A, et al. Deleterious, protein-altering variants in the transcriptional coregulator ZMYM3 in 27 individuals with a neurodevelopmental delay phenotype. *Am J Hum Genet.* 2022.
34. Philips AK, Sirén A, Avela K, Somer M, Peippo M, Ahvenainen M, et al. X-exome sequencing in Finnish families with intellectual disability--four novel mutations and two novel syndromic phenotypes. *Orphanet J Rare Dis.* 2014;9:49.
35. Leitão E, Schröder C, Parenti I, Dalle C, Rastetter A, Kühnel T, et al. Systematic analysis and prediction of genes associated with monogenic disorders on human chromosome X. *Nat Commun.* 2022;13(1):6570.
36. Cheng H, Capponi S, Wakeling E, Marchi E, Li Q, Zhao M, et al. Missense variants in TAF1 and developmental phenotypes: challenges of determining pathogenicity. *Hum Mutat.* 2019.
37. Wiczorek D, Bögershausen N, Beleggia F, Steiner-Haldenstatt S, Pohl E, Li Y, et al. A comprehensive molecular study on Coffin-Siris and Nicolaides-Baraitser syndromes identifies a broad molecular and clinical spectrum converging on altered chromatin remodeling. *Hum Mol Genet.* 2013;22(25):5121-35.
38. Tukiainen T, Villani AC, Yen A, Rivas MA, Marshall JL, Satija R, et al. Landscape of X chromosome inactivation across human tissues. *Nature.* 2017;550(7675):244-8.
39. Franco B, Ballabio A. X-inactivation and human disease: X-linked dominant male-lethal disorders. *Curr Opin Genet Dev.* 2006;16(3):254-9.

524

525

## 526 **ACKNOWLEDGMENTS**

527 We are grateful to the patients and their families for their participation in this study. We acknowledge  
528 Samplix for the technical support.

529

## 530 **AUTHOR CONTRIBUTIONS**

531 Conceptualization: C.G., S.T, A.B.; Data curation: C.G., S.T, A.B., E.S., F.P., D.C., A.M., T.F., G.M.,  
532 B.P., G.B.F; Formal analysis: C.G., S.T, L.P., S.C., V.P., S.C., A.R., A.B., P.D., A.B., T.P., M.T.;  
533 Investigation: C.G., S.T, L.P., S.C., V.P., S.C., L.B., A.F., P.S., S.D.R., J.B.; Methodology: C.G.,  
534 P.S., L.B., A.F., M.R., M.D., Visualization: Writing-original draft: C.G., S.T, A.B; Writing-review  
535 & editing: E.F., C.G., S.T, A.B, E.S., F.P., D.C., A.M., T.F., G.M., B.P., G.B.F, M.R., M.D.

536

## 537 **FUNDING SUPPORT**

538 This research received funding from the Italian Ministry for Education, University and Research  
539 (Ministero dell'Istruzione, dell'Università e della Ricerca - MIUR) PRIN2020 code 20203P8C3X to  
540 AB, “Associazione E.E. Rulfo per la genetica Medica” to AB, Fondazione Cassa di Risparmio di  
541 Torino to AB, and Fondazione Bambino Gesù (Vite Coraggiose) to MT. Sample collection was  
542 supported by the NIMH (U01MH111661 to JDB).

543

## 544 **ETHICS DECLARATION**

545 All individuals and families from the different institutions agreed to participate in this study and  
546 signed appropriate consent forms. The Ethics Committee of Città della Salute e della Scienza  
547 University Hospital (n. 0060884) and University of Skopje (n. 03-6116/7) approved this study.

548

## 549 **DECLARATION OF INTERESTS**

550 The authors declare no competing interests.

551

552 **Figure legends**

553 **Figure 1. Pedigree and variant analysis in the three families with XCI-skewed female cases.**

554 **A, F, J.**

555 Family trees of families 113, NWM24, and 237. We used X-Chromosome polymorphic  
556 microsatellites to reconstruct the haplotypes and to phase the pathogenetic variant on the  
557 inactive/active X chromosome (percentage indicated below the symbol of tested females; Xi and Xa  
558 indicate the less and the most active X chromosomes). The hyphen above each symbol indicates  
559 whenever DNA was available for genetic testing.

560 **B, G, K.** Sanger sequencing used to confirm the variants in *TAFI* (NM\_004606.5), *PHF6*  
561 (NM\_01015877.2) and *KDM5C* (NM\_004187.5). Representative electropherograms are shown: wild  
562 type (wt); mutant hemizygous (mut); mutant heterozygous (mut/wt). **C, H, M.** Multiple sequence  
563 alignment of the protein amino acid sequences in different species obtained using Marrvel software  
564 for the relevant changed aminoacids (highlighted in yellow; <http://marrvel.org/>)(hs: *Homo sapiens*;  
565 mm: *Mus musculus*; rn: *Rattus norvegicus*; xt: *Xenopus tropicalis*; dr: *Danio rerio*; dm: *Drosophila*  
566 *melanogaster*). **D, I, L.** Tolerance Landscape obtained using MetaDome Web Server visualizes  
567 regional tolerance to normal genetic variation (<https://stuart.radboudumc.nl/metadome/>). The  
568 position of the missense change is indicated for each gene. The Tolerance Landscape Y-axis is  
569 reported as a color scale from blue (position tolerant to variation, T), to yellow (position neutral to  
570 variation, N), to red (position intolerant to variation, I). Below the X- axis, a schematic representation  
571 of the known protein domains (pink). **E.** Localization of the pathogenic (red) and likely pathogenic  
572 (orange) variants reported in the literature for *TAFI* gene in male (upper panel) and female cases  
573 (lower panel). Our patient's variant is shown in black.

574

575 **Figure 2. Pedigrees and variants analysis in the three families with XCI skewed mothers of**  
576 **affected males.**



577 **A, E, H, I, K.** Family trees of families 236, NWM25, TF110, 234 and NWM127. See legend in figure  
578 2A, F, J. **B.** NGS Coverage of *ATRX* exons (schematized above) in ES data (upper panel) and with  
579 Xdrop enrichment (lower panel) in the II.1 proband from family 236. Xdrop enrichment primers (blue  
580 bars below) were designed 5' of the maximum estimated deletion. After enriching DNA for the  
581 region, and subsequent Illumina Sequencing, we were able to precisely identify a 5,971 bp deletion  
582 spanning exons 3 and 4. **C.** Sanger sequencing validation of the *ATRX* deletion in II.1 and his mother  
583 (I.2) using primers flanking the deleted segment (arrows). The deletion breakpoint is shown in panel  
584 **D, F, H.** Sanger sequencing validation of the identified variants. **J.** In family 234, we sequenced the  
585 genomic region (gDNA) and the corresponding transcript (cDNA) in one of the probands (II.1) and  
586 their mother (I.2). The wild-type allele only was detected in both cases in the cDNA, showing that  
587 the pathogenic variant was not expressed and thus located on the inactive X-chromosome. **G.**  
588 Multiple alignment of the protein amino acid sequences in different species as described in the legend  
589 for figure 2 **C, H, M.**  
590

Table 1- Comparison of skewed X-inactivation ratio in adult population, female NDD patients and mothers of male NDD patients.

				Percentage of population with XCI ratio of:		
	n.	Mean	S.D.	<80:20	>90:10	>95:5
<b>Adult Population *</b>	415	52:48:00	19.3	14.2%	3.6%	1.7%
<b>Female NDD</b>	90	68:32:00	14.2	22.2%	13.3%	6.7%
<b>Mother NDD</b>	186	68:32:00	12.8	18.3%	8.6%	3.8%

**Notes.** "Mean" indicates the mean X-chromosome inactivation (XCI) percentage within the group; S.D.: standard deviation. \* Control population data obtained from <sup>12</sup>.

Table 2. Variants found in the eight families with X-skewed females

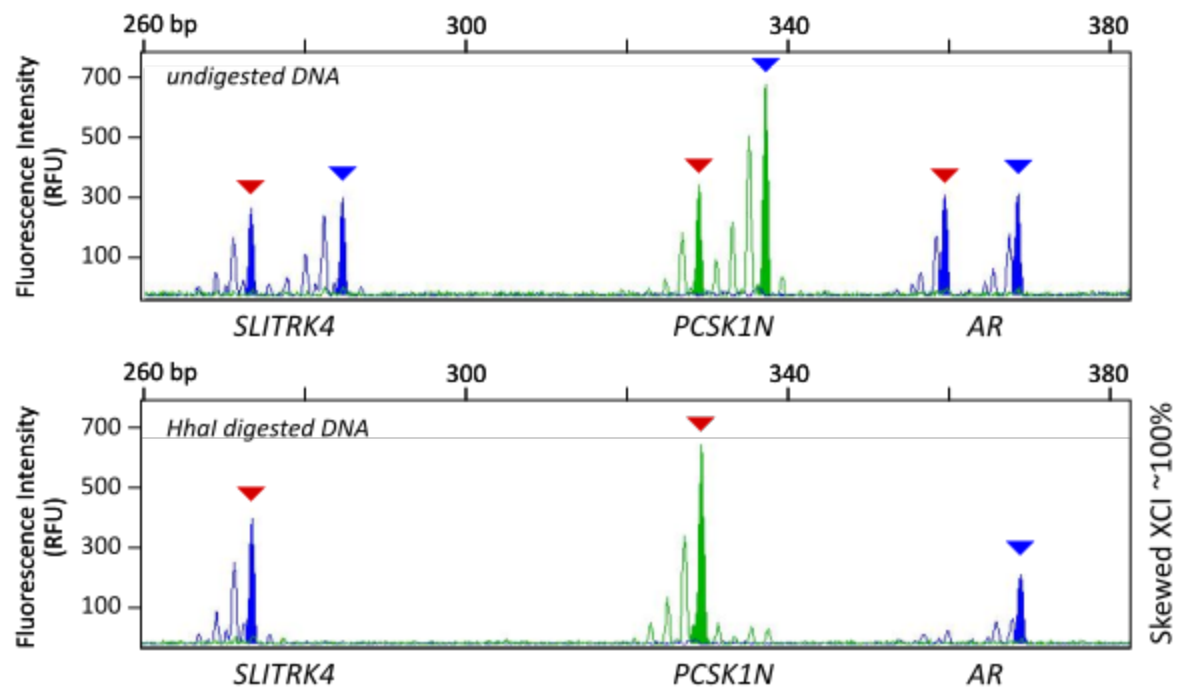
Family_ID	Sex	Diagnosis	OMIM	Inheritance	Gene	Variant c.DNA	Variant protein	SIFT DANN LRT Mutation Taster	ACMG Classification	XCI pattern	Evidence of X- skewing in the literature*	Escape gene**
113	F	MRXS33	300966	maternal	<i>TAF1</i>	NM_004606.5:c.745G>A	p.(Gly249Arg)	D 0.99 D D	Class3; PM2, PP1, PP2, PP3	100	yes	no
NWM24	F	BFLS	301900	<i>de novo</i>	<i>PHF6</i>	NM_001015877.2:c.890G>T	p.(Cys297Phe)	D 0.99 D D	Class5; PM1, PM2, PP2, PP3, PS2	90	yes	no
237	F	MRXSJ	300534	maternal	<i>KDM5C</i>	NM_004187.5:c.1204G>A	p.(Asp402Asn)	D 0.99 D D	Class4; PM2, PM5, PP2, PP1	90	yes	yes
234	M	MCAND	301056	maternal	<i>OTUD5</i>	NM_017602.4:c.1526C>T	p.(Pro509Leu)	D 0.99 N D	Class3, PM2, PP1, PP2	100	yes	no
236	M	MRXFH1	309580	maternal	<i>ATRX</i>	NC_000023.10:g.76953033_76959004del	p.(?)	/ / / /	Class5	100	yes	no
TF110	F		* 300634	<i>de novo</i>	<i>PDZD4</i>	NM_032512.5:c.10_16del	p.(Asn4Alafs*12)	/ / / /	Class4; PM2, PM4, PP3, PS2	96	N.A.	no

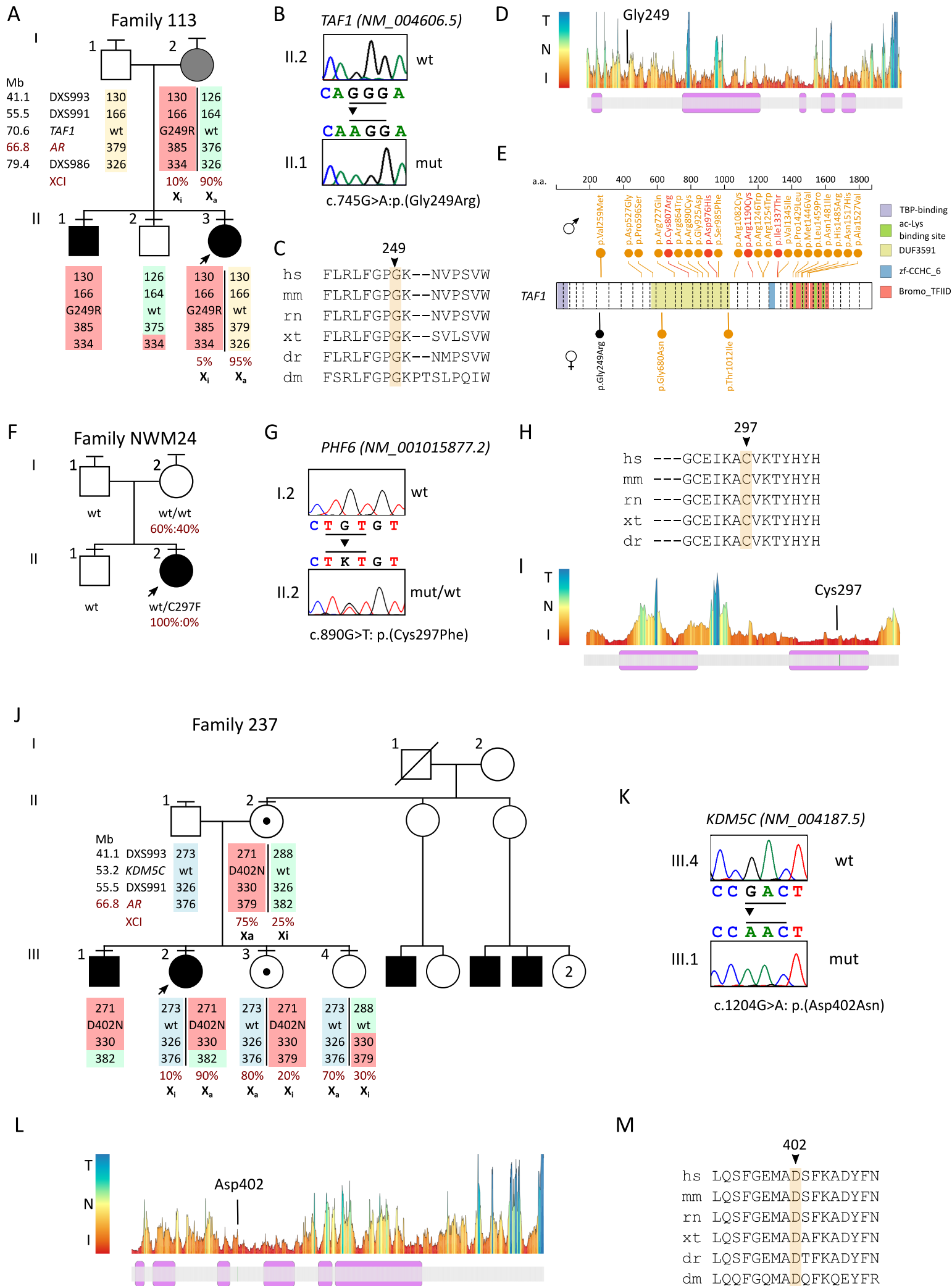
NWM25	M		* 300634	maternal	<i>PDZD4</i>	NM_032512.5:c.2190G>C	p.(Lys736Asn)	D 0.99 D D	Class3, PM2, PP3	91	N.A.	no
NWM127	M		* 300061	maternal	<i>ZMYM3</i>	NM_201599.3:c.1322G>A	p.(Arg441Gln)	T 0.99 / D	Class4, PS1, PM2	100	N.A.	no
Notes. *Data from(17); **Data from(38); D = Deleterious; N =Neutral. Variants submitted to ClinVar (SUB12130121)												

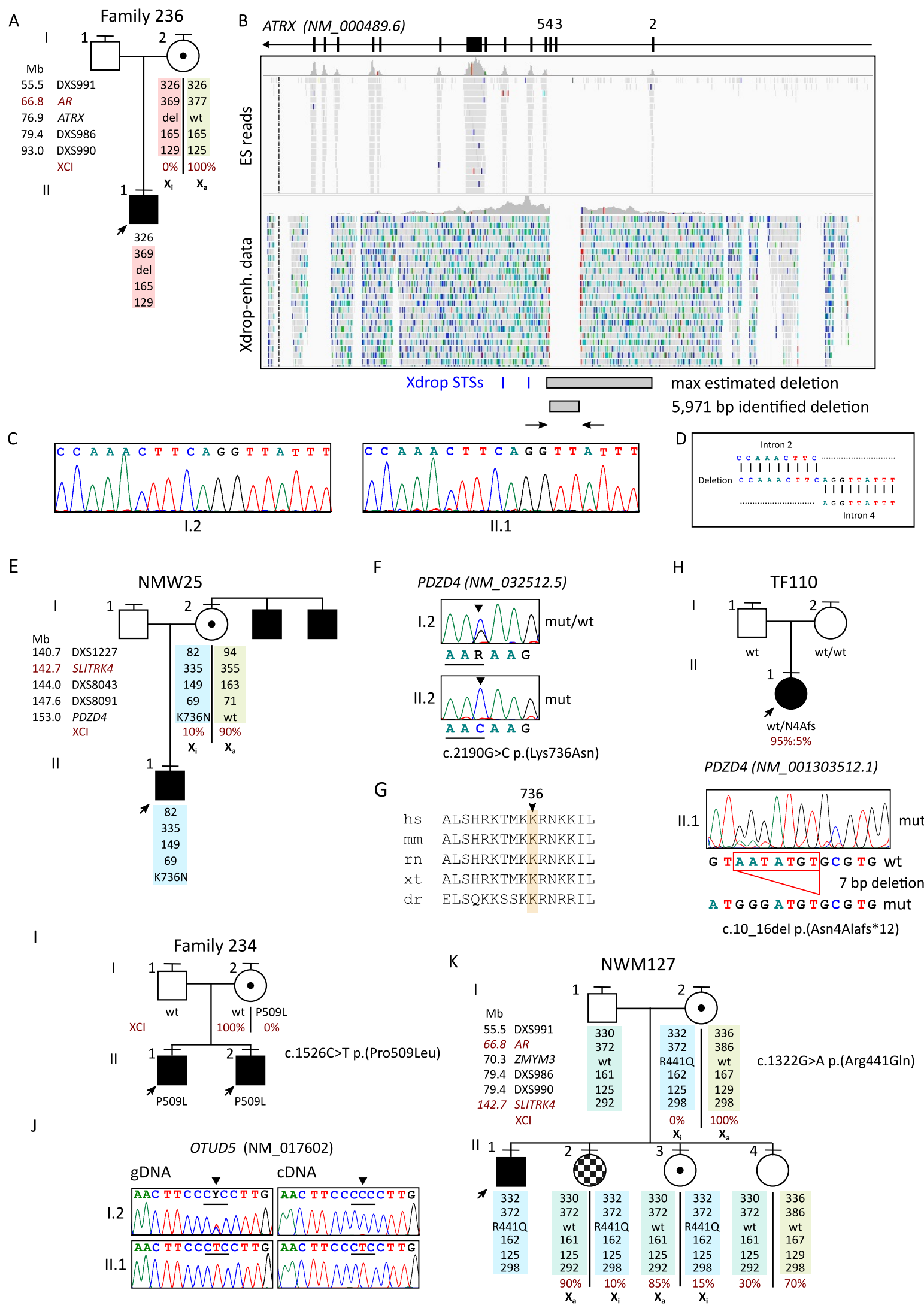
A



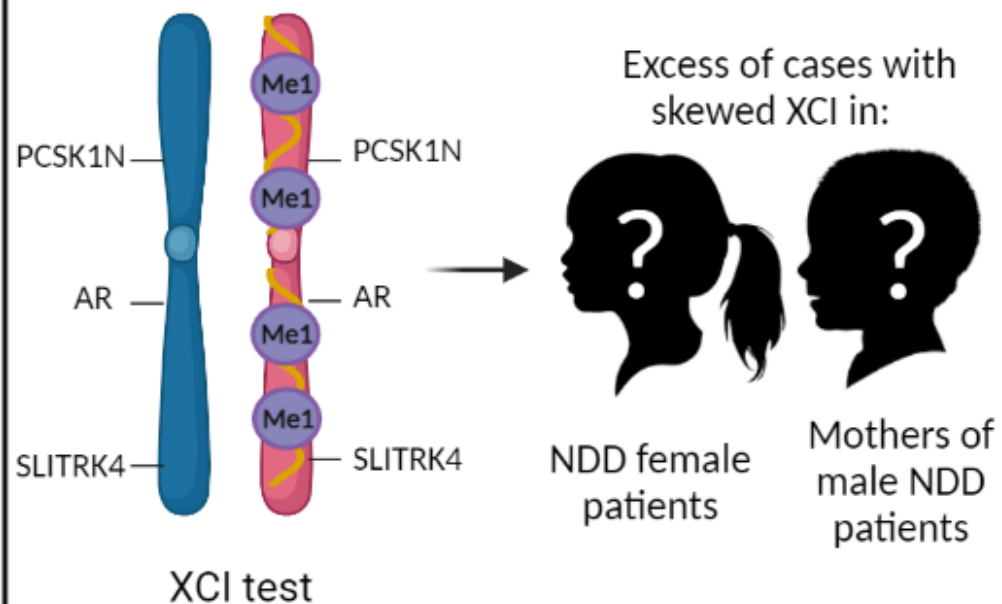
B







Screening for XCI pattern in  
undiagnosed NDD patients



XCI modulate the expression of a pathogenetic  
X-linked variant generating different scenarios

