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

SKEWED X-CHROMOSOME INACTIVATION IN UNSOLVED NEURODEVELOPMENTAL DISEASE CASES CAN GUIDE RE-EVALUATION FOR X-LINKED GENES

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Abstract

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIAL AND METHODS

Study cohort

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

X-chromosome inactivation analysis

XCI was tested using DNA extracted from whole blood using an in-house developed protocol. The XCI pattern was calculated using three independent microsatellite polymorphic markers on the X chromosome : (i) the CA repeat in the promoter region of the SLIT and NTRK Like Family Member 4 (*SLITRK4*) gene; (ii) the CAG repeat located in exon 1 of the androgen receptor (*AR*) gene(14); (iii) the CA and AG tandem repeats in the first intron of Proprotein Convertase Subtilisin/Kexin Type 1 Inhibitor (*PCSK1N*) gene (Supplemental Figure 1; supplemental materials and methods).

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

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

RNA extraction and RT-PCR

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

X-chromosome inactivation phasing by linkage analysis

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

RESULTS

X chromosome inactivation assay

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

methylation-sensitive PCR assay that simultaneously amplifies the *AR* and two additional independent polymorphic microsatellites within *SLITRK4* and *PCSK1N* (Supplemental figure 1). Firstly, we evaluated our assay in a female patient with a balanced Xq25;8q24 translocation and complete XCI skewing (100:0), previously assessed by HUMARA.(17) Complete XCI skewing was confirmed using the two additional informative loci (Supplemental table 2), demonstrating the validity of the test.

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

XCI screening in unsolved NDD cases

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

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

We observed 28 cases with extreme skewing (>90%) i: 12/90 female patients (13.3% vs. 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% 1.21-5.19) (Table1; Supplemental Figure 2-3, Supplemental Table 2).

Genetic analyses in XCI-skewed cases

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

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

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

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

residue, which is maintained from vertebrates to *Drosophila melanogaster* (Fig. 1C). The change was predicted to be intolerant by MetaDome(21) (Fig 1D) and deleterious by CADD (Phred: 24.7)(22). Most of the reported likely pathogenic/pathogenic *TAF1* variants are missense substitutions that cluster between exons 16-30, whereas p.(Gly249Arg) is located in exon 6 (Fig. 1E). However, using MutScore (which takes into consideration positional clustering of variants already detected in disease-associated genes and variants found in the population), we noted that the variant reached a predicted pathogenicity score of 0.96 (maximum 1).(23)

The pathogenicity of p.(Gly249Arg) was also supported by the predicted structural damage triggered by disallowed phi/psi alert in Missense 3D (24) (Supplemental Material and Methods, Supplemental figure 4A).Using a series of microsatellite markers on the X chromosome (DXS993, DXS991, DXS986), we analysed the segregation of the haplotype containing the c.745G>A *TAF1* variant in the family. Because the haplotype also spanned the AR microsatellite, we could determine the c.745G>A was located on the inactive X chromosome (X_i) in both the mother (I.2) and her daughter (II.3)(Fig. 1A).

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

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

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

A KDM5C variant with variable expressivity in family #237

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

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

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

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

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

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

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

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

PDZD4: a possible novel NDD gene in family NWM25

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

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

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

OTUD5 a novel recently identified gene in family #234

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

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

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

ZMYM3: a possible novel NDD gene in family NWM127

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

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

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

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

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

We previously missed seven variants on the X chromosome for one of three reasons, namely: (i) the gene was not associated with disease at the time of the analysis (*OTUD5*, *PDZD4*, and *ZMYM3*); (ii) the variant was a structural rearrangement missed by ES (*ATRX*) or (iii) the variant was overlooked because it was apparently inconsistent with X-linked segregation, since both males and females were affected (*TAF1*, *PHF6*, and *KDM5C*). In the first category, LINKage-specific-deubiquitylation-deficiency-induced embryonic defects (LINKED) syndrome was first associated with pathogenic *OTUD5* variants only in 2021,(32) whereas *PDZD4* and *ZMYM3* have been at present only proposed to be disease-causing genes. Variants predicted to damage protein structure or function in *ZMYM3* have been identified in patients with NDD in December 2022, (33) whereas *PDZD4* has been at present only proposed to be disease-causing gene. (35)the family with a microdeletion in *ATRX* highlights the importance of searching for genomic rearrangements, exploiting exome data, or performing genome sequencing. In this case the deletion was missed by CMA due to lack of array probes in the deleted tract. The strong clinical suspicion of ID-hypotonic facies syndrome (MIM# 309580) prompted us to analyze the coverage of all *ATRX* exons on ES data and to finally identify the deletion of exons 3-4. We also chose to locate the precise breakpoints using a novel method based on the enrichment for targeted resequencing by the Xdrop technology, which combines high-resolution droplet PCR (dPCR) with droplet sorting and Multiple Displacement Amplification in droplets (dMDA). This approach proved to be successful in fine-mapping the deletion breakpoints, narrowing them down from a large putative region of ~20 kb between exons 2 and 5. Given the flexibility of this technology, we expect it to be useful when analysing other similar cases where the large size of the involved region hampers the efficient use of traditional assays for the characterization of structural variations at the single-base resolution. Alternatively, achieving the same results would have required either genome sequencing (more expensive) or a very large sets of PCR-based assays and labor/time intensive work to map the whole 20 kb region, also because the deletion maps within a region rich in repeated sequences. Availability of the deletion boundaries allowed us to set up a simple PCR test to follow segregation of the variant in the family.

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

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

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

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

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

DATA AVAILABILITY

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

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AUTHOR CONTRIBUTIONS

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., 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.; 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., P.S., L.B., A.F., M.R., M.D., Visualization: Writing-original draft: C.G., S.T, A.B; Writing-review & 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.

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ETHICS DECLARATION

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

DECLARATION OF INTERESTS

The authors declare no competing interests.

Figure legends

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

A, F, J.

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

B, G, K. Sanger sequencing used to confirm the variants in *TAF1* (NM_004606.5), *PHF6* (NM_01015877.2) and *KDM5C* (NM_004187.5). Representative electropherograms are shown: wild type (wt); mutant hemizygous (mut); mutant heterozygous (mut/wt). **C, H, M.** Multiple sequence alignment of the protein amino acid sequences in different species obtained using Marrvel software for the relevant changed aminoacids (highlighted in yellow; http://marrvel.org/)(hs: *Homo sapiens*; mm: *Mus musculus*; rn: *Rattus norvegicus*; xt: *Xenopus tropicalis*; dr: *Danio rerio*; dm: *Drosophila melanogaster*). **D, I, L**. Tolerance Landscape obtained using MetaDome Web Server visualizes regional tolerance to normal genetic variation (https://stuart.radboudumc.nl/metadome/). The position of the missense change is indicated for each gene. The Tolerance Landscape Y-axis is reported as a color scale from blue (position tolerant to variation, T), to yellow (position neutral to variation, N), to red (position intolerant to variation, I). Below the X- axis, a schematic representation of the known protein domains (pink). **E**. Localization of the pathogenic (red) and likely pathogenic (orange) variants reported in the literature for *TAF1* gene in male (upper panel) and female cases (lower panel). Our patient's variant is shown in black.

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

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

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

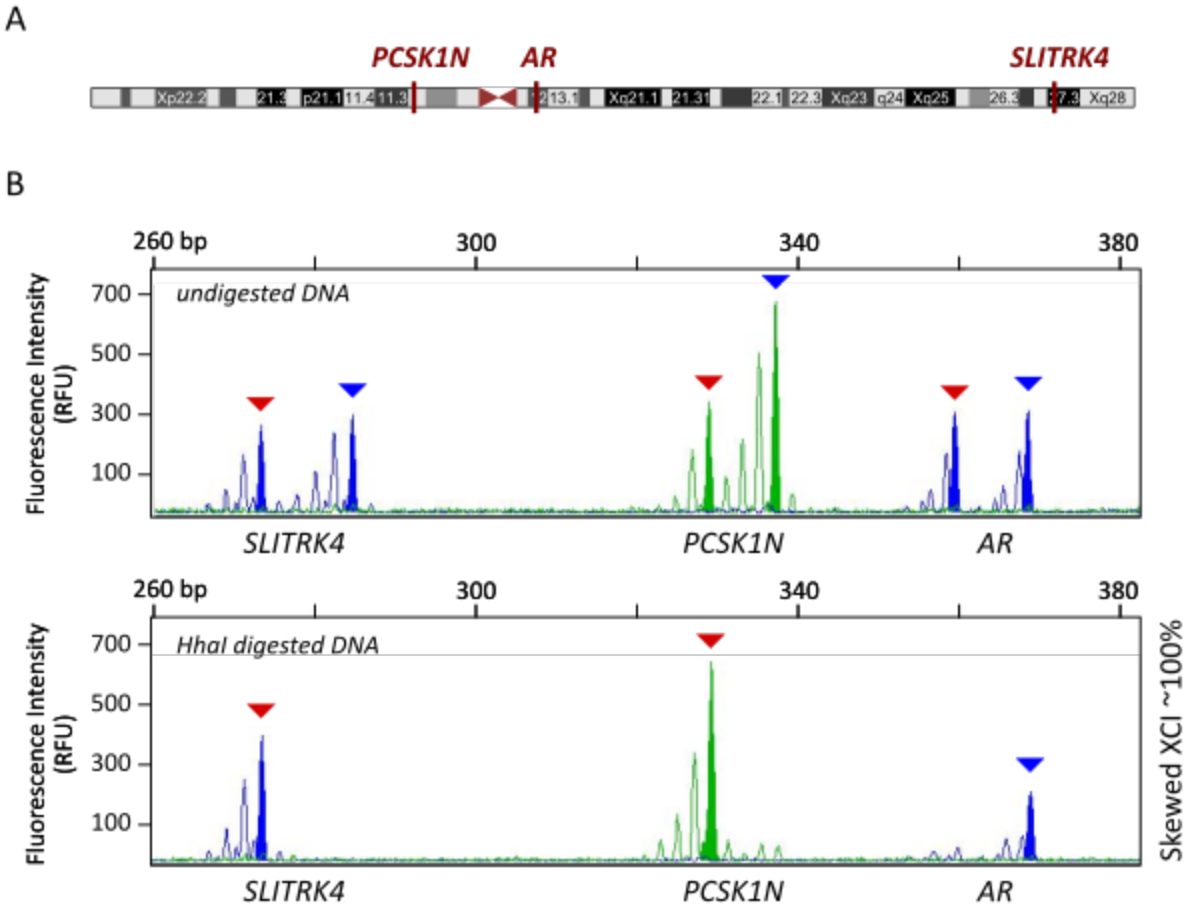
				Percentage of population with XCI ratio of:		
	n.	**Mean**	**S.D.**	**<80:20**	**>90:10**	**>95:5**
Adult Population *	415	52:48:00	19.3	14.2%	3.6%	1.7%
Female NDD	90	68:32:00	14.2	22.2%	13.3%	6.7%
Mother NDD	186	68:32:00	12.8	18.3%	8.6%	3.8%

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

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	*TAF1*	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	*de novo*	*PHF6*	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	*KDM5C*	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	*OTUD5*	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	*ATRX*	NC_000023.10:g.76953033_76959004del	p.(?)	/ / / /	Class5	100	yes	no
TF110	F		* 300634	*de novo*	*PDZD4*	NM_032512.5:c.10_16del	p.(Asn4Alafs*12)	/ / / /	Class4; PM2, PM4, PP3, PS2	96	N.A.	no

NWM25	M		* 300634	maternal	*PDZD4*	NM_032512.5:c.2190G>C	p.(Lys736Asn)	D 0.99 D D	Class3, PM2, PP3	91	N.A.	no
NWM127	M		* 300061	maternal	*ZMYM3*	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)												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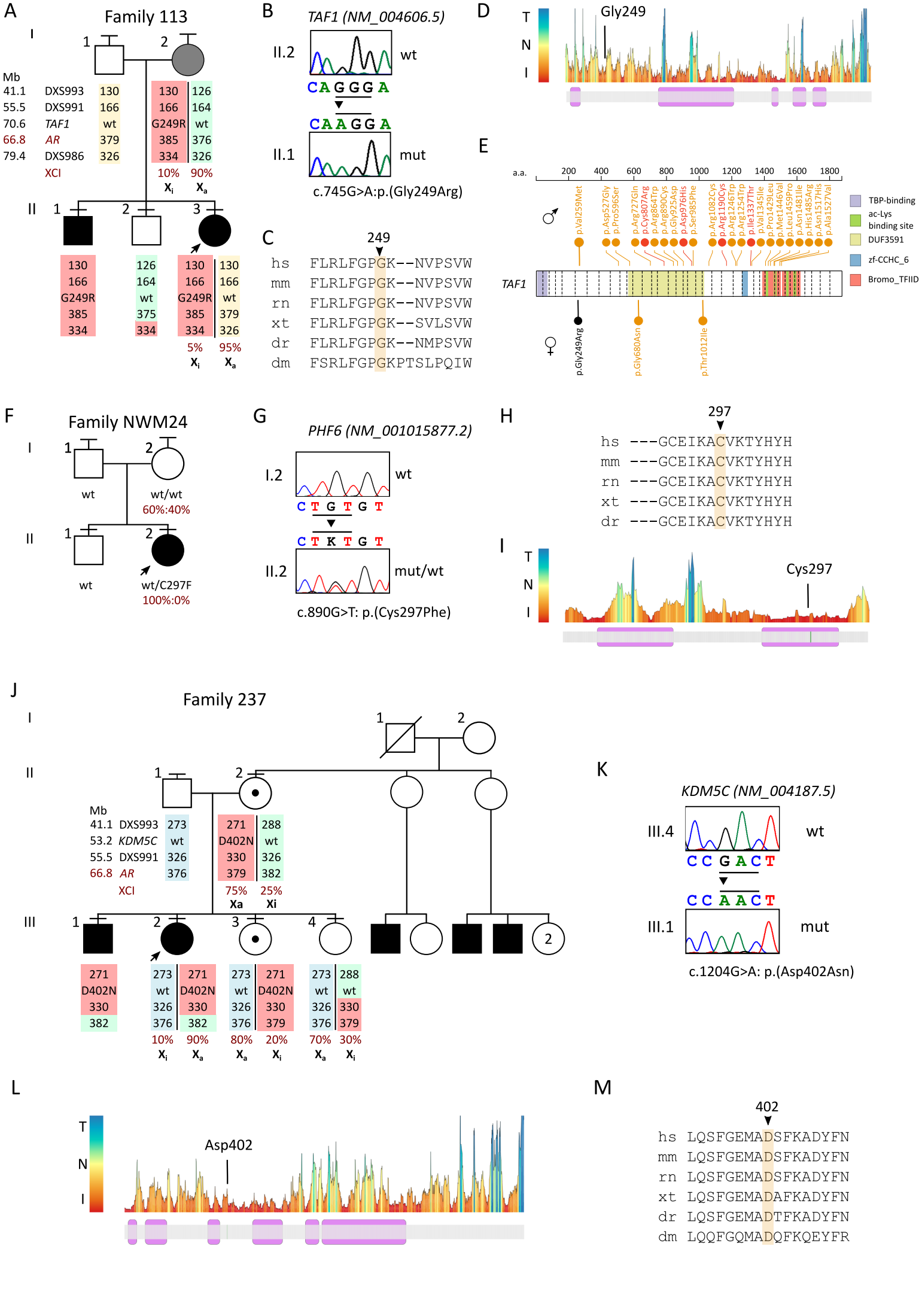
PCSK1N
AR
SLITRK4
B
260 bp
300
340
380
700
500
300
100
Fluorescence Intensity (RFU)
undigested DNA
SLITRK4
PCSK1N
AR
HhaI digested DNA
Skewed XCI ~100%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66.8 AR 379
79.4 DXS986 326
XCI
130 130 126
166 166 164
G249R wt
385 375
334 334
10% 90%
Xi Xa
II
1
2
3
130 126 130 130
166 164 166 166
G249R wt G249R wt
385 375 385 379
334 334 334 326
5% 95%
Xi Xa
B
TAF1 (NM_004606.5)
II.2
wt
C A G G G A
C A A G G A
II.1
mut
c.745G>A:p.(Gly249Arg)
C
249
hs FLRLFGPGK--NVPSVW
mm FLRLFGPGK--NVPSVW
rn FLRLFGPGK--NVPSVW
xt FLRLFGPGK--SVLSVW
dr FLRLFGPGK--NMPSVW
dm FSRLFGPGKPTSLPQIW
D
T
N
I
Gly249
E
a.a.
200 400 600 800 1000 1200 1400 1600 1800
p.Val259Met
p.Asp527Gly
p.Pro596Ser
p.Arg727Gln
p.Cys807Arg
p.Arg864Trp
p.Arg890Cys
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p.Ser985Phe
p.Arg1082Cys
p.Arg1190Cys
p.Arg1246Trp
p.Arg1254Trp
p.Ile1337Thr
p.Val1345Ile
p.Pro1429Leu
p.Met1446Val
p.Leu1459Pro
p.Asn1481Ile
p.His1485Arg
p.Asn1517His
p.Ala1527Val
TAF1
p.Gly249Arg
p.Gly680Asn
p.Thr1012Ile
TBP-binding
ac-Lys binding site
DUF3591
zf-CCHC_6
Bromo_TFIID
F
Family NWM24
I
1
2
wt
wt/wt
60%:40%
II
1
2
wt
wt/C297F
100%:0%
G
PHF6 (NM_001015877.2)
I.2
wt
C T G T G T
C T K T G T
II.2
mut/wt
c.890G>T: p.(Cys297Phe)
H
297
hs ---GCEIKACVKTYHYH
mm ---GCEIKACVKTYHYH
rn ---GCEIKACVKTYHYH
xt ---GCEIKACVKTYHYH
dr ---GCEIKACVKTYHYH
I
T
N
I
Cys297
J
Family 237
I
1
2
II
1
2
Mb
41.1 DXS993 273
53.2 KDM5C wt
55.5 DXS991 326
66.8 AR 376
XCI
271 288
D402N wt
330 326
379 382
75% 25%
Xa Xi
III
1
2
3
4
2
271 273 271 273 271 273 288
D402N wt D402N wt D402N wt wt
330 326 330 326 330 326 330
382 376 382 376 379 376 379
10% 90% 80% 20% 70% 30%
Xi Xa Xa Xi Xa Xi
K
KDM5C (NM_004187.5)
III.4
wt
C C G A C T
C C A A C T
III.1
mut
c.1204G>A: p.(Asp402Asn)
L
T
N
I
Asp402
M
402
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mm LQSFGEMADSFKADYFN
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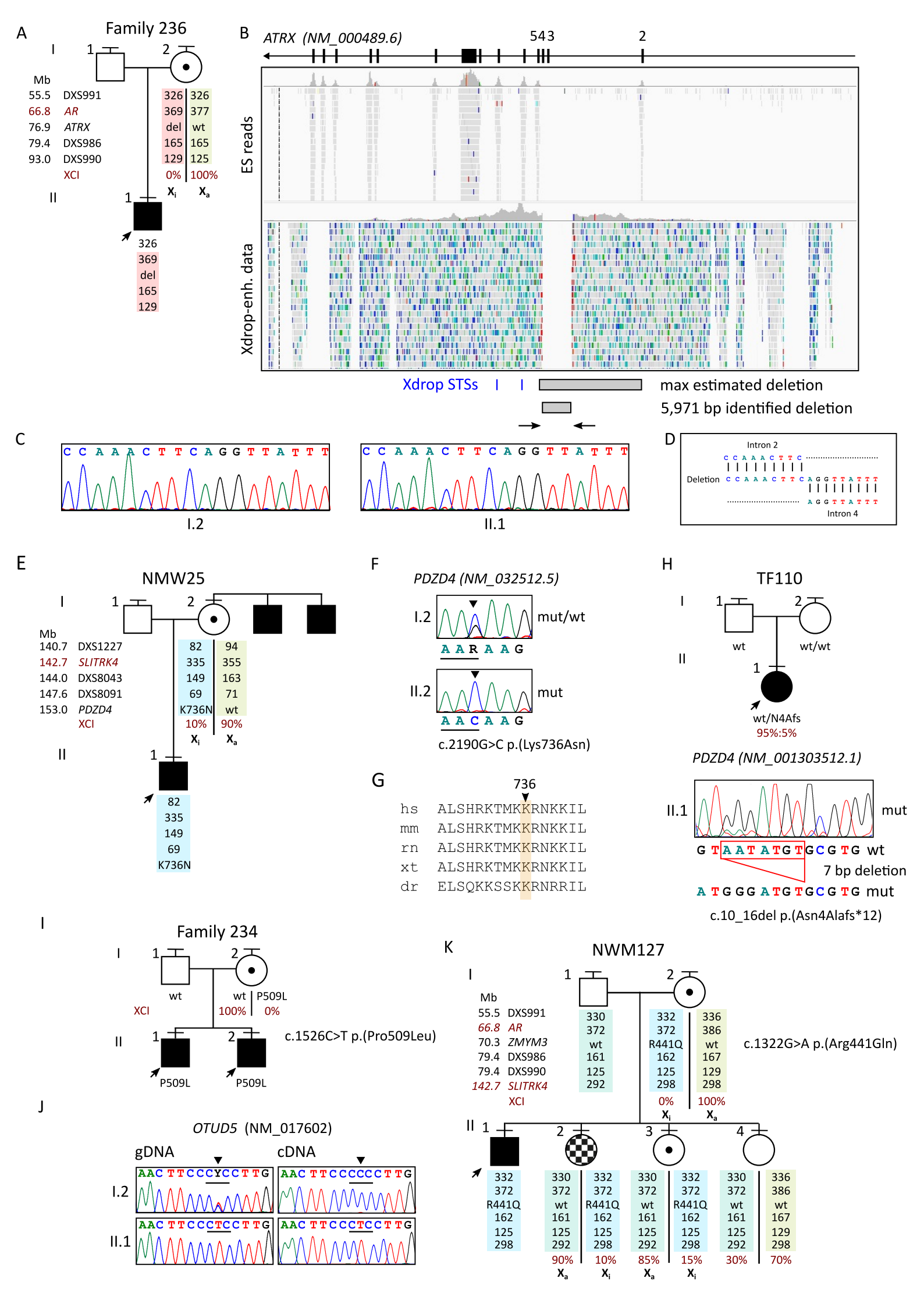
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369 377
del wt
165 165
129 125
0% 100%
Xi Xa
II
1
326
369
del
165
129
B
ATRX (NM_000489.6)
543
2
ES reads
Xdrop-enh. data
Xdrop STSs
max estimated deletion
5,971 bp identified deletion
C
C C A A A C T T C A G G T T A T T T
I.2
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II.1
D
Intron 2
C C A A A C T T C
Deletion
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A G G T T A T T T
Intron 4
E
NMW25
I
1
2
Mb
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142.7 SLITRK4
144.0 DXS8043
147.6 DXS8091
153.0 PDZD4
XCI
82 94
335 355
149 163
69 71
K736N wt
10% 90%
Xi Xa
II
1
82
335
149
69
K736N
F
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mut/wt
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II.2
mut
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mm ALSHRKTMKKRNKKIL
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dr ELSQKKSSKKRNRRIL
H
TF110
I
1
2
wt
wt/wt
II
1
wt/N4Afs
95%:5%
PDZD4 (NM_001303512.1)
II.1
mut
G T A A T A T G T G C G T G wt
7 bp deletion
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I
Family 234
I
1
2
wt
wt P509L
XCI
100% 0%
II
1
2
P509L
P509L
c.1526C>T p.(Pro509Leu)
J
OTUD5 (NM_017602)
gDNA
cDNA
I.2
A A C T T C C C Y C C T T G
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II.1
A A C T T C C C C T C C T T G
A A C T T C C C C T C C T T G
K
NWM127
I
1
2
Mb
55.5 DXS991
66.8 AR
70.3 ZMYM3
79.4 DXS986
79.4 DXS990
142.7 SLITRK4
XCI
330 332 336
372 372 386
wt R441Q wt
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292 298 298
0% 100%
Xi Xa
c.1322G>A p.(Arg441Gln)
II
1
2
3
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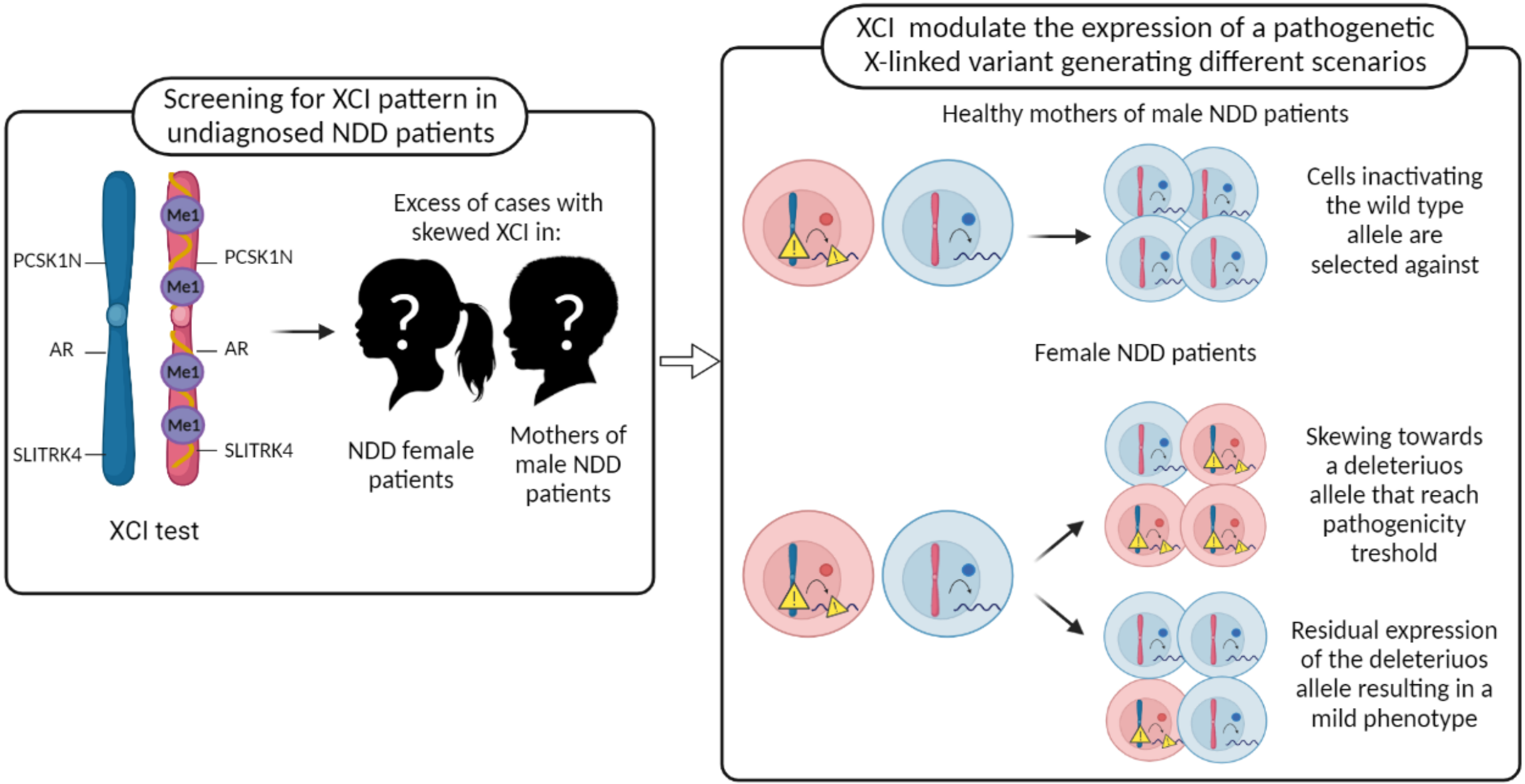
Screening for XCI pattern in undiagnosed NDD patients
PCSK1N
AR
SLITRK4
Me1
Me1
Me1
Me1
PCSK1N
AR
SLITRK4
XCI test
Excess of cases with skewed XCI in:
NDD female patients
Mothers of male NDD patients
XCI modulate the expression of a pathogenetic X-linked variant generating different scenarios
Healthy mothers of male NDD patients
Cells inactivating the wild type allele are selected against
Female NDD patients
Skewing towards a deleteriuos allele that reach pathogenicity treshold
Residual expression of the deleteriuos allele resulting in a mild phenotype