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1 *De novo MECP2* duplication in two females with random X-inactivation and moderate  
2 mental retardation

3

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7

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20

21 Running Title:

22 *MECP2* duplications in mentally retarded females

23

24 **Abstract**

1 Xq28 duplications including *MECP2* are a well known cause of severe mental  
2 retardation in males with seizures, muscular hypotonia, progressive spasticity, poor  
3 speech and recurrent infections that often lead to early death. Female carriers usually  
4 show a normal intellectual performance due to skewed X-inactivation. We report on two  
5 female patients with a *de novo* *MECP2* duplication associated with moderate mental  
6 retardation. In both patients, the *de novo* duplication occurred on the paternal allele, and  
7 both patients show a random X-inactivation, which can be assumed as the triggering  
8 factor for the phenotype. Furthermore, we describe the phenotype, which might be  
9 restricted to unspecific mild to moderate mental retardation with neurological features  
10 in early adulthood.

11

12 Keywords: Xq28 duplication, *MECP2*, X-inactivation, Mental Retardation in Females

13

#### 14 **Introduction**

15 The X-linked Rett syndrome (OMIM #312750) has been known for a long time as a  
16 mental retardation syndrome, with its first clinical description in 1966/1977, and the  
17 identification of its underlying cause with loss-of-function mutations of the *MECP2*  
18 gene in 1999<sup>1,2,3</sup>. The Rett syndrome is almost always sporadic due to *de novo*  
19 mutations and is seen almost exclusively in girls. Typical findings of classic Rett  
20 syndrome are a regression of motor skills, communicative abilities and especially a loss  
21 of purposeful hand movements, accompanied by secondary microcephaly after a period  
22 of (near to) normal development. The few affected males with deleterious *MECP2*  
23 mutations usually present with clinical features consisting of fatal seizures and severe  
24 mental retardation including microcephaly that often leads to death during the first

1 years. The genetically related Lubs syndrome (OMIM #300260) was first described in  
2 1999, and etiologically resolved in 2005 as the *MECP2* duplication syndrome<sup>4,5,6</sup>. Xq28  
3 duplications of different sizes spanning the *MECP2* gene have been identified to date as  
4 a common cause of mental retardation in males. In familial cases with X-linked  
5 pedigrees, the asymptomatic female carriers show a significant skewing of X-  
6 inactivation<sup>7,8,9</sup>. Besides mental retardation, the main features of the affected boys are  
7 muscular hypotonia, progressive spasticity, seizures, poor speech and recurrent  
8 infections. The reported duplications vary from 0.1 to 2.6 Mb with the minimal critical  
9 region containing the *IRAK* and *MECP2* genes<sup>7</sup>. In familial cases, carrier females might  
10 suffer from endocrinological abnormalities, autoimmune diseases as well as psychiatric  
11 disorders<sup>10</sup>. A phenotype with mental retardation has been reported only two times<sup>11,12</sup>.  
12 We report on two unrelated female patients with a *de novo* *MECP2* duplication and a  
13 random X-inactivation revealing a moderate but unspecific mental retardation in  
14 childhood and the development of neurological features in the second decade of life.

15

## 16 **Materials and Methods**

### 17 **Patients**

18 Both patients were examined for diagnostic evaluation because of mental retardation.  
19 Karyotype analysis was performed for each patient on metaphase chromosomes from  
20 cultured blood lymphocytes using Giemsa banding at 550 bands resolution with normal  
21 results. The parents of patient 1 also showed normal karyotypes. Informed consent for  
22 the investigation was obtained from both families.

23

### 24 **SNP Microarray**

1 Genomic DNA was isolated from peripheral blood leucocytes using routine procedures.  
2 In both samples, we used the Affymetrix Genome-Wide Human SNP Array 6.0  
3 (Affymetrix, Santa Clara, CA, USA) with an average distance of 1.3 kb between  
4 neighboring probes. Samples were processed according to the manufacturer's  
5 instructions. Genotypes were called with Affymetrix Genotyping Console Software  
6 v3(GTC) using the Birdseed algorithm with the default calling threshold of 0.5 and a  
7 prior size of 10000 bases in a simultaneous analysis of the patient-parents trio.  
8 Interpretation was based on Human Genome Build 36.

9

#### 10 Multiplex Ligation-Dependent Probe Amplification (MLPA)

11 In patient 2, *MECP2* duplication was confirmed by MLPA with the test kit P015D2  
12 Human Probe Mix by MRC Holland, Amsterdam, The Netherlands ([www.mlpa.com](http://www.mlpa.com))  
13 according to the manufacturer's instructions. MLPA profiles were obtained using  
14 normal and pathological DNA samples as controls.

15

#### 16 Quantitative PCR (qPCR)

17 In patient 2, the relative copy number of selected genomic regions was determined by  
18 quantitative PCR as described previously (data not shown).

19

#### 20 X-Inactivation (XCI)

21 The highly polymorphic (CAG)<sub>n</sub> motif within the human androgen receptor gene  
22 (*HUMARA*) was used to distinguish and compare the methylation activity of the X  
23 chromosomes in both patients as described previously<sup>13</sup>.

24

1 Fluorescence in situ hybridization (FISH)

2 To investigate the localization of the duplicated segment, FISH analyses were  
3 performed on metaphase spreads and interphase nuclei of both patients and the parents  
4 of patient 2. Using standard two-color FISH protocols, a BAC clone from the duplicated  
5 region Xq28 (RP11-218L14, kindly provided by Joris Vermeesch, Center for Human  
6 Genetics, Leuven, Belgium) was hybridized together with a whole or partial  
7 chromosome painting probe for chromosome X (wcpX or pcpXq)<sup>14</sup>. Microscopic  
8 evaluation of 20 metaphase spreads and 100 nuclei from each patient and the parents of  
9 patient 2 was performed using a DM RXA epifluorescence microscope (Leica  
10 Microsystems, Bensheim, Germany) equipped with a Sensys CCD camera  
11 (Photometrics, Tucson, AZ, USA).

12

13 Microsatellite analysis

14 The determination of the parental origin of the duplication was performed by  
15 microsatellite analysis, and the determination of the signal intensities for specific probes  
16 was performed on the 6.0 array. Microsatellite analysis was performed with four di-  
17 nucleotide sequences within the region of interest. Motifs were selected using the UCSC  
18 genome browser and repeat-spanning primers were designed with a 5'-fluorescence tag  
19 for the forward primer. PCR amplicons for trios were analyzed using capillary  
20 electrophoresis (Beckman Coulter CEQ8000) and amplicon lengths were determined.  
21 Besides genotyping, we measured peak heights for all duplication carriers compared to  
22 normal controls in order to identify duplicated and non-duplicated alleles as described  
23 earlier<sup>15</sup>.

1 The SNP-microarray data were evaluated considering the genotype and the  
2 corresponding probe signals within the duplicated region, thus providing a correlation  
3 of a specific genotype and the according signal height. The origin of the duplication was  
4 identified by an allele-specific elevation of signal intensity in the patient in comparison  
5 to the parental genotypes.

6

## 7 **Results**

8 All position coordinates given below are based on Human Genome Build 36.

9

### 10 Case reports

#### 11 Patient 1

12 The girl was born to healthy, non-consanguineous parents of Turkish origin at 39 weeks  
13 gestation via forceps delivery with a birth weight of 2900 g (-1.0 SD), a length of 48 cm  
14 (-1.3 SD) and a head circumference of 34 cm (-0.3 SD). She had constipation from birth  
15 on, which had to be treated with a laxative. During her first years of life, she presented  
16 with a high frequency of respiratory infections. She learned to walk and spoke her first  
17 words at the age of 19 months. Her further psychomotor development was delayed. The  
18 parents noted autistic behavioural features in her daughter, for example she mostly plays  
19 alone.

20 We examined patient 1 for diagnostic evaluation at the age of  $6\frac{2}{12}$  years and again at 7  
21  $\frac{6}{12}$  years. At second examination, her body measurements were still normal: her height  
22 was 128 cm (0.3 SD), her weight was 28.5 kg (0.9 SD) and her OFC was 52.5 cm (0.7  
23 SD). She visited a school for the mentally retarded, and was not able to write or count.  
24 Her non-verbal IQ was 58. Her constipation improved, but sometimes a drug therapy

1 was necessary. Adenoids were removed at the age of 5 years, but overall there was no  
2 history of recurrent or severe infectious diseases. She still displayed autistic behaviour,  
3 spending most of her time engaged in role-playing games with her dolls. Her  
4 psychomotor development was slow, but the parents did not report regression, and no  
5 epilepsy or stereotypies were reported. She had no facial dysmorphism, but facial  
6 similarity to her father, who also presented with synophrys. Bilateral simian creases as  
7 well as a sacral hemangioma were noted. No other abnormalities were present.

8

#### 9 Patient 2

10 Patient 2 was born at 40 weeks of gestation by spontaneous vaginal delivery as the 3<sup>rd</sup>  
11 of 4 children of non-consanguineous Turkish parents following an uncomplicated  
12 pregnancy. Birth measurements were normal with a weight of 3650 g (0.4 SD), a length  
13 of 52 cm (mean) and an OFC of 35.5 cm (0.5 SD). Her three brothers were healthy. She  
14 achieved normal motor milestones. Speech development was delayed and started at 3  
15 years. At the age of 5 years, the parents reported a stagnation of development, but no  
16 regression. She mostly played alone and revealed some autistic features. After a first  
17 year at regular school, she changed to a special school. She never learnt to read or write.  
18 At the age of 10 years, standardized non-verbal testing (SON) showed a developmental  
19 age of 6 years and a cognitive score of 55. On physical examination at the age of 10  
20 years, her weight was 36.8 kg (BMI: 17.6, 50<sup>th</sup>-70<sup>th</sup> centile), her height was 145 cm (0.9  
21 SD) and her OFC was 54 cm (1.2SD). She showed a primary enuresis until the age of  
22 12 years, and furthermore a vesicouretral reflux II° was diagnosed. The EEG at that age  
23 was irregular with alpha-rhythm and abnormal beta waves. The brain MRI was normal.

1 At the age of 18 years a spastic hypertonia of the legs appeared, which led to gait  
2 disturbances. EEG at the age of 20 was physiological, MRI of the myelon revealed no  
3 pathological aspects. With 20 years, the patient's weight was 67 kg (BMI: 26.2, 90<sup>th</sup>-  
4 97<sup>th</sup> centile), her height was 160 cm (-1 SD) and her OFC was 54 cm (mean). Patient 2  
5 did not present with any dysmorphic features. Her course of puberty was described as  
6 normal and no endocrinological problems were reported.

7

### 8 **Detection of *MECP2* duplications**

9 *De novo* duplication of Xq28 material, including the *MECP2* gene, was identified in  
10 both patients using array analyses. In patient 1, a 266 kb duplication (chrX:152 892  
11 781-153 158 642, 266 kb, 71 array markers) containing 7 reference sequence genes was  
12 detected (RefSeq, according to the Genome browser of the University of California in  
13 Santa Cruz, UCSC). The proximal breakpoint was located within the *TMEM187* gene,  
14 centromeric to the *IRAK* gene, and the telomeric breakpoint was located within the  
15 *TEX28* gene (Fig. 1). In addition, patient 1 showed a second *de novo* duplication of  
16 2q37.3 material (chr2:241 714 171-241 825 938, 112 kb, 71 array markers), including  
17 the genes *ANO7*, *HDLBP*, *PASK*, *PPP1R7*. The genotype for patient 1 according to the  
18 International System for Human Cytogenetic Nomenclature 2009 (ISCN) is arr  
19 2q37.3(241,714,171-241,825,938)x3 dn,Xq28(152,892,781-153,158,642)x3 dn.  
20 In patient 2, we found a duplication of chromosome Xq28 material (chrX: 152 925 421-  
21 153 403 154, 478 kb, 215 array markers) containing 22 RefSeq-genes (UCSC), with the  
22 proximal breakpoint centromeric to the *IRAK* gene and the distal breakpoint telomeric  
23 to the *FAM3A* gene (Fig. 1). The nomenclature according to ISCN for patient 2 is arr  
24 Xq28(152,925,421-153,403,154)x3 dn.

1

## 2 **Confirmation and delineation of *MECP2* duplications**

3 In patients 1 and 2, the duplication in Xq28 was confirmed by interphase FISH based on  
4 the presentation of three hybridization signals for the BAC clone RP11-218L14 (Fig. 2)  
5 A localization of the duplicated region on an autosome was excluded for both patients  
6 in metaphase spreads. In the parents of patient 2, FISH analyses for the Xq28 BAC  
7 clone revealed only normal signals on the X chromosomes. Furthermore, duplications of  
8 the genes *IRAK*, *MECP2* and *GDII* were confirmed in patient 2 according to the gene-  
9 specific probes from the MLPA kit, whereas a normal signal was obtained for the probe  
10 against the proximal flanking *IRAK* gene. In patient 2, the duplication of *MECP2* was  
11 confirmed by quantitative PCR (data not shown).

12 X inactivation studies showed a random X-inactivation in both patients with a status of  
13 61:39 and 71:29, respectively (Fig. 3). Microsatellite analysis revealed two alleles with  
14 different repeat lengths for the polymorphic marker in position chrX:153 050 087-153  
15 050 120 (17xTG) in the father's and in the mother's DNA of patient 2. Semi-  
16 quantitative analysis indicates a paternal origin of the *MECP2* duplication in patient 2  
17 (data not shown). This marker as well as all other tested markers were not informative  
18 for patient 1. The analysis of the SNP-microarray data regarding the correlation of  
19 genotype and signal height within the duplicated region indicates a paternal origin in  
20 both patients.

21

## 22 **Discussion**

23 There were a considerable number of reports on mentally retarded boys with *MECP2*-  
24 duplications during the years following the first publication<sup>6</sup>. However, to best of our

1 knowledge, only two mentally retarded females with an assured duplication have been  
2 reported<sup>11,12</sup>. This underrepresentation of girls may have different reasons. An  
3 investigation bias favouring affected boys to be investigated for *MECP2* duplications  
4 may have occurred, while girls were simply not investigated for this disorder. The fact  
5 that all females carrying the duplication were described as intellectually normal could  
6 have added to that effect. There is a recent report that considers certain clinical findings  
7 in those “carrier mothers” as an affinity to endocrinological abnormalities (e.g.  
8 hypothyroidism, menstrual irregularity and diabetes), autoimmune diseases as well as  
9 psychiatric disorders (especially depression, anxiety and compulsion)<sup>10</sup>. Nevertheless,  
10 the intellectual performance of all investigated females in that study was in the normal  
11 range (full scale IQ 81-123). During the last years, large cohorts of mentally retarded  
12 patients of both sexes were investigated with high resolution arrays for submicroscopic  
13 genomic imbalances through different array technologies. These should have shown  
14 *MECP2* duplications in females, and yet no further affected girls have been reported<sup>16</sup>.  
15 This contradicts the investigation bias as an explanation for the rarity of those cases. We  
16 conclude that *MECP2* duplications are a comparatively rare cause of mental retardation  
17 in females. This is confirmed by the identification of only 2 cases out of our cohort of  
18 1000 unselected patients with mental retardation (incidence of 0,002).  
19 In this study, we demonstrate two more female patients with an *MECP2* duplication,  
20 and further delineate the aspect that, as Reardon *et al* first suggested, the random X-  
21 inactivation (XCI) may be causative for the phenotype<sup>11</sup>. Most of the published carrier  
22 women who were informative for the XCI test presented a highly skewed X-inactivation  
23 with the duplication-bearing X chromosome being preferentially inactivated<sup>10</sup>. One can  
24 reasonably assume that the random X-inactivation is the crucial point leading to the

1 mental retardation phenotype in these patients, while a highly skewed XCI with a  
2 preferential inactivation of the duplication carrying X-chromosome is a protective  
3 factor. The impact of the XCI in our patients is further emphasised by the fact that a  
4 translocation of the duplicated segment to an autosome was excluded by FISH  
5 investigation. Another remarkable aspect of our patients, besides their retardation and  
6 the random XCI, is that the *MECP2* duplication occurred *de novo* in both cases. We  
7 propose that *de novo* occurrence of the *MECP2* duplication, as in our cases, might have  
8 an effect on the XCI by preventing a protective “mutation induced” skewed X-  
9 inactivation by a yet unrecognized mechanism. This would mean that there could be a  
10 different trigger for the methylation process from an inherited *MECP2* duplication than  
11 from one that occurred *de novo*. Furthermore, the analysis of the microsatellites and the  
12 signal intensities of the microarrays for our patients showed the *de novo MECP2*  
13 duplication had been derived from the paternal X chromosome, which is a novelty in the  
14 literature.

15 For all but one female cases reported so far, a duplication on the maternal X-  
16 chromosome can be assumed due to an X-linked pedigree. In this case the duplication  
17 occurred *de novo* on an autosome (chromosome 10), thus completely escaping X  
18 inactivation<sup>12</sup>.

19 A postzygotic mosaic occurrence of the duplication is a feasible explanation for a  
20 random XCI, and mosaicism can not be excluded by any means. However, both patients  
21 showed no clinical signs of mosaicism, such as pigmental abnormalities of the skin or  
22 asymmetry, and the SNP-Arrays gave no evidence for a mosaic state of the *MECP2*  
23 duplication. This contradicts a high-level postzygotic mosaicism as a cause for the  
24 random XCI in our cases.

1 It has to be mentioned that in patient 1 a second *de novo* duplication was detected  
2 spanning 112 kb of chromosome 2q37 material with the RefSeq-genes *PASK*, *PPP1R7*,  
3 *ANO7*, *HDLBP* (UCSC: NCBI36/hg18). Only weak *PASK* (OMIM \*607505)  
4 expression is found in human thymus and testis. *PPP1R7* (OMIM \*602877) is  
5 expressed in all human tissues including the brain. *ANO7* (OMIM \*605096) is  
6 expressed only in the prostate. Haploinsufficiency of two of the genes within this region  
7 has been connected with neuropsychiatric disorders (*HDLBP* and *PASK*<sup>18</sup>), but to our  
8 knowledge no phenotype has been demonstrated for a duplication of these genes. Thus,  
9 the significance of the 2q37 duplication remains unclear. An effect of the 2q37  
10 duplication on the phenotype of the patient seems to be unlikely, but cannot be  
11 completely excluded. Regarding that the phenotypic effect of a *MECP2* duplication is  
12 already well documented, and patient 1 presents no clinical signs of a neuropsychiatric  
13 disorder exceeding the phenotype of patient 2, we feel safe to assume that the phenotype  
14 is caused by the *MECP2* duplication in both patients.

15 The typical symptoms of affected boys, such as seizures, poor speech development and  
16 recurrent severe infections, are not present in the females. The clinical signs in female  
17 patients with *MECP2* duplication and random X-inactivation consist of unspecific mild  
18 to moderate mental retardation, with autistic features, recurrent infections in early  
19 childhood, constipation and late onset neurological features as variable symptoms.  
20 Facial dysmorphism was absent in all females (Tab. 1)<sup>19</sup>. This unspecific phenotype  
21 does not allow the diagnosis of females with a *MECP2* duplication and random X-  
22 inactivation on clinical grounds alone. Nevertheless the stagnation of development at  
23 the age of 5 years in patient 2 as well as the onset of spasticity in this patient at the age  
24 of 18 years seem remarkable, especially regarding the progressive spasticity in males.

1 This underlines the necessity to consider late-onset neurological features as a possible  
2 aspect in affected females.

3 In conclusion, we report on two female patients with a *de novo* *MECP2* duplication and  
4 a mental retardation. Both patients show a random X-inactivation, which can be  
5 postulated as the triggering factor for the phenotype. Furthermore, these are the first  
6 patients with a duplication derived from the paternal X chromosome and this report  
7 gives a description of the phenotype of affected females.

8

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19 project reference numbers 01GS08164, 01GS0816, 01GS08168, 01GS08162).

20

### 21 **Conflict of interest statement:**

22 The authors declare no conflict of interest.

23

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20

1 **Titles and Legends to Figures:**

2

3 Figure 1: Illustration of the genomic aberration of the *MECP2* region with the 6.0  
4 Affymetrix SNP Array and schematic representation of the duplicated region, a+b)  
5 image of the parents-patient trio analysis, showing the *de novo* occurrence of the  
6 duplication (red arrows) in both cases, c+d) mapping of the duplicated regions  
7 according to UCSC, e) Schematic representation of part of the genomic region Xq28  
8 with the location of the duplication of our two novel patients (blue arrows), the female  
9 patients of Reardon *et al*, 2010 and Makrythanasis *et al*, 2010 (grey arrows) as well as  
10 the minimal critical region (MCR) defined by Bartsch *et al*. 2010 (red arrow). Gene  
11 content of the region is shown from the Ensembl genome browser version 54.36p  
12 (NCBI36).

13

14 Figure 2: Metaphase and interphase FISH results

15 a) Metaphase spread of patient 1 hybridized with the BAC clone RP11-218L14  
16 localized in the duplicated chromosome region Xq28 (red) together with a partial  
17 chromosome painting probe for the long arm of the X chromosome (pcpXq, green).  
18 Signals are only present on both X chromosomes. b) Interphase nuclei of the same  
19 patient, presenting a normal hybridization signal and a duplicated signal for the Xq28  
20 BAC clone (red). c) Metaphase spread of patient 2 after hybridization of the Xq28-BAC  
21 clone (RP11-218L14, red) together with a chromosome painting probe for the X  
22 chromosome (wcpX, green). Signals are only present on both X chromosomes. d)  
23 Interphase nuclei of patient 2 showing three hybridization signals, a normal signal and a  
24 duplicated signal for the Xq28 BAC clone (red). e) Metaphase spread of the father of

1 patient 2 presenting only one signal on the X chromosome and f) a normal signal in the  
2 interphase nuclei with the Xq28 BAC clone. g) Metaphase spread of the mother of  
3 patient 2 showing a specific hybridization signal on both X chromosomes and h) two  
4 normal signals in the interphase nuclei.

5

6 Figure 3: XCI results

7 Results showing random X inactivation in both patients and prove of complete digestion  
8 with methylation-sensitive restriction endonuclease HpaII after PCR amplification of  
9 HUMARA (male control and female positive control, respectively). For patient 1  
10 (performed on ABI3100, Genotyper v3.7) trace a) shows the undigested sample, trace b)  
11 the digested fragments. The lower two traces show the male control c) undigested and  
12 d) after digestion. The XCI in patient 1 is 39:61. For patient 2 the upper two traces show  
13 the patient's sample a) undigested and b) digested (CEQ8000, BeckmanCoulter),  
14 revealing a random XCI of 29:71. The lower traces show a female positive control with  
15 complete skewing of X-inactivation in c) undigested and in d) digested.

16

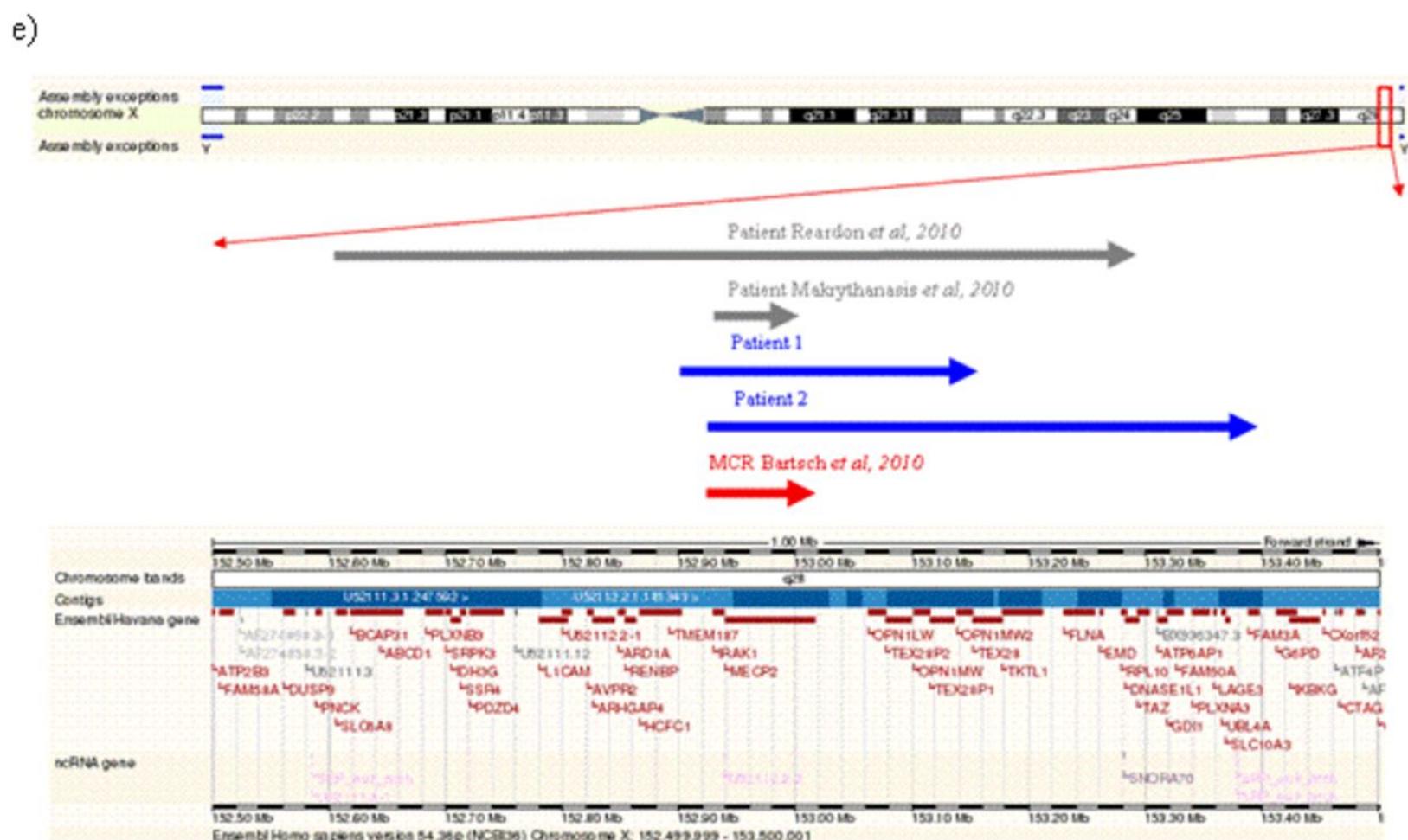
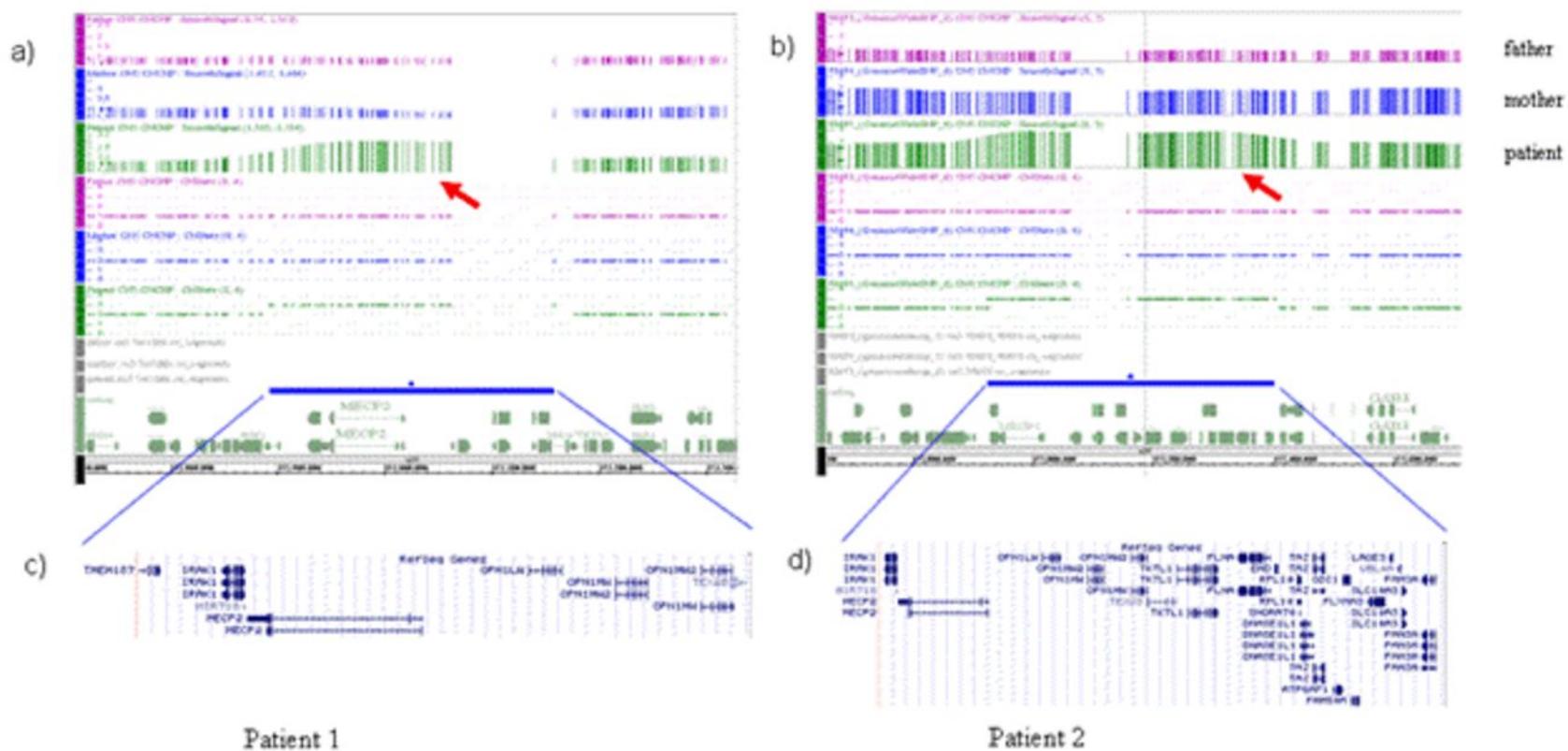


Figure 1

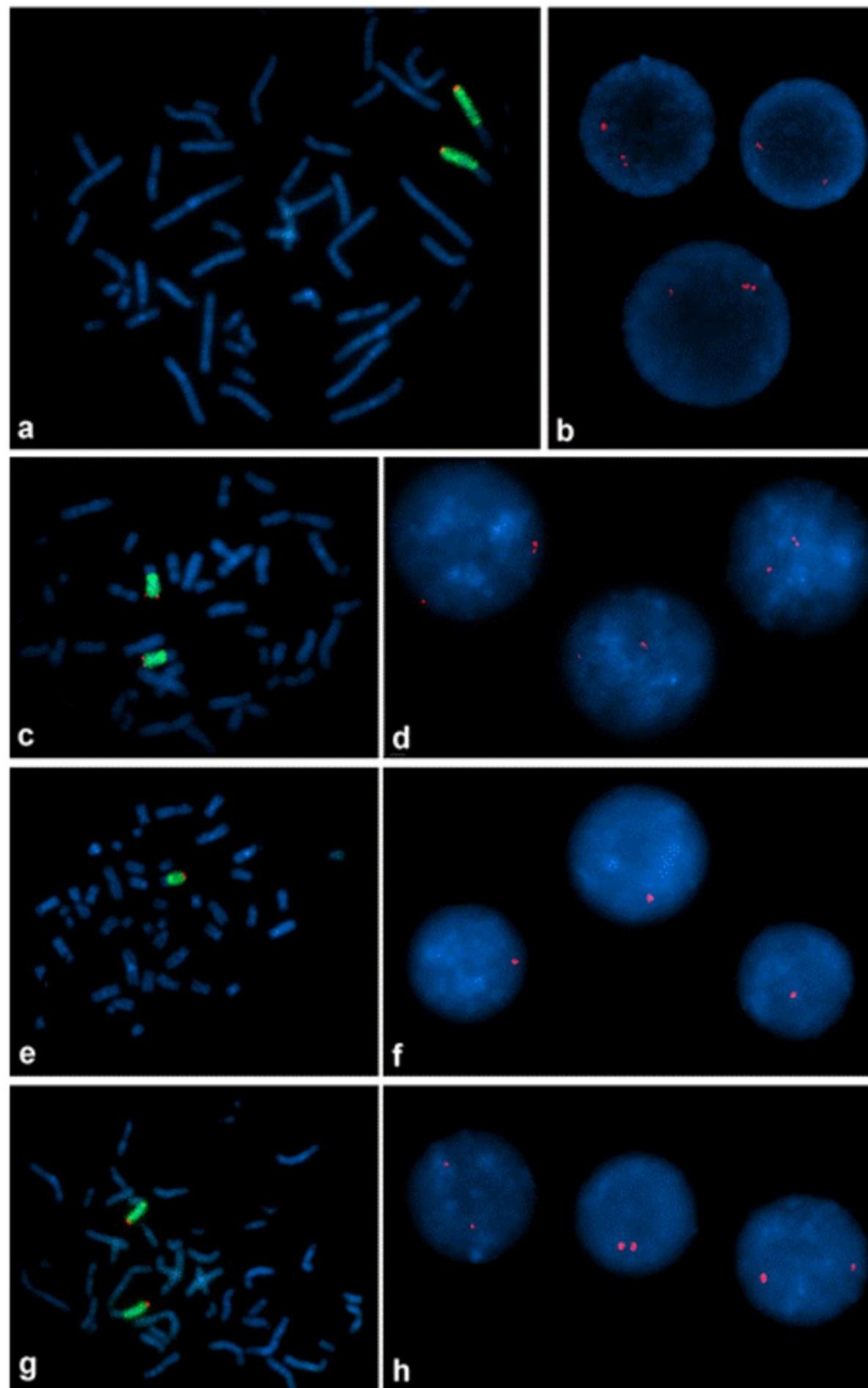
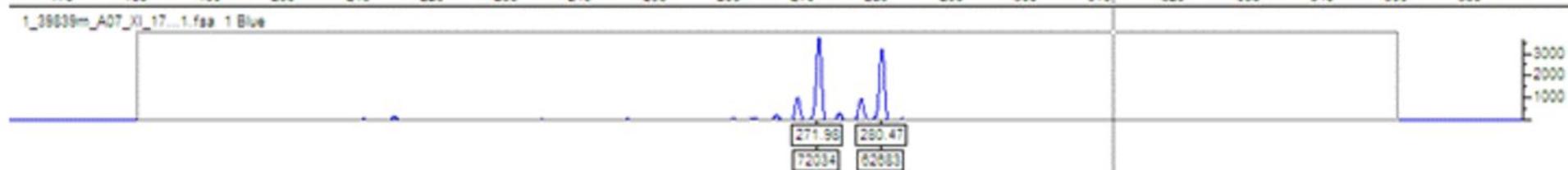


Figure 2

Patient 1

a)



b)



c)

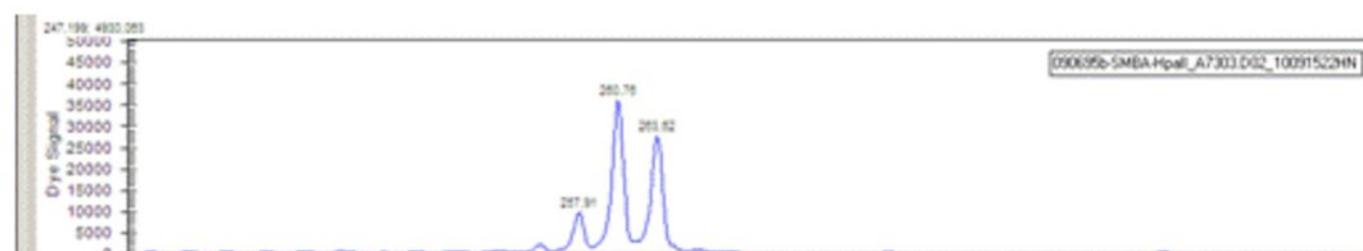


d)

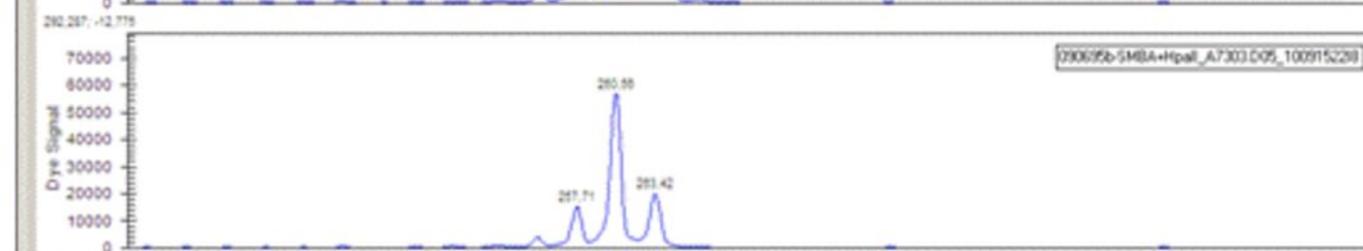


Patient 2

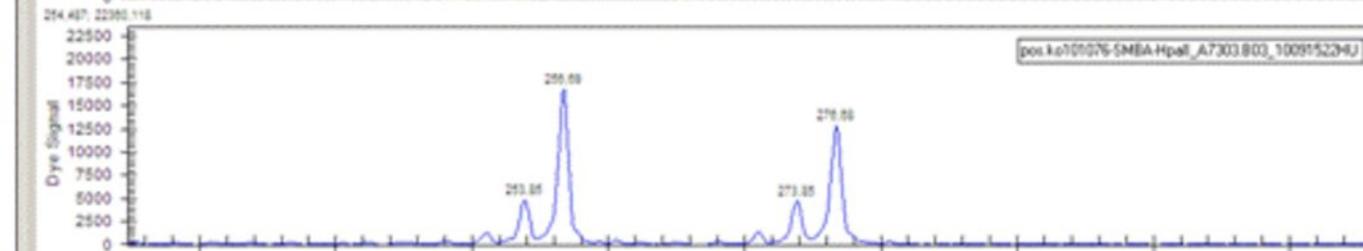
a)



b)



c)



d)

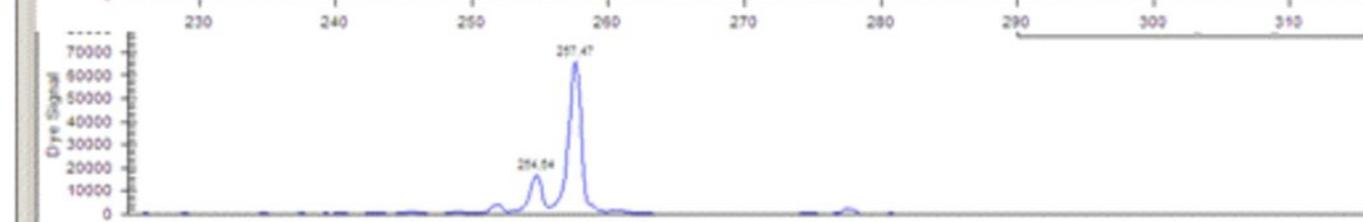


Figure 3

Table 1: Clinical findings in female patients with *MECP2* duplication and random X-inactivation

	Patient 1	Patient 2	Reardon <i>et al</i> <sup>12</sup>	Makrythanasis <i>et al</i> <sup>13</sup>
Age at last examination (years)	7 <sup>6</sup> / <sub>12</sub>	20	12	18
<i>MECP2</i> duplication	<i>de novo</i>	<i>de novo</i>	maternal	<i>de novo</i>
Mental retardation	moderate	moderate	mild	apparent, not specified
Autistic features	+	+	n.r.	-
Speech	good	good	good	simple
Seizures	-	-	-	-
Neurological symptoms	-	leg spasticity	-	poor coordination, eye movements
Facial dysmorphism	-	-	-	-
Constipation	+	-	n.r.	-
Frequent infections in early childhood	+	-	n.r.	n.r.
Bilateral simian creases	+	-	n.r.	-
Sacral hemangioma	+	-	n.r.	-
Vesicourethral reflux	n.r.	+	n.r.	-
Endocrinological problems	n.r.	-	n.r.	+

n.r.: not reported