

ONLINE MUTATION REPORT

NIPBL mutations and genetic heterogeneity in Cornelia de Lange syndrome

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Cornelia de Lange syndrome (CdLS, also called Brachmann de Lange syndrome; OMIM 122470) is characterised by pre- and postnatal growth retardation, microcephaly, severe mental retardation with speech delay, feeding problems, major malformations including limb defects, and characteristic facial features.¹ Facial dysmorphism includes arched eyebrows, synophrys, short nose with anteverted nares, long philtrum, thin upper lip, and micrognathia. Although few autosomal dominant forms of CdLS have been described,^{2,3} the large majority of cases are sporadic, and the scarcity of these familial forms has hampered the identification of the gene(s) underlying CdLS.⁴ Finally, rare cases of CdLS have been associated with balanced chromosomal translocations.⁵⁻⁷

A gene responsible for CdLS has been recently identified by two groups. Indeed, Krantz *et al* performed genome-wide linkage exclusion mapping in 12 CdLS families and identified a locus on chromosome 5p13.⁸ This locus mapped close to both a translocation breakpoint and a small de novo deletion associated with CdLS. Studying the 5p13 translocation breakpoint allowed both groups to identify a disrupted gene which they called *NIPBL*, for Nipped-B like.^{8,9} *NIPBL* is the human homolog of the *Drosophila Nipped-B* gene, the product of which belongs to the family of chromosomal adherins. The *Drosophila Nipped-B* protein is involved in chromatid cohesion processes and enhancer-promoter communication.^{10,11} The exact function of the human *NIPBL* gene product, called delangin, is unknown, but its wide expression pattern, including expression in embryonic limb bud, branchial arch, and craniofacial mesenchyme, is consistent with many of the anomalies observed in CdLS. *NIPBL* mutations have been identified in 20–50% of CdLS cases,^{8,9,12} suggesting that some mutations may have escaped detection and/or that CdLS is genetically heterogeneous.

To address this question, we performed a comprehensive clinical, cytogenetic, and molecular study in 14 affected children. Our results show that *NIPBL* mutations are present in only 35% of CdLS cases, strongly suggesting the genetic heterogeneity of this syndrome.

METHODS

Patients

The series included 14 children (seven boys and seven girls) ranging in age from 14 months to 17 years. All patients were regularly followed at the Department of Medical Genetics of the Necker - Enfants Malades Hospital, Paris. They were diagnosed with CdLS on the basis of four major criteria: (i) pre- and/or postnatal growth retardation, (ii) microcephaly, (iii) moderate to profound mental retardation with abnormal speech development, ranging from minimal speech to complete absence of speech, and (iv) facial dysmorphic features including arched eyebrows with synophrys, short nose with anteverted nares, long philtrum, and thin upper lip. In addition, at least two of the following four clinical

Key points

- Cornelia de Lange syndrome (CdLS) is characterised by facial dysmorphism, microcephaly, growth and mental retardation, and congenital anomalies including limb defects. Mutations in the gene *NIPBL*, the human homolog of *Drosophila Nipped-B*, have recently been found in 20–50% of CdLS cases.
- We extensively analysed a series of 14 children with CdLS. The study included a high resolution chromosome analysis, a search for small chromosome imbalances using array-CGH at 1 Mb resolution, and direct sequencing of the 46 *NIPBL* coding exons.
- Chromosomal or molecular anomalies most likely causing CdLS were found in 7/14 cases. Chromosome analysis detected an isochromosome 18p of paternal origin in a girl with a mild CdLS phenotype. Array-CGH analysis detected a de novo submicroscopic deletion of terminal chromosome 1q in a girl with severe manifestations of CdLS. We identified five heterozygous de novo *NIPBL* mutations including one splice site (IVS2+1G>A), one frameshift (95delT), two missense (N2236I, L2092V) mutations, and a deletion of three nucleotides (leading to N2218del).
- We conclude that *NIPBL* mutations account for ~35% of CdLS cases in our series. The absence of a *NIPBL* mutation in more than 50% of children with CdLS and the observation of two distinct chromosomal anomalies is highly suggestive of genetic heterogeneity.

signs or symptoms were required for diagnosis, namely: (i) behavioural problems including self injurious behaviour, hyperactivity, repetitive and autistic behaviour, (ii) feeding problems and/or gastroesophageal reflux, (iii) major or minor anomalies of the upper limbs, and (iv) skin anomalies, particularly hirsutism and cutis marmorata. Table 1 and fig 1 summarise our clinical findings in the 14 patients included in the study.

Two patients presented with unusual features, namely absence of corpus callosum (patient 2) and long palpebral fissures/prominent malar region (patient 6; fig 1), but both met the inclusion criteria including the typical behavioural phenotype.

Mean paternal and maternal age at birth of the affected child was 33 (range 24–45) and 30 (range 19–40) years, respectively. Three fathers and four mothers were over 40 and 35 years of age at the birth of their CdLS child, respectively.

Abbreviations: CdLS, Cornelia de Lange syndrome; DAPI, 4',6'-diamidino-2-phenylindole

Table 1 Clinical features in 14 CdLS patients

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total
Gender	F	F	F	F	M	M	F	F	M	M	M	F	M	M	7M/7F
Familial recurrence	-	-	Aff. brother	-	-	-	-	Aff. father	-	-	-	-	-	-	2
Age at last examination (years)	7	9	4	1	8	11	10	1	3	8	2	8	6	17	1-17
IUGR	+	+	+	+	+	+	+	-	+	+	+	+	+	-	12/14
Postnatal growth retardation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14/14
Microcephaly	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14/14
Mental retardation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14/14
Speech delay (+) or absent speech (++)	+	++	++	+	++	+	+	+	+	+	+	++	++	+	14/14
Facial dysmorphism															14/14
Arched eyebrows	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Synophrys	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Short nose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	
Anteverted nares	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Long philtrum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Thin upper lip	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Other															Long palpebral fissures, prominent malar region
Behavioural problems	AA HA SD	AD	HA	-	HA AA	HA	AA SD	-	-	HA HA AD	AD AA	AD	AD		11/14
Feeding problems	+	+	+	+	-	+	+	+	+	+	+	+	+	+	13/14
GER	+	+	+	+	-	-	-	+	-	-	+	-	+	-	7/14
Limb anomalies															14/14
Short hands	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Clinodactyly 5th finger	+	+	-	-	-	-	-	-	+	-	-	+	-	+	
Limitation elbow	-	-	-	-	+	-	-	-	+	-	-	+	-	-	
STC	-	-	-	+	+	-	-	-	-	-	-	-	-	-	
Short feet	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Syndactyly 2-3	-	-	-	-	+	-	-	+	-	-	-	-	-	-	
Hirsutism	-	+	-	-	+	-	-	+	+	+	+	+	+	+	9/14
Cutis marmorata	+	-	+	+	+	-	-	+	-	-	-	+	-	-	6/14
Ear and eye															
Deafness	-	+	-	-	-	-	NA	-	-	-	-	NA	Altered AEP	+	2/12
Strabismus	-	+	-	-	-	-	-	-	-	-	-	-	-	+	2/14
Ptosis	-	-	-	-	-	+	-	-	-	-	-	-	-	+	2/14
Nystagmus	-	-	-	-	-	-	-	-	+	-	-	-	-	-	1/14
Myopia	-	-	-	-	-	+	-	-	+	-	-	-	-	+	3/14
Neurologic anomalies															
Seizures	+	+	+	-	-	+	-	-	-	-	-	-	-	-	4/14
Cerebellar hypoplasia	+	-	-	-	-	-	-	-	+	-	-	-	-	-	2/14
ACC	-	+	-	-	-	-	-	-	-	-	-	-	-	-	1/14
Cleft palate	-	-	-	-	-	+	-	-	-	-	-	-	-	+	2/14
GI malformation	Hiatus hernia	-	-	-	-	-	-	-	-	Pyloric stenosis	-	-	Duodenal atresia	-	3/14
Heart defect	-	ASD	-	ASD	-	Mitral valve prolapse	-	-	-	-	-	VSD-	-	-	4/14
Genito-urinary anomaly	-	-	VUR	-	-	-	-	-	-	-	-	-	Cryptorchidism	Cryptorchidism	3/14
Chromosome anomaly	i(18p)	del 1qter	-	-	-	-	-	-	-	-	-	-	-	-	2/14
NIPBL mutation	-	-	N2218delN	N2236I	L2092V	IVS2+1G>A	95delT	-	-	-	-	-	-	-	5/14

AA, autoaggressive behaviour; ACC, absence of corpus callosum; AD, autistic disorder; AEP, acoustically evoked potentials; Aff., Affected; ASD, atrial septum defect; GER, gastroesophageal reflux; HA, hyperactivity; IUGR, intrauterine growth retardation; NA, not available; SD, sleep disturbances; STC, single transverse crease; VSD, ventricular septum defect; VUR, vesico-ureteral reflux.

All children were of French ancestry except cases 8 (Algeria) and 11 (France/Algeria). Two cases of familial recurrence were observed (families 3 and 8). In family 3, an older brother of the probanda was also diagnosed with CdLS based on prenatal growth deficiency, microcephaly, and typical facial dysmorphism. This boy died 1 week after birth. In family 8, CdLS was apparently transmitted as an autosomal dominant trait as the proband's father had short stature, a learning disability, congenital heart disease, and dysmorphic features reminiscent of mild CdLS (fig 1).

Cytogenetic studies and fluorescent genotyping

Blood samples were collected for chromosome analysis and DNA extraction with written informed consent of the parents. Standard karyotypes using RHG and/or GTG banding and high resolution chromosome analyses after synchronization and BrdU incorporation (RBG) were performed from peripheral blood lymphocytes of the patients following standard protocols.

For FISH analysis, we used the TelVysion probe panel mixture 12, containing in particular TelVysion 18p



Figure 1 Facial views of CdLS patients. The numbers correspond to the patient's number in the text and the tables. Patients 1 and 2 have a chromosome anomaly, and patients 3–7 a heterozygous *NIPBL* mutation. No anomaly was detected in patients 8–11 or 14. (Photographs are reproduced with consent.)

SpectrumGreen (telomeric clone) and CEP18 SpectrumAqua (alpha-satellite probe). Hybridisations were performed following the manufacturer's instructions (Abbott Diagnostics, Rungis, France). Chromosomes were counterstained with 4',6'-diamidino-2-phenylindole (DAPI). Image capture and analyses were performed using a Leica DMRXA epifluorescence microscope equipped with the appropriate filter combination for detecting SpectrumAqua, FITC, and DAPI. Images were captured by a cooled CCD camera using an image analysis system (Alphelys, Nikon). Fluorescent genotyping was performed as previously described.¹³ We genotyped the following chromosome 18p microsatellite markers in family 1: D18S59, D18S476, D18S54, D18S52, D18S391, D18S1150, D18S53, and D18S453 (in order from telomere to centromere).

***NIPBL* sequence analyses**

We designed 51 primer pairs to cover the 46 *NIPBL* coding exons (exons 2–47) and the corresponding exon-intron boundaries. The primers designed for exon 46 were chosen to include the 3' sequence specific for the *NIPBL* isoform B.⁹ Primer sequences and PCR conditions are available on request. PCR products were purified with Exo-SAP (Amersham, Orsay, France) and then directly sequenced in both directions on an ABI PRISM 3100 DNA sequencer (Perkin Elmer-Applied Biosystems, Courtaboeuf, France) using the dye terminator method according to the manufacturer's instructions.

Array-CGH

Because our study started prior to recent publications,^{8,9,12} a 1 Mb array-CGH search for submicroscopic deletions and duplications was performed in all 14 children. A total of 3523 clones were used for array construction, covering the genome with a 1 Mb resolution.¹⁴ DOP-PCR products of each clone were arrayed in duplicate onto amine binding slides. Details on all spotted clones can be obtained from the Ensembl database (http://www.ensembl.org/Homo_sapiens/cytoview). DNA labelling, array hybridisation, washing, and image and data analyses were performed as described previously.¹⁵

RESULTS

Chromosome imbalances

Two children were found to have a de novo chromosome imbalance. The karyotype of a girl with a mild CdLS phenotype (patient 1; fig 1 and table 1) detected a supernumerary marker chromosome in 100 metaphases analysed, consistent in size and banding pattern with an isochromosome 18p. FISH analysis confirmed this finding (fig 2A) and array-CGH detected a gain of chromosomal material consistent with a tetrasomy of the entire chromosome 18p (data not shown). No other associated chromosome imbalance was identified and no anomaly was detected in the parents' DNA suggesting a de novo event. Genotypes of seven microsatellite markers of chromosome 18p showed a stronger intensity of

Table 2 Analysis of polymorphic microsatellite markers in family 2

Marker	Position*	Father	Patient 2	Mother	Interpretation**
D1S321	238.1	159/161	157/161	157	Biparental
D1S2842	239.1	220/226	218	218/226	del pat
D1S2679	239.3	189/191	197	189/197	del pat
D1S2836	243.1	268/270	272	268/272	del pat
D1S2682	244.4	116/136	116	116/136	NI

Allele sizes are given in base pairs. Genotypes consistent with a deletion are shown in **bold**.

*The position of microsatellite markers is given according to the University of Santa Cruz Genome browser (www.genome.ucsc.edu, accessed May 2004) in Mb from the short arm telomere; **del pat, deletion of the paternal allele; NI, non informative.

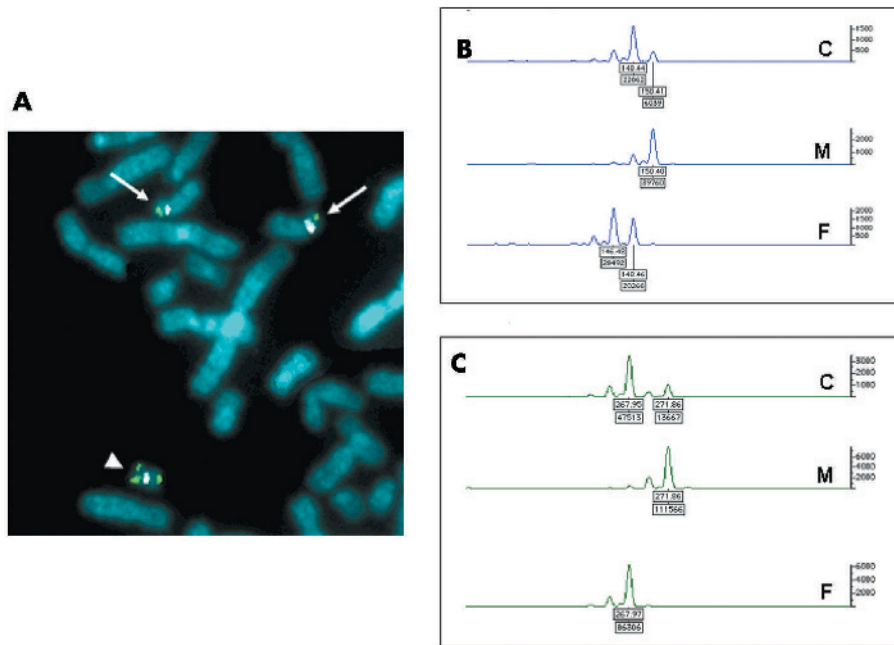


Figure 2 Detection of an isochromosome 18p of paternal origin in patient 1. (A) FISH analysis with centromeric (white) and telomeric (green) chromosome 18 specific probes. Arrows depict the two normal chromosomes 18. The monocentric isochromosome 18p is shown by an arrowhead. (B and C) Representative results of microsatellite analysis at loci D18S453 (B) and D18S476 (C) in family 1. C, M, and F stand for child, mother, and father genotypes, respectively. Numbers under the electropherograms indicate the length of the PCR products (in bp) and the area under the curve. Note the stronger intensity of the paternal allele compared to the maternal allele. Results for all other informative markers are comparable to those shown here.

the paternal allele, suggesting that the isochromosome 18p was of paternal origin (fig 2B and C).

In patient 2, a girl with severe manifestations of CdLS including severe mental retardation, autistic behaviour, seizures, and heart defect (fig 1 and table 1), array-CGH detected a subteleric deletion of chromosome 1q encompassing the six most telomeric clones (fig 3). Microsatellite analysis confirmed this deletion and showed that it had occurred on the paternal allele (table 2). The deletion spanned 6.5 Mb and had occurred de novo. Retrospective analysis of her high resolution karyotype failed to detect this deletion.

No chromosome imbalances were found in the remaining 12 CdLS patients, but six genomic imbalances of single clones were noted which correspond to either polymorphisms inherited from healthy parents or small variants (data not shown).

***NIPBL* mutations**

The microarray used for array-CGH contains a 188 kb long BAC clone (RP11-7M4) which encompasses the entire *NIPBL* coding sequence. The hybridisation pattern of this clone ruled out a large submicroscopic *NIPBL* deletion in the 14 CdLS children.

The direct sequencing of the 46 *NIPBL* coding exons detected five heterozygous mutations (patients 3–7): one splice site mutation (IVS2+1G>A), one frameshift mutation followed by a premature stop codon (95delT), one in-frame deletion of three nucleotides (leading to N2218del), and two missense mutations (N2236I, L2092V; table 3). The N2236I and L2092V mutations altered residues highly conserved across species (including rat, mouse, *Tetraodon*, and *Drosophila*) and were not detected in 110 and 280 alleles of control subjects, respectively. All mutations were found to

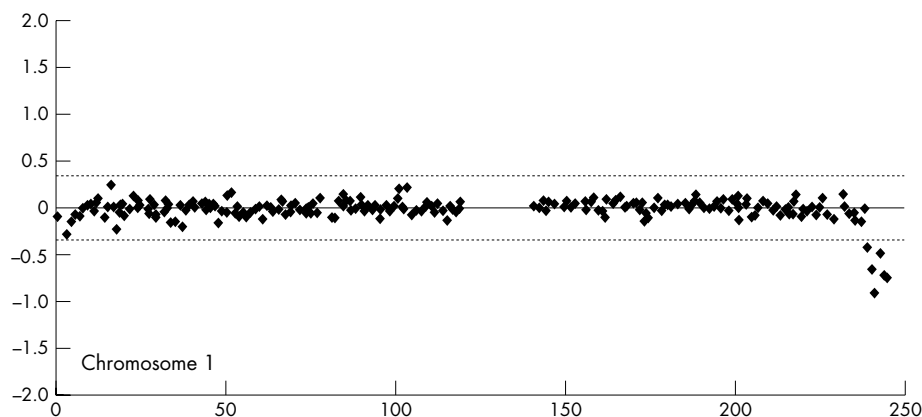


Figure 3 Identification of a subteleric deletion 1q in patient 2. Array-CGH profile of chromosome 1. The x axis marks the distance, in Mb, from the p telomere. The y axis marks the hybridisation ratio plotted on a \log_2 scale. Broken lines indicate thresholds for clone duplication and deletion (± 4 SD). The following six clones at the chromosome 1q telomere are deleted: RP11-411G13 (240.2–240.3 Mb), RP11-13L16, RP11-399B15, RP11-551G24, RP11-438H8, and CTB-160H23 (245.8–246.0 Mb).

Table 3 *NIPBL* mutations identified in CdLS patients

Individual	Location of the mutation	Nature of the mutation	Mutation*	Effect on mRNA/protein
6	Intron 2	Splice mutation (splice donor site)	IVS2+1G>A (c.64+1G>A)	Probable skipping of exon 2 which contains the translation initiation codon
7	Exon 3	1 bp deletion	c.95delT	Frameshift and stop codon 14 amino acids downstream
3	Exon 39	3 bp deletion	c.6653_6655 delATA	N2218delN
4	Exon 39	Missense	c.6707A>T	N2236I
5	Exon 36	Missense	c.6274C>G	L2092V

*Nucleotide numbering refers to the cDNA sequence specifying the 2804 amino acid isoform A (NM_133433) starting at position +1 of the translation initiation codon in exon 2.

have occurred de novo, as they were not detected in the parents of the affected individual. No *NIPBL* mutation was detected in the two children carrying a chromosome imbalance. Finally, we identified three sequence polymorphisms, including two already known and a novel intronic (IVS13–17A>G) polymorphism.

DISCUSSION

Here, we report the thorough cytogenetic and molecular investigation of a series of 14 CdLS cases using standard and high resolution karyotyping, 1 Mb resolution array-CGH, and *NIPBL* mutation screening. We found *NIPBL* mutations in 35% of our patients (5/14). Their clinical manifestations were typical of CdLS except for some dysmorphic features in patient 6. These results support the view that *NIPBL* is a major disease causing gene in CdLS.^{8,9,12} The mutant genotypes described here are de novo mutations including splice site, frameshift, and missense mutations, and a small in-frame deletion. The conservative L2092V substitution is most likely disease causing as it occurred de novo, affected a highly conserved amino acid residue, and was not present in a large control population. Interestingly, none of the mutations reported here have been described so far, supporting the broad allelic heterogeneity of *NIPBL* mutations in CdLS.^{8,9,12}

Among the five patients with *NIPBL* mutations, only one (patient 3) had a family history, but unfortunately no DNA from the other affected child was available. However, since the mutation was not found in their parents' DNA, the recurrence of CdLS in this family is most likely the consequence of a germinal mosaicism for the *NIPBL* mutation. Based on this observation, we suggest giving consideration to germinal mosaicism when counseling asymptomatic parents of children with CdLS.

It is important to note that we failed to detect *NIPBL* mutations in the majority of the cases in our series (9/14). Whether the disease causing mutation has been overlooked or another locus is involved in CdLS remains questionable. In support of the genetic heterogeneity of the disease, it is worth remembering that a recent linkage study detected positive LOD scores at single markers on chromosomes 2q37, 10p13, and 14q24.⁸ Our study further supports genetic heterogeneity as two distinct de novo chromosomal anomalies were detected in CdLS patients with no *NIPBL* mutation: a 6.5 Mb subtelomeric deletion of chromosome 1q and an isochromosome 18p. There is some phenotypic overlap between CdLS and terminal 1q deletions. Indeed, both syndromes share some non-specific dysmorphic features (such as long philtrum, downturned corners of the mouth, and micrognathia) as well as heart defects and gastroesophageal reflux.^{16,17} Interestingly, patient 2 presented a combination of monosomy 1qter features (such as absence of corpus callosum) and characteristic CdLS features (arched eyebrows with synophrys, hirsutism, thin upper lip, behavioural problems, complete absence of speech, and short hands and feet). This observation suggests that some children diagnosed

with CdLS may have a 1qter deletion or, alternatively, that a second CdLS locus could map to 1qter. It is therefore tempting to regard the genes mapping to this interval (genes involved in DNA repair (*KAB*), cell signalling, differentiation, and apoptosis (*AKT3*), transcriptional regulation (*ZNF283*, *ELYS*), and purine nucleotide biosynthesis (*ADSS*)) as candidate genes in CdLS patients with neither chromosomal anomaly nor *NIPBL* mutation.

Similarly, tetrasomy 18p shares common features with CdLS (namely low birth weight, microcephaly, mental retardation, feeding difficulties, small nose, long philtrum, strabismus, and seizures^{18–20}) and the behavioural phenotype of our isochromosome 18p patient is also consistent with CdLS (hyperactivity, sleep disturbances, repetitive behaviour, and minimal speech). By contrast, other features characteristic of isochromosome 18p, namely microstomia, prominent upper lip, pinched nose, asymmetric long face, long and narrow fingers/toes, clenched hand/camptodactyly, spasticity, slender habitus, and scoliosis were not observed in patient 1.¹⁸ Differences in either parental origin or mechanism of formation of the isochromosomes may provide a molecular explanation for the discordant phenotypes between patient 1 and the previously reported individuals with i(18p).^{19,20}

The observation of unusual dysmorphic features in one CdLS patient with a *NIPBL* mutation and the absence of any distinctive feature in CdLS patients with or without *NIPBL* mutation in our series make the establishment of genotype-phenotype correlations difficult. Our findings support the genetic heterogeneity of CdLS and emphasise the importance of molecular cytogenetic studies in CdLS patients without *NIPBL* mutation.

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ELECTRONIC-DATABASE INFORMATION



The following URLs are mentioned in this study: http://www.ensembl.org/Homo_sapiens/cytoview and www.genome.ucsc.edu.

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