

# Epigenetics, Copy Number Variation, and Other Molecular Mechanisms Underlying Neurodevelopmental Disabilities: New Insights and Diagnostic Approaches

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**ABSTRACT:** The diagnostic evaluation of children with intellectual disability (ID) and other neurodevelopmental disabilities (NDD) has become increasingly complex in recent years owing to a number of newly recognized genetic mechanisms and sophisticated methods to diagnose them. Previous studies have attempted to address the diagnostic yield of finding a genetic cause in ID. The results have varied widely from 10% to 81%, with the highest percentage being found in studies using new array comparative genomic hybridization methodology especially in autism. Although many cases of ID/NDD result from chromosomal aneuploidy or structural rearrangements, single gene disorders and new categories of genome modification, including epigenetics and copy number variation play an increasingly important role in diagnosis and testing. Epigenetic mechanisms, such as DNA methylation and modifications to histone proteins, regulate high-order DNA structure and gene expression. Aberrant epigenetic and copy number variation mechanisms are involved in several neurodevelopmental and neurodegenerative disorders including Rett syndrome, fragile X syndrome, and microdeletion syndromes. This review will describe a number of the molecular genetic mechanisms that play a role in disorders leading to ID/NDD and will discuss the categories and technologies for diagnostic testing of these conditions.

(*J Dev Behav Pediatr* 31:582–591, 2010) **Index terms:** epigenetics, intellectual disability, molecular genetics.

The underlying cause of intellectual disability (ID) and other neurodevelopmental disabilities (NDD) remains unknown in the majority of affected individuals. Previous studies have cited a prevalence of 1% to 3% for ID, with chromosomal anomalies accounting for the majority of currently identifiable causes.<sup>1–8</sup> The last decade has seen an explosion in new molecular genetic technologies, many of which have resulted from a better understanding of the mechanisms underlying brain development and function. More recent studies have resulted in a higher diagnostic yield, likely reflecting the use of newer cytogenetic and molecular technologies, especially microarray-based comparative genomic hybridization (array-CGH or aCGH).<sup>1,9</sup> This review focuses on genetic mechanisms underlying several causes of ID and other NDD that are amenable to diagnostic testing.

Common causes of ID include chromosome abnormalities, microdeletions, fragile X other X-linked syndromes, and single gene disorders including inborn errors of metabolism, Rubenstein-Taybi syndrome, and

Rett syndrome (RTT). Although the clinical and behavioral phenotypes of many of these disorders have been known for some time, the complex mechanisms underlying a number of them have only recently been fully elucidated. One example is copy number variation (CNV) of genes that occurs in fragile X syndrome, and can be detected by aCGH. Also, it has been appreciated in recent years that changes in gene expression can occur by mechanisms that do not permanently alter the DNA sequence,<sup>11</sup> a phenomenon termed epigenetics. Epigenetic mechanisms are important regulators of biological processes; they include genome reprogramming during embryogenesis/gametogenesis, cell differentiation, and maintenance of cell lineages. Important in developmental processes, epigenetic modification may have long-term effects on learning and memory formation or in the development of cancers<sup>12</sup> (Table 1). For the reason that these modifications may be reversible, research has focused on biological therapies to modify disease states in which epigenetics plays a role.<sup>12</sup>

## Epigenetic Mechanisms and ID/NDD

The most common epigenetic effects are DNA methylation and histone acetylation, both of which store information that controls heritable states of gene expression. These modifications are highly dynamic and may be both tissue specific and developmentally regulated. This is unlike heritable changes in the DNA sequence which are strongly conserved and stable. Histone acetylation

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**Table 1.** Examples of Neurodevelopmental Disorders That Involve Epigenetic Mechanisms

Disorder	Epigenetic Mechanism	Genetic Mechanism	DNA Methylation Status	Histone Alteration
Fragile X syndrome	DNA methylation alteration	Expanded CGG repeats cause gene silencing	Hypermethylation	
Rett syndrome	DNA methylation alteration	Loss of activity of MECP2	Hypermethylation	
Coffin Lowry syndrome	Histone alteration	Impairment of MAPK signaling		Hypophosphorylation
Rubenstein-Taybi syndrome	Histone alteration/ DNA methylation	CREB regulation	Hypertrimethylation	Hypoacetylation

MECP2, methyl CpG binding protein 2; CREB, cAMP response element-binding.

can open the chromatin structure and favor gene transcription (i.e., turning the gene on). In contrast, DNA methylation can cause condensation of the chromatin with resultant gene silencing (Fig. 1).<sup>13</sup> Epigenetic mechanisms were originally thought to be stable and irreversible processes; however, several recent studies have shown that they are dynamic and can be reversed even in fully differentiated cells such as neurons.<sup>14</sup>

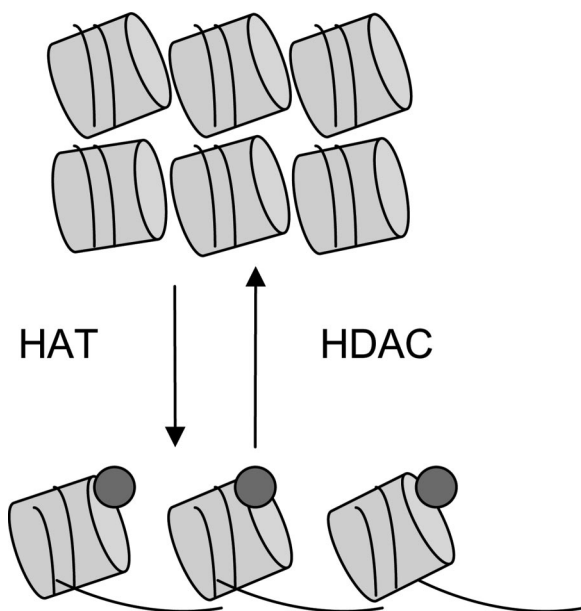
### DNA Methylation

DNA methylation is the most widely studied epigenetic mechanism. In eukaryotes, methylation consists of the covalent attachment of a methyl group (CH<sub>3</sub>) at the 5' position of cytosine residues. Methylation of cytosine forms CpG dinucleotides (the "p" in the CpG notation refers to the phosphodiester bond between the cytosine and the guanine), which are concentrated in regions called CpG islands that are found in promoter regions of genes (the

region promoting transcription). This modification is associated with turning off gene expression and is a mechanism observed in a number of NDDs (see Ref. 15). Methylation leads to silencing of imprinted genes (in which only 1 allele is expressed, either from the mother or father). This mechanism is the origin of most cases of Prader-Willi and Angelman syndromes (AS). Methylation also plays a role in X chromosome inactivation in females and, in accordance with the Lyon hypothesis, serves as a mechanism for dosage compensation (i.e., males have only 1 X chromosome but generally have the same level of X-derived gene products as females who have 2 X chromosomes). When X-chromosome inactivation is skewed, it can lead to expression of sex-linked conditions in females, such as ornithine transcarbamylase deficiency, a metabolic disorder associated with cognitive impairment related to hyperammonic crises.

### Histone Modification

Histone modification is another epigenetic mechanism that results in gene silencing.<sup>16</sup> DNA is condensed and packed in the nucleus of eukaryotic cells and wrapped around an octamer of proteins, termed histones (Fig. 1). Each octamer is comprised of 2 copies of each of the core histone proteins: H2A, H2B, H3, and H4.21. Several types of dynamic and reversible post-translational modifications in histones can occur. These include methylation of lysines and arginines, acetylation, phosphorylation, ubiquitination, ADP ribosylation, and SUMOylation.<sup>17,18</sup> There are 2 sets of antagonistic enzymes that either attach or remove the corresponding chemical group in a site-specific manner. The most commonly studied modification of histones involves acetylation, which is carried out by histone acetyltransferases (HATs), which promote gene expression, and can be reversed by histone deacetylases (HDACs), which inhibit gene expression. The changes in the post-translational chemical modifications of histones can also be used as targets for therapeutic intervention. As an example, HDAC inhibitors are gaining popularity in the treatment of childhood neurogenetic disorders by turning on important developmental genes that have been turned off by the mutation causing the disorder. There is some evidence that HDAC inhibitors such as suberoylanilide hydroxamic acid and trichostatin A ameliorate deficits in



**Figure 1.** Histone deacetylase (HDAC) inhibition—histone acetylation DNA (black) is wound around a core of histones (blue) to form the nucleosome, the smallest structural unit of chromatin. Nucleosomal histones are subject to acetylation (acetyl groups in gray) by histone acetyltransferases (HATs), which is associated with an open state of the chromatin. By this modification, correspondingly resulting from HDAC inhibition, DNA becomes transcriptionally active.

**Table 2.** Epigenetic Drug Treatment in Selected Pediatric Neurodevelopmental Conditions

Disorder	Type of Disorder	Type of Drug	Drug Actions
Adrenoleukodystrophy	Demyelination, white matter, degenerative	HDAC inhibitor	Restores peroxisome proliferation
Epilepsy	Gray matter, neurologic	HDAC inhibitor: valproic acid	Enhances GABAergic tone
Rubinstein-Taybi syndrome	Multiple malformation disorder with ID	HDAC inhibitor: SAHA Trichostatin A	Increases the level of late phase long term potentiation Improves memory deficit in CBP transgenic mice

HDAC, histone deacetylases; SAHA, suberoylanilide hydroxamic acid; ID, intellectual disability.

synaptic plasticity and cognition in mouse models of Rubinstein-Taybi syndrome and RTT<sup>19</sup> (Table 2).

### Small Interfering RNA

A third naturally occurring epigenetic modification involves small interfering RNA, which can suppress the activity of specific genes, causing changes in gene transcription and translation.<sup>20</sup> This mechanism may be involved in gene expression in fragile X syndrome, Down Syndrome, and Tuberous Sclerosis.<sup>21</sup> Although preliminary investigations of RNAi as a therapy using model organisms are encouraging, it is not currently in clinical trials for ID/NDD.

### Examples of NDD/IDs Associated With Epigenetic Phenomena

Certain NDDs associated with cognitive impairment can be attributed to disruptions in epigenetic function. These include single gene disorders and imprinted disorders include RTT, Rubinstein-Taybi syndrome, Coffin Lowry syndrome, Prader-Willi, and AS. Each of these disorders is caused by mutations in genes that encode proteins involved in the chromatin remodeling machinery.<sup>22</sup>

#### Rett Syndrome

RTT is an X-linked dominant neurodegenerative disorder. Affected patients have mutations in the gene encoding the methyl CpG binding protein 2 (MeCP2) that attaches to methylated DNA.<sup>23</sup> The majority of cases of RTT are the result of de novo mutations in this gene that can arise in either parent, but more frequently occur in the paternal gamete.<sup>24</sup> Mutations in the MECP2 gene account for half of girls with classic RTT. This mutation is prenatally lethal in males. A few surviving RTT male cases have been reported, but these were generally mosaic for MECP2 mutations.<sup>25</sup> In addition, germline MECP2 mutations can cause a severe neonatal encephalopathy leading to neonatal demise in males and to a milder X-linked ID syndrome in females.<sup>26</sup> Recently, a novel X-linked ID syndrome has also been associated with duplications of the MECP2 chromosomal region.<sup>27</sup> This suggests that careful regulation of the MECP2 gene is required for typical brain development and function, because both over expression and reduced expression are associated with ID.<sup>28</sup>

The MECP2 gene encodes a nuclear protein that acts as a transcriptional repressor, i.e., silences other genes.<sup>29</sup> Mutations in another gene, CDKL5 (which encodes cy-

clin-dependent kinase-like 5) have also been implicated in a Rett like syndrome phenotype.<sup>30,31</sup> MeCP2 and CDKL5 interact both in vivo and in vitro, suggesting that they belong to a common molecular pathway.<sup>32</sup>

#### Rubinstein-Taybi Syndrome

Rubinstein-Taybi syndrome is associated with the dysfunction of a HAT. An autosomal dominant disorder that is characterized by ID and physical anomalies, including broad radially deviated thumbs and halluces, postnatal growth deficiency that is later followed by excessive weight gain, characteristic dysmorphic facial appearance, and an increased risk for developing tumors.<sup>33,34</sup> Rubinstein-Taybi syndrome is caused by mutations in the cAMP response element-binding protein gene (CBP). As a coactivator of transcription, CBP has dual functions in mediating both gene activation and epigenetic modification. CBP recruits other transcription factors to the transcriptional machinery and, in addition, has HAT activity that alters chromatin structure. This has the potential to affect regulation of other genes, but the underlying mechanism linking this epigenetic regulation to brain development is currently unknown.

#### Coffin-Lowry Syndrome

Coffin-Lowry syndrome is a neurological disease caused by a deficiency in a histone phosphorylase. An X-linked disorder, this syndrome is associated with severe ID and a dysmorphic facial appearance, including a prominent forehead, down slanting palpebral fissures, orbital hypertelorism, thick lips, a thick nasal septum with anteverted nares, and irregular or missing teeth. Coffin-Lowry syndrome is caused by loss-of-function mutations in the RSK2 gene (also known as RPS6KA3) on chromosome Xp22.2.<sup>35</sup> RSK2 is a growth factor-regulated serine-threonine protein kinase that acts in the ras-mitogen-activated protein kinase signaling pathway. Coffin-Lowry syndrome is considered an epigenetic disorder because RSK2 affects chromatin structure through direct phosphorylation of histones as well as by interactions with cAMP response element-binding protein P.<sup>36,37</sup>

### Imprinting Disorders

Genomic imprinting is an epigenetic phenomenon in which the activity of the gene is modified depending on the sex of the transmitting parent. Typically, mammals inherit 2 complete sets of chromosomes (alleles), one

**Table 3.** Genetic Mechanisms Leading to Angelman Syndrome

Type of Mutation	Mechanism	Percentage	Comments
Class I	Deletion of chromosome 15q11–13; includes chromosome rearrangements	70 – 75	Most deletions are de novo and of maternal origin. Microdeletions occur because of unequal crossing over between low copy repeats (duplicons) within the chromosome region. The duplicons contain the <i>HERC2</i> gene, a highly conserved gene, which when mutated leads to developmental and fertility problems
Class II	Uniparental disomy for chromosome 15 and failure to inherit a maternal copy of UBE3A	2 – 3	
Class III	Abnormal methylation, consistent with a defect in imprinting	2	
Class IV	UBE3A mutations; (ubiquitin protein ligase—targets proteins for degradation)	5 – 10	
Class V	No genetic abnormality identified	12 – 15	

from the mother and the other from the father. Most autosomal genes are expressed in both maternal and paternal alleles. However, imprinted genes show expression from only 1 allele (the other is silenced), and this is determined during production of the gamete. Imprinting implies that the gene carries a “tag” placed on it during spermatogenesis or oogenesis. Imprinted genes are important in development and differentiation, and if expression from both alleles is not maintained, disturbances in development can result.<sup>38</sup> A genome wide search of imprinted human genes has identified >150 candidates. The first human imprinting disorder discovered was Prader-Willi syndrome (PWS). It is caused by a paternal deletion in chromosome 15 or maternal uniparental disomy (UPD) (in which both chromosome 15s come from the mother). Other examples of imprinted neurogenetic disorders include AS and Beckwith Wiedemann syndrome.<sup>39</sup>

### Prader-Willi Syndrome

PWS is characterized by severe hypotonia and feeding difficulties in early childhood, followed by an insatiable appetite and obesity by school age. PWS features significant motor and language delays in the first 2 years of life, borderline to moderate ID and severe behavioral problems, including compulsive and hoarding behaviors. Many affected children satisfy the criteria for the diagnosis of autism. Hypogonadism manifests as genital hypoplasia, incomplete pubertal development, and infertility in both males and females.<sup>40</sup>

The estimated prevalence of PWS is 1/10,000 to 1/22,000,<sup>41</sup> and the genetic defect involves 1 of 3 mechanisms: deletion, imprinting defect, or maternal UPD.<sup>40</sup> Approximately 75% of patients with PWS have a specific microdeletion, 15q11.2–13. This can be detected by high-resolution chromosomal analysis (i.e., greater than 550 band level), by fluorescence in situ hybridization (FISH) testing, or by aCGH. One percent of patients with PWS have a detectable chromosomal rearrangement re-

sulting in a deletion of 15q11.2-q13. Less than 1% of patients have a balanced chromosomal rearrangement with breakpoints within 15q11.2-q13. Less than 5% have an imprinting defect, and ~20% have UPD.<sup>42</sup>

### Angelman Syndrome

AS has a characteristic facial appearance (hypopigmented skin and eyes, prominent mandible, tongue thrusting, wide mouth, wide-spaced teeth), developmental delay, progressive microcephaly, ataxic gait, absence of speech, seizures, and inappropriate bouts of laughter.<sup>39</sup> The prevalence of AS is about 1:20,000. There are 4 genetic mechanisms that can cause AS, and a classification scheme has been developed to group patients according to etiology (Table 3). The basic underlying defect involves the UBE3A gene, which encodes ubiquitin protein ligase E3A. This enzyme is involved in targeting proteins for degradation within cells. Both copies of the UBE3A gene are active in most of the body's tissues, but in the brain only the maternal copy is normally active. If this copy is mutated or lost, it affects many developmental processes.

Genotype-phenotype correlations have been shown for AS. Patients with chromosome 15 deletions tend to be the most severely affected. They have a higher incidence of epilepsy, microcephaly, ID, absence of speech, and hypopigmentation.<sup>43</sup> Patients with UPD have a lower incidence of seizures, microcephaly, and hypopigmentation. They may have sparse speech, normal growth parameters, and less obvious dysmorphisms. Patients with imprinting defects are less likely to have seizures, microcephaly, and hypopigmentation, and show better motor and communication skills. Individuals with UBE3A mutations have features that fall between those of the UPD and deletion groups. They exhibit microcephaly and seizures but are not hypopigmented. Their speech and motor skills are generally better than those seen in the deletion group.<sup>44</sup> Many affected children satisfy the criteria for the diagnosis of autism.

## X-Linked Disorders Associated With ID

The expression of genes for X-linked disorders can be modulated by epigenetic control mechanisms leading to ID. Non-random X inactivation can result in tissues in which 1 cell type dominates in females. Here the X chromosome derived from the mother is the active one in most cells rather than there being equal number of cells containing maternally derived and paternally derived active X chromosomes. Skewed inactivation has been documented as the cause of NDD in heterozygous females with a number of X-linked conditions, including the urea cycle disorder ornithine transcarbamylase deficiency and certain forms of muscular dystrophy.<sup>45</sup> It is also possible that a functional disomy of X-linked genes can occur in females who have structurally abnormal X chromosomes that do not contain the X inactivation center.<sup>46</sup> Dose-dependent over expression of X-linked genes that “escape” X inactivation can also account for ID in individuals with X chromosome aneuploidic disorders (i.e., when there are extra or deleted copies of X chromosomes) such as Turner syndrome (X0) and Klinefelter syndrome (XXY).

## Copy Number Variation

There has been an increased recognition that copy number variability account for a number of NDDs.<sup>47,48</sup> This discovery has been enabled by advances in molecular cytogenetics, specifically microarray-based technologies that allow for high-resolution screening of the entire human genome simultaneously. CNVs involve either (1) DNA segments of 1 kilobase (Kb) or larger that are present in variable copy numbers in comparison to a reference genome, or (2) variations in the length of simple DNA triplet repeats, often referred to as “length dependent variations of triplet repeats,” which may involve only 150–180 bases (see also discussion below). CNV tend to be more dynamic than point mutations, and they often occur de novo. They cause NDD due to abnormal gene dosage.<sup>48,49</sup> One particular type of CNV is the trinucleotide repeat expansion which has been linked to a number of disorders that do not follow typical Mendelian inheritance. The first disorder discovered was fragile X syndrome and others include Huntington disease, myotonic dystrophy, Friedrich ataxia, Dentatorubropallidal atrophy, and the Spinocerebellar ataxias. Trinucleotide repeat disorders result from problems in recombination and replication during meiosis. The expansion length is linked to the phenotype, with the longer expansions presenting with the more severe clinical signs and symptoms. This is because the trinucleotide repeat expansion interferes with gene expression.

### Fragile X Syndrome [Fra (X)]

Fra (X) is the most common inherited cause of ID. Boys and girls with Fra (X) have a phenotype that includes a characteristic physical appearance, cognitive skills deficits, and impaired adaptive behaviors.<sup>50</sup> Many affected children satisfy the criteria for the diagnosis of

autism. The prevalence of Fra (X) for males with the full mutation is about 1:3,600. Prevalence of the full mutation in females is estimated to be at least 1:4,000 to 1:6,000.

Fragile X arises from an expansion of the number of cytosine-guanine-guanine (CGG) triplet repeats occurring within the initial 5' untranslated region of the *FMR1* gene (fragile X mental retardation protein gene). Inheritance of the instability in CGG regions leads to expansion from the normal number of repeats (6–40) to a premutation state (50–200) or from a premutation state to full mutation (>200 repeats). The stability of the CGG repeat depends on the length of the repeat as well as the sex of the individual passing on the mutation. The increased risk of CGG expansion from one generation to another is a phenomenon termed anticipation.

The full mutation state results in hypermethylation of the promoter region of the *FMR1* gene. Methylation of a CpG island in a promoter region of a gene usually prevents expression of the gene (i.e., prevents transcription and translation). The number of trinucleotide repeats as well as the methylation changes in *FMR1* can be detected by clinically available molecular genetic blood testing. There are rare cases of males with an unmethylated full mutation (i.e., expanded repeat but lacking CpG methylation in the *FMR1* gene) who do not have ID.<sup>51</sup>

## Microdeletion Syndromes

Microdeletion syndromes involve chromosomal deletions that span several genes but are too small to be detected using conventional cytogenetic techniques. Homologous recombination of flanking low-copy repeat gene clusters is the cause of many of these disorders.<sup>52</sup> Such low-copy repeats, now called duplicons, flank genomic regions that are prone to deletion, duplication, and inversion. The features of microdeletion syndromes such as Smith-Magenis syndrome (SMS), Williams syndrome (WS), and DeGeorge/velocardiofacial syndrome (VCFS) are because of these gene dosage effects. This involves the same mechanism as in imprinted disorders such as AS and PWS.<sup>39,40</sup>

### Smith-Magenis Syndrome

The SMS is a disorder manifest by multiple congenital anomalies and ID.<sup>53</sup> The clinical phenotype includes rather distinctive craniofacial and skeletal features that change with age, a history of infantile hypotonia, significant expressive language delay, ID, stereotypies, behavioral problems, and a sleep disorder because of an abnormal circadian secretion pattern of melatonin.<sup>54,55</sup> Many affected children satisfy the criteria for the diagnosis of autism. Recently, it has been appreciated that 2 genetic mechanisms contribute to SMS: an interstitial deletion involving chromosome 17p11.2 (including the retinoic acid-induced 1 gene) and a mutation in the retinoic acid-induced 1 gene.<sup>56,57</sup>

### Velocardiofacial Syndrome

VCFS is an autosomal dominant condition caused by a 3 Mb deletion of contiguous genes on chromosome

22q11.2. Multiple organ systems are affected including the face, palate, and heart. The condition has been referred to by various names including VCFS, Shprintzen syndrome, DiGeorge syndrome, conotruncal face anomalies syndrome, and CATCH 22 deletion. The VCFS syndrome presents with a highly variable clinical expression pattern that encompasses several different clinical presentations.

The prevalence of VCFS is estimated to be about 1 in 4,000, although this might represent an underestimation because of lack of case ascertainment. The 22q11.2 deletion is diagnosed in individuals who have a submicroscopic deletion of chromosome 22 that can be detected by FISH with DNA probes from the DiGeorge chromosomal region. Fewer than 5% of patients with clinical symptoms of the 22q11.2 deletion syndrome have normal routine cytogenetic analysis and negative FISH testing. At least 30 genes are encoded in the involved region of DNA. Research focusing on the neurobehavioral and cognitive aspects of VCFS delineate a non-verbal learning disability. Cognitive problems are present in the majority of individuals,<sup>58-60</sup> and many affected children satisfy the criteria for the diagnosis of autism.

### **Williams Syndrome**

WS is a chromosomal microdeletion syndrome characterized by a specific phenotype consisting of cognitive impairment in association with a characteristic cognitive profile, unique personality characteristics, distinctive facial features, and cardiovascular disease. In addition, a range of connective tissue abnormalities and multiorgan anomalies is observed. Hypercalcemia and hypercalciuria may be present in infancy.<sup>61</sup>

WS can be transmitted as an autosomal dominant disorder. Both females and males are affected equally. Most cases occur de novo, but parent-to-child transmission has been observed,<sup>61</sup> and there is a 50% risk of transmitting the deletion to offspring.

Clinical diagnostic criteria are available for WS; however, the most reliable means for diagnosis relies on detection of the contiguous gene deletion of the WS critical region on chromosome 7q11.2 that encompasses the elastin (*ELN*) gene as well as numerous genes believed to contribute to the unique phenotype. Virtually, all individuals with the clinical diagnosis of WS have been found to have this contiguous gene deletion, which can be detected using FISH.<sup>62</sup> The origin of the deletion can be paternal or maternal, without any parent of origin effect on the phenotype. Many of the clinical manifestations of WS are due to hemizygosity (one copy) of the elastin gene. Lim kinase 1 (*LIMK1*), a gene contiguous to *ELN*, is a second gene implicated in the WS phenotype.<sup>63,64</sup> These deletion disorders can be diagnosed by FISH or aCGH.

### **Technologies for the Diagnosis of the Child with an NDD**

How does the clinician approach the workup of the child with an NDD using the tools available in the cyto-

genetic and molecular genetics laboratories? Previous studies have demonstrated that genomic imbalance accounts for at least 3% to 4% of cases of idiopathic ID,<sup>65-67</sup> as determined by traditional cytogenetic methods, i.e., karyotyping and FISH analysis. In recent years, new screening methods for cryptic deletions including multiplex ligation-dependent probe amplification (MLPA) and aCGH have been developed. It is likely that they will significantly increase the detectable origins of ID.

### **Karyotype Analysis**

Cytogenetic evaluation will continue to have a high yield in children with ID who also have multiple congenital anomalies. A high-resolution karyotype (850-1000 band stage) usually requires cell synchronization methods or the addition of chemical agents to prevent chromosome condensation. Using this method, the smallest detectable imbalance (deletion, duplication) is ~2-3 Mb at standard metaphase resolution (500 bands) and 5-10 Mb at high resolution, including balanced translocations. The karyotype can reveal chromosomal deletions, rearrangements, translocations, or other abnormalities.

### **Fluorescence in Situ Hybridization**

Diagnosis of chromosomal anomalies has been improved with the use of FISH, which was the first molecular method for detection of submicroscopic genomic CNVs. FISH uses fluorescent-labeled chromosome-specific DNA segments or probes, which light up when exposed to ultraviolet light. The clinical use of FISH is in situations where the karyotype is normal, but there is clinical suspicion of a known deletion syndrome. FISH detectable deletions occur in ~1:7,000 live births. Most deletions are deleterious due to haploinsufficiency, because a single copy of genetic material cannot serve the same functions that are normally performed by 2 copies of the genes. Several of the deletion syndromes also involve imprinted genes. FISH has been used to detect PWS, AS, 22q11.2 deletion syndrome (VCFS), and Down syndrome.

A further improvement in this technology is called subtelomeric FISH that is combined with specific amplification methods, such as MLPA, that can probe for subtelomeric imbalances that are found in about 6% of individuals with idiopathic ID. MLPA relies on the use of progressively longer oligonucleotide probes to generate locus-specific amplicons of increasing size that can be resolved electrophoretically. These tests have served as precursors to the development of microarray-based technology, which is rapidly replacing these older methodologies.<sup>68,69</sup>

### **Microarray use in the Evaluation of ID/NDD**

aCGH is a new technology that can identify microscopic and submicroscopic chromosomal imbalances.<sup>48,70,71</sup> A DNA microarray is a multiplex technology that consists of an arrayed series of thousands of microscopic spots of DNA of a specific sequence, known as probes. Arrays with extended coverage at subtelomeric regions have replaced the subtelomeric probe testing described above.

As a result of array-based technologies, new ID genes and novel deletion and duplication syndromes have been identified such as deletions of 1p21.1 in patients with ID with or without other congenital anomalies<sup>72</sup>; recurrent rearrangements of 15q13.3 in patients with ID, dysmorphic features and/or seizures and autism<sup>73</sup>; deletions 16p11.2<sup>74</sup> in subjects with autism and neuropsychiatric conditions; and rearrangements of 17q21.3.<sup>75,76</sup>

The sensitivity of an array is determined by probe coverage (density), probe location, and choice of formats (i.e., targeted vs whole genome array). A targeted array will test for known areas of the genome that are associated with imbalances, whereas whole genome coverage is more expansive in its coverage. aCGH compares copy number of genomic loci between the patient and reference samples. These variations can range from an imbalance of an entire chromosome (aneuploidy, as in Down syndrome) to submicroscopic imbalances (deletions or duplications, as in WS). Coverage on aCGH is greater than in karyotype or FISH, with a yield close to 10% in the ID population.<sup>65,77</sup>

Many clinical laboratories now offer aCGH testing on a variety of platforms, but there is significant variability among laboratories in terms of the coverage offered on the probe. This can lead to different results for the same patient who is tested in 2 different laboratories. This lack of standardization has led to guidelines to address this issue.<sup>78</sup>

### **Bacterial Artificial Chromosome (BAC) Versus Oligonucleotide Arrays**

The first microarrays were based on BACs, which are typically 150 to 350 kbp in size but can be greater than 700 kb. BACs work by using a set of overlapping probes representing the entire genome, requiring as many as 32,400 clones to cover the human genome. In comparison, an oligonucleotide microarray is made with synthetic probes, usually 25 to 60 bases long, each designed to hybridize to a specific DNA. Oligonucleotide arrays offer several advantages over BAC arrays. Probe sequences for oligonucleotide arrays are based on the reference human genome sequence, so any sequence of interest can be a potential target. This contrasts with a BAC array that requires selecting BACs from existing libraries. Also, oligonucleotide probes are synthesized *in situ* on the array, allowing easy customization of content. Many commercially available arrays offer whole-genome coverage. It is also possible that an array can be custom designed to offer higher density coverage for a specific region of the chromosome, such as chromosome specific arrays that target the X chromosome.<sup>79,80</sup>

To be clinically useful, abnormal results obtained via arrays must be confirmed through a secondary method. This method should be able to detect copy number at the specific interval identified on the array. An accurate genetic diagnosis may require knowledge about chromosomal rearrangements. Genetic counseling should always be part of the analysis. For example, it may be

determined that the genomic gain or loss picked up on an array platform may be due to transmission of unbalanced genomic material from a balanced carrier parent. Only cytogenetic methods, such as FISH, can provide information about these chromosomal rearrangements.

### **Clinical Applications for Targeted Versus Whole-Genome Arrays**

Targeted arrays are designed to interrogate areas of the genome known to be associated with a disease or syndrome.<sup>80</sup> These originally included areas of known microdeletion/duplication syndromes and subtelomeric deletions but have now been expanded to include the pericentromeric regions and many Mendelian loci. Not all targeted arrays contain probes for Mendelian loci for which the mechanism of disease is typically a sequence variant rather than a deletion or duplication. This may lead some clinicians to assume that a negative test automatically rules out the associated condition. Despite these limitations, targeted arrays are advantageous because the sequence variants detected are usually well understood and their clinical relevance is more readily interpretable.

### **Variants of Unknown Significance Mandate Parental/Family Testing**

When a CNV is identified in a patient, the next step is to test the parents to determine whether it is *de novo* or inherited. Problems in interpretation include incomplete data due to non-paternity, adoption or one parent not being geographically available. An apparent *de novo* imbalance may be due to a balanced rearrangement from a parent. If this imbalance is found to be inherited, careful clinical examination of the parents is needed. Further evidence that the CNV is disease causing would be finding that the same or overlapping CNV seen in patients affected with similar phenotypes is absent in control populations (Table 4). CNVs in regions that are gene rich, are more likely to be clinically significant than those in gene poor regions. Deletions are generally considered more pathogenic than duplications, although this is not always the case. The size of genomic variants also influences pathogenicity, although relatively large variants (>500 Kb) can be familial, and many relatively small variants may be pathogenic.

Identification of novel variants is common in aCGH testing, and even more common with whole-genome

**Table 4.** Considerations in Determining Whether a CNV is Pathogenic

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The change is observed in affected individuals.
The change is not observed in unaffected parents or control populations.
Individuals with the same change manifest the same clinical features (phenotype).
The size of the deletion/duplication, even if present in an unaffected parent.

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CNV, copy number variation. Additional considerations include the gene content of the CNV.

arrays. Determining the clinical significance is the most challenging aspect of using aCGH testing. Information about CNV locations and clinical correlations are catalogued in several public databases, including the Database of Genomic Variants and the DECIPHER Database. Most companies will query these databases, and many clinical laboratories also maintain internal databases.<sup>81</sup>

### Microarray Analysis Versus Karyotype and FISH: Advantages and Limitations

Currently used as an adjunct to karyotyping, many proponents assert that the CGH should be the first line of evaluation for chromosomal imbalance. The value of array technology is that it is able to target multiple regions of the genome. FISH and karyotype analyses are typically ordered when there is a clinical suspicion of a specific disorder, whereas genome wide studies with aCGH can be performed without a specific suspicion of a diagnosis. This has the advantage of potentially identifying individuals with a disorder before the full manifestation are evident, allowing earlier intervention and potentially improved outcome. It may also streamline and limit the cost and number of tests that are conducted.<sup>82-84</sup> Limitations of CGH technology are its inability: (1) to detect balanced chromosomal rearrangements; and (2) to identify and subsequently interpret CNVs of unknown significance that may be identified. Practice guidelines for array CGH have recently been published by The American College of Medical Genetics.<sup>85</sup>

### Methylation Testing

Abnormal parent-specific methylation imprinting can be detected using methylation analysis.<sup>86</sup> For example, in PWS, if the methylation pattern is characteristic of maternal inheritance only, the diagnosis of PWS is confirmed. Importantly, DNA methylation analysis is able to detect virtually all cases of PWS whether caused by a deletion, UPD, or an imprinting defect.

### Can Therapeutics Be Developed for NDDs Based on Epigenetics?

One reason that understanding epigenetic mechanisms is important is that they may serve as targets for therapy in neurodevelopmental disorders. For example, DNA-demethylating drugs may have a potential role in several neurodegenerative and neurodevelopmental disorders, including fragile X syndrome and Rubinstein-Taybi syndrome. Currently, HDAC inhibitors (sodium butyrate, phenylbutyrate, trichostatin A, and suberoylanilide hydroxamic acid) are being studied in animal models of these disorders. As an example, CBP-negative mouse models of Rubinstein-Taybi syndrome that have been treated with HDAC inhibitors show improvement in long-term memory deficits. Here, HDAC inhibitors have increased histone acetylation sufficiently to compensate for absent CBP.<sup>34,87</sup>

### Summary

During the past two decades, a number of novel genetic mechanisms have been demonstrated to cause certain forms of ID/NDD. Many of these involve epigenetic changes in DNA that do not alter the sequence. Evidence from patients with neurodegenerative and neurodevelopmental disorders indicates that epigenetic mechanisms and chromatin remodeling need to be tightly controlled for proper cognitive function to occur and that their dysregulation can have devastating consequences on neurodevelopment. For the reason that they are dynamic, epigenetic mechanisms are also potentially reversible and may be manipulated by pharmacological interventions. Understanding these mechanisms has led to new strategies for diagnosis, the use of microarrays. aCGH can identify microscopic and submicroscopic chromosomal imbalances. It has led to the discovery of many new syndromes and is now recommended in the diagnosis of unknown causes of ID/NDD.<sup>88</sup>

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