Nuclear and Mitochondrial Genome Defects in Autism: Genomic Instability and Impact of Epigenetic and Environmental Factors

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Introduction

The term autism is applied to a triad of symptoms including communication deficits, impaired social interactions, and repetitive stereotypic behavior. Autism manifestations may occur in diseases with defined Mendelian genetic etiology, e.g., in tuberous sclerosis, fragile X mental retardation, and in specific metabolic conditions such as *phenylketonuria*. There is growing evidence that structural changes in the nuclear genome leading to deletions or duplications of specific chromosome segments are involved in the etiology of autism. These include microscopically visible changes and submicroscopic changes that alter copy number of specific segments and are detected through microarray analyses. *DNA sequencing* has revealed specific mutations at the nucleotide level in some cases of autism. There is also evidence that mitochondrial dysfunction, due potentially to changes in the nuclear or the *mitochondrial genome*, may lead to autism. Alteration in gene expression may arise from DNA changes or from dysregulation in epigenetic processes that modify DNA and impact gene expression. The goal of this chapter is to review genome changes observed in autism, the functions of key gene impacted by these changes. Specific environmental compounds that impact epigenetic factors and mitochondrial redox function will also be discussed.

Increased Frequency of Genomic Copy Number Variants in Autism

There is definite evidence that segmental chromosome changes including genomic duplications and deletions that lead to *gene dosage* changes occur with higher

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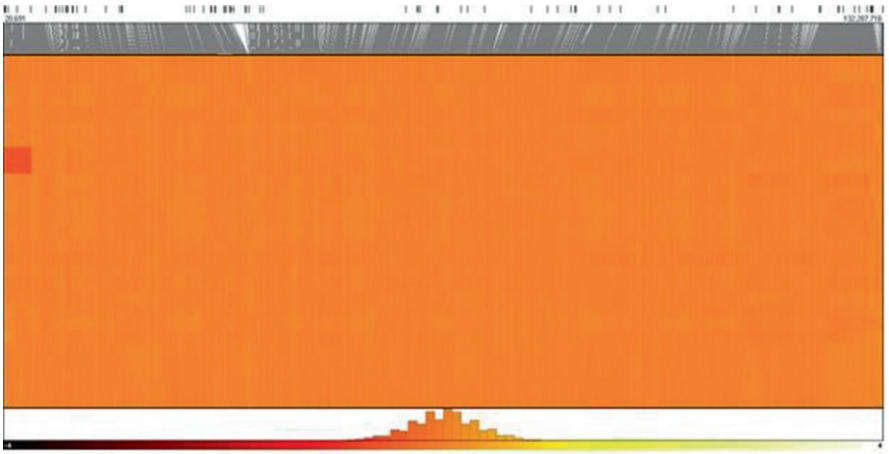


Fig. 1 Microarray *heat map* of chromosome 12. Image of microarray heat map of chromosome 12 derived using nuclear *DNA* from 27 individuals. In 2 of the 27 individuals, the hybridization signal intensity is less (*red signal*, *darker signal*) on the *left side* of the image that corresponds to the terminal portion of the short arm of chromosome 12 (12p)

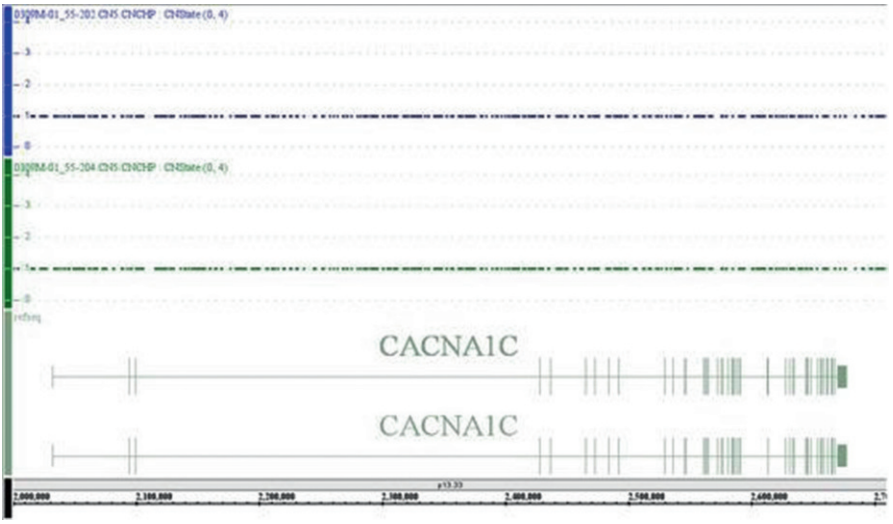


Fig. 2 Map of 12p gene deletion. This *map* indicates that the copy number of the ion channel gene CACNA1C is reduced to one in the individuals (siblings) shown on the microarray heat map to have deletion in the terminal region of 12p

frequency in autistic subjects relative to their siblings and relative to controls. These segmental changes are referred to as *copy number* variants (CNVs). These changes can be detected through microarray analyses. Examples from our studies of the use of microarrays to detect CNVs in autism probands are presented in Figs. 1 and 2.

The genomic approach to determining significance of specific rare copy number variations is to define the search parameters for microarray studies, e.g., number of *DNA markers*, including copy number markers and polymorphic *single-nucleotide* markers (SNPs); size of segments analyzed that are least likely to lead to *false-positive* results; and comparison of findings in test subjects and controls. Controls are individuals from the same population or siblings of autism subjects. It is also important that in comparing results from cases and controls, the cell source of DNA should be noted. There is evidence that copy number changes sometimes arise as sporadic events in cultured cells, particularly *lymphoblastoid cell lines*.

Sanders et al. 2011 carried out analysis of rare CNVs each detected by more than 20 markers and with no more than 50 % overlap with known polymorphic markers, in 872 probands and 872 matched siblings. They determined that the CNV frequency was significantly different in probands and siblings ($p = 3\times10^{-6}$). Rare CNVs occurred in 5.8 % of probands and in 1.7 % of siblings. Particularly striking was the fact that CNVs in probands impacted 1,153 genes, while in their unaffected siblings, CNVs impacted only 73 genes. The frequency of deletions was significantly different in probands versus controls ($p = 1\times10^{-4}$). In probands deletions occurred in 3.6 % of cases and impacted 625 genes. In siblings deletions occurred in 0.9 % of cases and together impacted 21 genes.

Girirajan et al. 2011 used custom microarrays and an analysis of CNVs each detected by at least ten probes. They determined that rare CNVs occurred in 11.1 % (10 of 90) of autism cases with *intellectual disability* and in 10.2 % (25 of 246) of autism cases without intellectual disability. They determined that the majority of CNVs were de novo in origin and were not transmitted from parents and that there was a trend of increased rates of de novo CNV origin and increased size of CNVs in cases with more severe intellectual impairment.

It is important to note that approximately 90 % of the subjects who met full diagnostic criteria for autism did not manifest *copy number* variants using the search criteria established in these studies.

Heterogeneity of Genomic Changes in Autism

There is evidence that defects in many different regions of the genome and in many different genes can lead to autism. Studies by Levy et al. 2011 revealed that rare segmental dosage changes or copy number variants (CNVs) at 300 or more different locations in the genome contribute to autism. Furthermore more than one rare CNV may occur in a specific person, and different CNVs may interact to cause the phenotype. It is also important to note that even for rare deleterious changes of large effect, e.g., 16p11.2 deletions, there is not a consistent correlation of genotype and phenotype.

Elucidation of the pathogenesis of autism is complicated by this *genetic heterogeneity* and by relative nonspecific nature of the autism diagnostic features. In a 2011 review, State and Leavitt questioned the phenotypic specificity of behavioral parameters used to diagnose autism. They noted that there are overlaps in symptoms that occur in a number of different psychiatric disorders.

Of particular interest is the fact that a specific genomic dosage change, i.e., a specific deletion or duplication, may lead to autism in some individuals and to cognitive impairment, *attention-deficit* hyperactivity disorders, or psychiatric manifestations in others (Guilmatre et al. 2009). These observations lead to the conclusion that specific genome dosage changes likely serve as risk factors for these disorders and that they interact with other factors to produce the autism symptom complex. Other risk factors may include nuclear or mitochondrial genomic mutations, epigenetic factors, and/or environmental factors.

The Role of Copy Number Variants in Autism Pathogenesis

Approaches to defining the pathogenesis of autism manifestations include analysis of the constellation of genome dosage changes present in a particular individual, delineation of the specific genes impacted by these dosage changes, and assessment of the functional pathways in which these genes act. An underlying currently unanswered question is whether only relatively large (greater than 100 kb) rare genome copy number dosage changes lead to autism or whether smaller rare copy number variations in specific combinations also play roles in autism pathogenesis.

State and Leavitt 2011 emphasized the importance of defining a sentinel event and then investigating if this specific event occurred in different cases with autism phenotype. Recurrent copy number variants that lead to autism reported in different studies are deletion or duplication of a region of 16p11.2, duplication of 15q11–q13, deletion or duplication of 22q11.2, and duplication of 7q11.23. Additional regions of recurrent autism-related CNVs occur in 1q21 and 17p21 and 15q13.3. In Figs. 3, 4, and 5, we present examples from our studies of de novo CNVs on chromosome 15 identified in autism subjects.

The impact of a specific deletion or duplication depends on whether the functions of the protein encoded by genes contained in the segments are dosage sensitive. In addition disruption of a portion of the sequence within a gene may alter the *reading frame* and generate abnormal products. In imprinted regions of the genome, the impact of dosage change varies depending on the parental origin of the altered chromosome. The status of the homologous chromosome may also influence the impact of a structural variant, e.g., a deletion on a specific chromosome may unmask a *recessive mutation* on the homologous chromosome.

Variable phenotypic *expressivity* of a specific copy number change may be due to occurrence of another CNV elsewhere. Veltman and Brunner 2010 proposed that two events, such as copy number variants at two different genomic sites, might act in concert to impact phenotype through additive or epistatic models. Girirajan et al. 2010 proposed that a two-hit model might be more generally applicable to neuropsychiatric disease. They identified second-hit CNVs in 6 out of 66 cases with 15q13.3 deletion and in 11 of 98 patients with 1q21.1 deletions. They proposed that the second-hit phenomenon might explain the *phenotypic variability* of recurrent *microdeletion syndromes*.

The question remains which specific genes within these chromosome regions impacted by copy number changes play significant roles in the etiology of autism.

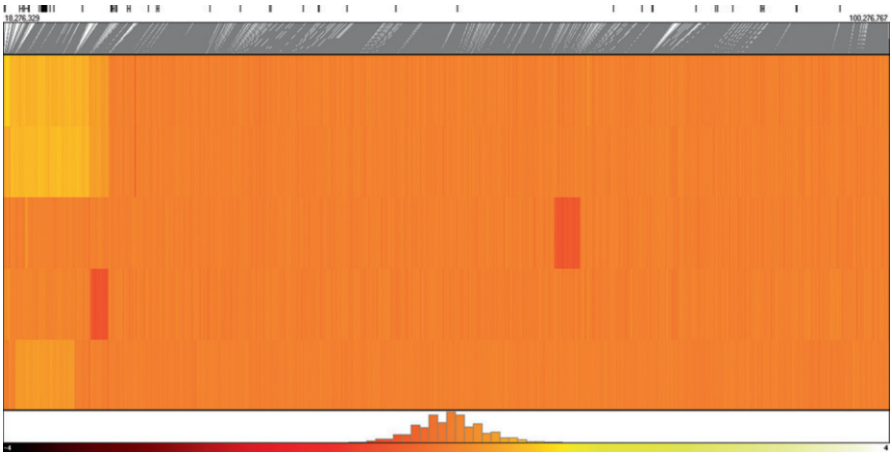


Fig. 3 Microarray *heat map* of chromosome 15. Image of microarray *heat map* showing hybridization signals on chromosome 15 derived using nuclear *DNA* from five different individuals. Three individuals have increased signals (*yellow signal*, *lighter signal*) due to increased *copy number* in the 15q11–q13.3 region on the *left side*. Two individuals have decreased signals (*red signal*, *darker signal*) due to deletion; one deletion involved the 15q13.2–q13.3 region, and the second deletion on the *right-hand side* involves 15q24.1–q24.2

Given the complexity of the *central nervous system* function and the nature of the symptoms in autism, it is likely that alterations in dosage or structure of gene products that function in a number of different functional pathways and gene products that encode structural synaptic elements potentially contribute to the manifestations.

It should be noted that in most reports of *copy number* variations in autism, researchers have concentrated on CNV that impacts protein-coding genes. Future studies should include analysis of CNV changes that involve regions of the genome from which nonprotein-coding RNAs are transcribed, including regulatory RNAs and microRNAs.

DNA Sequencing and Mutation Analysis in Autism

A number of genes identified as being present in recurrent rare CNVs in autism cases have also been found on *DNA sequencing* to have mutations of functional significance. These include neurexin1; SHANK1, 2, and 3; (SH3 *postsynaptic density* proteins); and the calcium ion channel gene CACNA1C, the GRIN2B glutamate receptor, and CNTNAP2, a protein involved in localization of ion channels (see Fig. 6).

In 2012 results of exome sequencing through next-generation sequencing approached on autistic subjects and family members were published by a several

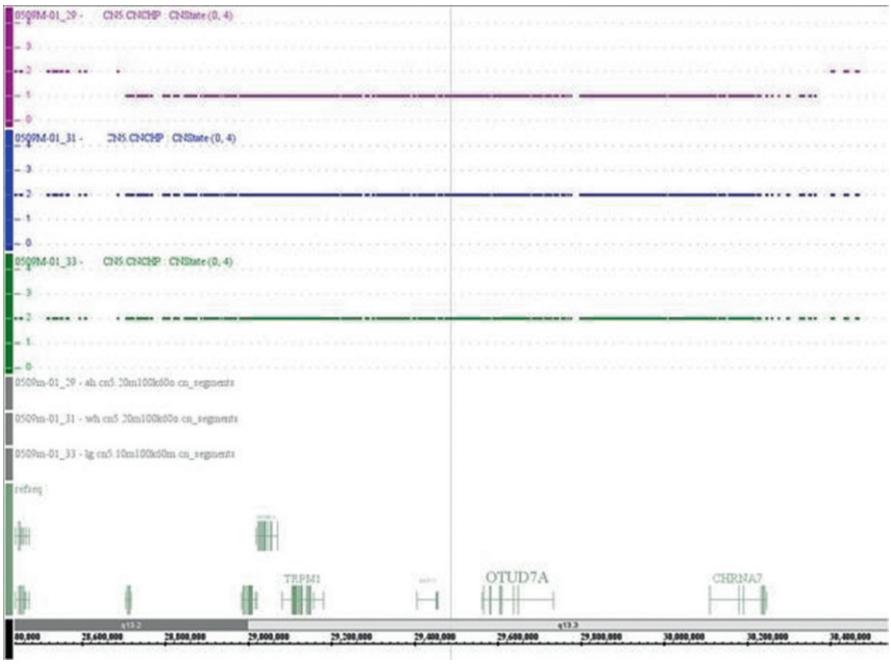


Fig. 4 Map of de novo deletion on 15q13.3. Map of chromosome 15 q13.2-q13.3 region. In the DNA derived from an *autistic* proband (*top*, 01–29), there is a deletion that reduced the copy number of this segment and the genes it contains, e.g., TRPM1 OTUD7A and CHRNA7, to one. This deletion was not present in the parents, 01–31 and 01–33, who each has two copies of this region. Deletion of CHRNA7 is likely to be a factor in the pathogenesis of the neurobehavioral disorder in this patient. CHRNA7 encodes the alpha-7 nicotinic cholinergic receptor subunit

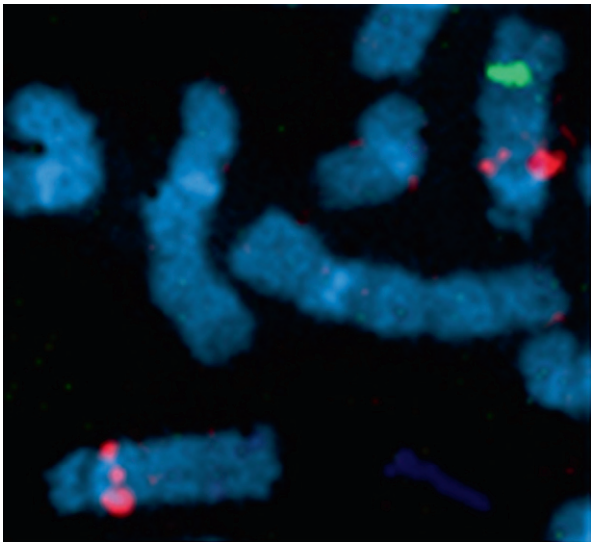
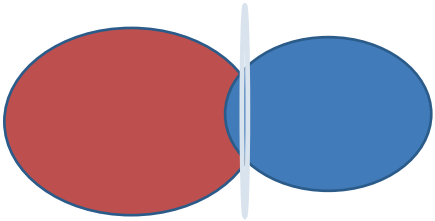


Fig. 5 Deletion of 15q24.1-q24.2. *Fluorescence in situ hybridization* and microscopy showing partial complement of chromosome in a cell. Two chromosomes have a *red* (*dark*) *signal* from a probe that maps close to the centromere. Only one of these two chromosomes has a *green-yellow signal* (*light*) from a probe that maps in the 15q24.1-q24.2 region



CACNA1C	Calcium ion channel
NRXN1	Synaptic cell adhesion
GRIN2B	Glutamate receptor
SCN1A	Sodium ion channel
SHANK1 SHANK2	Synapse-associated proteins
SHANK3	Synapse, regulation
CNTNAP1	Localization of ion channels

Fig. 6 Examples of genes impacted by either CNV or mutations in *autistic* probands. Specific genes that encode *synaptic proteins* are impacted by copy number variants in some patients with autism (*red oval*, *left*). These same genes have functionally significant mutations in other patients with autism (*blue oval*, *right*)

groups of investigators. O'Roak et al. 2011 reported the result of exome sequencing on 209 trios, comprised of autistic probands and parents. Only two different individuals carried mutations in the same genes, CDH8 (cadherin 8) and NTNG1 (netrinG1). In total they identified 49 protein-altering mutations and these mapped predominantly in an interconnected beta-*catenin* chromatin-remodeling network. They demonstrated that the de novo mutations were predominantly paternal in origin ratio (4:1, paternal to maternal) and that there was evidence of increased risk of de novo mutations in the children of older fathers. O'Roak et al. concluded that exome sequencing data and results of copy number variant analyses indicate extreme *locus heterogeneity* in autism.

Sanders et al. 2012 carried out sequencing in 200 families each with an autistic *proband* and an unaffected *sibling*. They determined that there was a significant difference between affected and unaffected siblings in the frequency of mutations in brain-expressed genes ($p = 0.01$). Non-synonymous and *missense mutation* occurred more frequently in autism-affected members of the sib-pairs. In the autistic probands, *loss of function mutations* occurred in 16 different genes. Only one gene SCN2A (voltage-gated sodium channel 2A) was mutated in two different probands. All other loss of function mutations occurred only in a single proband. Sanders et al. reported that mutations in KATNAL2 (*katanin* subunit A like 2) and CHD8 occurred in autistic probands in their study and that Neale et al. 2012 reported mutations in these genes in a different study of autism.

Neale et al. 2012 reported studies of exome sequencing in 175 autistic probands and parents. Their analysis of mutations in parents revealed a strong correlation between parental age and the number of mutations. In their initial cohort of 175 autistic probands, they identified mutations in three synaptic genes, KCN1 (voltage-gated potassium channel Kv1.8), KATNAL2, and SCN2A. They then

sequenced an additional 935 autistic cases and 870 controls. They identified three autistic cases with loss of function mutations; these occurred in KATNAL2 and CDH8. They concluded that their study revealed the challenge of identifying specific genes as risk factors in autism.

Results of the exome sequencing studies described above reveal significant heterogeneity if autism and few genes were found to be mutated in more than one study.

Targeted Exome Sequencing

Kelleher et al. 2012 carried out exome sequencing of genes that encode proteins that function in the glutamate receptor (mGluR) signaling pathway. They sequenced samples from 290 cases of nonsyndromic autism in the AGRE collection and from 300 ethnically matched controls. Following initial sequencing they validated findings on a second sequencing platform. They identified rare sequence variants that were found in cases but not in controls and classified these into four categories, benign, of unknown significance, possibly deleterious, and probably deleterious. Mutations defined as possibly or probably deleterious included rare nonsense mutations, splice donor or acceptor site mutations, and 3′ untranslated region mutations that could potentially impact *mRNA stability* or mutation. Nucleotide variants with allele frequencies less than 1 % in the population were considered rare variants.

Components of the mGluR signaling pathway analyzed in the study of Kelleher et al. included 18 genes. Included in the analysis were the *metabotropic glutamate receptors* mGluR1 and mGluR5, PSD *scaffolding proteins*, the fragile X mental retardation protein, five different kinases, the tuberous sclerosis genes *TSC1* and TSC2, EIF4 *translation initiation factor*, *UBE3A ubiquitin ligase*, endocytosis factor *r*, and HOMER1, a *metabotropic glutamate receptor* regulator factor. They identified an excess of potentially deleterious *single-nucleotide* variants in TSC1, TSC2, SHANK3, and HOMER1.

Kelleher et al. noted that their findings implicate TSC1 and TSC2 as risk genes for nonsyndromic autism. They noted further that none of the 8 TSC1 mutations and none of the 14 TSC2 mutations they found in autism cases have been previously reported in syndromic autism cases. In SHANK3 they identified 15 probably deleterious mutations; only one of these has been previously reported in autism. In five cases they identified potentially disruptive mutations in the HOMER1 gene. No disruptive mutations were found in this gene in controls. HOMER1 encodes a *postsynaptic density scaffolding protein*. One of the sequence variants they found is located in the 3′ untranslated gene region within a cluster of sequence elements that are *microRNA* binding sites. Kelleher et al. stated that there is growing evidence that sequence variants in 3′ untranslated region are possibly significant because of the density of cis-acting regulation elements in this region. In addition 3′ *untranslated* regions contain microRNA binding sites.

Relating Gene Defect to Behavioral Phenotype

Tuberous sclerosis due to mutations in either the TSC1 or the TSC2 gene leads to autism in a subset of patients. TSC1 and TSC2 gene products interact with each other and form together function as *Rheb GTPase* causing the Rheb *signaling molecule* to be present in its inactive form and to limit the function of *mTOR* (rapamycin-associated serine *threonine* kinase). Lewis et al. 2004 reported that autism occurred more frequently in patients with TSC2 gene mutations than in TSC1 mutation patient; *infantile spasms* occurred more commonly in TSC2 mutation patients ($p = 0.001$). They also established that intellectual impairment was more common in patients with truncating TSC2 mutations.

In Fig. 7, we illustrate a four-*base-pair* deletion leading to a frameshift mutation, in the TSC2 gene revealed in a patient with tuberous sclerosis by next-generation sequencing. The same deletion was subsequently identified in three other affected family members; two family members had seizures and intellectual impairment.

Numis et al. 2011 carried out a study to determine the genetic, electrophysiological, and neuroanatomic risk *factors associated* with *autistic behaviors* in patients with tuberous sclerosis. They reviewed medical records on 103 patients; 40 % of

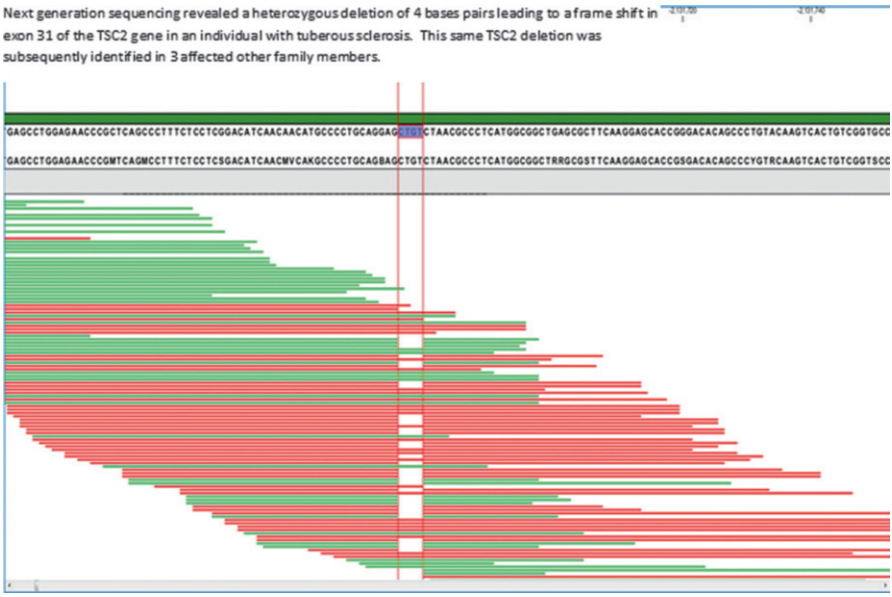


Fig. 7 Next-generation sequence analysis showing heterozygous deletion in TSC2. Four-base-pair deletion leading to a heterozygous frameshift mutation, in the TSC2 gene, revealed in a patient with tuberous sclerosis by exome capture and next-generation sequencing. Image shows multiple reads of the region from *left* 5′ and 3′ directions. The same deletion was subsequently identified in three other affected family members; two family members had seizures and intellectual impairment

the patients had a diagnosis of *autism spectrum disorders (ASD)*. They determined that the patients with *ASD* were more likely to have TSC2 mutations than *TSC1* mutations. They also determined that patients with tuberous sclerosis and autism had an earlier onset of seizures and more frequent seizures. In addition tuberous sclerosis patients with autism had a greater degree of temporal lobe interictal epileptiform activity. *Brain MRI* studies on these patients did not reveal regional differences in the *tuber* burden. Numis et al. concluded that persistent seizure-related activity in particular brain regions predisposes to autism disorders.

A key question that arises is whether heterozygous *TSC* gene mutations, independent of epilepsy and tubers, have a neurocognitive phenotype. van Eeghen et al. 2012 reported that mutations in the specific domain in either the TSC1 or the TSC2 gene product that determine the interaction of these proteins with each other are more likely associated with lower intellectual capacity in patients. In general missense or small in-frame deletions were found to be less deleterious to *intellectual development* than larger or out-of-frame mutations.

Mitochondrial Dysfunction in Autism

Evidence of mitochondrial dysfunction in autism, based on results of analysis of lactate and pyruvate in particular, has been reported by a number of different investigators. However, the question remains, whether mitochondrial dysfunction occurs secondarily or if it is a primary cause of autism.

Rossignol and Frye 2012a, b reviewed data from 536 children with autism in 18 different publications. In five of the studies that together included 114 autism-affected children and 114 controls pooled data revealed significant differences ($p = 0.0001$) between affected children and controls in blood levels of lactate and showed that levels were higher in cases than controls. Pyruvate, carnitine, *ubiquinone (coenzyme Q10)*, aspartate *aminotransferase*, and *alanine aminotransferase* were each examined in single studies that included between 15 and 147 *autistic* children and controls. In autistic children levels of pyruvate, aspartate aminotransferase, and alanine aminotransferase were elevated relative to controls, while levels of carnitine and ubiquinone were lower in autistic children. *Creatine kinase* was examined in two studies that together include 55 autism cases and 59 controls and levels were significantly higher in cases than controls. Rossignol and Frye 2012 reviewed clinical presentations and results of biochemical studies in cases of autism with evidence of mitochondrial dysfunction, compared with results in the general autism population. They noted that in children with autism and evidence of *mitochondrial disease*, developmental regression, motor delay, and gastrointestinal malfunction were more common, lactate and pyruvate levels were more elevated, and the male to female ratios were less skewed. In studies of electron transport complexes in children with autism and evidence of mitochondrial disease, levels of complex 1 were decreased in 53 % of cases and complex V levels were abnormal in some cases.

Rossignol and Frye noted that very few autism cases with *mitochondrial DNA* mutations have been reported. Reports include two cases with mutation in

mitochondrial *tRNA* (A3242A>G). It is important to note that mitochondrial dysfunction may arise due to nuclear gene mutations, structural changes, or *copy number* variations. Copy number variants associated with autism occur at many different sites in the nuclear genome. The current interpretation of these finding is that causative copy number variants impact specific genetic networks that contribute to optimal neurological function. The 2,000 nuclear genes required for mitochondrial assembly and functions constitute one such network (Smith et al. 2012).

Evidence of Increased Oxidative Stress in Autism

Chauhan et al. 2012 reported that higher levels of mitochondrial free radicals of oxygen were present in lymphoblastoid cells from autism cases versus controls. Studies of James et al. 2008 provided evidence of altered glutathione homeostasis in lymphoblastoid cells from children with autistic spectrum disorders. Chauhan et al. 2012 studied concentrations of oxidized and reduced glutathione in different brain regions from autistic subjects and controls. They determined that levels of reduced glutathione *(GSH)* were reduced in the temporal lobe and cerebellum in autism cases relative to controls. No significant concentration differences were found in the occipital, frontal, or parietal lobes. They concluded that glutathione redox imbalance existed in specific brain regions in autistic subjects. Decreased levels of expression of mitochondrial electron transfer complexes in cerebellum, frontal, and temporal region were found in brains of autism subjects in studies reported by Chauhan et al. 2011.

Oxidative Stress or Oxidative Shielding?

Naviaux 2012 postulated that accumulation of *reactive oxygen species* and chronic oxidative changes in membrane lipids and proteins result from a highly evolved oxidative shielding response that comes into play when cells are in a chemically hostile environment. He proposed that increased production of *reactive oxygen species (ROS)* is the result of the disease process rather than the cause of this process; therefore, the abnormal metabolic conditions that precede generation of *ROS* should be therapeutically targeted.

Dysregulation of Methylation and Redox of Pathways in Autism

Melnyk et al. 2012 investigated components in an integrated *metabolic pathway* that is essential for methylation and cellular antioxidant activity. They studied *autistic* children, their siblings, and controls.

This integrated pathway includes methionine regeneration from homocysteine and it involves activity of vitamin B12 and the transfer of *methyl groups* from 5-methyltetrahydrofolate through activity of methionine synthase. The second component of the pathway involves activation of methionine to generate

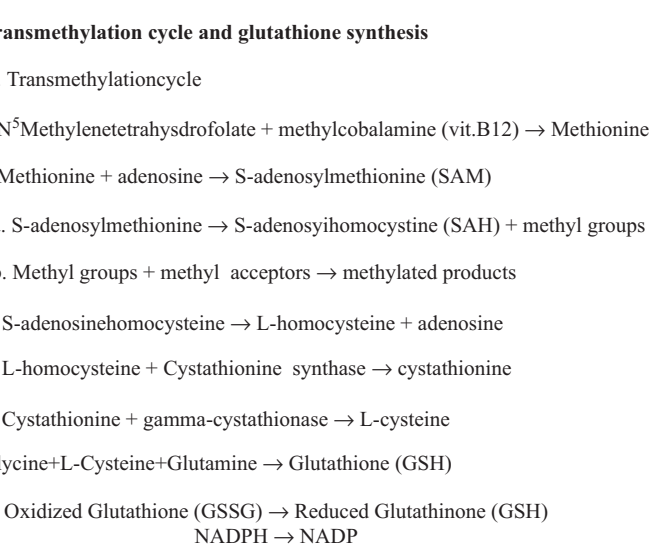


Fig. 8 Transmethylation cycle and glutathione synthesis. This is a schematic diagram of methionine synthesis and metabolism leading to release of methyl donors. Note also transmethylation cycle leads to generation of cysteine, a component of glutathione

S-adenosylmethionine (SAM). SAM acts as a methyl donor for a number of reactions, and methyl groups are transferred through the activity of methyltransferase. The demethylated form of SAM is S-adenosylhomocysteine (SAH). SAH is then converted to homocysteine and adenosine through the activity of the enzyme SAH hydrolase. Homocysteine may reenter the methylation cycle to form methionine or it may be converted to cystathionine through the activity of the enzyme cystathionine beta-synthase. Cystathionine is a component of the transsulfuration pathway and is converted to cysteine and to glutathione. Glutathione may be present in a reduced form GSH or it may be present in the oxidized form GSSG. The GSSG/GSH ratio is a reflection of the redox potential and is directly related to the antioxidant potential of the cell. In Fig. 8 we present a diagram of the *transmethylation* cycle and glutathione synthesis.

Melnyk et al. examined metabolites in fasting blood samples from 68 autism cases, 40 unaffected siblings, and 54 age-matched controls. Key results of their studies were statistically significant decreases in autistic cases versus siblings in levels of methionine, and SAM and in the SAM/SAH ratios ($p = <0.001$). There were statistically significant increases in levels of adenosine and SAH in autistic children versus siblings ($p < 0.001$). The only statistically significant difference between siblings and controls was that the levels of SAH were higher in siblings. Levels of cystine, the oxidized form of cysteine, were lower and the ratio of cystine to cysteine was lower in autism cases versus siblings. The levels of reduced glutathione *(GSH)* were lower in autistic cases, and ratio of GSH/GSSG was lower in autistic cases versus siblings.

Melnyk et al. concluded that the differences they observed could be due either to changes in the gene products involved in the reactions in this integrated pathway or to environmental exposures that impacted activity of key components. They emphasized that decreased activity of methyl donors and of methyl groups may contribute to dysregulation and to the genome-wide *hypomethylation* observed in autism. Furthermore impairments in the transsulfuration components of the integrated pathway may impair ability to maintain *redox homeostasis*.

Epigenetic Signatures in Autism

Epigenetic modifications impact gene expression. Methylation of chromatin involves methylation of *cytosine* residues in *DNA*, particularly cytosine methylation, at the 5′ position in cytosine guanine dinucleotide *(CpG)* and methylation of specific *amino acids* particularly lysine in histones. Shulha et al. 2012 characterized epigenetic signatures in the prefrontal neurons derived from postmortem samples from 16 cases of autism and 16 controls. They reported that in 4 of the 16 autism cases, there was excess spreading of the histone 3 lysine 4 methylation signal (H3K4me) downstream and upstream of from specific transcription start sites. Altered methylation signaling in autism cases versus controls was present in CACNA1 H (calcium channel, voltage-dependent, T type, and alpha 1H subunit) SEMA5A (SEMAPHORIN 5A), AUTS2 (autism susceptibility candidate 2), and PARK2 (PARKIN2).

Environmental Factors That Can Impact Methylation

Hou et al. 2012 reviewed environmental factors that impact chromatin methylation. *Hypomethylation of DNA* results from exposure to heavy metals, including cadmium and mercury, and from exposure to industrial chemicals such as benzene, *bisphenol*, and dioxin. In addition particular matter derived from vehicular emissions that includes black carbon alters *methylation status* of repetitive elements in DNA. Persistent exposures to organic pollutants have been shown to lead to alteration in methylation patterns in sperm. It is interesting to note that many industrial pollutants also increase production of *reactive oxygen species* that are DNA damaging.

Germline and Somatic Chromatin Hypomethylation and Genomic Instability

Hotspots of structural polymorphism in the human genome are usually rich in low copy DNA repeat sequences. These regions are associated with *genomic instability*. Li et al. 2012 generated maps of genomic regions of human DNA that are known to be hotspots of genomic rearrangement. Genomic structural variation was assessed

by analysis of *copy number* variants. They confirmed that a structural variant often originated in regions of *low copy repeats* and that recombination between low copy repeats led to recurrent deletions, duplications, and inversions.

They also derived maps of methylation through sequencing of sperm DNA. They determined that 20 % of the sperm genome showed evidence of poor methylation. Through comparison of the *methylome* map and the maps of structural variation hotspots, Li et al. determined that there is significant correlation between regions of hypomethylation and regions that are hotspots for structural variations. They determined that copy number variants originate twice as often in hypomethylated regions than in normally methylation regions of DNA.

Based on their studies, Li et al. concluded that rare and de novo CNVs in patients with autism and *intellectual disability* are concentrated within genomic regions of *hypomethylation*. The question then arises whether individual variation in genomic methylation results in an increased frequency of copy number variants.

Somatic genomic instability is a feature of cancer and *DNA* hypomethylation is commonly found in tumors. Lengauer et al. 1997 reported evidence for genomic instability and DNA hypomethylation in colon cancer cells. Howard et al. 2008 reported that mutations in the *maintenance methylase* Dnmt1 in mice were associated with DNA hypomethylation and tumor induction. *Chromosome instability* was also a feature of hypomethylated cells. These investigators determined that endosomal retroviral elements were activated in the Dnmt1-deficient hypomethylated cells. These activated elements inserted in specific genes and led to oncogenic activation. They concluded that spontaneous or chemically induced hypomethylation could lead to genomic instability through retroviral activation.

Chen et al. 1998 carried out studies on *embryonic stem cells* in which the Dnmt1 gene was deleted. They studied two genes, the *hypoxanthine* guanine phosphoribosyltransferase gene *(HGPRT)* and the thymidine kinase gene (TK), and determined that Dnmt1 depletion led to gene deletions at both loci. Abnormal *chromosome structure* is also a feature of cells treated with 5-aza-deoxycytidine a demethylating agent.

Transposable Element Activation and Genomic Instability

Barbara McClintock 1950, 1983 first reported that "traumatic events" led to unusual genome changes in maize. The events she described included abnormal conditions in cells, altered surroundings, exposure to *poisons*, and viral infections. McClintock demonstrated that chromosome breaks at a specific site served to activate transposable elements. These then entered different loci in the genome and led to structural changes at the level of single nucleotides or larger segments. Transposable elements can create insertion mutations. They can contribute to *genomic* instability by promoting nonallelic homologous recombination. Active transposable elements in the human genome include long and short interspersed elements (LINES and SINES and Alu elements). There is evidence that the frequency of nonallelic homologous recombination between *Alu elements* is particularly important in the

human genome (Belancio et al. 2009). Previously activity of transposable elements was thought to take place only in the germline and in early *embryogenesis*. There is now evidence that it also occurs in somatic tissues.

L1 transposable elements have two open reading frames (ORFs) that encode insertional endonucleases and reverse transcriptase. A very small proportion of all L1 elements in the genome are active, and these are sometimes referred to as HOT L1 elements. Brouha et al. 2003 reported that different L1 elements are active in different individuals and that the degree of L1 activity in different individuals varies. Coufal et al. 2009 reported that L1 *retrotransposition* is active in human neural progenitor cells. The activity of the L1 promoter is negatively impacted by methylation at its 5′ end. There is growing evidence that epigenetic factors impact retrotransposition. Muotri et al. 2010 reported that L1 retrotransposition in neurons is modulated by methyl *CpG* binding protein MECP2.

Tissue-specific transcription factors may impact L1 expression. Environmental agents that impact *transposable element* activity include ionizing radiation, heavy metals, specific air pollutants, and *DNA demethylation* agents. Heavy metals influence cellular enzymes responsible for suppressing transposable element activity.

Belancio et al. 2009 emphasized the important role of *Alu repetitive elements* in generating segmental duplications and deletions in the genome throughout life.

DNA Breakage and Genomic Instability

Mani and Chinnaiyan 2010 reviewed the roles of cellular and environmental factors in triggering *DNA* breakage and genomic rearrangements. Chemical compounds that lead to genotoxic stress include bioflavonoids present in food and dietary supplements. Increased generation of *reactive oxygen species* leads to increased frequency of double-stranded DNA breaks. Replication stress may occur due to deficiency of nucleotides or impaired activity of enzymes involved in *DNA replication*. Replication stress is sometimes associated with template switching during DNA replication and complex chromosome rearrangements.

Specific mechanisms involved in the *repair of DNA* breaks impact the frequency of genomic rearrangements. In *nonhomologous* end joining broken ends of DNA strands that result from double-stranded breaks are repaired by joining to adjacent nonhomologous ends. If double-stranded breaks are present on more than one chromosome at a specific *time point*, translocations may result. In *nonhomologous recombination* similar but nonidentical sequences are used for repair. L1 and Alu sequence are often involved in nonhomologous repair. Chromosome regions rich in repeat sequences, and fragile sites that are often rich in CGG repeats, are frequent locations of breaks.

Ledbetter in 1992 first described imbalances in telomeric and sub-telomeric regions and the role of these imbalances in cognitive impairment. He reported that the blocks of sequence homology that occur in sub-telomeric regions of different chromosomes predispose to *mis-pairing* and translocation. The mis-pairing and translocation predispose to DNA breakage and *genomic instability*.

Environmental Exposures and Autism

The increased frequency of autism may be related to changes in diagnostic criteria, inclusion of milder cases, and diagnosis at a younger age. However, Woods et al. 2012 considered these factors insufficient to explain the significant increase in the number of cases of autism. Furthermore studies in *monozygotic twins*, discordant for autism by Hallmeyer et al. 2011, indicate that there is a substantial environmental contribution to autism etiology.

Polybrominated Diphenyl Esters (PBDE) Exposure

Organic polybrominated *diphenyl* esters (PBDE) are used as flame retardants in textiles, carpeting, furniture electronics, and plastics. In a study of blood samples, Sjodin et al. 2008 reported that these compounds are detected in blood on 97 % of North Americans. Hites 2004 reported that blood levels of these compounds doubled every 4 years over several decades.

In a search for environmental factors that impact methylation, Woods et al. 2012 focused on PBDE since these compounds accumulate in lipophilic environments including serum, breast milk, and brain. They demonstrated that mice exposed to the PBDE BDE47 in the *perinatal period* subsequently showed reduced sociability that was independent of genotype. These mice also had reduced methylation in brain. Mutation in the Mecp2 gene, located on the X chromosome that led to partial deficiency of the Mecp2 protein, mitigated the effect of BDE47 exposure in female mice. Woods et al. attributed this to the fact that in the mutated mice, Dnmt1 and Dnmt3a *DNA* methyl transferases are upregulated and these enzymes promote *DNA methylation*.

Summary and Conclusions

There is evidence for increased genomic instability in *autistic* probands, leading to a higher frequency of segmental chromosomal deletions and duplications (*copy number* variants, CNVs) that lead to *gene dosage* changes. At least 300 different locations have been reported to be involved in de novo recurrent CNVs in autism. There is also evidence that a number of these rare recurrent CNVs are present not only in autism but also in other *neuropsychiatric disorders*. Results of *DNA sequencing* studies provide additional evidence for a higher frequency of nucleotide mutations in autism and for *genetic heterogeneity*.

Although many different genes are impacted by dosage changes and/or by functionally significant nucleotide mutations, there is some evidence that the impacted genes function in related pathways that together play roles in neuronal synaptic structure and function.

There is evidence that mitochondrial function is impaired in some cases with autism. This may be due to copy number alterations in one or more of

the 2,000 nuclear genes that encode enzymes and proteins that function within mitochondria. *Mitochondrial DNA* mutations are apparently not common in autism.

Epigenetic processes including chromatin methylation impact gene expression. *Hypomethylation of DNA* leads to *genomic instability*. Metabolic processes involved in the generation of methyl donors for methylation and processes involved in homeostasis of oxidation and reduction balance are impacted in some autism cases. Both of these pathways can be disrupted by environmental factors. Particular environmental toxicant, including flame retardants and black carbon, can lead to DNA methylation.

Analysis of sites of origin of copy number variants and maps of genomic methylation reveal that there is significant correlation between regions of *hypomethylation* and regions that are hotspots for structural chromosomal variation and CNVs.

Studies on transposable elements in the genome, especially L1 elements, reveal that activity of these elements is impacted by methylation and that demethylating agents increase this activity. Increased L1 activity and mobility lead to genomic instability.

It is possible that the increasing population frequency of autism and the evidence for higher rates of genomic instability in autism may be due to environmental factors that impact DNA methylation and *redox homeostasis*.

Key Terms

Copy Number Variants. Different number of copies of a specific chromosome segment.
Heterogeneity. Different underlying mechanisms lead to the same phenotype or disease.
Pathogenesis. Mechanism through which an underlying defect leads to disease manifestations.
Exome. Totality of exons (i.e., protein-coding segments) in the genome.
Oxidative stress. Imbalance in the production versus neutralization of reactive oxygen species.
Transposable elements. Mobile *DNA* sequence elements in the genome.
Retrotransposons. Transposable elements that can amplify and reinsert in the genome.
Epigenetics. Changes in the constitution of chromatin not due to nucleotide mutations.

Key Facts of Nuclear Genomic Changes in Autism

- Segmental chromosome deletions and duplication leading to *gene dosage* changes (*copy number* variants CNVs) occur more frequently in cases of autism than in controls.
- Nucleotide mutations in genes occur more frequently in autism cases than controls.
- In autism cases copy number variants leading to dosage changes and nucleotide frequently impact genes important to neuronal synapse function.

- In genomic regions that are prone to instability, DNA is frequently hypomethylated.
- Specific *environmental pollutants* lead to DNA hypomethylation.

Summary Points

- This chapter focuses on evidence for increased instability of the nuclear genome in autism.
- Evidence of nuclear genomic instability includes duplication and deletions of specific chromosome segments leading to altered gene dosage (copy number variants CNVs).
- Additional evidence for genomic instability is the higher frequency of nucleotide mutation in nuclear DNA in *autistic* probands relative to siblings or controls.
- At least 300 different regions in the nuclear genome are the site of autism-associated copy number variants.
- Different cases of autism differ with respect to the number and position of copy number variants and mutations indicating substantial *genetic heterogeneity* in autism.
- There are however at least eight different chromosome regions that are sites of recurrent rare copy number variants in autistic probands.
- Specific gene impacted by copy number variants and dosage changes in some individuals may be impacted by mutations in other cases.
- Genes impacted by copy number variants and/or mutations in autistic probands often encode products that are important to neuronal synaptic structure and function.
- There is evidence that epigenetic factors especially methylation processes modify chromatin (DNA and histones) and play roles in autism.
- DNA *hypomethylation* promotes *genomic instability* in part through activation of transposons and retroviral elements that are present in nuclear DNA.
- Specific environmental factors including environmental pollutants alter *DNA methylation* and promote genomic instability.
- It is possible that the increase in autism frequency is at least in part due to increased exposure to specific environmental *toxicants*.

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