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Mechanisms of Origin, Phenotypic Effects and Diagnostic Implications of Complex Chromosome Rearrangements

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Complex chromosome rearrangements · DNA double-strand break · Haploinsufficiency · Mixed mutation mechanisms · Structural genome variations · Triplosufficiency

Abstract
Complex chromosome rearrangements (CCRs) are currently defined as structural genome variations that involve more than 2 chromosome breaks and result in exchanges of chromosomal segments. They are thought to be extremely rare, but their detection rate is rising because of improvements in molecular cytogenetic technology. Their population frequency is also underestimated, since many CCRs may not elicit a phenotypic effect. CCRs may be the result of fork stalling and template switching, microhomology-mediated break-induced repair, breakage-fusion-bridge cycles, or chromothripsis. Patients with chromosomal instability syndromes show elevated rates of CCRs due to impaired DNA double-strand break responses during meiosis. Therefore, the putative functions of the proteins encoded by *ATM*, *BLM*, *WRN*, *ATR*, *MRE11*, *NBS1*, and *RAD51* in preventing CCRs are discussed. CCRs may exert a pathogenic effect by either (1) gene dosage-dependent mechanisms, e.g. haploinsufficiency, (2) mechanisms based on disruption of the genomic architecture, such that genes, parts of genes or regulatory elements are truncated, fused or relocated and thus their interactions disturbed – these mechanisms will predominantly affect gene expression – or (3) mixed mutation mechanisms in which a CCR on one chromosome is combined with a different type of mutation on the other chromosome. Such inferred mechanisms of pathogenicity need corroboration by mRNA sequencing. Also, future studies with in vitro models, such as inducible pluripotent stem cells from patients with CCRs, and transgenic model organisms should substantiate current inferences regarding putative pathogenic effects of CCRs. The ramifications of the growing body of information on CCRs for clinical and experimental genetics and future treatment modalities are briefly illustrated with 2 cases, one of which suggests *KDM4C (JMJD2C)* as a novel candidate gene for mental retardation.

Currently, structural genome variations (SVs) are operationally defined as alterations in the organization of genomic elements involving at least 50 bp [Alkan et al., 2011]. The rate of occurrence of SVs is inversely proportional to their size, the number of breaks and the number of genes they involve. Thus, smaller SVs, those that involve only 1 or 2 breaks and those that do not affect any gene, occur more frequently than larger ones, those that involve more than 2 breaks or those that affect at least one

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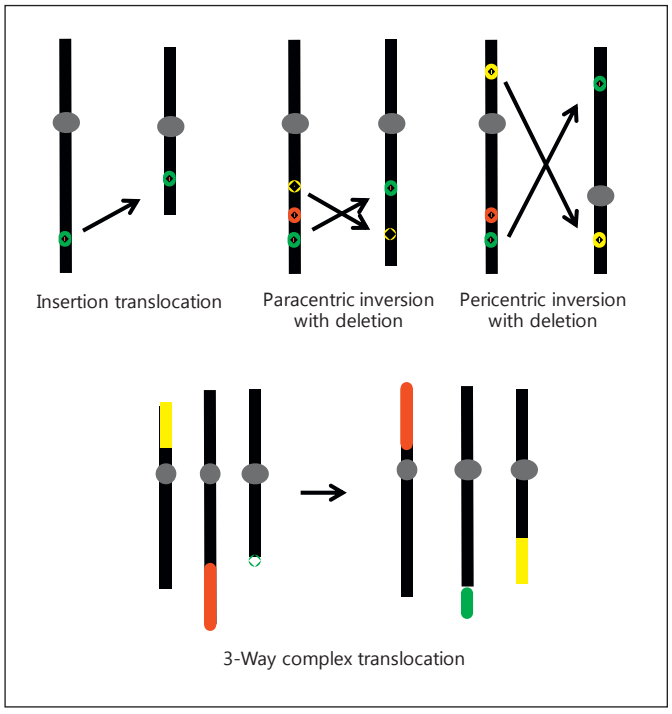

Fig. 1. Schematic depictions of examples of CCRs including an insertion-translocation, 2 types of complex inversions, and a 3-way translocation. Top: chromosomes are in black, and centromeres are represented by gray ovals. The arrows indicate the direction of transposition of chromosomal loci and segments. Small colored symbols represent loci affected by the CCR. Red circles represent lost loci. Bottom: colored bars represent segments that are exchanged by the 3-way translocation.

gene. This indicates that the phenotypic impact of an SV may provoke purifying selection, which will limit its frequency and persistence in the general population. For instance, CNVs larger than 100 kb, which by definition involve 2 breaks, arise de novo in the general population at an estimated rate of $\sim 1.2 \times 10^{-2}$ CNVs per meiosis [Itsara et al., 2010]. On the other hand, in ~14–18% of children with developmental delay, a CNV larger than 400 kb may be phenotypically significant [Cooper et al., 2011; Hochstenbach et al., 2011]. Taken together, all classes of germline SVs occur more frequently than germline single nucleotide variations (SNVs), they affect more nucleotides and may have a greater phenotypic impact than SNVs [Stankiewicz and Lupksi, 2010; Campbell and Eichler, 2013].

Complex chromosome rearrangements (CCRs) are a class of SVs that involve more than 2 chromosome breaks and result in exchanges of chromosomal segments. CCRs may be insertion-translocations, inversions associated with CNVs, translocations affecting more than 2 chromosomes, and combinations thereof (see fig. 1 for the major types of CCRs). Classically, CCRs were extremely rare events detected by karyotyping [Madan et al., 1997; Park et al., 2001]. The implementation of genome-wide assays for segmental aneuploidy, such as BAC, oligonucleotide and SNP arrays, flow karyotyping, and next-generation sequencing techniques, has revealed an increasing complexity of CCRs [Gribble et al., 2005; Fauth et al., 2006; De Gregori et al., 2007; Baptista et al., 2008; Higgins et al., 2008; Sismani et al., 2008; Schluth-Bolard et al., 2009; Gijsbers et al., 2010; Kang et al., 2010; Feenstra et al., 2011; Kloosterman et al., 2011; 2012; Nazaryan et al., 2014; Tabet et al., 2015]. Not only the detection rate of CCRs increased, they were also more often associated with CNVs than reciprocal translocations were [Feenstra et al., 2011]. An example of advancing insights into a CCR by progressively improving resolution is a case, which initially was reported as an 'interstitial loss 6q14 contained within a de novo pericentric inversion 6(p11.2q15)', but eventually became a 10-breakpoint CCR [Passarge, 2000; Poot et al., 2009; Kloosterman et al., 2012]. On the other hand, if genome aneuploidy screening is limited to arrays only, we may miss some of the CCRs or may misinterpret their nature and complexity [Hochstenbach et al., 2009; Brand et al., 2014]. Since the genome analysis method(s) used will affect the detection rate, estimates of population frequencies of CCRs are inherently subject to a technology-dependent ascertainment bias.

CCRs have been classified according to their structure, the number of chromosome breaks detected, whether a single or several chromosomes were involved, and the mode of inheritance, i.e. de novo versus transmitted CCRs [Pai et al., 1980; Kausch et al., 1988; Kousseff et al., 1993; Batista et al., 1994; Park et al., 2001; Pellestor et al., 2011]. Heterozygous carriers of CCRs have an elevated risk for spontaneous abortion or chromosomally abnormal offspring. Empirical risk estimates range from 50 to 100% for spontaneous abortion [Batista et al., 1994] and from 20 to 90% for phenotypic abnormalities [Gorski et al., 1988]. However, lack of a detailed understanding of the origin and nature of a CCR renders medical genetic and reproductive counseling difficult. An additional compounding factor is that male carriers of CCRs are often subfertile or sterile due to arrest of spermatogenesis.

An appreciable number of CCRs were found among healthy individuals [Pellestor et al., 2011; de Pagter et al., 2015]. Thus, some CCRs may be nonpathogenic. Therefore, it is essential to obtain information allowing us to identify potentially pathogenic characteristics of CCRs.

The prime pathogenic mechanism of a CCR is disruption of the genomic architecture either within a gene or between a gene and its regulatory elements [Yue et al., 2006; Klopocki and Mundlos, 2011]. If a CNV is associated with a CCR, haploinsufficiency or triplosufficiency of one or several genes may become an additional pathogenic mechanism [Yue et al., 2005; Poot et al., 2011a]. It is therefore cardinal to narrow down all breakpoint regions as much as possible, ideally to the single nucleotide level, and to determine whether CNVs are associated with a given CCR [Kloosterman et al., 2011, 2012]. Therefore, this review is limited to those CCRs that have been analyzed by molecular methods in sufficient detail regarding the sequences at their breakpoint regions. This information is crucial to address core questions in genetic counseling of carriers of CCRs: first, to infer the possible mechanism of origin and consequently recurrence risk and second, to determine whether disruption of gene-gene and gene-regulatory element architecture may be a possible pathogenic mechanism.

Definition and Classification of CCRs

CCRs have often been considered 'curiosities of nature', which, due to the limited resolution of classical karyotyping, were difficult to detect [Madan et al., 1997; Park et al., 2001]. In their pioneering study, Pai et al. [1980] referred to CCRs as 'translocations that are more complex than the well described reciprocal translocations'. For decades, the definition and classification of CCRs were based upon the number of chromosome breaks and the visible structure of the rearrangements. Both depend upon the level of resolution of the detection method. Advances in molecular cytogenetic technology have dramatically improved both the detection rates and the resolution of CCRs. Consequently, the criteria for both definition and classification of CCRs have to be reconsidered.

Initially, CCRs were classified according to the outcome after meiosis [Kausch et al., 1988]. These authors distinguished 3 groups. First, 2 or more independent translocations leading to 2 or more meiotic quadrivalents of which each can segregate in a balanced or an unbalanced way. These CCRs are generally familial and can be transmitted from one generation to the next [Meer et al., 1981; Farrell et al., 1994; Zahed et al., 1998]. Second, 'true' CCRs involving 'n' chromosomes with 'n' breakpoints leading to the formation of a meiotic multivalent (2n). This leads to 2 possibilities of balanced segregation of chromosomes, as compared to several distinct unbalanced segregation products depending upon the number of chromosomes involved, and third, CCRs involving more breakpoints than chromosomes [Bass et al., 1985; Batanian and Eswara, 1998]. In such cases, additional insertions or inversions have taken place, producing even more complicated meiotic configurations leading to further possible unbalanced segregation outcomes. These rearrangements can be highly complex with as many as 7 derivative chromosomes [Kousseff et al., 1987] and up to 15 breakpoints [Tupler et al., 1992; Houge et al., 2003]. Most of these CCRs occur de novo. While this classification accommodates reciprocal, double reciprocal and more complex translocations, it does not include 'simple' inversions or insertion-translocations. The latter 2 types of rearrangements can only be detected unequivocally by molecular cytogenetic techniques.

Alternatively, CCRs have been classified according to the number of chromosome breaks involved [Kousseff et al., 1993]. These authors divided CCRs in 2 broad groups: one group ≤4 breaks and the other group >4 breaks. They observed that most familial CCRs belonged to the group with up to 4 breaks and have been transmitted by a balanced female carrier, whereas most of the de novo CCRs belonged to the group with more than 4 breaks and were of paternal origin [Kousseff et al., 1993; Batista et al., 1994; Joyce et al., 1999; Lee et al., 2002; Grasshoff et al., 2003; Kuechler et al., 2005]. The majority of the reported cases had 4 or less breaks [Pellestor et al., 2011]. This may either reflect reduced viability or reproductive fitness of individuals with CCRs made up by more than 4 breaks or an ascertainment bias due to technical limitations.

A third classification was based upon both the location and the distribution of breakpoints, thus distinguishing CCRs with intrachromosomal rearrangements (i.e. insertions, inversions, deletions, or duplications) from those involving several chromosomes at once [Lurie et al., 1994].

Meanwhile, several authors described complex inversions and insertion-translocations, which, depending on the definition applied, may or may not have been labeled CCRs [Madan and Menko, 1992; Bernardini et al., 2008; Jiang et al., 2008; Lybaek et al., 2008; de Vree et al., 2009; Poot et al., 2009; Kang et al., 2010]. A special case of a CCR involving a single chromosome is the inversion-duplication-distal deletion rearrangement [Hulick et al., 2009; van Binsbergen et al., 2012; Kowalczyk et al., 2013; Trachoo et al., 2013]. These studies indicated that rearrangements involving only a single chromosome can also be 'complex'. Such 'borderline CCRs' prompted a reassess-

ment of the definition of a CCR. Due to improved resolution of molecular cytogenetic techniques, more and more 'simple' rearrangements, such as inversions and reciprocal translocations, prove to be unexpectedly 'complex'. Therefore, for the time being, the pragmatic approach of Madan [2013] may be the most appropriate. She advocated describing all possible CCRs as 'a CCR involving that and that chromosome, being familial or de novo, apparently balanced or unbalanced as appropriate'. With this purely descriptive approach, all possible CCRs should prompt detailed molecular cytogenetic analyses, which may provide the information needed for proper genetic counseling of the concerned patients and their families.

Mechanisms of Origin of CCRs

During meiosis, 2 consecutive cell divisions, which involve each one round of chromosome segregation, without intervening DNA replication, produce haploid germ cells. During the first cell division, homologous chromosomes pair and form Holliday junctions, which are resolved by multiple recombination events between the chromosomes. Thereupon, the chromosomes are separated and pulled to opposite poles. After this division, each cell contains a haploid number of chromosomes. In the second equational division, the sister chromatids segregate from one another, such that haploid nuclei with chromosomes consisting of single chromatids result. Due to recombination events during the first meiotic division, some genes will be exchanged between the 2 parental chromosomes. These meiotic recombination events are initiated by Spo11-catalyzed DNA double-strand breaks (DSBs), which prompt subsequent recombinational repair for cells to remain viable [Richardson et al., 1998; Romanienko and Camerini-Otero, 2000; Keeney, 2008; Andersen and Sekelsky, 2010; Wahls and Davidson, 2010; Phadnis et al., 2011]. The finding that *SPO11* is highly expressed in testes and ovaries and that spermatocytes and oocytes of $Spo11^{-/-}$ mice undergo cell cycle arrest, and elevated levels of apoptosis underscores that Spo11-catalyzed DSBs are the initial step required for meiosis [Romanienko and Camerini-Otero, 2000; Scott and Pandita, 2006].

Genes and Proteins

Although the biochemistry of DSB repair during meiosis is not completely understood, a number of human genetic disorders of defective DSB response may provide clues regarding the genes involved and their modes of action [O'Driscoll and Jeggo, 2006; Scott and Pandita, 2006]. Those are ataxia telangiectasia (OMIM 208900; gene: *ATM*), ataxia-telangiectasia-like disorders 1 and 2 (OMIM 604391 and 615919; genes: *MRE11* and *PCNA*), Bloom syndrome (OMIM 210900; gene *BLM*), Nijmegen breakage syndrome – NBS – (OMIM 251260; gene: *NBS1*), Seckel syndrome (OMIM 210600; gene: *ATR*), Fanconi anemia/*BRCA2* (OMIM 605724; gene: *FANCD1/BRCA2*), LIG4 syndrome (OMIM 606593; gene: *LIG4*), and Werner syndrome (OMIM 277700; gene *WRN*) [O'Driscoll and Jeggo, 2006; Scott and Pandita, 2006].

Some of these syndromes exhibit spontaneous chromosomal instability *(ATR*, *BLM*, *FANCD1/BRCA2*, *NBS1*, *WRN)* or elevated sensitivity to ionizing radiation *(ATM*, *LIG4)* and phenotypes such as hypogonadism and impaired spermatogenesis *(ATM)*, primary ovarian failure *(NBS1)*, hypospadias and cryptorchidism *(ATR)*. Also male patients with Bloom syndrome *(BLM)* show azoospermia and cryptorchidism, while females present with an overall reduced fertility. Male patients with Werner syndrome *(WRN)* present with complete azoospermia, while females show reduced fertility [Epstein et al., 1966].

Although the functions in meiosis of each of the genes involved in these syndromes is not yet fully understood, a picture begins to emerge. The ATM-encoded phosphatidylinositol 3-kinase cooperates with *TEL1* to limit the number of DSB to one per pair of sister chromatid and one per quartet of chromatids [Zhang et al., 2011]. $Atm^{-/-}$ mice are infertile because meiosis is arrested at the zygotene/pachytene stage of prophase I as a result of abnormal chromosomal synapsis and subsequent chromosome fragmentation [Xu et al., 1996]. In $Atm^{-/-}$ mice ATR, a protein related to ATM, DMC1, a RAD51 family member, and RAD51 show reduced localization to developing synaptonemal complexes in spermatocytes [Barlow et al., 1998]. ATM appears to act as a monitor of the prophase I meiosis and also to control DSB formation [Barlow et al., 1998; Lange et al., 2011]. Cooperation of ATM with SPO11 is required for the obligate XY crossover and also appears to control autosomal crossovers and chromosome integrity [Barchi et al., 2008]. In $Atm^{-/-}$ $Spo11^{+/-}$ mice, ATR rescues spermatogenesis by phosphorylating H2AX in response to DNA DSBs, while folliculogenesis remains partially defective [Bellani et al., 2005; Di Giacomo et al., 2005]. Also DSBs harboring oocytes may progress through meiosis in the presence of an active ATM-dependent DSB control pathway, since they appear to lack a G2-phase type of DNA damage control mechanism [Marangos and Carroll, 2012].

Although the *WRN* and the *BLM* helicases belong both to the family of RecQ helicases, defects of either during DSB response may lead to opposing outcomes [Suhasini and Brosh, 2013; Croteau et al., 2014; Keijzers et al., 2014; Kitano, 2014]. Loss of *WRN* is associated with a variegated translocation mosaicism and a reduction in DNA recombination [Salk et al., 1981; Dhillon et al., 2007; Melcher et al., 2000]. Loss of *BLM*, in contrast, provokes elevated rates of sister chromatid exchanges during meiosis I [Bischof et al., 2001]. The WRN-encoded protein activates the ATR-CHK1-induced S-phase checkpoint in response to topoisomerase-I-DNA covalent complexes, recruits and stabilizes Rad51 and limits the activity of the *MRE11*-encoded exonuclease [Patro et al., 2011; Su et al., 2014]. In somatic cells, absence of the *WRN*-encoded 3′-5′ helicase-3′-5′ exonuclease causes a prolongation of the S phase of the cell cycle, hypersensitivity to DNA cross-linking and DSBs provoking agents, elevated frequencies of micronuclei and a reduction in recombinational DSB repair [Poot et al., 1992, 1999, 2001, 2002, 2004; Ogburn et al., 1997; Honma et al., 2002; Kamath-Loeb et al., 2004; Dhillon et al., 2007]. The extent of DSB ligation is normal in WRN deficient cells, albeit that more deletions are formed at the fusion sites [Rünger et al., 1994]. Cultured fibroblasts from patients with Werner syndrome show a typical and diagnostic variegated chromosomal translocation mosaicism and spontaneous deletion formation [Salk et al., 1981; Fukuchi et al., 1989; Oshima et al., 2002].

The *BLM*-encoded 3′-5′ helicase prevents elevated formation of sister chromatid exchanges and quadriradial exchanges between homologous chromosomes during the S phase of the cell cycle [Bartram et al., 1976; Bischof et al., 2001]. Thus, BLM prevents chromosomal recombination and CCR formation [Bartram et al., 1976; LaRocque et al., 2011]. BLM recruits Rad51 and, in cooperation with DNA topoisomerase III, dissolves displacement loops that form during DNA recombination, thus ensuring that recombination intermediates are completely resolved during meiosis [Wu and Hickson, 2003; Fasching et al., 2015; Kaur et al., 2015; Tang et al., 2015]. As part of a complex with either WRN or BLM, Rad51 searches the entire genome to locate a region of homology that can be fused to the broken chromosome. These regions of homology can be located on a sister chromatid, on a homologous chromosome, or on an unrelated chromosome. Rad51 then opens the intact double-stranded DNA template to allow strand invasion and formation of a 3-stranded displacement loop in which the single-stranded broken-end base pairs with its complementary strand of the intact duplex. At this point, the cell can follow one of several alternative pathways. The cell can proceed through a synthesis-dependent strand annealing pathway that copies the template to seal the break without an accompanying crossover. Alternatively, the cell can repair the DSB by formation of a branched intermediate, i.e. a double Holliday junction, which has to be resolved subsequently by producing crossovers between the homologs [Haber, 2015]. In somatic cells, such crossovers may lead to the loss of heterozygosity. During meiosis, such crossovers generate tension between paired homologs, which assures the proper disjunction of chromosomes during meiosis I. Failure to do so, results in elevated rates of sister chromatid exchanges, the hallmark of Bloom syndrome [Bartram et al., 1976].

Thus, the proteins encoded by the genes mutated in BLM and WRN perform critical, albeit distinct, functions required for proper chromatid disjunction [Shen and Loeb, 2000; Mohaghegh et al., 2001; Compton et al., 2008; Monnat, 2010; Rossi et al., 2010; Kamath-Loeb et al., 2012]. It is, however, unlikely that these well-recognizable and extremely rare autosomal recessive syndromes with reduced fertility may account for a significant number of cases with CCRs. On the other hand, *WRN* heterozygotes occur at a rate of 1:200 in the general population. Individuals who are heterozygous for WRN mutations, e.g. the parents of WRN patients, show normal fertility but an elevated sensitivity to DNA damage induced by DNA cross-linking agents [Ogburn et al., 1997; Poot et al., 1999]. This indicates that hemizygosity for *WRN* may be pathogenic through haploinsufficiency. A likely molecular mechanism underlying haploinsufficiency is the formation of protein complexes with altered stoichiometries of the contributing partners [Poot et al., 2011a]. In the case of WRN, these may be Rad51, topoisomerase I and MRE11, while for BLM these would be Rad51, topoisomerase III, RPA, Rad54 and Rmi1 [Su et al., 2014; Fasching et al., 2015; Kaur et al., 2015; Tang et al., 2015]. Thus, perturbation of the delicate balance between pro-recombination activity of the WRN-pathway versus anti-recombination activity of the BLM pathway may either produce variegated translocation mosaicism (WRN) or elevated rates of sister chromatid exchanges (BLM). In addition, imbalanced functioning or complete inactivity of *ATM*, *ATR*, *MRE11*, *NBS1*, *PCNA*, *RAD51*, etc. during meiosis may conceivably provoke the formation of CCRs.

In addition, the PCNA protein appears to facilitate resolution of structures resembling double Holliday junctions that were postulated as intermediates of double-strand break repair during meiosis [Giannattasio et al., 2014]. Loss of both alleles of the *PCNA* gene caused a de-

fect in nucleotide excision repair, resulting in developmental delay, ataxia, sensorineural hearing loss, short stature, cutaneous and ocular telangiectasia, and photosensitivity [Baple et al., 2014]. Patients with mutations in *LIG4* show a clinical syndrome closely resembling NBS. In contrast to NBS cells, those from patients with LIG4 syndrome showed normal cell cycle checkpoint responses but impaired DSB rejoining [O'Driscoll et al., 2001]. Patients with mutations in *XRCC4*, the obligate partners of LIG4 in the nonhomologous end joining (NHEJ) pathway of DSB repair, show extreme global growth failure in utero, microcephalic primordial dwarfism and severe combined immunodeficiency [Murray et al., 2015]. In a single family, a male and female adult patient with short stature, early-onset metabolic syndrome and gonadal failure were found to carry *XRCC4* mutations [de Bruin et al., 2015]. By single-molecule fluorescence-resonance-energy-transfer analyses of the Ku/XRCC4/XLF/DNA ligase IV complex involved in NHEJ ligation, filament-forming proteins were found to bridge DSBs in vivo [Reid et al., 2015]. These filaments at either end of the DSB appeared to interact dynamically to achieve the optimal configuration and end-to-end positioning and ligation. Conceivably, a subtle defect in this process may lead to illegitimate repair of the DSB and subsequently to formation of a CCR. Overexpression of replication protein A1 (RPA1), for instance because of a recurrent duplication 17p13.3, provokes an abnormal S-phase transit, attenuated DSB-induced RAD51 retention, elevated rates of fused and triradial chromosomes, hydroxyurea-induced micronuclei, and increased sensitivity to camptothecin [Outwin et al., 2011]. These findings add to the growing genetic and phenotypic complexity of the biological pathways possibly involved in DSB response and CCR prevention.

Molecular Mechanisms: Fork Stalling and Template Switching, Microhomology-Mediated Break-Induced Repair and the Breakage-Fusion-Bridge Cycle

The above evidence suggests the involvement of these genes and proteins in assuring proper chromatid transmission during meiosis. Yet, no biological pathways leading to the formation of CCRs emerge. Based on studies of CNV formation, several mechanisms of CCR formation have been proposed [Hastings et al., 2009; Colnaghi et al., 2011; Liu et al., 2012]. These include the fork stalling and template switching mechanism (FoSTeS), microhomology-mediated break-induced repair (MMBIR) and the breakage-fusion-bridge cycle. The latter has been proposed after observations during classical karyotyping and involves NHEJ of the chromosomal pieces [Van Dyke et al., 1977; Colnaghi et al., 2011]. Finally, chromothripsis, i.e. shattering of chromosomes followed by randomly 'stitching together' of the pieces, has been proposed [Stephens et al., 2011]. Although it is impossible to observe these processes 'in the act' during meiosis, they leave specific patterns of sequence changes at the 'joining points', which may serve as 'molecular signatures' allowing us to infer the nature of the process that gave rise to the CCR in question.

In FoSTeS and MMBIR, active DNA replication forks are assumed. It is conceivable that similar processes may also occur during meiosis when Holliday junctions are being resolved [Hastings et al., 2009; Colnaghi et al., 2011]. Initially, FoSTeS assumed that if during DNA-lagging strand synthesis a DNA polymerase encountered a block, it would switch to a nearby active replication fork and continue nascent DNA synthesis [Hastings et al., 2009]. As a result, 2 simultaneously active replicons are fused in the nascent stretch of DNA. If this process would occur multiple times during attempts to resolve Holliday junctions, multiple crossovers, each involving a short stretch of DNA, would result. The eventual outcome of such a process would be a CCR. The molecular signature of such a FoSTeS-mediated CCR would be a set of closely interspersed joining points connecting multiple chromosomes or a set of interspersed duplications and triplications within a single chromosome, termed 'chromoanasynthesis' [Colnaghi et al., 2011; Hart and O'Driscoll, 2013; Plaisancié et al., 2014].

Alternatively, the DSB generated during meiosis may be subjected to MMBIR [Hastings et al., 2009; Colnaghi et al., 2011]. This model assumes that after exposure to high levels of endogenously generated DNA damage, e.g. nucleotide oxidation or DNA interstrand cross-linking, the DNA replication fork collapses. Thereupon, RAD51-mediated invasion of the 3′ end of the DSB into the double-stranded DNA template and 3′-5′ resection of one of the strands occurs. The *WRN*-encoded 3′-5′ helicase-3′-5′ exonuclease and the *BLM*-encoded 3′-5′ helicase may, together with the *MRE11*-encoded exonuclease, replication protein A and *NBS1*-encoded protein, be involved in such a process [Truong et al., 2013; Su et al., 2014]. On the one hand, the joining of 2 open DNA strands is based upon the microhomology between the 2 participating stretches of DNA. On the other hand, small deletions will be generated at the joining points during the exonuclease-mediated resection step [Colnaghi et al., 2011; Hart and O'Driscoll, 2013]. The molecular signature of MMBIR will be the concomitant presence of microhomologies

and small deletions at the joining points of the thus generated CCRs.

Recently, several cases of 'borderline CCRs' consisting of duplications and triplications associated with segments of copy-number-neutral loss of heterozygosity have been reported [Carvalho et al., 2015]. Detailed analyses suggest that these have resulted from a postzygotic MMBIR-like process involving template switches between sister chromatids [Carvalho et al., 2015].

The third proposed mechanism for CCR-formation is the breakage-fusion-bridge cycle prompting NHEJ [Colnaghi et al., 2011; Hart and O'Driscoll, 2013]. DNA NHEJ is the predominant pathway of DSB repair, which involves heterodimers of Ku70 and Ku80, and DNA-dependent protein kinases, DNA-PKcs. Heterodimers of Ku bind to the DSBs, which in turn recruit DNA-PKcs. The activated DNA-PKcs complex subsequently recruits artemis, XRCC4, LIG4, and DNA polymerase μ. The nuclease activity of artemis prepares the DNA ends for ligation and gap filling by LIG4 and DNA polymerase μ. NHEJ functions throughout the mitotic cell cycle and is the predominant DSB-rejoining mechanism in the G1 phase of the cell cycle [Scott and Pandita, 2006]. The molecular signature of NHEJ will be the formation of small deletions at the joining points in the absence of microhomologies.

Chromothripsis as a Possible Cause of CCRs

Recently, a fourth mechanism of CCR formation has been proposed: chromosome shattering, also known as chromothripsis, followed by random stitching together of the resulting chromosomal segments. Chromothripsis occurs frequently in many tumor types, but it has also been invoked to explain a number of CCRs in the germline [Kloosterman et al., 2011, 2012; Stephens et al., 2011; Nazaryan et al., 2014]. Chromothripsis results from multiple DSBs arising simultaneously in close proximity to each other, often on several chromosomes at once. Also a few likely cases of chromothripsis involving only a single chromosome have been described [Lybaek et al., 2008; Poot et al., 2009; Kloosterman et al., 2012]. The initiating event may be uncontrolled overactivity of SPO11, but also environmental insults, such as free radicals or ionizing radiation, leading to an overwhelming number of DSBs have been suggested [Lieber, 2010]. Furthermore, covalent DNA modification or other damages interfering with proper resolution of Holliday junctions or defective cell cycle checkpoint function have been invoked [Maher and Wilson, 2012; Pellestor et al., 2014].

Interestingly, CCRs resulting from chromothripsis involve either one or a few, but never all chromosomes [Maher and Wilson, 2012]. A CCR involving exchanges between multiple chromosomes may arise if several Holliday junctions are in close proximity to each other at the same point in time [Hansen et al., 2010]. This may be the case during the pachytene stage of meiosis I. In contrast, a CCR affects only a single chromosome, when it is either isolated, e.g. in a micronucleus, or in a highly condensed state, probably during early meiosis I [Crasta et al., 2012; Maher and Wilson, 2012]. Thus, the number of chromosomes being affected by a CCR may indicate at what precise stage of meiosis I chromothripsis may have taken place.

Chromothripsis was originally discovered in dividing somatic cells of tumors [Stephens et al., 2011]. In contrast to this form of chromothripsis, the kind of chromothripsis giving rise to CCRs should be termed 'constitutional' or 'germline chromothripsis', as it arises during meiosis and harbors a set of highly specific characteristics [Ballarati et al., 2009; Poot et al., 2009; Gijsbers et al., 2010; Kloosterman et al., 2011, 2012; Chiang et al., 2012; Korbel and Campbell, 2013; Nazaryan et al., 2014]. Based on a series of cases with CCRs, chromothripsis was inferred for those, which had at least one nonreciprocal exchange in their karyotype and, upon genome-wide CNV analyses, showed association of either diploid or haploid segments with the rearrangement sites. In a case with an insertion-translocation, the inserted fragment occurred in the triploid state, as was predicted from the karyotype. In general, the CNVs in cases with chromothripsis are only losses and not duplications. Since the diploid segments of the genome had retained heterozygosity for SNP markers, chromothripsis most likely took place immediately before or during meiosis I.

By paired-end and mate-pair sequencing the joining points of the rearrangements were investigated at nucleotide resolution [Kloosterman et al., 2011, 2012]. At these joining points, losses of a few base pairs up to several kb, and small insertions of non-templated sequences were found. In one case of a CCR caused by chromothripsis, 11 out of 12 joining points contained regions of microhomology, suggesting involvement of *BLM* and its partners [Truong et al., 2013; Nazaryan et al., 2014]. In another case, little or no microhomology was found, while in a series of cases, 45% of junctions displayed microhomologies, 29% no microhomology, and 26% had short insertions of 1–97 non-templated nucleotides [Kloosterman et al., 2011, 2012]. This indicates that after chromothripsis the chromosomal segments were either fused by NHEJ or by MMBIR [Liang et al., 1998; Holloway et al., 2010; Truong et al., 2013]. Remarkably, the segments

appeared to be randomly joined in head-to-tail, tail-to-tail and head-to-head orientations [Kloosterman et al., 2012]. This means that the joining process operates in both the forward and the reverse direction, which differentiates it from FoSTeS [Hastings et al., 2009; Liu et al., 2012]. Another differentiating hallmark of chromothripsis is the close clustering of breaks, analogous to the clustering of mutations (kataegis), resulting from the activity of editing cytosine deaminases of the APOBEC superfamily [Kloosterman et al., 2012; Lada et al., 2015], which can only be fully ascertained by paired-end and mate-pair sequencing. Chromothripsis-based rearrangements affect only a single haplotype, which suggests that it occurred just before or during meiosis I and indicates a possible involvement of *BLM* and its partners [Bischof et al., 2001; Holloway et al., 2010].

Chromothripsis took place in the paternal germline in all 4 of the cases that could thus be evaluated [Kloosterman and Hochstenbach, 2014]. As a causative mechanism for this sex bias, the higher number of cell divisions of sperm cells versus oocytes has been proposed [Gribble et al., 2005; Hehir-Kwa et al., 2011; Kloosterman et al., 2012]. If this were true, the risk for chromothripsis increases with the age of the father, as has been found for SNVs and CNVs [Hehir-Kwa et al., 2011; O'Roak et al., 2012; Buizer-Voskamp et al., 2013]. Alternatively, an increased burden of free radical-mediated DNA damage or less active free radical defense or repair mechanisms may be responsible for the paternal bias for chromothripsis [Kloosterman et al., 2012; Pellestor et al., 2014]. No definitive evidence supporting this hypothesis has yet been published. A single study of the antioxidant defense of round spermatids of the rat is in agreement with this hypothesis, however [Den Boer et al., 1990]. In addition, higher levels of abortive apoptosis, telomere erosion, mitotic errors, micronuclei formation, and p53 inactivation in the male versus female germline have been proposed [Pellestor et al., 2014]. Interestingly, all these mechanisms have been identified as potential causes for reproductive failure and chromosomal abnormalities, thus linking chromothripsis to the human germline once again [Pellestor et al., 2014].

Recently, a novel mechanism of chromothripsis was proposed [Zhang et al., 2015]. In cultured retinal pigment epithelial cells, one or several missegregated chromosomes may become separated into a micronucleus. During the subsequent S phase of the cell cycle, the chromosome(s) in the micronucleus undergo underreplication, and the resulting chromosomal segments are fused by a microhomology-dependent mechanism, akin to MMBIR. The resulting cell nucleus then contains one or several chromosomes that have undergone chromothripsis. If the fusion process involves several chromosomes at once, a CCR may emerge. This mechanism, which requires one round of DNA replication, is in agreement with several of the described cases of chromothripsis [Kloosterman et al., 2011, 2012; Nazaryan et al., 2014]. Most micronuclei accumulate DSBs, which are associated with the phosphorylated histone H2AX [Crasta et al., 2012] and the RAD51 protein [Haaf et al., 1999]. The latter is mobilized by the BLM helicase to sites of DSBs [Wu and Hickson, 2003; Fasching et al., 2015; Kaur et al., 2015; Tang et al., 2015]. If the micronucleus does not contain sufficient *BLM*-encoded helicase, the balance between *WRN*- and *BLM*-dependent activities may become tilted towards the hypo-recombinogenic WRN pathway, and a variegated translocation in association with deletions may result [Salk et al., 1981; Fukuchi et al., 1989; Dhillon et al., 2007; Melcher et al., 2000]. Thus, these observations provide a novel molecular mechanism linking chromothripsis to CCRs.

The molecular details of processes such as FoSTeS, MMBIR, breakage-fusion-bridge cycle and chromothripsis in the germline have not yet been fully elucidated [Hastings et al., 2009; Liu et al., 2012; Korbel and Campbell, 2013]. In particular, a transgenic model for germline chromothripsis has not yet been described. For clinical purposes, such as to infer the mechanism of origin and recurrence risk of the CCR, rigorous criteria to distinguish FoSTeS, MMBIR, breakage-fusion-bridge cycle and chromothripsis are a prerequisite. Nevertheless, operational definitions based on molecular signatures can be obtained by paired-end or mate-pair analyses at the nucleotide level. In table 1, the molecular mechanisms of CCR formation, the potentially involved genes, and the molecular signatures of joining points are summarized.

Pathogenetic Mechanisms of CCRs

Roughly, 70% of all reported CCRs were found in healthy individuals [Pellestor et al., 2011]. This means that CCRs, although they affect large numbers of nucleotides, do not necessarily elicit a phenotypic effect. Hence the need to define a set of characteristics distinguishing phenotypically neutral from truly pathogenic CCRs. More than a decade of experience with CNVs may guide our attempts at understanding the possible phenotypic effects of CCRs. Based on this experience, there are main-

Table 1. Mechanisms of CCR formation, involved genes and molecular signatures of joining points

	FoSTeS	MMBIR	Breakage-fusion-bridge cycle	Chromothripsis
Causes	stalled replication forks	DSBs	?	overactivity of SPO11, free radicals or ionizing radiation, covalent DNA damage interfering with resolution of Holliday junctions or defective cell cycle checkpoints
Basic mechanism	strand invasion by lagging DNA replication strand	joining of 2 open DNA strands	?	NHEJ and MMBIR
Enzymatic activities	DNA replication machinery	exonuclease-mediated resection	artemis-nuclease, gap-filling DNA polymerase, ligase 4	not studied
Associated CNVs	deletions, duplications, triplications	none	none	only losses, no duplications; pachytene stage of meiosis I
DNA fragment orientation	head-to-tail	head-to-tail	head-to-tail	randomly joined in head-to-tail, tail-to-tail and head-to-head orientations
Junction sequences	?	microhomologies, short deletions, non-templated insertions	small deletions due to NHEJ	losses of a few base pairs up to several kb, small insertions of non-templated sequences
Junction spacing	?	?	?	closely spaced
Key references	Hastings et al., 2009; Colnaghi et al., 2011	Hastings et al., 2009; Colnaghi et al., 2011	Scott and Pandita, 2006	Liang et al., 1998; Holloway et al., 2010; Truong et al., 2013

? = Not known.

ly 3 types of pathogenic mechanism for an SV: First, gene dosage-dependent mechanisms which have been extensively explored in the context of CNVs [Poot et al., 2011a], and second, mechanisms based on disruption of genomic architecture such that genes, parts of genes, and regulatory elements are relocated and their interactions perturbed. The phenotypic effects of this mechanism are mediated by alteration of gene expression rather than changes in the structure of the encoded protein(s) [Reymond et al., 2007; Henrichsen et al., 2009a; Klopocki and Mundlos, 2011; Spielmann and Klopocki, 2013; Spielmann and Mundlos, 2013], third, mixed mutation mechanisms (MMMs) in which a CNV or a disrupted gene on one chromosome is paired with a different type of mutation on the other chromosome. Genetically this constellation appears as recessive, but the underlying mechanism is distinct from a homozygous or compound heterozygous pair of variants in a single gene.

Gene Dosage-Dependent Mechanisms

In a diploid genome, CNVs affecting the phenotype based on a change in dosage of a gene exert a dominant effect [Poot et al., 2011a; Hart and O'Driscoll, 2013]. Basically, changes in gene dosage may be phenotypically significant if the encoded protein interacts with other proteins [Veitia, 2010; Veitia and Birchler, 2010]. That means haploinsufficiency or triplosufficiency for a gene due to loss or gain CNV, respectively, may alter the stoichiometry of the proteins in a complex and thus the function of the latter [Veitia, 2010; Veitia and Birchler, 2010; Poot et al., 2011a]. A prerequisite for this mechanism is that the change in gene dosage is translated into altered levels of mRNA. Only in a few cases this has actually been demonstrated. Those include de novo CNVs in patients with atrial septal defect, 22q11 deletions in patients with the velocardiofacial syndrome, deletions in patients with the Williams Beuren syndrome, duplications of the 1q21.1 region, and both deletions and duplications of the 16p11.2 region [Merla et al., 2006; Harvard et al., 2011; Luo et al., 2012; Blumenthal et al., 2014]. In a study of 129 individuals of European and Yoruba descent, complex relationships between size and population frequencies of CNVs with gene expression level have been found [Schlattl et al., 2011]. The data supported a causal role of CNVs in expression of quantitative trait loci and revealed a dosage compensation mechanism of some transcript levels [Schlattl et al., 2011]. This large-scale study generalizes the observation of a correlation of a mouse expression of

quantitative trait loci with corpus callosum thickness in patients with small deletions in the 1q44 region [Poot et al., 2011b]. Including fine-scale CNV information may thus complement association studies aimed at identifying phenotypically significant expression of quantitative trait loci [Schlattl et al., 2011].

Mechanisms Based on Disruption of the Genomic Architecture

A growing body of experimental evidence indicates that the genome consists of topologically associated domains (TADs), which are in the megabase range and separated by boundary regions [Dixon et al., 2012; Nora et al., 2012, 2013]. These TADs represent regulatory domains within which promoters and regulatory elements may interact with each other and protein-encoding genes [Spielmann and Klopocki, 2013; Spielmann and Mundlos, 2013]. Disruption of TADs by genomic rearrangements may then cause pathological interactions by perturbing gene expression [Spielmann and Klopocki, 2013; Spielmann and Mundlos, 2013; Lupiáñez et al., 2015]. In patients with skeletal dysmorphology (in particular of the hands), changes in noncoding elements have frequently been revealed by screening for CNVs [Klopocki et al., 2008, 2011; Benko et al., 2009; Dathe et al., 2009; Gordon et al., 2009, 2014; Kurth et al., 2009; Spielmann et al., 2012; Lohan et al., 2014; Tayebi et al., 2014]. This genomic mechanism is not necessarily limited to these disorders. Recently, losses of *cis*-regulatory elements, imbalance in coding and noncoding elements, illegitimate adoption of enhancer sequences and the like, resulting from CNVs have been proposed as a general mechanism of 'genomic' disease [Klopocki and Mundlos, 2011; Lettice et al., 2011; Poot and Kas, 2013; Spielmann and Klopocki, 2013; Gordon et al., 2014; Ibn-Salem et al., 2014; Lupiáñez et al., 2015]. CNVs may also cause 'side effects', i.e. they affect the level of expression of genes in the flanking regions [Reymond et al., 2007]. In mice, this effect extends to regions up to 250 kb for all tissues examined [Henrichsen et al., 2009b]. Systematic analysis of ENCODE data allowed establishing a map of regulatory domains consisting of sequences that enhance and/or inhibit the expression of CNV-flanking genes [Spielmann and Mundlos, 2013]. Changes in functioning of noncoding sequences as a mechanism of phenotypic effects of CCRs is a novel area of research for which the technological basis is still under construction [Lupiáñez et al., 2015].

A second mechanism by which disruption of the genomic architecture by CNVs or CCRs may affect organismal phenotypes is truncation or fusion of genes. Truncation of a gene may lead to expression of either a truncated mRNA or may, through loss of a legitimate transcription stop signal, activate nonsense-mediated mRNA decay [Wagner and Lykke-Andersen, 2002; Popp and Maquat, 2013]. A truncated mRNA may either be phenotypically inconsequential or exert an affect through a dominant-negative mode of action. Nonsense-mediated mRNA decay, on the other hand, will provoke a lowered level of gene expression, which amounts to a recessive mode of gene action, except for cases of gene haploinsufficiency or triplosufficiency [Poot et al., 2011a; Veitia, 2010; Veitia and Birchler, 2010]. In order to decide between these 2 possibilities, one needs to ascertain the level of transcription of the genes affected by the CCR.

If a CCR connects 2 genes with the same direction of transcription, a fusion transcript may result. In this way, the open reading frame of one gene, or part of a gene, will be fused with that of another gene or part of a gene. As a consequence, the level and the expression pattern of the fusion gene will come under the transcriptional control of one of the genes, which may lead to ectopic gene expression. To establish whether a CCR indeed provokes such a mode of perturbed gene expression, one needs to ascertain both the gene fusion points and whether the fusion gene is actually transcribed. In patients with autism and with schizophrenia, this mechanism as a result of deletions has already been demonstrated [Holt et al., 2012; Rippey et al., 2013]. Also duplications, either being tandem duplications, insertion-duplications, or triplications embedded within duplications, may cause disruption or fusion of genes [Newman et al., 2015]. Table 2 lists the currently reported CCRs, which produced gene fusions, together with the phenotypes of the patients, the involved chromosomal regions, and the affected genes. In 9 out of the 10 reported cases, 1–3 fusion transcripts were actually detected. In one case, such a fusion transcript was not detected, presumably because the direction of transcription of the 2 affected genes were opposite to each other [Malli et al., 2014]. In a patient with schizophrenia, the CCR apparently activated an alternative transcription mode, since the authors found 3 fusion transcripts [Eykelenboom et al., 2012]. While in 8 cases a fusion transcript was successfully demonstrated, other causes of the patient's phenotypes, such as haploinsufficiency of truncated genes, altered expression of unrelated genes, or additional CNVs, still have to be considered [Nothwang et al., 2001; Yue et al., 2005; Mansouri et al., 2006; Borsani et al., 2008; Backx et al., 2011; Eykelenboom et al., 2012; Kloosterman et al., 2012; Di Gregorio

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Table 2. Reported cases of gene fusion as a result of a CCR

Major patient phenotypes	Karyotype	Fusion transcripts: 5′ gene, exons; 3′ gene, exons	5′ gene breakpoint	3′ gene breakpoint	Truncated gene products	Additional SV, SNVs, or transcription changes	Fusion mechanism	Parental origin	Reference
DD, ataxia, brain atrophy	t(1;19)(q21.3;q13.2)dn	*PAFAH1B3*, exons 1–5; *CLK2*, exons 1–12	in intron 4 *(PAFAH1B3)*	5′ of exon 1 *(CLK2)*	*PAFAH13B* exons 1–5	?	AluSP repeats	?	Nothwang et al., 2001
DD, delay in expressive language, macrocephaly, kyphoscoliosis	t(7;10)(q33;q23)dn	*SEC8L1*, exons 1–11; *PTEN*, exons 3–9	in intron 11 *(SEC8L)*	in intron 2 *(PTEN)*	none	7-Mb deletion in 7q33q34 *(PTEN, TPK1)*	?	paternal germline	Yue et al., 2005
Hypospadias, anal atresia, recto-urethral fistula, hypoplastic kidney	t(6;17)(p21.31;q11.2)	182-FIP, exon 1; *LHFP5*, exon 4	in intron 1 *(FIP1)*	in intron 3 *(LHFP5)*	?	increased transcription of *SRPK1* and *TAOK1*	2-bp deletion and a 7-bp duplication	?	Mansouri et al., 2006
Left renal agenesis, neutropenia, recurrent pulmonary infections, long bone diaphysis broadening, growth and DD	t(2;7)(p13;p12)dn	*EXOC6B*, exon 1; *TNS3*, exon 16 and *TNS3*, exon 15; *EXOC6B*, exon 2	in intron 1 *(EXOC6B)*	in intron 15 *(TNS3)*	none	none; haploinsufficiency for both *TNS3* and *EXOC6B*	NHEJ?	?	Borsani et al., 2008
DD + agenesis of the corpus callosum	t(6;14)(q25.3;q13.2)dn	*ARID1B*, exons 1–5; *MRPP3*, exons 5–8; *MRPP3*, exons 1–4; *MRPP3*, exons 6–19	in intron 5 *(ARID1B)*	in intron 4 *(MRPP3)*	none	none; haploinsufficiency for both *ARID1B* and *MRPP3*	1. NHEJ? 2. non-templated insert	?	Backx et al., 2011
Schizophrenia	t(1;11)(q42.1;q14.3)dn	*DISC1*, exons 1–8; *DISC1FP1*, exons 4–7b; *DISC1*, exons 1–8; *DISC1FP1*, exons 3a–7b; *DISC1*, exons 1b–2; *DISCFP1*, exons 9–13	in exons 8 and 2 *(DISC1)*	in exons 4, 3a, and 9 *(DISC1FP1)*	none	haploinsufficiency for *DISC1*	NHEJ?	?	Eykelenboom et al., 2012
Psychomotor retardation, speech delay, facial dysmorphisms, preaxial polydactyly of the thumbs	t(1;12;7)(p21;q14;p21)dn	*DPYD*, exons 1–3; *ETV1*, exons 10–14; *FOXP1*, exons 1–11; unknown, exons 1–2	in intron 3 *(DPYD)*	in intron 9 *(ETV1)*	*FOXP1*, exons 1–11; *DPYD*, exons 1–3	decreased expression of *FOXP1* and *DPYD*	NHEJ?	?	Kloosterman et al., 2012; van Heesch et al., 2014
Psychomotor retardation, cerebellar hypoplasia	t(X;8)(q25;q24)dn	*PTK2*, exon 1; *THOC*, exons 2–36	in 5′ UTR *(PTK2)*	in intron 1 *(THOC)*	none	?	MER4/AluJ on 8q24 and SVA on Xq25	?	Di Gregorio et al., 2013
DD, speech impairment, dysmorphisms	t(5;6)(q12.3;q25.3)dn	no fusion transcripts expressed	in intron 10 *(ADAMTS6)*	in intron 2 *(ARID1B)*	none	haploinsufficiency for *ARID1B* and *ADAMST6*	NHEJ?	?	Malli et al., 2014
GTS, OCD, ADHD + comorbidities	t(3;9)(q25.1;q34.3)mat	*OLFM1*, exon 7; *TCONS*_12_00019929; *OLFM1*, exon 7; *TCONS*_12_00019930	in exon 7 *(OLFM1)*	in *TCONS*_12_00019929/30	*OLFM1*, exons 1–7	haploinsufficiency for *OLFM1*	NHEJ?	maternally inherited	Bertelsen et al., 2015

ADHD = Attention deficit hyperactivity disorder; DD = developmental delay; GTS = Gilles de la Tourette syndrome; OCD = obsessive-compulsive disorder; ? = not known.

et al., 2013; Malli et al., 2014]. In 2 cases, regions of homology have been identified at the fusion points, such that for the other 8 cases NHEJ seems to be the most likely mechanism of fusion.

Mixed Mutation Mechanisms

Disruption or deletion of one allele of a gene may also exert a phenotypic effect via a recessive mechanism. This occurs if, for the same locus as the deletion, an SNV or CNV residing on the chromosome from the other healthy parent is transmitted. This 'unmasking' of a recessive allele constitutes a mixed mutation mechanism (MMM). In table 3, all well-documented cases of 'unmasking' are compiled. Of the 24 cases with a deletion, 11 involved a known recurrent CNV. This is in agreement with frequent suggestions in the literature that the phenotypic variability of patients with a recurrent CNV may be due to an MMM, such as unmasking [Mefford et al., 2008; Hannes et al., 2009; Kumar et al., 2009; Bedeschi et al., 2010; Kunishima et al., 2013; McDonald-McGinn et al., 2013]. For 5 of the 11 de novo CNVs, the parental origin of the rearrangement could be determined: 4 occurred in the germ line of the father. Interestingly, in 2 cases a second overlapping deletion on the chromosome of the other healthy parent was discovered [Masurel-Paulet et al., 2010; Hochstenbach et al., 2012]. Thus far, only 2 cases of unmasking due to a de novo CCR have been reported. The phenotypes of 10 of the 11 cases with unmasking due to a de novo deletion could be classified as 'double syndromes', i.e. the patients exhibit features pertaining to 2 syndromes simultaneously [Flipsen-ten Berg et al., 2007]. No SNVs were unmasked in a series of 20 patients with random assortments of phenotypes, but in one case, an overlapping deletion was found in a genomic footprint of 1.53 Mb [Hochstenbach et al., 2012]. This is in agreement with the relative prevalence of potentially pathogenic SNVs and CNVs in the human genome [Stankiewicz and Lupksi, 2010; Campbell and Eichler, 2013]. The patients with a transmitted deletion and a concomitantly inherited SNV displayed a seemingly random assortment of phenotypes, not reminiscent of any Mendelian disorder. In contrast, genes associated with a Mendelian disease are less tolerant to phenotypically significant SNVs than genes that do not cause any known disease [Petrovski et al., 2013]. The loss of gene function due to gene disrupting mutations such as nonsense, coding indels, and splice acceptor/donor site mutations have been found at significantly elevated frequencies in the genomes of patients with severe ID, epilepsy and atrial septal defect [Petrovski et al., 2013]. As of yet, no clear phenotypic or genetic pattern allowing us to pinpoint an unmasking mechanism in patients with a CNV or a CCR has as yet emerged.

Multiple Possibly Pathogenic Mechanisms Provoked by CCRs: Two Cases

Since, by definition, a CCR is composed of multiple genomic alterations occurring simultaneously, it inherently is likely to provoke multiple pathogenic mechanisms, all operating at once in a single patient. It is a major challenge to determine to what extent each of these mechanisms may explain (part of) the patient's clinical phenotype(s). This is illustrated by the following cases.

The first case was a de novo CCR involving multiple deletions in association with a pericentric inversion of chromosome 6 [Passarge, 2000; Poot et al., 2009; Kloosterman et al., 2012]. Initially, this patient was described as a case with a pericentric inversion of 6p11.2;q15 with an additional deletion within 6q14, which upon SNP-array analysis was found to harbor 3, instead of 1, deletions within the de novo pericentric inversion (fig. 2) [Passarge, 2000; Poot et al., 2009]. The patient's phenotype consists of a dysmorphic face, nonprogressive deficit of motor control, lack of speech development, and a strongly reduced sensitivity to pain [Passarge, 2000; Poot et al., 2009]. Following a gene-centric approach, only haploinsufficiency of deleted genes or the disruption of the *MUT* gene by the leftmost breakpoint of deletion 1 (table 4) would be considered [Poot and Hochstenbach, 2010; Poot et al., 2011a]. In view of the ENCODE data, also regulatory domains consisting of sequences that enhance and/or inhibit the expression of breakpoint-flanking genes should be taken into account [Spielmann and Mundlos, 2013]. Thus, loss or disruption of the long noncoding RNA LOC101927048, binding sites for the transcription factors GATA1, GATA2, GATA3, p300 and c-*fos*, and the histone methylation marks of active promoter regions H3K4Me1 and H3K27AC (table 4), should also be considered when trying to explain the patient's phenotype.

The second case was initially described as a balanced insertion (9;12) translocation, which a mother transmitted such that it resulted in a trisomy of 9p22p24 in the proband [de Pater et al., 2002]. The mother, the proband and 2 other children, who inherited the balanced insertion (9;12) translocation all showed mental retardation. Therefore, the authors attributed the mental retardation in this 2-generation family to gene disruption brought about by the insertion (9;12) translocation [de Pater et al., 2002].

Table 3. Literature on unmasking of second SNVs or CNVs by inherited and de novo CNVs and CCRs

a Published cases of inherited recessive mutations unmasked by hemizygosity due to a de novo deletion

Phenotype of proband	Unmasked gene	Gene location	SNV	Parental origin of SNV	Chromosomal location	Deletion size, Mb	Parental origin of de novo deletion	Reference
Prader-Willi syndrome; oculocutaneous albinism	*OCA2*	15q12-q13.1	p.Val443Ile; c.1327G>A	mother is heterozygous carrier	del(15)(q11.2q13.1)	nd	paternal	Lee et al., 1994
VCF/DiGeorge syndrome; Bernard-Soulier syndrome	*GP1BB*	22q11.21	c.–133C>G	nd	del(22)(q11.2q11.2)	nd	nd	Ludlow et al., 1996
MR, retinoblastoma + Wilson disease	*ATP7B*	13q14.3	nd	maternal	del(13)(q14.2q21.1)	nd	paternal	Riley et al., 2001
Smith-Magenis syndrome; sensorineural hearing loss	*MYO15A*	17p11.2	p.Thr2205Ile; c.6614C>T	mother is heterozygous carrier	del(17)(p11.2p11.2)	nd	paternal	Liburd et al., 2001
Angelman syndrome; oculocutaneous albinism	*OCA2*	15q12-q13.1	c.647-?_807+?del (deletion of exon 7)	father is heterozygous carrier	del(15)(q11.2q13.1)	~5	maternal	Fridman et al., 2003
Williams syndrome; chronic granulomatous disease	*NCF1*	7q11.23	p.Tyr26fs; c.75-76del	nd	del(7)(q11.23q11.23)	nd	nd	Kabuki et al., 2003
Sotos syndrome; reduced coagulation factor 12 activity	*F12*	5q35.3	c.–4C>T (rs1801020)	nd	del(5)(q35.3q35.3)	2.2	nd	Kurotaki et al., 2005
22q11 del syndrome; psychotic disorder	*COMT*	22q11.21	p.Val158Met; c.472G>A	nd	del(22)(q11.2q11.2)	nd	nd	Gothelf et al., 2005
Wolf-Hirschhorn syndrome; Wolfram syndrome	*WFS1*	4p16.1	p.Gln366X; c.1096C>T	mother is heterozygous carrier	inv dup del(4) (:p15.33→p16.1::p16.1→qter)	8.3	paternal	Flipsen-ten Berg et al., 2007
22q13 del syndrome; metachromatic leukodystrophy	*ARSA*	22q13.33	p.Pro426Leu c.1277C>T	nd	del(22)(q13.2qter).ish cgh dup(13)(q22qter)	~8	nd	Bisgaard et al., 2009
Werner syndrome	*WRN*	8p12	p.Tyr57X; c.171C>G	nd	del (8)(p12p12)	0.553	nd	Friedrich et al., 2010

b Published cases of inherited recessive mutations unmasked by hemizygosity due to a deletion inherited from a healthy parent

Phenotype of proband	Unmasked gene	Gene location	SNV	Parental origin of SNV	Chromosomal location	Deletion size, Mb	Parental origin of deletion	Reference
MR; sensorineural hearing loss	probably *USH1E*	21q21	nd	nd	del(21)(q11.2q21.3)	~14	maternal	Wakui et al., 2002
Peters Plus syndrome	*B3GALTL*	13q12.3	p.?; c.660+1G>A	father is heterozygous carrier	del(13)(q12.3q13.1)	~1.5	maternal	Lesnik Oberstein et al., 2006
Growth retardation; autism; mild dysmorphic signs	*DIAPH3*	13q21.2	p.Pro614Thr; c.1840C>A	father is heterozygous carrier	del(13)(q21.1q21.31)	~10	maternal	Vorstman et al., 2011

Table 3b (continued)

Phenotype of proband	Unmasked gene	Gene location	SNV	Parental origin of SNV	Chromosomal location	Deletion size, Mb	Parental origin of deletion	Reference
DD/MR; ataxia; areflexia; macrocephaly; giant axonal neuropathy	*GAN*	16q23.2	p.Glu486Lys; c.1456G>A	father is heterozygous carrier	del(16)(q23.2q23.2)	0.057–0.131	maternal	Buysse et al., 2010
Van den Ende-Gupta syndrome	*SCARF2*	22q11.21	c.854 + 1G 1 T (intron 4)	maternal	del(22)(q11.2)	2.56	paternal	Bedeschi et al., 2010
Oculocutaneous albinism	*OCA2*	15q12q13.1	p.Gly651Glu; c.1952G>A	father is heterozygous carrier	del(15)(q13.1q13.1)	0.184 (intragenic deletion)	maternal	Rooryck et al., 2011
Oculocutaneous albinism	*OCA2*	15q12q13.1	p.AlaA334Val; c.1001C>T	nd	del(15(q13.1q13.1)	0.2	maternal	Rooryck et al., 2011
Cockayne syndrome	*ERCC6*	10q11.2	? frameshift	maternal	del(10q11.2)	5.0	paternal	Ghai et al., 2011
Cohen syndrome	*COH1*	8q22.2	p.Arg1696X; c.5086C>T	father is heterozygous carrier	del(8)(q22.2q22.2)	0.067 (intragenic deletion)	maternal	Rivera-Brugués et al., 2011
Cohen syndrome	*COH1*	8q22.2	p.Lys3835fsX43, c.11505delA	mother is heterozygous carrier	del(8)(q22.2q22.2)	0.193 (intragenic deletion)	paternal	Rivera-Brugués et al., 2011
Cohen syndrome	*COH1*	8q22.2	p.Thr1289Ser c.3866C>G + p.Asp3942_ Gly3943insAsp; c.11827_11828insATG	father is heterozygous carrier	del(8)(q22.2q22.2)	0.315 (intragenic deletion)	maternal	Rivera-Brugués et al. 2011
DD, retinitis pigmentosa + juvenile neuronal ceroid lipofuscinosis	*CLN3*	16p11.2	?	?	del(16)(p11.2)	220 kb (distal deletion)	?	Pebrel-Richard et al., 2014

c Published cases of CNVs or SNVs unmasked by a CCR

Phenotype of proband	Unmasked gene	Gene location	CNV or SNV	Parental origin of SNV	Chromosomal location	Deletion size, Mb	Parental origin of deletion	Reference
Primary amenorrhea, hypergonadotropic hypogonadism, disturbed folliculogenesis	*FSHR*	2p16.3	p.Pro587His; c.1760C4A	paternally inherited	t(2;8)(p16.3or21;p23.1)	?	maternally inherited	Kuechler et al., 2010
Short stature + congenital pulmonary alveolar proteinosis	*CSF2RA*	Yp11.32	gene deletion	maternally inherited	Xp22.33p22.2	?	de novo paternal	Auger et al., 2013

d Published cases of 2 overlapping deletions transmitted by healthy parents

Phenotype of proband	Affected gene	CNV location	CNV type	Parental origin	CNV location	CNV type	Parental origin	Reference
Severe epileptic encephalopathy, retinopathy, ASD and choreoathetosis	*CHRNA7*	15q13.3	recurrent deletion	maternally inherited	del(15)(q13.3)	recurrent deletion	paternally inherited	Masurel-Paulet et al., 2010
Severe MR, lack of speech, cheilognathopalatoschisis, microcephaly and bilateral hearing loss	*HSBP1*	16q23.3	unique deletion of 16 kbp	paternally inherited	del(16)(q23.3q24)	unique deletion of 2.166 Mb	maternally inherited	Hochstenbach et al., 2011

ASD = Atrial septal defect; DD = developmental delay; MR = mental retardation; nd = not determined; VCF = velocardiofacial syndrome; ? = not known.

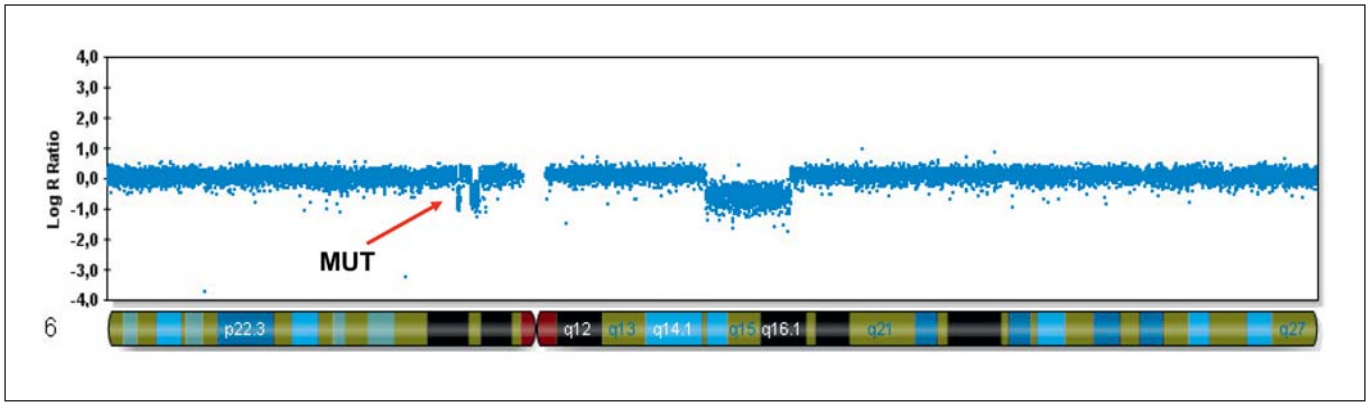

Fig. 2. SNP-array data of chromosome 6 of the proband with a de novo 46,XX,der(6)(pter/p12.3::p12.1→p12.1::q14.3→p12.1::p12.3→p12.2::q16.1→qter) [Passarge, 2000; Poot et al., 2009]. The arrow indicates the gene disrupted by the breakpoint.

Table 4. Breakpoints identified by SNP-array analysis of the proband with a de novo 46,XX,der(6)(pter→p12.3::p12.1→p12.1::q14.3→p12.1::p12.3→p12.2::q16.1→qter)

Deletion[a]	Breakpoint	Chromosome band	Defining SNPs		Disrupted gene	Breakpoint in gene	Histone modification[b]	DNAse I hypersensitivity	CpG island	DNA methylation sites	Transcription factor binding sites	Replication timing[c]
			left	right								
1	left	6p12.3	**rs6458687**	rs6458690	MUT	exon 12-intron 11	no	no	1	0	no	S2-S3
	right		rs6905366	**rs700002**	LOC101927048	no	no	no	0	0	no	S3-S4
2	left	6p12.2	**rs1342622**	rs10498786	no	no	H3K4me1H3K27ac	strong	1	1	GATA3, p300	G2
	right		rs9463802	**rs2063643**	no	no	H3K4me1 H3K27ac	weak	1	1	GATA1, GATA2, p300, c-*fos*	G1b-S1
3	left	6q14.2-6q12.3	**rs2324476**	rs4707016	no	no	no	no	0	no	no	S4-G2
	right		rs2799633	**rs2745650**	no	no	no	no	no	no	no	S4-G2

[a] Refers to the deletion regions shown in figure 2. [b] H3K27ac and H3K4me1 marks are often found near regulatory elements. [c] Replication timing data according to Hansen et al. [2010]. SNPs in bold are diploid; SNPs in normal font are deleted. Genes, motives or sites of interest within 1 kbp of the breakpoint regions [Passarge, 2000; Poot et al., 2009].

Upon genome-wide SNP-array analysis the patient's karyotype was described as: 46,XY,der(12)dir ins(12;9)(q23;p21p23)mat arr dup(9)(p21p23)(rs4740872×2,rs7860449×3,rs946452x3,rs1339284×2)(rs11790910×2,rs11793993×3,rs7038314×3,rs7863476×2)(rs10120052×2,rs13285154×3,rs2295797×3,rs3763630×2) arr del(12)(q23.1;q23)(rs11615348×2,rs10777975×1,rs10860431×1,rs7301622×2)(rs7965233×2,rs1007916×1,rs11111423×1,rs4764936×2), which involved 10 breakpoints (fig. 3). These breakpoints disrupted 5 candidate genes: *KDM4C*, *MPDZ*, *PLAA*, *TLN1* and *ANKS1B*, and may affect binding sites of the GATA3, CTCF, RFX5, NFYB, POLR2A and MAFK transcription factors (table 5). By mate-pair sequencing of the mother, a grand total of 23 fusion points were detected, of which 16 were transmitted to the son with the inherited mental retardation and the de novo dysmorphisms [Kloosterman et al., 2012]. The mate-pair data confirmed the breakpoints found by SNP-array genotyping of the son but also revealed breaks in regions 6q22.31 (disrupting *TRDN*), 7p14.2 (disrupting *AOAH*), 9p21.2 (disrupting *ITF74*), and in 12q24.21 (disrupting *TRPV4*) in the genome of the mother.

The breakpoint 1 in region 9p21 disrupts *KDM4C*, which is a histone demethylase of the Jumonji domain 2 (JMJD2) family. *KDM4C*, together with the histone demethylases of the same subclass exhibit distinct and combinatorial functions in control of the embryonal stem cell state [Das et al., 2014]. *KDM4C* is targeted to H3K4me3-positive transcription start sites, where it contributes to transcriptional regulation [Pedersen et al., 2014]. KDM4A/C-mediated control of histone methylation regulates intrinsic factors and signaling factors and may thus provide

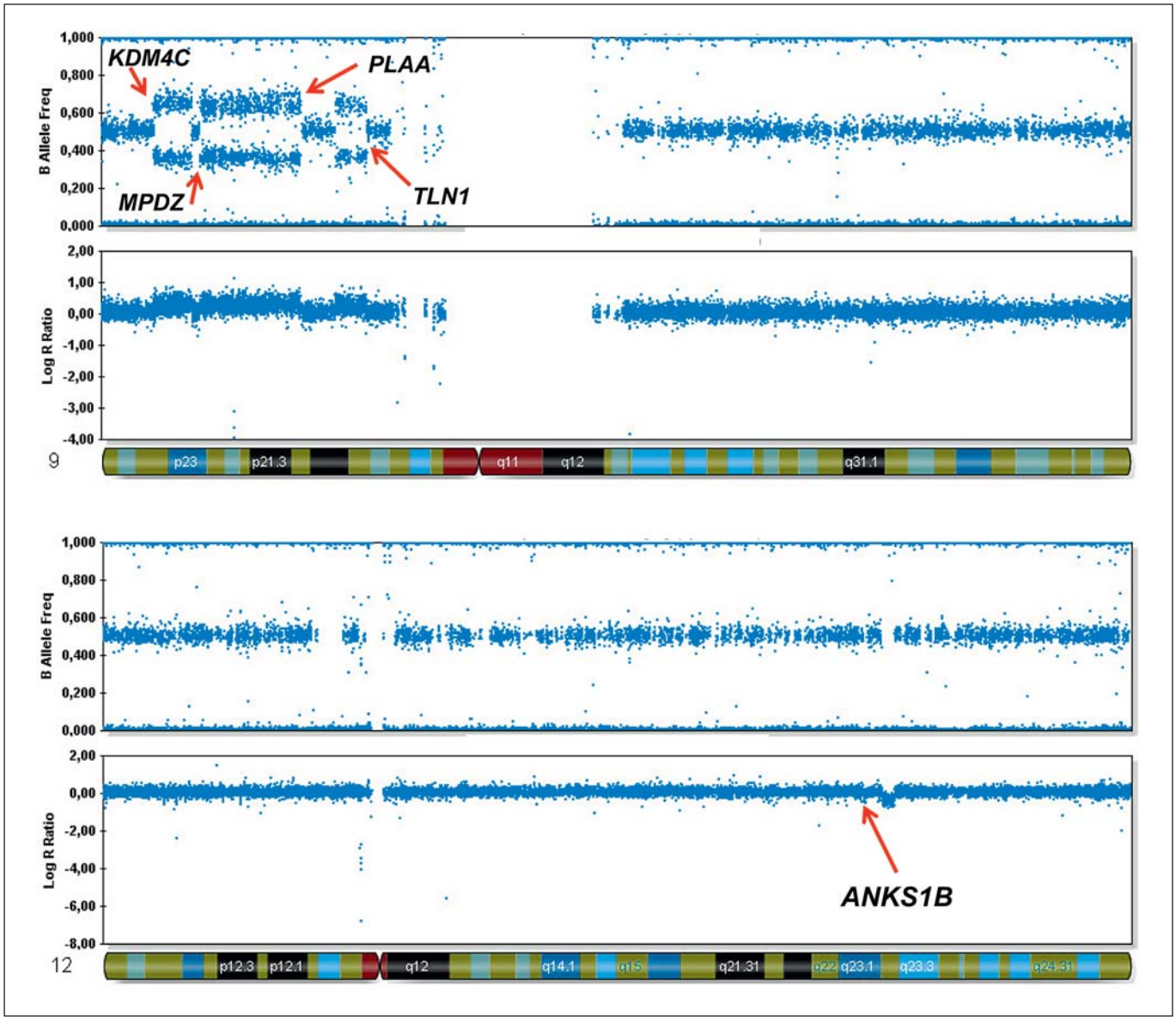

Fig. 3. SNP-array data of chromosomes 9 and 12 of the proband, who inherited a der(12)dir ins(12;9)(q23;p21p23) mat [de Pater et al., 2002]. The arrows mark the breakpoints identified by SNP-array and mate-pair sequencing analysis. The disrupted genes are indicated.

a novel control mechanism of lineage decision [Cascante et al., 2014]. Given this role in brain development, disruption of one copy of *KDM4C* may be involved in the transmitted form of mental retardation in this family.

Apart from detecting additional breakpoints beyond those seen by classical karyotyping, SNP-array genotyping and mate-pair sequencing revealed an additional level of complexity. In addition to CNVs and gene disruption, side effects and changes in noncoding sequences, e.g. DNA and histone methylation, and transcription factor binding sites may all occur simultaneously (table 5). Thus, the improved resolution of mate-pair sequencing showed that these 2 CCRs were even more complex than already revealed by SNP-array genotyping. Therefore, these CCRs must be considered as potential cases of MMMs operating simultaneously.

Table 5. Breakpoint regions identified by SNP-array analysis of the 46,XY,der(12)dir ins(12;9)(q23;p21p23)mat case

Breakpoint[a]	Chromosome band	Defining SNP		Disrupted gene	Breakpoint in gene	Histone modification[b]	DNAse I hypersensitivity	CpG island	DNA methylation sites	Transcription factor binding sites	Replication timing[c]
		left	right								
1		**rs4740872**	*rs7860449*	KDM4C	intron 17	no	no	no	no	no	S3-S4
2		*rs946452*	**rs1339284**	no	no	no	strong	no	no	no	S4-G2
3	9p23	**rs11790910**	*rs11793993*	*MPDZ*	intron 3	no	no	no	no	GATA3	S1-S2
4	9p23	*rs7038314*	**rs7863476**	*PLAA*	exon 14	no	no	no	no	no	S1-S2
5	9p23	**rs10120052**	*rs13285154*	no	no	no	no	no	no	no	S3-S4
6	9p23	*rs2295797*	**rs3763630**	*TLN1*	intron 1	H3K27ac	strong	no	no	CTCF, RFX5, NFYB, POLR2A	G1b
7	12q23.1	**rs11615348**	rs10777975	*ANKS1B*	intron 12	no	no	no	no	no	S4-G2
8	12q23.1	rs10860431	**rs7301622**	*ANKS1B*	intron 11	no	no	no	no	MAFK	S4-G2
9	12q23.2	**rs7965233**	rs1007916	no	no	no	no	no	no	no	S3-S4
10	12q23.2	rs11111423	**rs4764936**	no	no	no	no	no	no	no	S4-G2

[a] Refers to the deletion regions shown in figure 3. [b] H3K27ac mark is often found near regulatory elements. [c] Replication timing data according to Hansen et al. [2010]. SNPs in bold are diploid, italicized are duplicated and SNPs in normal font are deleted. Genes, motives or sites of interest within 1 kbp of the breakpoint regions [de Pater et al., 2002].

Diagnostic, Medical Genetic and Experimental Challenges

Facing patients with a complex set of phenotypes, not clearly fitting into a Mendelian syndrome, medical geneticists generally decide to first perform genome-wide segmental aneuploidy screening [Miller et al., 2010; Kearney et al., 2011]. This choice appears justified by the relatively high yield of potentially pathogenic CNVs in cohorts of patients with intellectual delay and neurodevelopmental disorders [Hochstenbach et al., 2009, 2011]. However, apparently 'balanced' SVs, which represent a small but significant number of cases, will thus escape detection [De Gregori et al., 2007; Hochstenbach et al., 2009]. With improving resolution of the CNV detecting arrays, the number of truly 'balanced' cases of SVs decreases, since the breakpoint regions of these SVs are increasingly found to contain small CNVs [Gribble et al., 2005; Fauth et al., 2006; De Gregori et al., 2007; Baptista et al., 2008; Higgins et al., 2008; Sismani et al., 2008; Schluth-Bolard et al., 2009; Gijsbers et al., 2010; Kang et al., 2010; Feenstra et al., 2011; Kloosterman et al., 2011, 2012; Tabet et al., 2015]. Also cases with multiple de novo or transmitted CNVs raise the suspicion of a CCR [Houge et al., 2003; Lybaek et al., 2008; Ballarati et al., 2009; Poot et al., 2009, 2010a; Schluth-Bolard et al., 2009; Tzschach et al., 2010]. Such cases should be further investigated by classical karyotyping or by paired-end or mate-pair sequencing [Korbel et al., 2007; Kloosterman et al., 2011, 2012; Hooper et al., 2012; Nazaryan et al., 2014].

The latter, highly laborious technique reveals the full complexity of the CCRs and will also allow determining the mode(s) of fusion of the chromosome breaks at nucleotide resolution [Kloosterman and Hochstenbach, 2014]. This information may contain clue(s) regarding possible mechanism(s) of origin of the CCR (see table 1). If these fusion points involve small deletions, microhomologies or non-templated insertions, this points towards biological pathways in which genes such as *ATM*, *BLM*, *WRN*, *RAD51*, *MRE11*, *ATR*, or *NBS1* participate. In such cases, the pedigree of the patient should be scrutinized for individuals with either CCRs, impaired fertility or recurrent miscarriages. Medical geneticists may then attempt to trace possible 'risk alleles', which cosegregate with such phenotypes in these pedigrees. Such transmitted risk alleles are by themselves not sufficient to cause CCRs, but may represent susceptibility factors, which may confer some degree of vulnerability towards CCR formation. If the fusion points do not contain any molecular signatures such as microhomologies, deletions or insertions, the CCR may have been stitched together by NHEJ. For the latter, no empirical or population data-based recurrence risk estimate is currently available.

A second aspect of the fusion point sequences is the respective orientation of the fused chromosomal fragments. If those are not exclusively head-to-tail, but also tail-to-tail, head-to-head, etc., they may have resulted from chromothripsis [Kloosterman et al., 2011, 2012]. This may indicate that male individuals in the family of the patient may harbor elevated levels of oxidative stress,

defective checkpoint functions, such as p53 activity, higher levels of abortive apoptosis, telomere erosion, mitotic errors, or micronucleus formation [Pellestor et al., 2014]. These suggested mechanisms of germline chromothripsis need further scrutiny before they can be incorporated into routine medical genetic counseling.

Since roughly 70% of all reported CCRs were found in healthy individuals, this type of SV does not necessarily elicit a phenotypic effect [Pellestor et al., 2011]. Hence, the need to distinguish phenotypically neutral from truly pathogenic CCRs. Again, obtaining information on the breakpoints at nucleotide resolution is a pivotal first step in identifying the possible pathogenic effects of a CCR. It will also allow ascertaining which genes and which additional architectural relationships within the genome are disrupted. The 2 cases described above illustrate this problem and show that CCRs inherently harbor multiple plausible mutational mechanisms, each of which need separate scrutiny (tables 4, 5).

As a first step, databases, such as ECARUCA (http://umcecaruca01.extern.umcn.nl:8080/ecaruca/ecaruca.jsp), DECIPHER (https://decipher.sanger.ac.uk/), ISCA (http://clinicalgenome.org/), and PubMed should be consulted for each of the disrupted genes to determine whether such a gene disruption has previously been discovered in a patient with a similar phenotype [Feenstra et al., 2006; Firth et al., 2009; Kaminsky et al., 2011; South and Brothman, 2011; de Leeuw et al., 2012; Riggs et al., 2012]. The Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home), on the other hand, summarizing data from 62 studies of healthy individuals, reports 3,024,212 CNVs and 2,335 inversions [MacDonald et al., 2014]. These databases are mainly 'populated' by CNVs, such that the reported SVs are biased towards those that contain dosage-sensitive genes, which may affect the individual's phenotype via haploinsufficiency or triplosufficiency [Poot et al., 2011a]. A recently developed database contains 2,643 individually curated breakpoints of congenital and somatic chromosomal rearrangements (dbCRID, http://dbCRID.biolead.org) [Kong et al., 2011]. Since there is no clinical need to karyotype randomly selected, healthy individuals, the number of true CCR carriers is underestimated, and no database of 'phenotypically neutral' CCR breakpoints, similar to the Database of Genomic Variants for CNVs, exists. In view of the recent advances in paired-end and mate-pair sequencing, there is clearly a need for such a more comprehensive database [Kloosterman and Hochstenbach, 2014].

A second way to assess the potential pathogenicity of CCRs is functional enrichment analyses of cohorts of patients with similar phenotypes. This approach has allowed us to relate phenotypes such as intellectual delay, autism, or attention deficit/hyperactivity disorder to certain biological pathways [Marshall et al., 2008; Webber et al., 2009; Hehir-Kwa et al., 2010; Pinto et al., 2010; Poot et al., 2010b; Webber, 2011; Noh et al., 2013; Andrews et al., 2015; Hawi et al., 2015]. To associate CCRs, CNVs, genes, and biological pathways with phenotypes, these have to be categorized systematically in so-called phenotype-ontologies [Robinson and Webber, 2014; Deans et al., 2015]. Next, the phenotypes of model organisms with disruptions of the orthologous genes, and features of breakpoint regions of the human CCRs, such as LINE elements, segmental duplications, 'side effects' on transcription levels of genes in the vicinity of the breakpoints, markers of active promoters (e.g. H3K4Me1, H3K27Ac), and adoption of enhancers or insulators have to be taken into consideration [Reymond et al., 2007; Hehir-Kwa et al., 2010; Spielmann and Mundlos, 2013; Ibn-Salem et al., 2014]. All these sophisticated methods of bioinformatic predictions notwithstanding, experimental confirmation of gene disruption or alteration of mRNA levels in a phenotypically relevant tissue of the patient remains necessary [Kloosterman and Hochstenbach, 2014]. The above highlights the need for both detailed clinical investigations of individuals with CCRs and complementary experimental studies to grasp the full impact of CCRs in the human genome. Induced pluripotent stem cells from patients with the 22q11.2 deletion syndrome, Down syndrome, and Prader-Willi syndrome have provided novel insights into the pathology of these disorders [Yang et al., 2010; Mou et al., 2012; Shcheglovitov et al., 2013; Paşca et al., 2014]. Transgenic animal models, such as mice and zebrafish, have allowed studying the effects of SVs at the organismal level and during development [Carvalho et al., 2014; Kloosterman et al., 2014; Portmann et al., 2014; Yang et al., 2015]. The novel genome editing CRISPR/Cas9 technology has opened an entirely new avenue for testing hypotheses on the functional impact of CNVs and CCRs, such as disruption of topological chromatin domains [Lupiáñez et al., 2015]. In addition, gene-gene interactions and novel treatment modalities can be studied in these models [Leblond et al., 2012; Schmeisser et al., 2012; Poot, 2013; Shcheglovitov et al., 2013]. In this way, the study of transgenic animals provides not only 'the proof of the pudding', it also opens up novel treatment avenues for otherwise difficult to study, rare disorders [Poot, 2013]. Eventually, investigation of CCRs may help to improve our insights into proper and disordered functioning and of our genome as a whole.

Acknowledgment

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URLs of websites

Database of Genomic Variants: http://dgv.tcag.ca/dgv/app/home.

DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER): https://decipher.sanger.ac.uk/.

European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA): http://umcecaruca01.extern.umcn.nl:8080/ecaruca/ecaruca.jsp.

International Standards for Cytogenomic Arrays Consortium (ISCA): http://clinicalgenome.org/.

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