CHROMOSOME BIOLOGY AS A KEY TO UNDERSTAND DISEASE MECHANISMS, GENOME ARCHITECTURE AND EVOLUTION

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CHROMOSOME BIOLOGY AS A KEY TO UNDERSTAND DISEASE MECHANISMS, GENOME ARCHITECTURE AND EVOLUTION

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REVIEW
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On the Complexity of Mechanisms and Consequences of Chromothripsis: An Update

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In the present review, we focus on the phenomenon of chromothripsis, a new type of complex chromosomal rearrangements. We discuss the challenges of chromothripsis detection and its distinction from other chromoanagenesis events. Along with already known causes and mechanisms, we introduce aberrant epigenetic regulation as a possible pathway to chromothripsis. We address the issue of chromothripsis characteristics in cancers and benign tumours, as well as chromothripsis inheritance in cases of its occurrence in germ cells, zygotes and early embryos. Summarising the presented data on different phenotypic effect of chromothripsis, we assume that its consequences are most likely determined not by the chromosome shattering and reassembly themselves, but by the genome regions involved in the rearrangement.

Keywords: chromothripsis, complex chromosomal rearrangements, epigenetics, cancer, benign tumour, chromosome pulverisation, constitutional chromothripsis

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INTRODUCTION

Complex chromosomal rearrangements have been found since introduction of cytogenetic techniques. At present, due to development of new molecular-cytogenetic and molecular methods, the nature of CCRs became apparent making possible their classification.

The first documented CCR case was a translocation affecting three chromosomes in a child with mental retardation and associated dysmorphic features (Nuzzo et al., 1968). In 1970, a team of Lund University researchers discovered another translocation involving three, or possibly, four chromosomes and characterised it as a "complex translocation" and "complex rearrangement" (Fredga and Hall, 1970). Subsequently, complex chromosomal translocations were given the definition that currently extends to the term "CCRs": complex chromosomal translocations involve more than a reciprocal exchange of segments between two chromosomes resulting in multiple derivative chromosomes (Pai et al., 1980). As molecular genetic techniques gained popularity, our understanding of the nature and origins of structural chromosomal abnormalities increased. As a result, the initial definition of CCRs is frequently updated in terms of the number of breakpoints and number of involved chromosomes. At present, CCRs are understood to be

Abbreviations: aCGH, array comparative genomic hybridisation; CC, constitutional chromothripsis; CCR, complex chromosomal rearrangement; DSB, DNA double-strand break; FISH, fluorescent *in situ* hybridisation; NHEJ, non-homologous end joining; SKY, spectral karyotyping; SNP array, single nucleotide polymorphism array.

structural chromosomal abnormalities that arise as a result of three or more breakpoints in one or more chromosomes, with the exception of inter- and intrachromosomal insertions (Madan, 2013; McGowan-Jordan et al., 2016).

In January 2011, Stephens et al. (2011) published a paper on CCRs in chronic lymphocytic leukaemia. Using paired-end DNA sequencing, they revealed 42 rearrangements affecting chromosome 4 and several rearrangements affecting chromosomes 1, 12, and 15 in the tumour cells of one patient. The detected rearrangements were characterised not only by numerous breakpoints in a relatively short genome region but also by multiple deletions in the almost complete absence of duplications. Subsequently, when studying similar genome alterations in a small-cell lung cancer cell line (SCLC-21H), the authors observed the formation of double minutes from fragments of derivative chromosome 8 (Stephens et al., 2011). Notably, the rearranged chromosomes and the double minutes comprised material from only one of the homologous chromosomes, the other remaining intact. The authors suggested the term "chromothripsis" to describe this phenomenon (from the Greek "chromos" – "chromosome" – and "thripsis" – "shattering" into small fragments) (Stephens et al., 2011).

Importantly, apart from chromothripsis, over the last 7 years two more CCR types have been described: chromoanasynthesis and chromoplexy. The three types of aberrations are covered by the umbrella term "chromoanagenesis" (from the Greek "anagenesis" – "rebirth"), which indicates a structural chromosome reorganisation (Holland and Cleveland, 2012). It is believed, however, that chromothripsis differs from other chromoanagenesis phenomena by the mechanisms of its occurrence and the nature of genetic alterations (Poot, 2018).

CHROMOTHRIPSIS AND OTHER TYPES OF CHROMOANAGENESIS

The results of whole genome sequencing, followed by mapping reads against a reference genome, lead us to believe that chromothripsis is based on the process of chromosome shattering triggered by double-strand DNA breaks (Stephens et al., 2011). The repair of double-strand breaks in a cell may occur through either a homologous recombination or NHEJ (reviewed in Ceccaldi et al., 2016). NHEJ is believed to be the primary repair mechanism in chromothripsis cases (Stephens et al., 2011). Once the DNA has been repaired through NHEJ, the reassembled chromosome may have errors in the order and orientation of segments. Fragments that do not ligate together with a centromere may be lost during subsequent cell divisions resulting in deletions (**Figure 1**; MacKinnon and Campbell, 2013). When double-strand breaks occur in two or more chromosomes, chromosome fragments may fuse, forming derivative chromosomes.

In theory, such CCRs may result from either chromosome pulverisation or sequential, independent rearrangements. The Monte-Carlo simulation method, which includes repeated random sampling and is traditionally used in stochastic process research, has established that the chromosome pulverisation model, which implies an absence of duplications, more accurately matches the genome alterations observed in chromothripsis. These data have given rise to an assumption that chromothripsis is the result of a single catastrophic event (Stephens et al., 2011).

The discovery of chromothripsis in the tumour cells of patients with chronic lymphocytic leukaemia was followed by a description of constitutional chromosomal rearrangements that are comparable with chromothripsis by number of breakpoints and breakpoint clustering but have different copy-number profiles. Microarray results have revealed that the karyotype of 17 patients with various developmental problems featured not only deletions but also multiple duplications and triplications, which could not have arisen as a result of NHEJ (Liu et al., 2011). This enabled the authors to hypothesise that such copy number alterations may result from replication and repair errors caused by DNA microhomology (MMBIR, microhomology-mediated break-induced replication; MMIR, microhomology/microsatellite-induced replication) (Payen et al., 2008; Hastings et al., 2009). Since chromothripsis does not fully reflect the characteristics of the observed genome alterations, the authors suggested replacing the term "chromothripsis" with "chromoanasynthesis," which stands for chromosome reconstitution or chromosome reassortment (Liu et al., 2011).

In their review article on the hypothetical mechanisms and consequences of chromoanagenesis, Holland and Cleveland (2012) contrast the terms "chromoanasynthesis" and "chromothripsis." According to the authors, chromoanasynthesis and chromothripsis are two independent phenomena with different underlying mechanisms. However, multiple chromosomal aberrations, which are observed in both, are most likely the result of a single catastrophic event, and not a successive series of rearrangements.

By contrast, chromoplexy, the third example of chromoanagenesis, is the result of an accumulation of chromosome rearrangements. The term "chromoplexy" (from the Greek "pleko" – "to weave") was introduced in 2013 to indicate complex rearrangements of prostate cancer genomes (Baca et al., 2013). To analyse the results of whole-genome sequencing and microarray-based comparative genomic hybridisation (aCGH), the researchers developed the ChainFinder algorithm, which identifies the chained rearrangements that resulted in the CCRs. They demonstrated that, in the majority of samples (50 out of 57), multiple deletions and translocations occurred successively, which is uncharacteristic of either chromothripsis or chromoanasynthesis. Chromoplexy is also characterised by fewer breakpoints and a larger number of rearranged chromosomes (up to eight) compared to chromothripsis (Baca et al., 2013). Importantly, the breakpoints are presumably localised in open chromatin regions (Berger et al., 2011). Therefore, the high transcription level of certain loci may serve as a chromoplexy trigger.

Recent study showed a novel potential mechanism of chromoanagenesis: DNA polymerase θ-dependent alternative homologous end joining (Masset et al., 2016). Thus, chromoanagenesis may be induced by a variety of mechanisms that lead to CCRs. In contrast to chromoplexy and chromoanasynthesis, chromothripsis is characterised by a larger

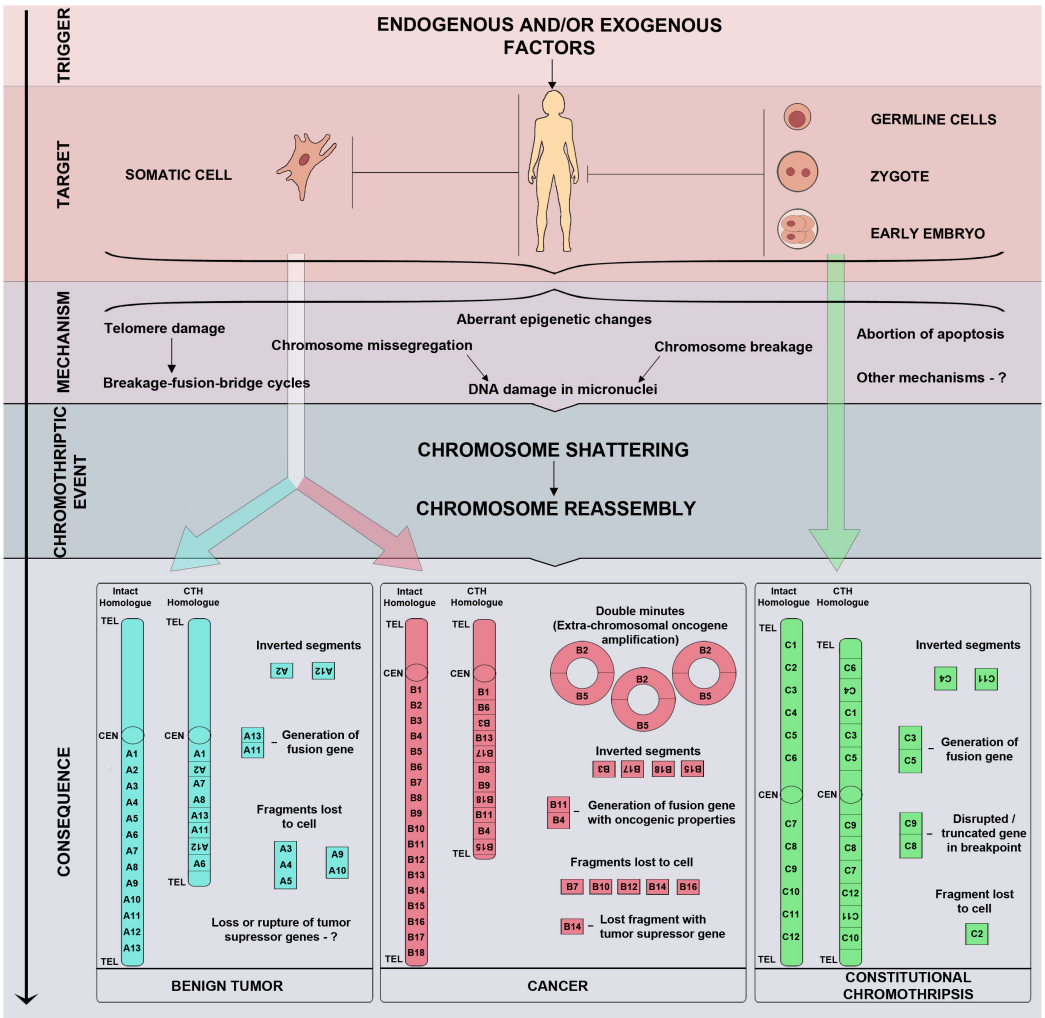

FIGURE 1 | Triggers, mechanisms, and consequences of chromothripsis. Chromothripsis may arise in any cell, including somatic cells, germline cells, zygotes, and blastomeres of preimplantation embryos, thus, determining the fate of an affected organ or the whole organism. Chromothripsis is induced by exogenous and/or endogenous factors which trigger chromosome shattering and sequential reassembly of fragments through micronuclei formation, breakage-fusion-bridge cycles, aberrant epigenetic regulation, abortive apoptosis, and other yet unknown mechanisms.

number of breakpoints and a random order and orientation of chromosome segments after reassembly. Chromothripsis features a high frequency of deletions in the almost complete absence of duplications in localised genome regions. However, chromothripsis identification among the multitude of CCRs is challenged by a lack of distinct limitations on the number of breakpoints and other features. The authors suggest six criteria to distinguish chromothripsis from other CCRs (Korbel and Campbell, 2013):

1. Clustering of breakpoints;
2. Oscillation of copy number states between one and two which is consistent with mono- or disomy;
3. A prevalence of regions with interspersed loss and retention of heterozygosity;
4. A prevalence of rearrangements affecting a single haplotype, i.e., one of two homologous chromosomes;
5. Randomness of DNA fragment joins and order, and;
6. Ability to "walk" the derivative chromosome by joining breakpoints.

The authors used statistical algorithms to justify some of the criteria, but they did not report the minimal number of breakpoints, and admitted the possibility of partial tri- and tetrasomies (Korbel and Campbell, 2013).

Initially, CCRs with over 50 breakpoints were classified as chromothripsis (Stephens et al., 2011). However, this criterion was not always fulfiled in subsequent works. In a number of cases, rearrangements with 20 (Molenaar et al., 2012), 10 (Northcott et al., 2012; Rausch et al., 2012), or fewer (Chiang et al., 2012) breakpoints were treated as chromothripsis. Kinsella et al. (2014) drew attention to this issue in 2014. Using statistical simulation, they demonstrated that chromothripsis-like rearrangements may result from sequential rearrangement. Importantly, these results

do not debunk the traditional hypothesis of the origins of chromothripsis but only emphasise the need for further research.

METHODS OF CHROMOTHRIPSIS DETECTION

It has been possible to describe the features of chromothripsis due to mate-pair sequencing and paired-end sequencing. These methods work for structural variant detection and CCRs, as well as genome assembly and *de novo* sequencing (Miller et al., 2010). In the case of a CCR, mate-pair and paired-end sequencing with subsequent verification by Sanger sequencing not only determine the precise localisation of breakpoints, but also gains data on nucleic acid sequences at breakpoint junctions (Gao and Smith, 2017). In spite of their high cost and challenging methodology, mate-pair sequencing and paired-end sequencing are widely used in chromothripsis studies.

Another efficient method of detecting and studying chromothripsis is microarray-based comparative genomic hybridisation (array CGH, aCGH), which is frequently referred to as "virtual karyotyping" or "chromosomal microarray analysis." Copy number analysis allows detection of deletions, duplications, and other aberrations as well as identification of their precise genome localisation and size. The resolution of this method is sufficient to detect submicroscopic aberrations. For higher resolution and information capacity, aCGH is combined with a single nucleotide polymorphism (SNP) array (Keren, 2014). As a method, aCGH is not without considerable limitations: it cannot detect balanced structural chromosomal aberrations or determine the order and orientation of derivative chromosome segments (Balajee and Hande, 2018).

For detection and localisation of a specific DNA or RNA sequence on a chromosome or in a cell, fluorescence *in situ* hybridisation (FISH) is frequently used. In chromothripsis studies, various FISH techniques are used, each of them addressing specific aspects in the identification of the derivative chromosome structure. SKY and multicolour FISH (M-FISH), with the use of whole chromosome probes conjugated with different fluorochromes, enables identification of chromosomes involved in a rearrangement. The multicolour-banding FISH technique (MCB-FISH) is a segment-specific variant of chromosome banding that allows one to determine the structure of an aberrant chromosome (Balajee and Hande, 2018). To map breakpoints on the chromosomes, locus-specific probes with known cytogenetic localisation may be used for FISH. A combination of SKY and *in situ* hybridisation with fluorescent locus-specific probes is used to determine the precise structure not only of derivative chromosomes but also of double minutes (Stephens et al., 2011).

In patients with hereditary diseases, chromothripsis may be detected by a conventional karyotyping of metaphases from peripheral lymphocytes. This technique allows identification of numerical and structural chromosomal abnormalities including translocations and inversions, which are frequently observed in CCR cases. However, the complex nature of CCRs makes their interpretation by conventional karyotyping alone difficult. Therefore, to precisely determine the structure of rearrangements in chromothripsis, it is necessary to use a complex approach that includes classical chromosome banding, visualisation of the aberrations on metaphase chromosomes by FISH and molecular genetic techniques.

CAUSES AND MECHANISMS OF CHROMOTHRIPSIS

The first assumptions regarding the mechanisms of chromothripsis were made by Stephens et al. (2011). The authors argue that DNA junction sequences and their localisation in the genome attests to chromosome pulverisation during mitosis at the stage of their highest condensation, not at the interphase stage. Today, several presumed causes of chromothripsis are listed (Meyerson and Pellman, 2011; Forment et al., 2012; Jones and Jallepalli, 2012; Maher and Wilson, 2012).

DNA Damage in Micronuclei

The most accepted hypothesis of chromothripsis occurrence is chromosome pulverisation in micronuclei. Chromosomes and their acentric fragments that lag during segregation in mitosis may be incorporated in a nuclear envelope outside of the main nucleus, which leads to the formation of micronuclei (Leibowitz et al., 2015). Certain features of the micronuclear envelope facilitate the access of cytoplasmic nucleases to the DNA (Géraud et al., 1989; Terradas et al., 2016). Micronuclei are characterised by abnormalities in chromatin condensation, which may lead to chromosome breaks (Terzoudi et al., 2015; Zhang et al., 2015). Experimental studies have shown the possibility of chromosome fragmentation and the formation of double minutes in micronuclei (Crasta et al., 2012; Hatch and Hetzer, 2015; Terradas et al., 2016). Using SKY, the authors determined that the majority of metaphases from cells with micronuclei feature multiple small fragments from one or two chromosomes (Crasta et al., 2012). The experiment on chromosome Y centromere inactivation also shed light on certain details of chromothripsis in micronuclei (Ly et al., 2017). The missegregated chromosome Y was included in a micronucleus and fragmented as a result of premature chromatin condensation. After the DNA breaks were repaired through NHEJ, the re-ligated chromosome Y showed typical characteristics of chromothripsis. It has been established that chromothripsis in micronuclei results from chromosome missegregation, their fragmentation, and the repair of breaks that occur during three cell cycles (Ly et al., 2017).

Aborted Programmed Cell Death

The abortion of apoptosis is regarded as one of the causes of chromothripsis (Tubio and Estivill, 2011; Tang et al., 2012). The first data on the association of chromothripsis with mutations of *TP53*, which encodes p53 protein – the key apoptosis regulator – were obtained in 2012. Chromothripsis was detected in Sonic Hedgehog (SHH) medulloblastoma cells in a patient with hereditary Li-Fraumeni syndrome (a germline mutation of *TP53*) (Rausch et al., 2012). In acute and chronic lymphocytic

leukaemia, TP53 mutations may co-occur with chromothripsis in tumour cells (Pei et al., 2012).

In 2015, the occurrence of chromothripsis in *TP53*$^{-/-}$ cells after doxorubicin treatment on a cell-based model system was confirmed (Mardin et al., 2015). Observing a higher frequency of chromothripsis in hyperploid medulloblastomas, as compared to diploid ones, the authors established an association between cell hyperploidisation and chromothripsis. In this regard, it has been suggested that hyperploidisation may serve as a risk factor for chromothripsis (Mardin et al., 2015).

Telomere Shortening and Formation of Dicentric Chromosomes

Highly localised rearrangements in chromothripsis can also be explained by breakage-fusion-bridge cycles in dicentric chromosomes, which arise from DNA damage or telomere fusion caused, in turn, by telomere shortening or loss (Stephens et al., 2011; Sorzano et al., 2013). When dicentric chromosomes segregate during mitosis, chromatin bridges are formed and undergo subsequent rupturing (McClintock, 1939). Having induced the formation of an envelope with an aberrant structure, the chromatin bridge is destroyed by cytoplasmic 3′-exonuclease TREX1 (Maciejowski et al., 2015; Maciejowski and de Lange, 2017). This may result not only in multiple losses and inversions of chromosome segments but also in the formation of double minutes. Breakage-fusion-bridge cycles may co-occur with fragment amplification, as demonstrated on regions of chromosome 21 (iAMP21) in a dicentric chromosome formed as a result of a Robertsonian translocation of chromosomes 15 and 21 (Li et al., 2014). The risk of iAMP21 acute lymphoblastic leukaemia in carriers of rob (15;21) is assessed to be ∼2700 times higher than in the population (Li et al., 2014). The presence of breakage-fusion-bridge cycles in cells with chromothripsis has also been demonstrated in studies of cancer genome alterations (Nones et al., 2014; Maciejowski et al., 2015; Ernst et al., 2016). These cycles, however, may be a part of neochromosome evolution and therefore, considering that neochromosomes arise through chromothripsis, may be the consequence, not the cause, of the phenomenon (Garsed et al., 2014).

Chromosome Pulverisation Caused by Exogenous Factors

Chromosome pulverisation is an extreme example of DNA fragmentation. Multiple double-strand breaks in the DNA may result from exposure to a range of DNA-damaging agents including drugs, therapeutic or environmental ionising radiation, oxidative stress and virus infections.

Despite the initial suggestion that ionising radiation may induce chromothripsis, experimental proof was not obtained until several years later. In their experiments, Morishita et al. (2016) used a focused vertical microbeam system designed to irradiate a spot within the nuclei – the Single Particle Irradiation system to Cell (SPICE) – on oral squamous-cell carcinoma cells. The authors then established irradiated monoclonal sublines from them and analysed genome abnormalities using SKY and SNP array. One of the 46 monoclonal sublines showed chromothripsis-like complex chromosomal alterations with 14 breakpoints. The involvement of 10 chromosomes in the rearrangement is explained by the exposure of the interphase nuclei to a powerful particle beam. The authors presume that cell irradiation during mitosis may induce chromosome missegregation and, as a result, lead to micronuclei formation (Morishita et al., 2016).

Another potential cause of chromothripsis is chromosome pulverisation in viral infections. A connexion has been established between chromosome pulverisation and fragmentation and infection of cell cultures with measles, herpes zoster, herpes simplex, and adenovirus types 4, 12, and 18 (Benyesh-Melnick et al., 1964; Nichols et al., 1965; O'Neill and Miles, 1970; Peat and Stanley, 1986). In addition, herpes simplex may induce cell polyploidisation, which is also a risk factor for chromothripsis (Chenet-Monte et al., 1986; Mardin et al., 2015). Tumour cells infected with the Epstein-Barr virus have an increased level of both transmissible and unstable chromosomal abnormalities (dicentric chromosomes, chromatid fragments, ring chromosomes, double minutes, satellite associations of acrocentric chromosomes, and chromatin breaks) (Kamranvar et al., 2007). However, only one of the studies (Schütze et al., 2016) confirms the association of chromothripsis with viral infections. In human foreskin keratinocytes culture infected with human papillomavirus, chromothripsis-like complex chromosomal alterations within chromosome 8 occurred after passage 30, were detected at passage 40, and resulted in a gain of *MYC*. Concurrently, immortalisation of the cell line *in vitro* with non-transformed phenotype was observed (Schütze et al., 2016).

While the listed causes of chromothripsis appear to be the most likely, it is necessary to consider other possible contributing factors such as mutations in DNA repair genes or abnormal chromatin condensation.

Aberrant Epigenetic Patterns as a Cause of Chromosome Damage

Chromothripsis is characterised by a high frequency of deletions, translocations and inversions (Stephens et al., 2011). These chromosomal aberrations result from multiple double-strand breaks (DSBs) possibly occurring during M or G1 phase. DSBs are most probably repaired by error-prone NHEJ or microhomology-mediated end joining (MMEJ) mechanisms (Jones and Jallepalli, 2012). In most cases, very short or no microhomology in the chromothripsis breakpoint junctions can be found (Stephens et al., 2011; Chiang et al., 2012; Kloosterman et al., 2012; Malhotra et al., 2013; Weckselblatt et al., 2015; Aristidou et al., 2018; Slamova et al., 2018). However, in a few cases of CC, DSBs were found in high-copy repeats (Nazaryan et al., 2014; Nazaryan-Petersen et al., 2016).

The chromatin conformation is of importance for occurrence of spontaneous DSBs. The transition from closed to open chromatin, which is necessary for transcription, makes DNA vulnerable to damage (Kuo, 1981; Falk et al., 2008; Meschini et al., 2015). Chromatin looping facilitates DNA cleavage by nucleases, including endogenous ones originating from transposable elements (Maniotis et al., 2005). In the study on

the events involved in the occurrence of stably segregating CC, DNA cleavage by catalytically active L1-endonuclease and translocations between distally located DNA regions were explained by Alu-mediated chromatin looping (Nazaryan-Petersen et al., 2016). Enhanced activation of transposable elements is associated with a response to environmental change and as well as with syndromes caused by *MeCP2* (methyl-CpG binding protein 2; involved in transcription regulation) and *ATM* (ataxia telangiectasia, mutated; involved in DNA repair machinery) mutations (Bundo et al., 2014).

A key role in the regulation of chromatin structure belongs to epigenetic mechanisms: DNA methylation, histone variants, and non-coding RNAs (Geiman and Robertson, 2002; Li, 2014). Both tumorigenesis and cell differentiation including embryonic and germline cells are characterised by extensive epigenetic changes (Yamaguchi et al., 2013; Efimova et al., 2015, 2017, 2018; Avgustinova and Benitah, 2016; Atlasi and Stunnenberg, 2017; Pendina et al., 2018). Epigenetic machinery provides fast response to environmental change through gene-specific and/or genome-wide alterations of DNA methylation with subsequent change in expression patterns of genes coding proteins and regulatory RNAs (Wang et al., 2017; West, 2017). Abnormal DNA methylation also may compromise genome integrity. *In vivo* increase of chromosome aberrations has been documented in tissues with reduced global DNA methylation caused by ionising radiation (Lee et al., 2015), oxidative stress (Tunc and Tremellen, 2009), or deregulated DNMTs (Gaudet et al., 2003). In blood cells of ICF patients having *DNMT3a* mutation, hypomethylation of 1q, 9q, 16q heterochromatin regions is associated with abnormal chromatin looping, telomeric associations, anaphase bridges, lagging chromosomes, chromosome breakage and micronuclei formation (Gisselsson et al., 2005). In addition, hypomethylation of pericentromeric heterochromatin may trouble kinetochore orientation and spindle attachment, resulting in chromosome missegregation and micronuclei formation (Luzhna et al., 2013). Thus, aberrant DNA methylation contributes to abnormal chromatin compaction and, as a consequence, to DNA damage.

The involvement of epigenetic mechanisms in the pathway between damaging agents and genome integrity has been established in the studies of the radiation-induced bystander effect. The bystander effect is a phenomenon whereby irradiated cells communicate damage to non-irradiated nearby bystander cells, thus destabilising their genome and contributing to carcinogenesis (Koturbash et al., 2007). In rodents, localised X-ray exposure modifies expression of DNA methyltransferases and 5-methylcytosine-binding protein MeCP2 genes leading to global hypomethylation both in irradiated and non-irradiated tissues *in vivo* (Koturbash et al., 2006, 2007; Tamminga et al., 2008). DNA damage in non-irradiated bystander tissues is associated with induction of apoptosis (Koturbash et al., 2008; Kovalchuk et al., 2010; Cordelli et al., 2012). Recent advances in bystander effect aetiology assumed that communication between irradiated and non-irradiated cells involves numerous microRNAs (Xu et al., 2015; Yuan et al., 2016; Cai et al., 2017). In addition to microRNAs, cell-free chromatin released from radiation-induced dying cells is involved in extensive chromosome instability of bystander cells (Kirolikar et al., 2018).

Summarising the abovementioned issues, it could be assumed that activation of the cellular mechanisms involved in the chromothripsis formation by exogenous and/or endogenous insult is epigenetically mediated. However, lack of experimental evidence directly linking disruption of epigenetic regulation to the initiation of chromothripsis substantiates further studies in this field.

CHROMOTHRIPSIS AND NEOPLASIA

In 2015, ChromothripsisDB[1] database was created (Yang et al., 2016) to categorise cases of chromothripsis in human and model organisms by disease, research method, and criteria that enabled the authors to classify the observed chromosomal abnormalities as chromothripsis. As of March 2018, the database counted 500 chromothripsis cases across 46 cancers. The authors of ChromothripsisDB update it on a regular basis and standardise the information on all the rearrangements that are treated as chromothripsis (Cai, 2018). At present, ChromothripsisDB is the most informative source of information for accessing and comparing the results of chromothripsis studies.

Chromothripsis in Cancers

Chromothripsis is typical for 2–3% of cancer types (Stephens et al., 2011). As of today, chromothripsis has been observed in blood cancers, central nervous system cancers, soft tissue tumours, and carcinomas (Rode et al., 2016).

The frequency of chromothripsis varies across tumour entities (**Table 1**). Chromothripsis occurs most frequently in bone cancers – osteosarcoma and chordoma (Stephens et al., 2011). It is associated with advanced stages of the disease and poor clinical outcomes (Forment et al., 2012). At times, chromothripsis is coupled with additional mutations in tumour cells, for instance, *IDH* mutations (Cohen et al., 2015). In addition, the occurrence of chromothripsis in cancers is considerably higher in patients with inherited genetic disorders that are linked to cell-cycle and DNA repair gene mutations: Li-Fraumeni and Louis-Bar syndromes (Rausch et al., 2012; Ratnaparkhe et al., 2017). The risk of chromothripsis also varies across different genome regions: chromosomes 17, 8, 12, and 11 are the most likely to be involved in such rearrangements. As it appears, the highest frequency of chromothripsis in chromosome 17 is predetermined by the presence of the *TP53* gene in its short arm (Cai et al., 2014).

Chromothripsis in Benign Tumours

Chromothripsis does not occur exclusively in malignant tumours; cases of chromothripsis have been observed in benign tumours as well. The year 2013 brought the first descriptions of chromothripsis in uterine leiomyoma (also called uterine fibroid) cells – a benign tumour of the uterine myometrium, which is characterised by a high frequency of chromosomal abnormalities. By various estimates, chromothripsis occurs in 13–42% of uterine fibroids (Mehine et al., 2013; Holzmann et al., 2014; Mehine et al., 2014).

[1] http://cgma.scu.edu.cn/ChromothripsisDB

TABLE 1 | Types of cancer with the highest occurrence of chromothripsis.

References	Cancer type	Cases with chromothripsis/ total number of cases	Chromothripsis frequency
Rausch et al., 2012	SHH medulloblastoma with *mut TP53*	10/10	100%
Rausch et al., 2012	SHH medulloblastoma with *wt TP53*	0/22	0%
Northcott et al., 2012; Rausch et al., 2012	Medulloblastoma, all subgroups	13/98; 139/1087	13%
Li et al., 2014	Acute lymphoblastic leukaemia with iAMP21	8/9	89%
Morrison et al., 2014	Invasive bladder carcinoma	81/150	60%
Zemanova et al., 2014	Myelodysplastic syndrome with CCR	77/157	49%
Rausch et al., 2012	Acute myeloid leukaemia with *mut TP53*	8/17	47%
Rausch et al., 2012	Acute myeloid leukaemia with *wt TP53*	1/91	1%
Przybytkowski et al., 2014	High-risk breast cancer	12/29	41%
Malhotra et al., 2013	Grade IV glioma (glioblastoma)	7/18	39%
Cohen et al., 2015	Grade IV glioma with *mut IDH*	9/24	37%
Cohen et al., 2015	Grade II–III glioma	5/45	11%
Malhotra et al., 2013	Lung adenocarcinoma	2/6	33%
Stephens et al., 2011	Osteosarcoma	3/9	33%
Nones et al., 2014	Esophageal adenocarcinoma	40/123	32%

iAMP, amplification of a chromosome 21 region; IDH, isocitrate dehydrogenase; SHH, Sonic Hedgehog.

Unlike malignant tumours, chromothripsis in uterine fibroid cells is characterised by fewer breakpoints (20 or more) and a larger number of affected chromosomes (up to four) (**Figure 1**). Such aberrations are normally observed in uterine fibroids without fibroid-specific *MED12* (mediator complex subunit 12) and *FH* (fumarate hydratase) mutations. They do not feature *TP53* mutations or histological signs of malignancy (Mehine et al., 2013; Holzmann et al., 2014; Mehine et al., 2014; Pendina et al., 2017). Furthermore, chromothripsis with large deletions (from 43 to 13,647 kbp) has been observed in non-cultured sample of uterine fibroid which demonstrated normal karyotype in culture conditions (Holzmann et al., 2014). This could be associated with a lower proliferative potential of tumour cells with chromothripsis *in vitro*. However, a case of unbalanced chromothripsis has been observed in both the cultured and non-cultured fibroid cells (Pendina et al., 2017). It is likely that the ability of fibroid cells with chromothripsis to proliferate *in vitro* is determined not so much by the size of deletions and number of breaks as by the genomic loci involved in rearrangement. It should be noted, however, that the absence of malignisation signs in fibroids with chromothripsis by no means implies that their growth and malignant potential does not require thorough study.

CONSTITUTIONAL CHROMOTHRIPSIS AS A CONSEQUENCE OF GENOME DAMAGE IN GERM CELLS AND PREIMPLANTATION EMBRYOS

Chromothripsis may also be a constitutional karyotype abnormality caused by chromosome damage in germline cells or preimplantation embryos. Cases of CC are extremely rare and usually coincide with congenital malformations or reproductive failure in the patient (**Table 2**; Kloosterman et al., 2011; de Pagter et al., 2015). In the virtually complete absence of any genetic imbalance, CC may co-occur with breakage of multiple genes or changes in their expression (**Table 2**; van Heesch et al., 2014; de Pagter et al., 2015; Bertelsen et al., 2016; Middelkamp et al., 2017). CC may include structural chromosomal abnormalities associated with genetic disorders (**Table 2**; Fontana et al., 2014; Genesio et al., 2015; Kurtas et al., 2018). In this case, the patient displays symptoms of an inherited disease. However, certain non-specific phenotypical features complicate the diagnosis and prognosis of the clinical outcome of the CC (**Table 2**).

Constitutional chromothripsis carriers may transmit the rearrangement to the offspring either stably or with *de novo* events (Gu et al., 2013; Weckselblatt et al., 2015; Bertelsen et al., 2016; Nazaryan-Petersen et al., 2016; Kurtas et al., 2019). Whereas the majority of *de novo* CC cases result from chromosomal aberrations arising from male gametogenesis (Pellestor et al., 2014), chromothripsis is inherited primarily from the mother (**Table 3**). To all appearances, it is determined by differences in DNA repair capacity and specific features of spermatogenesis and oogenesis.

Chromothripsis may arise during mitotic and meiotic divisions of spermatogenic cells as well as during spermiogenesis (round spermatid differentiation in spermatogonia) (Pellestor and Gatinois, 2018). Considering that spermatogonia undergo a succession of mitotic divisions, the replication stress may lead to errors during mitosis. Meiotic recombination may also feature double-strand break repair errors (Pellestor and Gatinois, 2018). The DNA breaks in spermatids that occur at the stage of histone-to-protamine transition during spermiogenesis can only be repaired through NHEJ because of the haploid chromosome number in cells at this stage (Gunes et al., 2015). In rodent male germ cells, scaffold/matrix-attached and differentially packaged chromatin regions are highly sensitive to endogenous nucleases, and, thus, to damage (Arpanahi et al., 2009; Grégoire et al., 2013). Accumulation of DNA strand breaks may be also caused

TABLE 2 | Clinical outcomes of constitutional chromothripsis.

References	Chromosome regions involved in chromothripsis	Chromothripsis detection method	Imbalance (size, copy number alterations)	Affected genes	Phenotype of a carrier(s)
Bertelsen et al., 2016; Nazaryan-Petersen et al., 2016	3q22.3-q23 5q23.1	Conventional cytogenetics, mate-pair sequencing	Four deletions (2–110 kb)	Truncated genes: *PPP2R3A*, *CLDN18*, *A4GNT*, *DBR1*, *HSD17B4*, *ATR* Fusion genes: *CLDN18-HSD17B4*, *HSD17B4-DBR1* Deleted genes: *DZIP1L*	No apparent association with a disorder
Anderson et al., 2016	13q33.1-q33.3 Xp11.22-p21.3 Xq21.31-q22.1	Conventional cytogenetics, FISH, aCGH	10 deletions (327 kb – 8 Mb): a total 4.4 Mb of chr. 13 material and 28.1 Mb of chr. X material	Deleted genes: Chr. 13 – *ERCC5*, *SLC10A2* Chr. X – *IL1RAPL1*, *DMD*, *GK*, *NROB1*, *CYBB*, *OTC*, *RPGR*, *TSPAN7*, *XK*, *ATP6AP2*, *BCOR*, *CASK*, *CFP*, *KDM6A*, *MAOA*, *NDP*, *NYX*, *RBM10*, *RP2*, *SYN1*, *UBA1*, *USP9X*, *ZNF81*, *BMP15*, *CACNA1F*, *CLCN5*, *FOXP3*, *HSD17B10*, *IQSEC2*, *KDM5C*, *PHF8*, *FGD1*, *HUWE1*, *HSD17B10*, *DIAPH2*, *SRPX2*	Developmental delay and dysmorphism
Weckselblatt et al., 2015	1q21 4q31 7p14.3 15q22	Conventional cytogenetics, FISH, targeted sequencing	530-kb deletion of chr. 1 material; 4,2-Mb duplication of chr. 7 material	No disrupted genes by the breakpoints	Developmental delay, autism, intellectual disability, and/or congenital anomalies
	3q25-q26 8q23 9p22-p24 11p14.1 3q21.1	Conventional cytogenetics, FISH, WGS	Mb-sized deletions of chr. 8 and 9 material; a total of 99 bp deleted of other chromosomes material	Disrupted genes by the breakpoints: *PTPRD*, *SH3GL2*	Developmental delay, autism, intellectual disability, and/or congenital anomalies
	2q32-qter 3q13 7q21.11-q22.1 10q21.3 11q14.1	Conventional cytogenetics, FISH, WGS	800-kb deletion of chr. 7 material, 2.2-Mb deletion of chr. 11 material; in addition, there are 55 total bp deleted at breakpoint junctions on other chromosomes	Disrupted genes by the breakpoints: *GRM3*, *KPNA1*, *DLG2*, *CACNA2D1*, *GULP1*, *COL5A2*, *KCNH7*, *PCLO*, *TRRAP*	Developmental delay, autism, intellectual disability, and/or congenital anomalies
Nazaryan et al., 2014	2p16.1-p22.1 5p14.2-p15.2 7p21.3-q31.1	Conventional cytogenetics, FISH, mate-pair sequencing	No copy number alterations	Truncated genes: *CDH12*, *DGKB*, *FOXP2*	Global developmental and psychomotor delay, severe speech disorder
Gamba et al., 2015	1p36.33-p35.3	Conventional cytogenetics, aCGH	Five deletions: 0.83, 0.94, 1.4, 1.7, 3.7 Mb 1 duplication: 5.9 Mb	No data	Multiple congenital malformations presenting some features overlapping the 1p36 deletion phenotype
Gu et al., 2013	5p13.3-p15.33 7p22 7q32 11q23 21q21	Conventional cytogenetics, FISH, aCGH	No copy number alterations	No data	Phenotypically normal
	5p13.3-p15.33 11q23	Conventional cytogenetics, FISH, aCGH	Three deletions: 2.89, 0.56, and 3.21 Mb	Deleted genes: *LOC340094*, *ADAMTS16*, *KIAA0947*, *FLJ33360*, *MED10*, *UBE2QL1*, *LOC255167*, *NSUN2*, *SRD5A1*, *PAPD7*, *MIR4278*	Phenotypically normal
	5p13.3-5p15.33	Conventional cytogenetics, FISH, aCGH	∼26.22-Mb deletion	No data	Developmental delay, dysmorphic and autistic features

(Continued)

TABLE 2 | Continued

References	Chromosome regions involved in chromothripsis	Chromothripsis detection method	Imbalance (size, copy number alterations)	Affected genes	Phenotype of a carrier(s)
Kloosterman et al., 2011	1p32.3 4q24 10q21.1	Conventional cytogenetics, SNP array, mate-pair sequencing	Small deletions and duplications (<50 bp)	Disrupted gene: *PCDH15*	Severe psychomotor retardation, speech delay, hypertelorism and kyphoscoliosis
Slamova et al., 2018	1q23-q25 6q15-q24 14q13? 18p11.2-p11.3 18q11.2	Conventional cytogenetics, FISH, aCGH, mate-pair sequencing	Two deletions: 0.7 and 2.5 Mb	Deleted genes: *DNM3*, *PIGC*, *C1ORF105*, *SUCO*, *NMBR*, *VTA1*, *ADGRG6*, *HIVEP2*, *AIG1*, *ADAT2*, *PEX3*, *FUCA2*, *PHACTR2*, *LTV1*, *ZC2HC1B*, *PLAGL1*, *SF3B5*, *STX11*, *UTRN*, *PAX9* Disrupted genes by the breakpoints: *FILIP1*, *PHIP*, *HMGN3*, *AK097143*, *GAREM*	Developmental and growth delay
Wang et al., 2015	19p13.13-p13.2 19p12 19q12 19q13.11-q13.12	Conventional cytogenetics, FISH, aCGH	Four duplications: 4.3, 0.98, 1.12, and 5.13 Mb	No data	Subtle dysmorphic features
Macera et al., 2015	3p24.3 5q14 7q35 9p23 18p11.31 18q21.31	Conventional cytogenetics, FISH, SNP array, NGS	No loss or gain of chromosomal material at any of the breakpoints	Disrupted genes by the breakpoints: *CNTN6*, *TBC1D5*, *CNTNAP2*, *PTPRD*, *L3MBTL4*, *LOC1001304840*, *WDR7*	Bilateral ventriculomegaly (13 and 15 mm), colpocephaly, with partial agenesis of the corpus callosum, and an absent left kidney and small right kidney
Kurtas et al., 2018	22q13.1-q13.3	Conventional cytogenetics, FISH, aCGH, WGS, WES	Two duplications: 2.4 Mb, 148 kb 1 deletion: 8.4 Mb	Disrupted genes by the breakpoints: *EP300*, *NFAM1*, *MYO18B*, *GTPBP1*	Phelan-McDermid syndrome
Genesio et al., 2015	9p21-q31	Conventional cytogenetics, FISH, aCGH	Two deletions: 176.56 kb, 7.44 Mb	Deleted genes: *RORB*, *TRPM6*, *NMRK1*, *OSTF1*, *GNAQ*, and the critical region of the 9q21.13 deletion syndrome	Platelet disorder and thyroid dysfunction in addition to the classical neurobehavioral phenotype of the 9q21.13 microdeletion syndrome
Del Rey et al., 2016	2q34-q37.3	Conventional cytogenetics, FISH, HR-CGH, MLPA	Deletion: 2.58 Mb duplication of 2q34q37.2	Deleted genes: *K1F1A*, *PASK*, *HDLBP*, *FARP2*	Multiple congenital disorders and intellectual disability
Fontana et al., 2014	1q41 1q43 9p24.3 21q22.12	Conventional cytogenetics, aCGH	Four deletions: 5.23, 1.33, 0.15871, and 0.826 Mb	Deleted genes: *SMYD2*, *PTPN14*, *CENPF*, *KCNK2*, *KCTD3*, *USH2A*, *ESRRG*, *SPATA17*, *RRP15*, *TGFB2*, *CHRM3*, *KANK1*, *RCAN1*, *CLIC6*, *RUNX1*	Loeys–Dietz syndrome, type 4; borderline mental impairment
Kurtas et al., 2019	3q22.3-q26.2	Conventional cytogenetics, FISH, aCGH, paired-end sequencing	Deletion: 6.8 kb	Disrupted genes by the breakpoints: *ROPN1B*, *NAALADL2*, *TF*	Healthy
	3q22.3-q26.2	Conventional cytogenetics, FISH, aCGH, paired-end sequencing	Duplication: 10 Mb deletion: 5 Mb	Disrupted genes by the breakpoints: *ROPN1B*, *NAALADL2*, *TF*	Multiple phenotypic abnormalities and psychomotor delay
	chr. 6 14q31.3	Conventional cytogenetics, FISH, aCGH, paired-end sequencing	Two deletions: 5.3 and 3.7 kb	Disrupted genes by the breakpoints: *OPRM*, *RNGTT*	Healthy
	chr. 6 14q31.3	Conventional cytogenetics, FISH, aCGH, paired-end sequencing	Deletion: 1 Mb	Disrupted genes by the breakpoints: *OPRM*, *RNGTT*	Healthy

(Continued)

TABLE 2 | Continued

References	Chromosome regions involved in chromothripsis	Chromothripsis detection method	Imbalance (size, copy number alterations)	Affected genes	Phenotype of a carrier(s)
	15q15.1 6p21.3-p25.1 6q14.2 6q21-q22.31 7q32.3	Conventional cytogenetics, FISH, SNP-CGH array, paired-end sequencing	Deletion: 6 kb	Disrupted genes by the breakpoints: *CASC5*, *RPF2*, *CHCHD3*, *CLVS2*	Healthy
	15q15.1 6p21.3-p25.1 6q14.2 6q21-q22.31 7q32.3	Conventional cytogenetics, FISH, SNP-CGH array, paired-end sequencing	Four deletions up to 100 bp 6-bp microduplication	Disrupted genes by the breakpoints: *CASC5*, *RPF2*, *CHCHD3*, *CLVS2*. One parental breakpoint junction is absent	Developmental and speech delay, dysmorphic features

aCGH, array comparative genomic hybridisation; FISH, fluorescent in situ hybridisation; HR-CGH, high resolution comparative genomic hybridisation; MLPA, multiplex ligation-dependent probe amplification; NGS, next generation sequencing; SNP array, single nucleotide polymorphism array; WGS, whole genome sequencing; WES, whole exome sequencing.

TABLE 3 | Chromothripsis inheritance.

References	Maternal inheritance, cases	Paternal inheritance, cases	*De novo* chromothripsis, cases
Kloosterman et al., 2011	–	–	1 (pat)
Kloosterman et al., 2012	1	–	7 (4/7 – pat; 3/7 – n/d)
Gu et al., 2013	1	–	–
Nazaryan et al., 2014	–	–	1
Fontana et al., 2014	–	–	1
Wang et al., 2015	–	–	1
Genesio et al., 2015	–	–	1
de Pagter et al., 2015	3	–	–
Gamba et al., 2015	–	–	1
Weckselblatt et al., 2015	1	1	1 (pat)
Del Rey et al., 2016	–	–	1
Anderson et al., 2016	–	–	1
Bertelsen et al., 2016	3	1	–
Collins et al., 2017	–	–	2
Kurtas et al., 2018	–	–	1
Kurtas et al., 2019	2	1	–
Total:	10	3	19

De novo chromothripsis cases include information on the parental origin of the rearranged chromosomes (if known). pat, paternal chromosomes; n/d, no data about derivative chromosome origin.

by the epigenetically mediated bystander effect in non-irradiated whole testis tissue (Tamminga et al., 2008). This phenomenon is also involved in the production of delayed DNA damage in mouse elongated spermatids due to upregulation of proapoptotic genes 21–33 days later after spermatogonia exposure to X-rays (Cordelli et al., 2012). However, the apoptotic elimination of spermatogenic cells with DNA damage may be aborted before completion (the so-called abortive apoptosis or anoikis), allowing such cells to continue to differentiate and participate in fertilisation (Tubio and Estivill, 2011; Tang et al., 2012). In addition, there are some evidence of aberrant DNA methylation and tissue-specific accumulation of chromosome aberrations in unexposed progeny of cranially irradiated rodents (Koturbash et al., 2006; Tamminga et al., 2008). These data indicate an epigenetic link between DNA damaging agents and occurrence of chromosome aberrations both in unexposed parental germline and offspring's somatic cells.

In contrast to male germ cells, oocytes may repair breaks through both homologous recombination and NHEJ (Marchetti et al., 2007). Consequently, chromothripsis during oogenesis appears to be less likely than during spermatogenesis. Aberrations in chromosome segregation and premature chromatid separation may cause chromosomal rearrangements during female gametogenesis (Pellestor and Gatinois, 2018). In addition, the DNA repair capacity of an oocyte is the crucial factor of zygote viability, because the repair of maternal and paternal chromosome damage after fertilisation and prior to embryo genome activation occurs through DNA repair factors accumulated in the oocyte cytoplasm.

De novo CC may also be induced by DNA damage during early embryogenesis. Preimplantation embryos typically demonstrate micronuclei formation, blastomere fragmentation, and abnormal mitosis at the cleavage stage (Chavez et al., 2012). This could be a consequence of imperfect repair in germ cells or DNA damage in embryo. In addition, asynchronous pronuclear development and resulting under-replication of the paternal DNA may induce chromosome pulverisation in a zygote (Eichenlaub-Ritter et al., 1995).

Importantly, CCRs are hardly ever detected during conventional karyotyping of chorion cells in a miscarriage, which is conducted starting from 4 to 5 weeks of gestation (i.e., after embryo implantation) (Pendina et al., 2014; Massalska et al., 2017; Soler et al., 2017; Pylyp et al., 2018). As of today, the literature describes only one case of CC in an embryo with multiple malformations (Macera et al., 2015). Apparently, most embryos with CCRs, including chromothripsis, are eliminated at the implantation stage. Despite the wide use of preimplantation genetic testing, the actual frequency and the specific mechanisms of chromothripsis occurrence in gametes and embryos at early stages of development are yet to be determined.

Constitutional chromothripsis is generally characterised by fewer chromosome breaks and almost complete absence of deletions in comparison with malignant tumours (**Figure 1**; Kloosterman and Cuppen, 2013). A number of studies treat CCR cases with duplications of chromosome regions as chromothripsis (**Table 2**; Gamba et al., 2015; Wang et al., 2015; Del Rey et al., 2016; Kurtas et al., 2018). It is yet to be established, however, whether such genetic abnormalities in patients are cases of true chromothripsis or variations of other CCRs.

CONCLUDING REMARKS

As is the case with any recently discovered phenomenon, the concept of chromothripsis is ambiguous. In our opinion, the most comprehensive definition of chromothripsis has been suggested by Ly and Cleveland: "Chromothripsis is a catastrophic event in which one or a few chromosomes are shattered and stitched back together in random order, producing a derivative chromosome with complex rearrangements within a few cell cycles" (Ly and Cleveland, 2017). Considering that chromothripsis is a highly complex genomic aberration, its reliable detection necessitates the use of a comprehensive approach, combining molecular genetic, molecular cytogenetic, and cytogenetic methods.

Chromothripsis was first detected in chronic lymphocytic leukaemia. To date, it is most frequently found in cancers, even though there are registered cases of chromothripsis both in benign tumours and as constitutional chromosomal abnormality. Both somatic and CC feature multiple rearrangements of one or more chromosomes with a random order and orientation of reassembled fragments, as well as alteration of regions with loss and retention of heterozygosity. However, these aberrations are less pronounced in CC, which normally has fewer breaks and shorter chromosome regions with copy number alterations or a complete absence of such.

The causes and mechanisms underlying chromothripsis remain a subject for discussion. The most probable are telomere damage, exposure to ionising radiation, and viral infections. Along with these already known causes and mechanisms, we suggest aberrant epigenetic regulation as a possible pathway to chromothripsis. The above-mentioned factors may directly destruct chromosomes or activate cell mechanisms associated with chromothripsis. To clearly understand chromothripsis mechanisms, it is necessary to develop models of chromosome pulverisation in micronuclei, reversible apoptosis, and dicentric chromosome breaks.

As of today, it is not clear whether somatic chromothripsis is the cause of tumours or a consequence of pathological processes in tumour cells. Considering that cases of chromothripsis are observed in both malignant and benign tumours, as well as in the karyotype of healthy individuals, it cannot be unambiguously associated with poor clinical outcomes. Apparently, what matters most for neoplasia pathogenesis and a chromothripsis carrier's phenotype are the genome regions involved in the rearrangement, their localisation, and the size of deleted or amplified fragments – not the presence of chromothripsis itself.

Regardless of the fact that chromothripsis was discovered over 7 years ago, we are still facing challenges in its differentiation from other multiple chromosomal rearrangements and in the understanding of its causes, mechanisms, and consequences – all of which requires further in-depth research.

AUTHOR CONTRIBUTIONS

AK, AP, OE, OC, TK, and VB contributed to the conception, writing, and checking of the manuscript for important intellectual content.

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REVIEW
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Methods for Enhancing Clustered Regularly Interspaced Short Palindromic Repeats/Cas9-Mediated Homology-Directed Repair Efficiency

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The evolution of organisms has provided a variety of mechanisms to maintain the integrity of its genome, but as damage occurs, DNA damage repair pathways are necessary to resolve errors. Among them, the DNA double-strand break repair pathway is highly conserved in eukaryotes, including mammals. Nonhomologous DNA end joining and homologous directed repair are two major DNA repair pathways that are synergistic or antagonistic. Clustered regularly interspaced short palindromic repeats genome editing techniques based on the nonhomologous DNA end joining repair pathway have been used to generate highly efficient insertions or deletions of variable-sized genes but are error-prone and inaccurate. By combining the homology-directed repair pathway with clustered regularly interspaced short palindromic repeats cleavage, more precise genome editing *via* insertion or deletion of the desired fragment can be performed. However, homologous directed repair is not efficient and needs further improvement. Here, we describe several ways to improve the efficiency of homologous directed repair by regulating the cell cycle, expressing key proteins involved in homologous recombination and selecting appropriate donor DNA.

Keywords: genome editing, clustered regularly interspaced short palindromic repeats, homologous-directed repair efficiency, double-strand break, nonhomologous end joining

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INTRODUCTION

Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated (Cas) Systems

Precise and efficient genomic modification is essential to biological processes, genetic engineering, and other various areas of study. In recent years, many techniques for mediating targeted genome editing have emerged throughout the world. The most important tools for genome editing are enzymes, including zinc finger nucleases, transcription activator-like effector nucleases, and engineered meganucleases (Richardson et al., 2016; Khadempar et al., 2018). A true revolution in genome editing occurred with the introduction of a programmable nuclease *via* the CRISPR-Cas system; Cas9 is one of the nucleases that plays a critical role during this process (Czarnek and Bereta, 2016). CRISPR-Cas technology can cleave specific DNA sequences

(Jinek et al., 2012); endonucleases cleave DNA fragments precisely and efficiently through fusion with transcriptional activators and inhibitors by targeting histone-modifying enzymes for epigenetic regulation as well as manipulation of chromatin topologies for gene regulation (Adli, 2018).

Ishino et al. first identified CRISPR in *Escherichia coli* in 1987. They considered CRISPR as a gene editor, a system used by bacteria to protect themselves against viruses (Czarnek and Bereta, 2016). Later, researchers found that it appeared to be a precise genetic tool that could be used to delete, add, activate, or inhibit target genes in other organisms including humans, mice, bacteria, and fruit flies (Cong et al., 2013). The CRISPR cluster is a family of specific DNA repeats that are widely found in the genomes of bacteria and archaea, consisting of a leader, multiple short, and highly conserved repeat regions and multiple spacers (Dahlman et al., 2015). The leader region is generally located upstream of the CRISPR cluster and is an AT-rich region with a length of 300–500 bp; this is considered to be a promoter sequence of the CRISPR cluster. The repeat sequence region has a length of 21–48 bp and contains a palindromic sequence which can form a hairpin structure. In addition, the repeat sequences are separated by a spacer of 26–72 bp that consists of captured extraneous DNA, which is related to immune memory. When DNA containing the foreign sequence is encountered, it can be recognized by the bacteria and cut to inactivate the sequence in order to protect itself (Czarnek and Bereta, 2016). By analyzing the flanking sequence of the CRISPR cluster, it was found that there is a polymorphic family gene in its vicinity. The proteins encoded by this family contain functional domains (having nuclease, helicase, integrase, and polymerase activities) that interact with nucleic acids and work together with the CRISPR region; they are named CRISPR-associated (Cas) genes. Cas genes have been discovered, including Cas9. The Cas gene and CRISPR cluster have evolved together to form a highly conserved system, known as the CRISPR-Cas system (Salsman et al., 2017).

Subsequent studies showed that CRISPR and Cas9 endonuclease forms a complex, the gene encoding the Cas9 protein is located near the CRISPR locus, and that Cas9 creates a gap in the target DNA or RNA sequences. In addition, their genomes are protected from attack from phage nucleic acids and integrating plasmids by the CRISPR-Cas9 systems. In fact, CRISPR-Cas9 coordinates with the immune system and targets a wide range of invading proteins and nucleic acids such as RNA and DNA (Hale et al., 2009). Cas nucleases break down the invasive foreign DNA, part of which is placed in the CRISPR site between two repeated sequences (referred to as a spacer). The sequences of the spacer are further used as templates to produce short CRISPR RNAs (crRNAs; Jinek et al., 2012). These two sequences appear to act as a guide sequence to promote the binding of the Cas9 protein to the foreign DNA. Upon their successful binding, Cas9 protein cleaves invading DNA strands complementary to the crRNA sequence and its opposite sequence through the nuclease domains of HNH and RuvC, respectively (Jiang et al., 2016).

This system of genome editing can be used to select certain genetic products that have therapeutic potential. However, the editing of the specific sequences depends on the type of repair strategy being used by a cell, such as nonhomologous end joining (NHEJ) or homologous directed repair (HDR), as presented below in detail and summarized in **Figure 1**. The advantage of CRISPR technology is that it is very accurate,

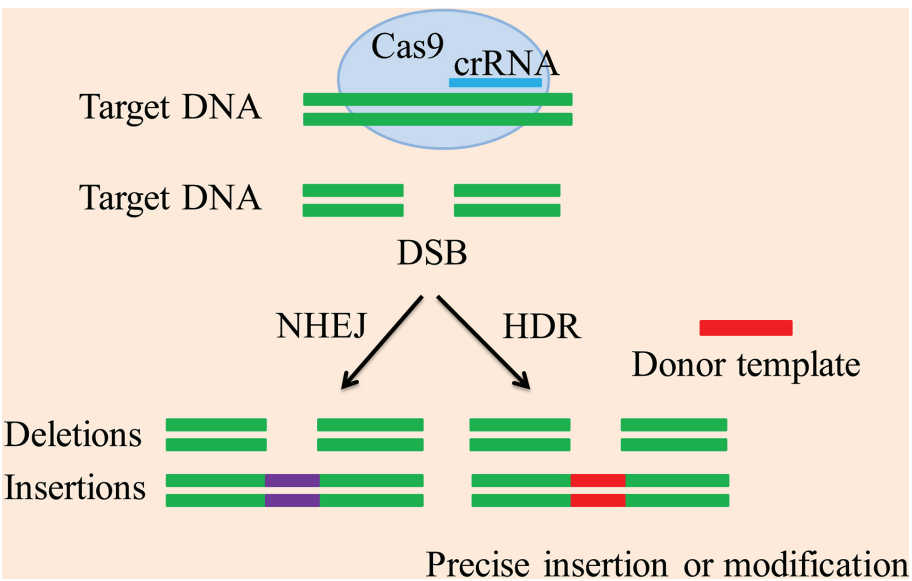

FIGURE 1 | CRISPR/Cas9-mediated DSB repair mechanism. The CRISPR-associated enzyme Cas9 breaks down the target DNA to create a DSB, the two repeated sequences are further used as templates to produce short crRNAs. Methods for DSB repair include the NHEJ and HDR pathway. The NHEJ pathway creates accurate deletions and insertions. The HDR pathway uses homologous donor DNA sequences from sister chromatids or foreign DNA to create accurate insertions, base substitutions between DSB sites or two DSBs, and other modifications.

but the Cas9 protein sometimes removes sequences that are similar (not including the target sequence and off-target sequences). More precise control is required and is an area of further study (Hussain et al., 2018).

Nonhomologous End Joining

The CRISPR-associated enzyme Cas9 achieves site-specific genomic engineering by introducing a double-strand break (DSB) at the chromosomal site specified by the guide RNA (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). Cells repair the DSB using the NHEJ or HDR pathway. NHEJ is a major form of mammalian DNA repair machinery that successfully joins broken DNA together (Mao et al., 2008). The low fidelity of NHEJ, which is prone to errors, may result in a base deletion or insertion (indel) after repair, resulting in a frameshift mutation (Bernheim et al., 2017). Ultimately, the goal of gene knockout is achieved. Gene knockout model animals can be prepared by using a targeted nuclease to efficiently cause frameshift mutations at the fertilized egg level. The emergence of CRISPR/Cas9 technology makes it possible to prepare gene knockout model organisms without using the embryonic stem cell (ESC) line of the corresponding species and has been successfully applied to mice, rats, fruit flies, and the like (Gonzalez, 2016; Arnoult et al., 2017).

The NHEJ pathway is further divided into two pathways: classical and alternative NHEJ pathways. However, since NHEJ is error-prone, in many settings, the end product of this pathway usually contains added or missing DNA sequences which may result in a nonfunctional coding sequence (Hug et al., 2016).

NHEJ is the predominant DSB repair pathway and is responsible for most DSB repairs throughout the cell cycle (Arnoult et al., 2017). NHEJ is dependent on Ku to thread onto DNA termini and thus enhancing the affinity of NHEJ enzymatic components which contain a nuclease, a ligase, and two polymerases (Mateos-Gomez et al., 2017). Intriguingly, each of these enzymatic components is unique for its capability in working on a broad range of incompatible DNA ends coupled with flexibility in loading order, leading to several possible junctional consequences from one DSB. The DNA end configurations can be directly ligated. However, if these ends are incompatible, they may be processed until configurations that are ligatable are achieved that are usually stabilized by no more than 4 bp of terminal micro-homology. DNA ends processing causes the addition or loss of nucleotides, accounting for the fact that original DNA sequences can rarely be restored after NHEJ repair of DSBs. Collectively, NHEJ is a DSB repair pathway with various enzymes and can result in multiple repair outcomes (Pannunzio et al., 2018).

Homology-Directed Repair

The second DSB repair pathway is HDR. This mechanism has high fidelity but low incidence. An exogenous repair template is utilized to direct cleavage of the DNA by the targeting nuclease. This can increase the probability of homologous recombination (HR) by about 1,000-fold. Notably, HDR can be used to accurately edit the genome in various techniques, including conditional gene knockout, gene knock-in, gene replacement, and point mutations (Arnoult et al., 2017). The HDR pathway uses homologous donor DNA sequences from sister chromatids or foreign DNA to create accurate insertions, base substitutions between DSB sites or two DSBs, and other modifications. This kind of precise modification is significant to genomic engineering in order to achieve the desired effect (Lin et al., 2014). Sequences of sister chromatids or homologous chromosomes form the basis of HDR. Sister chromatids are only available in the S and G2 phase; thus, HDR is limited to these phases of the cell cycle (Branzei and Foiani, 2008).

Much research has been done on proteins involved in the HDR pathway. Ataxia telangiectasia mutated (ATM) phosphorylates H2A histone family member X (H2AX), then DNA damage checkpoint protein 1 (MDC1) binds to this making γH2AX a site of accumulation at the area of DNA damage (Marechal and Zou, 2013). The MRN complex is then localized to the DSB, which exerts a stabilizing effect and inhibits chromosome breaks. After the initial stabilization of the DSB, the 5′ exonuclease activity of C-terminal-binding protein-interacting protein (CtIP) or exonuclease 1-Bloom helicase (Exo1-BLM) creates 3′ single-stranded (3′SS) overhangs, and human replication protein A (RPA) binds to these 3′SS overhangs (Symington, 2014). Rad51 works in conjunction with breast cancer 1 and 2 proteins (BRCA1 and BRCA2) and BRCA2 molecular chaperones (Salsman et al., 2017) to replace RPA and forms filaments on the DNA. The reconstitution process is initiated by looking for repair templates or sister chromatids through the 3′ overhang of Rad51 (Buisson et al., 2014). With the aid of proliferating cell nuclear antigen (PCNA), to synthesize the deleted DNA fragment. After the formation of the new DNA fragment, a Holliday junction is also formed, after which ligation is completed and the original DNA sequence is restored, as shown in **Figure 2** (Jasin and Rothstein, 2013).

The mechanism for repairing a DSB is not random; election of any repair mechanism will affect the results of genome editing. HDR is an uncommon form of DSB repair compared to NHEJ, but proper use of this repair mechanism for targeted genome editing can have a significant impact (Rothkamm et al., 2003). However, the availability of the HDR pathway is limited in undivided cells, which includes most cells *in vivo*. Therefore HDR-mediated genome editing methods are limited to *in vivo* applications (Nami et al., 2018).

METHODS FOR ENHANCING CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS/CAS9-MEDIATED HOMOLOGOUS-DIRECTED REPAIR EFFICIENCY

Cell Cycle-Determining Pathway Components Determine Homologous-Directed Repair Selection and Efficiency

The key factor in selecting the repair pathway for the DSB is the phase of the cell cycle. Cells utilize the NHEJ method to repair DSBs occurring in G1, S, and G2 phases, while the

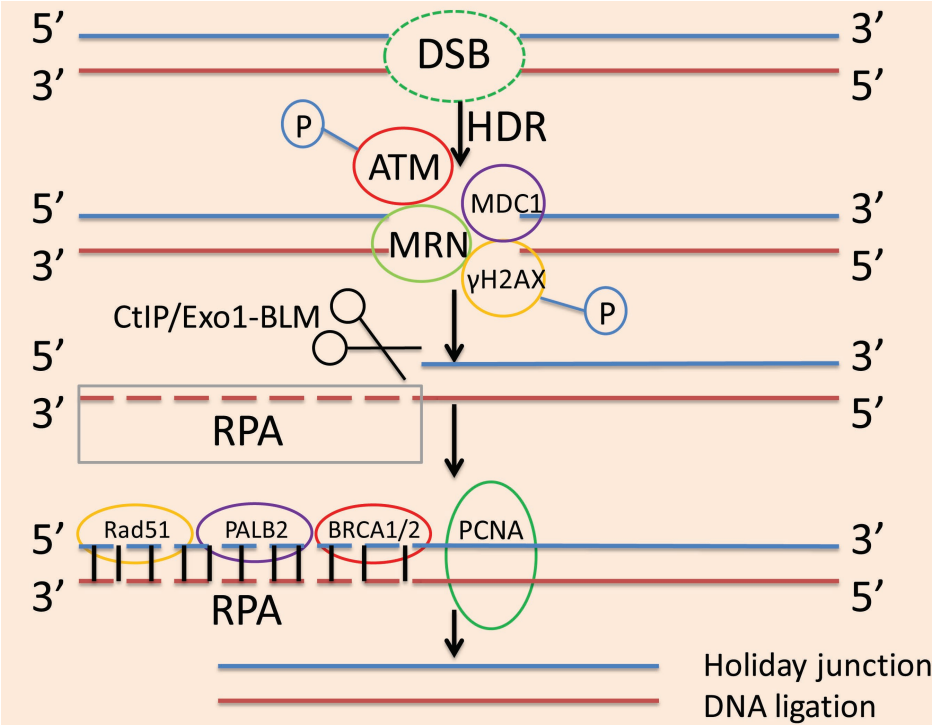

FIGURE 2 | The process of the HDR pathway. In the HDR pathway, ATM phosphorylates H2AX, then MDC1 binds to this making γH2AX a site of accumulation at the area of DNA damage. The MRN complex localizes to the DSB, which exerts a stabilizing effect and inhibits chromosome breaks. After the initial stabilization of DSB, the 5′ exonuclease activity of CtIP or Exo1-BLM creates 3′SS overhangs, and RPA binds to these 3′SS overhangs. Rad51 works in conjunction with BRCA1 and BRCA2 as well as PALB2 to replace RPA and form filaments on the DNA. With the aid of PCNA, to synthesize the deleted DNA fragment. After the formation of the new DNA fragment, a Holliday junction is also formed, after which ligation is used to complete the reconstruction and restore the original DNA sequence.

HDR method is only available during the S and G2 phases (Sartori et al., 2007), with sister chromatids used as repair templates (Heyer et al., 2010; Gonzalez, 2016). Because these two repair strategies compete with each other, theoretically, suppressing NHEJ will improve the incidence of HDR (Arnoult et al., 2017). Based on this hypothesis, researchers blocked NHEJ either by chemical substances and small interfering RNA (siRNA) key proteins or knocked out key NHEJ effectors with siRNA and short hairpin RNA (shRNA) to inhibit the NHEJ pathway and increase the likelihood of the HDR pathway (Chu et al., 2015; Li et al., 2017, 2018a). In 2012, Srivastava and colleagues identified a small-molecule inhibitor of NHEJ, 5,6-bis-((E)-benzylideneamino)-2-mercaptopyrimidin-4-ol (SCR7) (Srivastava et al., 2012). Mechanistically, SCR7 blocks the NHEJ pathway by binding to DNA ligase IV, a key enzyme of the NHEJ pathway, in a concentration-dependent manner (Vartak and Raghavan, 2015). Specifically, it works by reducing the affinity of DNA ligase IV for DSBs (Gerlach et al., 2018). SCR7 binds to the DNA-binding domain of DNA ligase IV, thereby preventing DNA ligase IV from binding to the DNA ends, resulting in the elimination of the NHEJ pathway (Vartak and Raghavan, 2015). However, for other NHEJ proteins such as KU70/KU80, DNA-PKcs, and artemis, no suitable inhibitors have been found. Interestingly, experiments have shown that the addition of SCR7 does not improve HDR efficiency during genome editing. In contrast, coordinated expression of Cas9 in the HDR-dominant cell cycle is more efficient in inducing HDR than inhibition of NHEJ (Gerlach et al., 2018). Nonetheless, existing studies have shown that these manipulations may be difficult to perform, or the process of manipulation may cause greater damage to cells. This was evidenced by a study where the site cut by CRISPR-Cas9 was destroyed when using HDR to repair the DSB and could no longer be cut by CRISPR-Cas9 (Wang et al., 2015).

In turn, the cell cycle-determining pathway also affects the efficiency of HDR. A study found that Cas9-directed RNA ribonucleosides synergistically bind the cell cycle proteins to the pre-assembled Cas9 ribonucleoprotein (RNP) complex for direct nuclear transfection to improve the likelihood of HDR. In this approach, timed delivery of protein complexes can be controlled during the cell cycle phase of HDR (Jinek et al., 2013). This method can simultaneously transfect multiple Cas9 RNPs and donor DNAs with higher cell viability than DNA transfection (Kim et al., 2014; Zuris et al., 2015). These features enable powerful genome editing while reducing off-target effects. Importantly, this system maximizes the efficiency of HDR.

Nocodazole, an anti-tumor drug, acts to depolymerize microtubules, which are essential for cell mitosis; it can also interfere with the polymerization of microtubules and keep cells in the G2 or M phase of the cell cycle. Nocodazole treatment resulted in higher HDR selection when the Cas9 RNP dose was reduced (Lin et al., 2014). HDR is more prone

to be selected with nocodazole treatment. One possibility to explain the higher efficiency is that Cas9 RNP targets multiple cells after synchronization with nocodazole. Another possibility is that the nuclear membrane is destroyed and Cas9 RNP can easily obtain DNA, leading to higher HDR efficiency. The high HDR efficiency upon treatment with nocodazole has no off-target editing and provides important advances in the development of scar-free genetic modification (Lin et al., 2014).

According to the current research, HDR of the Cas9 system has been used to knock-in genetic material. For example, *CXCR4* can be knocked in and knocked out by electroporation of Cas9 RNPs (Schumann et al., 2015). In addition, the work of Tu et al. has demonstrated that CRISPR/Cas9 nickase genome editing can efficiently result in a deletion of the RB1 gene in human embryonic stem cells (Tu et al., 2018). However, HDR of the Cas9 system does not guarantee successful genetic knock-in all the time. For example, although Cas9 RNP can mediate successful knock-in of specific nucleotides to *CXCR4* and *PD-1* in primary T cells, this is accompanied by a relatively higher incidence of off-target effects, rendering the efficiency of such genetic knock-in less significant in T cells compared with other cells (Schumann et al., 2015). Therefore, a detailed evaluation of the off-target effects of Cas9 RNP is needed. In addition, further investigation to resolve these off-target effects to make the Cas9 system more efficient is also deserved.

Improving Homologous-Directed Repair Efficiency by Expressing Key Proteins of Homologous Recombination

Subsequent studies have found that further improvements in directing HDR selection may require regulation of related proteins or key factors in the HDR or NHEJ pathways (Humbert et al., 2012). These related proteins, referred to as key HDR factors, can switch DNA repair from NHEJ to HDR by stimulating these key HDR factors (Bozas et al., 2009). Moreover, it seems that HDR stimulation is a more effective way of precise knock-in than NHEJ inhibition.

Recombination Protein A (Rad) Family Members

When foreign DNA is integrated into the chromosome, members of the Rad family (Rad50, Rad51, Rad52, etc.) play an indispensable role (Shao et al., 2017). Rad52 is an important homologous recombinant protein, and its complex with Rad51 plays a key role in HDR, mainly involved in the regulation of foreign DNA in eukaryotes (Di Primio et al., 2005; Kalvala et al., 2010). Key steps in the process of HR include repair mediated by Rad51 and strand exchange. The current model assumes that the formation of Rad51 requires the interaction of Rad52 (Ma et al., 2018). In particular, researchers suggest that co-expression of Rad52 with CRISPR/Cas9 nucleases can significantly enhance the likelihood of HDR (Di Primio et al., 2005; Shao et al., 2017; Van Chu et al., 2018). As detected by genome editing assays, co-expression of these proteins increased the likelihood of HDR by approximately three-fold. Studies have shown that a Rad52-Cas9 fusion is a better choice for enhancing CRISPR/Cas9-mediated HDR and may be helpful for accurate genome editing studies. However, the Rad52-Cas9 fusion mediated by different donor templates showed different HDR enhancement efficiencies (Shao et al., 2017). In addition, RAD52 motif protein 1 (RDM1) is similar to RAD52; RDM1 can repair DSBs caused by DNA replication, prevent G2 or M cell cycle arrest, and improve HDR selection (Tong et al., 2018).

Fanconi Anemia Core Complex

Fanconi anemia (FA) is a recessive hereditary disease caused by a biallelic mutation in at least one of 22 genes (Palovcak et al., 2017). The FA core complex includes eight Fanconi anemia core complex (FANC) proteins that are members of the translesion synthesis polymerase family (Ceccaldi et al., 2016). Under many circumstances, DSBs can be repaired by HR proteins, including FANC proteins (Crossan et al., 2011; Stoepker et al., 2011). When there is no HR repair factor, a DSB can be joined by NHEJ repair (Palovcak et al., 2017).

FANC proteins are components of the FA core complex (Wang and Smogorzewska, 2015) with two biochemical activities: strand exchange (SE) and single-strand annealing (SSA). The published data suggest that the SE and SSA activities of FANC are closely associated and play a critical role in DSB repair, and cell-based DSB repair assays clearly demonstrate that FANC contributes to the DSB repairs (Benitez et al., 2018). In addition, the data also indicate that FANC plays a role in DSB repair by catalyzing SSA and/or SE (Leung et al., 2012; Benitez et al., 2018).

FANC itself has different affinities to DNA, with high affinity to single-stranded DNA (ssDNA) and relatively low affinity to double-stranded DNA (dsDNA) (Yuan et al., 2012). Through its high affinity to ssDNA, FANC takes two ssDNAs together to form a dsDNA. Once dsDNA is formed, the low affinity of FANC results in the release of dsDNA products from dsDNA, triggering subsequent catalytic processes (Benitez et al., 2018).

Tumor Suppressor *p53*

The tumor suppressor gene *p53* can cause the production of mutant protein, usually in the DNA-binding domain, and is one of the most common mutant genes in cancer. *p53* acts as a transcription factor to activate or inhibit the target gene (Haigis and Dove, 2003). It also performs downstream regulation processes such as apoptosis, DNA repair, and DNA recombination. *p53* plays a direct role in DNA repair, including HR regulation; it affects the extension of new DNA, thereby affecting HDR selection (Gottifredi and Wiesmuller, 2018). *In vivo*, p53 binds to the nuclear matrix and is a rate-limiting factor in repairing DNA structure (Wiesmuller et al., 1996). The tumor suppressor *p53* regulates DNA repair processes in almost all eukaryotes *via* transactivation-dependent and -independent pathways, but only the transactivation-independent function of *p53* is involved in HR regulation. Thus, *p53* can act as a "molecular node" located at the intersection of the upstream signal cascade and downstream DNA repair and recombination pathways (Sengupta and Harris, 2005).

Current research indicates that the wild-type (WT) p53 protein can link DSBs to form intact DNA (Tang et al., 1999),

as well as also exerting a role in inhibiting NHEJ (Akyuz et al., 2002). A study found that p53 interacts with HR-related proteins, including Rad51; p53 controls HR through direct interaction with Rad51 (Linke et al., 2003; Sengupta et al., 2003). The interaction between HR proteins (such as RAD51 and RAD54) and HR-DNA intermediates indicates that p53 acts directly in HR in the early and late stages of recombination (Sengupta and Harris, 2005). This direct effect of p53 can maintain the stability of the genome. In 1996, Mummembrauer et al. found that the core domain of p53 has intrinsic 3′-5′ exonuclease activity (Mummenbrauer et al., 1996), and according to the damage of the DSB, p53 can play a role in correcting the mismatch of nucleic acids and exchanging incomplete homologous sequences (Sengupta and Harris, 2005).

C-Terminal-Binding Protein-Interacting Protein

In order to improve the efficiency of genome editing *via* elevated HDR selection, researchers around the world have developed many different strategies to date. Research on the development of genome editing technology involving the CRISPR-Cas9 system usually includes DSBs introduced by endonucleases; these are then repaired by HDR or the like (Rouet et al., 1994).

CtIP is a key protein in the early stages of HR. The minimal N-terminal fragment of CtIP is called the HDR enhancer, which is used to stimulate HDR (Gutschner et al., 2016). Increased rates of HDR can be achieved by fusing Cas9 to the N-terminal domain of CtIP, allowing CtIP to enter the cleavage site and increase transgene integration *via* HDR. HDR stimulation with Cas9 results in a two-fold or more increase in the frequency of targeted transgene integration, facilitating HDR-accurate genome editing (Charpentier et al., 2018).

The Choice of Donor DNA Determines the Efficiency of Homologous-Directed Repair

Donor DNA is optional and can be either single-stranded or double-stranded. Studies have shown that the efficiency of HDR is determined by the donor DNA selected. If the donor DNA is double-stranded, after pairing with the invading genomic strand, it can begin to replicate by the action of the polymerase. If the donor DNA is single-stranded, the process of repairing the DSB is relatively easy (Song and Stieger, 2017). However, HDR is not highly efficient in all cells and is very inefficient in certain types of cells, such as induced pluripotent stem cells (iPSCs). To increase the HDR efficiency of these particular types of cells, cyclin D1 (CCND1) is involved in G1 to S conversion during the cell cycle, while nocodazole is a G2 to M phase synchronizer. The addition of these two components increased HDR efficiency by 30% in a study. In summary, the study found that the choice of DNA donor is closely related to HDR efficiency (Zhang et al., 2017).

Studies have also shown that if the donor DNA selected is single-stranded, the process of repairing a DSB will be relatively easy; however, the choice of the single-stranded donor will also have a significant impact on HDR efficiency. It has been proven that when Cas9-initiated HDR is used with a short single-stranded oligodeoxynucleotide pair, it can act on many genes. However, conditional null alleles are produced at the locus and are less efficient when applied on a large scale (Lanza et al., 2018). Conversely, long single-stranded oligodeoxynucleotides are matched for efficient high-throughput processes of large numbers of conditional alleles. Of course, no matter which single-stranded DNA is used as a donor, it is necessary to first screen for sequence errors in the HDR locus and randomly insert the donor sequence into the genome (Lanza et al., 2018). In addition, researchers also found that using overlapping single guide RNA (sgRNA) and single-stranded oligonucleotide-mediated HDR can improve HDR efficiency (Jang et al., 2018).

CONCLUSIONS AND PERSPECTIVES

In view of the CRISPR/Cas9-mediated genome editing strategy, the CRISPR/Cas9-NHEJ genome editing method is common, but CRISPR/Cas9-HDR is infrequent (Gerlach et al., 2018). The main reasons for this are the low efficiency of HDR and the poor availability of exogenous DNA as a repair template, which seriously affect HDR as an accurate method of genome editing (Li et al., 2018b). Current methods of enhancing HDR selection include using chemicals, inhibiting NHEJ, and regulating the cell cycle. However, these methods face many challenges (Ye et al., 2018).

HDR and NHEJ are different types of genome editing methods, but both are genome editing techniques for repairing DSBs. Previous research has shown that NHEJ is more error-prone when repairing DSBs, but in fact, recent studies have demonstrated that NHEJ repair is performed after Cas9 cuts the target position, and the process is repeated until an error occurs, which prevents Cas9-mediated DNA cleavage (Zaboikin et al., 2017). Therefore, errors that are prone to occur in NHEJ are not errors that occur at the outset. In fact, NHEJ is a key strategy for stabilizing the genome, which plays an important role in the repair of DSBs (Lu et al., 2018). Although HDR is more accurate, it has a specific cycle limit. When it is unavailable, NHEJ is still relied on to repair the DSB, but it is converse in the time period when the HDR method can be used, which implies it is the obvious advantage. Hence, in order to improve HDR selection, studies need to be done how to direct selection of HDR as the most effective way to repair the DSB in the future studies (Hu et al., 2018).

In addition to the commonly used CRISPR/Cas9-NHEJ and CRISPR/Cas9-HDR genome editing methods, there are also genome editing methods such as CRISPR/Cas12a-, CRISPR/Cas13-, and CasX-NHEJ/HDR. The CRISPR/Cas12a system may provide a means for inducing genomic alterations through HR, and complementation can convert repair from NHEJ to HR. Cas13 has been used to degrade mRNA and thus antagonize viral RNA replication (Schindele et al., 2018). In addition, CasX is a fundamentally distinct RNA-guided genome editing system that uses unique structures to generate staggered DSBs in DNA at sequences complementary to a 20-nucleotide

segment of its guide RNA, making it a third enzyme family that is functionally distinct from Cas9, Cas12a, and Cas13 (Liu et al., 2019).

Of course, we still need a relatively neutral and critical attitude toward these technologies. Although they are widely used in many scenarios, other genome editing technologies have also been used in certain circumstances. For example, researchers have found that homology-mediated end joining (HMEJ)-based methods produce higher knock-in efficiency in HEK293T cells and primary astrocytes. Also, this method achieved transgenic integration in monkey and mouse embryos, which is more effective than NHEJ and HR methods (Yao et al., 2017). Therefore, in future genome editing applications, in addition to considering the Cas9-mediated HDR system for knocking in genetic material, HMEJ-based strategies can also be considered for a variety of applications to generate animal models and targeted gene therapy.

AUTHOR CONTRIBUTIONS

X-DT, W-TM, and D-KC designed the structure of this review. X-DT, FG, and M-JL wrote the first version of the manuscript. Q-LF helped revise the manuscript. All authors have reviewed the final version of the manuscript.

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Comparative Cytogenetics and Neo-Y Formation in Small-Sized Fish Species of the Genus *Pyrrhulina* (Characiformes, Lebiasinidae)

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Although fishes have traditionally been the subject of comparative evolutionary studies, few reports have concentrated on the application of multipronged modern molecular cytogenetic techniques (such as comparative genomic hybridization = CGH and whole chromosome painting = WCP) to analyze deeper the karyotype evolution of specific groups, especially the historically neglected small-sized ones. Representatives of the family Lebiasinidae (Characiformes) are a notable example, where only a few cytogenetic investigations have been conducted thus far. Here, we aim to elucidate the evolutionary processes behind the karyotype differentiation of *Pyrrhulina* species on a finer-scale cytogenetic level. To achieve this, we applied C-banding, repetitive DNA mapping, CGH and WCP in *Pyrrhulina semifasciata* and *P. brevis*. Our results showed 2n = 42 in both sexes of *P. brevis*, while the difference in 2n between male and female in *P. semifasciata* (♂41/♀42) stands out due to the presence of a multiple X_1X_2Y sex chromosome system, until now undetected in this family. As a remarkable common feature, multiple 18S and 5S rDNA sites are present, with an occasional synteny or tandem-repeat amplification. Male-*vs*.-female CGH experiments in *P. semifasciata* highlighted the accumulation of male-enriched repetitive sequences in the pericentromeric region of the Y chromosome. Inter-specific CGH experiments evidenced a divergence between both species' genomes based on the presence of several species-specific signals, highlighting their inner genomic diversity. WCP with the *P. semifasciata*-derived Y (PSEMI-Y) probe painted not only the entire metacentric Y chromosome in males but also the X_1 and X_2 chromosomes in both male and female chromosomes of *P. semifasciata.* In the cross-species experiments, the PSEMI-Y probe painted four acrocentric chromosomes in both males and females of the other tested *Pyrrhulina* species. In summary, our results show that both intra- and interchromosomal rearrangements together with the dynamics of repetitive DNA significantly contributed to the karyotype divergence among *Pyrrhulina*

species, possibly promoted by specific populational and ecological traits and accompanied in one species by the origin of neo-sex chromosomes. The present results suggest how particular evolutionary scenarios found in fish species can help to clarify several issues related to genome organization and the karyotype evolution of vertebrates in general.

Keywords: fishes, molecular cytogenetics, sex chromosome, chromosomal painting, comparative genomic hybridization (CGH), karyotype evolution

INTRODUCTION

South American miniature freshwater fishes cover, by definition, the species that do not exceed 26 mm in the standard length, yet most of them reach the maturity with a length of 20 mm (Weitzman and Vari, 1988). Such a small size limited or hampered especially cytogenetic investigations in these fishes over years. It is also the case of the Lebiasinidae family (Characiformes), whose representatives generally range from 16 to 70 mm in length. They are mainly distributed in the isolated streams of Central America (Panama and Costa Rica) and in almost all South American countries, except for Chile (Weitzman and Weitzman, 2003). Lebiasinidae branches to two subfamilies: Lebiasininae and Pyrrhulininae, which comprise seven genera and 77 recognized species (Froese and Pauly, 2018). Lebiasininae is formed by three genera: *Lebiasina* (18 recognized species), *Piabucina* (nine species), and a monotypic *Derhamia* (Weitzman and Weitzman, 2003; Froese and Pauly, 2018). Pyrrhulininae is considerably more diverse group on the species level (Netto-Ferreira and Marinho, 2013), encompassing four genera: *Nannostomus* (20 species), *Pyrrhulina* (18 species), *Copella* (6 species), and *Copeina* (2 species) (Weitzman and Weitzman, 2003; Froese and Pauly, 2018). Fishes from this subfamily experienced gradual decrease in the body size during their evolution, resulting in many miniaturized taxa (Netto-Ferreira and Marinho, 2013).

Lebiasinidae was formerly considered to be phylogenetically related to Erythrinidae, Ctenoluciidae, and Hepsetidae, due to sharing of some particular morphological similarities (Buckup, 1998). Nonetheless, more recent robust molecular phylogenetic analyses indicated that Erythrinidae and Hepsetidae are in fact not closely related to Lebiasinidae; instead, the close relationship between Lebiasinidae and Ctenoluciidae was demonstrated (Arcila et al., 2017; Arcila et al., 2018). However, while providing significant advances to this issue, these relationships still require complementary studies for the comprehensive understanding of the evolutionary history among its evolutionary lineages. In this context, conventional and molecular cytogenetic studies have brought valuable contributions to clarify the evolutionary relationships among phylogenetically related fish lineages (reviewed in Cioffi et al., 2018). However, like many other Neotropical fish groups with many representatives of small to miniature body size, the Lebiasinidae family was subject of only limited cytogenetic effort conducted thus far. The very small size of its species, especially the Pyrrhulininae ones, pose a significant challenge as it is notoriously difficult to obtain satisfactory chromosomal preparations, and therefore, most of the available data are limited only to the description of the haploid and/or diploid chromosome numbers (n/2n) in some species, with particularly 2n ranging from 22 in *N. unifasciatus* to 46 in *N. trifasciatus* (Scheel, 1973; Oliveira et al., 1991; Arai, 2011). However, a recent study employing the combined conventional and molecular cytogenetic approach in the two *Pyrrhulina* species (*P. australis* and *Pyrrhulina* aff. *australis*) has been conducted. Despite the fact that both species have been found to share the same 2n (40), without any karyotype differentiation between the sexes, interspecific CGH experiments were convincing enough to demonstrate some degree of genomic divergence, as inferred from a range of non-overlapping species-specific signals (Moraes et al., 2017).

In recent years, modern molecular cytogenetic techniques including whole chromosome painting (WCP) and comparative genomic hybridization (CGH) have been effective in broadening our understanding of the genome evolution and organization in fishes, allowing us to gain more detailed insights into a number of evolutionary issues. Specifically, both techniques have been used for the investigation of genomic divergence among related species (Nagamachi et al., 2010; Symonová et al., 2013; Moraes et al., 2017; Sember et al., 2018b) and to track the origin and evolution of sex chromosomes (Phillips et al., 2001; Henning et al., 2011; Cioffi et al., 2013; Freitas et al., 2018; Oliveira et al., 2018).

The present work aims to extend our understanding of the chromosomal evolutionary processes within the Lebiasinidae family, particularly by a deep investigation of the evolutionary relationships within the *Pyrrhulina* genus. For this, we applied C-banding, repetitive DNA mapping, CGH, and WCP in two species of *Pyrrhulina* – *P. semifasciata* and *P. brevis*. Our results strongly indicated the presence of a multiple X_1X_2Y sex chromosome system in *P. semifasciata*, which clearly emerged from a relatively recent centric fusion event with the signs of emerging male-specific region around the fusion point. In addition, the data obtained also highlight the high chromosomal dynamics within the investigated *Pyrrhulina* species, probably driven by the small population sizes and/or certain ecological properties of these small fishes.

MATERIALS AND METHODS

Animals

The number and sex of individuals investigated, collection sites, and the respective deposit numbers are presented in **Figure 1** and **Table 1**. The individuals were collected with the authorization of the Brazilian environmental agency ICMBIO/SISBIO (license no. 48628-2) and SISGEN (A96FF09). All species were properly identified by morphological criteria, and specimens

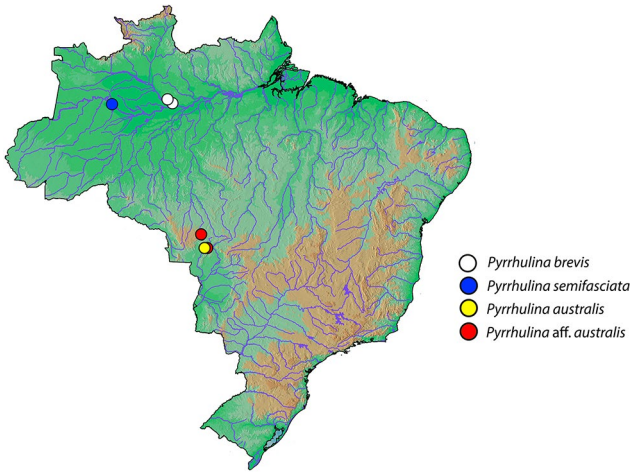

FIGURE 1 | Brazilian map showing the collection sites of *Pyrrhulina brevis* (white circle), *Pyrrhulina semifasciata* (blue circles), *Pyrrhulina australis* (yellow circle), and *Pyrrhulina* aff. *australis* (red circles). The last two species were cytogenetically investigated in Moraes et al. (2017).

TABLE 1 | Brazilian collection sites of the *Pyrrhulina* species analyzed, with the sample sizes (*N*).

Species	Collection site	N
Pyrrhulina australis	- Branco river (MT) – Paraguai river Basin	(30♀; 18 ♂)
Pyrrhulina aff. *australis*	- St. Antônio stream (MT) – Amazon river Basin	(22 ♀; 16 ♂)
Pyrrhulina aff. *australis*	- Branco river (MT) – Paraguai river Basin	(09 ♀; 20 ♂)
Pyrrhulina brevis	- Adolfo Ducke Rerserve- Igarapé from Barro Branco	(13 ♀; 17 ♂)
Pyrrhulina semifasciata	- Tefé River (AM) – Amazon river Basin	(07 ♀; 12 ♂)

were deposited in the fish collections of the Museu de Zoologia da Universidade de São Paulo (MZUSP) under the voucher numbers (119077, 119079, 123073, 123077, and 123080). The experiments followed ethical and anesthesia conducts and were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (process number CEUA 1853260315).

Chromosome Preparation and Analysis of Constitutive Heterochromatin

Mitotic chromosomes were obtained from kidney cells by the protocol described in Bertollo et al. (2015). Visualization of the amount and distribution of constitutive heterochromatin was done by C-banding according to Sumner (1972).

Preparation of FISH Probes Derived From Repetitive Sequences

The 5S rDNA probe included 120 base pairs (bp) of the 5S rDNA gene coding region and the 200 bp long non-transcribed spacer (NTS) (Pendás et al., 1994). The 18S rDNA probe corresponded to a 1,400-bp-long segment of the 18S rDNA coding region (Cioffi et al., 2009). The 18S and 5S rDNA probes were directly labeled with the Nick-Translation Mix (Roche, Mannheim, Germany) – 18S rDNA with Spectrum Green-dUTP and 5S rDNA with Spectrum Orange-dUTP (both Vysis, Downers Grove, USA), according to the manufacturer's instructions. $(CA)_{15}$ and $(GA)_{15}$ microsatellite probes were directly labeled with Cy3 during the synthesis according to Kubát et al. (2008).

Fluorescence *In Situ* Hybridization (FISH) for Repetitive DNA Mapping

Fluorescence *in situ* hybridization (FISH) was performed under high-stringency conditions as described in Yano et al. (2017a). Briefly, the chromosome preparations were incubated with RNase A (40 µg/ml) for 1.5 h at 37°C. After denaturation of the chromosomal DNA in 70% formamide/2x SSC at 70°C, slides were dehydrated in an ethanol series (70, 85 and 100%), 2 min each. 20 µl of the hybridization mixture (100 ng of each probe, 50% deionized formamide and 10% dextran sulfate) were dropped on the slides, and the hybridization was performed for 14 h at 37°C in a moist chamber containing 2x SSC (pH = 7.0). The post-hybridization wash was carried out with 1x SSC for 5 min at 42°C. Finally, the chromosomes were counterstained with DAPI (1.2 µg/ml) and mounted in antifade solution (Vector, Burlingame, CA, USA).

Preparation of Probes for Comparative Genomic Hybridization (CGH)

The gDNAs of males and females of *P. semifasciata* and *P. brevis* were extracted from liver tissue by a standard phenol-chloroform-isoamyl alcohol method (Sambrook and Russell, 2001). Two different experimental designs were used for this study. In the first set of experiments, we focused on intraspecific comparisons. In this case, male and female gDNAs of *P. semifasciata* and *P. brevis* were labeled and hybridized against the chromosomal background of males from *P. semifasciata* and *P. brevis,* respectively. Male gDNAs were labeled with digoxigenin-11-dUTP using DIG-Nick Translation Mix (Roche, Mannheim, Germany), while female gDNAs were labeled with biotin-16-dUTP using BIO-Nick Translation Mix (Roche, Mannheim, Germany). The final hybridization mixture for each slide contained 500 ng of each male- and female-derived labeled gDNA and 25 µg of unlabeled female-derived C_0t-1 DNA (to block the shared repetitive sequences; prepared according to Zwick et al., 1997), dissolved in 20 µl of the hybridization buffer (50% formamide, 2× SSC, 10% SDS, 10% dextran sulfate and Denhardt´s buffer, pH 7.0). In the second set of experiments, we focused on the interspecific genomic comparisons; hence, we co-hybridized 500 ng of male-derived gDNA of *P. semifasciata* (labeled with digoxigenin-11-dUTP) with 500 ng of male-derived gDNA of *P. brevis* (labeled with biotin-16-dUTP) on the chromosomal background of both species. In this case, the final probe cocktail for each slide contained also 15 µg of

female-derived C_0t-1 DNA from *P. semifasciata* and 15 μg of female-derived C_0t-1 DNA from *P. brevis*.

FISH Used for CGH

CGH experiments were performed according to Symonová et al. (2015). Briefly, the slides were aged for 1–2 h at 60°C, followed by a treatment with RNase A (200 μg/ml; 90 min at 37°C in a wet chamber) and with pepsin (50 μg/ml; 3 min at 37°C). Chromosomes were denatured in 75% formamide in 2xSSC at 74°C for 3 min, while the probes were denatured at 86°C for 6 min, chilled on ice (10 min) and then applied on the slides. Hybridization was done for 3 days in a humid chamber (37°C). Subsequently, non-specific hybridization was removed by a stringent washing at 44°C, twice in 50% formamide/2xSSC (10 min each) and three times in 1xSSC (7 min each), and then rinsed in 2xSSC at room temperature. The hybridization signals were detected with Anti-Digoxigenin-Rhodamin (Roche, Mannheim, Germany) diluted in 0.5% bovine serum albumin (BSA) in PBS, and avidin-FITC (Sigma, St. Louis, MO, USA) diluted in PBS containing 10% normal goat serum (NGS). Four final washes were performed at 44°C in 4xSSC/0.1% Tween, 7 min each. Finally, the chromosomes were counterstained with DAPI (1.2 μg/ml) and mounted in an antifade solution (Vector, Burlingame, CA, USA).

Chromosome Microdissection, Probe Preparation, and Labeling

Twenty copies of the Y chromosome from *P. semifasciata* (hereafter designated as PSEMI-Y) were manually microdissected using the glass needles, under an inverted microscope (Zeiss Axiovert 135). The chromosomes were amplified by degenerate oligonucleotide primed-PCR (DOP-PCR), following the protocol described in Yang et al. (2009). Then, 1 μl of the primary amplification product was used as a template DNA for a secondary labeling DOP-PCR with Spectrum Orange-dUTP (Vysis, Downers Grove, USA) in 30 cycles, following Yang and Graphodatsky (2009). The final probe mixture for one slide contained 500 ng of the PSEMI-Y probe and 30μg of C_0t-1 DNA isolated from *P. semifasciata* female genome.

FISH Used for Whole Chromosome Painting

Chromosomal preparations of males and females of *P. semifasciata, P. brevis,* and two other *Pyrrhulina* species (*P. australis* and *P.yrrhulina* aff. *australis*) were used for Zoo-FISH experiments with the PSEMI-Y probe. The hybridization procedures followed Yano et al. (2017a). Hybridization was performed for 48 h at 37°C in a moist chamber. The post-hybridization wash was carried out with 1xSSC for 5 min at 65°C, and in 4xSSC/Tween (RT), and the chromosomes were mounted with DAPI (1.2 μg/ml) in antifade as described above.

Microscopy and Image Processing

At least 30 metaphase spreads per individual were analyzed to confirm the 2n, karyotype structure and the FISH results. Images were captured using an Olympus BX50 epifluorescence microscope (Olympus Corporation, Ishikawa, Japan) with the CoolSNAP system software and the images were processed using Image Pro Plus 4.1 Software (Media Cybernetics, Silver Spring, MD, USA). Final images were optimized and arranged using Adobe Photoshop, version 7.0. Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a), according to their arm ratios (Levan et al., 1964).

RESULTS

Karyotype Analysis and Heterochromatin Distribution

The karyotype of *P. semifasciata* was composed of 2n = 41, 1m + 4st + 36a in males, and 2n = 42, 4st + 38a in females (**Figure 2**). However, all *P. brevis* individuals displayed 2n = 42 and the karyotype composed of 2sm + 4st + 36a, both in males and females (**Figure 3**). The distribution of constitutive heterochromatin was restricted to the centromeric and telomeric regions of several chromosomes in both species, but the intensity of C-bands was more pronounced in *P. semifasciata*. *P. brevis* further displayed conspicuous interstitial C-bands, which were absent in *P. semifasciata* (**Figures 2** and **3**).

Chromosomal Mapping of Repetitive DNA Markers

Dual-color FISH with 5S and 18S rDNA probes revealed that the investigated species differ notably by their patterns of distribution for both multigene families, yet they share a presence of multiple sites for both ribosomal clusters. In *P. semifasciata*, the 18S rDNA cistrons were found to cover short (*p*) arms of the largest (st) chromosome pair in the karyotype as well as the *p*-arms of three acrocentric pairs (nos. 3, 6, and 11), while 5S rDNA signals occupied the *p*-arms of five acrocentric pairs (nos. 4, 7, 8, 9, and 15), with yet another sixth acrocentric pair (no. 21) bearing an interstitial 5S cluster (**Figures 2** and **4**). In contrast to *P. semifasciata*, where none of the rDNA signals occured in synteny or even in the adjacent regions, two out of three pairs of 18S-bearing chromosomes in *P. brevis* (st pair no. 3 and a pairs nos. 11 and 14) bore also an adjacent 5S rDNA site on their *p*-arms (pairs nos. 3 and 14). At the same time, pair no. 3 exhibited a remarkable 18S rDNA site amplification accompanied by an extensive size heteromorphism between homologs (**Figures 3** and **4**). Besides chromosome pairs nos. 3 and 14, there were another three acrocentric pairs bearing 5S rDNA tandem repeats (pairs nos. 7, 8, and 10). Interestingly, pairs 7 and 10 encompassed double 5S rDNA sites—one occupying *p*-arms and the second being placed interstitially on the long (*q*) arms on both chromosome pairs (**Figures 3** and **4**).

The chromosomal mapping of the microsatellite motif $(CA)_{15}$ showed a prominent clustering in the telomeric sites of all chromosomes, especially on *q*-arms, while few distinct interstitial accumulations were also apparent, especially in *P. brevis*. On the other hand, $(GA)_{15}$ motif displayed more scattered distribution along the chromosome complement of both species, though a strong preference for telomeric regions can be also inferred for this motif (**Figure 5**).

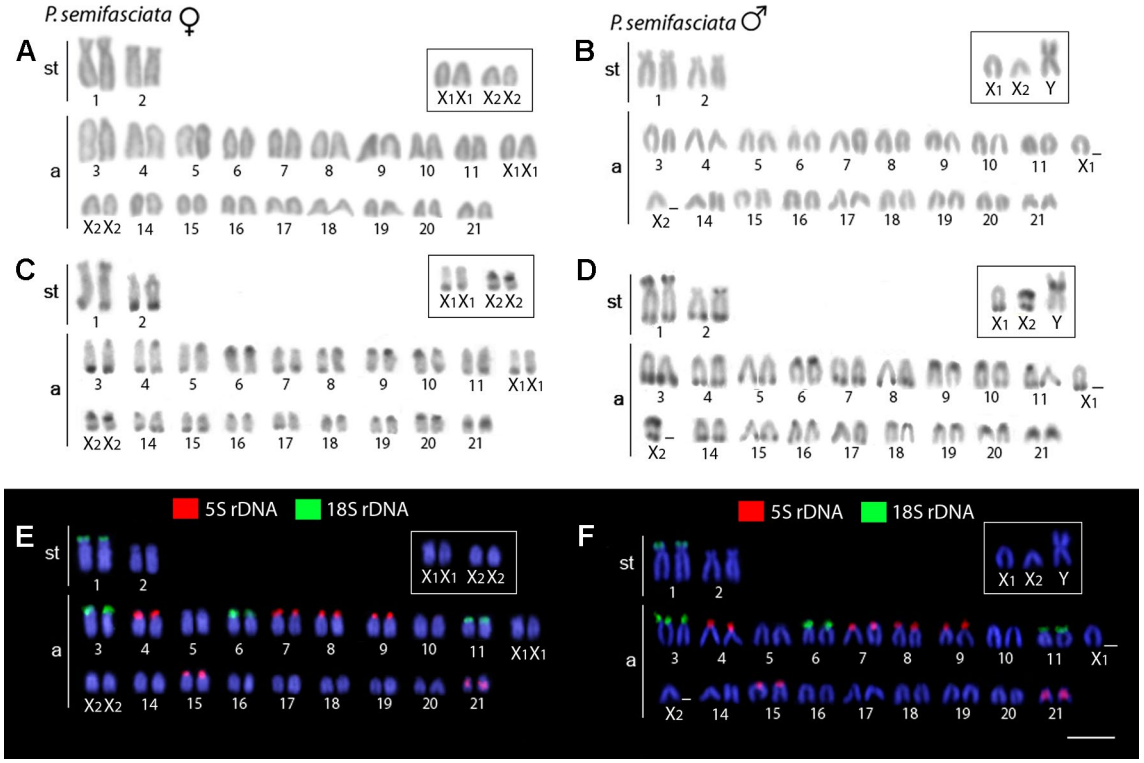

FIGURE 2 | Karyotypes of *Pyrrhulina semifasciata* (female and male) arranged from chromosomes after different cytogenetic procedures. Giemsa staining in female **(A)** and male **(B)**, C-banding in female **(C)** and male **(D)**, dual-color FISH with 18S (green) and 5S (red) rDNA probes in female **(E)** and male **(F)**. Chromosomes are counterstained with DAPI (blue). Bar = 5 μm.

Detection of the Male-Specific Region and Interspecific Genomic Divergence by CGH

The intraspecific genomic hybridization between males and females of *P. semifasciata* revealed a strong binding preference for the male-derived probe to the pericentromeric region of the neo-Y chromosome, while the female-derived probe produced only a weak hybridization signal in this segment (**Figures 6A–D**). The intraspecific genomic hybridization between males and females of *P. brevis* did not show clustering of sex-specific sequences on any chromosome (**Figures 6E–H**).

The interspecific CGH experiments performed to compare the genomes of *P. semifasciata* and *P. brevis* on the level of repetitive DNA divergence yielded a range of non-overlapping species-specific signals as a consequence of their specific evolutionary history. Preferential hybridization of the *P. brevis*-derived probe to the terminal regions of some chromosomes highly likely overlaps with the rDNA sites (**Figures 6I–L**).

WCP With a PSEMI-Y Probe

The WCP experiments with the PSEMI-Y probe prepared from the neo-Y chromosome of *P. semifasciata* (**Figure 7A**) entirely painted four chromosomes (named X_1 and X_2, two homologs of each) in females and three elements (named X_1, X_2, and neo-Y chromosome) in males of *P. semifasciata*, confirming the occurrence of a multiple X_1X_2Y sex chromosome system in this species (**Figures 7B, C**). In the cross-species experiments, the PSEMI-Y probe painted two independent chromosome pairs in both males and females of *P. brevis, P. australis,* and *P.* aff. *australis* (**Figures 7D–F**).

DISCUSSION

Karyotype and Repetitive DNA Patterns in the Genus *Pyrrhulina*

In many fish groups with taxa of the small-sized body, the lack of cytogenetic data impairs the knowledge about the chromosomal relationships and it prevents to make any meaningful inferences about the impact of chromosome dynamics on their evolutionary history (Liu et al., 2012). The present study brings new insights into the karyotype dynamics of two *Pyrrhulina* species (*P. brevis* and *P. semifasciata*) using conventional and molecular cytogenetic procedures. The karyotype analyses showed the predominance of acrocentric chromosomes in both species, thus documenting a common pattern found in all other studied species from the Lebiasinidae family (Oliveira et al., 1991; Arai, 2011; Moraes et al., 2017). In addition, the observed 2n (41 or 42) fits the conserved 2n found in *Pyrrhulina* species to date, as it ranges from 40 to 42 chromosomes (Oliveira et al., 1991; Arai, 2011;

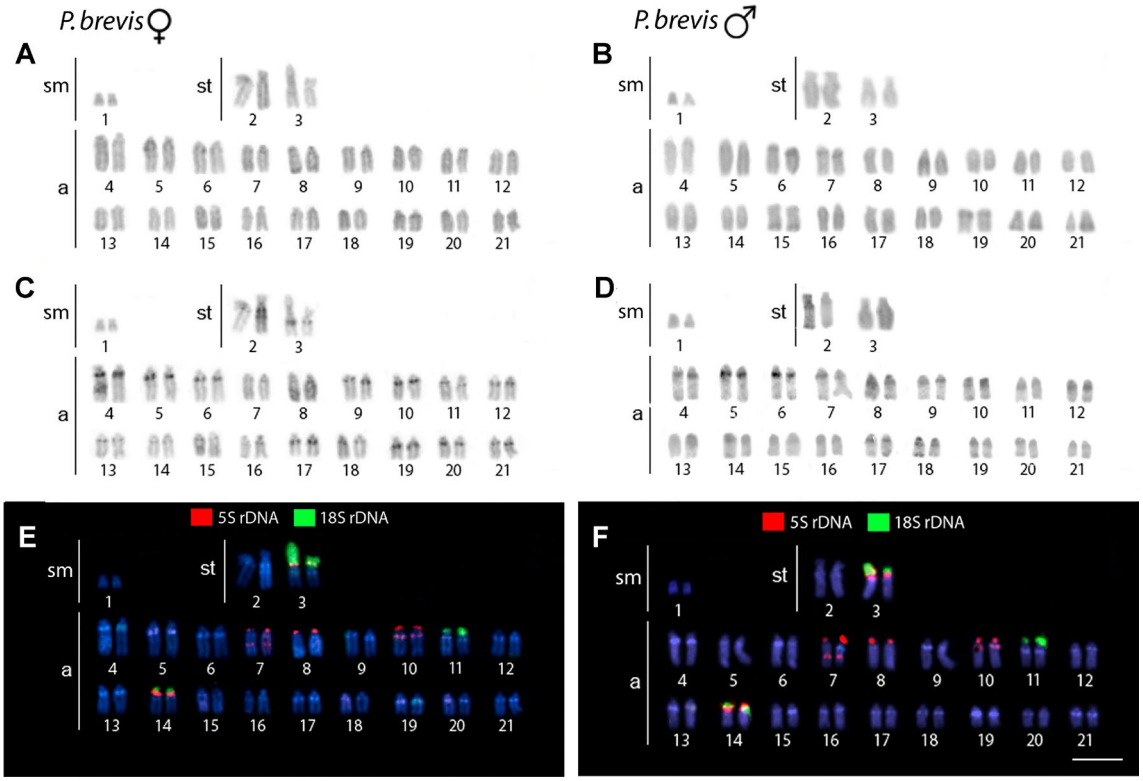

FIGURE 3 | Karyotypes of *Pyrrhulina brevis* (female and male) arranged from chromosomes after different cytogenetic protocols. Giemsa staining in female **(A)** and male **(B)**, C-banding in female **(C)** and male **(D)**, dual-color FISH with 18S (green) and 5S (red) rDNA probes in female **(E)** and male **(F)**. Chromosomes are counterstained with DAPI (blue). Bar = 5 μm.

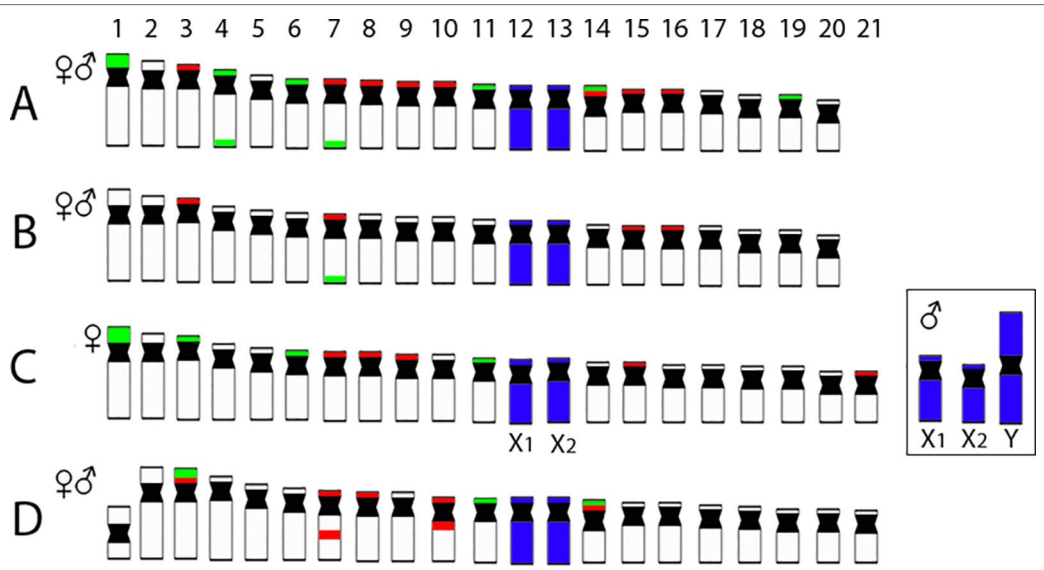

FIGURE 4 | Representative idiograms of *Pyrrhulina* species showing the distribution of 18S (green) and 5S rDNA (red) sites on the chromosomes of *P. australis* **(A)**, *Pyrrhulina* aff. *australis* **(B)** (based on our previous study; Moraes et al. (2017), and *P. semifasciata* **(C)** and *P. brevis* **(D)** (this study). Dark blue indicates the chromosomes painted with the PSEMI-Y probe. Bar = 5 μm.

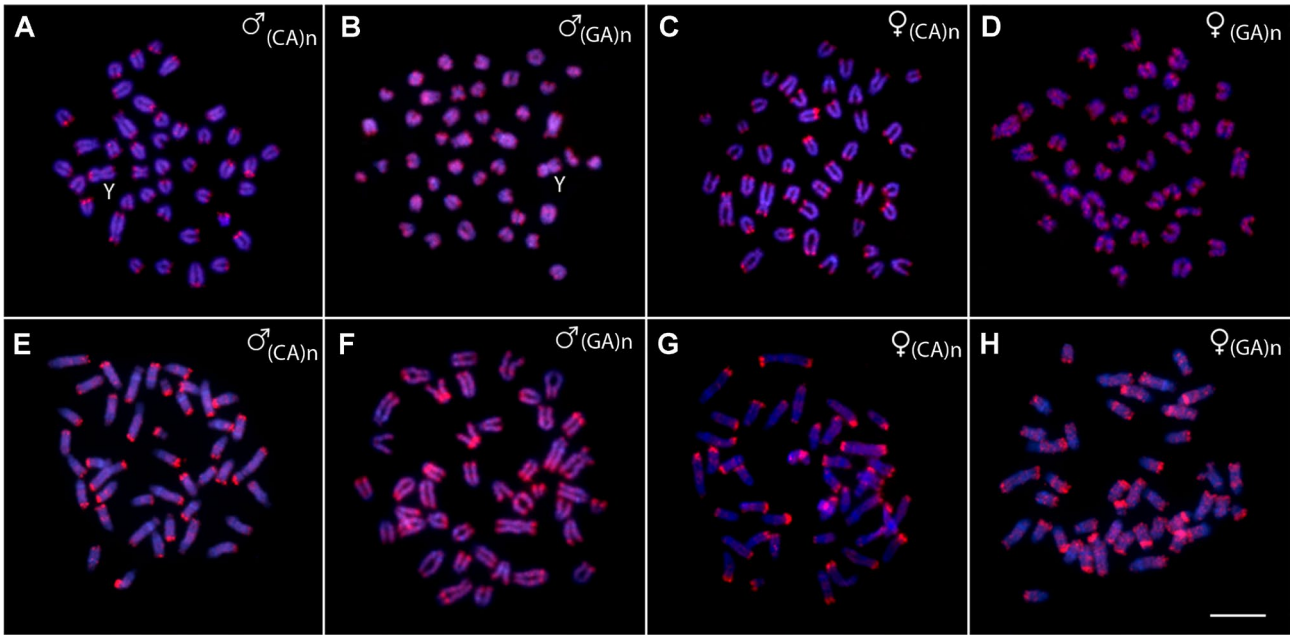

FIGURE 5 | Metaphases from males and females of *Pyrrhulina semifasciata* **(A-D)** and *Pyrrhulina brevis* **(E-H)** hybridized with the microsatellite probes $(CA)_{15}$ and $(GA)_{15}$, showing the general distribution pattern of these repetitive DNAs on the chromosomes. Bar = 5 μm.

Moraes et al., 2017). Nonetheless, the difference in 2n between male and female in *P. semifasciata* (♂41/♀42) stands out due to the presence of multiple X_1X_2Y sex chromosome system, until now unique for this genus. Based solely on the Giemsa-stained karyotypes, an apparent Robertsonian (Rb) translocation gave rise to the largest metacentric Y sex chromosome in the male karyotype. Karyotypes of both analyzed species are otherwise very similar, being composed of 4st + 38a in *P. semifasciata* females and 2sm + 4st + 36a in both sexes of *P. brevis*, as well as in other two *Pyrrhulina* species (*P. australis* and *P.* aff. *australis*), whose karyotypes were revised by us recently, both presenting 4st + 38a (Moraes et al., 2017). This scenario thus points to the involvement of structural chromosome rearrangements such as pericentric inversions in the differentiation of *Pyrrhulina* karyotypes.

Reciprocal interspecific CGH patterns encountered in *P. semifasciata* and *P. brevis* showed that a certain degree of the genome divergence is apparent between both genomes despite close evolutionary relationships between these congeners – on the level of repetitive DNA distribution, manifested by a presence of certain species-specific CGH signals. In addition, such divergent evolutionary features are also supported by the patterns of C-banding and repetitive DNA mapping, in which an advanced stage of sequence divergence is observed, except for the bright signal, corresponding to C-positive heterochromatic/NOR sites (**Figure 6**).

In fact, the presence of interstitial C-bands differentiates *P. brevis* from *P. semifasciata*, in addition to a pool of repetitive elements in such regions that are not shared between these two species, as evidenced by CGH experiments. On the contrary, interstitial C-bands represent a shared trait between *P. brevis* and *P.* aff. *australis* (Moraes et al., 2017) and their presence supports our view about the probable action of intrachromosomal rearrangements of the peri/pericentromeric inversion type in these genomes. Interestingly, a conspicuous polymorphic block of constitutive heterochromatin found previously on the chromosome pair no. 5 in both males and females of *P.* aff. *australis* is not present in the species analyzed herein.

Our hypothesis about the involvement of peri- or paracentric inversions in the karyotype differentiation of *P. semifasciata* and *P. brevis* is further strengthened by the patterns of rDNA distribution. More specifically, the presence of two 5S rDNA sites on the same specific chromosomes in *P. brevis* might indicate that a portion of an original 5S rDNA cluster might have been shifted by inversion to a different location, resulting in a secondary site, similarly to what has been proposed in other (not only) fish groups (Fernandes et al., 2017; Sember et al., 2018a). Nonetheless, bearing in mind that i) the region between doubled 5S rDNA sites encompasses the centromere (thus favorizing pericentric inversions as the underlying mechanism of rDNA mobility) and that, ii) both studied species exhibit very similar karyotypes, an alternative explanation operating with the spreading of 5S rDNA sites through (retro-) transposition is equally probable, especially when taking into account previously reported association of *Rex3* non-LTR retrotransposon with amplified 5S rDNA loci in *P. australis* and *P.* aff. *australis* (Moraes et al., 2017). Taken together, *Pyrrhulina* species deviate from the prevalent patterns of rDNA distribution in fish genomes, where the most of species often bear a single pair of 5S and/or 45S rDNA sites (Gornung, 2013; Sochorová et al., 2018). Nevertheless, multiple rDNA loci are not uncommon in fishes

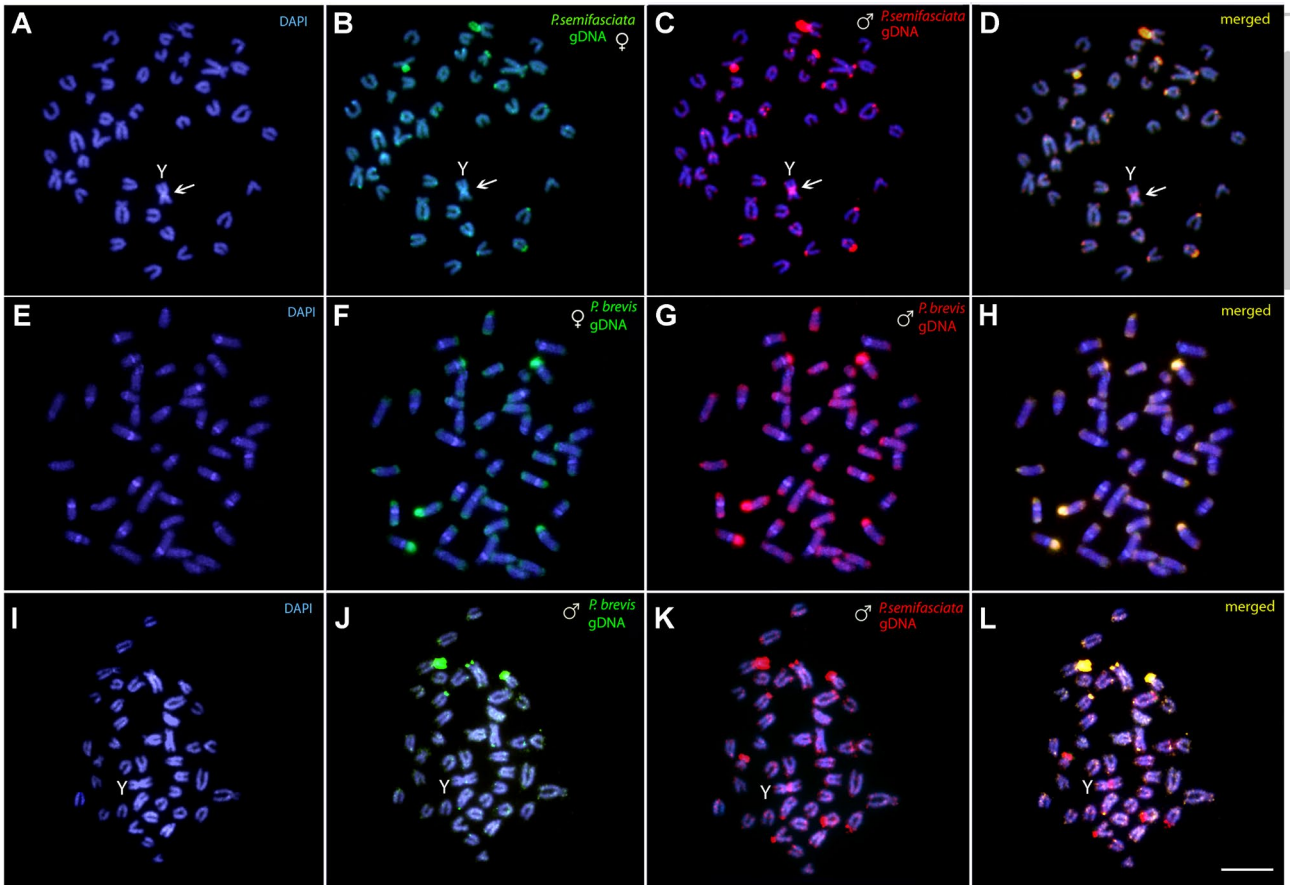

FIGURE 6 | Comparative genomic hybridization (CGH) for intra- and interspecific comparisons. **(A–D)** Male- and female-derived genomic probes from *P. semifasciata* mapped against the male chromosomes of *P. semifasciata*. **(E–H)** Male- and female-derived genomic probes from *P. brevis* mapped against the male chromosomes of *P. brevis*. **(I–L)** Male-derived genomic probes from both *P. semifasciata* and *P. brevis* hybridized together onto male chromosomes of *P. semifasciata.* The common genomic regions of both compared karyomorphs are depicted in yellow and the arrows indicate the male-specific region located on the Y chromosome of *P. semifasciata.* Bar = 5 μm.

and they might eventually point to elevated genome dynamics, possibly associated with an ongoing interspecific divergence or with the fast fixation due to genetic drift in small populations (Symonová et al., 2013; Sember et al., 2015; Symonová and Howell, 2018). Similar syntenic association of both rDNA classes, as revealed in *P. brevis*, is repeatedly emerging across the teleost phylogeny, being likely rather a by-product of sub-chromosomal dynamics, though bearing potentially some significance with respect to spatial gene co-expression in interphase nuclei, organized into active and inactive domains (Cavalli and Misteli, 2013; Fraser et al., 2015).

Microsatellites are also repetitive elements useful for analyzing the biodiversity and evolutionary processes among fishes (reviewed in Cioffi et al., 2012a). In fact, clustering of microsatellites might help to trace the level of sub-chromosomal dynamics (Basset et al., 2006) and it might also provide important insights into the processes of sex chromosome differentiation (e.g., Kubát et al., 2008; Pokorná et al., 2011; Kejnovský et al., 2013; Poltronieri et al., 2014). In this study, the $(CA)_{15}$ and $(GA)_{15}$ microsatellite motifs showed similar distributional patterns among *P. semifasciata* and *P. brevis* and this holds true also for other species of the *Pyrrhulina* genus already analyzed (Moraes et al., 2017). More specifically, in all four species, the accumulation of $(CA)_{15}$ motif appeared to be almost exclusively telomere-specific, while $(GA)_{15}$ showed rather a dispersed distribution throughout the analyzed chromosome complements, in addition to a higher affinity for telomeric regions. From this data, it might be inferred that microsatellite motifs utilized herein are not resolute for tracking the sub-chromosomal dynamics in *Pyrrhulina,* as their distribution seems to be largely conserved within the analyzed species. Furthermore, they do not show any significant sex chromosome-specific accumulations.

In summary, cytogenetic data accumulated for *Pyrrhulina* species (Moraes et al., 2017, this study) point on largely conserved karyotype macrostructure, yet evidencing extensive dynamics on the sub-chromosomal level, i.e., divergence in the accumulation of certain repetitive DNA classes and highly probable presence of genome-specific repeats. The sub-chromosomal dynamics might be likely facilitated by divergent

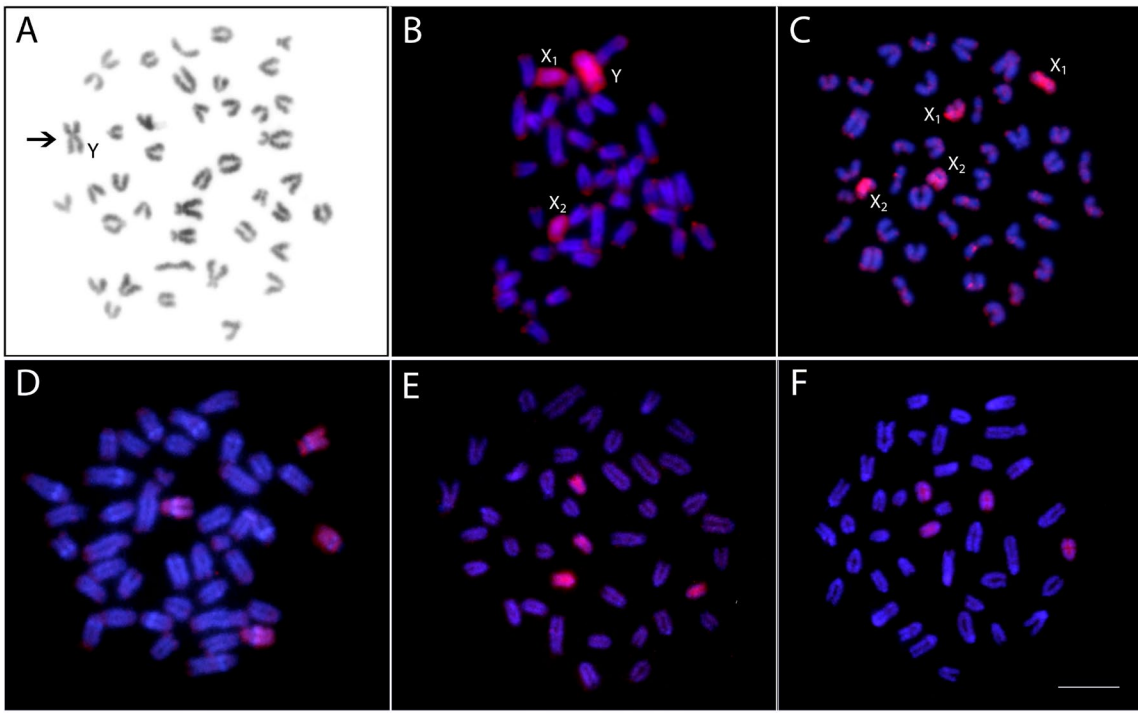

FIGURE 7 | Zoo-FISH with the *PSEMI-Y painting probe* derived from the Y chromosome (arrow) of *P. semifasciata* **(A)** hybridized on the metaphase plates of *P. semifasciata* male **(B)**, *P. semifasciata* female **(C)**, *P. brevis* male **(D)**, *P. australis* male **(E)**, and *Pyrrhulina* aff. *australis* male **(F)**. Bar = 5 μm.

evolutionary histories of *Pyrrhulina* species and by their common endemic status (Netto-Ferreira and Marinho, 2013).

Origin and Differentiation of the X_1X_2Y Sex Chromosome System in *Pyrrhulina semifasciata*

Although the different 2n present in males (41) and females (42) could also indicate the occurrence of an X0 sex system, our CGH and particularly WCP results confirmed the occurrence of a multiple X_1X_2Y sex chromosome system in *P. semifasciata*. Though fishes possess an amazing variety of sex determination and differentiation mechanisms (Devlin and Nagahama, 2002; Herpin and Schartl, 2015; Schartl et al., 2016; Guiguen et al., 2019), sex chromosomes have been described only in about 5% of cytogenetically analyzed species (based on Arai, 2011). It is, however, increasingly apparent that this information is skewed by frequent presence of homomorphic (i.e., cytogenetically unrecognizable) fish gonosomes, which goes hand in hand with their relative evolutionary "youth" and predisposition to frequent sex chromosome turnovers in closely related species or even within species (Kitano and Peichel, 2012; Pennell et al., 2015; Gamble, 2016; Schartl et al., 2016). In spite of that, at least eight sex chromosome systems (XY and ZW and their variations) are known to occur in certain fish species, fully represented also in Neotropical ichthyofauna (Cioffi et al., 2012b; Cioffi et al., 2017). Among them, X_1X_2Y gonosomes represent the most frequent multiple sex chromosome system (Kitano and Peichel, 2012; Pennell et al., 2015). In most of the fish taxa with X_1X_2Y sex chromosomes, centric or tandem fusions are hypothesized to be the underlying mechanism of their origin, giving rise to a large neo-Y chromosome. Examples of this scenario can be found in several fish species, such as *Harttia punctata* (Loricariidae) (Blanco et al., 2014), *Eigenmannia trilineata* (Sternopygidae) (Fernandes et al., 2010), *Achirus achirus* (Achiridae) (Bitencourt et al., 2016), *Erythrinus erythrinus* and *Hoplias malabaricus* (Erythrinidae) (Bertollo et al., 2004; Cioffi et al., 2013), and *Gymnotus pantanal* (Gymnotidae) (Margarido et al., 2007), among others. Based on available data, it seems likely that chromosome rearrangements are often the fully sufficient mechanism to establish the recombination arrest in different fish neo/multiple sex chromosomes, without need for additional repetitive DNA and heterochromatin accumulation (Almeida-Toledo and Foresti, 2001; Oliveira et al., 2008; Fernandes et al., 2010; Cioffi et al., 2011a; Soares et al., 2014; Cardoso et al., 2015; Sember et al., 2015; Bitencourt et al., 2016; Sember et al., 2018b). This scenario sharply contrasts with several examples in animal or plant kingdom, where massive repetitive DNA accumulations are observed on nascent neo-sex chromosomes (e.g., Mariotti et al., 2009; Bachtrog, 2013).

Bearing in mind its general principle, CGH method might represent a useful tool also for delimitation and gross molecular characterization of sex-specific regions on sex chromosomes and, in many cases, it was also sensitive enough to reveal morphologically homomorphic sex chromosomes (Traut et al., 1999; Symonová et al., 2015; Montiel et al., 2017; Yano

et al., 2017b; Freitas et al., 2018; Oliveira et al., 2018; Sember et al., 2018b; Zrzavá et al., 2018). In this study, CGH revealed a notable bias in the accumulation of male-specific or male-enriched repetitive DNA in the pericentromeric region of the Y chromosome, when compared to the intensity of female probe hybridization in the same region. We suppose that this pattern might reflect the incipient stage of differentiation inside the male-specific region, similarly to what has been supposed for karyomorphs C and F of the wolf fish *H. malabaricus* (Freitas et al., 2018; Oliveira et al., 2018; Sember et al., 2018b). It is of interest to note that the region in question encompasses the area around the fusion point on Y. As the recombination in the rearranged region might be significantly reduced or abolished due to sterical constraints, this region gradually accumulates sequence divergence (Faria and Navarro, 2010; Guerrero and Kirkpatrick, 2014). One of the consequences might be a selective advantage, especially if the rearrangement brings into close proximity two (or more) loci whose maintained linkage disequilibrium is favorable to contribute to local adaptation and/or perhaps to speciation (Kawakami et al., 2011) or to resolve genomic conflict (through the linkage of sexually antagonistic genes to male-specific region) (Charlesworth et al., 2005; van Doorn and Kirkpatrick, 2010). The evidence is currently mounting for such scenarios, especially in conjunction with emerging neo-sex chromosomes (Kitano et al., 2009; Yasukochi et al., 2011; Nguyen et al., 2013; Smith et al., 2016; Bracewell et al., 2017). It would be therefore interesting to further investigate, whether the formation of X_1X_2Y sex chromosomes was a selected event providing an advantage for a species or whether a genetic drift, highly likely acting in small isolated *P. semifasciata* populations, drove fast fixation of this sex chromosome system randomly just in this species (Charlesworth and Wall, 1999). Importantly, analogous male-*vs*.-female CGH experiments in other *Pyrrhulina* species failed to show any sex-specific region (data not shown).

Lastly, we employed WCP with the PSEMI-Y probe in order to evaluate our hypotheses about the origin of the sex chromosome system present in *P. semifasciata* and to map the orthologous regions in other *Pyrrhulina* species in an attempt to predict potential homomorphic sex chromosomes in these species. Indeed, this approach facilitated many times a finer-scale survey of fish sex chromosomes with a common (Machado et al., 2011; Parise-Maltempi et al., 2013; Pansonato-Alves et al., 2014; Scacchetti et al., 2015; Yano et al., 2017b; Barros et al., 2018) or independent (Reed et al., 1995; Phillips et al., 2001; Henning et al., 2008; Henning et al., 2011; Cioffi et al., 2011b; Cioffi et al., 2013; Oliveira et al., 2018) origin within the frame of certain family, genus, or species/species complex. Here, WCP with the PSEMI-Y probe applied back against its own chromosome complement painted not only the entire metacentric Y chromosome in males but also the entire acrocentric X_1 and X_2 chromosomes in both male and female karyotypes. In the cross-species experiments, the PSEMI-Y probe marked four acrocentric chromosomes in both males and females of the other tested *Pyrrhulina* species. These results not only strongly support the proposed origin *via* centric fusion between two non-homologous acrocentric chromosomes, but also that this event might have been fixed in *P. semifasciata* relatively recently, as WCP revealed preservation of all orthologous chromosomes in related *Pyrrhulina* species without apparent major divergence or rearrangements. As CGH results did not show clustering of sex-specific sequences on any chromosome of the *P. brevis* complement, it remains to be investigated, whether any of four PSEMI-Y labeled chromosomes represent cryptic (homomorphic) sex chromosomes with a sex-specific region being under resolution limit of the CGH method, or whether sex chromosomes are not present at all in this species and the sex determination is governed by other means (Herpin and Schartl, 2015; Guiguen et al., 2019).

CONCLUSION

Despite methodological difficulties, sufficient chromosomal preparations were obtained in miniature fishes of the genus *Pyrrhulina* in the present study. It was possible to demonstrate that chromosomal markers are useful cytotaxonomic tools in characterizing the biodiversity of these fishes, highlighting their evolutionary relationships. Among the obtained results, a discovery of multiple sex chromosome system in our *P. semifasciata* stands out and its investigation have led us to the following conclusions: i) the neo-Y chromosome arose likely from a centric fusion between two non-homologous acrocentric chromosomes or, possibly, between former (proto) sex chromosomes and an autosomal pair; ii) this event might have been fixed in *P. semifasciata* relatively recently, as revealed by CGH and WCP; the latter technique revealed all orthologous chromosomes in related *Pyrrhulina* species without apparent major divergence or rearrangements; iii) formation of neo-Y in *P. semifasciata* might be driven by genetic drift, while direct selective/adaptive advantage resulting from close association of formerly unlinked genetic content cannot be ruled out; and iv) despite presumably short evolutionary time, CGH revealed considerable accumulation of male-enriched sequences in the pericentromeric region of neo-Y. Whether the origin of multiple sex chromosomes was driven by positive selection or by genetic drift and whether related cryptic sex chromosomes occur in sibling species, remains to be shown. Nonetheless, a nascent male-specific region on Y in *P. semifasciata* as might be inferred from CGH suggests fast sequence evolution, with the area around the fusion point potentially hosting candidate genes for the sex determination.

The present study further underscores the importance of analyzing data from so-called lower vertebrates such as fishes, as the evolutionary scenarios uncovered in these lineages may provide important clues about the fundamental processes behind the genome organization and the karyotype evolution of vertebrates in general. It may particularly increase our cytogenetic knowledge in so-called higher vertebrates, especially when we take into account that intra- and interchromosomal rearrangements are potent drivers of evolution in Hominoidea, with gibbons of the family Hylobatidae representing the most spectacular example (Weise et al., 2015, Sangpakdee et al., 2016). The same holds true for the dynamics of repetitive DNA, which significantly

contributes to karyotype divergence among fishes, but is rarely studied in detail in higher vertebrates (Mrasek et al., 2001; Liehr et al., 2016), despite it might play a relevant role in species' divergence here as well. Finally, also complex sex-chromosome systems, such as the one described in the present study, hold a great potential to build up the reproductive barriers among different populations of the same species and can be occasionally found also in Hominoidea, as exemplified, e.g., by *Trachypithecus cristatus* (Xiaobo et al., 2013).

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

ETHICS STATEMENT

The experiments followed ethical and anesthesia conducts and were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (Process number CEUA 1853260315).

AUTHOR CONTRIBUTIONS

RM and AS carried out the cytogenetic analysis and drafted the manuscript. TH, EO, AA-R, and PV helped in the cytogenetic analysis, drafted and revised the manuscript. TL, PR, EF and MM drafted and revised the manuscript. MC and LB coordinated the study, drafted and revised the manuscript. All authors read and approved the final version of the manuscript.

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Chromosome Instability in the Neurodegenerating Brain

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Chromosome instability (CIN) is a hallmark of cancer (Heng, 2015; Rangel et al., 2017; Machiela, 2019; Simonetti et al., 2019). Additionally, a number of neurodegenerative diseases (NDD) demonstrate CIN, which mediates neuronal cell loss and appears to be a key element of the pathogenic cascade (Iourov et al., 2009a; Iourov et al., 2009b; Arendt et al., 2010; Jeppesen et al., 2011; Driver, 2012; Bajic et al., 2015; Leija-Salazar et al., 2018; Nudelman et al., 2019). Moreover, CIN is repeatedly associated with aging and aging-related deterioration of the brain (Yurov et al., 2010; Kennedy et al., 2012; Andriani et al., 2017; Vijg et al., 2017; Zhang and Vijg, 2018). Despite numerous studies dedicated to CIN in NDD, there is still no clear understanding of differences between "cancerous" and "neurodegenerative" CINs. Here, we propose a theoretical model, which seems to highlight the differences between these CIN types.

Oncogenic parallels have long been observed in NDD. More specifically, CIN manifesting as aneuploidy (gains or losses of whole chromosomes) has been systematically identified in the brain of individuals with NDD. The Alzheimer's disease brain has been found to demonstrate high rates of spontaneous aneuploidy (Iourov et al., 2009b; Iourov et al., 2011; Yurov et al., 2014; Bajic et al., 2015; Arendt et al., 2017; Yurov et al., 2018). Furthermore, Alzheimer's disease genes are involved in molecular pathways, alterations to which result in chromosome mis-segregation and aneuploidy (Granic et al., 2010). Similarly, CIN syndromes and/or mutations in genes involved in cell cycle/mitotic checkpoint pathways exhibit brain-specific CIN associated with neurodegeneration. Thus, CIN has been demonstrated to underlie neurodegenerative processes (Iourov et al., 2009a; Caneus et al., 2018; Leija-Salazar et al., 2018). Additionally, submicroscopic CIN producing structural rearrangements of the *APP* gene (21q21.3) has been shown to be involved in neurodegenerative pathways to Alzheimer's disease (Bushman et al., 2015; Lee et al., 2018). It is important to note that numerical CIN (aneuploidy) is shown to be implicated in the neurodegeneration pathway inasmuch as the neurons affected by CIN/aneuploidy are susceptible to selective cell death (Arendt et al., 2010; Fricker et al., 2018; Iourov et al., 2019). Finally, DNA repair deficiency (Jeppesen et al., 2011) and DNA replication stress (Yurov et al., 2011) have been identified as possible mechanisms for neurodegeneration.

Another body of evidence for the contribution of CIN to neurodegeneration is provided by brain aging studies. Actually, CIN and related phenomena (aneuploidization, somatic mutagenesis, etc.) are considered to be elements of a global pathogenic cascade resulting in aging phenotypes (Kennedy et al., 2012; Vijg, 2014; Andriani et al., 2017). Progressive accumulation of somatic chromosomal mutations (aneuploidy) causing numerical CIN is suggested to be implicated in cellular senescence and tissue aging (Yurov et al., 2010; Zhang and Vijg, 2018; Iourov et al., 2019). For instance, rates of X chromosome aneuploidy increase with age in the Alzheimer's disease brain (Yurov et al., 2014). It is to note that X chromosome aneuploidy (loss/monosomy) is a cytogenetic biomarker of human aging (Vijg, 2014; Zhang and Vijg, 2018; Iourov et al., 2019). Genome instability at the

chromosomal level (numerical and structural CINs) has been determined as a conserved mechanism for aging, as a whole, and, more particularly, for aging of the brain, a post-mitotic tissue with an extremely limited potential of cell renewal (Yurov et al., 2010; Andriani et al., 2017; Vijg et al., 2017). It appears that aging-related CIN leads to aging-related deterioration of the brain producing phenotypes similar to NDD (Andriani et al., 2017; Zhang and Vijg, 2018). Functionally, CIN is supposed to be an underlying cause of cellular (neuronal) senescence (Yurov et al., 2010; Arendt et al., 2017; Zhang and Vijg, 2018; Iourov et al., 2019). The latter has been recently demonstrated to represent a mechanism for both brain aging and NDD (Baker and Petersen, 2018). Therefore, one may conclude that the pathogenic pathways are likely to be shared by brain aging, neurodegeneration, and cancer.

NDD (e.g., Alzheimer's disease) have been consistently shown to share biological hallmarks with cancer, which are, but not limited to, alterations to genome stability maintenance pathways (mitotic checkpoint, cell-cycle regulation, DNA replication/ repair, programmed cell death, etc.) and CIN/genome instability (for review, see Driver, 2012, Arendt et al., 2017, Nudelman et al., 2019). More precisely, numerical CIN (aneuploidy) leading to chromosomal mosaicism is a mechanism for a variety of brain diseases including NDD. Somatic mosaicism and increased rates of aneuploidy and structural CIN have been identified in the neurodegenerating brain (Alzheimer's disease and ataxia telangiectasia), schizophrenia brain, and individuals with intellectual disability and autism spectrum disorders. Mutations of specific genes implicated in genome stability maintenance pathways have been associated with NDD (Iourov et al., 2009a; Iourov et al., 2009b; Arendt et al., 2010; Iourov et al., 2011; Jeppesen et al., 2011; Yurov et al., 2014; Bajic et al., 2015; Caneus et al., 2018; Rohrback et al., 2018; Yurov et al., 2018; Iourov et al., 2019). Aneuploidy is a common feature of cancer cell populations and is likely to influence cancer behavior (for review, see Simonetti et al., 2019). Moreover, chromosomal mosaicism is a susceptibility factor for cancer (Schick et al., 2013; Vijg, 2014; Machiela, 2019). Genetic alterations to the genome stability maintenance pathways produced by copy number and sequence variations of the implicated genes are observed both in cancer and in the neurodegenerating brain (Granic et al., 2010; Bushman et al., 2015; Heng, 2015; Caneus et al., 2018; Lee et al., 2018). As noted before, a possible mechanism of neurodegeneration is DNA repair deficiency (Jeppesen et al., 2011). The later commonly leads to CIN and karyotypic chaos in a wide spectrum of cancers (Driver, 2012; Heng, 2015; Rangel et al., 2017). DNA replication stress seems to lie at the origins of CIN in the neurodegenerating brain of individuals with Alzheimer's disease (Yurov et al., 2011). Likewise, this phenomenon negatively impacts chromosome segregation producing CIN during tumorigenesis (Zhang et al., 2019). Finally, cellular senescence is able to contribute both to neurodegeneration (brain aging deterioration) and to cancer (Yurov et al., 2010; Vijg, 2014; Baker and Petersen, 2018; Machiela, 2019). It appears that either neurodegeneration or cancer is more likely to result from complex genetic-environmental interactions, in which CIN plays a key role in the pathogenic cascade (Iourov et al., 2013; Heng, 2015). However, taking into account diverse consequences of "neurodegenerative" and "cancerous" CINs, there should be a number of differences between these types of chromosome/genome instability. For instance, the lack of convincing evidence for comorbidities such as NDD and brain cancers suggests that brain cells affected by CIN may have at least two alternative fates: (i) to become malignant (i.e., cancerization) and (ii) to be cleared by cell death (i.e., neurodegeneration). Therefore, there should be a striking difference in molecular pathways to cancer and NDD.

Since somatic mosaicism and CIN in the brain are more likely to have developmental origins (Yurov et al., 2007; Rohrback et al., 2018; Yurov et al., 2018; Iourov et al., 2019), alterations to programmed cell death may be an explanation of the presence of cells with abnormal chromosome complements (genomes) in the diseased brain (Arendt et al., 2010; Yurov et al., 2010; Fricker et al., 2018; Iourov et al., 2019). More precisely, abnormal neural cells generated during the development are not cleared throughout gestation and antenatal period. As a result, CIN-affected (abnormal) cellular populations alter brain functioning after birth (for more details, see Yurov et al., 2007; Yurov et al., 2010; Rohrback et al., 2018; Iourov et al., 2019). Thus, programmed cell death acts differently in the neurodegenerating brain and in cancer. The former demonstrates excessive neuronal cell loss probably mediated by CIN, whereas the latter is characterized by astonishing tolerance of cell populations to programed cell death (Heng, 2015; Fricker et al., 2018; Iourov et al., 2019). Therefore, cancer cells are likely to be affected by abnormal cell-death checkpoint in contrast to neuronal cells affected by "neurodegenerative CIN," in which the checkpoint probably acts to an abnormal environmental trigger. Interestingly, CIN/aneuploidy is usually chromosome-specific in the diseased brain. In the Alzheimer's disease brain, CIN commonly involves chromosome 21, whereas the selectively degenerating cerebellum of ataxia-telangiectasia individuals exhibits CIN commonly involving chromosome 14 (Iourov et al., 2009a; Iourov et al., 2009b; Arendt et al., 2010; Granic et al., 2010). This is generally not the case for the overwhelming majority of cancer cells expressing genetic defect specific for a cancer/ tumor type, karyotypic chaos, or numerical and structural CINs (Heng, 2015). The natural selection pressure against cells affected by non-specific CIN types and observations on patterns of CIN in the neurodegenerating brain suggest that neuronal cell populations affected by neurodegeneration possess primary genetic defects without progressive clonal evolution (Iourov et al., 2009a; Arendt et al., 2010; Yurov et al., 2011; Iourov et al., 2013; Arendt et al., 2017; Leija-Salazar et al., 2018). The latter, however, is shown to be an underlying cause of cancer (Driver, 2012; Heng, 2015; Rangel et al., 2017; Simonetti et al., 2019). Taking into consideration the aforementioned differences between cancer and NDD, we have proposed a theoretical model for CIN to mediate either cancer or neurodegeneration. Thus, "cancerous CIN" is likely to result from genetic-environment interactions and genetic defects, which render cells with unstable genomes tolerant to clearance (i.e., programmed cell death) and advantageous for proliferation over other cells. The malignancy is then achieved by clonal evolution. Alternatively, CIN and aneuploidy may possess a detrimental effect on cell growth under the normal growth conditions. In this case, cancerization is achieved through an adaptation of a subclone of cells to aneuploidy and CIN, which further evolves to a cell

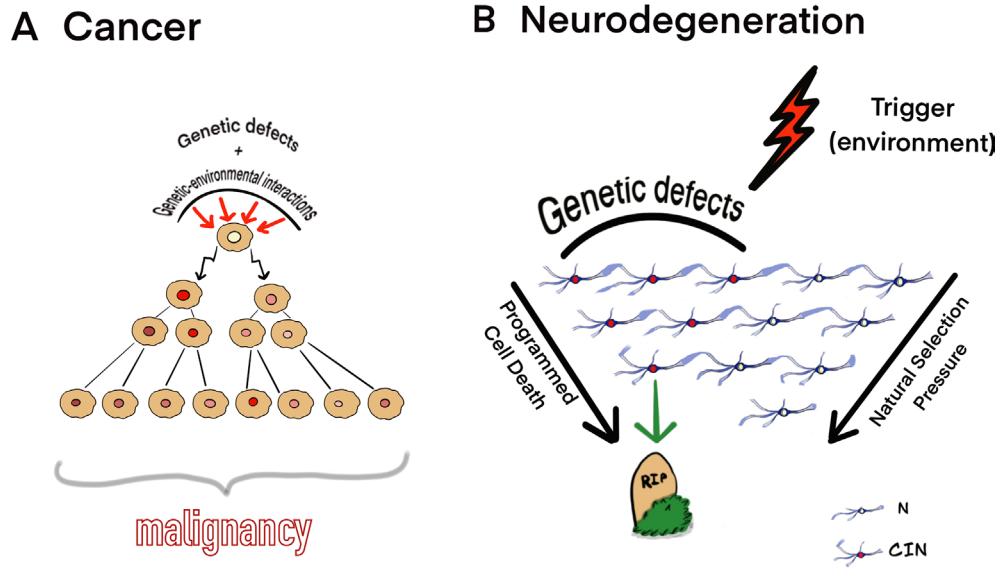

FIGURE 1 | Theoretical model for CIN mediating **(A)** cancer and **(B)** neurodegeneration. **(A)** Genetic defects and genetic-environmental interactions may cause chromosomal/genomic changes, which produce CIN; alternatively, cell populations may adapt to aneuploidy and CIN evolving to a cell population with a fitness advantage. Cells affected by CIN and tolerating deteriorating effects of CIN on cellular homeostasis are able to evolve clonally to produce malignancy. **(B)** CIN/somatic mosaicism affecting a significant proportion of cells interacting with environmental triggers may result into progressive neuronal cell loss (neurodegeneration) under natural selection pressure and through the programmed cell death (N, normal neurons; CIN, neuronal cell affected by CIN). The model is based on the observations of CIN in the neurodegenerating brain and cancers (Iourov et al., 2009a; Iourov et al., 2009b; Arendt et al., 2010; Granic et al., 2010; Iourov et al., 2011; Jeppesen et al., 2011; Yurov et al., 2011; Driver, 2012; Kennedy et al., 2012; Vijg, 2014; Yurov et al., 2014; Bajic et al., 2015; Heng, 2015; Arendt et al., 2017; Rangel et al., 2017; Caneus et al., 2018; Leija-Salazar et al., 2018; Yurov et al., 2018; Machiela, 2019; Simonetti et al., 2019).

population with a fitness advantage (Vijg, 2014; Heng, 2015; Zhang et al., 2019). As a result, cells tolerating CIN without the loss form a stable cell population causing cancer invasion and metastasis (Loeb, 2010).

In contrast to cancer, neurodegeneration is likely to start because of the interaction between environmental trigger and CIN/genetic defects persisting in an appreciable proportion of brain cells. The interactions may launch a kind of "neuroprotective program" for clearance of CIN-affected cells. It appears that such "neuroprotective program" exists in the developing mammalian brain, which loses the majority of cells affected by CIN throughout gestation. It has been hypothesized that CIN/aneuploidy serves as an initiator of cell death (i.e., mitotic catastrophe) under natural selection in the developing brain (Yurov et al., 2007; Yurov et al., 2010; Rohrback et al., 2018; Iourov et al., 2019). Since CIN affects the critical number of neuronal cells (Iourov et al., 2009a), progressive loss of these cells would produce brain dysfunction leading to NDD phenotypes. **Figure 1** schematically shows our model for CIN contribution to cancer and neurodegeneration according to observations on CIN in the neurodegenerating brain in cancers (Iourov et al., 2009a; Iourov et al., 2009b; Arendt et al., 2010; Granic et al., 2010; Iourov et al., 2011; Jeppesen et al., 2011; Yurov et al., 2011; Driver, 2012; Kennedy et al., 2012; Vijg, 2014; Yurov et al., 2014; Bajic et al., 2015; Heng, 2015; Arendt et al., 2017; Rangel et al., 2017; Caneus et al., 2018; Leija-Salazar et al., 2018; Yurov et al., 2018; Machiela, 2019; Simonetti et al., 2019).

Understating the role of CIN in the neurodegeneration pathway is important for successful therapeutic interventions in NDD. Certainly, there is a need for further studies dedicated to analysis of the applicability of the "neurodegenerative CIN" model to describe molecular and cellular mechanisms for neurodegeneration. If the model is applicable, new opportunities for NDD prevention and treatments through the external control of CIN will be available.

AUTHOR CONTRIBUTIONS

All authors conceived the idea and made theoretical contributions to the manuscript. II wrote the manuscript.

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MINI REVIEW
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What Is Karyotype Coding and Why Is Genomic Topology Important for Cancer and Evolution?

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While the importance of chromosomal/nuclear variations vs. gene mutations in diseases is becoming more appreciated, less is known about its genomic basis. Traditionally, chromosomes are considered the carriers of genes, and genes define bio-inheritance. In recent years, the gene-centric concept has been challenged by the surprising data of various sequencing projects. The genome system theory has been introduced to offer an alternative framework. One of the key concepts of the genome system theory is karyotype or chromosomal coding: chromosome sets function as gene organizers, and the genomic topologies provide a context for regulating gene expression and function. In other words, the interaction of individual genes, defined by genomic topology, is part of the full informational system. The genes define the "parts inheritance," while the karyotype and genomic topology (the physical relationship of genes within a three-dimensional nucleus) plus the gene content defines "system inheritance." In this mini-review, the concept of karyotype or chromosomal coding will be briefly discussed, including: 1) the rationale for searching for new genomic inheritance, 2) chromosomal or karyotype coding (hypothesis, model, and its predictions), and 3) the significance and evidence of chromosomal coding (maintaining and changing the system inheritance-defined bio-systems). This mini-review aims to provide a new conceptual framework for appreciating the genome organization-based information package and its ultimate importance for future genomic and evolutionary studies.

Keywords: chromosomal instability (CIN), fuzzy inheritance, genome chaos, genome theory, karyotype or chromosomal coding, missing heritability, non-clonal chromosome aberrations (NCCAs), system inheritance

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INTRODUCTION

Sequence-driven and gene-focused molecular research has surprisingly revealed its key limitation: the predictive value between content of individual genes and cellular or organismal phenotype is not strong, especially when dealing with many common and complex diseases like cancer (Heng, 2015). This limitation is at odds with many promises that rationalized the need of various large-scale sequencing and -omics projects (van Karnebeek et al., 2018). Moreover, combined with missing heritability (Eichler et al., 2010; Zuk et al., 2012), these limitations fundamentally challenge the gene theory where the inheritance of a group of individual genes is the key causative factor of phenotype

(Heng, 2009; McClellan and King, 2010; Boyle et al., 2017), even though this issue is rarely discussed in public.

Since the 10th-anniversary celebration of the completion of the Human Genome Project, different reasons have been offered to explain these limitations (Check Hayden, 2010). The article "Genomics is not enough" (Chakravarti, 2011) called into question how adequate is the concept of the gene and the general genomic mechanism of diseases based on sequencing data.

Unfortunately, when highly heterogeneous data do not fit the expectation of pattern identification, the data are often blamed. One general conclusion is that the current genomic data are either not enough (quantity) or not good enough (quality). Logically, the future research should focus on the data: how to generate and collect more data and how to improve data analyses. Suggested approaches include: 1) collect additional data sets from more clinical samples and develop better computational platforms to filter out the "noise" and to identify the patterns; 2) incorporate epigenetics, gene–environment interactions, microbiota, and metabolic profiles into the analyses; and 3) use the combinatorial approach of systems biology (Ao et al., 2010; Palsson, 2015).

Others are less certain about how to move the field forward (Weinberg 2014). The complexity in genomic medicine requires a new framework to understand the heterogeneous data and its implications. Our group considers biosystems as adaptative systems and focuses on evolutionary mechanisms rather than specific molecular mechanisms. While it is challenging to understand the common mechanism of genomics through reductionist approaches (focusing on genetic parts characterization), it can be achieved by studying the evolutionary mechanism (tracing the pattern of evolution and system emergency). Clearly, studying the genome-mediated somatic evolution will be a better strategy than characterizing gene-based mutations or pathways, as many diverse pathways can lead to the same evolutionary end-products, and each "run" of somatic evolution will likely produce different genomic landscapes.

Recently, cancer genome projects have validated our main predictions about the importance of genome-mediated somatic evolution and limitations of gene-focused research. The increased sample size in most cancer types confirmed the high degree of genomic heterogeneity as a general rule (one which cannot simply be eliminated by bioinformatics tools). The chromosomal profile provides better clinical predictions than gene mutation profiles (Jamal-Hanjani et al., 2017), and the genome chaos, including chromothripsis, can be detected from many cancer types, challenging the stepwise gene mutation theory of cancer (Ye et al., 2018a; Ye et al., 2018b). Furthermore, genome-mediated evolution has received increased attention, as it is linked to system stress, immuno-response, transcriptional dynamics, and cancer evolutionary potential (Horne et al., 2014). Chromosomal changes, including mosaicism, are a universal feature in many common and complex diseases (Iourov et al., 2008a; Iourov et al., 2008b; Iourov et al., 2012; Heng et al., 2016; Iourov et al., 2019). Equally important, the integrity of the karyotype has been linked to the function of sexual reproduction and is the main system constraint of macro-evolution for organisms (Heng, 2007b; Wilkins and Holliday, 2009; Gorelick and Heng, 2011); the genome organization has been considered the organizer of network interaction (Heng, 2009). Such realization has established the core genome or karyotype, rather than individual genes, as the evolutionary selective package.

Altogether, chromosomal-related research is regaining its popularity. As mentioned by editors of this special issue, chromosome biology represents the key to understanding disease mechanisms, genome architecture, and evolution, as genetic inheritance relies on the proper organization of chromosomes and the genome. However, influenced by the gene-centric tradition, recent chromosomal studies are still focusing on gene-defined "parts inheritance." Rather than address the mechanism of how chromosomes organize the expression and interaction of individual genes, many still consider chromosomes the vehicles or helpers of genes (e.g., contributing to epigenetics by modifying the gene's function).

Here, a newly realized key concept—order of DNA sequence on chromosome serving as a system code—is briefly discussed. Although "system inheritance" has been previously promoted (Heng, 2009), it has failed to transform the field, possibly due to the dominance of gene-centric concepts and the high hopes for various large-scale sequencing projects. With the recent discussions of karyotype or systematic chromosome-sets-coded inheritance (Heng, 2019), the time is ripe to embrace this new framework, which should serve as a foundation for future genomic research.

KARYOTYPE CODING DEFINES SYSTEM INHERITANCE

Rationale and Metaphor for Searching for New Genomic Inheritance

A key rationale of searching for new types of inheritance is because gene-based inheritance has several limitations. First, missing heritability is a real phenomenon rather than a methodological limitation caused by insufficient samples or technologies, which challenges both current technical strategy and the concept of genes. Second, many case studies have illustrated a lack of correlation between gene profile and phenotype, with strong correlation only being detectable from exceptional cases. Third, for cancer research, chromosomal alterations are abundant, most of which differ from gene mutation (except in the cases of gene fusion caused by chromosomal translocation). Furthermore, gene mutation and epigenetic effects do not explain macro-cellular evolution. Fourth, while interesting, most epigenetic regulation involves fine-tuning of gene regulation, which is not sufficient to explain the missing inheritance.

Since multiple levels of genomic organization comprise eukaryotic systems, and a given chromosomal change often can impact many genes, chromosomal alteration represents information change at a higher system level. Equally important, the bio-topological features serve as an important form of bio-information. The specific chromatin distribution within 3D nuclei highlights the topological significance regarding the gene's relationship along and among chromosomes.

Thus, studying inheritance as defined by chromosome sets should be the priority, especially knowing that this level has been traditionally ignored and deserves timely and systematical study. The topological context of the chromosome set likely serves as the context of gene interaction at the scale of an entire genome.

To illustrate the importance of bio-topology in defining the function of the system, the relationship between building materials (e.g., bricks) and the overall structure of buildings (e.g., architecture) can serve as a metaphor, where the information encoded within architecture can be independent from information encoded within the materials, and the same materials can be used to build different structures with different functions. This metaphor serves to show that sequencing all genes to decode the genomic blueprint will not work. The coding of how genes interact rather than how an individual gene makes protein is the blueprint. The topological relationship among genes likely serves as the genomic information.

Karyotype Coding: Hypothesis, Model, and Prediction

By considering the genomic topology as a new type of information, we have hypothesized that chromosome sets carry organizational information of genes (system inheritance) that is distinct from the information created strictly by individual gene sequences (parts inheritance). The system inheritance is unique for most species and is maintained by sexual reproduction through meiosis (the main function of chromosomal pairing in meiosis serves as a major checkpoint to maintain the correct order of the chromosomal coding) (Heng, 2007a). Chromosomes are not just the vehicle of genes but the organizers of gene interaction (by providing the physical platform of the genetic network). We have additionally posited that this genomic information can be reshuffled *via* chromosomal rearrangement to create a new emergent genome with new system inheritance.

A model has been introduced (**Figure 1**) to summarize ideas behind karyotype coding. This model illustrates the relationship

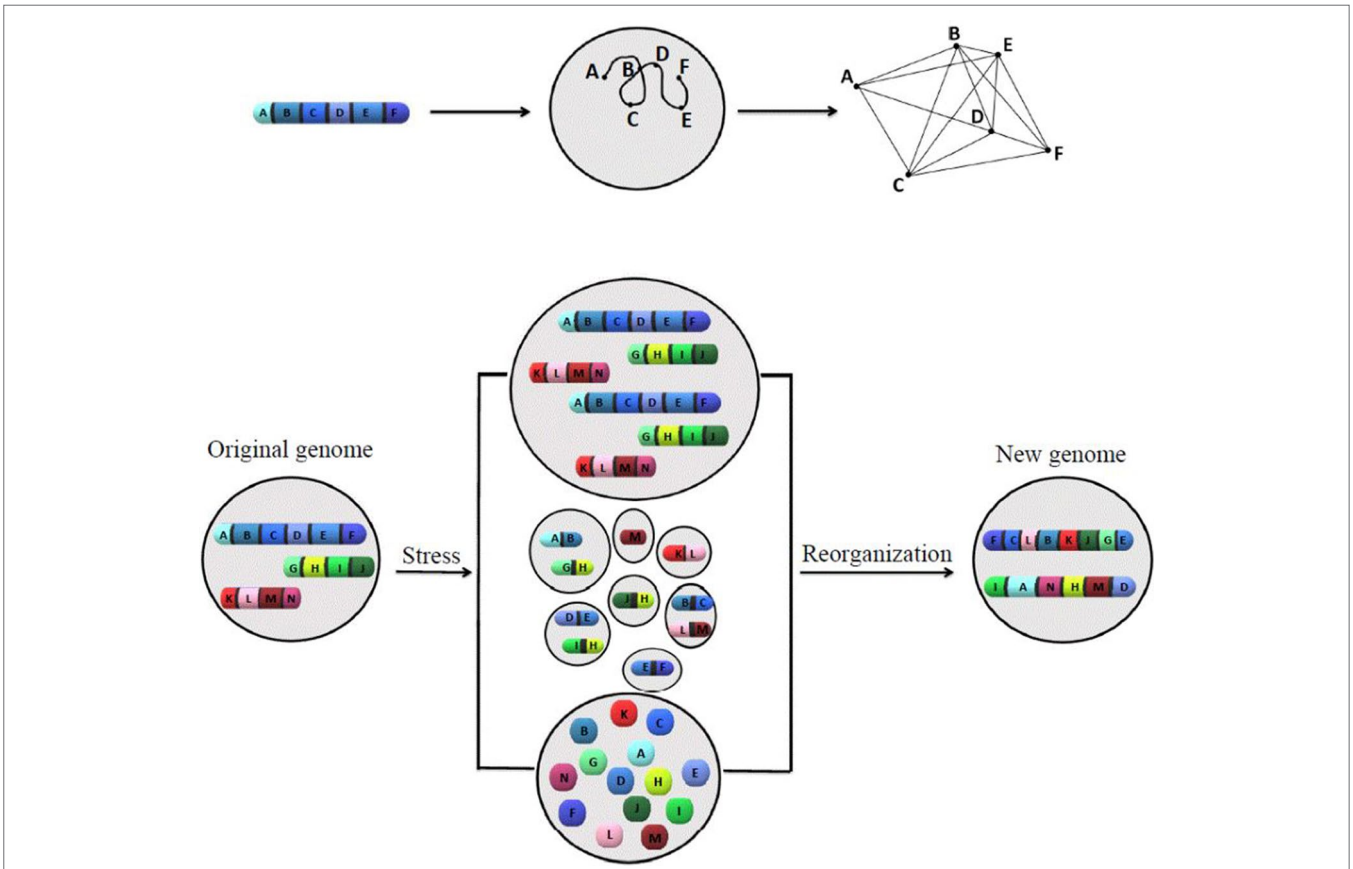

FIGURE 1 | The model of how karyotype or chromosomal coding defines the network structure, and how chromosomal/nuclear variation changes the chromosomal-coded system inheritance. The proposed models to illustrate the relationship between order of genes along chromosomes, network structure (upper panel), and how stress-induced genome re-organization creates a new genome through genome chaos (lower panel). The upper panel illustrates one chromosome with a gene order of A to F, its chromatin domain in interphase nuclei, and a defined network structure (from left to right). For simplicity, only one chromosome is shown. The pattern of interaction among multiple chromosomes would be more complicated. The lower panel illustrates the process of new genome emergence (from the original genome through different types of chromosome/nuclear re-organization under crisis). Only three chromosomes are presented for the original genome. Under high levels of cellular stress, genome chaos occurs as an effective survival strategy. Among many types of genome re-organization (including different types of genome chaos), only polyploidy (upper), micronuclei clusters (middle), and chromosomal fragmentation (lower) are shown. Additional types of genome chaos can be found in Heng et al. (2013a), Liu et al. (2014), and Heng, 2019. The result of genome re-organization (not dependent on the mechanism in which it proceeds) is the formation of new genomes with a higher chance of survival and new chromosomal codes reflected by two newly formed chromosomes with new gene order, providing new network structures.

between the order of genes along chromosomes and its defined network structure, as well as how stress responses change the system coding.

The following are key features, observations, and predictions supporting karyotype-coded system inheritance (see also **Table 1**)

a. The majority of cancer cells and natural species display different karyotypes. Different species have their own unique gene order along and among chromosomes.
b. Alteration of gene order along the chromosome is biologically significant. The synteny relationship (conservation of gene order) among different species is well known, and

TABLE 1 | Terminology/rationales/evidences/implications of karyotype-coded system inheritance.

I.	**Terminology**
a.	"Karyotype coding" or "chromosomal (set) coding" functions as an organizer of gene interactions within the entire genome. Its biological effect is not just on individual genes but on the entire genomic network. As opposed to gene coding or vague ideas that chromosomes carry additional information, karyotype coding is defined by specific features: 1) the physical organization of the chromosome codes system information; 2) genomic topology provides context for individual genes; and 3) since different species display unique karyotypes or core genomes, karyotype coding is often species-specific. The key is that the order of gene and non-coding sequences along a chromosome represents a new "system inheritance," much like how the order of base pairs codes for "parts inheritance" in mainstream "gene coding."
b.	Although the physical location of individual genes along a chromosome has previously been linked to gene expression (such as the position effect), karyotype coding has long been ignored. However, there have been efforts to search for inheritance above the gene coding level. For example, the "genome system architecture" concept proposed a model based on how a computer program or operating system is organized (Shapiro, 2005). Specifically, the distribution pattern of repetitive DNA was suggested as a key architectural factor. Karyotype coding is described by the order of genes and non-gene genomic sequences, including repetitive sequences and sequences for chromatin architecture such as topoisomerase associate domains (TADs).
II.	**Brief rationales/history of search for new inheritance**
a.	Chromosomal position effect has long been observed to impact chromatin behavior and function of genes (Heng et al., 2004; Elgin and Gunter, 2013; Heng, 2019)
b.	Missing heritability is real and a search for inheritance beyond genes is urgently needed (Eichler et al., 2010; Heng et al.,2011; Zuk et al., 2012)
c.	Studying cancer evolution illustrates the distinctive roles of inheritance between gene and genome, and the emergence of new karyotypes is key for cancer evolution (Heng et al., 2006; Heng, 2007a)
d.	A collection of gene sequences does not equal the blueprint. Considering biological systems as multiple levels of interaction/control systems requires a higher level of genomic coding (Heng, 2009; Heng et al., 2011)
e.	Genomic topology likely functions as the coding of the genomic blueprint or gene interaction (Heng, 2019)
f.	The function of sex represents a mechanism of preserving chromosomal coding (Heng et al., 2006; Gorelick and Heng, 2011; Heng, 2015; Heng, 2019)
III.	**Evidences to support chromosomal coding**
a.	Each chromosome has its physical domain within a nucleus, and the genomic topology is related to a gene's function (Cremer and Cremer, 2001; Heng et al., 2004)
b.	The importance of gene clusters in development (Hox genes) (Gehring, 1998)
c.	Chromosomal synteny is preserved among plants and animals (Eckardt, 2001; Murphy et al., 2005)
d.	The formation of new gene clusters contributes to specific pathways (Wong and Wolfe, 2005)
e.	Different karyotypes among species suggest that genomic topology (order of genes/regulation elements) is species specific (Heng 2009)
f.	Chromosomal alterations represent the most common driver for cancer evolution (Erenpreisa et al., 2005; Walen, 2005; Stevens et al., 2007; Heng 2007a; Wallen, 2008; Zhang et al., 2014; Horne et al., 2015; Bloomfield and Duesberg, 2016; Hamann et al., 2017; Hamann et al., 2017; Bakhoum et al., 2018; Salmina et al., 2019)
g.	Changing the chromosomal number by fusing them into one single yeast chromosome can effectively establish reproduction barriers (Luo et al., 2018; Shao et al., 2018)
h.	Chromosomal alterations can rescue yeast following key gene knockout (Rancati et al., 2008)
i.	Individual chromosomal alterations can impact the entire transcriptome (Stevens et al., 2013; Heng, 2015)
j.	The linkage between genome alterations and various diseases is common (Heng 2009; Heng et al., 2016; Heng 2019), and chromosomal mosaicism is a common phenomenon (Iourov et al., 2012; Heng et al., 2013a; Iourov et al., 2008a; Iourov et al., 2008b; Iourov et al., 2019)
k.	Both TADs and position effects are examples of data where the expression of coding information is sensitive to physical location in the genome.
l.	NCCAs, an index of genome instability, have been linked to "fuzzy inheritance," which is essential for evolutionary potential (Heng 2009; Heng 2019). NCCAs contribute to the emergence of new genomes under crisis, including outlier-mediated drug resistance, various types of cellular survival, and adaptations (Rangel et al., 2017; Poot, 2017a; Frias et al., 2019). Interestingly, other types of genome dynamics, such as small supernumerary marker chromosomes and transposable elements, can influence chromosomal coding under certain conditions (Liehr et al., 2013; Liehr 2016; Poot, 2017a; Poot, 2017b). Equally important, the pattern and dynamics of NCCAs should be used to study somatic mosaicism (Lourov et al., 2008a; Lourov et al., 2008b; Lourov et al., 2012; Biesecker and Spinner, 2013; Heng et al., 2013a; Lourov et al., 2019) and multiple levels of core genome-associated genomic interactions (Heng et al., 2013a; Heng et al., 2016; Shapiro, 2017; Shapiro, 2019; Heng 2019), including minimal genome variations at germline, somatic alteration and mosaicism, and host microbiome. Such genome–environment interactions play an important role for evolutionary adaptation and survival.
III.	**Implications**
a.	Reconcile "parts inheritance" and "system inheritance" and prioritize the importance of the true blueprint for eukaryotic systems
b.	Emphasize the importance of using chromosomal dynamics to study cellular evolution, and applying chromosomal aberrations (rather than individual gene mutation profiles) as a biomarker
c.	Understanding the genomic basis of information inheritance in macro- and micro-evolution
d.	Illustrate the emergence of phenotype based on genomic mosaicism and its interactions with all involved genomes and the environment

More examples can be found in Heng (2009, 2019).

the positional effect and the importance of order of genes within a gene cluster is well appreciated (e.g., Hox cluster, topoisomerase associate domains (TADs), and position effect). Recently, the significance of order of genes has been illustrated in synthetic biology (Heng, 2019).

c. Chromosomal alterations (e.g., translocations, aneuploidy, and polyploidy) can alter system inheritance as reflected by the transcriptome and the phenotypes. It also can trigger genome instability to produce further chromosomal changes.
d. Changing the coding is a common mechanism for new genome formation for both organismal and somatic evolution. Chromosomal re-organization creates new emergent information and is the most effective way of creating new and sometimes drastically different phenotypes.
e. A given gene can have different functions within different genomic topology, exhibited through increased or reduced activity, as well as new genomic interaction with other genes, which can change its function. The same protein can have different functions when located in different regions of the cell, with different partners, or when involving different pathways. It is also possible that different cellular sites of protein synthesis are function specific. Nevertheless, most genes are known to work in this context-dependent manner. The genomic topology serves as such context.
f. Different karyotypes can have similar phenotypes as long as some functional modules are preserved within an altered genome. Alternatively, different genomes can display different phenotypes in different environments (many new phenotypes only occur in altered "future" environments).
g. There is a gene and karyotype interaction (both collaboration and conflict). The genome can control or influence an individual gene's function. The change of genomic context also includes gene–promoter interaction. For example, the capture of an aerobic promoter by *Escherichia coli* with a previously anaerobic or unexpressed citrate transporter leads to a novel phenotype (van Hofwegen et al., 2016).
h. The gene's key evolutionary involvement is mainly at the micro-evolutionary phase.
i. Fuzzy inheritance can be detected from the chromosomal coding level as well. Furthermore, the heterogeneity of the karyotypes can be explained by the "core genome" concept (Heng, 2019).

Significance and Evidence: Maintaining and Changing System Inheritance-Defined Bio-Systems

Significance:

a. Inheritance is a key feature for all biosystems. Establishing the correct mechanism for how biosystems create, and then pass on, their information is of utmost importance for both basic genomic research and its application for medicine. It is long accepted that the gene defines bio-inheritance. Now with the realization that chromosome-mediated system inheritance organizes the parts inheritance, many bio-concepts based on the understanding of parts inheritance need to be modified, including genomic/evolution studies and molecular medicine.
b. The concept of karyotype coding effectively addresses the issue of missing heritability. This key genome factor likely accounts for a large portion of the missing heritability, even though the fuzzy inheritance at gene level is also contributing to the phenomenon. In addition, system inheritance also defines the boundary of the epigenetic regulation; equally important, there is a gap between germline-defined inheritance and the environmental-influenced somatic inheritance (such emergent properties are highly dynamic and constantly changing in response to development, aging, and cellular stress).
c. Karyotype coding unifies organismal evolution and somatic evolution, as both evolutions need to pass system inheritance and involve macro- and microevolution. They share the same two phases of macro- and microevolution despite the different mechanisms used to maintain system inheritance. It also explains why cancer can happen within 20–30 years while organismal evolution takes much longer (though initial speciation can be quick, it often takes a long time to form a stable population). Without the genome constraint ensured by sexual reproduction, the genome chaos can fast become dominant in somatic evolution, leading to cancer (Heng, 2015). In contrast, the function of sex provides the strong genome constraint in organismal evolution. For a successful speciation, it requires three highly rare events: genome re-organization to produce survivable individuals with altered genome; the availability of other mating partners with a matching genome (producing fertile offspring); and the initial small population growing into a visible population (Heng, 2019).
d. The model (**Figure 1**) unifies diverse molecular mechanisms of genome variations. Although different molecular mechanisms can be linked to each type of chromosomal/nuclear abnormality, they can all be unified under the evolutionary mechanism of re-organizing chromosomal coding. For example, from aneuploidy and/or simple translocation to chaotic genomes, including chromosome fragmentations, micronuclei cluster, polyploidy, entosis, and budding/bursting/fusion, they all can be explained by changes to the genomic information (Walen, 2005; Stevens et al., 2007; Stevens et al., 2011; Zhang et al., 2014; Ye et al., 2019). Evolutionary selection acts on new emergent genomes with new phenotypes and "cares" less which molecular mechanisms are responsible.

Evidence:

Examples of supporting evidences are listed in **Table 1**. More examples can be found in the book *Genome Chaos* (Heng, 2019).

FUTURE DIRECTION

In 2011, the journal *Cell* asked a few leading genomic researchers "what's been most surprising" for the human genome? The answers were: "let's remember the chromosomes"; "variation and complexity"; "a hidden ecosystem"; and "huge heterogeneity." Interestingly, all issues are directly related to the chromosomal coding-defined system inheritance (Leading Edge, 2011).

Recently, the importance of chromosomal research has become more obvious. For example, chromosomal abnormalities are

copious in cancer including various types of genome chaos, and predicting clinical outcomes based on chromosomal data is much better than based on DNA sequencing data (Davoli et al., 2017; Jamal-Hanjani et al., 2017). In addition, chromosomal and nuclear aberrations have been linked to immune response (Mackenzie et al., 2017; Santaguida et al., 2017). The stochastic chromosomal changes, such as non-clonal chromosome aberrations (NCCAs), are used to measure chromosomal instability (CIN) and to explain treatment outcomes (Heng et al., 2006; Heng et al., 2013a; Heng et al., 2013b). Now, it is increasingly clear why high levels of NCCAs should not be ignored, as they reflect the system instability. Furthermore, the evolutionary meaning of altering the chromosomal coding is also applied to the study of other disease types, and organismal evolutionary studies (Heng, 2009; Heng, 2019).

With the introduction of the chromosomal-coding concept, the following tasks need to be achieved to maturate this concept:

a. Further illustrate the molecular details of karyotype coding:

As illustrated in **Figure 1**, the model of genome-topology based inheritance does not offer molecular details of how karyotype coding works. We know that altering the order of genes along a chromosome can change species and/or phenotypes (like how changing the order of the Hox gene cluster leads to abnormal development and chaotic genome changes in the overall transcriptome); however, little is known about which specific mechanisms are involved at whole genome scale. Unlike how DNA codes for proteins, where there is a direct correlation between a three-nucleotide codon in a nucleic acid sequence and a single amino acid in a protein (which is with high certainty), chromosomal coding is more like "gene regulatory codes," which determine when, where, and what amount of specific proteins are to be produced (which involve diverse mechanisms and less predictability). Further, chromosomal coding may involve more complicated mechanisms due to the large-scale organization, which likely involves emergent behavior. Nevertheless, studies are needed to link the order changes among genes on chromosomes (including translocation, aneuploidy) to interphase changes (dynamics and/or behavior) and specific pathway changes. Moreover, the altered evolutionary potential needs to be studied with these changes. These studies will likely help people accept the concept of chromosomal coding, even though, similar to mechanisms of "gene regulatory codes" (such as control of chromatin packaging), these mechanisms could be less specific when compared to "gene-protein codes".

b. Illustrate the relationship among different types of bio-inheritance:

To illustrate the significance of karyotype coding, quantitative and comparative studies are needed to rank the contribution of different types of inheritance under different bioprocesses and environments. The following solutions are needed when systematically comparing different types of bio-inheritance: separating germline and somatic cells (germline with the highest constraint, the somatic cell with highest dynamic changes) to compare the germline profile with tissue-specific somatic cell profiles; separating profiles of individual cells and cellular populations; separating the two phases of cancer evolution (cancer formation by creating new genome systems; microevolution to increase the number of cancer systems, by stochastically capturing the oncogenes) (Ye et al., 2018a; Ye et al., 2018b); separating average populations and outliers; and separating normal physiological conditions and pathological conditions.

c. Study mechanisms of organismal macro-evolution and how changes in karyotype coding can create new species:

While the model of how karyotype change leads to speciation has been proposed (Heng, 2007b; Heng, 2019), it has a long way to go before the research community accepts it. Many questions need to be addressed, for example: How universal is chromosomal coding to define species knowing that it is rather common in angiosperms and in animals (Murphy et al., 2005; Heng, 2009; Dodsworth et al., 2016)? How are we to define species without typical chromosomal coding? Answering these questions requires an understanding of how genome-based information is packaged and regulated. The following approaches are useful: 1) creation of a testable model for the chromosomal code, 2) mechanistic study of chromosomal reshuffling to create new emergent information in evolution, and 3) development of working models where the new emergent genomic topology (with the same gene materials) drives a phenotype. In fact, the suggested chromosome shuffling experiments were already partly performed in yeast (see **Table 1**).

d. Clinical implications

Studying karyotype coding has clinical significance. Besides cancer prediction, it can potentially be used in many common and complex diseases. For example, chromosome instability has been proposed as a new general feature for diseases caused by cellular adaptation and its trade-off (see Horne et al., 2014; Heng et al, 2016). Somatic mosaicism needs to be considered as well as it can alter the phenotypes. Equally important, the combination of system inheritance and the fuzzy inheritance will provide a deep understanding of how environmental interaction contributes to disease phenotype based on the genome–environment interaction.

AUTHOR CONTRIBUTIONS

CY, AM, and HH, drafted the manuscript. LS and GL participated in the discussion, literature search, and editing of the manuscript.

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Recombinant Chromosomes Resulting From Parental Pericentric Inversions—Two New Cases and a Review of the Literature

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A balanced pericentric inversion is normally without any clinical consequences for its carrier. However, there is a well-known risk of such inversions to lead to unbalanced offspring. Inversion-loop formation is the mechanism which may lead to duplication or deletion of the entire or parts of the inverted segment in the offspring. However, also partial deletion and duplication may be an effect of a parental inversion, depending on the size of the inversion and the uneven number of crossing over events, also suggested to be due to an inversion loop. Here we describe two new cases of recombinant chromosomes and provide a review of the literature of comparable cases. Interestingly, this survey confirmed the general genetic principle that gain of copy numbers are better tolerated than losses. Furthermore, there is a non-random distribution of all human chromosomes concerning their involvement in recombinant formation, which is also discussed.

Keywords: balanced pericentric inversion, recombinant chromosomes, dosage sensitive genes, duplication, deletion

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INTRODUCTION

As reviewed by Martin (1991) one can find pericentric inversions in human in 1–2% of general population. Normally these balanced chromosomal rearrangements do not cause any problem for the carrier, but during meiosis there is a certain risk of inversion loop formation leading to *de novo* duplication (e.g. Malinverni et al., 2016), deletion (e.g. Lacbawan et al., 1999) or a combination of both in the offspring (**Supplementary Table 1**) when an uneven number of crossing over events occur within the inversion loop. If the latter happens, this is denominated as the formation of a recombinant chromosome. As explained by Morel et al. (2007) for male gametogenesis: "an odd number of crossovers within the loop results in one spermatozoon bearing the normal chromosome, one the inverted chromosome and two recombinants with both duplicated and deficient chromosome segments including the regions distal to the inversion [duplication q/deletion p (dup q/del p) or del q/dup p]". Besides, other rare rearrangements may be due to a parental inversion, like unequal crossing over (Yang et al., 1997), U-loop-formation (Ashley et al., 2007), breakage and unequal reunion of sister chromatids within the inversion loop (Phelan et al., 1993), or even ring-chromosome formation (Hu et al., 2006). Also, recombinants have been seen in triploid fetuses (Ekblom et al., 1993).

It has been originally suggested that viable, but mentally and physically impaired offspring can only result if the inversion includes less than 30% of the length of the affected chromosome (Martin, 1991). Before, it was proposed to determine the possibility for viable unbalanced offspring by measuring the percentage of haploid autosomal length of the chromosomal segments distal to the inversion-breakpoints (Daniel, 1981). Later on, Morel et al. (2007) suggested that no recombinants can be produced when the inverted segment size is <30%, a few recombinants when inverted segment size is within 30–50% and significant numbers are produced when the inverted segment size is >50% of the total length of the affected chromosome. Nonetheless, also examples were found for recombinants less than 100 Mb in size (Malan et al., 2006) or large families without any recombinant offspring not fitting to that suggested rules (Van der Linden et al., 1975; Honeywell et al., 2012).

Here we report two new cases with pericentric inversion and offspring with recombinant chromosomes and provide a review of overall 210 such cases {plus >100 cases with "recombinant chromosome 8 syndrome" [Online Mendelian Inheritance in Man (OMIM) 179613]}. This data includes also the 56 cases reviewed in 1997 by Ishii and colleagues. As preferential maternal origin of recombinant chromosomes was already shown by Ishii et al. (1997) this was not recapitulated in the present study. Similar effects are well known for small supernumerary marker chromosomes (Liehr 2006) and passing on of other kinds of chromosomal aberrations in human (Liehr et al., 2018). For clinical impact and impact of large and small to submicroscopic paracentric inversions, the latter being part of normal variance in humans, see Pettenati et al. (1995); Liehr et al. (2018) and database of genomic variants (http://dgv.tcag.ca/dgv/app/home).

MATERIAL AND METHODS

Cases Studied

The cases include here were identified during routine (molecular) cytogenetic diagnostics and informed consent for publication were provided.

Family 1: Here a healthy female had two affected children with two different male partners. The first son, 17y, showed slight mental impairment, dwarfism, sensorineural hearing loss, and facial dysmorphism; the second son, 6y, also had slight mental impairment, microcephaly, sensorineural hearing loss, dysmorphic signs. Blood samples were available from mother and the two children.

Family 2: Blood and amnion cells of a healthy pregnant female were studied due to sonographic abnormalities detected during routine diagnostics at 16 weeks of gestation.

Molecular Cytogenetic Tests

Blood and/or amnion from both families were subjected to routine cell culture or DNA-extraction using standard procedures. Metaphase preparation was performed according to standard procedures and karyotypes were analyzed by G-banding at a ~450 band level. Fluorescence *in situ* hybridization (FISH) was done using probes for subtelomeric regions of chromosome 18 (Abbott, Vysis, Wiesbaden, Germany), partial chromosome paints for the same chromosome (home brewed probes of Liehr and Claussen, 2002) or a multicolor banding probe set for chromosome 11 (Liehr et al., 2002). Array-comparative genomic hybridization (aCGH) was done as previously reported (Coci et al., 2017).

Literature Search

Born and unborn cases with recombinant chromosomes were put together based on Ishii et al. (1997); Schinzel (2001), and search in https://www.ncbi.nlm.nih.gov/pubmed and https://www.google.de/. *De novo* cases or such with not clarified parental origin were not included here. Definitely cases reported only on genetic meetings were missed, as those are neither provided systematically by any libraries not being available online.

RESULTS

Cases Studied

Family 1: The mother was identified to be carrier of a pericentric inversion in chromosome 18; karyotype: 46,XX,inv(18)(p11.22q22.3), while both children had the same recombinant chromosome 18; karyotype: 46,XY,rec(18)(pter->q22.3::p11.22->pter).arr[GRCh37] 18p11.32p11.22(118760_9774819)x3,18q22.3q23(69934975_78010032)x1 (**Figure 1A**).

Family 2: in the mother a karyotype 46,XX,inv(11)(p14.3q24) was identified and the unborn child was carrier of a karyotype: 46,XX,rec(11)(pter->q23?.3::p14.3->pter) (**Figure 1B**).

Literature Search

Literature search revealed overall 210 families/cases with recombinant chromosomes due to an inherited balanced pericentric inversion [**Supplementary Table 1**—plus >100 cases with "recombinant chromosome 8 syndrome" (OMIM 179613)]. Examples for all chromosomes were found, apart from Y-chromosome.

Recombinant chromosomes provide terminal deletions and duplications to the human genome. Based on **Supplementary Table 1** a scheme was drawn in **Figure 2** highlighting the terminal deletions and duplications being compatible with human live.

DISCUSSION

Two new cases of recombinant chromosomes (rec) were added to the yet reported 210 comparable cases [plus >100 cases with "recombinant chromosome 8 syndrome" (OMIM 179613)], which all are due to a parental pericentric inversion. In contrast to the review of Ishii et al. from 1997 now there are examples available for all human chromosomes, apart for Y-chromosome. However, rec(Y) chromosomes should only be possible in case of men with 2 Y-chromosomes, one with pericentric inversion; and such an instant was not reported yet.

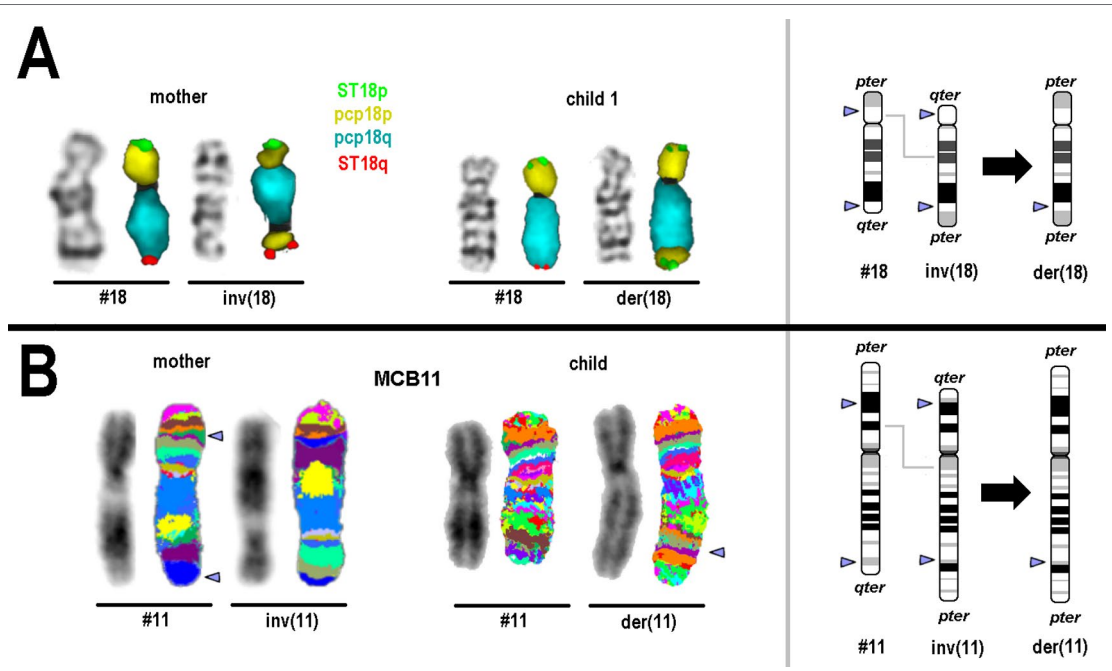

FIGURE 1 | Results of molecular cytogenetics performed for families 1 and 2. On the left side GTG-/inverted 4′,6-diamidino-2-phenylindole-banding result and FISH result of the corresponding normal and derivative chromosome is depicted. On the right schematic depictions of normal, inverted and derivative/recombinant chromosome is shown; breakpoints are highlighted by arrow-heads. **(A)** Normal and derivative chromosomes 18 of mother and child 1 after GTG-banding and FISH are visible. For FISH subtelomeric probes for 18pter (ST 18p) and 18qter (ST18q) and partial chromosome paints (pcps) for 18p and 18q were used. **(B)** Normal and derivative chromosomes 11 of mother and unborn child after inverted 4′,6-diamidino-2-phenylindole-banding and FISH are visible. For FISH a chromosome-11specific multicolor banding probe set was used—results are depicted in two different pseudocolor bandings; the latter had to be applied, due to different preparation qualities of blood and amnion derived chromosomes. Arrowheads highlight the chromosomal breakpoints.

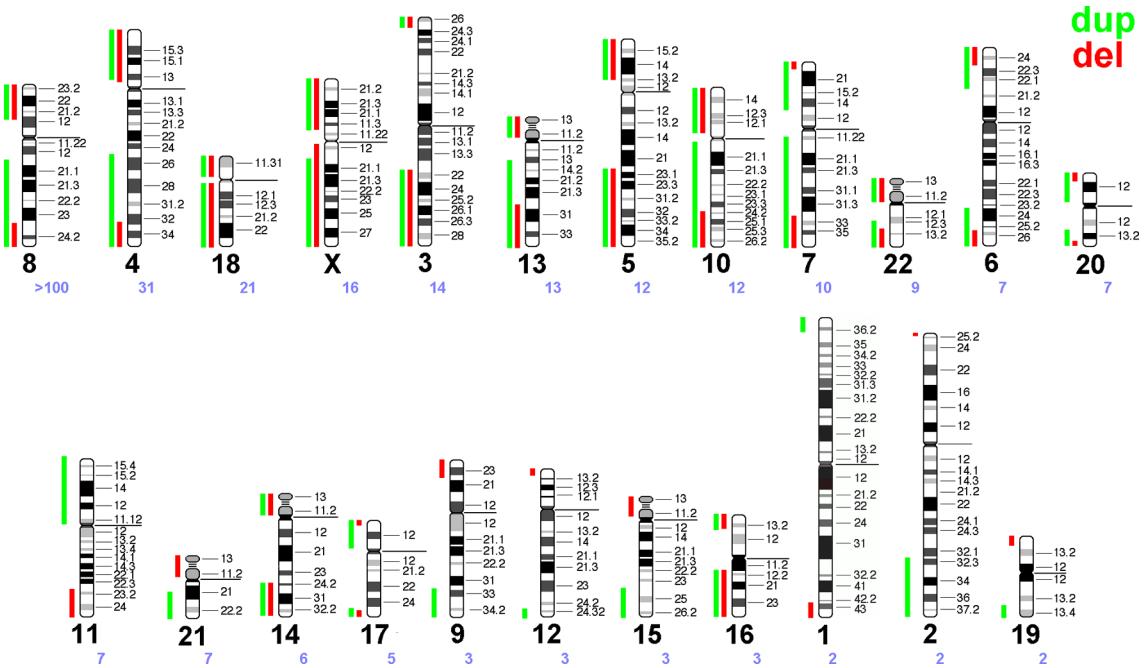

FIGURE 2 | Summary of the literature survey (see **Supplementary Table 1**). Maximal regions of terminal gains or losses along each human autosome and the X-chromosome are entered as green and red vertical lines, each. Chromosomes are sorted according to the number of cases reported with a corresponding recombinant chromosome due to a parental pericentric inversion—the chromosome number is given as a large black and the number of reported cases as a small violet number below each idiogram.

Interestingly, a well-known principle of copy number variants (CNVs) can also be deduced from **Figure 2**: gains of copy numbers are better compensable by human genome than losses; examples are microdeletion/microduplication syndromes or the fact that only trisomies 13, 18, and 21 are viable but not monosomies of those three chromosomes (Weise et al., 2012). In this study (**Figure 2**) for practically all chromosomes the regions compatible with live are lager for gains than for losses of copy numbers (see also **Table 1**).

The observed frequency of recombinants is chromosome-specific, as well as differences concerning arising of viable recombinant chromosomes are different:

- Chromosomes with more than 50 reports summarized in this study:Chromosome 8 is the only one with >100 reported cases and even an own OMIM number for a syndrome caused by this kind of rearrangement. Most likely this is due to a high frequency of inv(8)(p23.1q22.1) in Hispanic population in USA (Sujansky et al., 1993).
- Chromosomes with more than 7-10 reports summarized in this study:Cases involving chromosomes 4 and 5 may have been more frequently observed due to more detailed cytogenetic studies in patients with Wolf-Hirschhorn- (OMIM 194190) and Cri-du-Chat-syndrome (OMIM 123450), respectively. Chromosomes 13, 18, and 21 are the gene-poorest human chromosomes, thus, partial deletions in them are more tolerable than for other autosomes. Chromosomes 7 and 11 underlay imprinting and thus are also connected with well-known imprinting disorders [Wiedemann-Beckwith- (OMIM 130650) and Silver-Russel-syndrome (OMIM 180860)]; thus, patients with these disorders also may be studied more likely in detail than others. X-chromosome aberrations may lead to problems with sex-determination and/or infertility—thus also such aberrations are more likely to be picked up than other (autosomal) ones. For chromosomes 3 and 10, where recombinants are also regularly observed (**Figure 2**) these two chromosomes have in some populations regularly appearing large pericentric inversions [inv(3)(p25q23), inv(3)(p25q25), or inv(10)(p11q26) (Gardner and Amor 2018)], like reported for chromosome 8 in Hispanic population in USA.
- Chromosomes only rarely observed summarized in this study: Among remainder chromosomes some are relatively gene-rich (like chromosomes 1 and 19) or acrocentrics (in which pericentric inversions are quite rare, chrs. 13,14, 15, 21, and 22).

TABLE 1 | Percentage of maximally deleted and duplicated regions per chromosome arm, compatible with live.

chr.	del/p	dup/p	del/q	dup/q
X	82	83	95	83
1	n.a.	11	11	n.a.
2	4	n.a.	n.a.	10
3	10	10	63	63
4	90	83	16	58
5	75	75	50	50
6	24	57	13	31
7	8	70	28	94
8	65	65	22	80
9	35	n.a.	n.a.	29
10	87	87	33	98
11	n.a.	95	32	n.a.
12	15	n.a.	n.a.	10
13	n.a.	n.a.	41	85
14	n.a.	n.a.	35	35
15	n.a.	n.a.	n.a.	32
16	36	38	80	80
17	20	98	9	15
18	88	88	95	95
19	27	n.a.	n.a.	28
20	23	90	13	43
21	n.a.	n.a.	n.a.	73
22	n.a.	n.a.	43	57
average	**53**	**68**	**40**	**55**

Besides the yet discussed factors potentially influencing the frequencies of recombinant chromosomes in viable human offspring, different recombination rates and recombination hot-spots along each chromosome and in dependence of the gender meiosis is going through as nicely outlined by Bhatt et al. (2014) may also have an impact here.

According to Gardner and Amor (2018) formation of recombinant chromosomes in gametes of pericentric inversion carriers is a function of the size of the inversion: the larger the inversion, the more frequently recombinants are observable in the gametes. Also, p-deletion/q-duplication- appear about in same frequencies as q-deletion/p-duplication-recombinants. However, as already suggestable from data of Ishii et al. (1997), p-deletion/q-duplication is about double as frequent than q-deletion/p-duplication in viable forms of recombinant chromosomes. Considering the before discussed difference of CNVs, being present as gains or as losses, one would have to consider in general lower numbers of dosage sensitive genes in the p-arm of the human chromosomes than in the q-arms.

Overall, here an up to date review of pericentromeric inversion based viable recombinants is provided. Considering also recent new insights into influence of chromosomal rearrangements on interphase architecture (keyword: topologically associated domains = TADs) (Schrank and Gautier, 2019), as well as of overlap of evolutionary conserved breakpoints (important in speciation) and breakpoints observed in clinical cases (Liehr et al., 2011), the importance of gross cytogenetic aberrations to provide a better understanding of general principles of the human genome is highlighted.

DATA AVAILABILITY STATEMENT

The datasets for this study can be requested from the authors.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation

and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TL drafted the paper, and did the literature search. AW did the cytogenetic analyses of family 1. KM did the aCGH analyses of family 1. MZ, NP, and AA-R did the FISH analyses of both families. KW provided family 1 with clinical information.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01165/full#supplementary-material

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CASE REPORT
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Reproductive History of a Woman With 8p and 18p Genetic Imbalance and Minor Phenotypic Abnormalities

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We report on the phenotype and the reproductive history of an adult female patient with an unbalanced karyotype: 8p23 and 18p11.3 terminal deletions and 8p22 duplication. The indication for karyotyping of the 28-year-old patient was a structural rearrangement in her miscarriage specimen: 45,XX,der(8;18)t(8;18)(p23;p11.3). Unexpectedly, the patient had the same karyotype with only one normal chromosome 8, one normal chromosome 18, and a derivative chromosome, which was a product of chromosomes 8 and 18 fusion with loss of their short arm terminal regions. Fluorescence *in situ* hybridization revealed that derivative chromosome was a pseudodicentric with an active centromere of chromosome 8. Array comparative genomic hybridization confirmed 8p and 18p terminal deletions and additionally revealed 8p22 duplication with a total of 43 OMIM annotated genes being affected by the rearrangement. The patient had minor facial and cranial dysmorphia and no pronounced physical or mental abnormalities. She was socially normal, had higher education and had been married since the age of 26 years. Considering genetic counseling, the patient had decided to conceive the next pregnancy through *in vitro* fertilization (IVF) with preimplantation genetic testing for structural chromosomal aberrations (PGT-SR). She underwent four IVF/PGT-SR cycles with a total of 25 oocytes obtained and a total of 10 embryos analyzed. Only one embryo was balanced regarding chromosomes 8 and 18, while the others were unbalanced and demonstrated different combinations of the normal chromosomes 8 and 18 and the derivative chromosome. The balanced embryo was transferred, but the pregnancy was not registered. After four unsuccessful IVF/PGT-SR cycles, the patient conceived naturally. Non-invasive prenatal testing showed additional chromosome 18. The prenatal cytogenetic analysis of chorionic villi revealed an abnormal karyotype: 46,XX,der(8;18)t(8;18)(p23;p11.3)mat,+18. The pregnancy was terminated for medical reasons. The patient has a strong intention to conceive a karyotypically normal fetus. However, genetic counseling regarding this issue is highly challenging. Taking into

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account a very low chance of balanced gametes, emotional stress caused by numerous unsuccessful attempts to conceive a balanced embryo and increasing age of the patient, an IVF cycle with a donor oocyte should probably be considered.

Keywords: 8p deletion, 18p deletion, 8p duplication, genotype–phenotype correlation, miscarriage, PGT-SR, prenatal karyotyping, NIPT

BACKGROUND

Terminal deletions are among frequent karyotype abnormalities in the adult population. Deletions of the terminal regions have been described for every human chromosome. The genetic imbalance caused by terminal deletions is considered to be a major source of multiple congenital anomalies and mental retardation (Gardner et al., 2011). Telomeric deficiencies cause a number of syndromes including 1p36 deletion (1p-), Wolf-Hirschhorn (4p-), Cri-du-chat (5p-), Miller-Dieker (17p-), 18q-, and 22q- (Schinzel, 2001). In many cases, apparently simple terminal deletions are accompanied with submicroscopic duplications, triplications, and inversions (Ballif et al., 2003; Ballif et al., 2007; Rossi et al., 2008). Patients with such a genetic imbalance rarely have reproductive attempts; genetic counseling regarding the way of conception and adequate approaches for genetic diagnostics of their embryos is challenging.

Here, we report on the phenotype and the reproductive history of a female patient with 8p23 and 18p11.3 terminal deletions and a 8p22 duplication.

CASE PRESENTATION

The patient is 28-year-old woman who was referred to D.O. Ott Research Institute of Obstetrics, Gynecology and Reproductology (St. Petersburg, Russia) for cytogenetic analysis of a miscarriage specimen. The pregnancy was lost at 5/6 weeks of gestation. Conventional karyotyping revealed an abnormal karyotype in chorion with a total of 45 chromosomes, only one normal chromosome 8, one normal chromosome 18, and a derivative chromosome that resulted from a translocation of the chromosomes 8 and 18 with a loss of their short arm terminal regions: 45,XX,der(8;18)t(8;18)(p23;p11.3). The patient and her spouse underwent karyotyping which revealed a normal male karyotype in the spouse (46,XY) and an unbalanced female karyotype in the patient—45,XX,der(8;18)t(8;18)(p23;p11.3), the same as the one detected in the miscarriage specimen. The patient's parents were karyotypically normal.

The following genetic counseling revealed the patient's unremarkable family history. The patient was born after the second uneventful pregnancy (following the first ectopic pregnancy) of non-consanguineous parents of Russian ethnicity—a 29-years-old mother and a 34-year-old father. During the preconception period and the first trimester, the parents lived in Luanda, Angola—a territory with an elevated level of radiation because of uranium mines. The patient's mother worked as a translator; the father, as a radio operator on a MI-8 helicopter. In the second trimester, the parents returned to Russia. The patient was born in term by spontaneous vaginal delivery complicated by footling presentation and fetal asphyxia. Her birth weight was 3,200 g, her length—49 cm.

During the first year of life, the patient was followed up by a neurologist because of hypotonia and received vitamin therapy and therapeutic massage sessions. During childhood and adolescence, the patient was followed up by an endocrinologist because of obesity. The patient never demonstrated either behavioral or mental health problems. She completed a general primary school, received general secondary education and higher education. The patient works as a child psychologist. The patient had been married since the age of 26 years. Taken together, these data strongly indicate her good social adaptation.

At the genetic counseling, the patient had first-degree obesity: her height was 158 cm (the mother's height—160 cm, the father's height—168 cm), weight—78 kg, BMI 31.24 kg/m^2. The patient had minor facial and cranial dysmorphia: flattened superciliary arches, a narrow palpebral fissure, hanging eyelids, a wide nasal arch and a fleshy nose tip. She had a bit shortened arm length, a slight valgus deformity, and disproportionately large feet (**Figure 1A**). In general, the patient looks like her mother.

The patient had menarche at the age of 11 years, regular menstrual cycles of 28 days with menses lasting 4 to 5 days. By the time of the genetic counseling, the patient had experienced two naturally conceived pregnancies; both ended in a miscarriage at 5/6 weeks of gestation. Cytogenetic analysis was performed only for the second miscarriage specimen and revealed the above-described karyotype. An ultrasound examination of the patient's pelvic organs showed an unremarkable uterus and ovaries of normal size with 11–13 antral follicles. Her hormonal status was normal: follicle-stimulating hormone (FSH)—7.43 mIU/ml, luteinizing hormone (LH)—1.8 IU/l, anti-müllerian hormone (AMH)—1.31 ng/ml, prolactin—281 ng/ml, dehydroepiandrosterone (DHEA)—7.0 ng/ml, 17-OHP—4.6 ng/ml, thyroid-stimulating hormone (TSH)—1.32 mU/l. Genetic testing for common *CYP21A2* genetic variants revealed heterozygosity for V281L

Abbreviations; aCGH, array comparative genomic hybridization; AMH, anti-müllerian hormone; BMI, body mass index; CNV, copy number variation; DHEA, dehydroepiandrosterone; ER, estrogen; FISH, fluorescent *in situ* hybridization; FSH, follicle-stimulating hormone; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; GnRH, gonadotropin-releasing hormone; GV, germinal vesicle; LH, luteinizing hormone; NIPT, non-invasive prenatal testing; OMIM, Online Mendelian Inheritance in Man; PGT-SR, preimplantation genetic testing for structural chromosomal aberrations; PHA, phytohemagglutinin; PR, progesteron; QFH/AcD, quinacrine fluorescence Hoechst/actinomycin D.

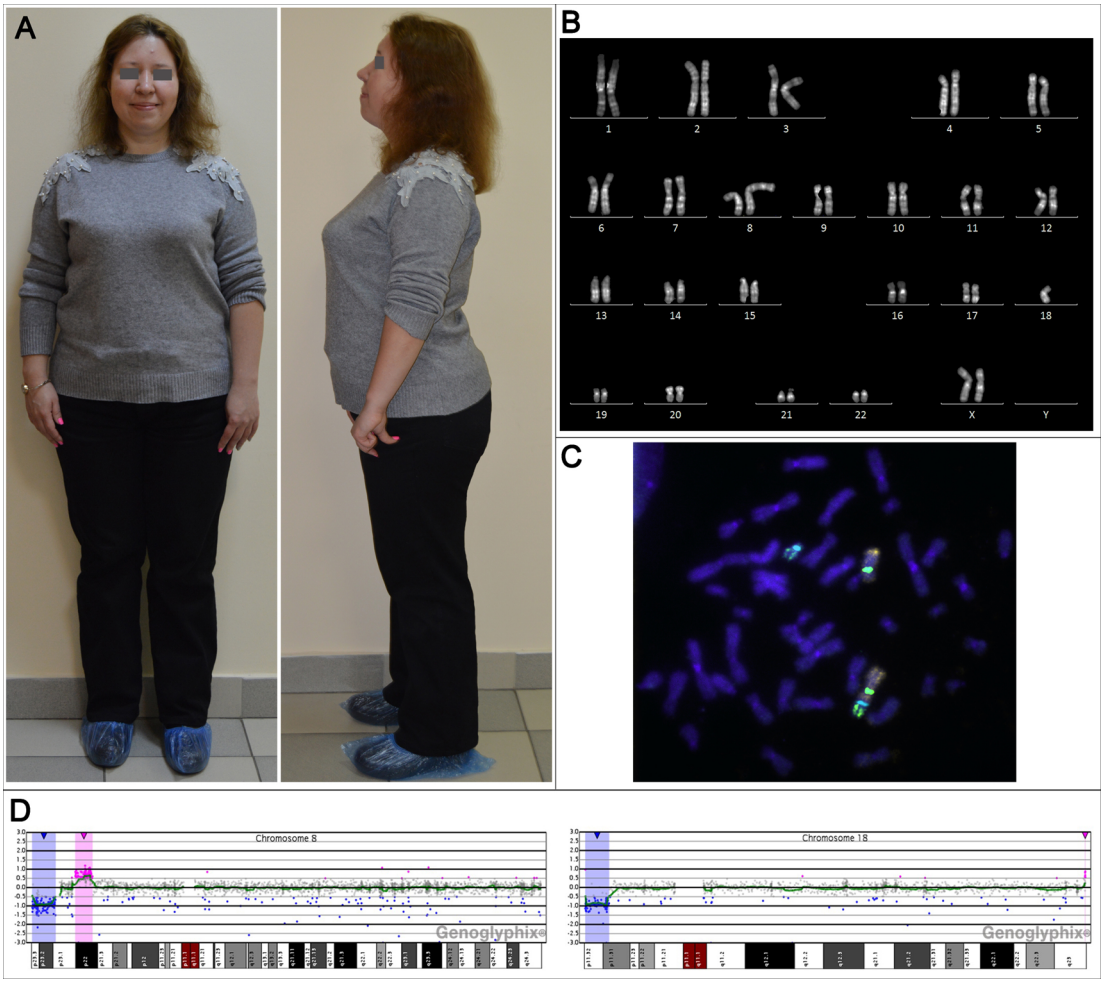

FIGURE 1 | The phenotypic and genotypic characteristics of the adult female patient with unbalanced karyotype 45,XX,der(8;18)t(8;18)(p23;p11.3). **(A)** The patient's appearance at the age of 31 years. **(B)** The karyogram of QFH/AcD stained chromosomes from the patient's PHA-stimulated lymphocyte. **(C)** Metaphase chromosomes from the patient's PHA-stimulated lymphocyte after fluorescence *in situ* hybridization with DNA probes specific to chromosomes 8 and 18: Vysis CEP 8 (D8Z2) (green), CEP 18 (aqua) (Abbott Molecular), whole chromosome 8 painting probe (orange), whole chromosome 18 painting probe (green) (Applied Spectral Imaging). **(D)** aCGH results for chromosomes 8 and 18; deleted regions are marked with blue, duplicated regions are marked with red.

substitution; the patient's spouse had no *CYP21A2* genetic variants. Considering the genetic counseling, the patient had decided to conceive the next pregnancy through *in vitro* fertilization (IVF) with preimplantation genetic testing for structural chromosomal aberrations (PGT-SR).

During the subsequent 2-year period, the patient underwent four IVF/PGT-SR cycles. In one cycle, an embryo transfer was performed; the pregnancy was not registered. After another two months, the patient conceived naturally. At early stages of gestation, the patient had an embryonic delay and a threatened miscarriage and was successfully treated with progestogens. At a gestational age of 9/10 weeks, the patient was recommended chorionic villus sampling for prenatal karyotyping. In spite of the known increased risk of having a karyotypically abnormal fetus, she refused to undergo invasive prenatal diagnosis due to the fear of pregnancy loss. As an alternative, she was recommended non-invasive prenatal testing (NIPT). The NIPT showed an additional copy of chromosome 18 material. An ultrasound examination of the fetus at 12/13 weeks of gestation showed total edema and an increased nuchal translucency (5.2 mm). The patient was repeatedly recommended prenatal karyotyping, and chorionic villus sampling was performed. The cytogenetic analysis revealed an abnormal karyotype with a maternally inherited derivative chromosome and an additional chromosome 18: 46,XX,der(8;18)t(8;18)(p23;p11.3)mat,+18. The patient was recommended to terminate the pregnancy for medical reasons. Dilation and curettage was performed. A subsequent histological and immunohistochemical analysis of the obtained endometrium showed no hormonal pathology: the expression of ER and PR receptors was in accordance with the gestational age. Four months after the curettage, the patient had normal results of the pelvic organs ultrasound examination with 10 antral follicles in ovaries and normal endometrial thickness.

LABORATORY INVESTIGATIONS AND DIAGNOSTIC TESTS

Genetic Characterization of the Patient

Conventional karyotyping was performed on PHA-stimulated patient's peripheral blood lymphocytes. Preparation of metaphase chromosomes from fixed cell suspension and QFH/AcD staining was performed according to the standard technique with minor modifications described previously (Grigorian et al., 2010). The patient's karyotype was unbalanced, with a total of 45 chromosomes. There was only one normal chromosome 8 and only one normal chromosome 18. The aberrant chromosome was a product of chromosomes 8 and 18 fusion with apparent loss of their short arm terminal regions (**Figure 1B**). The karyotype was designated 45,XX,der(8;18)t(8;18)(8qter→8p23::18p11.3→18qter)dn.

To investigate the structure of the aberrant chromosome, fluorescence *in situ* hybridization (FISH) was performed on metaphase preparations using DNA probes specific to chromosomes 8 and 18: TelVysion 8p, TelVysion 18p, Vysis CEP 8 (D8Z2), CEP 18 (Abbott Molecular), whole chromosome 8 painting probe, and whole chromosome 18 painting probe (Applied Spectral Imaging). FISH signals were analyzed using a Leica DM 2500 microscope with a Leica DFC345 FX camera and the Leica Application SuiteV.3.8.0 software. The aberrant chromosome consisted of chromosome 8 and 18 material and contained FISH signals of both centromeres. Morphologically, only the centromere of chromosome 8 formed a constriction, indicating that the aberrant chromosome was a pseudodicentric with one active centromere—that of chromosome 8 (**Figure 1C**). The aberrant chromosome lacked both 8p and 18p subtelomeric regions. The breakpoints were in 8p23 and 18p11.3. Thus, conventional karyotyping and FISH showed a double partial monosomy involving 8p and 18p subtelomeric regions. The resulting karyotype was designated 45,XX,psu dic(8;18)(8qter→8p23::18p11.3→18qter)dn.

To determine the precise size of the deletions, array comparative genomic hybridization (aCGH) was performed (CGXv1.1 8x60K, PerkinElmer) using the protocol recommended by the manufacturer. DNA was extracted from peripheral blood lymphocytes. aCGH revealed a 6.718 Mb deletion in 8p23.1–p23.3, a 3.693 Mb deletion in 18p11.31–p11.32 and, in addition to karyotyping and FISH results, a 4.937 Mb duplication in 8p22 and a 0.060 Mb duplication in 18q23 classified as a copy number variation (CNV) (**Figure 1D**). Thus, the results of aCGH were as follows:

1. arr[GRCh37] 8p23.1p23.3(202133_6920415)x1
2. arr[GRCh37] 8p22(12582909_17519858)x3
3. arr[GRCh37] 18p11.31p11.32(146484_3839773)x1
4. arr[GRCh37] 18q23(77954106_78013620)x3.

The affected chromosome regions contained 44 OMIM annotated genes: 16 genes in 8p deleted region, 12 genes in 8p duplicated region, 15 genes in 18p deleted region, and 1 gene in 18q duplicated region.

Outcomes of *In Vitro* Fertilization Protocols

The patient underwent four standard GnRH antagonist protocols. The outcomes of the protocols are summarized in the **Table 1**. In total, ultrasound monitoring showed 32 preovulatory follicles. A total of 25 cumulus–oocyte complexes were obtained by transvaginal aspiration. Of them, only 13 reached MII, 3 reached MI and the remaining ones degraded or were at the germinal vesicle (GV) stage. Intracytoplasmic sperm injection (ICSI) was performed for 16 oocytes (13 at MII and 3 at MI stage). After 20 h, two pronuclei were registered in 12 oocytes, three pronuclei—in 1 oocyte, and no pronuclei—in 3 oocytes. The embryos were cultured for 4–6 days under standard conditions. Since the third day of development, the embryos showed developmental

TABLE 1 | The outcomes of four IVF/PGT-SR cycles undergone by the female patient with unbalanced karyotype 45,XX,der(8;18)t(8;18)(p23;p11.3).

IVF/ICSI cycle	Treatment protocol	Follicles expected, n	Cumulus-oocyte complex, n	MII oocytes	Biopsied embryos on 3 day, n	Genetically balanced embryos regarding chromosomes 8 and 18, n	Outcome
1	GnRH antagonist + corifollitrophin alfa/HMG	11	4	1	0	–	No embryo transfer
2	GnRH antagonist + rFSH	9	9	4	4	1	Transfer of one embryo; no pregnancy
3	GnRH antagonist+ corifollitrophin alfa/HMG	8	8	5	4	0	No embryo transfer
4	GnRH antagonist + rFSH	4	4	3	2	0	No embryo transfer

delay, fragmentation, insufficient or absent compaction, and impaired blastulation.

Blastomere biopsy was performed on day 3 for 10 embryos. PGT-SR was performed by FISH with DNA probes specific to the p and q subtelomeric regions and the centromeric regions of chromosomes 8 and 18: TelVysion 8p, TelVysion 18p, TelVysion 8q, TelVysion 18q, Vysis CEP 8 (D8Z2), CEP 18 (Abbott Molecular). In 9 out of 10 cases, the PGT-SR results were informative. In all but one case, the embryos were genetically unbalanced. The most probable chromosome combinations based on the revealed FISH signal patterns are shown on **Figure 2**. A total of seven genetic imbalance variants were registered among eight abnormal embryos. This advocates for a variety of chromosome disjunction patterns with no obvious prevalence of either variant (**Figure 2**). The only genetically balanced embryo developed to a 3CC blastocyst (Gardner grade) by day 6 and was transferred. The pregnancy was not registered.

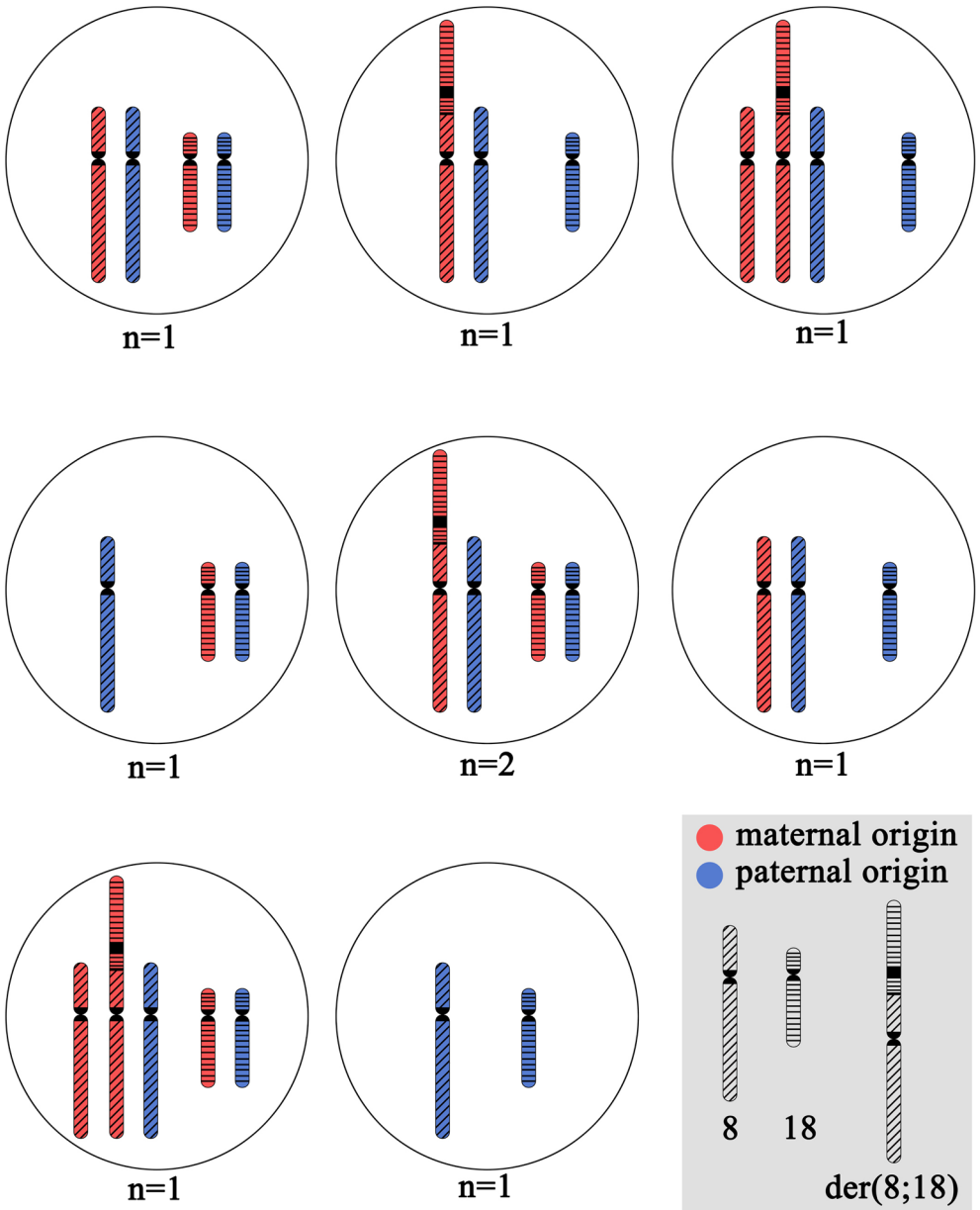

FIGURE 2 | The cytogenetic picture of preimplantation embryos from the patient with unbalanced karyotype 45,XX,der(8;18)t(8;18)(p23;p11.3). The shown chromosome combinations are based on the revealed FISH signal patterns during PGT-SR with DNA probes specific to p and q subtelomeric regions and centromeric regions of chromosomes 8 and 18: TelVysion 8p, TelVysion 18p, TelVysion 8q, TelVysion 18q, Vysis CEP 8 (D8Z2), CEP 18 (Abbott Molecular). In all cases, the inheritance of normal chromosome 8 and normal chromosome 18 from the karyotypically normal farther is assumed. N indicates the number of embryos with detected variant of chromosome combination.

Outcomes of the Patient's Naturally Conceived Pregnancies

The patient experienced three naturally conceived pregnancies: two pregnancies prior to and one after the IVF/PGT-SR cycles. The first naturally conceived pregnancy ended in a miscarriage at 5/6 weeks of gestation. The miscarriage specimen was not karyotyped. The second naturally conceived pregnancy also ended in a miscarriage at 5/6 weeks of gestation. The miscarriage specimen obtained by curettage of the uterine cavity was sent for karyotyping. Chorionic villi were selected and released from the maternal decidua and blood clots under a Leica M125 stereomicroscope. Metaphase chromosomes were prepared from the chorionic villi by the direct technique (without culturing) according to the protocol developed for tissues containing dividing cells (Baranov et al., 1990) with modifications (Pendina et al., 2014; Efimova et al., 2017; Efimova et al., 2018). Karyotyping was performed on QFH/AcD-stained metaphases. An unbalanced karyotype with structurally rearranged chromosome was revealed: 45,XX,der(8;18)t(8;18)(p23;p11.3) (**Figure 3A**). The detection of this chromosome abnormality in chorionic villi initiated the story as it was the only indication for karyotyping of the patient and her spouse.

After four unsuccessful IVF/PGT-SR cycles (see *Outcomes of in vitro fertilization protocols*), the patient conceived the third natural pregnancy. NIPT was performed at the gestational age of 9/10 weeks. Blood sample was collected to an EDTA-K2 tube. The plasma was separated by two-step centrifugation. DNA was extracted from 2 ml of plasma using the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific Inc., USA). DNA library was prepared using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific Inc.) according to a modified protocol. The library was then templated by an Ion Chef system and sequenced on an Ion Torrent S5 system (Thermo Fisher Scientific Inc.) The sequencing depth of the sample was 0.03×. The reads were aligned to GRCh37 reference and filtered by mapping quality (> 10) and read length (70–130 bp). The reads were divided into 50 kbp bins and Z score was estimated for each bin as described previously (Johansson et al., 2017). According to the previously described protocol (Ivashchenko et al., 2019), the sample was considered to be aneuploid if the average Z score for a chromosome was above 3 or below -3. The NIPT of the patient's sample revealed additional material of chromosome 18 (**Figure 3B**).

To verify the NIPT results by cytogenetic analysis, chorionic villus sampling was performed. The preparation and staining of metaphase chromosomes was made as described above. An abnormal karyotype was detected, as there was only one normal chromosome 8, two normal chromosomes 18, and an aberrant chromosome der(8;18) (**Figure 3C**). Most likely, the fetus inherited the aberrant chromosome der(8;18) together with one normal chromosome 18 from the patient; one more normal chromosome 18 came from the patient's spouse thus forming a karyotype with in fact three copies of chromosome 18. The pregnancy was terminated for medical reasons.

DISCUSSION

A genotype–phenotype correlation is of high importance for genetic counseling of unbalanced karyotype carriers. In certain cases, the same genetic imbalance may cause different phenotypes including normal ones and severely pathological ones.

Clinical picture is highly variable in patients with small terminal deletions of 8p. Based on phenotype severity, three groups of 8p deletion carriers may be distinguished. The first group is the most numerous and includes children karyotyped during the first year of life because of facial dysmorphism, neurological symptoms (convulsions) and congenital malformations including heart, genitourinary, diaphragmatic and central nervous system defects (Hutchinson et al., 1992; Wu et al., 1996; Pehlivan et al., 1999; Shimokawa et al., 2004). Patients of the second group are referred to genetic counseling and karyotyping because of severe behavioral disturbances with outbursts of aggressiveness and destructiveness and mild mental defects in childhood (Gardner et al., 2011). In some cases, the clinical manifestation is similar to that of fragile X chromosome syndrome (Wu et al., 1996). The third group is the smallest one; it includes patients who are referred for genetic counseling because of reproductive disorders. These patients are mentally normal and have no malformations or dysmorphia (Pettenati et al., 1992; Reddy, 1999). In some cases, 8p terminal deletions are associated with autism (Chien et al., 2010).

A variability of the clinical picture is also typical for carriers of 8p duplications. In some cases, children who inherit the duplication from a phenotypically normal parent manifest a number of anomalies including minor facial anomalies (a wide nasal arch), moderate mental retardation and autistic behavior (https://decipher.sanger.ac.uk/). A small duplication limited to the 8p22 region is associated with developmental delay (Buysse et al., 2009). Therefore, patients with 8p terminal deletions as well as with 8p duplications have no common behavioral or phenotypic features that can be classified as specific syndromes.

In contrast, deletion of the chromosome 18 short arm is known as the 18p- syndrome (OMIM #146390). Patients with a deletion of almost the entire 18p feature short stature, microcephaly, dysmorphism, round face, mild to moderate mental retardation and behavioral disturbance (de Grouchy, Turleau, 1984). However, if the deletion does not affect the whole arm, the clinical picture may be less pronounced and may include only some or even none of the listed abnormalities.

The patient reported in this study has a 8p22 duplication and terminal deletions both in 8p and 18p. Surprisingly, she has no physical, mental or behavioral abnormalities. The only reason for karyotyping was a structural chromosome rearrangement detected in the patient's miscarriage specimen. Of all the clinical traits associated with an 8p and 18p imbalance, the patient features only a minor dysmorphism that, in fact, does not exceed that of normal physiology—flattened superciliary arches, a wide nasal arch and a fleshy nose tip. The underlying basis for this highly unexpected genotype-phenotype correlation remains obscure. Although rare cases of phenotypically normal patients with 8p or 18p imbalance are described (Pettenati et al., 1992; Reddy, 1999; https://decipher.sanger.ac.uk/; Liehr, 2019), it is almost unbelievable that a combination of both 8p and 18p terminal deletions accompanied by 8p22 duplication may not cause serious clinical manifestations.

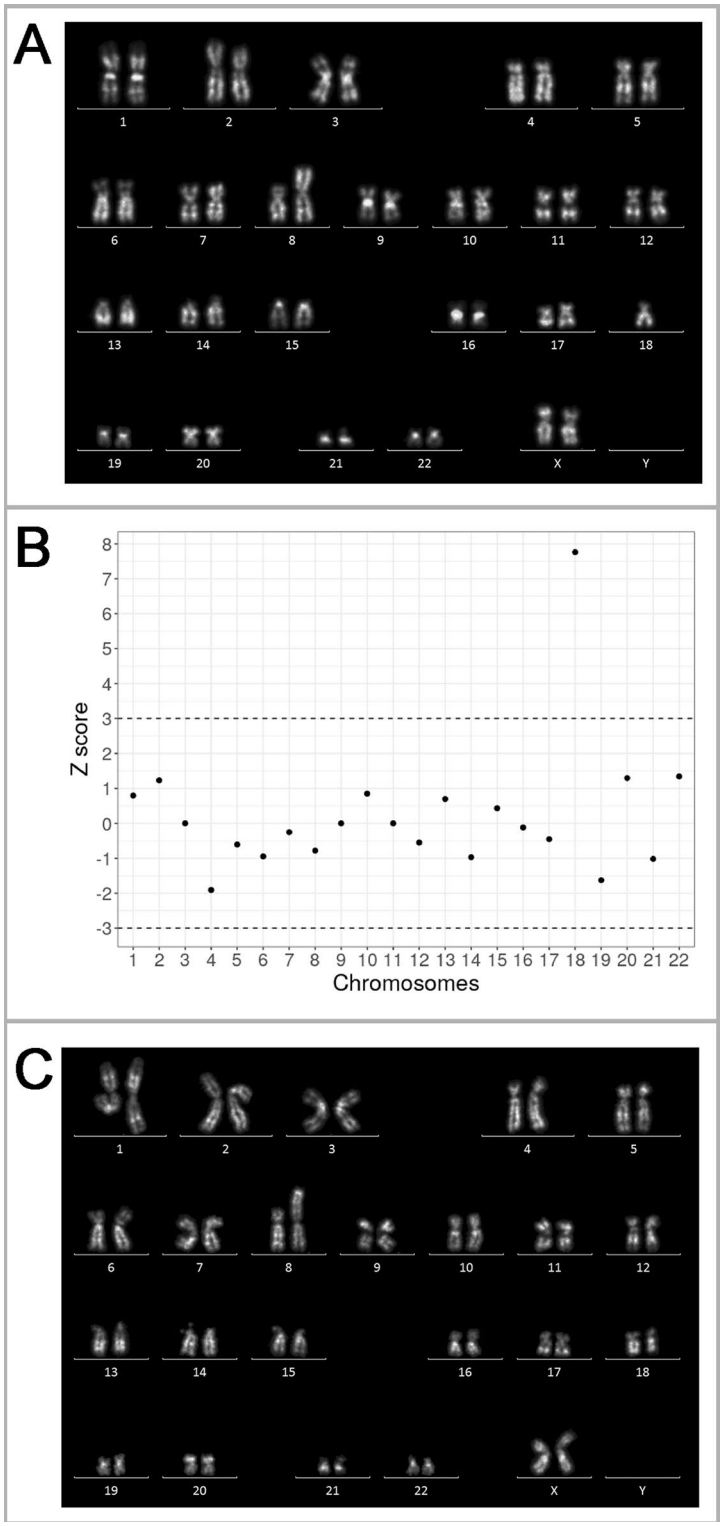

FIGURE 3 | Genetic outcomes of the natural pregnancies in the patient with unbalanced karyotype 45,XX,der(8;18)t(8;18)(p23;p11.3). **(A)** The karyogram of QFH/AcD banded chromosomes from chorionic villi of the miscarriage specimen at 5/6 weeks of gestation, karyotype 45,XX,der(8;18)t(8;18)(p23;p11.3)mat. **(B)** Summary of non-invasive prenatal testing (NIPT) results in progressing pregnancy at 9/10 weeks of gestation. Z scores for the corresponding chromosomes are present on the y-axis. The dash-lines represent the upper and lower limits of the z score for euploidy. The 18 chromosome score is classified as trisomy. **(C)** The karyogram of QFH/AcD banded chromosomes from chorionic villi in progressing pregnancy with trisomy 18 detected by NIPT. The karyotype is 46,XX,der(8;18)t(8;18)(p23;p11.3)mat,+18.

The patient is socially normal, and it is her reasonable wish to realize her reproductive rights. However, the genetic counseling regarding this issue is highly challenging. She is likely to have a good reproductive potential, as could be concluded from her normal hormonal status, normal endometrium and her capability to conceive naturally with no specific effort. Her unusual karyotype abnormality and the resulting increased frequency of abnormal gametes, seems to be the only cause of the patient's reproductive failures.

Carriers of structurally rearranged chromosomes are at high risk of having genetically unbalanced offspring (Gardner et al., 2011). However, the theoretically expected ratio of balanced and unbalanced gametes depends on the type of structural rearrangement. In the patient reported in this study, in the case of alternative segregation, the balanced gametes are only those with a normal chromosome 8 and a normal chromosome 18. The counterparts receiving der(8;18) chromosome are unbalanced. All other segregation types form unbalanced gametes. Therefore, the patient theoretically has two times lower chance of balanced gametes than the carriers of reciprocal translocations. Moreover, segregation of dicentric chromosome may result in *de novo* chromosomal rearrangements including complex ones such as chromothripsis (Koltsova et al., 2019). Indeed, among 11 genetically analyzed products of conception (one miscarriage, 9 preimplantation embryos and one prenatally analyzed embryo), only one was balanced regarding the rearrangement. The other embryos were unbalanced; they demonstrated different combinations of normal chromosomes 8 and 18 and the rearranged one with no prevalence of any of the variants.

It should be emphasized that inheritance of the patient's derivative chromosome by her offspring together with normal chromosomes 8 and 18 from the patient's spouse is not desirable. Even though in this case the fetus would have the same karyotype as the patient, the phenotypic effect of the aberration may be unpredictable. Pettenati et al. reported on a family where a brother and a sister inherited a 8p23 deletion from their phenotypically normal father. The boy had mental retardation, behavioral problems, convulsions and hydrocephalus. His sister had no mental problems, but was hyperactive (Pettenati et al., 1992). In another study, a 6-month-old girl who inherited a 8p23.1~23.2 deletion from her asymptomatic father also had no clinical manifestations (Reddy, 1999).

Considering the very low chance of balanced gametes, the emotional stress caused by numerous unsuccessful attempts to conceive a balanced embryo and the increasing age of the patient, which in turn elevates the risk of other aneuploidies, the patient has been offered an IVF cycle with a donor oocyte. The patient has a strong intention to conceive a karyotypically normal fetus, but she has not yet decided whether to make another attempt of the IVF/PGT-SR cycle with her own oocytes or to use donor cells.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of D.O. Ott Research Institute of Obstetrics, Gynecology and Reproductology. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Design and conception of the study: AP, YVS, OT, OE, IK. Laboratory investigations and diagnostic tests: AP, YVS, OT, OC, OM, VD, OE, LP, AK, AT, TT, NO, MK, AP-K, TK, TI, GT, OG, OR, AS, SU, VT, YE, SS, YMS, IM, EK, EV, PK, ND, ASG, AMG, IK. Genetic counseling: EAS, ESS. Writing of the manuscript: OE, AP. Checking of the manuscript for important intellectual content: OC, VB, AMG, IK. All authors read and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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New Insights Into Chromomere Organization Provided by Lampbrush Chromosome Microdissection and High-Throughput Sequencing

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Giant lampbrush chromosomes (LBCs) typical for growing oocytes of various animal species are characterized by a specific chromomere-loop appearance and massive transcription. Chromomeres represent universal units of chromatin packaging at LBC stage. While quite good progress has been made in investigation of LBCs structure and function, chromomere organization still remains poorly understood. To extend our knowledge on chromomere organization, we applied microdissection to chicken LBCs. In particular, 31 and 5 individual chromomeres were dissected one by one along the macrochromosome 4 and one microchromosome, respectively. The data on genomic context of individual chromomeres was obtained by high-throughput sequencing of the corresponding chromomere DNA. Alignment of adjacent chromomeres to chicken genome assembly provided information on chromomeres size and genomic boarders, indicating that prominent marker chromomeres are about 4–5 Mb in size, while common chromomeres of 1.5–3.5 Mb. Analysis of genomic features showed that the majority of chromomere-loop complexes combine gene-dense and gene-poor regions, while massive loopless DAPI-positive chromomeres lack genes and are remarkably enriched with different repetitive elements. Finally, dissected LBC chromomeres were compared with chromatin domains (topologically associated domains [TADs] and A/B-compartments), earlier identified by Hi-C technique in interphase nucleus of chicken embryonic fibroblasts. Generally, the results obtained suggest that chromomeres of LBCs do not correspond unambiguously to any type of well-established spatial domains of interphase nucleus in chicken somatic cells.

Keywords: chromatin domains, lampbrush chromosome, chromomere, chromosome microdissection, chicken

INTRODUCTION

In highly extended chromosomes, such as polytene chromosomes, lampbrush chromosomes (LBCs), and pachytene chromosomes, a chromomere is defined as a universal unit of chromatin packaging (Vlad and Macgregor, 1975). While our understanding of structure and function of chromomeres in polytene chromosomes has considerably grown in recent years, chromomere organization in LBCs still remains poorly understood.

Chromomeres of lampbrush chromosomes, being typical for animal growing oocytes, are regarded as condensed and apparently transcriptionally inactive chromatin domains (Vlad and Macgregor, 1975; Macgregor, 2012). LBC chromomeres can be seen in both fixed and living chromosome preparations. An array of chromomeres constitutes an axis of each LBC, with neighboring chromomeres being connected by thin decondensed chromatin threads (interchromomeric fibers). Generally, chromomeres are unevenly distributed along the chromosome axis: arrays of massive and prominent chromomeres alternate with regions of small and medium-sized ones (Callan, 1986; Rodionov et al., 2002; Galkina et al., 2006). Besides, apart from chromomeres with numerous pairs of extended loops, there are some chromomeres lacking recognizable loops. Since chromomeres constantly appear in the same positions one can develop cytological maps reflecting the number, size, and general pattern of distribution of chromomeres along LBC's axes (Galkina et al., 2005; Galkina et al., 2006; Daks et al., 2010; Zlotina et al., 2012). One of the notable examples is LBC W of the domestic chicken that consists of seven distinct chromomeres (Solovei et al., 1993). From structural point of view LBC chromomeres are thought to represent a rosette of microloops, which are connected by protein clips at their bases. In particular, cohesin and condensin complexes that were found in LBC chromomeres can serve as such longitudinal and transverse clips (Beenders et al., 2003; Krasikova et al., 2005; Austin et al., 2009). Intriguingly, LBC axes lack any linker histones H1 (Hock et al., 1993).

Being compact chromatin domains, the overwhelming majority of chromomeres are enriched with epigenetic landmarks typical for inactive chromatin: 5-methylcytosine-modified DNA and the methylated DNA-binding protein MeCP2, histone H3 trimethylated at lysine 9 or lysine 27, as well as heterochromatin protein HP1β (Krasikova et al., 2009; Morgan et al., 2012; Krasikova and Kulikova, 2017). Certain chromomeres are less compact and looser in appearance and also comprise some amount of hyperacetylated histone H4 (Sommerville et al., 1993), which can be explained by transcriptional activity of certain microloops being a part of a chromomere. Nevertheless, while there is some data on overall structure, protein composition, and epigenetic status of LBC chromomeres, their genomic context has not been a focus of previous studies.

Morphologically discrete chromomeres can be mechanically dissected from a single copy of LBC by glass needles. Moreover, DNA fragments from individual isolated chromomeres can be deciphered by one of the next generation sequencing (NGS) approaches and assigned to certain regions in the reference genome assembly (Zlotina et al., 2016).

To extend our knowledge on chromomere organization and genomic context, we performed microdissection of all prominent chromomeres from lampbrush macrochromosome 4 and one of the microchromosomes in a chicken lampbrush chromosome set. The data on cytogenetic and genomic features of individual chromomeres were obtained by high-resolution fluorescence *in situ* hybridization (FISH) and high-throughput sequencing procedure. Finally, LBC chromomeres were compared with chromatin domains earlier identified by Hi-C technique in interphase nucleus of chicken embryonic fibroblasts. Generally, the results obtained in the present study suggest that chromomeres of LBCs do not correspond unambiguously to any type of well-established chromatin domains of interphase nucleus of somatic cells.

MATERIALS AND METHODS

Chromosome Preparation and Needle-Based Microdissection Procedure

Chicken lampbrush chromosomes (LBCs) were manually isolated from growing oocytes, fixed in 2% formaldehyde and dehydrated as described elsewhere (https://projects.exeter.ac.uk/lampbrush/). Mitotic metaphase chromosomes were obtained from chicken embryonic fibroblasts according to standard protocols. All institutional and national guidelines for the care and use of laboratory and farm animals were followed. The animal studies received approval #131–03-2 of the Ethical committee of Saint-Petersburg State University.

Glass needle-microdissection of LBC chromomeres was performed according to the previously published protocol with some modifications (Zlotina et al., 2016; Zlotina et al., 2019). In brief, individual chromomeres were dissected one after another along the length of macrochromosome 4 and one of the microchromosomes under phase contrast microscopy. The microdissected fragments were transferred into micropipettes with a collection drop solution (30% glycerol, 10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 0.1% SDS, 1 mM EDTA, 0.1% Triton X-100, 1.44 mg/ml proteinase K) followed by incubation at 60°C for 1–2 h. Primary amplification of the isolated DNA material was performed using DOP-PCR (degenerate oligonucleotide-primed PCR) with a degenerate universal primer 5′-CCG ACT CGA GNN NNN NAT GTG G-3′ as previously described (Zlotina et al., 2016).

Fluorescence *In Situ* Hybridization

Probes Preparation

The primary DOP-PCR products were differentially labeled with biotin or digoxigenin by PCR with the same degenerate primer (for details see Zlotina et al., 2016). Labeled PCR products were dissolved in a standard hybridization buffer (50% deionized formamide [ICN], 2×SSC, 10% dextran sulphate [Sigma]) to a final concentration of 20–40 ng/μl with a 50-fold excess of salmon sperm DNA (Invitrogen).

In Situ Hybridization

The obtained FISH-probes were applied to mitotic metaphase and LBCs. Metaphase chromosomes were pre-treated with 0.01% pepsin and post-fixed with 1% formaldehyde in 1×PBS. FISH on LBCs was performed according to a DNA/(DNA+RNA) hybridization protocol without any pretreatments. Chromosomes and DNA-probes were co-denatured on a slide under a coverslip at 78°C for 5 min followed by hybridization at 37°C in a humid chamber for 16–20 h. Post-hybridization washings included two changes of 0.2×SSC at 60°C and two changes of 2×SSC at 45°C. Avidin-Alexa 488 (Molecular Probes Inc.) and mouse antibody against digoxigenin conjugated with Cy3 (Jackson ImmunoResearch Laboratories) were used to detect biotin- and digoxigenin-labeled probes, respectively. To amplify the signals, we performed an additional incubation with biotinylated anti-avidin (Vectorlabs) followed by the second round of incubation with avidin-Alexa 488 for biotin-labeled probes, and incubation with Cy3-conjugated goat anti-mouse IgG+IgM (H+L) (Jackson ImmunoResearch Laboratories) for digoxigenin-labeled probes. All preparations were dehydrated, air-dried, and mounted in antifade solution containing 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI).

DNA-Library Preparation and High-Throughput Sequencing

The DNA-library preparation was performed according to the manufacturer's recommendations with some modifications (Ion Torrent, Life Technologies). In particular, primary DOP-PCR products of the dissected material were re-amplified and barcoded using a panel of Ion-Torrent primers. Quality and quantity of the fragments were evaluated by high-resolution capillary electrophoresis using Shimadzu MultiNA (Japan). In average, fragment length distribution was 150–350 bp with a target pick at ~200 bp. To get rid of dimers of primers, the samples were purified using magnetic beads Agencourt AMPure XP (Beckman Coulter) followed by a capillary electrophoresis analysis. Final concentrations of the DNA-libraries were assessed using Qubit 2.0 Fluorometer (Invitrogen/Life Technologies USA), after that all samples were diluted to ~ 26 pM and equimolarly pooled. Sequencing run was carried out with Ion Torrent PGM genome analyzer (Life Technologies); single-end sequencing was performed. Procedures of emulsion PCR, Ion Sphere Particle Enrichment, and loading of Ion 318 Chip v2 were carried out according to the manufacturer's instructions.

Sequencing Data Processing and Analysis

The sequencing data was processed and analyzed using the web-based bioinformatic platform Galaxy (https://usegalaxy.org/, Giardine et al., 2005) as earlier described (Zlotina et al., 2016). In brief, input files were converted to an appropriate FASTQ format using sff converter (version 1.0.1) and FASTQ Groomer (version 1.0.4) tools, after that the quality of the data was evaluated using the FastQC tool. To get rid of terminal adapter sequences and remove poor quality base calls from the end, the sequence reads were trimmed and filtered by length. The reads were mapped to chicken reference genome assembly (https://www.ncbi.nlm.nih.gov/genome/111, Gallus_gallus-5.0) using a short-read aligner Bowtie2 (Langmead and Salzberg, 2012). The data visualization and analysis were carried out with genome browser Integrative Genomics Viewer (IGV) (Robinson et al., 2011). The mapped chromosome regions were also evaluated with regard to some genome characteristics (such as gene-density, repeats content) using corresponding imported tracks downloaded from the UCSC Genome Browser (http://genome.ucsc.edu).

Coordinates of topologically associated domains (TADs) and A/B compartments in chicken embryonic fibroblasts (CEF) were imported from http://icg.nsc.ru/ontogen/ (Fishman et al., 2019). For comparative analysis the chromomere borders were defined according to the alignment of sequenced reads to the reference genome and by excluding single reads distant from the main cluster of reads. The chromomeres with ambiguous borders were excluded from the analysis. The comparison of genomic coordinates of chromomeres and A/B compartments was performed by visual matching and by counting switches of the compartment type within 500 kb distance from left and right chromomere borders. The number of TADs per chromomere was counted using JuiceBox heatmaps and the defined chromomere borders.

RESULTS

Microdissection of Individual Chromomeres From Chicken Lampbrush Chromosomes and Their Mapping on Mitotic Metaphase Chromosomes

To analyze the genomic organization of LBC chromomeres, we applied mechanical microdissection to chicken lampbrush chromosomes followed by preparation of DNA-libraries of isolated chromomeres. In particular, all prominent chromomeres were dissected one by one along the chicken lampbrush macrochromosome 4 starting from the q-ter chromosome region: chromomeres ##1–31 (**Supplementary Figure S1**). In chicken karyotype, chromosome 4 has an interesting evolutional background being a result of centric fusion of ancestral macrochromosome 4 (GGA4q) and a microchromosome (GGA4p) (Shibusawa et al., 2004; Griffin et al., 2007). Additionally, we isolated chromomeres constituting one of chicken microchromosomes in the lampbrush form.

The microdissected material was used for preparation of DNA probes for FISH. To verify the quality and specificity of the dissected samples, the DNA fragments were mapped on chicken metaphase chromosomes. Among 31 DNA probes marking chicken LBC4, 27 probes demonstrated bright and specific hybridization signal on a corresponding pair of homologous chromosomes in metaphase plates (**Figures 1A–C**). The remainder four probes gave a major hybridization signal on GGA4 as well as additional minor signals dispersed across the karyotype, which might be due to excess of interspersed DNA-repeats in microdissected material. All 5 FISH-probes marking

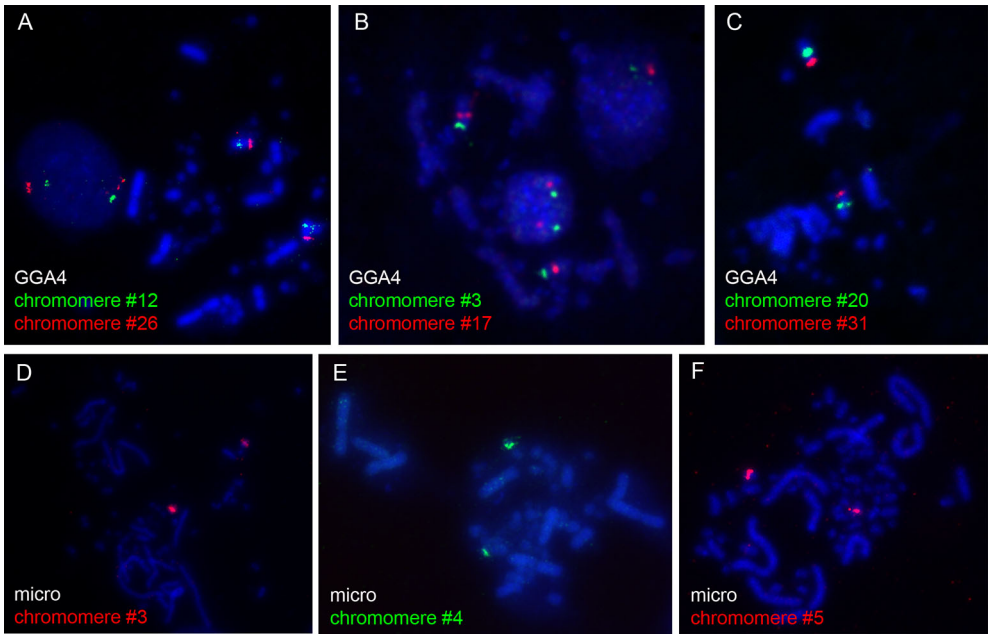

FIGURE 1 | FISH-mapping of microdissected lampbrush chromosome chromomeres on chicken metaphase chromosomes. Examples of FISH with DNA material of individual chromomeres on metaphase macrochromosome 4 **(A–C)** and microchromosome 11 **(D–F)**. Chromomere ID numbers are indicated. Chromosomes are counterstained with DAPI.

the microchromosome also proved to be bright and very specific (**Figures 1D–F**).

High-Resolution Mapping and Analysis of Transcriptional Activity of DNA Fragments From Microdissected Chromomeres on Lampbrush Chromosomes

Using a DNA/DNA+RNA hybridization protocol we mapped all DNA sequences from microdissected individual chromomeres on chicken LBCs (**Figure 2**). In most cases, a hybridization signal was observed in a single chromomere similar in size and morphology to the dissected chromomere indicating a tendency of chromomeres to maintain their integrity as individual chromatin domains. At the same time, in some cases we observed a FISH signal in several neighboring chromomeres, which can be explained by different degrees of LBC's condensation during the oocyte growth. That is, chromatin of an individual chromomere dissected from a more compact lampbrush chromosome may be included into several smaller chromomeres in less compact chromosomes.

The majority of DNA probes hybridized to small and medium-sized loose chromomeres, with the hybridization signal being also revealed in RNP-matrix of extended lateral loops (**Figures 2B, C**). Thus we conclude that obtained DNA probes in fact correspond to chromomere-loop complexes of LBC4. In contrast, dissected material of massive marker chromomeres of chicken LBCs 1–3 had been previously revealed in loopless DAPI-positive chromomeres (**Figure 2A**) (Zlotina et al., 2016).

Investigation of Genomic Context of Individual Lampbrush Chromosome Chromomeres

To investigate the genomic context of LBC chromomeres, we applied high-throughput sequencing of individual chromomeres microdissected from chicken lampbrush chromosomes. Earlier we had deciphered several massive DAPI-positive chromomeres dissected from chicken LBCs 1, 2, and 3 (Zlotina et al., 2016). Such marker chromomeres are typical for certain regions of the largest chicken lampbrush marcrochromosomes. In the present study, we sequenced DNA-material of 24 neighboring chromomeres covering the LBC 4 along its length (samples ## macro1–6, 11–23, 27–31), and five chromomeres constituting one of the chicken microchromosomes (samples ## micro1–5). In case of LBC4, all 24 samples of individual chromomere-loop complexes were successfully assigned to GGA4 reference genome assembly with neighboring dissected chromomeres being mapped to adjacent genomic regions (**Figure 3**). Besides, the results of genome mapping allowed identifying the dissected lampbrush microchromosome as chromosome 11.

Further NGS analysis allowed to evaluate the chromomeres' size and borders. In particular, according to our assessments the amount of DNA in the majority of small and medium-sized chromomeres is about 1.5 to 3.5 Mb, while DNA content of large marker chromomeres is 4 to 5 Mb (**Figure 3**, Zlotina et al., 2016). These results are consistent with previous estimation of chromomere size based on the analysis of cytological maps of a chromomere-loop pattern of LBCs (Galkina et al., 2006).

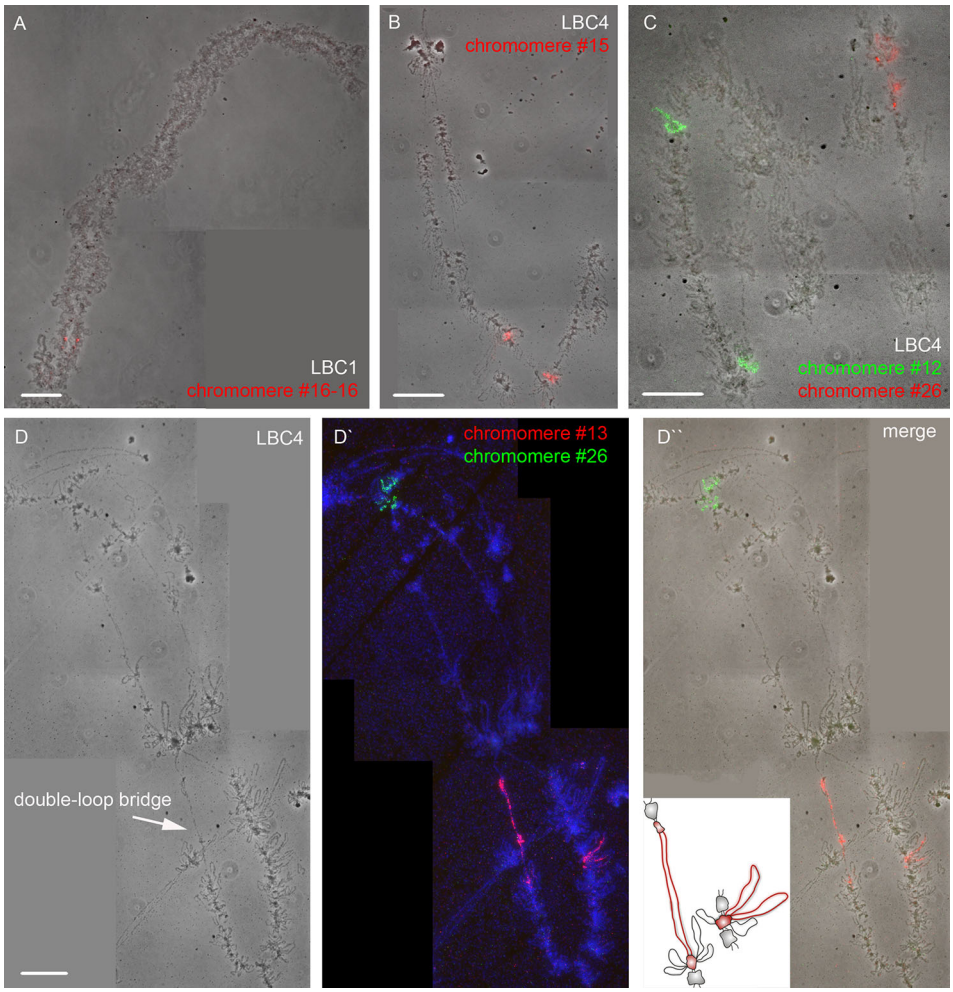

FIGURE 2 | High-resolution FISH-mapping of DNA fragments from microdissected chromomeres on chicken lampbrush chromosomes. Examples of FISH with chromomere DNA probes to LBC 1 **(A)** and LBC 4 **(B, C, D–D'')**. Arrow points to a "double-loop bridge" (DLB) **(D)**; the insert shows a schematic drawing of the DLB region with the mapped FISH-probe to chromomere #13 (red, **D'**). FISH signals are shown on the top of LBC phase contrast images **(A, B, C, D'')**. FISH was carried out according to a DNA/DNA+RNA hybridization protocol. Chromomere ID numbers are indicated. Chromosomes are counterstained with DAPI. Scale bars = 10 μm.

The microdissected chromomere samples were also analyzed with regard to gene density and repeat content (**Figure 3**). Besides, the sequencing data was compared with the results of FISH-mapping on lampbrush chromosomes. The majority of simple chromomeres had a mixed genomic context and comprised both gene-rich/repeat-poor DNA as well as gene-poor and repeat-rich DNA (for instance, chromomeres of LBC4 ## 11, 14, 15, 19, 20). Based on the FISH data, such DNA sequences were revealed both in chromomere cores and arising lateral loops i.e. chromomere-loop complexes (**Figure 2B**). Some chromomeres demonstrated relatively higher gene density and lower content of repetitive sequences (chromomeres of LBC11, chromomeres #22 and #13 of LBC4). It is worth noting that according to FISH mapping, the DNA probe generated from dissected chromomere #13 hybridized to a so-called «double loop bridge» (a chromosomal region with broken chromomere structure), namely to chromatin fiber and flanking halve-chromomeres (**Figures 2D–D''**). Thus the genomic coordinates of the double loop bridge region were determined precisely, which provides prospects to determine the DNA sequences underlying the formation of such structures. Besides, high-throughput sequencing data demonstrated that DNA of massive loopless chromomeres was significantly enriched by repetitive DNA-elements of different nature and comprised smaller amount of genes as compared to neighboring regions. In particular, the DNA of such chromomeres is enriched by chicken LINE element CR1 (Zlotina et al., 2016).

We conclude that the described complex approach combining cytological, cytogenetic, and genomic analysis allows to correlate morphologically distinct chromatin domains—lampbrush chromosome chromomeres in complex with arising lateral loops—with particular deciphered genomic regions.

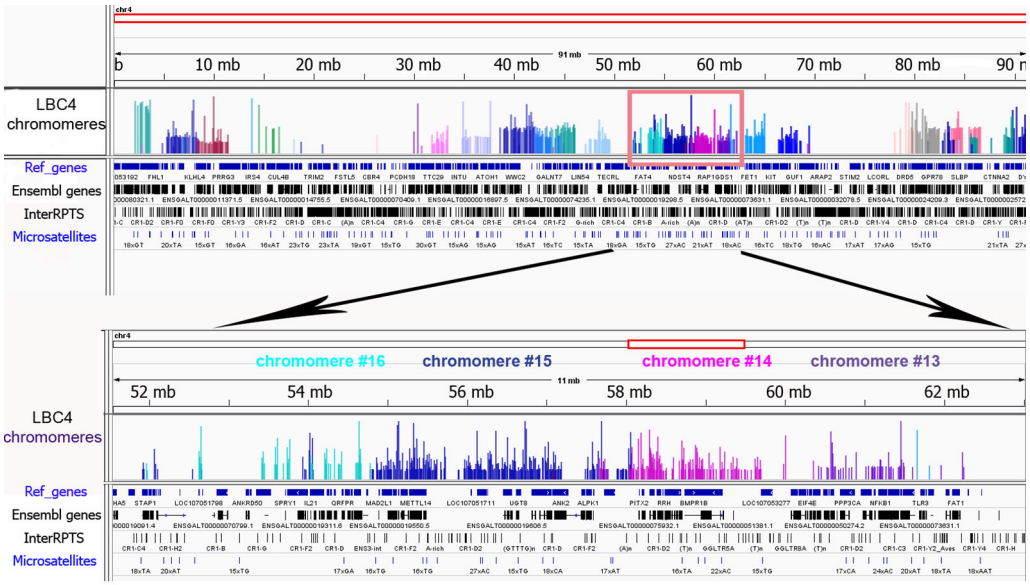

FIGURE 3 | Genomic context of individual lampbrush chromosome chromomeres. Genomic mapping of LBC4 chromomeres to chicken reference genome assembly (Gallus_gallus-5.0). The sequencing data was visualized in the «Integrative Genomics Viewer» (IGV) genomic browser. The sequencing reads corresponding to individual chromomeres are shown in different colors. An upper panel is «the chromosome» view; a lower panel—a zoomed target region encompassing chromomeres #13–16. Imported tracks with annotated genes (Ref_genes, Ensembl genes) and various types of repetitive DNA elements (InterRPTS from the RepeatMasker program which displays interspersed repeats and low complexity DNA sequences, Microsatellites) are shown.

Comparative Analysis of Lampbrush Chromomeres and Chromatin Domains of Interphase Nucleus

Information on genomic coordinates of an array of LBC chromomeres allowed to compare chromomeres with spatial hierarchical chromatin domains earlier characterized by Hi-C in chicken somatic cells (Fishman et al., 2019). In particular, similar to other vertebrates chicken genome proved to be folded into large-scale epigenetically distinct domains: A-compartments containing open and transcriptionally active chromatin and B-compartments with silent chromatin. Within compartments the chromatin is packaged into submegabase-sized topologically associated domains (TADs), which represent local contact-enriched self-interacting chromatin domains.

At first, we compared the genomic regions corresponding to the dissected LBC chromomeres with an A/B compartments profile obtained for chicken embryonic fibroblasts at 100 kb resolution of a contact matrix. By visual matching the genomic coordinates of chromomeres and A/B compartments, we concluded that chromomere borders do not correspond to the boundaries of A/B compartments (**Figure 4A**). In other words, a single chromomere may contain chromatin belonging to both A and B compartments of interphase nucleus. For more thorough analysis, we estimated the ratio of "somatic" A/B compartments in every sequenced chromomere of LBC4 and LBC11 as well as in earlier deciphered marker chromomeres of LBC1, 2, and 3 (**Figure 4B**). The majority of dissected chromomeres contained different proportions of A and B compartments with only single chromomeres being fully overlapped by a compartment of one type. In particular, a prominent marker chromomere from LBC1, which was shown to be gene-poor and highly enriched with repetitive DNA elements (sample #16–16, Zlotina et al., 2016), had an unambiguous «B» status (**Figure 4C**).

Then we analyzed how LBC chromomere borders correlate to the borders of "somatic" A/B compartments (**Figure 4D**). It should be taken into account that genomic borders of microdissected chromomeres were mapped with some precision, which was determined by the accuracy of chromomere identification and isolation during microdissection and by the sequencing depth. We counted switches of the domain type (A→B or B→A) throughout the genomic regions at 500 kb distance from the chromomere borders. We have not found the preferred "switching" of the compartments between A/B types near the chromomeres boundaries. Borders of A/B compartments were found near the chromomere borders in 51% of left and right chromomere borders.

Finally, we compared LBC chromomeres with TADs of chicken somatic cells. We used the genomic coordinates of TADs identified by the directionality index algorithm (DI, Dixon et al., 2012) and Armatus algorithm (Armatus, Filippova et al., 2014) (Fishman et al., 2019). We found that LBC chromomeres generally correspond to several somatic TADs (**Figure 4E**). That is, we analyzed 27 deciphered chromomeres and estimated that one chromomere may comprise from 0.5 to 8.5 DI TADs and from

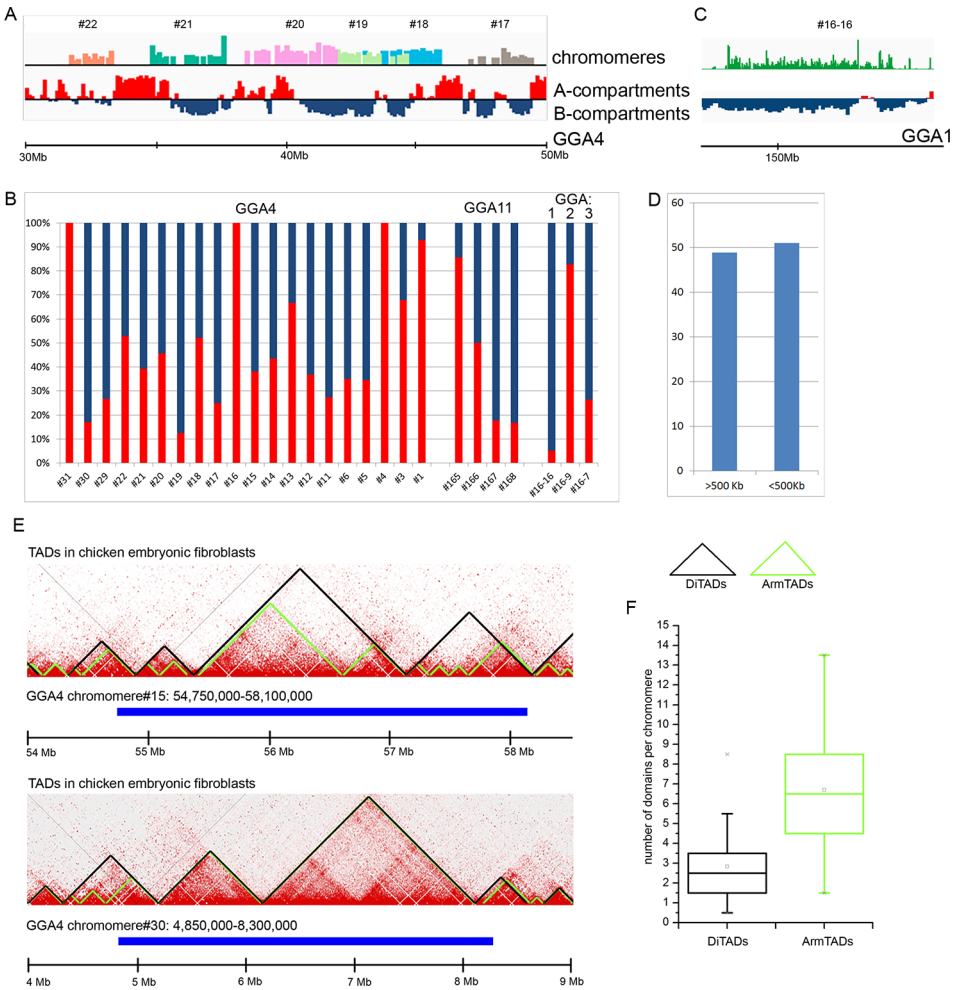

FIGURE 4 | Comparison of chicken lampbrush chromosome chromomeres with A/B compartments and topologically associated domains (TADs) of interphase nucleus. **(A, C)** Alignment of the LBC chromomeres sequences with a profile of A/B-compartments of embryonic fibroblasts. Individual chromomeres are shown in different colors and numbered according to chromomere ID. A- and B-compartments are shown in red and blue, correspondingly. **(B)** The ratio of somatic A- and B-compartments in individual chromomeres from LBC4, LBC11, and LBCs 1–3 (the sequencing data on LBCs 1–3 was described in detail in Zlotina et al., 2016). **(C)** Marker chromomere (#16–16 from LBC1) with pronounced B-status. **(D)** Proportion of switches between the domain type (A→B or B→A) 500 kb upstream or downstream from the right and left chromomere borders. **(E)** Comparison of genomic coordinates of individual LBC chromomeres and somatic TADs. The heatmaps of spatial interactions show TADs, identified by different algorithms: DiTADs (black) and ArmTADs (green). The genomic regions corresponding to LBC chromomeres are shown in blue. **(F)** Boxplots illustrating the number of somatic DiTADs (black) and ArmTADs (green) per chromomere (n = 27).

1.5 to 13.5 Armatus TADs (**Figure 4F**). On the average, chromomeres contained 2.5 DI TADs and 6.5 Armatus TADs.

In general, our data suggest that lampbrush chromosome chromomeres do not correspond unambiguously to any type of spatial genomic domains previously identified in the interphase nucleus of somatic cells.

DISCUSSION

To get a deeper insight into organization of LBC chromatin domains, we applied the approach that combines mechanical microdissection of individual chromomeres from chicken lampbrush chromosomes, preparation of DNA-libraries from the dissected material followed by high-resolution FISH-mapping and high-throughput sequencing of chromomere DNA (Zlotina et al., 2016). The described approach allowed us to correlate particular chromomere-loop complexes with the deciphered genomic regions.

Until this study, the DNA composition of lampbrush chromosome chromomeres has remained unknown with few exceptions. In particular, some data were obtained for a small number of chromomeres consisting of massive arrays of tandemly repeated sequences. For instance, it was found that

the majority of chromomeres on the chicken LBC W are occupied by specific families of DNA repeats (Solovei et al., 1998; Komissarov et al., 2018). Another example includes prominent dense chromomeres in the centromere regions of lampbrush chromosomes that were demonstrated to contain (peri) centromeric DNA repeats (Solovei et al., 1996; Saifitdinova et al., 2001; Krasikova et al., 2006; Krasikova et al., 2012; Zlotina et al., 2019). Additionally, non-centromere clusters of tandem repeats were shown to constitute some interstitial chromomeres (Krasikova et al., 2006; Zlotina et al., 2010). However, as early as in 1980 H. Macgregor suggested that while some chromomeres bear highly uniform DNA (such as clusters of repetitive sequences), the others have a less uniform content (Macgregor, 1980). In the present study, we for the first time established genomic properties of an array of regular chromomeres from chicken LBCs including all morphologically distinct chromomeres from macrochromosome 4 and microchromosome 11. Previously we had also microdissected several individual marker chromomeres from chicken LBCs 1, 2, and 3 (Zlotina et al., 2016). Analysis of the genetic context of all dissected chromomeres allowed us to confirm the Macgregor`s hypothesis. Indeed, we found that regular chromomere-loop complexes generally have a mixed composition and combine genomic regions enriched in genes/depleted for DNA repeats with regions lacking genes/enriched in repetitive elements. At the same time, individual marker DAPI-positive chromomeres typical for the largest chicken LBCs seem to be more homogeneous and demonstrate a high content of repetitive DNA.

Apparently, chromomeres of meiotic lampbrush chromosomes have little in common with chromomeres of polytene chromosomes. In polytene chromosomes that form in interphase nuclei, homologous chromomeres fuse forming a transverse band (Zykova et al., 2018). In Drosophila, the positions of bands and interbands in polytene chromosomes can be predicted by Hi-C technique and confirmed by FISH. Bands and interbands were demonstrated to be equivalent to TADs and the regions between them respectively, with a high degree of conservation between polytene TADs and diploid TADs (Eagen et al., 2015; Ulianov et al., 2016). On the contrary, the data obtained in our study imply that chromomeres of chicken LBCs generally do not correspond to TADs identified in chicken embryonic fibroblasts. In particular, LBC chromomeres are larger structural units of chromatin organization, and genomic regions corresponding to several somatic TADs are involved in their formation. Moreover, along the whole length of GGA4 and GGA11, chromomere borders do not match to the borders of A/B chromatin compartments typical for interphase nuclei of chicken fibroblasts. There are three possible explanations for the lack of correspondence between the boundaries of lampbrush chromomeres and interphase A/B compartments: difference in genomic borders of A/B compartments in interphase nucleus and diplotene oocyte nucleus, the uncertainty of the identification of chromomere borders, or absence of correspondence between the chromomere positions and A/B compartments.

A pattern of transcription during the lampbrush stage of meiosis dramatically differs from one in somatic cells due to a supposed role of LBCs in accumulation of maternal RNAs in growing oocytes. Such a peculiar pattern of transcription leads to a distinctive pattern of untranscribed regions gathering in chromomeres. This can underlay the discrepancy in organization between LBC chromomeres and compact chromatin domains of somatic cells. It was previously suggested that lampbrush chromosome chromomeres appear as a result of massive transcription taking place on the lateral loops (Callan, 1986). That is, lateral loops with RNP-matrix consisting of nascent transcripts and associated RNA-binding factors push apart dense transcriptionally inactive chromatin domains leading to their insularity. The question on the role of CTCF insulator protein in establishing the borders between neighboring LBC chromomeres remains open-ended.

Further single-cell Hi-C studies of oocyte nucleus with a lampbrush chromosome set together with high-resolution FISH-mapping are required to determine chromatin domains with higher frequency of self-interactions and their correspondence to lampbrush chromomeres.

DATA AVAILABILITY STATEMENT

We have uploaded all DNA sequencing data (for 29 samples) from our study to European Nucleotide Archive (ENA) repository, the study accession number is: PRJEB36087.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical committee of Saint-Petersburg State University (approval #131–03-2).

AUTHOR CONTRIBUTIONS

AK conceived the study and supervised the project. AK, AZ, NK, AA-R, and TL performed microdissection procedure. AZ planned and carried out most of the principal cytogenetic experiments. AZ and OP performed high-throughput sequencing. AZ and AM performed bioinformatic analysis. AZ, AM, and AK drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00057/full#supplementary-material

SUPPLEMENTARY FIGURE S1 | Microdissection of individual chromomeres along the chicken lampbrush chromosome 4. **(A)** Phase-contrast image of chicken LBC 4 subjected to microdissection procedure. Totally, as much as 31 chromomeres were isolated from one of the halve-bivalents starting from the *q*-arm terminal region (chromomeres ##1–31). **(B)** Cytological map of a chromomere-loop pattern of chicken LBC4 (according to [Galkina et al., 2006] with modifications). Cen, centromere position. Arrows point to microdissected chromosome regions.

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Chromatin Landscaping At Mitotic Exit Orchestrates Genome Function

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Chromatin architecture is highly dynamic during different phases of cell cycle to accommodate DNA-based processes. This is particularly obvious during mitotic exit, where highly condensed rod-like chromatids need to be rapidly decondensed. Such chromatin structural transitions are tightly controlled and organized as any perturbance in this dynamic process can lead to genome dysfunction which may culminate in loss of cellular fitness. However, the mechanisms underlying cell cycle-dependent chromatin structural changes are not fully understood. In this mini review, we highlight our current knowledge of chromatin structural organization, focusing on mitotic exit. In this regard, we examine how nuclear processes are orchestrated during chromatin unfolding and compartmentalization and discuss the critical importance of cell cycle-controlled chromatin landscaping in maintaining genome integrity.

Keywords: chromatin compaction, mitosis, decondensation, cell cycle, transcription, replication

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INTRODUCTION

The cell cycle of proliferating cells is defined by two major events, first, error-free duplication of the genome during synthesis phase and second, faithful transmission of genetic material into the daughter cells during mitosis. Since genetic material is packaged in the form of chromatin, the proper execution of nuclear processes is critically dependent on cell cycle regulated chromatin organization and restructuring (Ma et al., 2015). This process is orchestrated by a variety of factors notably histone PTMs (posttranslational modifications) and chromatin protein complexes (Antonin and Neumann, 2016). In this mini review, we highlight how daughter cells inherit proper chromatin structure and discuss its importance in the execution of genome-wide nuclear functions.

CHROMATIN STRUCTURE IN INTERPHASE

The fundamental repeating unit of chromatin is the nucleosome, which is formed by ~147 bp of DNA wrapped around an octamer histone core (Luger et al., 1997). Individual nucleosomes are connected by linker DNA and organized into long linear arrays, which interact with nucleosomes in the neighboring arrays to create a chromatin fiber (Luger et al., 2012; Belmont, 2014; Hansen et al., 2018). Interactions among adjacent chromatin fibers may contribute to increased folding, finally reaching the maximal degree of compaction (~10,000 fold) observed in the metaphase chromosome (Tremethick, 2007; Batty and Gerlich, 2019). It is now widely established that chromosome

territories/domains are positioned in a non-random manner within the nucleus where gene density, chromosome size and morphology play a major determining role in their organization (Nagano et al., 2017; Nozaki et al., 2017; Finn et al., 2019; Mirny et al., 2019). Gene-rich areas tend to locate at the center of the nucleus whereas gene-poor regions tend to be located at the periphery where they are associated with the nuclear lamina (NL) (Nunez et al., 2009; Van Steensel and Belmont, 2017; Lochs et al., 2019; Sivakumar et al., 2019).

High-throughput sequencing based approaches have markedly advanced the understanding of chromatin folding patterns and their relevance to nuclear functions. In particular, different versions of chromosome conformation capture-based methods (Denker and De Laat, 2016; Sati and Cavalli, 2017; Han et al., 2018) were developed to measure the frequency at which two genomic loci physically associate in 3D space (Sati and Cavalli, 2017). The most recent of such methods termed Hi-C measures frequencies of all the possible genomic contacts (all-versus-all). This method has been used to identify three primary landscapes of chromatin folding: i) loops, ii) TADs (topologically associating domains) and, iii) compartments (Denker and De Laat, 2016; Beagrie and Pombo, 2017; Nagano et al., 2017). Chromatin loops are formed when two small genomic regions typically 100 to 750 kb (kilobases) apart come in close proximation through association with CTCF (the CCCTC-binding factor) (Rao et al., 2014). Hi-C mapping at a higher resolution has annotated ~2,000 sharply defined regions as TADs (Dixon et al., 2012). TADs are relatively isolated genomic regions around 100 kb to 2 Mb (megabases) in size that exhibit preferential intra-domain contacts. Finally, at the Mb scale, chromosomes are segregated into A-type (active) and B-type (inactive) compartments that are defined by their transcriptional activity (Simonis et al., 2006) (**Figure 1A**). In the context of this review, we focus on the major components of the B-type compartment, including LADs (lamina-associated domains), NADs (nucleolar-associated domains), as described in **Box 1**.

CHROMATIN STRUCTURE DYNAMICS AROUND MITOSIS

Chromatin Condensation During Mitosis

The massive structural reorganization of chromatin during mitosis is mediated by the eukaryotic members of SMC (structural-maintenance-of-chromosomes) protein complexes, namely condensins and cohesins (Belmont, 2006; Wood et al., 2010; Houlard et al., 2015; Piskadlo and Oliveira, 2017). Together with topoisomerase II and other non-histone proteins, condensins and cohesins help orchestrate higher order chromatin folding and chromosome-wide compaction leading to cytologically distinct and longitudinally compacted chromosomes (Hirano, 2012; Antonin and Neumann, 2016; Piskadlo and Oliveira, 2017; Schalbetter et al., 2017). Whereas condensins organize and condense large-scale chromosome rearrangements by loop formation and lateral/axial compaction, histone PTMs generally promote inter-nucleosomal association and hence, drive close-range chromosome compaction (Antonin and Neumann, 2016). Among the various histone PTMs, phosphorylation of several of the histone H3 amino acid residues surge during different stages of mitosis (Sawicka and Seiser, 2012; Wang and Higgins, 2013), however, the exact mechanism by which they contribute to mitotic chromosome condensation in mammalian cells remains elusive.

In addition to histone H3 phosphorylation, different methylation states of histone H4 lysine 20 (H4K20me1/2/3) have also been implicated in chromatin compaction (Houston et al., 2008; Oda et al., 2009). However, the mechanistic details of their role in chromosome condensation are not well understood. SET8, the enzyme responsible for genome-wide deposition of H4K20 monomethylation, is tightly regulated during the cell cycle and peaks around G2 phase (Tardat et al., 2010; Jorgensen et al., 2013). Majority of H4K20me1 is subsequently converted into H4K20me2 and H4K20me3 by the action of SUV4-20H1 and SUV4-20H2 enzymes during M and G1 phases (Nishioka et al., 2002). However, about 10% of H4K20me1 persists and is found to be significantly enriched in the gene bodies of highly transcribing genes (Barski et al., 2007; Van Nuland and Gozani, 2016). The presence of H420me1 in transcriptionally active and hence, more open chromatin regions suggest that chromatin compaction functions are most likely regulated by H4K20me2 and H4K20me3 states. These observations support the idea that both SMC and histone PTM-mediated chromosome structural changes may function in parallel, albeit at different levels of chromosome architecture. It is highly likely that mitotic chromosome condensation requires a cross-talk between both these mechanisms (**Figure 1B**).

Chromatin Decondensation After Mitosis

Mitotic exit is characterized by two major nuclear events, first, the nuclear envelope is reformed to provide an enclosed space for the segregated genomic material. Second, the re-establishment of functional interphase chromatin within the nuclear envelope, where rod-shaped chromatids rapidly decondense into more loosely arranged, non-random structures, fully competent for DNA-based processes. Indeed, simulation on a mitotic chromosome-like polymer shows that the large-scale 3D organization of TADs and A/B compartments during mitotic exit occurs simply as a result of partial decondensation in an inflation-like process (Kumar et al., 2019). In this regard, while TADs and loops are established rapidly following mitotic exit, the larger A/B compartments form more slowly and continue to grow as cells advance through the cell cycle (Abramo et al., 2019).

In the context of this review, we focus on how the major B-type compartment components i.e. LADs and NADs, are organized at mitotic exit. During interphase, LADs interact dynamically with the NL however, they move only within a layer <1 μm thick (Kind et al., 2013). Furthermore, there is a degree of both cooperativity and stochasticity in the positioning of LADs within individual cells (Jurisic et al., 2018). Intriguingly, the nuclear positioning of majority of LADs is not inherited following mitosis but instead some LADs (termed facultative LADs or fLADs) are stochastically reshuffled between other repressive environments. However, around 30% of LAD

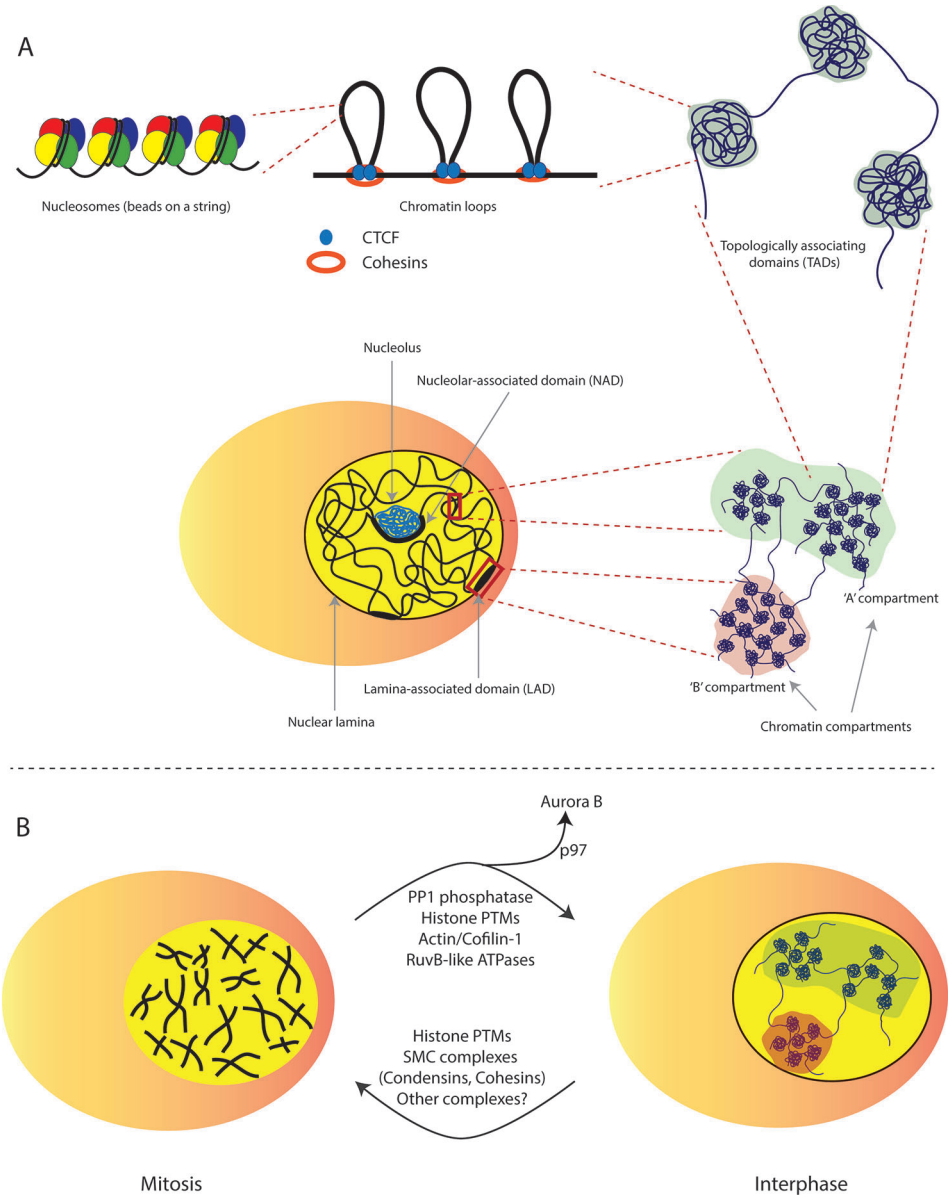

FIGURE 1 | Chromatin structural organization during interphase and mitosis. **(A)** Levels of chromatin structural organization. At the most basic level, an histone octamer makes a nucleosome with ~147 bp of DNA resembling a beads-on-a-string structure, which is then folded with neighbouring nucleosomes to make a chromatin fiber. Individual fibers help establish structural chromatin loops through co-anchorage with CTCF and cohesion by means of loop extrusion. Self-interacting chromatin loops then assemble together into TADs (topologically associating domains). Several TADs then spatially organize to become specific nuclear compartments i.e., the A-type (active) enriched in active genes or the B-type (inactive) compartments that mainly comprise of repressed genomic regions including but not limited to LADs (lamina-associated domains) and NADs (nucleolar associated domains). **(B)** Chromatin structure transition from mitosis to G1 phase of the cell cycle. At the onset of mitosis, interphase chromatin is organized into highly condensed rod-shaped chromatids organized by SMC complexes (condensins and cohesins) and several phosphorylation events on histone H3. At the mitotic exit chromosomes rapidly decondense into more loosely packed, non-random interphase chromatin structures. The phosphatase PP1 plays a major role in dephosphorylation of H3S10 residue and this is deemed critical for decondensation. Nuclear targeting of actin filaments by Cofilin-1 also facilitates nuclear volume expansion presumably through structural reorganisation of the nuclear compartment. Additionally, RuvB-like ATPases are required for chromatin decondensation through as yet unknown mechanism. Finally, eviction of Aurora B kinase by the p97 ATPase is essential for chromosome decompaction as cells enter the next cell cycle.

regions, termed cLADs (constitutive LADs) appear to be cell-type invariant in their association with the nuclear periphery and may serve to anchor chromosomes to the NL (Kind et al., 2015). Anchoring of cLADs, that display the highest NL contact frequencies and form the most stable NL contacts, likely contributes to the overall organization of interphase chromatin after mitotic exit (Falk et al., 2019). In this regard, H3K9me2 (histone H3 lysine 9 dimethyl) has recently been shown to be

BOX 1

Lamina-associated domains (LADs)

Condensed chromatin regions corresponding to B-type domains that lie in proximity to the nuclear lamina (NL) are termed as lamina-associated domains (LADs) (Van Steensel and Belmont, 2017). There are approximately 1,000–1,500 LADs, typically 0.1–10 Mb in size that cover more than one-third of the genome and are distributed along all chromosomes (Guelen et al., 2008). LADs have sharply defined borders enriched for active promoters leading away from the LADs, CpG islands and CTCF binding sites.

Nucleolar-associated domains (NADs)

Nucleolar-associated domains (NADs) are heterochromatic regions that associate with the nucleolus (Nemeth et al., 2010; Van Koningsbruggen et al., 2010). NADs are relatively gene-poor, enriched in satellite repeats and approximately 0.1–10 Mb in size. NADs are formed by active processes through tethering proteins in addition to mere physical proximity to the nucleolus (Potapova and Gerton, 2019). There is substantial overlap between NADs and LADs with some studies showing that these loci could switch positions following mitotic exit (Kind et al., 2013; Ragoczy et al., 2014). Additionally, NADs are also found to locate near to the NL in a subset of cells.

preserved across mitosis and is required for the re-establishment of LADs in the daughter cells (Poleshko et al., 2019).

NADs are also found to locate in the proximity of NL in a subset of cells. In this regard, there may be a substantial overlap between NADs and LADs with some studies showing that these loci could switch positions following mitotic exit (Van Koningsbruggen et al., 2010; Kind et al., 2013; Ragoczy et al., 2014). A recent study identified two distinct classes of NADs in mouse embryonic fibroblasts, which differ primarily in their frequency to associate with the nucleolar periphery and with the NL (Vertii et al., 2019). While type I NADs display characteristics of constitutive heterochromatin and associate with both nucleolar periphery and NL, type II NADs are more specifically associated with the nucleolus. Considering a substantial overlap between type I NADs and LAD regions, their mode of inheritance is expected to be largely the same (Kind et al., 2013; Vertii et al., 2019). However, it is unclear at the moment how type II NADs are inherited in the daughter cells.

At the nucleosomal level, the chromatin landscaping at mitotic exit is marked primarily by reappearance of histone acetyl marks and loss of histone phosphorylation (Wang and Higgins, 2013). The phosphatase PP1 plays an essential role in removing mitotic H3 phosphorylation, including H3T3p, H3S10p, H3T11p, and H3S28p. In this regard, Repo-Man, the principal PP1-recruiting factor is targeted to anaphase chromosomes and is required for timely removal of H3T3p and H3T11p (Qian et al., 2011; Vagnarelli et al., 2011). Although Repo-Man is dispensable for chromatin decondensation at mitotic exit, it has been shown to play a role in nuclear envelope reformation in a PP1-independent manner (Vagnarelli et al., 2011). In this regard, the nucleolar protein Ki-67 might function redundantly with Repo-Man to target PP1 onto anaphase chromosomes (Booth et al., 2014). Further into mitosis, PNUTS, the PP1 nuclear targeting subunit localizes PP1 to the reforming nuclei and its loading onto chromatin has been linked to decondensation (Landsverk et al., 2005). However, as this occurs following dephosphorylation of H3S10, the exact mechanism by which PNUTS facilitates chromosome decompaction is not currently understood. Removal of H3S10p leads to the dissociation of the chromosome passenger complex and promotes re-establishment of HP1 (heterochromatin protein 1) binding to H3K9me3 (histone H3 lysine 9 trimethyl) to maintain heterochromatin at mitotic exit. Establishment of the H3K9me3-HP1 axis facilitates loading of cohesin by the histone H4K20 methyltransferase SUV4-20H2 that is itself targeted through HP1 binding. This initial loading of cohesin seems to be crucial for the establishment of pericentromeric heterochromatin as cells enter interphase (Hahn et al., 2013). Furthermore, H4K20 methylation is in itself important for finetuning chromatin compaction states during mitotic exit. In this context, we have uncovered that loss of H4K20me leads to abnormal chromatin decompaction in cells exiting mitosis, which has significant functional implications in terms of DNA replication and genome stability during the next cell cycle (discussed below) (Shoaib et al., 2018).

Additional protein complexes have also been shown to play a role in chromatin decondensation at the mitotic exit. Firstly, removal of the mitotic kinase Aurora B from the chromatin seems to be a prerequisite for chromatin decondensation and nuclear envelope reformation. This is carried out by the hexameric ATPase p97 that binds to the ubiquitylated form of Aurora B and evicts it from the chromatin thereby, facilitating chromatin decondensation (Ramadan et al., 2007). Apart from p97, a second class of ATPases, RuvBL1 and RuvBL2, seems to be essential for chromatin decondensation. Using purified chromatin and Xenopus egg extracts to recapitulate mitotic exit events in a cell free system, Magalska et al. showed that decompaction of metaphase chromosomes is an active process that requires the activity of these AAA-ATPases (Magalska et al., 2014). Finally, nuclear actin filament (F-actin) polymerization during early G1 phase of the cell cycle is thought to aid nuclear volume expansion and chromatin decondensation (Baarlink et al., 2017). In this context, the nuclear targeting of actin-disassembling factor Cofilin-1 during mitotic exit spatiotemporally controls the assembly and turnover of F-actin polymers in turn regulating chromatin reorganization and nuclear architecture of the newly formed daughter cells (**Figure 1B**).

Establishing proper 'ground state' chromatin structure entails massive structural reorganization of the chromatin. Using single-cell Hi-C analysis, Nagano et al. compared chromatin structure in different cell cycle phases, starting from mitotic exit (Nagano et al., 2017). As the cells exit mitosis, a dramatic expansion of TADs containing active genes was observed, which subsequently decreases as cells enter S phase. On the contrary, compartmentalization increases as the cells progress through the cell cycle and reaches its peak before next mitosis (Nagano et al., 2017). More recently, detailed Hi-C mapping at defined time points following mitotic exit was presented to describe the reorganization of chromatin landscape specifically at the M-G1 transition (Abramo et al., 2019; Zhang et al., 2019). Similar to Nagano et. al., the authors observed that TADs and A/B

compartments establish rapidly after mitosis and continue to strengthen through the cell cycle. Local compartmentalization is accompanied by contact domain formation in a "bottom-up" manner where smaller sub-TADs are the first to form followed by their convergence into multi-domain TAD structures. Interestingly, Zhang et al. found that CTCF is strongly retained at a significant proportion of its binding sites in mitotic chromosomes, whereas, cohesin is completely evicted during mitosis and is only loaded onto chromatin with delayed kinetics. Intriguingly, cohesin binding is followed by the formation of structural chromatin loops co-anchored with CTCF. Furthermore, the authors showed that chromatin loops can also be formed through contact between cis-regulatory elements (promotor-enhancer loops). These data suggest that a dynamic hierarchical network of mutually influential, yet distinct forces drive post-mitotic chromatin landscaping.

CHROMATIN LANDSCAPING AT MITOTIC EXITS MEETS NUCLEAR FUNCTION

The large-scale spatial segregation of locally folded loops, TADs, compartments that define interphase 3D chromatin organization is largely absent in mitotic chromosomes (Nagano et al., 2017; Abramo et al., 2019). Thus, during mitotic exit, chromatin is not simply decondensed but also needs to be landscaped into hierarchically folded chromatin domains. Additionally, the *de novo* establishment of functional chromatin domains needs to be well coordinated with the genome-wide execution of DNA-based processes in particular transcription and replication. In this regard, it is unclear at the moment whether nuclear functions (transcription, replication etc.) drive chromatin domain unfolding or vice versa. Below we discuss how cells coordinate chromatin reorganization and nuclear processes during their transition to the next cell cycle.

Coordinated Transcription Around Mitosis

To achieve maximum chromatin condensation during mitosis, the landscape of interphase chromatin including intra- and inter-chromosomal contacts is lost. In this regard, many chromatin modifiers and transcription factors are dissociated from chromatin, facilitating segregation of genomic material into the daughter nuclei (Kadauke and Blobel, 2013; Naumova et al., 2013; Festuccia et al., 2017; Raccaud and Suter, 2018; Zaidi et al., 2018). In contrast to previous reports that all bound proteins are evicted from chromatin during mitosis, the histone H3K4 methyltransferase MLL1 (Mixed Lineage Leukemia 1) seems to retain its chromatin association during mitosis and its loss impairs the rapid reactivation of its target genes (Blobel et al., 2009; Black et al., 2016). Thus, a comprehensive analysis of mitotic chromosome bound proteome is required to identify whether other chromatin modifying complexes similar to MLL1 remain on the mitotic chromosome and facilitate inheritance of transcriptional competence in the daughter cells.

Additionally, recent evidence indicates ongoing transcription of many genes during mitosis albeit at low levels, with a transient surge at the mitotic exit (Palozola et al., 2017). The initial transcriptional activity following mitosis primarily relates to the genes that are involved in growth and restoration of daughter cells besides establishing the transcriptional amplitude to be later maintained during interphase. Intriguingly, around 50% of active genes exhibit this transcriptional spike, which constitutes the maximum transcriptional output per DNA copy observed at any point during the cell cycle (Blobel et al., 2009; Black et al., 2016). In terms of histone modifications, mitotic levels of histone H3 lysine 27 acetylation at the individual loci best predict the transcriptional spike seen during the M-G1 transition. These observations support the idea of 'mitotic bookmarking', where retention of key chromatin factors during mitosis contributes to maintenance of epigenetic memory for rapid establishment of transcriptional and structural states of the genome in the daughter cells (Kadauke and Blobel, 2013; Wang and Higgins, 2013; Ma et al., 2015; Zaidi et al., 2018). It is yet to be established how these bookmarking factors drive the formation of TADs and facilitate compartmentalization into active and inactive compartments at the mitotic exit.

Establishing adequate chromatin compaction during G1 phase is also necessary for preventing unregulated transcription. Using DNMT (DNA methyltransferase) and HDAC (histone deacetylase) inhibitors, Brocks et al. showed that disruption of repressive chromatin environment induces cryptic transcription start sites encoded within long terminal repeat retrotransposons (Brocks et al., 2017). Recent work has further shown that condensed chromatin may not necessarily impair transcription initiation but instead leads to inefficient elongation resulting in accumulation of RNA polymerase II at transcription site (Vankova Hausnerova and Lanctot, 2017a). Upon decompaction, release of the RNA polymerase II leads to a transient increase in transcriptional activity. This transient outburst of transcription in cells undergoing mitotic exit occurs likely as a result of the rapid decondensation of chromatin before cells establish ground state chromatin (Vankova Hausnerova and Lanctot, 2017b). Hence, controlled decompaction during mitotic exit critically prevents increased &/or untoward transcriptional activity until cells have advanced further into the interphase (**Figure 2A**). This is in line with the notion that regulated chromatin decompaction during M-G1 transition is essential to ensure well-controlled DNA-based processes and thereby critical to maintainance of genomic stability (Nair et al., 2017).

Chromatin Decondensation at Mitotic Exit Is Coupled to DNA Replication Licensing

DNA replication is a tightly controlled chromatin process that ensures faithful duplication of genetic material once per cell cycle. Replication is temporally divided into two-steps, where first the future replication origins are 'licensed' by loading of pre-RC (pre-replication complex) starting in late telophase and continued through G1 phase, followed by 'firing' of origins at the start of S phase (Remus and Diffley, 2009; Fragkos et al., 2015; Yeeles et al., 2015). Pre-RC assembly starts with loading of

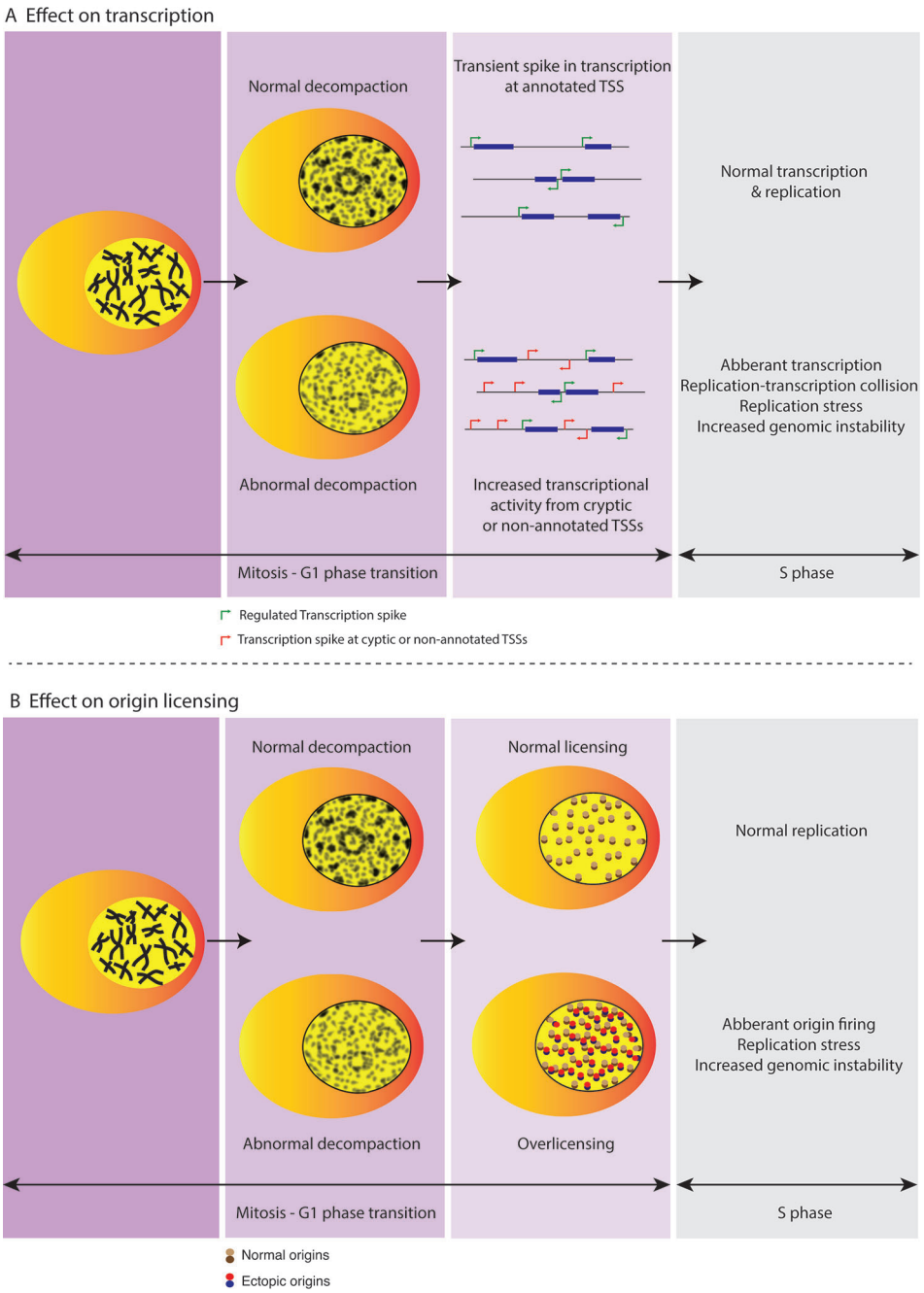

FIGURE 2 | Regulated decompaction at M-G1 transition preserves genome stability. **(A)** A transient spike in transcriptional output from annotated TSSs (transcription start sites) is observed during M-G1 transition while chromatin undergoes regulated decompaction and before cells enter interphase. However, increased decompaction at this point could lead to dysregulation of gene activity wherein unplanned transcription at cryptic promoters or non-annotated TSSs could lead to replication-transcription collisions in turn causing replication stress and genomic instability further into the cell cycle. **(B)** Regulated decompaction at M-G1 transition facilitates restricted licensing of origins in preparation for DNA replication during the following S phase. However, in case of abnormal decompaction, increased chromatin accessibility is accompanied by over-licensing that can lead to replication stress and genome instability arising from aberrant origin firing at the start of subsequent S phase.

ORC1-6 (origin recognition complex subunits 1-6), whose binding in higher eukaryotes is largely sequence independent (Mechali et al., 2013). Later, CDC6 (Cell Division Cycle 6) and CDT1 (Chromatin Licensing and DNA Replication Factor 1) act to recruit the replicative helicase MCM2-7 (minichromosome maintenance protein complex 2-7) to ORC-bound genomic loci. Since the assembly of ORC complex starts in late mitosis, it needs to be coupled with chromosome decondensation and chromatin

reorganization into functional territories and domains. The exact mechanism of recruitment of ORC complex to chromatin is not yet elucidated, however, it has been shown that ORC1, the largest subunit of ORC complex, is the first subunit to bind to mitotic chromosomes at the start of mitosis, followed by the rest of the subunits in late mitosis (Kara et al., 2015). The absence of DNA sequence specific binding of ORC complex allows a more chromatin-regulated recruitment and loading process (Cayrou et al., 2015). In this regard, the N-terminal BAH domain of ORC1 has been shown to specifically recognize H4K20me2, which in itself is being established on histone H4 around late mitosis and early G1 phase (Kuo et al., 2012). ORC complex has also been shown to interact with three repressive chromatin marks namely, H3K9me3, H3K27me3 and H4K20me3 (Bartke et al., 2010; Vermeulen et al., 2010). These findings strongly argue for a key role of specific chromatin environment that stabilizes ORC1 at human replication origins during mitosis and early G1 phase. However, it is not clear at the moment if ORC complex loading and eventually pre-RC loading is dictated or affected by chromatin loops and TADs in the daughter nuclei.

Chromatin enforces specificity of replication initiation by restricting non-specific ORC binding to origins (Devbhandari et al., 2017; Kurat et al., 2017). Thus, tightly regulated chromatin compaction threshold limits replication licensing at the M/G1 transition. In particular, recent data from our group showed that the H4K20me pathway plays a key role in establishment of ground-state chromatin compaction upon mitotic exit (Shoaib et al., 2018). In the absence of proper H4K20me levels, aberrant loading of ORC and MCM2-7 complexes promotes single-stranded DNA formation and DNA damage in the ensuing S phase. Importantly, restoration of chromatin compaction at the cellular transition from mitosis to G1 restricts uncontrolled replication licensing and thus preserves genome stability. In line with this, Kurat et al. previously showed that while chromatin does not completely inhibit assembly of CMG (CDC45/MCM/GINS) complex, DNA synthesis is strongly restricted due to the presence of chromatin and requires additional factors for replisome progression (Kurat et al., 2017). Additionally, "open chromatin" can induce replication stress by facilitating activation of dormant replication origins further threatening the fidelity of DNA replication (Conti et al., 2010) (**Figure 2B**). Finally, re-establishment of interphase chromatin domains is important for maintaining replication timing. TADs represent stable regulatory units of replication timing in a cell-type specific manner and follow characteristics of active and repressed compartments of the genome (Pope et al., 2014). In this regard, DNA replication is synchronized with transcription, initiating within the TADs permissive for transcription and later advance into repressive TAD regions. Aberrant chromatin reorganization at mitotic exit could effectively abolish TAD boundaries and hence, may endanger genomic integrity through replication-transcription conflicts.

CONCLUSIONS/PERSPECTIVES

To coordinate and regulate various nuclear functions, chromatin inherited by the daughter cells during mitotic exit maintains the structural organization of their predecessor. It is an important mechanism for cells to maintain their cellular identity. This inevitably requires highly regulated chromatin decondensation, which is dictated by both chromatin modifications and non-histone chromatin structural proteins. The molecular events leading to reversal of highly condensed chromosomes into loosely organized interphase chromatin are not fully elucidated. In this regard, several key questions require further investigations. A comprehensive analysis of chromatin factors that remain on mitotic chromosomes during cell division is lacking. Also, it is unresolved at the moment how much mitotic bookmarking contributes to reestablishment of interphase chromatin states and how extensive it is throughout the genome. For certain genomic regions such as LADs, there is a *de novo* establishment of chromatin state (cLADs vs fLADs) at mitotic exit. It is unclear how cells push certain LAD regions to the nuclear periphery while others remain in the nuclear interior. The question remains if chromatin landscaping at mitotic exit is largely a stochastic process or there is a method to this randomness. By employing high throughput 'omics' approaches, future studies will shed light on chromatin landscaping at mitotic exit and how it regulates nuclear processes thereby maintaining genome integrity and cell identity.

AUTHOR CONTRIBUTIONS

MS, NN, and CS contributed to the conception, writing, and checking of the manuscript for important intellectual content.

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PERSPECTIVE
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De Novo Small Supernumerary Marker Chromosomes Arising From Partial Trisomy Rescue

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Small supernumerary marker chromosomes (SMCs) are rare cytogenetic abnormalities. *De novo* small SMCs, particularly those combined with uniparental disomy (UPD), are assumed to result from incomplete trisomy rescue. Recently, a one-off cellular event designated as chromothripsis was reported as a mechanism for trisomy rescue in micronuclei. This *Perspective* article aims to highlight a possible association among trisomy rescue, chromothripsis, and SMCs. We propose that chromothripsis-mediated incomplete trisomy rescue in micronuclei underlies various chromosomal rearrangements including SMCs, although other mechanisms such as U-type exchange may also yield SMCs. These assumptions are primarily based on observations of previously reported patients with complex rearrangements and our patient with a small SMC. Given the high frequency of trisomic cells in human preimplantation embryos, chromothripsis-mediated trisomy rescue may be a physiologically important phenomenon. Nevertheless, trisomy rescue has a potential to produce UPD, SMCs, and other chromosomal rearrangements. The concepts of trisomy rescue, chromothripsis, and micronuclei provide novel insights into the mechanism for the maintenance and modification of human chromosomes.

Keywords: chromothripsis, embryo, genomic rearrangement, micronucleus, supernumerary chromosome, uniparental disomy, U-type exchange

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INTRODUCTION

Small supernumerary marker chromosomes (SMCs) are defined as structurally abnormal chromosomes whose size is smaller than or equal to that of chromosome 20 on the same metaphase spreads (Liehr et al., 2011; Liehr, 2019). Small SMCs can have ring, centric minute, or inverted duplication structures and result in copy-number gain of the affected genomic regions (Liehr et al., 2011; Liehr, 2018; Liehr, 2019). Small SMCs can be present in both mosaic and non-mosaic statuses (Liehr et al., 2011; Al-Rikabi et al., 2018). Reportedly, ~70% of cases of small SMCs are *de novo* and are most frequently derived from chromosome 15 (Liehr, 2012).

Recent studies have suggested that *de novo* non-recurrent small SMCs result from incomplete trisomy rescue (Kurtas et al., 2019). Trisomy rescue is a physiological phenomenon to eliminate excessive chromosomes from trisomic cells (Fenech et al., 2011; Jones et al., 2012). When trisomy rescue is interrupted before complete elimination of target chromosomes, it may create various

chromosomal rearrangements including small SMCs (Liehr, 2010; Kurtas et al., 2019). In this *Perspective* article, we discuss the underlying mechanisms and clinical significance of trisomy rescue in association with SMCs and other complex chromosomal rearrangements (CCRs). To this end, we introduce several patients, including our patient, who carried *de novo* CCRs.

TRISOMY RESCUE OCCURS IN MICRONUCLEI

Human preimplantation embryos frequently contain trisomic cells (Los et al., 2004; Liehr, 2010; Irani et al., 2018). Previous studies have suggested that ~1.7% of such trisomic cells have a chance to be corrected through trisomy rescue (Los et al., 2004; Irani et al., 2018). Trisomy rescue is assumed to take place predominantly between the first and fourth postzygotic cell divisions and result in a mosaic status of trisomic and disomic cells (Los et al., 1998; Liehr, 2010). Subsequently, the proportion of trisomic cells in the body would decrease over time because aneuploid cells are at a growth disadvantage compared to diploid cells. Thus, trisomic cells become undetectable in several postnatal clinical samples.

Although trisomy rescue can normalize the number of chromosomes in aneuploid cells, 1/3 of the corrected cells exhibit uniparental disomy (UPD) (**Figure 1A**). For example, when a trisomic cell containing one paternally derived and two maternally derived chromosomes 15 is subjected to trisomy rescue and thereby loses the paternally derived chromosome 15, this cell develops maternal UPD(15). Indeed, trisomy rescue represents the major cause of UPD, although other mechanisms such as monosomy rescue and gamete complementation can also produce UPD (Shaffer et al., 2001). UPD caused by trisomy rescue is comprised of either complete heterodisomy or a combination of isodisomy and heterodisomy (Robinson et al., 1998; Kotzot and Utermann, 2005).

Trisomy rescue is assumed to occur in micronucleus, an aberrant cellular component frequently observed in cancer cells and, to a lesser extent, in normal cells (Zhang et al, 2015; Marcozzi et al., 2018). A micronucleus encapsulates one or a few chromosomes to separate them from the other chromosomes in the main nucleus (**Figure 1A**) (Zhang et al., 2015). Micronuclei have been implicated in the degradation of mis-segregated or supernumeral chromosomes (Jones et al., 2012;

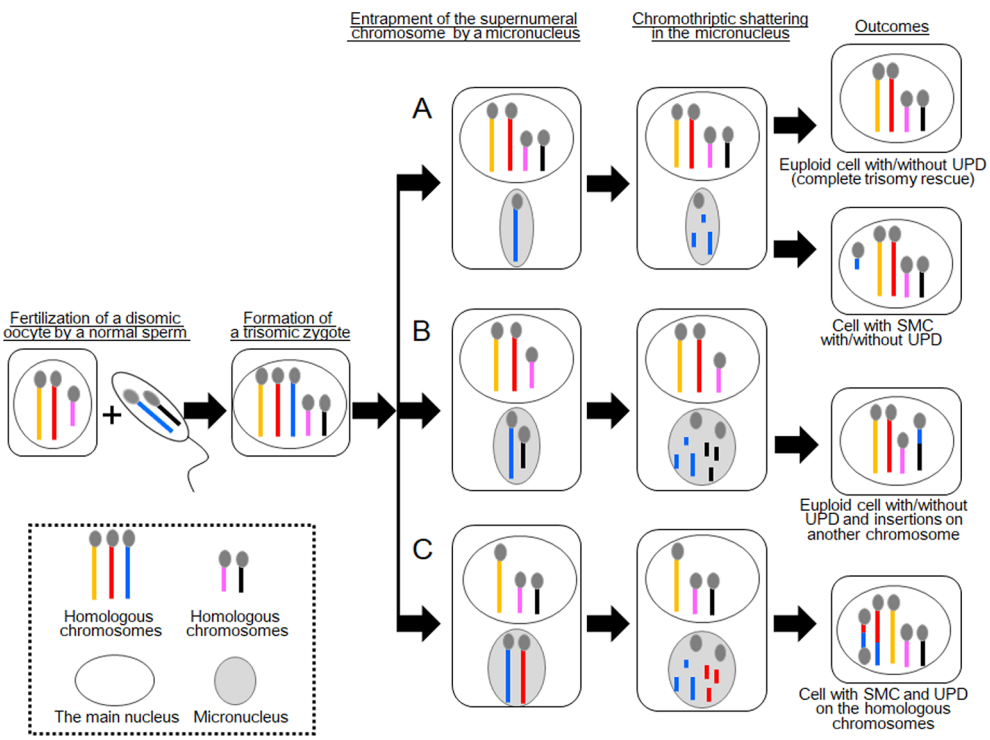

FIGURE 1 | Schematic of the consequences of chromothripsis-mediated trisomy rescue in micronuclei. Two sets of homologous chromosomes are shown as yellow, red, and blue lines, and pink and black lines. During trisomy rescue, trisomic chromosomes (blue lines) are trapped into micronuclei (gray circles) and subjected to chromothriptic shattering. Micronuclei are capable of containing additional chromosomes. **(A)** Complete trisomy rescue creates a karyotypically normal cell, while partial trisomy rescue can generate a cell with a small supernumerary marker chromosome (SMC). One third of these cells develop uniparental disomy (UPD). **(B)** A normal chromosome (black line) may also be encapsulated in a micronucleus together with a supernumerary chromosome. In this case, partial trisomy rescue creates complex chromosomal rearrangements (CCRs) on multiple chromosomes, in addition to UPD. **(C)** Partial trisomy rescue can also produce SMC and UPD, when two homologous chromosomes (red and blue lines) are jointly trapped in a micronucleus.

Zhang et al., 2015; Marcozzi et al., 2018). Jones et al. (2012) performed fluorescence *in situ* hybridization (FISH) analyses for micronuclei in leukocytes of 59 individuals, focusing on sex chromosome-containing micronuclei, and found that most micronuclei in young individuals are involved in trisomy rescue, whereas those in elder individuals are frequently associated with cellular errors leading to the degradation of an intact chromosome.

MICRONUCLEI ARE KNOWN AS THE PLATFORM FOR CHROMOTHRIPSIS

Micronuclei are known as the platform of chromothripsis, a recently discovered one-off cellular event that produces catastrophic structural changes exclusively on a single or a few chromosomes (Zhang et al., 2015). Chromothripsis was first discovered in the cancer genome and was soon after detected in the germline (Kloosterman et al., 2011; Liu et al., 2011). In addition, *de novo* occurrence of chromothripsis in postzygotic cells has also been documented (Kato et al., 2017). Constitutional chromothripsis usually creates CCRs with 3–20 breakpoints, while chromothripsis in cancer cells frequently generates rearrangements with more than 100 breakpoints (Liu et al., 2011). More recently, other one-off mutagenic events, such as chromoanasynthesis and chromoplexy, have been reported as underlying mechanisms for CCRs (Masset et al., 2016; Marcozzi et al., 2018). However, it remains to be clarified whether chromothripsis and these events are completely different phenomena. In this manuscript, we use the word "chromothripsis" to refer to all catastrophic cellular events leading to CCRs.

Presumably, chromothripsis in micronuclei follows the following processes (Poot and Haaf, 2015; Marcozzi et al., 2018); (i) a supernumeral or mis-segregated chromosome is encapsulated in a micronucleus, (ii) the trapped chromosome undergoes aberrant DNA replication and premature DNA condensation, (iii) the uncoordinated replication/condensation processes trigger DNA double-strand breaks and resultant chromosomal shattering, (iv) the contents of the micronucleus (DNA fragments) are incorporated into the main nucleus of a daughter cell, and (v) the fragments are randomly assembled and fused through non-homologous end joining and/or microhomology-mediated break-induced replication. Alternatively, when the components of the micronucleus fail to enter the main nucleus, these DNA fragments are degraded in the cytoplasm and lost.

Defective DNA replication in micronuclei may reflect their abnormal membrane structures (Holland and Cleveland, 2012). Specifically, the envelopes of micronuclei possess only a limited number of nuclear poles and, therefore, are predicted to provide an insufficient protein supply for DNA replication and repair (Holland and Cleveland, 2012). Moreover, micronuclei seem to be prone to irreversible nuclear envelope collapse during the interphase (Ly and Cleveland, 2017).

VARIOUS CHROMOSOMAL REARRANGEMENTS CAN BE CREATED IN MICRONUCLEI

Zhang et al. (2015) performed live-cell imaging and single-cell genome sequencing and elegantly confirmed that entrapment of one chromosome in a micronucleus can produce a spectrum of genomic rearrangements. Such rearrangements include catastrophic genomic crisis, as well as small circular chromosomes. Furthermore, because a micronucleus is capable of encapsulating more than one chromosome, a micronuclear chromothriptic event can create CCRs on multiple chromosomes (Storchová and Kloosterman, 2016).

In terms of trisomy rescue, micronucleus-mediated chromothripsis contributes to the elimination of excessive chromosomes to generate euploid cells (**Figure 1A**). However, when the process of chromothripsis is interrupted, it can cause partial chromosomal loss and/or rearrangements with or without UPD (**Figure 1A**) (Liehr, 2010). Co-occurrence of *de novo* CCRs and UPD, particularly those involving a set of homologous chromosomes, is indicative of incomplete trisomy rescue (Liehr et al., 2011; Kurtas et al., 2019). Previous studies have shown that a substantial proportion of UPD cases were accompanied by additional genomic or cytogenetic abnormalities (Liehr, 2010). Of these, SMCs accounted for ~4% of previously reported cases.

TWO CASES WITH CCRS INDICATIVE OF PARTIAL TRISOMY RESCUE

In 2017, Kato et al. reported an interesting case with CCRs and UPD [case 1 in Kato et al. (2017)]. This case involved segmental UPD(4) and *de novo* CCRs on chromosome 14. The results of cytogenetic analysis and SNP genotyping suggested that this patient initially had trisomy 4 consisting of one paternally and two maternally derived chromosomes. Then, one of the maternally derived chromosomes 4 was fragmented into several pieces, some of which were inserted into the paternally derived chromosome 14 to create an interstitial deletion on the recipient chromosome. The remaining pieces of the maternal chromosome 4 seemed to be lost. All other autosomes and X chromosomes remained structurally intact. The most likely explanation for this case is that a cell with trisomy 4 went through partial trisomy rescue, during which one of the three chromosomes 4 and one normal chromosome 14 were trapped into a micronucleus and subjected to chromothripsis (**Figure 1B**). The chromosome 14 seems to have been accidentally trapped in the micronucleus because it was not associated with any numerical or structural abnormalities. The mutagenic processes appear to have occurred during early-postzygotic stages because both the maternally and paternally derived materials were involved in the CCRs, and this case showed no evidence of somatic mosaicism.

Furthermore, Kato et al. reported a case with CCRs and partial trisomy 5 [case 2 in Kato et al. (2017)]. This case

comprised *de novo* CCRs on chromosomes 5 and 8 and a partial trisomy of chromosome 5. It appeared that chromosome 5 of the paternal origin was fragmented and some of the DNA fragments were inserted into the paternally derived chromosome 8, thereby deleting original sequences of that chromosome. Remarkably, the inserted fragments were derived from the nontransmitted chromosome 5 of the father. These results suggest that in this case, the zygote inherited two chromosomes 5 from the father and one from the mother. Subsequently, one of the two paternally derived chromosomes 5 was trapped into a micronucleus together with the paternally derived chromosome 8 and subjected to chromothripsis. The majority of the trapped chromosome 5 appeared to be lost during chromothripsis.

The results of these two cases imply that incomplete trisomy rescue can create *de novo* CCRs on multiple chromosomes, when one normal and one trisomic chromosomes are jointly incorporated into a micronucleus (**Figure 1B**). Such CCRs develop with or without UPD, as seen in cases 1 and 2, respectively. Considering that both cases 1 and 2 carried large insertions, micronucleus-mediated chromothripsis may favor amplification of genomic materials. Indeed, Kato et al. (2017) proposed that micronuclei formed from anaphase-lagging chromosomes may predispose a pulverized insertion.

OUR CASE WITH UPD AND A SMALL SMC

We recently encountered a patient with a small SMC, who possibly represents a novel case of atypical partial trisomy rescue. The patient was a 2-year-old boy with a 47,XY,+mar, ish dic(q13;q13)(D15Z1++, SNRPN++) karyotype. He presented with developmental delay, hypotonia, feeding difficulties, and epilepsy. We performed comparative genomic hybridization (CGH) using a catalog human CGH + single nucleotide polymorphism (SNP) array containing ~120,000 CGH probes and ~60,000 SNP probes for the entire genome (Sureprint G3; catalog number, G4890; 4 × 180 K format; Agilent Technologies, Palo Alto, CA, USA). The procedure was as described in the manufacturer's instructions. The data were analyzed using the default settings of the Genomic Workbench Software (Version 6.5, Agilent Technologies). The copy-number of each genomic region was assessed from log2 ratios of the corresponding probes; log2 ratios between +0.4 and +0.6 and between +0.9 and +1.1 were considered the signs of 3-copy and 4-copy regions, respectively. Copy-number for the centromeric part of 15q11.2 could not be determined because of the presence of repetitive sequences. To determine the parental origin of chromosomal segments, we genotyped 20 microsatellite markers on chromosome 15 (**Supplementary Table 1**) using genomic DNA samples from the patient and his parents. PCR amplification was performed using fluorescently labeled forward primers and unlabeled reverse primers, as described previously (Matsubara et al., 2011). Subsequently, the PCR products were analyzed using the CEQ8000 sequencer (Beckman Coulter, Fullerton, CA, USA).

The results revealed that the boy harbors a *de novo* SMC comprising a dicentric chromosome 15, in addition to two structurally normal chromosomes 15 (**Figure 2**). The two chromosomes 15 exhibited maternal heterodisomy, except for the terminal region of the long arm which showed a biparental pattern (**Figure 2** and **Supplementary Table 1**). The SMC was an asymmetric dicentric chromosome containing both maternal and paternal materials. Collectively, this patient appeared to have a partial tetrasomy combined with segmental maternal UPD (15). No copy-number abnormalities were detected in the other chromosomes.

These findings indicate that this case initially had a trisomy due to the inheritance of two chromosomes 15 from the mother and one chromosome 15 from the father. We speculate that this individual have been subjected to chromothripsis-mediated partial trisomy rescue. If this is the case, a micronucleus encapsulated the paternally derived chromosome 15 and one of the two maternally derived chromosomes 15 (**Figure 1C**). Then, the trapped chromosomes were shattered and reassembled to form one structurally normal chromosome 15 and one SMC.

However, one may argue against chromothripsis having occurred in this case because the boy lacked chaotic rearrangements characteristic for chromothripsis. The other potential explanation of this case is U-type exchange (Liehr, 2019). It is possible that the genomic structure of this case has been generated through prezygotic or postzygotic U-type exchange and a subsequent homologous recombination (**Supplementary Figure 1**). Indeed, the breakpoints of this SMC resided within the 15q11–q13 region, a hotspot for homologous recombination (Pujana et al., 2002; Cassidy and Driscoll, 2009). Furthermore, previous studies have suggested that homologous recombination actually occurs in postzygotic cells, although its frequency is much lower than that in prezygotic cells (Kotzot, 2001; Roehl et al., 2010; Sasaki et al., 2013), and no studies have demonstrated that trisomic zygotes are associated with a particularly high frequency of postzygotic recombination. Thus, from this single case, we could not conclude that chromothripsis is actually involved in the development of small SMCs and UPD.

CASES WITH SMCS INDICATIVE OF CHROMOTHRIPSIS

Recently, Kurtas et al. (2019) provided evidence that chromothripsis-mediated partial trisomy rescue accounts for certain percentage of the etiology of *de novo* small SMCs. The authors performed whole-genome sequencing and trio-genotyping for 12 cases with *de novo* small SMCs. The authors found that eight of the 12 individuals carried SMCs comprising non-contiguous portions of one chromosome, whereas the remaining four individuals had SMCs consisting solely of pericentromeric portions of a chromosome. In the eight patients, multiple portions of the SMCs were aligned in a disordered manner, suggesting that they have been subjected to

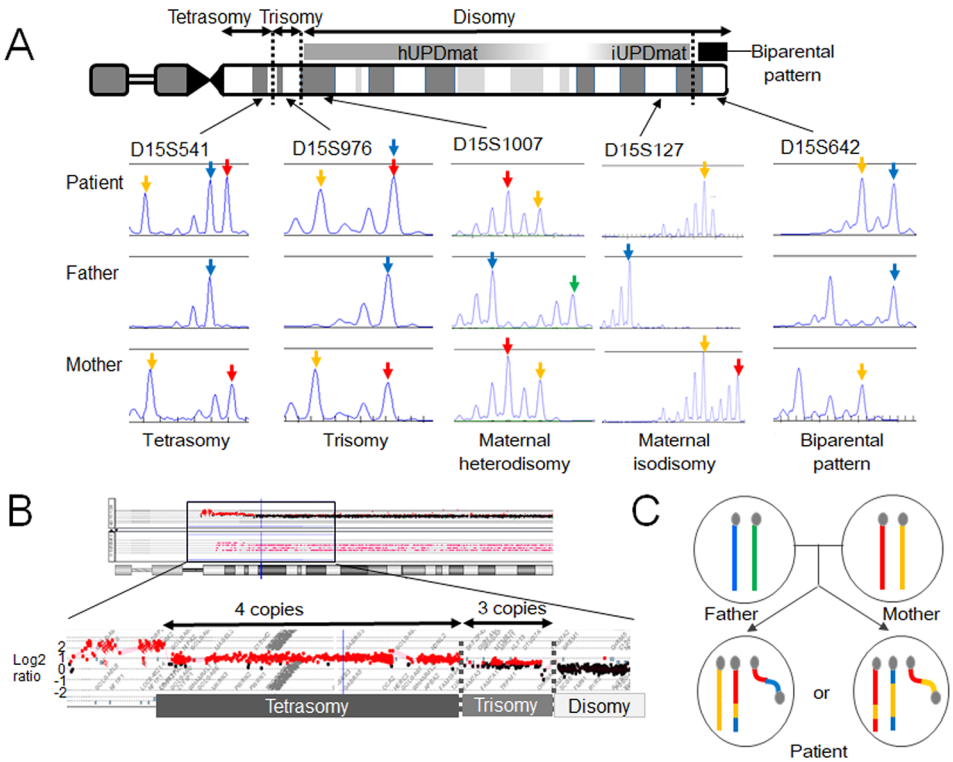

FIGURE 2 | Molecular findings of our case. **(A)** Microsatellite analysis revealed segmental maternal disomy of chromosome 15. The red and yellow arrows depict alleles present in the mother, and the blue and green arrows indicate alleles present in the father. **(B)** Array-based comparative genomic hybridization detected copy-number gains in the 15q11-13 region. The breakpoints resided within low copy-number repeat regions. **(C)** Two most likely structures of chromosomes 15 in this case. One of the two structurally normal chromosomes 15 and the supernumerary marker chromosome are likely to comprise both maternal (red and yellow) and paternal (blue) materials. hUPDmat, maternal heterodisomy; iUPDmat, maternal isodisomy.

chromothripsis. Moreover, these eight cases were likely to result from trisomy rescue because two of the eight cases showed a paternal origin of SMCs combined with maternal UPD of the homologous chromosomes, while the other six cases showed a maternal origin of the SMCs together with biparentally inherited homologous chromosomes. These findings indicate that the SMCs in the eight cases originated through maternal meiotic nondisjunction and subsequent chromothripsis-mediated degradation of excessive chromosomes. Indeed, most of these cases were associated with advanced maternal age, which is known as the major risk factor for meiotic nondisjunction (Jones, 2008). Al-Rikabi et al. (2018) also reported individuals who carried chromothripsis-consistent SMCs.

PATHOPHYSIOLOGICAL SIGNIFICANCE OF TRISOMY RESCUE IN HUMAN EMBRYOS

Complete trisomy rescue in micronuclei increases the viability of human fetuses by reducing the negative effects of chromosomal aneuploidy. Considering the high frequency of trisomic cells in early-stage human embryos (Los et al., 2004; Liehr, 2010; Irani et al., 2018), trisomy rescue appears to be a physiologically important event. On the other hand, trisomy rescue frequently creates UPD. Although UPD is not necessarily pathogenic, it can cause imprinting disorders and unmask recessive mutations. It has been proposed that the frequency of UPD among newborns is as high as ~1/3,500 (Robinson, 2000; Yamazawa et al., 2010), and a substantial percentage of UPD cases result from trisomy rescue (Liehr, 2010; Liehr, 2019).

Moreover, partial trisomy rescue underlies various types of genomic rearrangements involving one or more chromosomes. For example, Bonaglia et al. (2018) demonstrated that the primary driver for *de novo* unbalanced translocations is maternal meiotic disjunction and subsequent partial trisomy rescue of the supernumerary chromosome. CCRs created by partial trisomy rescue often lead to congenital malformations and developmental delay by altering the copy-number or cis-regulatory machinery of the genes on the affected chromosomes (Poot and Haaf, 2015). Collectively, chromothripsis-mediated trisomy rescue in micronuclei appears to be a physiologically and pathologically important phenomenon.

CONCLUSIONS

Trisomy rescue seems to be a physiologically indispensable event in human fetuses to reduce the deleterious effects of chromosomal aneuploidy. Nevertheless, trisomy rescue can produce UPD and genomic rearrangements. Recent studies, including our own, suggested that chromothripsis-mediated partial trisomy rescue is involved in the formation of small SMCs, although other mechanisms such as U-type exchange and postzygotic recombinations may also contribute to the development of such SMCs. The concepts of trisomy rescue, chromothripsis, and micronuclei provide novel insights into the origin of complex structural changes in the genome. Further studies on cases with various types of SMCs will clarify the molecular machineries involved in the maintenance and modification of chromosomal architectures.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in no repository but are available from the corresponding author upon reasonable request (no accession number).

ETHICS STATEMENT

Our case study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. The guardian of the patient gave written informed consent in accordance with the Declaration of Helsinki for the participation in the study and the publication of the results.

AUTHOR CONTRIBUTIONS

KM, MK, and MF contributed to study design. KM and MK performed molecular analysis. KY and TN performed clinical analysis. KM, MK, and MF contributed to the writing of the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00132/full#supplementary-material

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