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Chromosomes in the DNA era: Perspectives in diagnostics and research

Introduction

Chromosomal disorders have existed as long as life has existed and will exist in the future—because of our genome architecture. The latter is the fundamental dynamic link driving technical developments from basic research into routine diagnostics and back. Understanding the peculiarities of each available approach for studying the human genome can help to combine these techniques properly to provide the maximal diagnostic yield for individual patients and affected families. At the same time, by bringing all the data and sometimes apparently insignificant results together, a better understanding of higher-order genomic architecture, genomic disease mechanisms, and genome evolution can be achieved.

The 2m of human nuclear DNA are divided into 46 densely packed portions in each cell, wound around complex, higher-order protein structures such as chromosomes, which are organized in a well-defined hierarchical three-dimensional way. This higher-order compaction is driven by the necessity and functionality of the nucleus and was denominated "chromosomics" in 2005 [1]. To address the link between basic research and clinical diagnostics in the field of chromosome biology, we divided the manuscript into two subparts: (i) current clinical applications and diagnostic strategies for approaching chromosomal aberrations and (ii) integrative aspects of structure, function, and evolution of the human genome towards a better understanding of human chromosome biology.

(i) Chromosomes in genetic diagnostics

To unveil the nature and role of chromosomal aberrations in syndromic disease, banding cytogenetics is most often the first essential part of current genetic diagnostics [2]. Chromosomal aberrations are microscopically visible large-scale genomic alterations resulting in imbalances that lead to gene dosage effects (aneuploidies, deletions, duplications), may cause deregulation of gene expression by disrupting genes, leading to gene fusions, position effects or a predisposition for larger imbalances in the next generation (due to translocations, inversions). Besides, imprinting effects due to unequal parental inheritance of some chromosomes or chromosomal segments can be another easily forgotten consequence (uniparental disomy, UPD [3]). If such chromosomal aberrations affect germ cells this may cause inherited genomic diseases based on microdeletions and microduplications, or can lead to infertility and/or abortions due to imbalanced transmission of the rearrangement to the next generation. On the other hand, if somatic cells are affected, such alterations can contribute to cancer and genetic aging [4–6]. In addition to classical and banding cytogenetic approaches, chromosomes are nowadays studied for diagnostic purposes in situ by molecular cytogenetics and in vitro by molecular karyotyping, next-generation sequencing, and multiple polymerase chain reaction (PCR)-based approaches [7].

Chromosomes visualized by classical and banding cytogenetics

Walter Flemming, a German anatomist, was the first to analyze structures and processes in the interphase nucleus in a systematic way using a light microscope. He described the process of "mitosis," and visualized and denominated "chromatin" for the first time in 1879. Therefore, he is recognized as the founder of cytogenetics. In 1956, Tjio and Levan correctly determined that the constitutional human chromosome number is 46. Accordingly, from 1956 onward several well-known clinical syndromes were assigned to have their underlying cause in aneuploidy (e. g., trisomy 21 leading to Down's syndrome). However, structural aberrations were (almost) not detectable until the introduction of banding cytogenetics in 1971 by Lore Zech. She also contributed to the field of tumor cytogenetics, where the link between abnormal chromosomes and cancer had been established since 1914 owing to a seminal paper by Theodor Boveri presenting his "chromosome theory of tumor development" [8, 9].

The highest incidence of constitutional chromosomal aberrations in humans is found in first-trimester spontaneous abortions. An overall rate of >50% prenatal fatal abnormalities can be seen in these abortions, and the observed spectrum of aberrations is similar in natural and assisted reproduction [10, 11]. Compared with the general population, incidences of chromosomal aberrations are higher in patients with mental retardation, dysmorphic features, pre- and postnatal growth abnormalities,

absent/preterm puberty, infertility, and recurrent abortions. Taken together, the incidence of gross chromosomal abnormalities is estimated to be 1 in 135 live-born children, around 40% of them being phenotypically affected [12]. A cytogenetically unresolvable or an apparently balanced *de novo* aberration found in a phenotypically affected individual is the starting point for further analysis; molecular cytogenetics and/or molecular karyotyping to identify possible causative submicroscopic imbalances, complex rearrangements or disrupted genes are nowadays routinely applied in such cases [13]. However, if banding cytogenetic analysis has been skipped and a chromosomal imbalance (>50 kb) is detected, e. g., by CMA, it is extremely important to verify the molecular karyotyping results at the chromosomal level. This is necessary to distinguish an unbalanced insertion from an unbalanced translocation or a small supernumerary marker chromosome from a simple duplication. Also, subsequent analyses of the parents is necessary to estimate recurrence risks.

Furthermore, identification and characterization of chromosomal aberrations play an important role in tumor cytogenetic diagnostics of leukemia, lymphoma, and solid tumors. Acquired, cytogenetically detectable aberrations are frequently observed in these conditions and can be the most complex results of human genome reshuffling [6, 14].

In general, the prerequisites for cytogenetic approaches are living cells that still divide or may be stimulated to divide again. The huge advantage of this technique is the "whole genomic view," which enables screening for any kind of numerical and gross structural aberration at a single cell level; the latter is a unique opportunity to also detect low-level mosaicism [15]. Additionally, banding cytogenetic analyses can be performed at relative low equipment and consumables costs compared with higher resolution techniques (**Table 1**). For acquired somatic clonal chromosomal abnormalities in tumor cytogenetics, the result can help to classify the tumor type, may influence tumor therapy and prognosis, and can be used to monitor the disease course in the patient.

Chromosomes visualized by molecular cytogenetics

Molecular cytogenetics originally comprised two approaches: primed in situ hybridization (PRINS) and fluorescence in situ hybridization (FISH). Nowadays, PRINS is only rarely used in research as it turned out to be exclusively suitable for repetitive regions. FISH was established between 1986 and 1989 for human chromosomes as a single-, two- or three-color approach. Previously, ISH was only available as a radioactive methodology. In 1992, chromosome-based comparative genomic hybridization (CGH) was developed. CGH was later developed toward molecular karyotyping (i. e., array CGH). Since 1996, various multicolor FISH probe-sets have been established. First, only whole chromosome paints were used. Afterward, all kind of probes such as partial chromosome paints, centromeric probes and locus-specific probes were combined for subtelomeric, (peri-)centromeric and/or FISH-banding probe sets [7]. Since the 2000s, it has become possible to apply FISH to study genomic architecture of 3D-preserved interphase nuclei. This approach is still only used for research purposes, even though a possible impact on clinical consequences was reported (see also part (ii) of this article).

FISH is nowadays routinely applied in tumor cytogenetics, including interphase-directed approaches in solid tumors and leukemia [6]. For constitutional genetics, it has recently been shown that FISH is the only routine diagnostic approach capable of detecting the parental origin of disease-causing submicroscopic inversions relevant for the offspring. Such submicroscopic events may lead to microdeletion or microduplication in the putative progeny of such inversion carriers [16]. Nonetheless, patients with a suspected microdeletion syndrome (including subtelomeric imbalances) are nowadays tested using molecular karyotyping rather than

Abstract · Zusammenfassung

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Chromosomes in the DNA era: Perspectives in diagnostics and research

Abstract

Chromosomes were discovered more than 130 years ago. The implementation of chromosomal investigations in clinical diagnostics was fueled by determining the correct number of human chromosomes to be 46 and the development of specific banding techniques. Subsequent technical improvements in the field of genetic diagnostics, such as fluorescence in situ hybridization (FISH), chromosomal microarrays (CMA, array CGH) or next-generation sequencing (NGS) techniques, partially succeeded in overcoming limitations of banding cytogenetics. Consequently, nowadays, higher diagnostic yields can be achieved if new approaches such as NGS, CMA or FISH are applied in combination with cytogenetics. Nonetheless, high-resolution DNA-focused techniques have dominated clinical diagnostics more recently, rather than a "chromosomic view," including banding cytogenetics as a precondition for the application of higher resolution methods. Currently, there is a renaissance of this "chromosomic view" in research, understanding chromosomes to be an essential feature of genomic architecture, owing to the discovery of (i) higher order chromosomal sub-compartments, (ii) chromosomal features that influence genomic architecture, gene expression, and evolution, and (iii) 3D and 4D chromatin organization within the nucleus, including the complex way in which chromosomes interact with each other. Interestingly, in many instances research was triggered by specific clinical diagnostic cases or diseases that contributed to new and fascinating insights, not only into disease mechanisms but also into basic principles of chromosome biology. Here we review the role, the intrinsic value, and the perspectives of chromosomes in a molecular genetics-dominated human genetics diagnostic era and make comparison with basic research, where these benefits are well-recognized.

Keywords

Karyotyping · FISH · CMA · Fragile sites · Interphase architecture

Chromosomen im DNA-Zeitalter: Perspektiven für die Diagnostik und Forschung

Zusammenfassung

Vor mehr als 130 Jahren wurden Chromosomen erstmals beschrieben. Die Analyse menschlicher Chromosomen in der humangenetischen Diagnostik etablierte sich rasch, nachdem die Anzahl der menschlichen Chromosomen mit 46 richtig bestimmt und mit Einführung der Bänderungszytogenetik vollzogen worden war. Nachfolgende technische Fortschritte der genetischen Diagnostik, wie Fluoreszenz-in-situ-Hybridisierung (FISH), „Molekulare Karyotypisierung" (CMA, array CGH) oder „Next Generation Sequencing" (NGS) trugen dazu bei, einige der Einschränkungen der Bänderungszytogenetik zu überwinden. Letztlich kann aber eine verbesserte Diagnostik dann erzielt werden, wenn die genannten neuen Ansätze wie NGS, CMA oder FISH in Kombination mit zytogenetischen Methoden angewandt werden. Nichtsdestotrotz dominieren heutzutage hochauflösende DNA-fokussierte Techniken die humangenetische Diagnostik und weniger die Sichtweise, die als „Chromosomics" beschrieben wurde, d. h. unter anderem auch die Durchführung der Bänderungszytogenetik als Grundvoraussetzung für die Anwendung von Methoden mit höherer Auflösung. Eine gänzlich gegensätzliche Entwicklung lässt sich hingegen im Bereich der Forschung beobachten. Hier rücken die Chromosomen als ein essenzieller Bestandteil der Genomarchitektur mehr und mehr in den Mittelpunkt, spätestens seit der Entdeckung (i) chromosomaler Sub-Kompartimente, welche sich klar in einer „höheren Ordnung" präsentieren, (ii) von Eigenschaften der Chromosomen, welche die Architektur des Genoms, die Genexpression und die Evolution beeinflussen, sowie (iii) der 3D- und 4D-Chromatinorganisation innerhalb des Zellkerns, einschließlich der komplexen Art und Weise, wie Chromosomen miteinander interagieren. Interessanterweise wurde die hier genannte Forschung in vielen Fällen durch spezifische klinische Einzelfälle aus der Diagnostik oder das Studium bestimmter Erkrankungen gefördert, die nicht nur zum Verständnis beitrugen wie Krankheiten entstehen können, sondern oftmals zu neuen und faszinierenden Einblicken in die Chromosomenbiologie führten. In der vorliegenden Arbeit betrachten wir die Rolle der Chromosomenstruktur und die Gründe, warum sie berücksichtigt werden muss, sowie die Perspektiven von Chromosomenstudien/„Chromosomics" in einem diagnostikorientierten, molekulargenetisch dominierten humangenetischen Umfeld und stellen sie der Welt der Grundlagenforschung gegenüber, in der diese Aspekte den Akteuren voll bewusst sind.

Schlüsselwörter

Karyotypisierung · FISH · CMA · Fragile sites · Interphasekernarchitektur

FISH. Although in the past, FISH was used as a "prenatal quicktest" to screen for the most frequent second-trimester aneuploidies, this is nowadays most often examined using molecular genetics tests via microsatellite analysis. However, interphase FISH performed in somatic tissues other than blood or fibroblasts is an important tool for ruling out the possibility of cryptic mosaicism, e. g., in the case of female infertility and a banding cytogenetic karyotype of 45,X[2]/46,XX[27]/47,XXX[1], FISH in buccal mucosa may confirm X-chromosome mosaicism [7].

Metaphase directed FISH is especially important for the characterization of acquired and constitutional chromosomal rearrangements, not being resolvable by banding cytogenetics alone. Cryptic complex rearrangements, orientation of inserted DNA fragments and submicroscopic deletions or duplications may be detected and characterized by FISH [7]. Another important field addressable by FISH is the characterization of heterochromatic variants, which may also be distinguished from euchromatic balanced rearrangements [5]. Finally, FISH is often used as the "second method" to

Table 1 Comparison of currently available routine diagnostic approaches

Feature	GTG banding	FISH	CMA	NGS
Principle	Monochrome-stained chromosomes are screened for numerical, structurally balanced and unbalanced aberrations	DNA-specific fluorescent probes are hybridized on patient chromosomes/nuclei to resolve structural and numerical aberrations	Patient DNA is applied to a microarray to screen for genomic gains or losses (and if SNP based for LOH) at high resolution	Panel-specific and/or genome-wide detection of causal mutations in monogenic disorders by high-throughput sequencing techniques
Material	Living cells	Metaphases and interphase	DNA	DNA
		Tissue sections, primary fixed cells		
Equipment	Incubator	FISH probes	Microarray	NGS platform
	Light microscope	Fluorescence microscope	Scanner	Bioinformatic data processing
Range	Genome-wide	Genome-wide (M-FISH)	Genome-wide	Genome-wide (WGS)
	Single cell level	Locus- and region-specific	Pool of cells	Gene panel-specific
		Single cell level		Pool of cells
Resolution for routine diagnostics	>5–10 Mb	>50 kb	>50 kb	1–300 bp
Mosaic detection (minimum range)	Depends on the number of analyzed cells (5–25%)	Depends on the number of analyzed cells (0.1–1%)	Depends on the mosaic ratio in the sample (20–30%; SNP array <5%)	Depends on the mosaic ratio in the sample and coverage and the analysis software (>0.1% with MuTect; >10% with HaploTyper)
Minimal turnaround time	Depending on culture time: 1–15 days	Depending on FISH probe type: 3 h to 3 days	Depending on platform: 1–4 d	Depending on platform and panel: 5–14 d
Applications	First-line screening test for gross structural and numerical aberrations	Second-line test for specific rearrangements	Second-line screening for genomic imbalances after normal GTG/FISH	Second-/third-line panel-specific or genome-wide screening for DNA mutations
	Tumor cytogenetic	Detects small mosaics	Defining size of chromosomal imbalances	First-line test as panel in defined clinical subgroups
	(Metaphases for FISH)	Verification of GTG and CMA results	Verification in parents to interpret VUS in offspring	Tumor-specific targeted panels
		Resolves complex rearrangements		Predictive testing and verification of familial variants
		Parental screening for predisposing rearrangements		(CNV detection)
		Tumor-specific FISH panels		
Advantage	Low costs for equipment and consumables	Widely used for commercial probes	Widely used	High throughput lowers single-run/-case costs
	Widely used	Sensitive mosaic detection	~100 × higher resolution than GTG	Widely used
	Detection of heterochromatic regions	Predisposing rearrangements in parents detectable, genomic location and orientation resolvable	CNV detection	Single base resolution on genome-wide scale
	Global whole genome view with information on genomic location	CNV detection (especially deletion, inversion, translocation events) at higher resolution than GTG		Low-level mosaics detectable
		Verification method		
		Use of frozen or FFPET sections		

Table 1 (Continued)

Feature	GTG banding	FISH	CMA	NGS
Pitfalls and disadvantages	Viable cells and culture artefacts	Possible cross hybridization	Heterochromatic regions are not covered	Short reads limit CNV and structural aberration detection
	Small and/or rare rearrangements can be overlooked	No commercial FISH probe for region of interest	Location of an imbalance within the genome is unknown	Heterochromatic/repetitive regions are unsatisfactorily/not covered (artefact variants)
	Experienced staff is needed	High costs of FISH probes	Balanced aberrations are not detectable	Fidelity at low coverage
	Resolution	Small duplication events are hard to verify	Low-level mosaics escape	High rate of VUS, interpretation problems, secondary findings
		FISH assay and lab-specific cut-offs need to be established	High rate of VUS	No standards for software pipelines and data processing
			Costs for equipment and consumables	Costs for equipment and consumables
			Polyploidy detection	Bioinformatic trained stuff is needed

GTG G-bands by trypsin using Giemsa, **FISH** fluorescence in situ hybridization, **CMA** chromosomal microarray, **NGS** next-generation sequencing, **CGH** comparative genomic hybridization, **SNP** single-nucleotide polymorphism, **LOH** loss of heterozygosity, **Mb** mega base pair, **kb** kilo base pair, **bp** base pair, **VUS** variant of uncertain clinical significance, **CNV** copy number variation, **FFPET** formalin-fixed paraffin-embedded tissue, **WGS** whole-genome sequencing

confirm a molecular karyotyping result (**Table 1**).

Chromosomal micro-arrays (CMA)/ molecular karyotyping

For around 10 years chromosomal microarrays (CMA), based on the original CGH method (see above), have been routinely used to screen for genome-wide imbalances. In addition, single-nucleotide polymorphism (SNP)-based techniques are used to test for loss of heterozygosity (LOH). The resolution depends on the platform used and the diagnostic indication and can be as low as 5–10 kb. CMAs and banding cytogenetics are both genome-wide techniques, but the resolution of CMA is 100–1,000-fold higher. Thus, CMA is also referred as molecular karyotyping, even though no *in situ* view on the chromosome and no information on ploidy or heterochromatic genomic regions are available [5]. Based on CMA, numerous new recurrent microdeletion and microduplication syndromes were reported, e. g., Weise et al. [4]. At the same time, more and more copy number variations with uncertain clinical significance and combinatorial effects have been identified. As CMA utilizes DNA from a relatively large cell pool, small mosaics within the sample may stay undetected. Furthermore, CMA in routine diagnostic settings is able to identify neither balanced aberrations (translocation, inversion, insertion) that might have an effect on the recurrence risk or have a position effect, nor DNA sequence or epigenetic changes including imbalances below the specific resolution of the array platform. Finally, the exact nature of an imbalance cannot be resolved by CMA alone (**Fig. 1**) and needs subsequent (molecular) cytogenetic analyses. Moreover, the investigation of the parental or de novo origin of a CMA-detected imbalance can help to estimate recurrence risks in single families [11].

Using CMA, the highest diagnostic yield is available in mentally retarded individuals with a normal karyotype; here, an additional detection rate of around 20% has been reported [17]. Other patients with submicroscopic genomic imbalances include individuals with

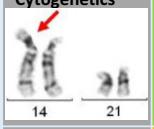
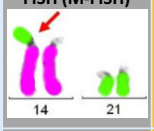
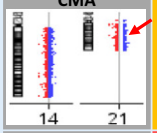
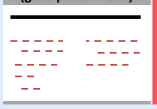
Detected by	EBM in €	Missed diagnostic information	Additional diagnostic information
Cytogenetics 	197	-	• Indication for parental testing to distinguish familiar DS from de novo, estimate recurrence risk
FISH (M-FISH) 	954	-	• Indication for parental testing to distinguish familiar DS from de novo, estimate recurrence risk
CMA 	945	• No differentiation between free and translocation trisomy • Parental CMA will give normal results although one parent might be a carrier with increased recurrence risk	• When SNP-array is used additional information on UPD / LOH • Additional clinical relevant CNVs might be detected
NGS (gene panel <25kb) 	2626	• No CNV detection by NGS pipeline → completely missed diagnosis • CNV detection by NGS pipeline → no differentiation between free or translocation trisomy → Parental NGS will give normal results although one parent might be a carrier with increased recurrence risk	• Additional clinical sequence mutations might be detected • CNV detection by NGS pipeline →additional clinical relevant CNVs might be detected

Fig. 1 ▲ Example of a postnatal case with translocation trisomy 21 due to a rob(14;21). This example is chosen because it illustrates the different levels of diagnostic information for different methods and is an example where (molecular) cytogenetics gives the highest diagnostic value. Depending on other diagnostic indications and causative mutations other techniques might be more comprehensive. Different detection techniques are compared, along with the reimbursement by the German health system according to the current "EBM" catalogue per case and the missed and additional diagnostic information when each technique is used as a stand-alone.
GTG G-bands by trypsin using Giemsa, **FISH** fluorescence in situ hybridization, **CMA** chromosomal microarray, **NGS** next-generation sequencing, **CNV** copy number variation, **DS** Down Syndrome, **UPD** uniparental disomy, **LOH** loss of heterozygosity, **EBM** Einheitlicher Bewertungsmaßstab

autism, epilepsy, dysmorphic features, developmental delay, and congenital malformations, or a combination of the aforementioned characteristics. However, up to ~80% of infertile patients with a small supernumerary marker chromosome would be missed in CMA [18].

Nonchromosome-directed molecular diagnostic strategies

The main driving forces for developing new techniques in genetic diagnostics are the limitations of the established ones (**Table 1**). This also holds true for PCR-based approaches developed over the last decades (e.g., multiplex ligation-dependent probe amplification—MLPA), and for the latest achievement: high-throughput next-generation sequencing (NGS) techniques. NGS dramatically reduced sequencing costs and time when entering routine diagnostics to replace Sanger sequencing of single genes. However, the most comprehensive strategy for finding new causative mutations by NGS is to run whole-genome (WGS) or whole-exome (WES) sequencing (ideally in family trios). Targeted gene panels are straightforward for reducing time and costs for the health system in routine diagnostics of defined clinical subgroups (e.g., certain malformations, neurological phenotypes, malignancies, etc.). Consequently, targeted NGS-based tests have been developed that can deal with very small amounts of cell-free DNA and DNA ratio differences, as in non-invasive prenatal testing (NIPT). Currently, NIPT is applied globally as a prenatal screening test focusing on trisomies 13, 18, and 21, and on gonosomes [19]. This led to the secondary phenomenon that the incidence of children born with inborn disease-related copy number variations (CNVs), which were formerly picked up by GTG banding, FISH, and/or CMA, became epidemic [20, 21]. In fact, this has had drastic consequences not only for individual families, insufficiently informed about the drawbacks of the NIPT test, but also for national health systems [19–21].

Hence, besides having a high diagnostic yield, NGS has limitations (**Table 1**). A recent retrospective study by Höchstenbach et al. [22] nicely illustrates the spectrum of missed diagnoses when WGS might be used as a "one fits all" test. At least 8.1% of GTG/FISH/CMA-detected abnormalities are missed: 73.3% in the premature ovarian failure due to low-level gonosomal mosaicism group, 25.6% in couples with recurrent miscarriages because of undetected Robertsonian translocations (**Fig. 1**), and 0.35% in mentally retarded patients. Thus, clinicians, clinical laboratory geneticists, and the patient/family need to be aware of expected pick-up rates and the type of abnormalities that can escape the applied

diagnostic methods. Another side effect when new state-of-the-art techniques become routine is the decreasing number of analyses of "old-fashioned" methods that might lead to fading competency in the neglected field [23]. Therefore, traditional methods should not be bypassed just because newer approaches become available. The choice of stepwise diagnostics (e. g., from low to high resolution) may be preferable. An example is the need for subsequent diagnostics in defining the type of trisomy found by CMA, which can either be a free trisomy and most likely sporadic, or arise from a (parental) translocation being connected with an enhanced probability of recurrence in further offspring and a UPD risk for future pregnancies. To elucidate the underlying type of trisomy, only cytogenetics can help (**Fig. 1**).

Interim conclusions for diagnostics

In conclusion, reasonable (preferably stepwise) combinations of well-established new high-throughput methods will lead to a maximum diagnostic yield for the patients and their families, while keeping in mind methodological limitations, advantages, and disadvantages (**Table 1**). Even a simple karyotype may resolve the diagnosis (**Fig. 1**). On the other hand, WGS may serve as a first-line test to find causative single-gene mutations, LOHs, CNVs, and structural rearrangements, given that this can be accompanied by a third-generation sequencing revolution providing long read sequence information. Nevertheless, karyotyping will still be helpful and necessary, as heterochromatic regions are barely covered by sequencing approaches.

However, all patients, in industrial countries and in developing nations, deserve the best and most straightforward strategies for a quick and comprehensive diagnosis. Therefore, clinically customized and cost-minimizing analyses should be used before starting with novel "one fits all" methods (**Fig. 1**).

Interim conclusions for research

Owing to the application of high-throughput settings and cytogenetic approaches in clinical diagnostics, there is a tremendous output of large amounts of diagnostic data and metadata collected in open databases. This is not only fruitful for better genotype–phenotype correlation and comprehensive genetic counseling, but also provides new and deeper insights into fundamental genome biology and disease mechanisms. One example is the identification of recurrent microdeletion and -duplication syndromes by CMA collected in several databases (e. g., Decipher, ISCA, ECRUCA) and the identification of the underlying pathomechanism of non-homologous recombination triggered by low copy repeats. Subsequently, predisposing rearrangements such as inversions were discovered in parents of affected individuals and are now the subject of evaluation in routine FISH diagnostics to estimate the recurrence risk for those families [16]. Even combining all available standard approaches in human genetic diagnostics may be insufficient to solve the disease-causing gene. But putting all information together would still be helpful in terms of understanding general principles such as genome, chromosomal, and nuclear organization. For example, the recent characterization of a disease mechanism facilitated by a deletion acting in trans was only accessible by taking interphase architecture into account [24]—see the next part of this paper.

(ii) Chromosome biology as a key to understanding genome architecture

Chromosomes are not only a bundled storage structure of the primary DNA sequence but instead present an additional layer of information for the living cell. More recently, because of the new technical developments mentioned in the first part of this article, this basic fact tends to be forgotten in human genetic diagnostics. However, at least in research the focus is heading toward understanding genomic architecture so as to understand functional substructures of the nucleus, how DNA is folded into chromosomes, and how chromosomes—and the genes located on them—are functionally arranged and interact. Overall, a combination of DNA sequence, epigenetic modifications, chromosomal sub-compartmentalization, and spatial and chronological organization within the nucleus orchestrates the symphony of life; this is all summarized in the concept of "chromosomics" [1].

Interphase and metaphase architecture

The spatial and temporal fine tuning of nuclear architecture is critical for replication, transcription, DNA repair, and cell cycle progression. Recent technical developments include chromosome conformation capture and chromatin immunoprecipitation with subsequent sequencing methods (summarized in Schmitt et al. [25]), together with super resolution microscopy (summarized in Cattoni et al. [26]). Thereby, these methods provide an alternative view into the highly dynamic and complex organization of the nucleus.

The anatomy of the nucleus came into spotlight on the discovery that chromosomes are not fully decondensed in the interphase nucleus [27] and are randomly placed but occupy a preferred area that is also known as chromosome territory [28]. One key method for accessing the nucleus architecture is sequence-specific multicolor FISH. Another modification for reaching a more in vivo situation was to perform studies in 3D-preserved interphase nuclei. Accordingly, there is strong evidence that defined chromosomal positioning is a prerequisite for the correct functioning of living cells. Thus, the comparison of the chromosomal constitution in healthy and disease-affected human brains [29], of sperm in healthy and infertile individuals [30, 31] or in leukemic and normal bone marrow [32, 33] can enlighten the as yet not understood pathomechanisms of many human diseases. Additionally, the impact of extra chromosomes on the nuclear architecture has been studied using this technique in humans [34] and other species

[35], in addition to the general position of chromosomes in species other than humans [36, 37].

Recently, it has been confirmed that the precise order of chromosomes is not only restricted to the interphase and prophase nucleus [38] but also seems to be conserved with respect to the parental origin of homologous chromosomes down to the metaphase stage; a functional relevance cannot be neglected any longer [39].

As outlined above for diagnostic chromosome analysis (in the first part of this article), different techniques for studying the human genome involve specific limitations. This also holds true for studies in 3D nuclear architecture, where no single technique is able to address all the questions to be studied. This is mainly because of remarkable differences in throughput, resolution, and reproducibility of the available approaches. In addition, most technical possibilities are static snapshots without temporal dynamic chromatin information, lacking information on cell context and cell variability within a population. The recently launched 4D nucleome project is aimed at overcoming that kind of limitation [40], but a strong need for standards and guidelines is required to ensure data reproducibility [41].

Chromosomal aberrations and altered "chromosome kissing"

Although chromosomal aberrations can have a direct effect on the phenotype (by altered gene dosage as outlined in the first part of this article) a frequently discussed and barely accessible mechanism is the so-called "position effect" [42]. It is known that genes are aligned linearly along a chromosome. Whereas the influence of enhancers or inhibitors in front of or near to a gene on its functionality is well-accepted, close proximity to a heterochromatic region or direct interaction of genetic regions located Mbs apart from each other (in cis) or even in trans, has long been debated. Meanwhile, it is well-known that the expression of genes distant from a given breakpoint can be affected because of disturbed higher-order chromatin organization (e. g., topologically associated domains, TADs; reviewed in Spielmann et al. [43]). Apart from the structural organization of the nucleus and folding of chromosomes into local aggregates such as TADs, chromosomes can directly interact with each other. This was first observed as the "intermingling" of chromosome territories and is also referred as "chromosome kissing" or "nonhomologous chromosomal contacts" [44, 45]. Such interchromosomal interactions contribute to the formation of nuclear compartments, e. g., when the short arms of human acrocentric chromosomes build up the nucleolus, and/or form a gene expression regulation network with regulatory elements interacting in trans (summarized in Maass et al. [46]). The close proximity between nonhomologous chromosomes facilitates not only the exchange of chromosomal material, appearing in as many as 1:1,000 for Robertsonian transloca-

tions [12] but also drives partner selection for other (partly recurrent) translocations [47]. This phenomenon could be tissue-specific, illustrated by recurrent tumor-specific translocations [33, 48]. The direct influence of a chromosomal aberration on 3D nuclear architecture was recently shown for a *HDAC4* deletion located in 2q37, resulting in altered "chromosome kissing" among chromosomes 2, 12, and 17 [24].

Fragile sites as "drivers" of gene und genome evolution

The common bases for acquired or constitutional chromosomal rearrangements and for chromosomal changes between species are DNA double-strand breaks. One specific class of cytogenetically visible breaks of decondensed chromatin are fragile sites (FSs) that are considered as regions of chromosomal instability with overlapping signatures for breakpoints repeatedly observed in tumors [49–51], in constitutional rearrangements [4, 52, 53], and also as evolutionarily conserved breakpoints [54–58]. In addition, those breakage-prone regions are conserved beyond the mammalian linage [59] and seem to be a general and conserved feature of chromosome biology. Therefore, FSs are exemplary structures for focusing deeper into mechanisms of chromosome, gene, and genome evolution.

Fragile sites can be induced under different culture conditions inhibiting proper DNA replication and resulting in unreplicated stretches of DNA visible as chromosomal breaks or gaps on a cytogenetic view. Up to now, more than 230 different FSs have been described at a genomic resolution of 5–10 Mb [60]. So far, only few have been mapped at the molecular level. This is because these so-called common FSs can only be observed in low frequencies (mostly below 0.1%) and are not linked to a specific DNA sequence but rather reflect regions of enhanced breakage probability with variable sizes (up to several Mb). Besides these "common FSs," which apparently contribute to regular chromosome structure and biology and are not of clinical relevance, "rare FSs" exist, which break at specific repeats and can segregate within families or are associated with certain syndromes [54]. It has become evident that within common FSs, large stretches of DNA-activated replication origins are lacking [61]. Instead, origin scarcity is connected to the even greater effect of replication hindrance on the timely replication of this region. Under-replicated DNA, therefore, parts will be left behind, somehow escaping check point activation before mitosis onset, as treatment with low doses of a potent replication inhibitor can do [62]. During the metaphase such zones of incomplete replication can appear as relaxed chromosomal parts that are reminiscent of gaps or breaks present within each human individual. A recent study on molecular features of fine-mapped FS [63] revealed that these regions are in general gene-poor but at the same time enriched in disease related and Online Mendelian Inheritance in Man(OMIM)-annotated genes. Additionally, they comprise an increased enrichment of CNVs [64, 65], most likely mediated by the imperfect repair of these breaks. Those CNVs include euchromatic gene-carrying sequences, leading to copy gains and losses, and are therefore a substrate for population variability and evolutionary processes. This could be identified as an enrichment of single gene and pseudogene family members located at different genomic FS regions [63].

In conclusion, FSs seem to be not only reused genomic puzzle edges in karyotype evolution but also provide the infrastructure to spread gene (copies) over the genome as a source of evolutionary adaptation. Collectively, this postulated model of "FS-driven gene and genome evolution" awaits further exciting insights into the trade-off between the risk for genomic diseases and cancer on the one hand and genetic variability and flexibility for evolutionary adaptation on the other.

Interim conclusions for research and diagnostics

The "chromosomic view," including nuclear architecture, inter- and intrachromosomal interactions, and FS as underlying principles of chromosomal evolution (including the formation of new genes) and disease, is the unifying tool for understanding all these different aspects of genetics. This applies to future research directions and to the most urgent integration of three-dimensional nuclear organization into human genetic diagnostics.

Concluding remarks

A truism must be mentioned at the end of this perspective on chromosomes in clinical diagnostics and basic research—the more we learn, the less we know, and the more questions arise. Accordingly, being called "dead," not being of interest, and not worthy of being studied several times during the last few decades, it turns out once more that an understanding of chromosomal functions is essential to gain insights into mechanisms of constitutional and acquired genomic diseases, in addition to the adaptability of living beings.

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Präzise Veränderung des Erbguts – mit Licht

Wissenschaftler*innen aus Heidelberg und Berlin um Roland Eils haben ein Proteinkonstrukt names CASANOVA entwickelt, das die CRISPR Genschere im Dunkeln abschaltet.

Casanova, ein italienischer Schriftsteller aus dem 18. Jahrhundert, wird aufgrund seiner zahlreichen Liebschaften mit den Damen der Zeit noch heute im Volksmund zitiert. Das gleichnamige molekulare Präzisionswerkzeug, das Wissenschaftler*innen aus Heidelberg und Berlin entwickelt haben, hat auf den ersten Blick durchaus Gemeinsamkeiten mit seinem Namensvetter. Es sucht sich eine Partnerin und geht eine enge Bindung mit ihr ein, gibt diese aber auch ebenso unbefangen wieder frei. Allerdings ist die Partnerin hier keine Dame, sondern die programmierbare Genschere CRISPR/Cas9, die es erlaubt, das Genom in menschlichen Zellen gezielt zu verändern. Ihre Ergebnisse haben die Forscher*innen nun in *Nature Methods* veröffentlicht.

Optogenetisches Verfahren

Genau gesagt, steht CASANOVA für „CRISPR/Cas Aktivierung durch ein neues, optogenetisches Verfahren basierend auf Anti-CRISPR Proteinen". Anti-CRISPR Proteine sind kleine Eiweiße aus Bakterien-infizierenden Viren, die in der Lage sind, die CRISPR Genschere zu binden. Im gebundenen Zustand ist die Genschere blind und nicht mehr in der Lage, ihre Zielsequenz im Erbgut zu erreichen. Dadurch ist das virale Erbgut vor den Angriffen durch die Genschere geschützt. Die Forscher*innen um Dr. Dominik Niopek, Institut für Pharmazie und Molekulare Biotechnologie/Bioquant-Zentrum der Universität Heidelberg, und Prof. Dr. Roland Eils, Berliner Institut für Gesundheitsforschung (BIH)/Charité Universitätsmedizin/Heidelberger Universitätsklinikum, bauten Anti-CRISPR Proteine mit Hilfe gentechnischer Verfahren so um, dass diese von außen an- und abgeschaltet werden können – und zwar mit Licht. Dazu integrierten sie einen molekularen Lichtsensor aus der Haferpflanze in ein Anti-CRISPR Protein. Anschließend brachten die Forscher*innen das so erzeugte Hybrid – genannt CASANOVA – zusammen mit der CRISPR Genschere in humane Zellkulturen ein. „Im Dunkeln bindet CASANOVA effizient an die CRISPR Genschere und schaltet diese dadurch ab", erläutert Niopek. „Trifft jedoch blaues Licht auf das Proteinpaar in der Zelle, so hat die Romanze ein jähes Ende. Die Genschere löst sich vom Anti-CRISPR Protein und wird dadurch aktiv."

Mit ihrer Methode konnten die Forscher*innen um Niopek und Eils die Erbgutsequenz in menschlichen Zellen durch Beleuchtung von außen gezielt verändern. CASANOVA ermöglichte es außerdem, Gene auf Knopfdruck an- und wieder abzuschalten. Sogar die Bindungsdynamik der CRISPR Genschere an ihre Zielsequenz im Erbgut lebender Zellen konnten die Wissenschaftler*innen live unter dem Mikroskop verfolgen. „CASANOVA ist nicht nur ein innovatives Werkzeug für die Grundlagenforschung, z.B. um das Zusammenspiel zwischen der Aktivität von Genen und dem Verhalten von Zellen zu studieren. Die Methode könnte in Zukunft auch für besonders präzise Therapien genetischer Erkrankungen relevant werden", sagt Eils. „Die Vielfältigkeit und einfache Anwendbarkeit von CASANOVA ist dabei ein entscheidender Vorteil gegenüber vorhergehenden Methoden zur Kontrolle von CRISPR/Cas9", ergänzt Felix Bubeck. Gemeinsam mit Mareike Hoffmann, Doktorandin am Deutschen Krebsforschungszentrum, hat er viele der entscheidenden Experimente in Niopeks und Eils' Labor durchgeführt. Bubeck ist Student im Masterstudiengang Molekulare Biotechnologie an der Universität Heidelberg und Ko-Erstautor der Publikation.

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