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Extrachromosomal DNA: An Emerging Hallmark in Human Cancer

Sihan Wu,[1] Vineet Bafna,[2] Howard Y. Chang,[3] and Paul S. Mischel[4,5]

[1]Children's Medical Center Research Institute, University of Texas Southwestern Medical Center, Dallas, Texas, USA; email: sihan.wu@utsouthwestern.edu

[2]Department of Computer Science and Engineering, University of California, San Diego, La Jolla, California, USA

[3]Center for Personal Dynamic Regulomes and Howard Hughes Medical Institute, Stanford University, Stanford, California, USA

[4]Department of Pathology, Stanford University School of Medicine, Stanford, California, USA; email: pmischel@stanford.edu

[5]Chemistry, Engineering, and Medicine for Human Health (ChEM-H), Stanford University, Stanford, California, USA

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Abstract

Human genes are arranged on 23 pairs of chromosomes, but in cancer, tumor-promoting genes and regulatory elements can free themselves from chromosomes and relocate to circular, extrachromosomal pieces of DNA (ecDNA). ecDNA, because of its nonchromosomal inheritance, drives high-copy-number oncogene amplification and enables tumors to evolve their genomes rapidly. Furthermore, the circular ecDNA architecture fundamentally alters gene regulation and transcription, and the higher-order organization of ecDNA contributes to tumor pathogenesis. Consequently, patients whose cancers harbor ecDNA have significantly shorter survival. Although ecDNA was first observed more than 50 years ago, its critical importance has only recently come to light. In this review, we discuss the current state of understanding of how ecDNAs form and function as well as how they contribute to drug resistance and accelerated cancer evolution.

INTRODUCTION

The dynamic and rapid evolution of tumors presents one of the greatest challenges for developing more effective and durable treatments for cancer patients. Breakthroughs in genomic and epigenomic technologies, coupled with computational advances, have yielded unprecedented capacities to map the landscape of cancer, including within an individual patient. These capabilities have promised to usher in an era of precision cancer medicine. However, that promise has yet to be fulfilled for many patients with aggressive forms of cancer, because their tumor genomes seem to change rapidly in response to treatment at rates that are difficult to understand. These highly aggressive cancers are driven primarily by oncogene amplification, and recent research suggests that extrachromosomal DNA (ecDNA) may be the pivotal reason for this rapid tumor evolution. ecDNA is a remarkably effective way to cheat the system: It lacks centromeres and therefore is subject to non-Mendelian inheritance. Consequently, tumors with ecDNA evolve in unanticipated ways and at unexpected rates, because they change their genomes rapidly in response to changing selection pressures.

THE DISCOVERY AND REDISCOVERY OF EXTRACHROMOSOMAL DNA IN CANCER

The first series of tumor cases reporting the presence of ecDNA was documented in 1965 (1, 2). At that time, it was given many names: double fragments of chromosome, double minutes, double bodies, minute chromatin bodies, and accessory chromatin. These early accounts already captured and presumed some of the crucial features of ecDNA, which are validated by modern biology:

- It was not found in matched blood cultures and was observed only in mitotic tumor cells.
- Its size varies.
- It may be subject to random segregation during mitosis.
- It could be circular, reminiscent of a ring chromosome.

Although the persistence of extrachromosomal particles in metaphases raised a debate regarding the presence of a functional centromere, subsequent research in 1978 showed that ecDNA does not have detectable centromeric activity (3). Notably, only 30% of ecDNAs occur as classic double-minute pairs (4); therefore, we use the term ecDNA, which describes both singlet ecDNA particles and double minutes.

In the late 1970s to 1980s, the content of ecDNA in cancer gradually became clear. The Schimke group (5) was the first to recognize that a stable and unstable increase in dihydrofolate reductase gene (*DHFR*) copy number contributed to murine cancer cells' resistance to methotrexate. They soon realized that the unstable amplification of *DHFR* was associated with ecDNA, whereas the stable amplification was related to large chromosomes (6–9). Subsequently, more and more ecDNA species carrying important driver oncogenes were identified, including *MYCN* and *MYC* ecDNA, in various cancer types (10, 11).

Although research into ecDNA in cancer has continued since its discovery, the literature has been relatively sparse in the past 50 years. With the development of contemporary genomic profiling technologies such as comparative genomic hybridization array and whole-genome sequencing (WGS), old-school cytogenetics seems to be left out in the cancer research field. However, the trade-off for high throughput and high sequence resolution is loss of spatial resolution, in which ecDNA information is buried underneath the rich body of data. In the past 20 years, many cancer genome sequencing projects identified hundreds of amplified oncogenic drivers. Yet, the question of where exactly these oncogenes are located was challenging to answer, as the sequencing data from a corrupted cancer genome were mapped to a normal reference genome to infer a DNA

segment's coordinate, assuming that every gene sits in a specific chromosomal location according to a traditional analysis pipeline.

In 2017, the first systematic investigation of ecDNA prevalence in human cancer was conducted on an NCI60 cell line panel, patient-derived cancer cell cultures, and clinical tumor tissues (4). This study combined WGS to call and analyze amplicon structure with microscopic examination of the metaphase cell in an unbiased way to directly visualize ecDNA, yielding a highly accurate ecDNA landscape in common cancer cell lines (4). Unlike a previous report based on the Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer stating that double minutes were present in only 1.4% of all cancers (12), the 2017 study revealed that ecDNA is surprisingly prevalent in cancer cells of various pathological types: Nearly 40% of tumor cell lines and nearly 90% of patient-derived brain tumor cultures were positive for ecDNA, which was almost undetectable in normal cells. The contradiction here is possibly due to differing definitions of ecDNA. The Mitelman database enumerates only the frequency of the double minutes, yet 70% of ecDNAs are in singlet form rather than in paired double minutes (4). Notably, there is no evidence showing that singlet ecDNA is biologically distinct from double minutes, and double minutes may simply represent the replicated form of singlet ecDNA found in early metaphase prior to segregation (13, 14). More recently, a computational analysis of WGS data of more than 3,000 clinical cancer samples from The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) cohorts again revealed a high prevalence of ecDNA, especially in the most aggressive and common tumor types—including brain, esophageal, ovarian, bladder, lung, breast, gastric, and cervical cancers—in which the presence of ecDNA is tightly associated with worse clinical outcomes (15).

Notably, although ecDNA is rarely observed in normal cells, both historical literature and modern research show evidence that ecDNA may exist in the normal tissue of cancer patients at an extremely low frequency, including in leukocytes and lymphocytes (4, 15–18). However, whether the ecDNA represents contamination from circulating or metastatic tumor cells is not known, nor is the content of ecDNA in normal cells. In addition, ecDNA has been found in benign hyperplasia or precancerous cells at a low frequency, including in endometrial polyps and fibroblasts from patients with Bloom's syndrome (19, 20). However, whether the presence of ecDNA is associated with outcome in precancers remains to be explored.

MOLECULAR FEATURES AND FUNCTIONS OF EXTRACHROMOSOMAL DNA

Extrachromosomal DNA Is Circular

Since the discovery of ecDNA, the quest to reveal its physical shape has continued. Early ultrastructural studies using transmission electron microscopy uncovered some essential features of ecDNA. First, it is composed of nucleosomal chromatin and is organized into a certain degree of high-order fibers typical of the chromosome. Second, ecDNA appears to be circular, as no visible free ends were detected (21–23). Attempts to use polymerase chain reaction and mapping by restriction enzyme digestion of an *MYCN* ecDNA in a human neuroblastoma cell line also suggest a head-to-tail configuration in the nucleotide sequence (24). Scanning electron microscopy and atomic force microscopy were later used to reveal the ultrastructure. However, possibly due to resolution limitations, these images showed a spherical shape for ecDNA (14, 25, 26).

The most definitive proof establishing the circular shape was obtained in a study from late 2019 (27). This study combined the strength of DNA sequencing and high-resolution imaging. First, short-read WGS with amplicon architecture analysis revealed a circular configuration of several DNA segments joined together with breakpoints in between. Second, long-range optical mapping,

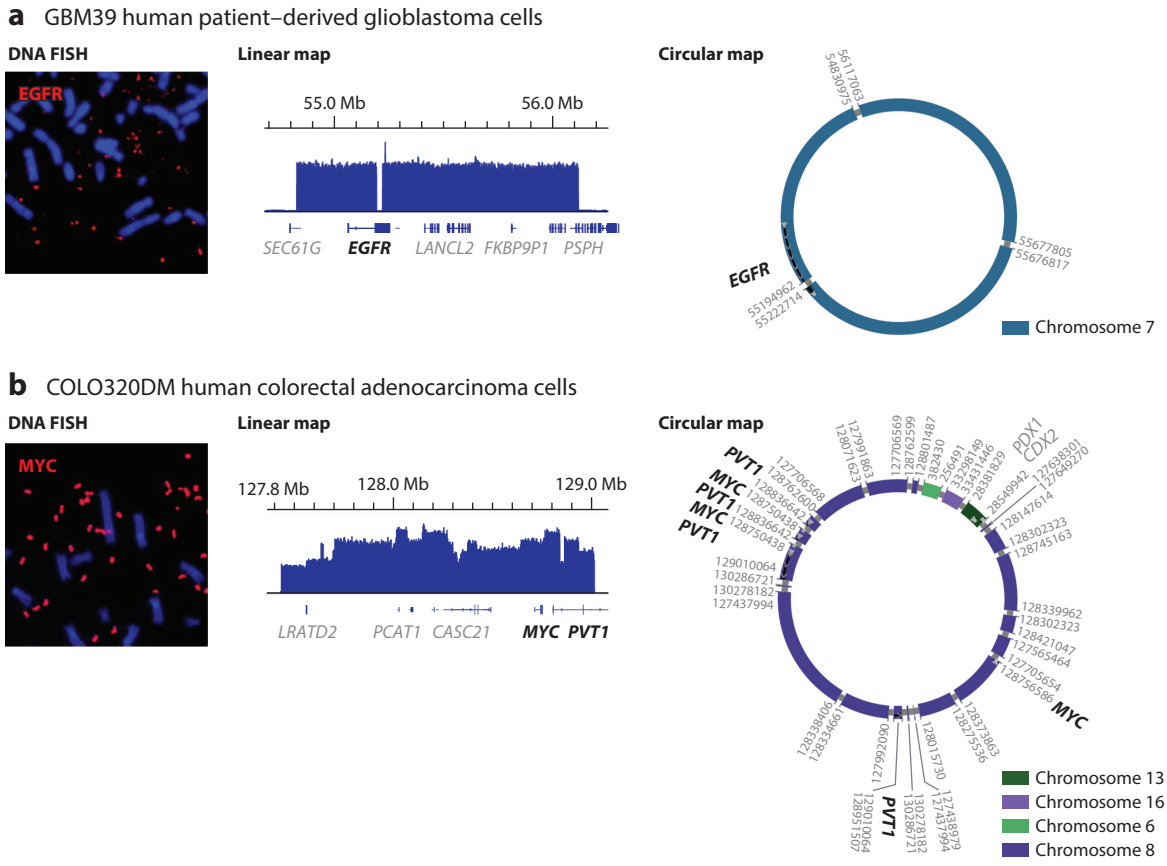


Figure 1

A circular map of circular extrachromosomal DNA (ecDNA). Current evidence unequivocally shows that ecDNA is circular. However, traditional genome browsers still use linear maps, which cannot show the true nature of ecDNA. (*a*) For ecDNA with a simple structure (e.g., *EGFR* ecDNA in GBM39 cells), a linear map may still be useful. (*b*) However, ecDNA with complicated rearrangements (e.g., *MYC* ecDNA in COLO320DM cells) is difficult to visualize with a linear map, while a circular map helps disambiguate the orders and orientations of rearranged genomic segments, including material that is duplicated within an ecDNA. Abbreviation: FISH, fluorescence in situ hybridization. Linear and circular maps were created on the basis of publicly deposited whole-genome sequencing data (27).

revealed a continuous contig that spans across all breakpoints of ecDNA, further supporting the circular shape of the ecDNA particle. Third, all ultrastructural imaging data, including scanning and transmission electron microscopy as well as 3D structured illumination microscopy, showed a ring shape, indicating that ecDNA is unequivocally circular. The confirmation that ecDNA is circular, coupled with techniques to map its sequence content, has made it possible to produce individualized maps of ecDNA particles, including in individual tumor samples (**Figure 1**).

Extrachromosomal DNA Drives Massive Oncogene Expression Due to High Copy Number and Decompacted Chromatin

Circular ecDNA serves as a template to direct gene transcription, which is revealed by allele-specific RNA-sequencing analysis (27). More importantly, genes encoded on ecDNA, especially oncogenes, are usually highly expressed; the abundance of some transcripts can reach the top 1%

of the tumor's transcriptome, which indicates that ecDNA may play a critical role in amplifying oncogenic signaling in cancer (27).

ecDNA promotes high oncogene expression through at least two mechanisms. First, ecDNA can be detected at high copy number levels that are not observed with other forms of gene amplification (27). It is not uncommon to see dozens to more than a hundred ecDNA particles in a cancer cell. Compared with chromosomal amplification, the copy number of ecDNA is usually higher, sometimes even higher than that observed in breakage-fusion-bridge (BFB) amplicons (15, 27). The ability to acquire such a high copy number may relate to the unequal segregation of ecDNA during mitosis, which is discussed in the section titled Extrachromosomal DNA Mediates Rapid Tumor Evolution, below.

Yet, the high copy number explains only part of the mechanism. Recent studies have demonstrated that ecDNA transcribes higher levels of oncogenes compared with copy-number-matched chromosomal DNA (15, 27). The human genome is segmented into hierarchical chromatin structures with differential accessibility and organizes transcriptional activity into individual compartments, whose configuration is tightly linked to the chromatin's epigenetic landscape (28). Quantitative assessments of ecDNA accessibility by ATAC-seq (assay for transposase-accessible chromatin with high-throughput sequencing) and ATAC-see (assay of transposase-accessible chromatin with visualization) have revealed that ecDNA contains the most accessible chromatin in the cancer genome and lacks the higher-order compaction typical of heterochromatin, thereby allowing higher transcriptional activity (15, 27).

Circular Extrachromosomal DNA Forms a Novel *Cis*-Regulatory Circuit

DNA also codes information in its shape. Once circularized, DNA segments of ecDNA form a new chromatin domain distinct from their chromosomal counterparts. The most significant outcome is that DNA elements farther away are brought into proximity due to DNA circularization, forming novel *cis*-regulatory circuits that are not possible in chromosomal DNA (27). Therefore, ecDNA is a powerful enhancer hijacking vector in cancer.

There are two enhancer hijacking models in ecDNA: local and distal. In the local hijacking model, a cluster of enhancers, up to a few megabases (the upper limit size of an ecDNA) distal to an oncogene, are brought into proximity by DNA circularization. The enhancer cluster can be isolated from the oncogene by an insulator in the chromosomal DNA. However, once this enhancer cluster is coamplified with the oncogene and circularized as ecDNA, the enhancers can trespass the insulator and become accessible to the oncogene (**Figure 2*a***). In glioblastoma, the oncogenic driver gene *EGFR* often coamplifies with the upstream enhancer and forms ecDNA, creating new enhancer–oncogene contacts and contributing to cancer cell growth (29). Local enhancer hijacking is also found in neuroblastoma ecDNA containing the oncogene *MYCN* (30).

The distal enhancer hijacking model refers to the mechanism by which enhancer segments and oncogene segments that are physically far apart are joined together to form an ecDNA circle. These DNA segments could originate from the same chromosome, or even different chromosomes. Therefore, this model enables ecDNA to create complicated and heterogeneous domains to regulate oncogene expression that expand the possibilities for oncogene hijacking (**Figure 2*a***). ecDNA-directed distal enhancer hijacking is common in neuroblastoma and is associated with worse clinical outcomes (30, 31).

Extrachromosomal DNA Is a Mobile *Trans*-Acting Element

Eukaryotic DNA in the nucleus is folded into chromosomes and subsequently organized into chromosomal territories, where each chromosome occupies a specific space inside the nucleus.

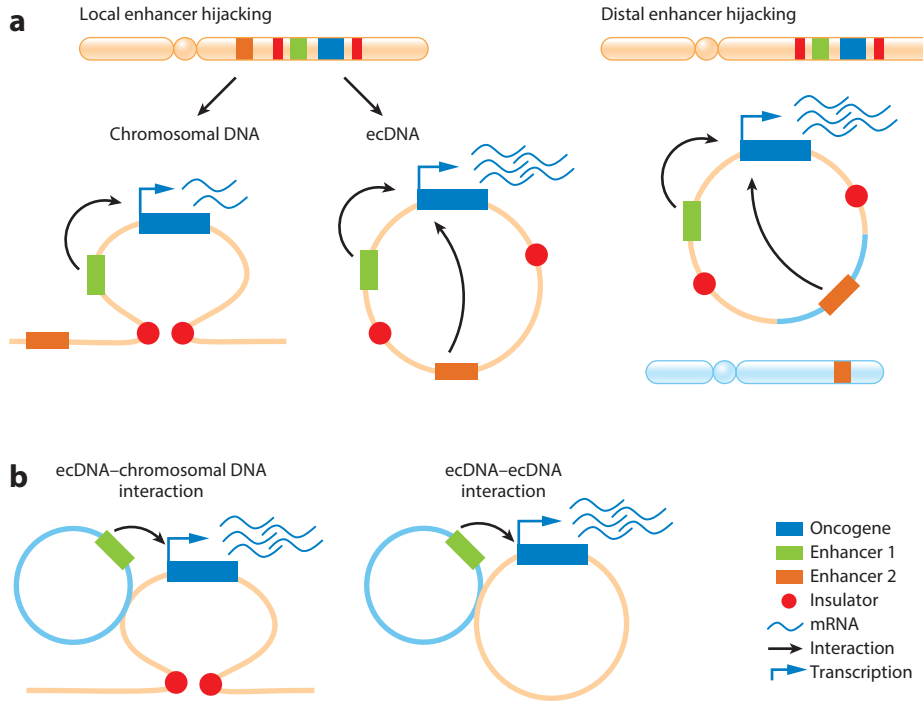


Figure 2

Extrachromosomal DNA (ecDNA) acts in *cis* and in *trans*. (*a*) ecDNA is a vector of enhancer hijacking. By incorporating an enhancer from an adjacent topologically associating domain into a circle, or from a distal region such as a different chromosome by chimeric circularization, the oncogene encoded on ecDNA can access a variety of enhancers through *cis*-interactions that are not possible in chromosomes. (*b*) ecDNA can act in *trans* with other DNA, including ecDNA–chromosomal DNA interaction and ecDNA–ecDNA interaction.

This spatial organization is critical to the stability and physiological function of the chromosome, including acting as an architecture for chromatin interaction within a chromosome, thereby limiting interchromosomal contact within specific regions of the chromosome exteriors (32, 33). Although it is unclear whether ecDNA in cancer is also well organized spatially, microscopic imaging has shown that ecDNA is scattered throughout the nucleus. Furthermore, this observation suggests that, due to its high copy number and small size, ecDNA is mobile and may freely interact with other DNA.

Recent research mapping ecDNA–chromosomal DNA interactions suggests that ecDNA can act as a mobile *trans*-acting element (**Figure 2*b***). The interaction of ecDNA with chromosomal DNA appears to be genome-wide. Furthermore, hundreds of *trans*-interaction sites have been identified by RNA polymerase II ChIA-PET (chromatin interaction analysis with paired-end tag sequencing), showing that interactions produce an elevated transcriptional potential. More importantly, ecDNA is enriched for enhancers, which interact with chromosomal DNA more frequently. These findings are the basis of a new concept, that ecDNA can function as a mobile enhancer to regulate chromosomal gene expression, including that of oncogenes (34).

As ecDNA is highly amplified and mobile, it is not difficult to imagine that one ecDNA particle can interact with another (**Figure 2*b***). A recent study (35) demonstrates that approximately 10–100 ecDNA particles can cluster together to form an interaction hub and promote oncogene

expression. For example, a subspecies of *MYC-PVT1* ecDNA lacking enhancers can access another ecDNA subspecies carrying enhancers within the ecDNA hub.

Extrachromosomal DNA as a Source of Somatic Rearrangement

Historically, ecDNA has been thought of as an unstable form of gene amplification that is sensitive to different microenvironments with different selection pressures. The modern concept regarding instability can expanded to structural and spatial dynamics, in which an ecDNA particle can change in its sequence and localization during cancer evolution (7, 9, 36).

Accumulating evidence suggests that the complexity of ecDNA can increase during tumor evolution. In the late 1980s, an episome model was proposed wherein ecDNA can arise from small, submicroscopic extrachromosomal particles (episomes) that eventually enlarge into microscopically visible ecDNA (36). Although not all ecDNAs observed to date are derived from episome aggregation and fusion (37, 38), amplicon structural analyses suggest that DNA segments can be added into or deleted from an existing ecDNA, creating new ecDNA species in cancer (39, 40). The molecular mechanism governing ecDNA rearrangement is unclear. However, because ecDNA widely interacts with chromosomal DNA and naturally forms ecDNA hubs (34, 35), it is possible that these ecDNA interaction foci may be functional units for ecDNA rearrangement. Further research is needed to complete the missing pieces of this puzzle.

More importantly, ecDNA can reintegrate into the chromosome. Building on earlier observations made using Southern blots (9, 36), modern sequencing technologies and bioinformatic analyses have revealed several important aspects of the reintegration:

- Cellular stress, including drug treatment and DNA double-strand breaks, can drive reintegration (4, 41, 42).
- Several ecDNA particles can aggregate together and then reintegrate into the chromosome, echoing the episome model (4, 36).
- The integrated element can disrupt the integrity of the chromosomal genes in the integration site. For example, in neuroblastoma, ecDNA reintegration can destroy the gene body of the tumor suppressor gene *DCLK1*, leading to decreased expression, and enhance the expression of *TERT*, potentially by enhancer hijacking (31).

In addition, the birth of an ecDNA may enable gene fusion due to chimeric circularization of DNA segments from one or more chromosomes (31, 38, 39). Pan-cancer analyses show that transcript fusions occur in ecDNA with a fivefold frequency compared with their occurrence in other types of amplification (15). However, further studies are needed to understand the functional consequence of ecDNA-driven gene fusion in cancer.

EXTRACHROMOSOMAL DNA MEDIATES RAPID TUMOR EVOLUTION

The first functional impact of ecDNA discovered is probably how it mediates drug resistance, thanks to a series of studies by the Schimke group during the late 1970s and early 1980s. Schimke and colleagues first documented that *DHFR* is selectively amplified in methotrexate-resistant cell lines, which existed in stable and unstable forms (5). Subsequent research linked ecDNA to unstable drug resistance, which was rendered stable by reintegration of ecDNA particles into the chromosome, creating homogeneous staining regions (HSRs) (6–9). More recently, BRAF-V600E mutant gene amplification as ecDNA was found to govern ERK inhibitor resistance in PDX models (43).

The response of *EGFR* ecDNA–carrying glioblastoma cells to EGFR-targeted therapy (41) presents a stark contrast to the above examples. Drug treatment using the kinase inhibitor erlotinib led to a rapid decline in *EFGR* ecDNA copy number, resulting in resistance. Some of the ecDNA even reintegrated into the chromosome to form an HSR-like structure with low gene activity. With the loss of the drug target, the cancer cells no longer responded to the treatment. Upon drug removal, the *EGFR* ecDNA rapidly reemerged and drove tumor growth (41). All of these studies highlight the powerful and disparate mechanisms by which ecDNA causes therapeutic resistance through dynamic copy number changes.

ecDNAs are acentric, as revealed by imaging and sequencing (3, 4). Notably, lack of a functional centromere does not substantially affect ecDNA's segregation efficiency, because ecDNA can hitchhike with chromosomes to segregate into daughter cells (44, 45). Nevertheless, there is no mechanism to ensure equal ecDNA segregation during mitosis. The segregation direction of ecDNA is a coin toss, which leads to a binomial distribution of ecDNA in daughter cells, freeing ecDNA from the constraints of Mendelian inheritance (46, 47).

The random segregation of ecDNA drives rapid tumor evolution by at least two mechanisms. First, it allows a portion of cancer cells to acquire high oncogene copy numbers rapidly, and second, it creates an enormous pool of genetically heterogeneous cancer cells to promptly adapt to the ever-changing microenvironment and selection pressures (4). It is also possible that the unique *cis*-interactions within an ecDNA particle (27, 29–31) and the prevalent ecDNA–ecDNA and ecDNA–chromosomal DNA *trans*-interactions may contribute to accelerated tumor evolution (34, 35). Furthermore, potential ongoing mutagenesis of ecDNA may also have an effect on tumor evolution.

If we imagine that there are three identical ecDNA particles in a mother cell that become six particles after DNA replication in S phase, upon mitosis the ecDNA particles will randomly segregate into two daughter cells, which may receive from zero to six particles. Therefore, ecDNA can create genetic heterogeneity in just one cell cycle (**Figure 3**). If a higher oncogene copy number favors advantaged cell growth, the daughter cells with more ecDNA will expand more rapidly and will continue to increase oncogene copy number until hitting some threshold that limits cellular fitness. This explains why methotrexate and ERK inhibitor treatment increases the copy number of *DFHR* and *BRAF-V600E* mutant genes, respectively. Similarly, if selection pressure opposes high oncogene copy number, such as EGFR kinase targeting in glioblastoma, a tumor type rarely containing point mutations in the EGFR kinase domain, the *EGFR* ecDNA copy number declines rapidly through negative selection, and *EGFR* ecDNA may reintegrate into a chromosome and deactivate its transcriptional activity through an unknown mechanism (41).

The foundational principles of Darwinian evolution are variation, selection, and identity by descent. However, the impact of nonchromosomal oncogene inheritance—random identity by descent—is not well understood. Recent research integrating mathematical modeling, unbiased image analysis, CRISPR-based ecDNA tagging, and live-cell imaging has revealed a set of basic "rules" for how random ecDNA inheritance drives oncogene copy number and distribution, resulting in extensive intratumoral ecDNA copy number heterogeneity and rapid adaptation to metabolic stress and targeted cancer treatments (48).

Segregation of ecDNA in cancer cells is reminiscent of plasmid-directed hereditary mechanisms in bacteria, which are potent drivers of the genetic variation that underlies the rapid appearance of cells with a selective advantage (49). In cancer, ecDNA may be even more powerful because multiple species of ecDNA can coexist in one cancer cell and even interact with other DNA, adding another layer of complexity to the random segregation mechanism (27, 34, 35). With the increase of ecDNA species in the cancer cell population, the heterogeneity increases more dramatically, facilitating even faster tumor evolution.

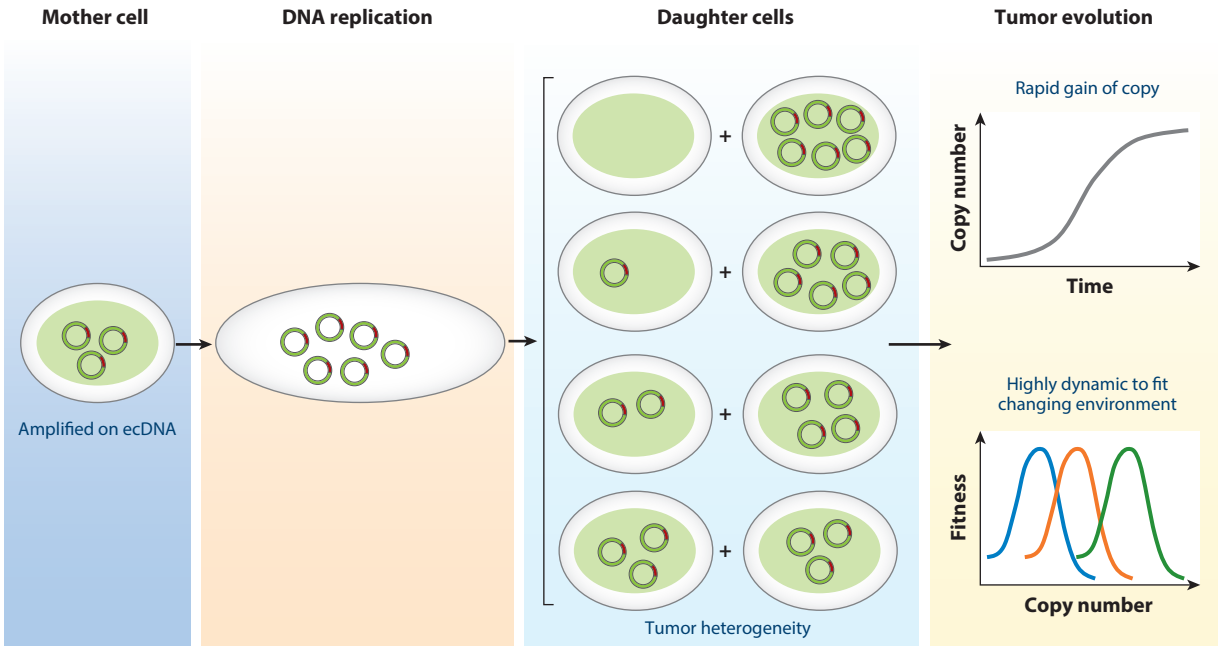


Figure 3

Unequal segregation of extrachromosomal DNA (ecDNA) drives rapid tumor evolution. Acentric ecDNA segregates unequally to two daughter cells during cell division. Therefore, each cell division generates genetic heterogeneity in the cancer population, allowing a portion of cancer cells to gain oncogene copy number rapidly. Furthermore, this process creates a pool of genetically heterogeneous cancer cells for microenvironmental selection, increasing cancer fitness and promoting rapid cancer evolution.

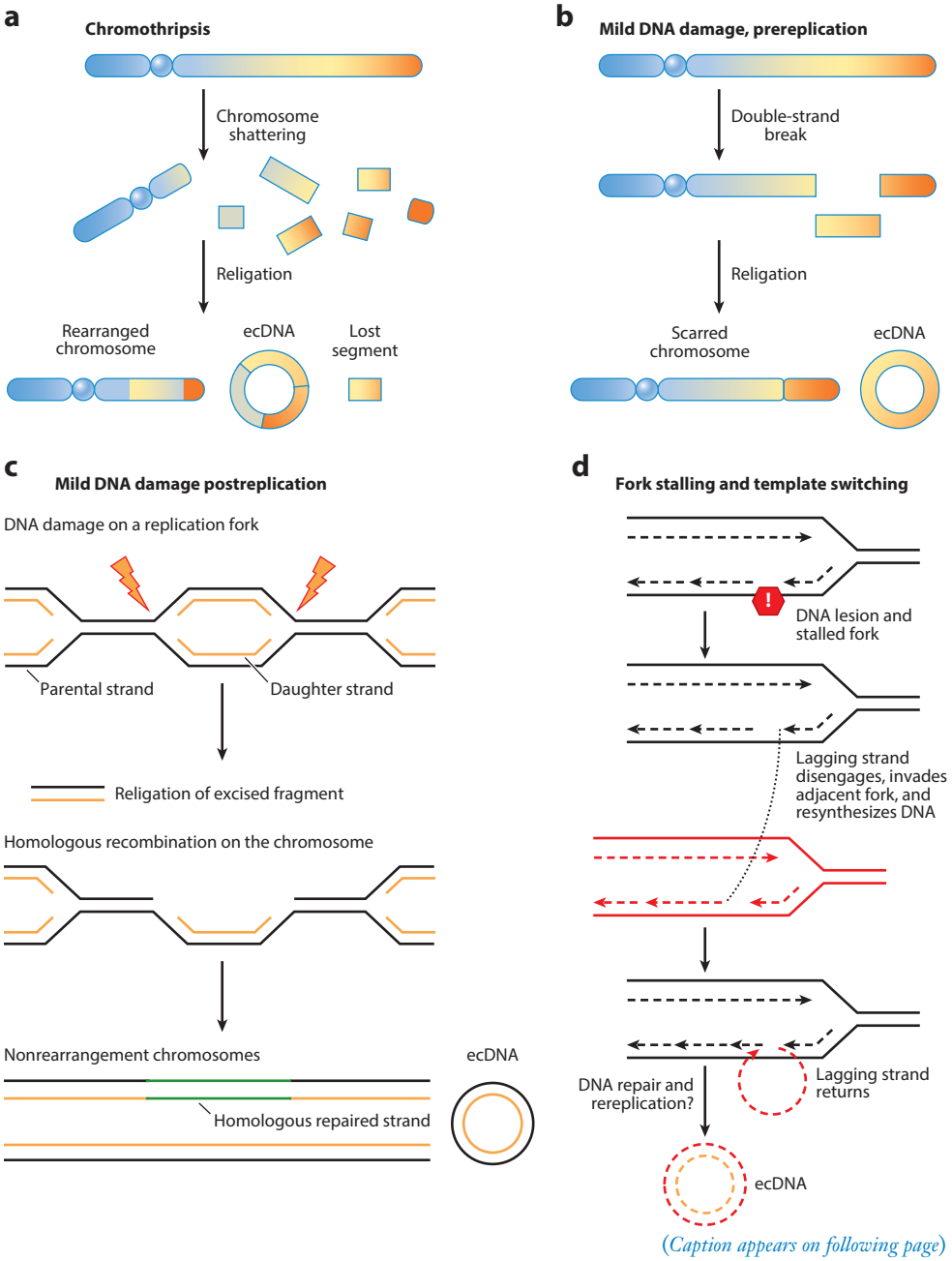
THE ORIGIN OF EXTRACHROMOSOMAL DNA

How does ecDNA originate? There appear to be multiple paths toward ecDNA formation, each of which involves some type of DNA damage, usually occurring in the context of tumor suppressor losses. However, the types of DNA damage and the factors triggering them are diverse.

Chromothripsis

The best-known origin of ecDNA is chromothripsis, in which a chromosome is broken into small pieces due to catastrophic DNA damage and undergoes massive DNA rearrangement by religation, acting as a powerful driver of karyotype evolution in cancer (50). In some cases, a few shattered DNA fragments can be stitched back together as circular ecDNA (**Figure 4*a***). Early evidence has shown that chromosome missegregation can lead to micronucleus formation, where chromothripsis occurs and generates megabase-scale circular chromosomes based on sequencing (51). A recent study using a model system that selectively induces lagging Y chromosome formation has also demonstrated that the chromothriptic Y chromosome can give rise to ecDNA particles, along with many structural aberrations (52). More importantly, multiple studies have documented that the fragments that form circular amplicons are missing from the chromothripsis-derived chromosome, further supporting the idea that the reassembly of DNA fragments can generate ecDNA (51, 53–55). In addition, continuous BFB cycles, which can generate >100-Mb chromosome arms, are also responsible for ecDNA formation, as the abnormal chromosome is prone to becoming trapped within interphase bridges and shattered, generating ecDNA by chromothripsis (42).

However, on the basis of DNA copy number and structural variation analysis in the pan-cancer data set, although half of the ecDNA-positive cases have chromothripsis in any part of the genome, only ~36% of the DNA segments forming ecDNAs show a chromothripsis signature (15). These data show that a substantial fraction of ecDNAs originate through a mechanism other than catastrophic chromosome damage and religation.



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Potential pathogenesis pathways of extrachromosomal DNA (ecDNA). (*a*) Religation of shattered DNA segments from chromothripsis can form ecDNA. (*b*) Two DNA double-strand breaks in one arm of a chromosome can create ecDNA by religation of the excised segment, leaving a scarred chromosome. (*c*) If mild DNA damage occurs between two replication foci, the chromosome may be repaired through a homologous recombination mechanism while generating an ecDNA particle. (*d*) Speculative model for ecDNA generation through fork stalling and template switching. In this model, a DNA lesion occurs in the template strand, stalling the lagging strand in DNA replication. The lagging strand can disengage from the current template and invade the adjacent replication fork through microhomology to continue DNA synthesis. Strand disengagement and invasion can occur over multiple rounds until the strand returns to the original template. Although the mechanism is unclear, this process may generate ecDNA through DNA repair.

Mild DNA Damage and Religation

In theory, if a piece of the linear double-stranded DNA segment is ligated head-to-tail, circular DNA forms. Could this actually happen inside the cell nucleus? A recent study using CRISPR to fragment and circularize a fluorescent biosensor (CRISPR-C) provides an answer. This approach generates endogenous extrachromosomal circular DNAs (eccDNAs) in sizes ranging from 10^2 to 10^5 bp, and even a 47.4-Mb ring chromosome, by nonhomologous end-joining and blunt end-joining repair processes (56). More importantly, although the DNA circles generated by CRISPR-C gradually disappear, the kinetics is slower than in the theoretical dilution model, indicating that a replication mechanism is involved (56). This study suggests that a DNA double-strand break creating a free DNA segment is sufficient to generate eccDNA, which replicates during the cell cycle (**Figure 4*b***). On the basis of these findings, we hypothesize that if the DNA circle contains an oncogene that enhances cellular fitness, the cell with the DNA circle will be preserved and gain a fitness advantage. Due to the unequal segregation that creates a pool of cells with various copy numbers of circles, a population with an optimal range of DNA copy numbers will rapidly emerge, ultimately becoming cancer if other regulators such as tumor suppressors are disabled.

Studies focusing on ecDNAs and their chromosomal origins have shed light on the mild DNA damage model. In the 1980s, when comprehensive genomic profiling was not available, Wahl and colleagues (36) used Southern blotting to study the episome dynamic in an artificial system. Their results suggested that ecDNA formation is associated with the loss of chromosomal sequence. More recently, fluorescence in situ hybridization (FISH) in a leukemia case revealed that the presence of *MYC* ecDNA is accompanied by the deletion of one *MYC* locus on chromosome 8, suggesting that *MYC* ecDNA arises from the lost copy of chromosomal *MYC* (57). Furthermore, chromosome walking and microarray analyses indicate that, in some glioma cases, the chromosomal origins of ecDNAs do not have the extensive DNA copy number oscillations that are typical of chromothripsis (58). More importantly, the chromosome of origin may or may not have a deleted locus corresponding to the ecDNA segment (58, 59). These observations raise two potential ecDNA pathogenesis pathways:

- One or a few DNA segments are excised from the same chromosome or different chromosomes and fused to form ecDNA, leaving scars on the chromosome of origin (**Figure 4*b***).
- A double-stranded DNA segment is excised between two replication forks and forms ecDNA, and the chromosomal scar is healed by rereplication (59) (**Figure 4*c***).

Interestingly, one study suggests that a V(D)J-like recombination mechanism may be involved in ecDNA formation, as some of the ecDNA junctions were found to have ectopic recombination signal sequences (58).

Fork Stalling and Template Switching

A homology analysis at the ecDNA breakpoints suggests that fork stalling and template switching during DNA replication is another possible origin of ecDNA (60). In this model, a DNA replication fork stalls at the lesion, where the lagging strand disengages from the current template, invades and anneals to an active adjacent replication fork by microhomology, and continues to synthesize nascent DNA. Strand invasion and resynthesis could happen multiple times before the strand eventually returns to its original template. Therefore, the nascent DNA synthesized during template switching is not entirely complementary to the original template, resulting in a single-stranded DNA bulge in either strand (61). Although this model has not yet been experimentally proven, the single-stranded DNA could be a source of ecDNA (**Figure 4*d***).

Genome Instability

Emerging studies have begun to determine which genetic background is permissive for ecDNA pathogenesis. One hypothesis is that loss of genomic guardians, including cell cycle checkpoint and DNA repair pathways, is essential to initiate ecDNA formation. In mouse mammary tumors harboring *Brca1* and *Trp53* deletions, the oncogene *Met* is frequently amplified as ecDNA in vivo. Interestingly, these *Met* ecDNAs are gradually lost after a few passages in culture, suggesting that the in vivo microenvironment is required for ecDNA maintenance (62). Mouse NIH-3T3 cells transformed through overexpression of the cell cycle promoter *Sertad1* (formerly called *Sei-1*) can also generate *Met* ecDNAs in vivo that gradually disappear during in vitro culture (63, 64).

Functional integrity of *SIRT1* is essential to prevent DNA breakage upon replication stress. Cells with compromised *SIRT1*, including loss of expression and inability to phosphorylate its T530 site, are prone to generate extrachromosomal elements. Treatment with aphidicolin, an agent that induces replication stress, can further increase the number of extrachromosomal elements that appear in cells with compromised *SIRT1* (65). However, the physical shape, size, content, and stability of these extrachromosomal elements have not yet been characterized. Therefore, whether these extrachromosomal particles resemble functional ecDNA in cancer or are transient products of DNA breakage remains unclear.

The sequence and structure of ecDNA can continue to evolve after it is formed. Historically, the episome model proposed that large, microscopically visible ecDNA is derived from small, submicroscopic circular episomes, which can gradually enlarge in cultured cells (36). Although later research showed that episomal evolution is not essential (37), it does not rule out the possibility that the structure and content of ecDNA are capable of changing over time. Modern structural analyses using WGS found, in a specimen from a patient with esophageal carcinoma, that a large *KRAS* ecDNA was composed of two *KRAS*-containing DNA segments assembled in a mirror-repeat fashion, suggesting that two smaller ecDNAs merged together (27). Continuous evolution of ecDNA structures has been detected by tracking amplicon structural variation during tumor progression. For example, in a glioblastoma case, new ecDNA species emerged at tumor relapse that were generated by integration of new oncogene-containing segments into the ecDNA found at diagnosis (66).

MAINTAINING EXTRACHROMOSOMAL DNA IN CANCER CELLS

ecDNA imposes a barrier to traditional oncogene targeted therapy due to its heterogeneity and dynamic ability to rapidly change tumor genomes. Therefore, targeting the mechanisms that regulate ecDNA itself may be necessary to yield benefit. To this end, we need to understand (*a*) the

molecular mechanisms of ecDNA formation (discussed above), so that we can prevent the appearance of ecDNA up front during therapy, and (*b*) the maintenance mechanisms of ecDNA, so that we can tackle these pathways to disarm ecDNA in cancer cells.

To date, it is still not very clear what cellular contexts are required to maintain ecDNA in cancer cells. As mentioned above, multiple studies have shown that primary isolated cancer cells will lose ecDNA over time during culture but tend to retain ecDNA if maintained as xenografts, suggesting that the in vivo microenvironment is critical in producing selection pressure favoring ecDNA (62–64, 67–70). Even ecDNAs that are generated in vitro, such as methotrexate-driven *DHFR* ecDNAs, could disappear without selection pressure (5, 8, 71). However, many established cancer cell lines under regular culturing conditions in vitro also stably host ecDNA (4), presumably because in vitro culturing environments maintain selection pressure. But what exactly are these selection pressures? Do they represent specific microenvironments interacting with specific metabolic programs, genetic contexts, and cellular signals?

Hydroxyurea treatment and radiation, both of which damage DNA, can lower ecDNA levels in some contexts (72–78), but the mechanism of damage has not been established. Its relative specificity for ecDNA and its potential differential efficacy against ecDNA-driven cancers remain open questions. One consistent finding is that, once damaged, ecDNA particles often aggregate and subsequently form micronuclei (76–78). It is still unclear why aggregation happens in this situation and whether it is linked to ecDNA associated with homologous repairing templates, though ecDNA hubs form naturally and participate in cooperative transcriptional regulation (35). Interestingly, silencing the homologous recombination repair gene *BRCA1* was reported to decrease the *DFHR* ecDNA number in one methotrexate-resistant cancer cell line (79).

Several other studies have attempted to address what genetic background and signals are required for ecDNA maintenance. One report suggests that *SIRT1* is responsible for stabilizing extrachromosomal particles, as knocking out *SIRT1* results in the loss of these particles (80). However, this study was conducted with a transfected episomal plasmid in a COLO320DM cell line that already carries native ecDNAs (27). While the plasmid episomes were lost in the *SIRT1*-null background, it will be necessary to investigate whether *SIRT1* is required to maintain endogenous ecDNA. Another study showed that inhibiting Met signaling reduced ecDNA numbers in *Sertad1*-transformed NIH-3T3 cells (64). However, because these ecDNAs carry *Met*, the observed phenomenon may also be explained by selection pressure against *Met*. Specifically, under therapy, the cell population with a high *Met* copy number that is addicted to Met signaling may be eliminated by Met inhibition, regardless of the form of *Met* amplification. Therefore, Met signaling may not represent a universal mechanism underlying ecDNA maintenance.

CURRENT TOOLBOX FOR EXTRACHROMOSOMAL DNA RESEARCH

Imaging-Based Approaches

Seeing is believing. Microscopic examination of cells at metaphase remains the gold standard to identify the existence of ecDNA. The classic protocol involves enriching the cell population at metaphase by drug treatment (e.g., with colcemid) or by mitotic shake-off, swelling mitotic cells with hypotonic buffer, fixing with 3:1 methanol acetic acid fixative, dropping the cells onto a humidified slide, and finally staining with DNA dye. With downstream FISH, this approach can unequivocally determine whether a gene is amplified on ecDNA.

Algorithms and software have recently been developed to detect and quantify the number of ecDNAs across large cell populations. ecDetect uses computer vision–based methods to identify and quantify ecDNA in 4′,6-diamidino-2-phenylindole (DAPI)-stained images in a

semiautomated fashion. It was designed to minimize false positives and has very high precision but also somewhat lower sensitivity than visual counting, leading to an undercounting of ecDNA, particularly ecDNA particles close to chromosomes (4). A more recent method, ecSeg, utilizes a deep neural network approach to improve the sensitivity of DAPI-stained ecDNA identification in metaphase cells and allows for the integration of FISH signals (81). While the models for these methods were trained largely using an image set generated by a single laboratory using established cancer cell lines, the availability of additional training data sets should provide the community with a robust analysis tool for microscopic images generated by different labs from different sample sources.

However, imaging-based technology has limitations:

- Metaphase cell preparation is not always feasible because it requires viable, cultured cells.
- Some ecDNAs may be too small to visualize with DNA dyes alone, though FISH can significantly enhance the sensitivity of visual detection.
- Without prior sequencing or other profiling information to determine the amplified region, it is not possible to perform FISH.
- Throughput is low.

Therefore, universal, unbiased, and high-throughput solutions are urgently needed.

Sequencing-Based Approaches

Sequencing and computational technologies have become popular options to determine the structure, content, copy number, and diversity of ecDNA in cancer. Importantly, the development of sequence-based methods is critical for analyzing ecDNA scope, scale, and sequence content from publicly available cancer genome databases and tumors excised from patients who will be studied in clinical trials, a context in which live tumor cells are often unavailable.

The first strategy is to directly sequence the whole genome and look for discordant reads that support a circular architecture. In this scenario, short-read WGS is possibly the most cost-effective and widely used way to detect ecDNA genome-wide. Generally, 10× coverage of short-read WGS is sufficient to identify ecDNA. Even shallow sequencing with coverage down to 1× is feasible, as the genomic segments composing ecDNAs are amplified to very high levels, often 10–100× (4). AmpliconArchitect software, used with the preprocessing script PrepareAA and a postprocessing classification tool called AmpliconClassifier, is a powerful tool for analysis of amplicons and extraction of ecDNA information from short-read WGS data. AmpliconArchitect explores an amplified region using discordant read mapping to reconstruct an amplicon graph that describes the architecture (the order and orientation of amplified genomic segments) of ecDNA (82). ecDNA prediction using AmpliconArchitect was recently validated by comparisons to metaphase FISH in multiple cancer cell lines, including NCI60, with a positive predictive value of 85% and a sensitivity of 83% (4, 15, 27). The high accuracy of ecDNA prediction led to an exploration of the ecDNA landscape using short-read WGS data from more than 3,000 cancer patients in the TCGA and ICGC cohorts (15). Notably, results from WGS with AmpliconArchitect are almost 100% concordant with those from Circle-seq, a library enrichment method optimized for circular DNA detection, further supporting the fidelity of this approach.

However, although short-read WGS has the highest base-pair resolution, the read length is usually 100–200 bp, limiting the capacity to resolve the complicated structural rearrangement of an amplicon of the size of ecDNA, which ranges from hundreds of kilobases to several megabases. Technologies such as nanopore long-read sequencing and optical mapping can mitigate this limitation and are proven approaches to resolving ecDNA architecture (27, 30). Using a newly

developed algorithm, AmpliconReconstructor, recent research has demonstrated that the combination of short-read WGS and optical mapping provides a high-fidelity, cost-effective way to resolve complicated amplicon architectures (83).

Investigators have proposed alternative direct sequencing and analyzing tools, such as ATAC-seq with Circle_finder software, that can identify both the small circular DNA and the large, oncogene-containing ecDNA in cancer (84). Additionally, the genomic fragments sequenced after enrichment often do not contain paired edges connecting different junctions, resulting in a loss of information as to whether different genomic segments are part of the same or different ecDNA. A side-by-side benchmarking of WGS and ATAC-seq with the corresponding analysis software will be necessary for the field to select the optimal tool to identify ecDNA by sequencing.

The second strategy used to search for ecDNA is to enrich for circular DNA before sequencing. Traditionally, circular DNA isolation involves cesium chloride–ethidium bromide density gradient centrifugation and 2D gel electrophoresis (85, 86). Column- or magnetic bead–based methods to isolate high-molecular-weight DNA, followed by removal of chromosomal DNA by exonuclease, have recently become popular. One of the representative methods is Circle-seq, which has been used to characterize small, nonamplified, usually non-gene-containing eccDNAs (87, 88) as well as large, highly amplified, oncogene-enriched circular ecDNAs in cancer. Circle-seq has been applied to neuroblastoma, revealing the structure of crucial oncogenes such as *MYCN* that are frequently amplified as ecDNA in this neoplasm (31).

Compared with direct sequencing approaches, enrichment strategies theoretically improve the specificity of identifying circular ecDNA by eliminating amplicons generated by BFB or found in homogeneously staining regions. However, the key to success is to protect the integrity of high-molecular-weight ecDNA from shearing before exonuclease digestion, which is still technically challenging, especially for large ecDNAs that are several megabases long. Technologies including automatic liquid handling and slow pipetting may improve sample integrity and consistency during preparation. In addition, most enrichment protocols involve rolling-circle amplification of ecDNA before sequencing. Although the fidelity of the polymerase Phi29 is high, amplification bias and the potential introduction of mutations during amplification of ecDNA remain to be evaluated.

CONCLUDING REMARKS

The 23 pairs of chromosomes are the blueprint of human life. They are profoundly altered in cancer, driving uncontrolled growth. The discovery and rediscovery of ecDNA have shed new light on how cancers can hack genetic codes and rapidly change their genomes by changing the location and the topology of organization of their DNA, in addition to revealing some of the other well-known mechanisms involving changing their sequence and abundance.

ecDNA imposes a barrier to precision oncology and targeted cancer therapy. Future studies will be needed to better understand the molecular pathogenesis mechanisms of ecDNA formation, function, maintenance, and vulnerability, as well as their interaction with the microenvironment, including the immune system, to overcome these barriers and develop more effective treatments for patients with some of the most aggressive cancers. Although ecDNA was first observed more than 50 years ago, this prescient work on its potential importance and its scale, scope, and impact was not well understood until recently. Powerful new integrative molecular approaches have shown us that ecDNAs are present in nearly half of all human cancer types and likely in at least a quarter of all cancer patients. They have taught us that ecDNA is, indeed, one of the most urgent problems facing patients with cancer. Our new knowledge of ecDNA has catalyzed a shift in our understanding of gene amplification in cancer, lending a powerful insight into the accelerated

evolutionary trajectory of some tumors that have surprised and foiled so many modern treatment strategies. The presence of ecDNA also makes us recognize, once again, that DNA conveys information not only in its sequence but also in its shape, and that we will need to come to grips with the fact that altered DNA topology is a central feature of cancer pathogenesis. Finally, ecDNA challenges the successful implementation of targeted cancer therapies, clearly indicating that it is a problem worthy of its nomination as a Cancer Grand Challenge (89). We look forward to the collective engagement of the field to develop new fundamental understandings of ecDNA in human cancer, and to develop and deploy new treatments for patients with some of the most aggressive forms of cancer.

DISCLOSURE STATEMENT

V.B. is a cofounder, consultant, and Scientific Advisory Board member of and has equity interest in Boundless Bio, Inc. (BB) and Abterra Bio, Inc. The terms of this arrangement have been reviewed and approved by the University of California, San Diego, in accordance with its conflict-of-interest policies. H.Y.C. is a cofounder of Accent Therapeutics and BB and an advisor for 10× Genomics, Arsenal Biosciences, and Spring Discovery. P.S.M. is a cofounder of BB. He has equity in the company and serves as the chair of its Scientific Advisory Board, for which he is compensated.

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