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Review article

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Extrachromosomal DNA in cancer

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Abstract

Extrachromosomal DNA (ecDNA) has recently been recognized as a major contributor to cancer pathogenesis that is identified in most cancer types and is associated with poor outcomes. When it was discovered over 60 years ago, ecDNA was considered to be rare, and its impact on tumour biology was not well understood. The application of modern imaging and computational techniques has yielded powerful new insights into the importance of ecDNA in cancer. The non-chromosomal inheritance of ecDNA during cell division results in high oncogene copy number, intra-tumoural genetic heterogeneity and rapid tumour evolution that contributes to treatment resistance and shorter patient survival. In addition, the circular architecture of ecDNA results in altered patterns of gene regulation that drive elevated oncogene expression, potentially enabling the remodelling of tumour genomes. The generation of clusters of ecDNAs, termed ecDNA hubs, results in interactions between enhancers and promoters in *trans*, yielding a new paradigm in oncogenic transcription. In this Review, we highlight the rapid advancements in ecDNA research, providing new insights into ecDNA biogenesis, maintenance and transcription and its role in promoting tumour heterogeneity. To conclude, we delve into a set of unanswered questions whose answers will pave the way for the development of ecDNA targeted therapeutic approaches.

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Introduction

The foundation of our understanding of eukaryotic genetics, including cancer genetics, has long rested on the principle of chromosomal inheritance. This concept has been pivotal in elucidating the location and distribution of oncogenes within tumour cell nuclei, their transmission to daughter cells during cell division, and the dynamics of cancer genome alterations and resistance to treatment. From constructing maps of cancer genomes to developing tumour phylogenetic trajectories, the assumption of chromosomal inheritance has underpinned these fundamental aspects of cancer research. However, the discovery of extrachromosomal DNA (ecDNA) has initiated a reevaluation of the assumptions tied to pure chromosomal inheritance, introducing an additional stratum of complexity to our understanding of cancer biology. In the 1960s, ecDNA was observed for the first time, as megabase-sized, double-stranded circular DNA molecules that reside within the nuclei of cancer cells[1,2]. Notably, ecDNA can harbour oncogenes akin to those found on chromosomes[3–5], yet it adheres to an entirely distinct inheritance mechanism[6,7]. Operating autonomously, ecDNA undergoes replication and segregates randomly during cell division[6,7]. This unique mode of inheritance imparts upon it the capability to swiftly modulate its copy number within cancer cell nuclei, presenting an alternative mechanism for rapid genome evolution that can either foster the amplification of oncogenes to promote cancer progression or confer resistance to therapeutic agents[7].

Since its early identification in the 1960s, ecDNA has been referred as various names, such as double minutes[8–10], minute chromatin bodies[1,11], submicroscopic circular DNA[3,12] and autonomously replicating episomes[12,13]. Contrary to initial beliefs that these particles often manifested in pairs[1,10], the majority of them do not exhibit such paired arrangements[14], leading to the recent renaming as ecDNA. Subsequent molecular and structural studies have unveiled ecDNA as centromere-free, telomere-free circular DNA located within the cell nucleus, spanning a size range from 100 kilobases (kb) to several megabases (Mb)[4,15,16]. Sequencing and imaging studies have demonstrated that ecDNA frequently harbours specific oncogenes[3–5], drug-resistance genes[12], immunomodulatory genes[17] or other regulatory elements[18,19], and it is commonly subjected to amplification — a characteristic probably driven by its fitness advantage[7,14]. In addition, ecDNA is frequently distinguished by a more open chromatin structure, further enhancing its transcriptional proclivity[4]. This unique structural feature of ecDNA introduces a new paradigm in gene regulation and expression[4,15,18]. Although ecDNA was identified decades ago, the limited throughput of imaging detection and inaccurate estimations from sequencing data initially led to the perception that ecDNA was exceedingly rare, accounting for less than 1.4% of cancers, according to the Mitelman database. Owing to the prevailing belief in its infrequent occurrence and an initial lack of clarity regarding its significance across a broader spectrum of cancers, research efforts were confined to a few research groups. However, these early studies have established a solid foundation for ongoing investigations into ecDNA by demonstrating that gene amplification on ecDNA occurs independently of chromosomes and contributes to various types of cancer[3,8,12,20–25].

Through the use of pan-cancer whole-genome sequencing (WGS) data, supported by advanced computational detection algorithms and validated through DNA fluorescence in situ hybridization (DNA FISH) (Table 1), ecDNA has now been identified in most human cancer types, including glioblastoma, neuroblastoma, sarcoma and medulloblastoma, as well as head and neck, lung, oesophageal, stomach, pancreatic, liver, colon, bladder, breast, ovarian and prostate cancers[5,14]. The pervasive presence of ecDNA across these cancer types suggests a potentially pivotal role in cancer pathogenesis. Through years of investigation, the critical involvement of ecDNA in various aspects of cancer biology has been elucidated. Notably, studies have demonstrated its role in upregulating oncogene expression[4,18], fostering intra-tumoural genetic heterogeneity[7,14] and contributing to resistance against cancer treatments[26]. Furthermore, the presence of ecDNA has been associated with shorter survival durations than those of patients with cancer lacking ecDNA[5], highlighting its critical role in propelling cancer progression. A recent study by Luebeck et al. has used WGS data from patients with biopsy-validated Barrett oesophagus to explore the relationship between the presence of ecDNA in pre-cancerous samples and subsequent cancer outcomes, suggesting that ecDNA may even contribute to the transition from pre-cancer to cancer[17].

It is important to note that ecDNA differs from another closely related term, eccDNA (extrachromosomal circular DNA), which describes either circular DNA particles separated from linear chromosomes in general or a group of much smaller (<1 kb), non-clonal circular DNA that is found in normal tissues. The differences between ecDNA and eccDNA have been thoroughly outlined elsewhere[27,28]. This Review will centre its focus on the pivotal role played by large oncogenic ecDNAs in tumour formation, progression and drug resistance. Finally, we will highlight the importance of ecDNA in the development of innovative methodologies for cancer diagnosis and propose avenues for novel treatments.

Aberrant gene expression by ecDNA

Oncogene amplification

The presence of ecDNA and/or homogeneously staining regions (HSRs) on specific chromosomes has been utilized as a cytogenetic indicator of gene amplification in cancer[29,30]. Increasing evidence suggests that numerous amplified oncogenes, particularly those present at high copy numbers in cancer genomes, are localized on ecDNA, especially in primary tumour cells[14,23,31] (Fig. 1). This encompasses well-known cancer driver genes, such as *MYC*, cyclin-dependent kinase 4 (*CDK4*), *MDM2, AKT1*, epidermal growth factor receptor (*EGFR*) and SRY-box transcription factor 2 (*SOX2*)[3,5,14,30–36].

Similar to oncogenes within chromosomes, RNA sequencing has demonstrated that ecDNA also serves as a template for mRNA transcription[4]. In cancer cells harbouring ecDNAs, the number of ecDNAs containing the same oncogene can exceed one hundred copies per cell, providing an abundant pool for high-level oncogene RNA transcription[7]. For instance, in *MYC*-driven colon cancers, the patient-derived cell line Colo 320DM was found to contain, on average, 50–100 ecDNA per cell, with *MYC* amplified on over 95% of the cellular ecDNA[4]. Although an oncogene can also be amplified to similar degree on chromosomes, for example, through processes such as breakage–fusion–bridge (BFB) cycles, this occurs generally at a much slower pace. Because ecDNA lacks the typical centromere structure found in chromosomes[15], it undergoes random inheritance during cell division[6,7], leading to a more rapid increase in oncogene copy numbers than other amplification mechanisms[7]. This rapid adaptation is crucial for cancer cells to respond to a constantly changing environment and establish drug resistance during treatments.

Although other mechanisms may contribute to oncogene upregulation in cancers containing ecDNA, copy number increase stands out as the most significant mechanism for high oncogene expression in cancer cells[4,7,18] (Fig. 1). In a recent study in *EGFR*-containing

Table 1 | Useful techniques in ecDNA research

Purpose	Technique	Description	Refs.
De novo ecDNA generation	CRISPR-C	This induces double-strand breaks flanking the region of interest; ecDNA can be generated by self-circularization in cells	7,64
	Cre-mediated circularization	By inserting two loxP sites flanking the region of interest in cancer or primary cells, ecDNA can be induced by expressing Cre recombinase; the use of fluorescent protein has been incorporated in this technique to indicate successful ecDNA circularization and allows sorting to further enrich ecDNA contained cells	106
Fixed sample imaging	Metaphase spread	This involves preparing chromosomes in a dispersed arrangement on a microscope slide, capturing a snapshot of the condensed chromosomes and ecDNA during cell division; this technique allows the detection of ecDNA existence and the counting of ecDNA copy numbers in mitotic cells	15
	DNA FISH	A molecular cytogenetic technique that uses fluorescently labelled DNA probes to specifically bind to target DNA sequences in fixed cells, allowing the visualization and localization of specific genomic regions under a fluorescence microscope; because DNA FISH applies probes targeting ecDNA-specific or ecDNA-enriched sequences such as amplified oncogene, it is frequently used on metaphase spread or unsynchronized cells to examine the behaviour and spatial organization of specific ecDNA species	18
	Optical mapping	An imaging-based genomic technique that stretches and labels DNA with fluorescently labelled probes, enabling high-resolution visualization and mapping of the physical structure; this provides insights into genome organization, structural variations and genomic features	4,107
Live cell imaging	Array-based ecDNA targeting	By inserting a repetitive array (for example, TetO, LacO or CuO) into ecDNA, live cell imaging can be achieved through tracking fluorescent-bound array-specific binding proteins, allowing dynamic tracing of ecDNA molecules in live cells	18
	ecTag	This approach utilizes CRISPR technology; by recruiting fluorescent proteins to specific sequences on ecDNA with the guidance of endonuclease-deficient Cas9–single guide RNA, ecTag enables real-time visualization of ecDNA within live cells; to implement this technology effectively, it is necessary to identify ecDNA-specific sequences, often located at breakpoints in the ecDNA structure	6
Image analysis	ecSeg	A deep learning-based imaging analysis tool to facilitate ecDNA calling and counting on DNA FISH images performed on metaphase spread	108
ecDNA isolation	2D separation	Separating extracted DNA by neutral–neutral 2D agarose gel analysis allows detection of circular DNA segments	109
	CRISPR-CATCH	By introducing ecDNA-specific double-strand break with CRISPR–Cas9, linearized ecDNA can be separated from genomic DNA by gel electrophoresis	16
Sequencing	Whole-genome sequencing (WGS)	WGS enables in-depth sequencing analysis of ecDNA contents and structural variations	54
	ATAC-seq	A method used to analyse the accessibility of chromatin regions in the genome, which can be applied to study ecDNA at single-cell level	4,110
	Circle-seq	Circular DNA is column purified, filtered of remaining linear chromosomal DNA using exonuclease, then rolling-circle amplified and subsequently deep sequenced, and mapped; this method can also be separately used for isolation of circular DNA, including ecDNA	111
	scEC&T-seq	A single-cell sequencing method enabling isolation and sequencing of both mRNA and circular DNA at the single-cell level	57
	ChIA-PET, ChIA-Drop, Hi-C, Hi-ChIP and 3C	Sequencing-based methods to evaluate chromosomal interactions, which can be applied to ecDNA to determine the 3D organization and chromosomal interactions	18,43,112
	Long-read sequencing	This enables the detection of longer reads ranging from 10,000 to 100,000 base pairs enabling comprehensive investigations of ecDNA and its structural features	52,113
Sequencing analysis	AmpliconArchitect	A computational tool used for analysing and visualizing the complex amplicon structures, particularly in cancer genomes, aiding in the identification and characterization of ecDNA and other genomic alterations	54
	ECCsplorer and Circle-Map	Software to detect circular DNA that was specifically designed for analysing circular DNA-sequencing datasets, including Circle-seq	114
	FitMS	An algorithm facilitating mutation signature analysis	115
	FLED	An algorithm reconstructing circular DNA using long-read sequencing data	113

Other analysis methods are available, however, here we have selected and included only the well-established and structured methods to enable easy accessibility to each technique listed. ATAC-seq, the assay for transposase-accessible chromatin with sequencing; ChIP, chromatin immunoprecipitation; Circle-seq, illumina sequencing of amplified circular DNA; CRISPR-CATCH, in vitro CRISPR–Cas9 treatment and pulsed field gel electrophoresis of agarose-entrapped genomic DNA; ecDNA, extrachromosomal DNA; FISH, fluorescent in situ hybridization; FitMS, signature fit, multistep; FLED, full-length eccDNA detection; scEC&T-seq, single-cell extrachromosomal circular DNA and transcriptome sequencing.

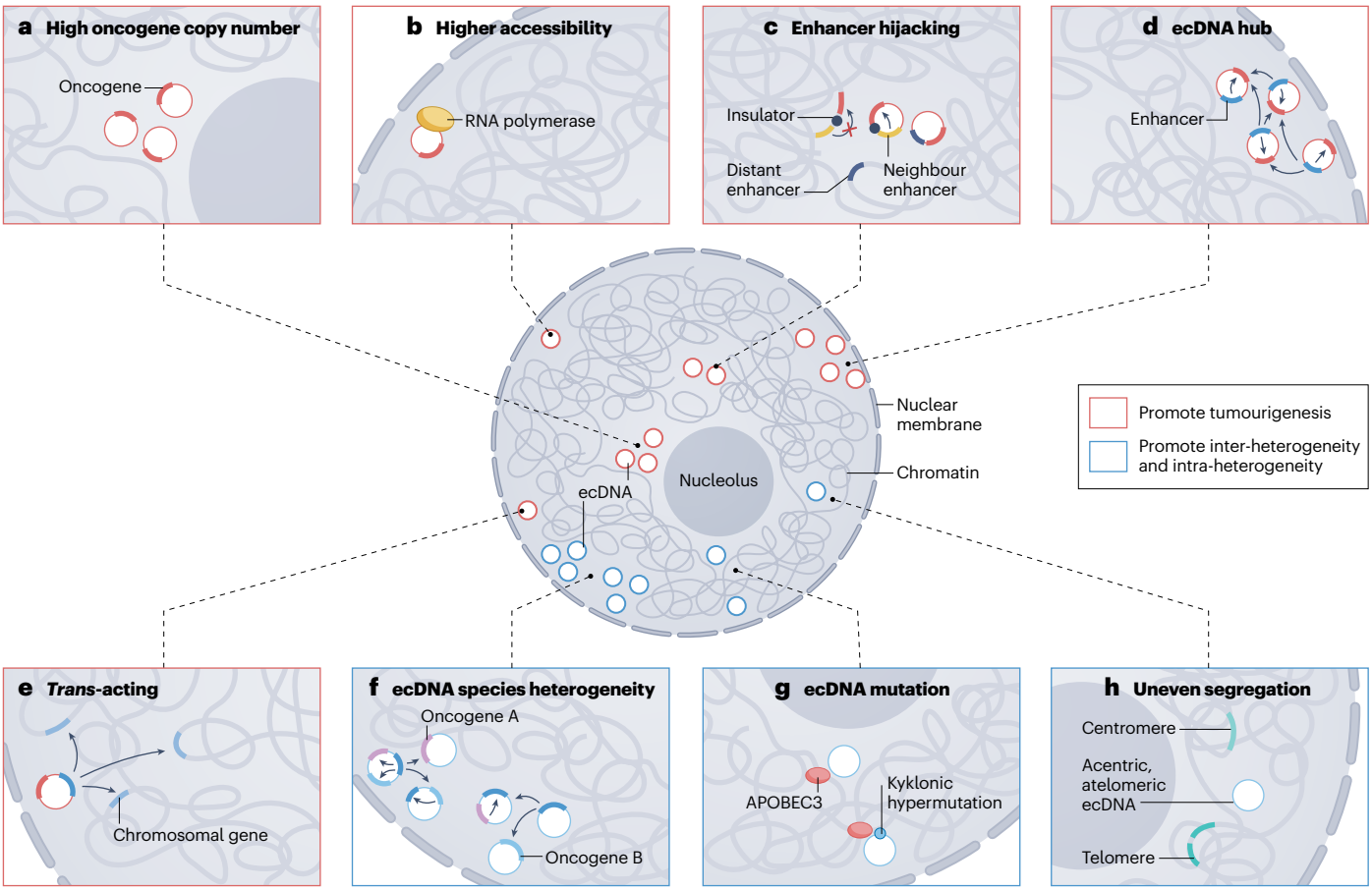

Fig. 1 | ecDNA promotes tumoreginesis and tumour heterogeneity. Extrachromosomal DNAs (ecDNAs) are double-stranded circular DNA molecules that are frequently observed within the nucleus of cancer cells. The common features of ecDNA that have pivotal roles in promoting tumorigenesis (parts **a**–**e**, highlighted in red) and promoting inter-tumour and intra-tumour heterogeneity (parts **f**–**h**, in blue) are depicted here. The placement within the nucleus is not representative of where it functions. **a**, ecDNA is often detected with high copy number in cancers, increasing oncogene expression. **b**, Owing to the structure of ecDNA, oncogenic regions are more accessible, enabling increased transcription. **c**, Enhancers are frequently detected on ecDNA to promote transcription; this includes those neighbouring and distant to the gene on its original chromosome. This is often termed as enhancer hijacking. **d**, ecDNAs form hubs to boost transcription across neighbouring ecDNAs by sharing regulatory elements such as enhancers. **e**, ecDNA can modulate genes on chromosomal DNA functioning as a mobile *trans*-acting element. For instance, it can augment chromosomal gene expression by interacting with enhancers located on ecDNA. **f**, The population of ecDNA within a cell, within a tumour or across tumours can harbour different oncogenes, non-oncogene regulatory elements and oncogenic structural variants to increase cellular and tumoural heterogeneity. **g**, ecDNA also contains various mutations, including kyklonic hypermutation, which is a distinctive characteristic resulting from the action of apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3 (APOBEC3) enzymes. These mutations often intersect with known cancer driver genes, promoting ecDNA evolution. **h**, ecDNA is acentric and atelomeric and, therefore, segregates unevenly during cell division, promoting cellular heterogeneity.

glioblastoma (GBM)-derived glioma stem cells, the copy number of ecDNA containing *EGFR* was identified as the sole driver of high levels of *EGFR* transcription[37]. Therefore, ecDNA offers an effective mechanism for the selection of tumour-promoting genes and a potent avenue for promoting oncogene expression.

ecDNA has high transcriptional activity

In both cancer cell lines and clinical tumour samples, it has been observed that oncogenes encoded on ecDNA produced more RNA transcripts than the same sequence located at their endogenous chromosomal locus, even after copy number normalization[4,18]. This transcriptional advantage of ecDNAs is probably attributable to the accessibility of ecDNA structure, the ability of ecDNA to hijack enhancers, and the formation of ecDNA hubs that boosts transcription (Fig. 1).

ecDNA is more accessible. Firstly, circular ecDNA typically exhibits a more open chromatin structure, granting ecDNA a higher level of transcriptional activity[4] (Fig. 1). Through the utilization of chromosomal accessibility assays, including the assay for transposase-accessible chromatin with sequencing (ATAC-seq), micrococcal nuclease digestion with deep sequencing (MNase-Seq) and the assay of transposase-accessible chromatin with visualization (ATAC-see) (Table 1) in colon cancer and glioblastoma, ecDNA was shown to display greater chromatin accessibility than the corresponding oncogene

located on linear chromosomes[4]. In another study, reduced DNA methylation was also detected on ecDNA at regulatory elements, including the *EGFR* promoter in comparison to the same elements on chromosomal DNA[16]. This aligns with the notion of a more active chromatin state of ecDNA in GBM39 cells[4]. Importantly, data from colon cancer and glioblastoma suggest that ecDNAs are organized into nucleosomes, much like chromosomal DNA, but the higher-order compaction of nucleosomes that suppresses gene transcription is lacking on ecDNAs[4].

Enhancer hijacking upregulates oncogene on ecDNA. Secondly, the circularization of ecDNA enables the emergence of new physical chromosomal contacts, facilitating the formation of novel regulatory networks[19,38] (Fig. 1). Owing to the circular topology, ecDNA can come into contact with enhancer elements that would typically be too far apart[19]. Moreover, insulator elements that would normally suppress these interactions within chromosomal topologically associated domains (TADs) are prevented from doing so by the circular structure of ecDNA[19]. This rewiring of the regulatory network can occur by hijacking previously insulated neighbour enhancers[19]. For instance, in glioblastoma, *EGFR* on ecDNA is preferentially co-amplified with its neighbouring non-coding region, establishing new enhancer–oncogene contacts that promote tumorigenesis[39]. Additionally, ecDNA can join different oncogenes with remote non-coding fragments[40]. In colon cancer, the amplified oncogene *MYC* on ecDNA is often found in fusion form, creating a fusion gene that reconnects the long non-coding RNA gene Pvt1 oncogene (*PVT1*) with *MYC*[18]. Structural analysis of MYCN proto-oncogene (*MYCN*) ecDNA in neuroblastoma has also revealed two classes of *MYCN* ecDNAs[19]. One class is co-amplified with a proximal enhancer, whereas the other contains distal chromosomal fragments harbouring remote enhancers, compensating for the loss of local enhancer[19].

ecDNA hubs further boost transcription. Thirdly, in some cancers, ecDNAs tend to cluster into micrometre-sized physical structures of a few to 100 copies of ecDNA, known as ecDNA hubs[18], which significantly amplify oncogene expression within the ecDNA[27,41] (Fig. 1). These hubs exhibit higher transcription activity, and the dispersal of ecDNA hubs with BET inhibitor JQ1 has been linked to reduced oncogene expression[18]. ecDNA hubs enable more cooperative sharing of regulatory elements among ecDNAs owing to their spatial proximity, particularly interactions between distinct enhancers and promoters. In the gastric cancer cell line SNU16, two distinct ecDNAs coexist, each carrying a different oncogene (*MYC* and fibroblast growth factor receptor 2 (*FGFR2*)). Within this context, five enhancers on the ecDNA containing *FGFR2* have been identified as capable of activating *MYC* expression in *trans*, which tends to require an ecDNA hub[18]. Further sequencing analysis has revealed the existence of ecDNA that exclusively contains oncogene-coding genes or enhancers, which individually cannot contribute to oncogene expression[16]. However, because ecDNAs can interact with one another in *trans* within a hub, these specialized ecDNAs are able to cooperate and leverage each other to further increase oncogene expression[16].

ecDNA acts as a mobile enhancer

In eukaryotic cells, chromosomes are widely accepted to fold into discrete chromosome territories within interphase nuclei[42]. Although this organization is essential, it constrains interactions between different chromosomes. By contrast, ecDNA, being an isolated circular molecule with a high copy number, is believed to possess the capability to explore a significantly larger nuclear space compared with its original chromosomal locus. Recent studies utilizing techniques such as chromatin interaction analysis with paired-end-tag sequencing (ChIA-PET) and ChIA-Drop (Table 1) have suggested that gene regulation within chromosomal regions might be influenced by interactions with ecDNA, especially in regions that are transcriptionally active[43]. These primary interaction sites are characterized by a broad increase in the activation histone mark H3K27ac, both on ecDNA and chromosomes[43]. Consequently, in glioblastoma and prostate cancer cells, chromosomal genes that spatially associated with ecDNA tend to exhibit higher expression levels, supporting the notion that ecDNA acts as a mobile enhancer[43] (Fig. 1). This intriguing role of ecDNA as a mobile enhancer also offers an additional explanation for why ecDNA is often enriched with numerous non-coding regulatory elements[16,43], and in some cases, ecDNAs exclusively contain regulatory elements without any coding genes[16]. Some of these ecDNAs encompass amplicons of enhancers, referred to as enhancer-only ecDNA[16]. The specific roles of these ecDNA species have not been comprehensively explored. However, given that enhancers co-amplified with oncogenes have been demonstrated to contribute to oncogene expression through ecDNA hubs and increase chromosomal gene transcription via *trans*-interaction, it is plausible that enhancer-only ecDNAs may also have the capacity to promote transcription of genes on other ecDNAs or chromosomes[44] (Fig. 1).

ecDNA promotes cancer evolution

Copy number heterogeneity

The foundational principles of Darwinian evolution, which include inheritance, variation, selection and time, have been instrumental in explaining how cancers evolve, including ways that enable them to develop resistance to treatment[45,46]. The concept of genetic identity by descent is crucial for our understanding of cancer evolution, and the assumption of chromosomal inheritance during cell division serves as the basis for explaining the clonal evolution of many tumours[47]. However, when we compare cancers with ecDNA with those without, we find that ecDNA-containing cancers evolve at accelerated rates that cannot be solely explained by the principles of chromosomal inheritance[14,48]. Our research, along with that of others, has integrated theoretical models of random segregation, unbiased image analysis, CRISPR-based ecDNA tagging combined with live-cell imaging and CRISPR-C techniques (Table 1) to demonstrate that random ecDNA inheritance during cell division drives extensive intra-tumoural ecDNA copy number heterogeneity (Fig. 1). This enables rapid adaptation to metabolic stress and targeted treatment by tuning the copy number either up or down[6–8,26]. For instance, in a few in vitro glioblastoma cell models, we have observed significant, dose-dependent decreases or increases in the number of ecDNAs per cell in response to selective pressures, such as glucose restriction or the administration of a targeted anticancer drug, only in the tumour cell lines with ecDNA[7]. This fundamental characteristic may explain why tumour cells with ecDNA possess the capacity to resist targeted treatment or metabolic stress[6–8,26].

Genetic and epigenetic heterogeneity

ecDNAs with distinct DNA sequences are generally referred to as different ecDNA species. The classification of ecDNA species is commonly based on the most critical gene contained within the ecDNA, mostly oncogene, such as *MYC* ecDNA in colon cancer[18] or *EGFR* ecDNA in

glioblastoma[4]. Although many oncogenes are found as individual species, it is also possible for multiple oncogenes to be amplified on the same ecDNA molecule[49,50] (Fig. 1). Furthermore, ecDNAs can exhibit various structural variants besides encoding full-length open reading frames[4,18,26]. These variants include fusions, truncations and splicing variants, which significantly increase ecDNA heterogeneity[40,51,52]. For example, in colon cancer, both full-length *MYC* and the fusion form of *PVT1-MYC* can coexist in the same cell[18].

Mutational studies heavily rely on sequencing analysis, but sequences of ecDNA share high similarity with those on chromosomes. This makes it extremely difficult to know whether to map short reads back to ecDNA or the corresponding chromosomal region. Computational methods have been developed in recent years to reconstruct fragments of WGS reads into circular structures, taking advantage of sequence features surrounding breakpoints[53,54] (Table 1). The successful reconstruction of a circular structure then suggests the presence of ecDNA, enabling mutational studies on ecDNA (Table 1). Recently, Bergstorm et al. characterized clustered mutations in 2,583 cancer genomes from over 30 types of cancer, including samples from patients whose tumours contain ecDNAs[55]. This unveiled an unexpectedly high occurrence of apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3 (APOBEC3) mutational pattern on ecDNA[55,56]. Notably, 41% of all kyklonic events (co-occurrence of APOBEC3 kataegis on ecDNA) were found to intersect with known cancer driver genes, resulting in various mutations in these driver genes[55] (Fig. 1). These data suggest a significant contribution of APOBEC3 to ecDNA evolution.

In addition to the advancements in computational methods for distinguishing ecDNA and chromosomes, the development of CRISPR–Cas9-assisted targeting of chromosome segments (CRISPR-CATCH) (Table 1) represents a significant breakthrough, enabling the physical isolation of specific ecDNA species from other genomic regions[16]. By accurately sequencing and comparing ecDNA with other chromosomal regions, King et al. utilized CRISPR-CATCH to isolate ecDNA in glioblastoma cancer cell lines and flash-frozen metastatic melanoma tumours, revealing that activating oncogenic mutations in *EGFR* or neuroblastoma RAS viral oncogene homologue (*NRAS*) predominantly reside on ecDNA, whereas the same chromosomal allele is mainly wild type[16]. Furthermore, in combination with long-read sequencing, the authors analysed DNA methylation patterns of isolated ecDNA and observed a significant hypomethylation of gene promoters on ecDNA compared with chromosomes[16]. This suggests that, beyond sequence-level genetic heterogeneity, ecDNA may also exhibit differential epigenetic regulation which requires further investigation.

The advancement of single-cell-based technologies, including single-cell sequencing and imaging, has significantly facilitated the examination and exploration of tumour heterogeneity along with its underlying mechanisms. Detection of oncogene copy number heterogeneity and oncogene expressional heterogeneity in ecDNA-containing cancers has been greatly aided by the application of single-cell metaphase imaging and single-cell RNA sequencing[4,7,14,26] (Table 1). Although populational assays remain valuable[16,55], the significance of studying ecDNA heterogeneity at a cellular level cannot be overstated. The advent of the newly developed technology, single-cell extrachromosomal circular DNA and transcriptome sequencing (scEC&T-Seq) (Table 1) allows parallel sequencing of both circular DNAs and full-length mRNAs from individual cells[57]. This enables a matched investigation of both ecDNA structural heterogeneity and their transcriptional impact[57]. Applying this technology to two neuroblastoma cancer lines, CHP-212 and TR14, the authors confirm the critical role of ecDNA in driving both intercellular copy number and transcriptional heterogeneity at a single-cell level[57]. Furthermore, by reconstructing and comparing different ecDNA structures in individual cells, the authors hypothesize that circular recombination is a possible mechanism driving ecDNA structural changes and consequently ecDNA evolution[57]. With the ongoing development of more single-cell-based technologies, the investigation of ecDNA heterogeneity at the cellular level is expected to deepen our understanding of ecDNA dynamics and contribute to evaluating the functional heterogeneity that contributes to tumour progression.

ecDNA biogenesis

As a community, our understanding of ecDNA biogenesis primarily stems from structural analyses of ecDNA sequences obtained through WGS data. Through the examination of the chromosomal sites that give rise to ecDNA, scrutiny of the characteristics surrounding these breakpoints, and the assessment of ecDNA molecule diversity, several hypotheses have emerged to elucidate the mechanisms underpinning ecDNA formation in the context of cancer.

Excisional model

The excisional model, previously known as the episomal model, is a straightforward hypothesis for the origin of ecDNA (Fig. 2). According to this model, ecDNA originates from chromosomal breakages followed by the circularization of the excised fragment, resulting in the formation of ecDNA with a relatively simple structure and low diversity[30,58–61]. When chromosomal DNA breakages happen without a nearby homologous template, the chromosomal copy tends to be permanently lost, leaving a genetic scar[13,30,62,63]. This concept finds support in studies conducted in Chinese hamster ovary cells, wherein ecDNA formation coincided with deletions on the original chromosome[13]. Furthermore, an investigation into acute promyelocytic leukaemia (APL) revealed cases wherein the *MYC* gene was deleted from its original chromosomal location (chromosome 8) and amplified on ecDNA[62]. The development of CRISPR-C technology (Table 1) has facilitated the replication of this process and successfully generated ecDNA molecules in normal cell models[64]. By using CRISPR–Cas9 technology to induce double-strand breaks (DSBs) on the same chromosomal arm, circular DNA fragments are generated and can be enriched through the application of specific drugs that favour the excised region[7]. This not only lends support to the viability of the excisional model but also furnishes experimental evidence for the occurrence of ecDNA formation through this mechanism. Importantly, DNA breakages can also occur when a homologous template is nearby, particularly during replication stalling when replisomes encounter obstacles or nucleotide sources are limited[65]. In such cases, the excised fragment can be ligated to form ecDNA without causing a corresponding chromosomal deletion, as the excised segment can be filled in and restored[58,63,66] (Fig. 2).

A critical step in the excisional model involves the re-ligation of the excised fragment into a circular molecule. In human melanoma tumours, non-homologous end joining (NHEJ) emerges as a prominent contributor to driving ecDNA generation, with approximately 70% of mapped breakpoints exhibiting the NHEJ signature[56]. Notably, in human melanoma cell lines and patient-derived xenografts, the inhibition of NHEJ results in a reduction in the copy number of ecDNA[56]. In other scenarios, such as in a case study that included five patients with glioma and two patient-derived xenografts, the majority of ecDNAs with an amplified *EGFR* gene were found to be

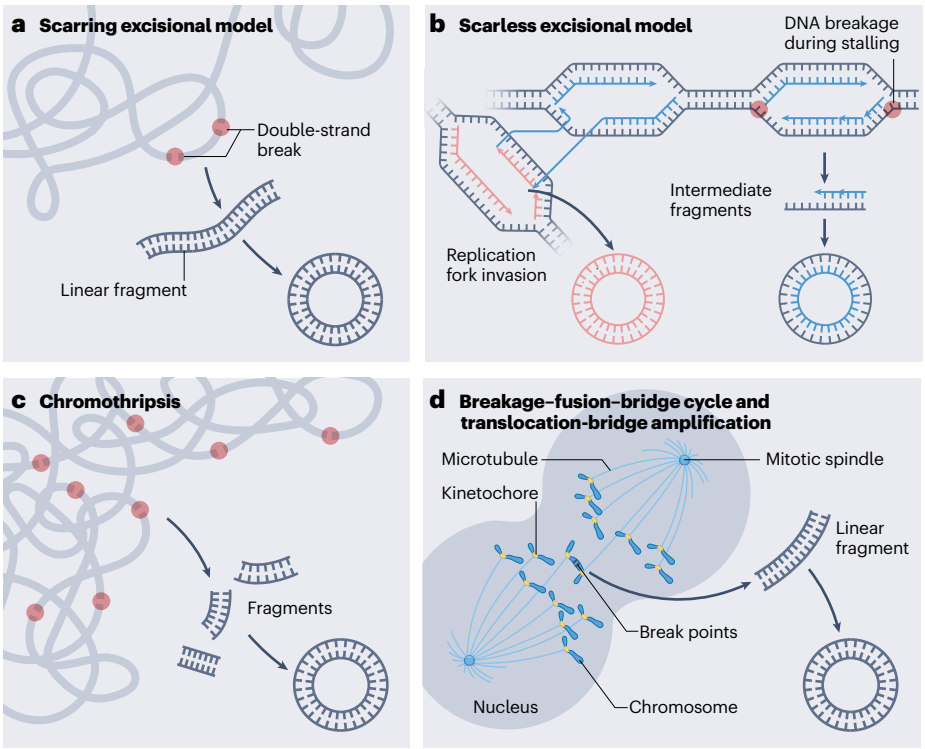

Fig. 2 | Mechanisms that facilitate ecDNA biogenesis. a, The scarring excisional model suggests that ecDNA is formed following the re-joining of linear fragments generated through occasional double-strand breaks of chromosomal DNA. If the DNA breaks occur before or after replication (but not during), ecDNA is generated, leaving a scar at the original chromosomal region. **b**, Alternatively, if DNA breaks occur during replication, ecDNA can form while the chromosome is restored through homologous recombination, leaving no scar on the chromosome and an additional copy of the gene on ecDNA. Various methods have been proposed. These include the circularization of intermediate fragments resulting from DNA double-strand breaks generated during replication fork stalling (right) or replication from inflated single-strand DNA during template switching (left). **c**, ecDNA can also be generated through catastrophic DNA damage events, such as chromothripsis. ecDNA can form from the fragmented pieces that fail to reintegrate into the chromosome. The precise mechanism is unknown. **d**, Alternatively, DNA fragments originating from chromosomal bridges, a consequence of the breakage–fusion–bridge (BFB) cycle or chromosomal translocation, can be made into ecDNA. The BFB cycle occurs when telomeres are lost, resulting in the fusion of sister chromatids and the creation of a dicentric chromosome. Simultaneously, chromosomal translocation can directly instigate the formation of a dicentric chromosome. These events give rise to chromosomal bridges during anaphase, causing DNA double-strand breaks, thus, contributing to the formation of ecDNA. The precise mechanism is unknown.

single-fragment circles formed through a microhomology-based mechanism[58]. Microhomology was found at six out of seven cases, emphasizing the importance of microhomology-based mechanism in ecDNA formation in gliomas[58]. However, in a different study involving more *EGFR*-amplified glioblastoma samples, both microhomology and breakpoints that lack homology were observed, suggesting that some of the initial circularization in glioblastoma could also be generated by non-homology-based mechanisms[63]. The precise contribution of different DNA repair mechanisms to this process remains inconclusive. However, it is evident that both non-homologous-based and homologous-based mechanisms contribute to ecDNA formation[30,56,58,63].

Chromothripsis

ecDNA can also emerge during more catastrophic DNA damage events, such as chromothripsis (Fig. 2). Chromothripsis is a chromosomal shattering process whereby a chromosome undergoes multiple random breaks and rearrangements in a single event, and results in intricate chromosomal rearrangements, such as deletions, inversions, duplications and translocations within a localized region of a chromosome[67]. During chromothripsis, it has been suggested that ecDNA can be generated when the fragmented pieces fail to correctly reintegrate into the chromosomes[68], and a correlation between chromothripsis and the presence of ecDNA has been identified in various cancer types[17,68–71]. In DLD-1 in vitro colorectal cancer cell line model, the induction of chromothripsis results in a spectrum of genetic rearrangements, including the formation of ecDNA[68]. Another study conducted in HeLa cells by Shoshani et al. has utilized paired-end WGS to analyse clones selected after methotrexate treatment, finding ecDNA and signatures of chromothripsis, further supporting chromothripsis as a mechanism for ecDNA generation[51].

Notably, DNA repair pathways also have a pivotal role in ecDNA formation during chromothripsis. In the context of ecDNA generation during the acquisition of methotrexate resistance, NHEJ and poly(ADP–ribose) polymerase (PARP) dependent pathways appear to be of significance, as the inhibition of either pathway led to a substantial decrease in the frequency of resistant colony formation and ecDNA generation[51]. While these findings shed light on the mechanisms at

Glossary

Breakage–fusion–bridge (BFB) cycle
A mechanism of chromosomal instability wherein broken ends of different chromatids or chromosomes fuse.

ChIA-Drop
A chromatin conformation capture technique that combines chromatin immunoprecipitation with droplet-based single-cell sequencing, allowing the investigation of chromatin interactions at the single-cell level.

Chromatin interaction analysis with paired-end-tag sequencing
(ChIA-PET). A genomic technique that enables the identification and mapping of long-range chromatin interactions by combining chromatin immunoprecipitation with paired-end high-throughput sequencing.

Chromosome territories
The specific region of the nucleus that a certain chromosome tends to occupy.

CRISPR-C
A technique that allows in vitro ecDNA generation in cells by inducing double-strand breaks flanking the region of interest upon the delivery of pairs of CRISPR–Cas9 guide RNAs.

Double minutes
(DM). A traditional term for ecDNA that is still indicated in names of most ecDNA-containing cell lines, for example Colo 320DM.

Gene amplification
A process by which the copy number of a specific gene is increased in a cell, leading to heightened expression and contributing to the development and progression of cancer when oncogene is amplified.

Genetic identity by descent
A genetic term indicating the sharing of a specific DNA segment between two or more individuals owing to inheritance from a common ancestor.

Homogeneously staining region
(HSR). A large repetitive region in a chromosome that displays a homogeneous staining pattern when targeted with probes; in this Review, HSR is used to mostly refer to oncogene amplification regions in chromosomes.

Microhomology
The presence of short, identical or nearly identical sequences (typically 2 to 20 base pairs) at or near the ends of two DNA fragments, typically arising during DNA repair or rearrangment to facilitate precise alignment.

Micronuclei
Small, additional nuclei that can form during cell division and contain fragments of chromosomes or entire chromosomes that were not incorporated into the main nucleus.

Non-homologous end joining
(NHEJ). DNA repair mechanism whereby double-stand breaks are ligated without the need for a homologous template.

Nucleosomes
Basic structural units of eukaryotic DNA packaging, consisting of a segment of coiled DNA around eight core histone proteins.

Repli-seq
A genomic technique that involves sequencing the DNA obtained from cells at different stages of the S phase to map DNA replication patterns and identify regions undergoing replication.

Topologically associated domain
(TAD). A genomic region that spatially interacts with itself.

play, further comprehensive studies are warranted to establish a systematic understanding of the various DNA repair pathways involved and their respective contributions to ecDNA formation during chromothripsis.

BFB cycle and translocation bridge amplification

Signatures of the BFB cycles are commonly observed in cancer genomes, including those with ecDNA[5]. The BFB cycle commences when sister chromatids fuse owing to telomere loss and are subsequently separated during anaphase. Because the fused chromosome does not necessarily break at the points of initial fusion, this process generates uneven chromatid and chromosomal duplications[72]. In the absence of functional telomeres, the BFB process continues in subsequent generations until all chromosomal ends receive telomeres, leading to extensive chromosomal rearrangements over time[73].

Although signatures of the BFB cycle are prevalent in ecDNA-containing cancers, the direct link between the BFB cycle and ecDNA formation remains unclear[5]. In the same study conducted by Shoshani et al., BFB events were identified in methotrexate-resistant clones that subsequently underwent chromothripsis, suggesting that the BFB cycle may contribute to ecDNA generation by destabilizing the genome, favouring chromosomal bridge formation, chromosome shattering and chromothripsis[51] (Fig. 2). Additionally, mechanisms such as inter-chromosomal translocations, which lead to the formation of chromosomal bridges, have also been demonstrated to contribute to ecDNA formation, a phenomenon termed translocation-bridge amplification[74] (Fig. 2). Translocation-bridge amplification accounts for a significant portion of the amplification observed in key oncogenes such as Erb-B2 receptor tyrosine kinase 2 (*ERBB2*) and cyclin D1 (*CCND1*) in breast cancer, characterized by inter-chromosomal translocations at specific amplicon boundaries[74]. However, a more comprehensive understanding of the detailed mechanisms underlying processes like the BFB cycle or translocation-bridge amplification is still required. For instance, in post-treatment biopsies from a patient with colorectal cancer, both ecDNA and BFB events without chromothripsis were detected, suggesting that BFB cycles can independently contribute to ecDNA generation[51].

Another noteworthy aspect to consider is the relationship between ecDNA and HSR (Fig. 3). It has long been hypothesized that ecDNA might originate from pre-existing HSR[75]. Cytogenic observations of ecDNA in a patient-derived neuroblastoma cell line reveal two classes of cells characterized by the presence of either ecDNA or HSR[75]. The existence of two additional marker chromosomes in all the cells suggests a common ancestor, implying a potential connection between ecDNA and HSR[75]. Although our previous discussion primarily centred on the potential role of BFB cycles in ecDNA generation, it is crucial to recognize that the BFB cycle can lead to gene amplification, often resulting in the formation of HSR structures[76]. Therefore, it is plausible that BFB events, along with their downstream effects such as the formation of chromosomal bridge or even chromothripsis, might contribute to the generation of ecDNA from HSR. Nevertheless, more targeted and comprehensive studies are required to unravel the intricacies of this relationship and elucidate the mechanisms underlying this process.

ecDNA maintenance

Self-replication

The initial belief that all ecDNA was exclusively attributed to de novo generation shifted with the discovery that ecDNA is also capable of undergoing replication[75] (Fig. 3). Since then, researchers have hypothesized and examined whether distinct mechanisms of ecDNA replication exist and contribute to the biological diversity associated with ecDNA[77]. These hypotheses include but are not limited to when ecDNA replication can happen and how many times a single ecDNA can be replicated per cell cycle. Intriguingly, despite the unique behaviour of ecDNA compared with linear chromosomes, ecDNA appears to follow similar replication principles[9,77]. Investigations using [3H]deoxythymidine ([3H]dThd) and bromodeoxyuridine (BrdU) labelling revealed that ecDNA replicates exclusively during the S phase, synchronizing with the rest of the genome[9,77]. Notably, euchromatin generally undergoes replication earlier than heterochromatin[78]. In line with this observation and the understanding that ecDNA is structurally more open, ecDNA predominantly replicates during the midde and early S phase but not in the late replicating phase[9,77]. Furthermore, ecDNA undergo replication only once throughout the entire cell cycle[9,77]. Therefore, in contrast to the earlier hypotheses, ecDNA does not undergo replication outside of S phase or replicate disproportionally from the rest of the genome during S phase, indicating its role in ecDNA maintenance rather than contributing to ecDNA heterogeneity. Interestingly, in colon cancer line COLO 320DM, ecDNA relocates to the nuclear interior region during S phase, preceding the replication of peripheral heterochromatin[79], suggesting the existence of a replication mechanism unique to ecDNA. Whether this relocation is necessary for ecDNA replication remains unclear. Using modern techniques such as Repli-seq might provide more comprehensive insights into ecDNA replication.

Positive selection

Positive selection is a fundamental concept underlying the maintenance of ecDNA (Fig. 3). This is supported by the frequent amplification of specific oncogenes on ecDNA in different cancers, such as *MYC* in colon cancer and *EGFR* in glioblastoma[18]. Studies involving resistance to dihydrofolate reductase (DHFR) inhibitor methotrexate in various mouse cell models have demonstrated that ecDNA-amplified *DHFR* is progressively diminished when cells are cultured without the drug[21,22,80,81]. This demonstrates that as selective pressure is removed, ecDNA is gradually lost, highlighting the role of positive selection in ecDNA maintenance.

Tethering

In contrast to the segregation of mitotic chromosomes, which is orchestrated by spindle microtubules that attach to the centromeres, ecDNA maintenance should involve mechanisms for retaining acentric ecDNA in the nucleus during and after open mitosis when the nuclear envelope completely disassembles[10]. Using a combination of light, electron and contemporary fluorescent microscopy techniques, researchers have consistently observed a close spatial association between ecDNA and chromosomes during the mitotic phase even after the nuclear membrane has been completely broken down[10,15,18,82–85]. This is often described as ecDNA tethering, but has also been termed mitotic hitchhiking (Fig. 3).

It is often assumed that ecDNA tethering is critical for maintaining ecDNA by preventing its loss even when it cannot associate with the mitotic spindle apparatus when a functional centromere is absent[83]. Yet the precise molecular nature of the tether remains unknown. Electron microscopy experiments have revealed that chromatin fibres interact with ecDNA and chromosomes, suggesting a direct interaction between ecDNA and chromosomes, possibly involving DNA or RNA[84,85]. However, whether proteins also contribute to ecDNA tethering remains unknown.

In a closely related field focusing on the biology of acentric chromosomes, two recent studies have explored the inheritance of acentric fragments resulting from chromothripsis in mammalian cells[86,87]. These studies have independently highlighted the role of DNA repair proteins DNA topoisomerase 2-binding protein 1 (TOPBP1) and cellular inhibitor of protein phosphatase 2A (CIP2A) in facilitating the tethering of acentric chromosomes to the main chromosomes during mitosis[86,87]. Disruption of CIP2A–TOPBP1 leads to the dispersion of acentric fragments during mitosis, followed by aberrant accumulation of cytoplasmic DNA[86,87], suggesting that DNA repair proteins aid in mitotic tethering of acentric DNA fragments. However, whether DNA repair proteins also have a role in ecDNA tethering has not been explored. Given that acentric fragments resulting from chromothripsis tend to be significantly damaged, it is plausible that TOPBP1 and CIP2A may primarily contribute to tethering of damaged acentric DNA and, thus, not interact with undamaged ecDNA. Further research is required.

In other episomal systems that associate with mitotic chromosomes to facilitate maintenance[88], such as polyomavirus SV40 (ref. 89), bovine papillomavirus (BPV)[90] or Epstein–Barr virus (EBV)[91], various

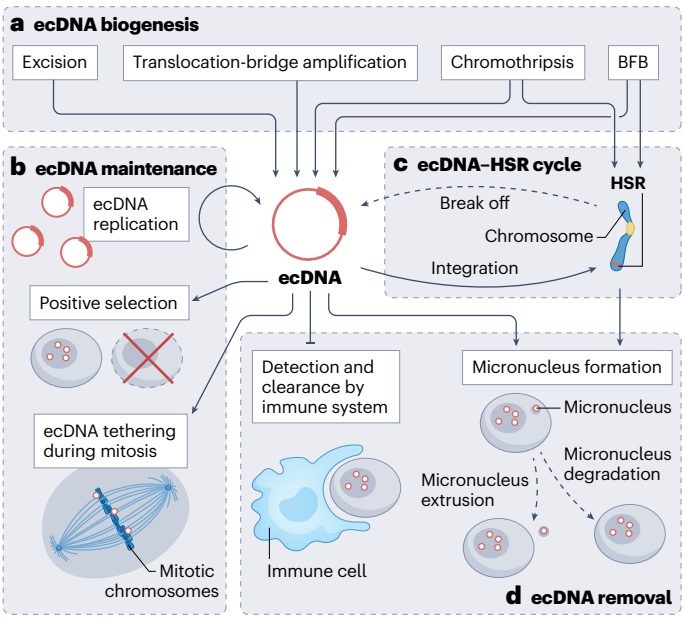

Fig. 3 | Life cycle of ecDNA. The relationship between extrachromosomal DNA (ecDNA) biogenesis, maintenance, removal and conversion with homogeneously staining regions (HSRs) is depicted. **a**, Various mechanisms are suggested to promote ecDNA biogenesis, including excision, translocation-bridge amplification, chromothripsis and breakage–fusion–bridge (BFB) cycle (further outlined in Fig. 2). **b**, ecDNA is maintained through active replication, through positive selection for traits improving cellular fitness, and by tethering itself to mitotic chromosomes during cell division. **c**, It is hypothesized that an ecDNA–HSR cycle may exist; repeated sequences of HSRs are hypothesized to recombine to form ecDNA, and that concomitantly ecDNA could reintegrate back into chromosomes to create HSRs. **d**, Additionally, ecDNA can potentially be removed through cellular clearance by the host immune system or through the formation of micronuclei which could be extruded or degraded. A dashed line is used here to indicate a lack of direct evidence.

proteins, SV40 large T-antigen, E1 and E2 proteins, and EBV nuclear antigen 1 (EBNA1), respectively, have been identified as crucial components of the tethering process[86,90]. Intriguingly, these bridging proteins are viral proteins encoded by the DNA viruses themselves, rather than being derived from the host genome[88]. This observation suggests that the human genome may not inherently encode proteins for bridging acentric DNA with mitotic chromosomes and may, therefore, need to repurpose specific cellular machinery. However, whether proteins contribute to ecDNA tethering remains an unanswered question.

Integration

As previously mentioned, ecDNA might originate from HSR. It is worth noting that the relationship between ecDNA and HSR can be dynamic and bidirectional. There is evidence to suggest that the reverse process can also occur, wherein ecDNA reintegrates back into the host genome, leading to the formation of HSR[22,25,92] (Fig. 3). For instance, in the human colorectal cancer cell line Colo 320DM, the primary cultures initially contained cells with ecDNA but no HSR; however, after continued passage in vitro, cells with HSR were observed[25]. Most recently, a study has analysed different structural variants in gastric cancer and has identified ecDNA as a driver of oncogene amplification[93]. In this study, numerous structural variants consistent with ecDNA reintegration were identified[93]. These events were characterized by features such as the presence of the amplicon edges matching ecDNA junctions and the manifestation of ultra-large structural variants that corresponded to reintegrated sequences located at a considerable distance from their original chromosomal coordinate[93]. This highlights the complexity of ecDNA dynamics and its potential to reintegrate into the genome, contributing to genetic alterations in cancer cells.

DNA damage appears to also have a pivotal role in triggering ecDNA integration processes[51,94]. Early research in the ecDNA field demonstrated that exogenous plasmids containing replication initiation regions and matrix attachment regions have the capability to integrate into existing ecDNA or induce de novo generation of HSR through reintegration events[95]. It was found that DNA damage resulting from conflicts between replication and transcription processes is a significant trigger for HSR formation in this system[94]. Notably, engineering the plasmids to prevent collisions between replication and transcription machineries completely eliminated HSR formation[94]. More recently, through the utilization of ionizing radiation, doxorubicin treatment or CRISPR–Cas9 to randomly induce DSBs in HeLa cells[51], ectopic HSR formation was observed in approximately 30% of all cells studied[51]. Interestingly, treatment with a PARP inhibitor, but not a DNA-dependent protein kinase catalytic subunit (DNA-PKcs) inhibitor, significantly increased the frequency of ectopic HSR formation (up to 78%)[51], suggesting that PARP suppresses ecDNA integration. These findings underscore the intricate relationship between DNA repair mechanisms and the integration of ecDNA into the genome.

So far, two frequent integration preferences have been identified, and one of them is the tendency of ecDNA to integrate in sub-telomeric regions elucidated by cytogenic examination and Hi-C sequencing techniques[13,24,30,51] (Table 1). However, the underlying rationale for this preference remains enigmatic. Despite concerted efforts to identify shared characteristics among these insertion sites through bioinformatic analysis, no consistent patterns or commonalities have emerged thus far[30]. Recent investigations have illuminated the potential role of DNA damage in influencing ecDNA integration[51]. It has been observed that ecDNA integrates at specific damage sites induced through CRISPR–Cas9-mediated DSBs in approximately 17% of the studied cells[51]. This intriguing finding suggests a possible link between ecDNA integration and genomic regions susceptible to DNA damage. Because sub-telomeric regions are prone to DNA damage through replication fork stalling[96], it is possible that DNA damage might also contribute to the terminal integration of ecDNA.

The second integration tendency of ecDNA is to insert itself into or near coding sequences within the genome. An in-depth WGS analysis of potential ecDNA integration sites in neuroblastoma has unveiled a striking enrichment of ecDNA integration into or near genes with direct relevance to cancer[40]. For instance, the integration of ecDNA into doublecortin like kinase 1 (*DCLK1*) disrupts its coding sequence and, thus, downregulates its expression[40]. This aligns with the role of *DCLK1* in neuroblastoma, wherein its expression levels exhibit an inverse correlation with patient prognosis[40]. Additionally, the integration of ecDNA in proximity to the oncogene telomere reverse transcriptase (*TERT*) has been associated with an upregulation of *TERT* expression[40]. This effect is probably mediated through the rewiring of the local regulatory network, facilitated by the provision of new regulatory elements, including enhancers from inserted ecDNA. Although the observed enrichment of cancer-related genes may be attributed to post-integration fitness selection, the possibility that ecDNA integration-mediated genome reorganization serves as a mechanism to either repress tumour-suppressor genes or activate oncogenes remains, potentially contributing to tumorigenesis. Nonetheless, it is crucial to emphasize that further experimental evidence is required to substantiate this intriguing hypothesis.

Removal

A fundamental aspect of ecDNA biology involves understanding mechanisms driving its elimination (Fig. 3). ecDNA could either be removed from individual cells, or cells that harbour ecDNA could be removed.

This first approach focuses on reducing the copy number of ecDNA within cells. DNA damage appears to have a significant role in the rapid loss of ecDNA, particularly following the administration of low-dose cytotoxic drugs such as hydroxyurea and gemcitabine[97–99]. In this context, the formation of micronuclei has been posited as a potential mechanism facilitating the removal of ecDNA[97–99]. This is substantiated by observation of reduced ecDNA copy numbers and increased micronuclei formation in human colorectal and ovarian cancer cells[97,98]. Although it has been suggested that micronuclei may either undergo degradation or be extruded entirely from the cells, the precise mechanisms responsible for ecDNA removal through micronuclei in cancer cells containing ecDNA remain to be fully elucidated[100].

The second approach necessitates the collaboration of the immune system to detect and subsequently eliminate cells containing ecDNA (Fig. 3). According to the analysis of WGS and gene expression information from 1,684 patient samples across various cancers, the presence of ecDNA in cancer cells is positively correlated with tumour immune evasion[101]. One potential mechanism explaining this association is the reduced expression of both major histocompatibility complex I (MHC-I) and MHC-II antigen presentation genes in tumours with ecDNA[101]. Notably, a recent case study has unveiled the identification of ecDNAs encoding immunomodulatory proteins, such as suppressor of cytokine signalling 1 (SOCS1), suggesting a direct role for ecDNA in immune escape[17]. Presently, research in this area largely relies on correlation studies using data from high-throughput sequencing[17,101]. The precise mechanisms governing the interplay between ecDNA-containing cancer cells and the host immune system remain unclear, and mechanistic studies are constrained by the absence of suitable in vivo model systems that

have both a functional immune system and have been screened for the presence of ecDNA. The establishment of such an in vivo ecDNA model system would be of great importance.

Perspectives and conclusions

The application of modern molecular techniques that shed light on the spatial organization of cancer genomes has led to a paradigm shift in cancer biology, suggesting that ecDNA is a fundamental driver of altered cancer genomes. ecDNA is able to explain inheritance of genes that in some cancers could not be explained by existing models of clonal evolution. It further explains the variation of genetic heterogeneity across tumours, how that heterogeneity is maintained, and why they are able to rapidly resist treatment. Current research is actively underway to try to better understand the mechanisms for this.

Future studies will be needed to better understand the intrinsic and environmental contexts that may contribute to ecDNA formation and maintenance. What are the relationships between cell lineage and ecDNA content? What is the role of DNA damage and DNA damage response in ecDNA formation, propagation and evolution? Considerable data suggests that it may happen in different ways in cancer cells from different origins[30,56,58,63,94,97–99]. Moreover, whether ecDNA particles are inherited purely by hitchhiking on chromosomes during cell division or whether other mechanisms are required remains largely unknown. Utilizing advanced live cell imaging techniques in future investigations will be essential for addressing these questions and ultimately developing treatments for ecDNA-containing cancers.

Data showing that ecDNA architecture affects gene regulatory processes both in *cis* and in *trans*[4,14,18,19,38,43] force us to rethink our assumptions about oncogenic transcription. Further studies are needed to better understand these processes and, thus, to identify potential actionable therapeutic strategies. Given that ecDNAs also appear to suppress the immune system[101], understanding the specific mechanisms by which this occurs could be of therapeutic value. Moreover, the presence of immunomodulatory elements on ecDNA indicates that certain genes can be selected to create a tumour permissive environment[17]. This also opens the possibility to include immunotherapeutics to re-engage the immune system as a weapon against ecDNA-containing tumours.

The relative paucity of longitudinal studies from pre-cancer to cancer for which WGS is available, together with the challenges of interpreting clonality in the face of non-Mendelian genetics, has made it difficult to determine whether ecDNAs might be involved in cancerous transformation or whether they are largely a later manifestation of genome instability. Although the aforementioned study of Barrett oesophagus suggested that ecDNA might contribute to the transition from pre-cancer to cancer[17], a broader examination of the role of ecDNA in pre-cancers is necessary. This is particularly important for the purpose of using ecDNA detection as a new diagnostic approach and stratification metric when ecDNA targeted treatments become available. Addressing this will also require new tools that enable detection of ecDNA in clinical samples, including blood, so that patients with cancer can be stratified for ecDNA targeted therapies and monitored for the effectiveness of these treatments. It is also interesting to note that in virally induced cancers such as HPV, positive head and neck cancer, and cervical cancer, the formation of hybrid viral–human ecDNAs can be found[102,103]. This implies a potential close relationship between ecDNA and viral infection, raising important questions about whether viral infection facilitates ecDNA generation and about the role ecDNA plays in virally induced cancers.

Lastly, researchers are now identifying actionable ecDNA targets and drugs that may more effectively treat ecDNA-driven cancers. Initially released from a conference abstract, a CRISPR genetic screen, using a methotrexate-induced ecDNA amplification model in HeLa cancer cells, has successfully identified checkpoint kinase 1 (*CHK1*) as an essential target for the survival of ecDNA-dependent cancer cells[104]. This has also been confirmed in other ecDNA-containing cell lines, including Colo 320DM[104]. Consequently, a small-molecule *CHK1* inhibitor has been developed and is now the first orally available ecDNA directed therapy, BBI-335, to be in phase I clinical trial (NCT05827614)[105]. The safety profile is being tested in various ecDNA-containing cancers, including liposarcoma and lung, head and neck, oesophageal, gastric, breast, bladder, ovarian and endometrial cancers[105]. Moreover, various therapeutic strategies targeting ecDNA are actively under investigation. These strategies include the development of drugs specifically designed to target the spatial and structural characteristics of ecDNA, with the goal of reducing oncogene transcription. Additionally, there is an emphasis on counteracting the broad immunosuppressive effects associated with ecDNA-containing cancers. A collaborative endeavour spanning diverse disciplines, such as cancer biology, genetics, bioinformatics, chemical biology and clinical oncology, is imperative to further our understanding of ecDNA. This interdisciplinary effort is vital for the development of more innovative therapeutic strategies. Considering the prevalence and crucial roles of ecDNA in various cancers, therapeutically targeting it holds great promise. This may pave the way for the success of next-generation cancer therapies by developing more ecDNA-specific drugs to treat a diverse range of cancers.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

H.C. is a co-founder of Accent Therapeutics, Boundless Bio, Cartography Biosciences and Orbital Therapeutics, and is an adviser to 10x Genomics, Arsenal Biosciences, Chroma Medicine and Spring Discovery. P.M. is a co-founder of, chairs the scientific advisory board (SAB) of and has equity interest in Boundless Bio. P.M. is also an adviser with equity for Asteroid Therapeutics. X.Y declares no competing interests.

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