Demystifying extrachromosomal DNA circles: Categories, biogenesis, and cancer therapeutics

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\section*{Abstract}
Since the advent of sequencing technologies in the 1990s, researchers have focused on the association between aberrations in chromosomal DNA and disease. However, not all forms of the DNA are linear and chromosomal. Extrachromosomal circular DNAs (eccDNAs) are double-stranded, closed-circled DNA constructs free from the chromosome that reside in the nuclei. Although widely overlooked, the eccDNAs have recently gained attention for their potential roles in physiological response, intratumoral heterogeneity and cancer therapeutics. In this review, we summarize the history, classifications, biogenesis, and highlight recent progresses on the emerging topic of eccDNAs and comment on their potential application as biomarkers in clinical settings.

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1. Introduction

The ring/disk shaped atypical chromosomes in Crepis tectorum [1] and maize[2] have long been reported in the early 1930’s. Unlike ordinary rod-like chromosomes, these chromosomal derived extrachromosomal circular DNAs (eccDNAs) were modified in their organization and number in different cells, yielding in a typical variegation. Several decades later, Yasuo Hotta and Alix Bassel discovered various sizes of eccDNAs in isolated wheat nuclei and boar sperm by sedimentation analysis and electron microscopy[3], which provided one of the early evidences to support Stahl’s idea that DNA might be circularized in higher organisms [4]. Contemporaneously, Cox et al. encountered various number of small double chromatin bodies neighboring intact chromosomes while karyotyping embryonic tumors and bronchial carcinoma tumor[5], which enhanced the credibility of the existence of small double fragments reported preceding in a primary lesion of medulloblastoma[6]. Although it was unrealizable to trace their origin to any chromosomes, Cox et al. suggested the acentric ring-like chromatin bodies were not caused by random chromosomal fragmentation[5]. The foremost discovery of eccDNAs was later recapitulated in several other organisms such as the fly[7], hamster[8], mice[9], yeast[10], roundworms[11], pigeons[12], and Arabidopsis[13], suggesting that eccDNAs are prevalent and likely influence cellular processes in eukaryotic cells. While eccDNAs have been identified in both normal and cancer cells, variations in their size distribution[14,15] and frequencies have been reported[16,17]. In general, circular structures as large as $10^4$-$10^7$ base pairs that carry oncogenes were rarely detected in normal tissues, whereas smaller structures such as small polydispersed circular DNAs (spcDNA) were found in both normal and cancer cells[16], albeit their amount was lower in healthy individuals. Previous attempts to identify and resolve the complex eccDNA elements were constrained by low throughput methods[18]. For example, while both electron microscopy and metaphase 4’,6-diamidino-2-phenylindole (DAPI) could recognize the intactness of these molecules, they were compensated for their low sensitivity and their inability to resolve molecular architecture. Recent advances in next-generation sequencing technologies and third-generation sequencing platforms have revolutionized the way researchers decipher the complex genetic landscape of eccDNAs. Using whole genome sequencing (WGS), cytogenetic and semi-automated image analyses, Turner et al. identified eccDNAs in approximately half of the 17 different cancer types tested, howbeit their frequencies varied based on the tumor types[19]. Similarly, more recently, Kumar et al. used chromatin accessibility assays (i.e. ATAC-seq) to discover thousands of eccDNAs in various cancer types, which were further validated by inverse PCR and metaphase fluorescence in situ hybridization (FISH)[20]. EccDNAs can also be identified by Circle-seq purification and enrichment[21] coupled with long-read sequencing technologies such as long-read Nanopore and single-molecule real-time sequencing (SMRT-seq)[22]. Further, another method called CRISPR-CATCH, which does not require DNA amplification to purify targeted megabase-sized eccDNAs, was invented to overcome limitations of Circle-seq (such as the need for intact DNA circles and the fragility of large eccDNAs)[23]. More recently, a third-generation sequencing technology-based method was developed to enable detection of eccDNAs at a single-cell whole-genome level[24]. Together, the use of parallel paired-end next-generation sequencing by these studies suggest that the architecture of eccDNAs are significantly more complex than previously considered.

1.1. Classification and biogenesis of extrachromosomal DNA species

EccDNAs are categorized into multiple groups depending on their size and sequence[25], the four[26] that are commonly found in cancer are described below and their method of detection has been summarized in Table 1.

### 1.1.1. Small polydispersed circular DNA (spcDNA)

Despite their discovery and isolation based on their buoyancy in alkaline solutions[27], these heterogeneously sized DNA species ranging from 0.2 to 2 μm remained unnamed until 1972, when their name was coined by Smith et al.[28]. Since then, various spcDNA of more than 0.5 μm or 1.5 kb have been identified using mica-press-adsorption for electron microscopy[29]. Although spcDNA have long been suggested to be associated with genomic

### Table 1

| Types       | First discovered | Size                      | Properties                                                                                      | Methods of detection                                                                 |
|-------------|------------------|---------------------------|-------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| spcDNA      | 1967             | 500bp-10kb (occasionally >10kb) | Resistant to denaturation, enhanced sedimentation velocity in neutral and alkaline solution, enhanced buoyant density in alkaline solution | Isolation: Buoyant-density method, e.g., CsCl containing ethidium bromide followed by centrifugation, Rolling circle amplification (RCA), e.g., Circle-Seq, Mobilome-Seq, CIDER-Seq |
| t-circle    | 1995             | Multiple units of 738bp    | Highly supercoiled                                                                                | Library types: WGS, ATAC, CRISPR-CATCH                                                |
| microDNA    | 2012             | 80-2000bp (>50% in 200-400bp) | Derived from nonrepetitive genomic sequences, enriched in 5’-UTR of genes, exons, and CpG islands | Imaging: Giemsa, acetoorcein, Feulgen, and DAPI staining, FISH, Electron microscopy     |
| eccDNA      | 1965 (as DM)     | $10^5$-$10^7$ bp          | Exists as pairs (DM) and singletons                                                              | Image processing: ECDetect, EGSq                                                        |
instability, their varied size and sequence content implies potentially different mechanisms of generation[17]. For example, preferential formation of spcDNA from Alu-rich regions in HeLa cells may be attributed to the juxtaposition of poly(A) sequence at the 5′ and the 3′ end of the Alu element[30]. Alternatively, spcDNA circularization could also result from the recombination mechanism. Homologous intrachromosomal recombination was proposed by Jones and Potter to explain the 9 bp direct repeats that occur in spcDNA[31]. Repeated circularization of multiple recombination events in the V_{α} and J_{α} regions of T-cell receptor α-chain could also explain the high copy number of spcDNA[32]. Whilst spcDNA are generated through hitherto unknown processes, mechanisms may exist that enable elements to loop out of the chromosomes and promote the joining of flanking DNA by illegitimate recombination[30].

1.1.2. Telomeric circles (t-circles)
In 1995, Nosek et al. reported the discovery of inverted terminal repeats that were made up of tandemly repeating units in yeast type 2 linear mitochondrial genomes (mtDNA)[33]. Taking Candida parapsilosis as an example, the terminus is comprised of a 738 bp repeating unit, with a 5′ single-stranded extension of about 110 nucleotides that is accessible to the enzymes. Later, Tomaska et al. used electrophoresis and electron microscope to reveal that the super-twisted circular conformation of these extragenomic molecules was derived from mitochondrial telomere repeats[34]. Although minicircular structures in mitochondrial DNA have been reported in several phylogenetically distinct species[39–43], their precise mechanism of generation and functions remains unexplored. While t-circles could be generated either by intramolecular recombination within the telomeric array or via telomeric loop extrusion, it was believed that t-circles maintain telomeric arrays of linear DNA through recombination. As DNA ends need to be protected against nucleolytic attacks and improper DNA metabolism, Tomaska et al. suggested that the various types of t-elements represent alternative strategies to tackle these obstacles[44]. For example, t-circles are believed to maintain telomeres integrity through alternative lengthening of telomeres (ALT) in 15 % of telomerase-negative cancers[45–47], thereby providing an alternative strategy for aberrant upregulation of cancer cell activity.

1.1.3. MicroDNA
A new form of eccDNA – microDNA was first found in 2012 in various tissues and cell lines[35]. Spanning 200 to 400 base pair long, these microDNA are enriched in 5′ UTR, exons, and CpG islands, and have a short region of micro-homology at the beginning and the end of the circles, suggesting the likelihood of microdeletions from the source genomic loci[35]. Unlike spcDNA, which typically span a few kilobase pairs and seem to originate from repetitive regions[7–9,48–50], microDNA originate from non-repetitive sequences, preferentially from high gene density areas. In 2015, Dillon et al. revealed microDNA were originated from DNA breaks or replication slippage following mismatch repair and loop excision[51]. The same study upon profiling microDNA from chicken DT40 cell lines lacking various crucial DNA repair proteins involved in non-homologous end joining (NHEJ), homologous recombination (HR), and microhomology-mediated end-joining (MMEJ), observed that microDNA were produced by all proteins involved in non-homologous end joining. This results in mature microRNA (miRNA) and repressed endogenous targets[52]. Since microDNA that carry miRNA genes are functional, and miRNAs in turn are indispensable to animal development, cell differentiation and homeostasis[53], and tumor progression[54], it is likely that microDNA are key regulators of biological processes.

1.1.4. Extrachromosomal DNA (ecDNA)
In 2017, Turner et al. introduced yet another new type of eccDNA – ecDNA, a mega base pair amplified circular DNA that is visible in optical microscopy[19]. Accordingly, multiple image-based analysis tools were developed to identify ecDNA from DAPI-stained metaphases[19,38]. Unlike other ecDNA, ecDNA are almost never found in normal cells, but are large enough to carry driver oncogenes[19]. A subsequent study using AmpliConArchitect found that oncogenes amplified on ecDNA had higher transcripts compared to when the same genes were not amplified on ecDNA, even after normalization of their copy numbers, suggesting alterations to the genetic structure, such as enhanced chromatin accessibility[55]. Although the underlying mechanisms of ecDNA biogenesis are not yet fully elucidated, four models have been proposed, including breakage-fusion-bridge cycle, translocation-excision-deletion-amplification, episome, and chromothripsis. Details of these four models are further examined in the next section.

1.1.5. Breakage-fusion-bridge cycle
This concept was first introduced in 1951 by McClintock while studying the mechanisms responsible for mutable loci in maize[56]. The breakage-fusion-bridge cycle is initiated when newly broken ends of chromosomes at a meiotic mitosis cause the fusion between sister chromatids (Fig. 1A), resulting in a bridge configuration followed by separation of centromeres of the dicentric chromatid[56]. Because the break could occur anywhere between the two centromeres and the cycle continues in successive mitoses during development, breakage-fusion-bridge cycle results in extensive DNA ladder-like focal amplifications and large deletions, and potentially the formation of double minutes (DM)[57–59], a term often used in early studies to describe extrachromosomal structures.

1.1.6. Translocation-excision-deletion-amplification
Translocation and amplification are two important cytogenetic categories associated with tumor etiology[60]. The chromosomal translocations studied in various cancers suggested two routes to activate oncogenes: the activation of a proto-oncogene juxtaposed to a T-cell receptor gene or an immunoglobulin protein, and the creation of a fusion gene by the breaks of two coding regions[61]. DNA amplification is a frequent genetic abnormality in tumors, which are manifested as DM and homogeneously staining regions (HSR) as cytogenetic hallmarks[62]. In some cases, translocation could concert with amplification to promote tumorogenesis. In 1996, Barr et al. employed FISH, RT-PCR and Southern blot to show the amplification of PAX3-FKHR or PAX7-FKHR fusion genes in 20 % of fusion-positive alveolar rhabdomyosarcomas[60], substantiating a sequential process through which oncogenes were activated. Another study unraveled the mechanism of non-syntenic co-amplification of MYC and ATBF1 in a neuroblastoma cell line, which involved multiple double-stranded breaks accompanied by a reciprocal t(8;16) translocation and deletion near the breakpoints[63]. In line with other findings[64,65], extra replication or loop formation could result in a DM configuration (Fig. 1B).

1.1.7. Episome
The concept of episome was first introduced in 1987 when Carroll et al. found a subclone of T5 transformant gave rise to a CAD episome containing donated CAD genes[68]. Gel electrophoresis showed these extrachromosomal molecules were 250 to 300 kilo-
base pairs in size and were covalently closed. Like DM, these circular elements contain a functional origin of DNA replication and can replicate autonomously. To explore the episomal formation mechanism, Carroll et al. grew CAD episome containing T5 cells under nonselective conditions and found that the loss of episome was correlated to the loss of donated CAD genes, suggesting the formation of episome by corresponding chromosomal region deletion [67]. Two mechanisms were speculated by the authors, recombination model and recombination across the looped replication domains[67]. While the prediction of the first model implies a rereplicated chromatin strand forming a “loop” structure, the latter model involves recombination of donated chromosomal sequences of sufficient size bearing origins of replication[68]. Another study on MYC carrying a DM has shown the amplified region was deleted at 8q24 in 68 % of the cases, which favored the episome model[69]. Interestingly, breakage across replication bubbles at stalled forks could also result in ecDNA formation (Fig. 1C)[70,71]. Consequently, episomal amplification could result in palindromic amplifications[71], or tyrosine kinase activation by the fusion between NUP214 and ABL1, promoting the pathogenesis of T-cell acute lymphoblastic leukemia (T-ALL)[72].

1.1.8. Chromothripsis
A remarkable phenomenon whereby extensive genomic rearrangements occur in a single catastrophic event was termed as chromothripsis by Stephens et al. in 2011[73]. Although the prevailing cancer evolution dogma indicated gradual acquisition of driver mutations, which resulted in increasing malignancy[74], somatic mutation outbursts might be a one-time event which promotes cancer development[73]. Importantly, chromothripsis could facilitate ecDNA generation (Fig. 1D)[73,75,76]. For example, the MYC containing DM was found to be generated by the shattering of chromosome 8 in a small cell lung cancer cell line[73]. While the mechanism for chromothripsis is unknown, a recent study showed that the process was dependent on poly(ADP-ribose) polymerases (PARP) and DNA-dependent protein kinase (DNA-PKcs) [77]. An error resulting in chromosome mis-segregation or intact chromatin bridge during the interphase could also pulverize the chromosomes[78], causing the scars in genome and DNA rearrangements, usually resulting in DNA circularization.

2. Functional characteristics of ecDNA
The diverse molecular and physiological functions of EccDNA summarized in Fig. 2 are described in detail this section.

2.1. Spatiotemporal dynamics of ecDNA
Antibiotic resistance (ABR) genes reside on mobile genetic elements (MGEs) such as plasmids, integrative and conjugative elements (ICEs), and various transposons[79]. Like plasmid DNA, ecDNAs carry critical genes that offer selective advantages in varying selective pressures (reviewed below in the section Adaptation of ecDNAs under therapeutic response). Since MGEs are mobile through various mechanisms, it is likely that ecDNAs are locomotive[80].

One aspect of ecDNA motility is its elimination by micronucleation[81–83], which was exemplified by the spontaneous extrusion of supernumerary MYCN amplified ecDNA into micronuclei [82]. It was shown that in a small percentage of cells, hybridization signals distributed peculiarly in clusters, adhered to nuclear membrane, and aggregated in nuclear protrusions[82,84], suggesting a spontaneous elimination process. The idea of an ecDNA hub was confirmed in a later study[85] where the authors labelled MYC ecDNAs with TetR-eGFP/TetR-eGFP(A206K) in COLO320-DM cells. Interestingly, treatment with 500 nM of a bromodomain and extraterminal (BET) protein inhibitor, JQ1, dispersed ecDNA hubs in COLO320-DM cells but did not alter the signal distribution of MYC in COLO320-HSR cells, implying the involvement of BET in hub maintenance[85]. Subsequently, Yi et al. established a CRISPR-based tracking technique which utilized sequences covering ecDNA-specific breakpoints to uncover disjointed ecDNA inheritance pattern during mitosis[86]. The authors found that the fluorescent signal was diluted during cellular cytoplasmic division and was reestablished once the two daughter cells entered the interphase; thereby providing direct visual evidence of the spatiotemporal dynamic feature of ecDNA[86].

Another aspect of ecDNA mobility is exemplified by the HIV-1 DNA integration into and disintegration from the host genome. 1-long terminal repeat (1-LTR) and 2-LTR circles are the two types of episomal HIV-1 DNAs that are particularly found in acutely infected cells such as the effector memory CD4+ T cells[87]. Although these elements contain sequences for viral replication, their functions remain unknown and were considered as by-products of the reverse transcription. A recent study suggested that 2-LTR circles could serve as reservoirs for proviral integration due to their palindromic junctions being recognized by integrase. Interestingly, the cleavage was specific and could be improved by the integrase cofactor LEDGF/p75[88]. It is likely that 2-LTR could serve as a main source of substrate for integration when its number surpasses that of linear DNA. For example, in the presence of...
a HIV integrase inhibitor raltegravir[88]. In a recent study, clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) was used to excised HIV proviral DNA in NL4-3/Luc-transduced 293 T cells[89]. Upon ablation, circular DNA with full-length LTRs formed via intermolecular, and sense-sense joining could be detected for up to 14 days. These concatemers upregulated integrase and p24 production upon pTat and pRev cotransfection, suggesting that they could be transcriptionally active[89].

Like retroviruses, retrotransposons also involve reverse transcribed eccDNAs as part of their lifecycles[90]; therefore, eccDNAs could potentially characterize the reservoir of active transposons [13,36,90,91]. Interestingly, transposon display revealed integration of ONSEN transposons into the genome in Arabidopsis under heat stress and drugs, suggesting eccDNAs may contribute to genome evolution[90].

2.2. EccDNA as a mobile regulatory element

Due to the absence of centromeres, eccDNA are subjected to loss during nuclear envelope break down. However, studies revealed that eccDNA were tethered to chromosomes, which enabled acen- tric eccDNA to be efficiently passed onto daughter cells during mitosis[92–94]. Interestingly, eccDNA were associated with the periphery of prometaphase chromosome rosettes and were localized far away from the spindle poles, suggesting their dependence on antipolar forces[93]. Although the mechanisms of eccDNA-chromosomal adherence remains unclear, a study on the interac-

tion between the origin of plasmid replication (oriP) and the viral protein EBNA-1 in Epstein-Barr virus (EBV) offered an unique insight[95]. The trans-activator protein EBNA-1 interacted with oriP and was thought to facilitate the anchorage of viral genomes on cellular chromosomes[96]. Analogously, Baiker et al. have shown the interaction between the origin of replication in simian virus 40 (SV40) genome, which attached to scaffold/matrix attachment region (S/MAR), and the chromosome scaffold, providing a mechanistic explanation of episomal stability and retention[97].

Although the idea that eccDNA might interact with chromosomes or with each other is not new[17,98], it was speculative and no concrete evidence was revealed. Recently, a model proposed ecDNA functioned as mobile regulatory elements that promoted the activity of chromosomal genes[99]. In this study, the authors performed Hi-C and RNAPII-associated ChIA-PET analysis on multiple GBM-patient-derived neurospheres and found ecDNA broadly contact the whole genome. The enrichment of transchromosomal interaction frequencies (nTIF) compared to the average genome-wide nTIF at 50 kb resolution was still significant after adjusting for the copy number. Furthermore, by comparing Histone H3 Lysine 27 acetyl (H3K27ac) peaks detected in the interacting loci on the ecDNAs, their chromosomal partners, and the genome-wide regions not contacting with ecDNAs, the authors showed ecDNA-chromosome interactions were associated with transcriptionally active sites[99]. Multiple lines of evidence have also corroborated the ecDNA-chromosomal interactome including: the significantly higher RNA transcription of chromosomal genes contacting ecDNAs, multi-color FISH validation, the higher interac-
tion frequencies mediated from ecDNA by comparing adjusted nTIF of subsampled regions on ecDNA and chromosomes with matched H3K27ac fold enrichment, the higher RNA expression level of genes connecting with ecDNA but with comparable RNAPII binding enrichment than those without connections. Although the dynamics of eccDNA diffusion and the stability of trans-interaction remain unknown, ecDNA could potentially act as mobile enhancers (Fig. 3) that greatly expand the transcriptional plasticity of a cell population[100].

2.3. Epigenetic landscape of eccDNAs

Although oncogene bearing eccDNAs could facilitate gene over-expression by merely increasing their copy number[19], the amount of DNA template was not the only factor that contributed to gene transcription[55 101 102,103]. Wu et al. integrated ATAC-seq profile with WGS data and found that the ATAC-seq signal was significantly higher in circular amplicons even after normalization of the DNA copy number[55]. In addition to the less compacted nucleosomal organization, co-amplification of the proximal enhancer and hijacking of the ectopic enhancer into highly rearranged MYCN amplified ecDNA have also been reported[101]. This finding was in line with an earlier study that found significant co-amplification of non-coding DNA beyond amplified oncogenes on ecDNA across several tumor types[102]. Interestingly, a recent study showed guide RNAs targeting an intergenic region near MYC- amplified ecDNAs significantly impaired cell growth[104]. Together these studies highlight new mechanisms by which ecDNA contribute to cancer progression.

2.4. EccDNA association with immune response

A recent study suggested that eccDNAs could function as potential innate immunostimulants in a manner which was dependent on the circular structure but not the underlying sequence[105]. The authors generated bone marrow-derived dendritic cells and bone marrow-derived macrophages, and compared their immune response (i.e. production of IFNα, IFNβ, IL-6, and TNF) between linear genomic DNA, eccDNA, and poly(dG:dC). Surprisingly, all aforementioned cytokines were significantly generated by eccDNA compared to linear DNA at varying concentrations. Similarly, these cytokines were significantly upregulated by eccDNA compared to poly(dG:dC) at lower concentrations, implicating the potency of the circular DNA on immune response. Furthermore, generation of linearized eccDNA by introducing one nick per circular DNA revealed that these linearized eccDNA behaved like linear DNAs and failed to activate cytokines; thus supporting the strong immunostimulant activity of eccDNA[105] and their potential induction of primary B cell and T helper type 2 responses[106].

Circularization of viral DNA by NHEJ pathway, on the other hand, has been suggested to alleviate the apoptotic effect in retroviral infected cells, albeit without excluding indirect mechanisms.

Fig. 3. EccDNAs are mobile. A. DMs entrapped in micronuclei could be eliminated upon hydroxyurea treatment, suggesting opportunities to improve chemotherapeutic regimen. B. Clusters of eccDNAs could form a hub which promotes intermolecular interactions. BET protein such as BRD4 facilitates such interaction, which could be disrupted by the BET inhibitor JQ1. C. Proviral DNA circles could be used as substrates for host genome integration. Upon CRISPR/Cas9 ablation, DNA circles with LTR are accumulated. D. EccDNAs are associated with the telomeric regions of chromosome rosettes at the onset of anaphase, and the tethering process may mimic viral vector behavior. E. EccDNAs could function as mobile enhancers that globally amplify chromosomal transcription. On the contrary, synthetic eccDNAs not carrying enhancer regions do not increase genome-wide RNA expression. F. The loss of EGFRvIII carrying DMs are associated with EGFR TKI resistance, suggesting eccDNAs’ adaptation under environmental pressure.
for suppression[107]. Although unintegrated viral DNA expression could not downregulate human leukocyte antigen complex on resting CD4+ T cells, it sensitized infected cells to be targeted and killed by functional cytotoxic T cells[108]. Moreover, episomal HIV-1 DNA has been shown to be transcriptionally active, and could generate functional viral proteins such as Tat and Nef[109–112], which may contribute to the CC-chemokine production in infected macrophages and the recruitment of lymphocytes[113].

2.5. Adaptation of eccDNAs under therapeutic response

The capability of eccDNAs to dynamically regulate themselves under environmental pressures is well known. In 1979, Kaufman et al. observed the unstably amplified dihydrofolate reductase gene (DHFR) on DM in higher concentrations of methotrexate[114]. As DHFR converts dihydrofollic acid to tetrahydrofollic acid, which is indispensable for synthesizing purines and pyrimidines, the presence of DHFR-carrying DM may partly explain methotrexate resistance. Similarly, later studies also identified an association between amplified DHFR and small circular DM in various cell types[104,115–117]. Surprisingly, DHFR was altered spontaneously and exhibited a 270-fold reduction in binding affinity for methotrexate[115], which further contributed to the inhibitor insensitivity by reducing the enzymatic activity. When methotrexate was absent, the growth rate of methotrexate-resistant murine S-180 cell was inversely correlated to the copy number of DHFR carrying DM[118], implying that the cells lacking DHFR amplification had a growth advantage when the drug was removed, and were likely selected during uneven segregation. Interestingly, the resistant S-180 line lost most of its DM after continued selection in methotrexate-containing medium and acquired DHFR genes on a few chromosomes[118]. This phenomenon was also reported in other studies[119–121], which could be revealed by Giemsa-banding.

In addition to the association between DHFR carrying eccDNA and antifolate resistance, the loss of oncogene bearing eccDNA was also correlated with the substantial reduction of tumorigenicity in several human tumor cell lines[81]. Von Hoff et al. showed that the treatment of a low concentration of hydroxyurea on HL60, COLO 320, NB4, and SF188 cells promoted the loss of MYC amplified DM, which is involved in the entrapment of DM within the micronuclei[81]. In a clinical trial conducted in 2001, researchers investigated whether a low dosage of hydroxyurea could down-grade DMs in 16 patients with advanced ovarian carcinomas[122]. Results revealed that 45 % of the patients showed more than 50 % reduction of the number of spreads with DM containing tumor cells, while one patient demonstrated 52 % reduction of the c-myc copy number after hydroxyurea treatment[122]. Similar mechanism of eccDNA extrusion was also proposed in another study[123], where 2 Gy fractions up to a total radiation dose of 28 Gy resulted in the reduction of MDR1 and MYCC bearing eccDNA via entrapment in micronuclei in multidrug-resistant lines.

Other chemotherapeutic agents have also been reported to regulate gene amplifications on eccDNAs. For example, lower levels of mitoxantrone induced ABCG2 amplification via DM in the SF295 glioblastoma cell line[124]. Gemcitabine at a 7500X lower concentration of hydroxyurea effectively reduced DM in an ovarian cancer cell line by incorporating the ampiclons and γH2AX signals into micronuclei[125]. The sensitivity of cisplatin induced apoptosis could be reversed by introducing antisense oligonucleotides targeting against MDM2 mRNA in human glioblastoma cells[126]. As the development of resistance to chemotherapeutic drugs is considered as one of the reasons for the failure of cancer treatment, and both DM and HSR were shown as cytogenetic manifestations in cancer cells, studying the role of eccDNAs in chemotherapeutic drug response may offer mechanistic insights into acquired resistance.

Targeted cancer therapies that interfere with tumor cell growth by interacting with specific molecules have shown some promising results as evident from the high response rates demonstrated by patients on these regimens. However, the prolonged benefit of majority of these therapies are limited by the eventual development of resistance of the tumor cells[127–129]. Nathanson et al. have found that resistance to erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), in glioblastoma was conferred by the reversible loss of eccDNAs containing the active oncogenic variant EGFRVIII, which bestowed an optimal cellular state for growth and survival[130]. This finding was later recapitulated in another study[104]. Interestingly, EGFR+ HSRs were observed throughout the entire stages of naïve, drug resistant, and drug retraction, whereas EGFR+ eccDNAs were only completely lost in erlotinib-resistant GBM cells, suggesting an adaptive route by which tumors can evade targeted therapy. Similarly, Song et al. recently showed that BRAF amplification also causes challenges to targeted therapy dosages[131]. The authors developed a melanoma model of dual MAPK inhibitor (MAPKi; specifically, vemurafenib [BRAFi] and selumetinib [MEKi]) resistance that bore BRAFV600 amplifications either through DM or HSR. They found that drug-resistant plasticity was coupled with focal amplifications, and that inconstant drug dosage prevented the switch from DM to HSR. Moreover, a different form of cell death, ferropotosis, occurred during BRAF amplification mediated MAPKi resistance, extending the resistant mechanisms beyond cellular dedifferentiation[131].

2.6. EccDNA confer resistance to herbicide

EccDNA dynamics has also been suggested to promote plant evolution. Koo et al. reported that eccDNA based EPSPS amplification was associated with rapid glyphosate resistance in the crop weed Amaranthus palmeri through adaptive evolution[132]. Interestingly, a sexual transmission study that crossed a female A. palmeri lacking eccDNA and a male A. palmeri carrying eccDNA showed positive signals associated with mitotic metaphase chromosomes in the descendants, indicating the successful transmission of herbicide resistance to the offspring via eccDNA.

2.7. EccDNA and aging

Recently, Hull et al. demonstrated the transcription of tandem CUP1 copies stimulated CUP1 encoding eccDNA in yeast that were aged under environmental exposure to copper[133], a process triggered by factors such as Sae2, Mre11 and Mus81 that are involved in DNA repair. In addition to facilitating adaptive evolution, eccDNAs accumulation in the nuclei may also promote ageing and tumorigenesis. Current hypothesis suggests the transport of damaged DNA via eccDNA out from the nucleus into the cytosol, where a cell-autonomous nucleic acids (NA) degradation machinery is triggered to keep NA below the immunostimulatory threshold[134]. However, during ageing, the increase of dysfunctional nuclear pore complexes (NPCs) may result in the accretion of eccDNA in the nucleus[135]. The consequent accumulation of DNA damage in turn promotes cellular senescence and apoptosis[136], further indicating a strong link between eccDNAs and ageing.

2.8. EccDNAs may potenti ate intercellular crosstalk

Circular RNAs (circRNAs), recently found to be amply supplied and stable in exosomes[137], were suggested to mediate intercellular crosstalk in the tumor microenvironment by involving cancer and stromal cells[138]. For example, hepatocellular carcinoma...
(HCC) cells with high metastatic capability could transfer their metastatic potentiality to other HCC cells with low or no metastatic capability by secreting exosomes with circPTGR1[139], exosomal circ–CACAC1 in cholangiocarcinoma was transmitted to endothelial cells, which promoted angiogenesis by downregulating junctional proteins[140]. Similarly, could eccDNA trapped micronuclei represent an entity facilitating intercellular network? Despite extracellular micronuclei with DM being reported in some studies[83,141,142], which suggested micronuclei could be expelled from the cell and serve as a repertoire of DNA elements, the impact on intercellular genetic communication remains unclear. Interestingly, the content of micronuclei could be shuttled into multivesicular bodies via direct contact[143]. Nevertheless, mitochondrial circular genome could not only be transferred through direct cell–cell contact[144,145], but also via circulating extracellular vesicles[146], suggesting circular DNAs as potent paracrine/endocrine signaling factors. However, as studies on eccDNAs as communicators between cells are limited and still speculative, additional are needed to determine this potential role of eccDNAs.

2.9. EccDNAs as potential biomarkers

Episomal HIV-1 DNAs were found in patients with advanced central nervous system damage[147], and patients on antiretroviral therapy (ART)[148–150]. Although the roles of these episodes are still debatable, a study comparing the envelop sequences in episomal and proviral genomes before viral rebound upon treatment interruption with those in emergent viral RNA[150] suggested that episomal HIV-1 could fuel viremia rebound. Moreover, episomal HIV-1 genomes could be used as a marker to monitor ART, due to its lability in vivo, and given that traditional methods were not sensitive in viral reservoirs detection[151].

A recent study using four paired primary and metastatic tissues of high grade serous ovarian cancer (HGSOC) highlighted the association between DNMT1 circle-10302690-10302961 downregulation and HGSOC metastasis[152], suggesting certain eccDNA element could be considered as a prognostic marker.

Liquid biopsy, which includes cell-free DNA (cfDNA), circulating tumor cells and exosomes, has made great progress in recent years due to its non-invasiveness, high concentration and low quantity in plasma[154], which suggested that cell-free microDNA present in tumor lung tissue specimens were longer than those in paired normal lung samples; moreover, serum and plasma samples collected prior to surgery were enriched with longer microDNA compared with that obtained from the same patients following surgical tumor resection[15]. Interestingly, the formation of eccDNAs was found to be dependent on the lineage of cancer[51].

Although unique eccDNAs were able to be identified in some diseases, characteristics such as high GC content, repetitiveness, and low quantity in plasma[154] may hamper their application in clinical setting by increasing the difficulty in primer design and detection. While common eccDNA detection methods involves RCA, which is an efficient isothermal DNA amplification procedure, the synthesis cost is high, and primers are still in need. Nevertheless, a recent study using a label-free fluorescent biosensor to detect circRNA provides an ultrasensitive alternative to identify eccDNA[155].

While circRNA was reported to degrade completely within 15 s in 25 % serum[156], the average half-life of fetal eccDNA in the maternal blood was found to be 29.7 min[157]. The half-lives of extracellular microRNAs (ex-miRNAs), on the other hand, varied between ex-miRNA entities and the species[158,159]. It is important to note that while the stability of ex-miRNAs were measured in cell culture[158,159], fetal eccDNA kinetics were determined from blood samples collected from pregnant women before delivery and at multiple time points postpartum[157]. Serial time point collection of blood was important as it correctly reflects the half-lives of eccDNA in the biological system. While long non-coding RNAs (IncRNAs) have drawn attention as molecular biomarker for cancer prognosis[160], their rate of decay has also only been measured in in vitro models[161,162]. While the half-lives of circRNA, eccDNA, ex-miRNA, IncRNA are yet to be directly compared from genome-wide analyses, based on eccDNAs’ size distribution, sequence, resistance to exonuclease or ribonuclease, and their stability compared to RNA, growing evidence indicates that eccDNAs could serve as potent biomarkers for disease surveillance.

2.10. EccDNA databases

To date, three eccDNA databases have been introduced (Fig. 4) [163–165], which compiled eccDNAs from different resources and are focused on diverse aspects of eccDNAs. While CircleBase,
the first integrated platform for eccDNA functional interpretation, comprises 601,036 eccDNAs collected from 13 papers[163], both eccDNAdb[164] and eccDB[165] profiled eccDNAs based on different library types and computational methods. To characterize potential eccDNA function, six modules comprising of various functional databases were used, and the diffusion algorithm PageRank was used to prioritize genes interacting with eccDNAs based on three of the modules[163]. While eccDNAdb focused on the prognostic value of eccDNA genes[164], eccDB complemented CircleBase database by incorporating eccDNA interchromosomal interactions and evolutionary relationships through multi-species sequence comparisons[165].

3. Summary and outlook

In this review, we summarize the current state of understanding of eccDNAs pertaining to their discovery, prevalence across multiple species and cancer types, classification and associated formation mechanisms, and physiological characteristics. Although the knowledge that eccDNAs contribute to cancer progression has been known for decades, gaps in our knowledge of eccDNAs remain and continue to be one of the intractable challenges faced by cancer researchers[166]. Although the overall frequency of DM in primary cancer has been reported to be 1.4% based on the Mitelman database[167,168], a recent study suggests its frequency is far greater than previously inferred[19].

1. Future research on eccDNA integration and dissociation is needed. Since sequence and structure could contribute to the selective fragility of the genome[169,170], what are specific features of the boundaries. Are there specific chromatin organization or underlying DNA sequences?

2. While the prominent mechanism of how eccDNAs promote evolution of cancer cells relies on competitive advantage offered by uneven segregation of oncogene amplified eccDNAs during mitosis, the detailed molecular mechanisms are only now beginning to emerge and will require detailed mechanistic studies[84,104,171–173]. An important contributor to this puzzle could be that eccDNA can behave like enhancer elements and may traverse the nucleus to enable global chromosomal contacts thus introducing transcriptional plasticity in a cell population. Colocalization of eccDNAs which form a hub and recruit RNA polymerase not only provides a plausible mechanism of high oncogene transcriptional rate and intratumoral heterogeneity, but also offers promising avenues for the eccDNA hub directed cancer therapies.

3. Another important outstanding question is if DMs replicate independently. While some studies suggested DMs replicate synchronously with chromosomal in S phase[174,175], others suggested they were derived de novo from HSR fragmentation[176]. Nevertheless, the separation of DM's sister elements during G1 phase prevents the formation of quadruple chromatids[177], and likely alleviate their numerical heterogeneity between cells which results from their anomalous segregation during cell cycle. The mechanism of separation is unclear because the chromatin fiber organization of DMs is currently unknown[177]. Furthermore, DMs attach to nucleolar matter near the end of chromosome arm during metaphase[92], but what force drives scattered DMs inside the cytoplasm in prophase to relocate to the chromosome ends in metaphase?

4. Finally, aside from the elucidation of the eccDNA replication mechanism during cellular division, more studies on eccDNAs’ roles in clonal dynamics, their targetable vulnerabilities, and their half-lives are needed to fully realize their translational value.

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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