Extrachromosomal circular DNA in cancer
history, current knowledge, and methods
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Extrachromosomal circular DNA (eccDNA) is a closed-circle, nuclear, nonplasmid DNA molecule found in all tested eukaryotes. eccDNA plays important roles in cancer pathogenesis, evolution of tumor heterogeneity, and therapeutic resistance. It is known under many names, including very large cancer-specific circular extrachromosomal DNA (ecDNA), which carries oncogenes and is often amplified in cancer cells. Our understanding of eccDNA has historically been limited and fragmented. To provide better a context of new and previous research on eccDNA, in this review we give an overview of the various names given to eccDNA at different times. We describe the different mechanisms for formation of eccDNA and the methods used to study eccDNA thus far. Finally, we explore the potential clinical value of eccDNA.

Healthy human somatic cells contain 23 pairs of chromosomes in the form of long, linear, condensable chromatin fibers. Besides the mitochondrial genes, our chromosomes contain all the genetic information needed for a cell to carry out all functions. During mitosis, all chromosomes are replicated once and the resulting sister chromatids are equally segregated, ensuring the formation of two genetically identical daughter cells.

This normally tightly regulated mechanism is often disrupted in the genomes of cancer cells. Cancers progress by a sequence of mutational events including nucleotide substitutions, translocations, and gene copy number gains or losses that result from an environment of genomic instability [1]. One of the most common genetic changes in tumorigenesis is oncogene copy number gains [2], leading to overexpression of oncogenic gene products, which provides the cancer cells with growth advantages. The mechanisms leading to oncogene amplification are not thoroughly understood, although it is widely acknowledged as an underlying cause of cancer development. A major challenge for current cancer therapies such as chemotherapy is the development of resistance to therapeutic drugs, ultimately leading to therapy failure. Therapeutic resistance depends on biological properties such as tumor heterogeneity, cell populations with stem-cell-like properties, regulation of the therapeutic target molecules’ expression, and activation of prosurvival pathways, which can all result from gene amplification [3].

Circularization of otherwise linear chromosomal DNA is one of the keys to understanding how gene amplifications arise. Circular DNA molecules, which are not plasmids, are found in the nuclei of all eukaryotic cells studied. This eccDNA has been described in the scientific literature under various names, which are covered below. eccDNA sequence content is homologous to the nuclear chromosomal DNA, which it derives from [4–9]. eccDNA can vary in size from less than 100 bp to several megabases, and can contain any element found in the human genome from small, noncoding regions to entire genes [7–9]. eccDNA was observed for the first time in boar sperm and wheat embryos in 1964 when Hotta and Bassel investigated using electron
microscopy the theory that chromosomes of higher organisms are made of DNA circles [10]. Later in the 1960s and 1970s, eccDNA was observed in filamentous fungi and yeast as well as birds and a variety of mammalian tissues, suggesting that eccDNA is a common phenomenon in eukaryotic cells [5]. The majority of eccDNA identified in these studies was too small (<500 bp) to contain whole protein-coding regions [11]. In cancer cells, much larger extrachromosomal DNA structures were discovered at approximately the same time through staining and light microscopic examination of metaphase chromosomes [12]. These structures were initially denoted double minutes (DMs) due to their small size (in relation to chromosomes) and distinct pairing in metaphase. DMs were large enough that their circular structures were observed by light microscopy [13]. Later, sequencing of the junction points supported the circularization relative to the linear chromosomal sequence [6].

Pioneering work from Wahl and others revealed how oncogenes are amplified on DMs in tumors [4]. The close link between eccDNA and most cancers has recently become even more clear. In an influential paper from 2017, it was revealed how a large proportion of tumors from different cancer types carry megabase-size eccDNA, specifically called ecDNA [8]. This was complemented by reports showing how ecDNA evolves in different cancers of neurological origin [14,15] and how tumors with ecDNA amplifications of some oncogenes are associated with higher mortality [16,17].

Although cancers can carry many eccDNAs in different sizes and with different genetic elements, according to some reports [7,16,18], most research has focused on the large ecDNAs that amplify genes, including oncogenes [4,8,14,19]. We still have little understanding of how mixtures of thousands of different eccDNAs arise in tumors and affect cancer progression, and whether eccDNA can be used as a cancer biomarker. In this review, we first give an overview of how eccDNA was discovered and named several times in different scientific contexts. We then describe the current knowledge of how eccDNA is generated and maintained in cells. We describe the methods used in the field and finally discuss the potential use of eccDNA as a marker for diagnosis, prognosis, and treatment of cancer.

**Nomenclature and definitions**

**eccDNA**

Historically, eccDNA has been isolated by a number of methods and in many different organisms and cell types, which has led to a number of different names. eccDNA was suggested as a term to cover all nuclear, extrachromosomal circular DNA of endogenous chromosomal origin in 1990 [5].

**Covaletly closed circular DNA**

In the earliest literature describing eccDNA, the term covalently closed circular DNA was sometimes used. This was used to describe all known double-stranded circular DNA including viral genomes, bacterial plasmids, mitochondrial DNA, and eccDNA [5,20], but the term is now mostly used in the field of virology.

**Small polydisperse circular DNA**

The name small polydisperse circular DNA (spcDNA) was first used to describe eccDNA isolated from HeLa cells by density separation from the chromosomal DNA and visualized by electron microscopy in 1972 [11]. spcDNA was used to describe eccDNA at the smaller end of the size spectrum (<100–10 000 bp) until the 2000s. The name comes from their heterogeneous size distribution and sequence content [5,11]. spcDNA was described as mainly containing repetitive genome sequences [5], although this could reflect the limited DNA sequence analysis methods available at the time rather than the true frequency of repeat sequences on spcDNA. It was found to be common in normal eukaryotic cells, but much more abundant in genetically unstable cells such as cancer cells and cells from patients with Fanconi’s anemia [21,22].
microDNA
The term microDNA arose in 2012 when small, circularized DNA were isolated from mouse and human cell lines by density purification [7]. The vast majority of these were determined to be between 200 and 3000 bp [23]. Thus, the terms microDNA and spcDNA cover circular DNA molecules with similar sizes and physical properties. Sequencing of microDNA revealed that they arise from all parts of the genome, though microDNA from the 5′ and 3′ termini of genes and regions with a higher GC content appears to be overrepresented when compared to the whole genome [7]. The functions of microDNA and spcDNA in eukaryotic cells are not well-elucidated. The small size of these DNA molecules makes them unable to carry full protein-coding gene sequences and promoter regions. A 2019 study found that microDNA can express functional small regulatory RNA, including microRNA (miRNA) and small interfering RNA [24]. The authors also found that microDNA molecules could be transcribed without a canonical promoter. These results suggest that microDNA can regulate gene expression through transcription of regulatory RNA. Although the formation of microDNA is common in healthy individuals, the length distribution of microDNAs varies in different sample types such as tissue, plasma, or cancer cell lines [9,25,26].

Telomeric circles
Telomeric circles are a specialized group of eccDNA that have been found to be important in immortalization of telomerase-negative cancers though the alternative lengthening of telomeres (ALT) mechanism [27]. Telomeric circles serve as templates for telomere elongation and the ALT mechanism is reported to be responsible for telomere maintenance in 10–15% of all cancers [28]. Telomeric circles are found in the form of t circles, which are fully double-stranded and contain telomeric repeats, or c circles, which have a partially single-stranded C-rich region. Electron microscopy has shown that the telomeric circles vary in size from 100 to 30 000 bp [29]. ALT has been observed in a variety of tumors including osteosarcoma, soft tissue sarcoma, glioblastoma multiforme (GBM), renal cell carcinoma, adrenocortical carcinoma, breast carcinoma, non-small cell lung carcinoma, and ovarian carcinoma [27].

DMs
Large eccDNA in the megabase range was first described by Cox et al. in 1965, when they examined metaphase spreads of chromosomes from childhood cancer cells by light microscopy and discovered small, paired chromatin bodies, which they named DMs [12]. DMs are a DNA species without recognizable telomeres and centromeres [4,30] and serve an important role in oncogene amplification and overexpression. They tend to accumulate in malignant tumor cells when they amplify genes that provide a growth advantage [4].

Episomes
In the 1980s, it was observed that tumor cells also contain autonomously replicating circular DNA in the submicroscopic size range. These could still be large enough to carry whole genes, and they were named episomes [31]. Episomes were isolated by denaturation and renaturation of DNA and visualized by gel electrophoresis. This work led to the development of an episome model in cancer genetics that states that episomes are formed by excision of linear DNA from chromosomes followed by circularization and amplification [4,32–34].

ecDNA
Next-generation sequencing (NGS)-based studies have in recent years allowed scientists to study the sequences of circular DNA from tumors in-depth. This also led to a new definition of mega-base-pair amplified circular DNA in cancer, namely ecDNA [8]. ecDNA with oncogenes is reported in a broad variety of tumors and in 46% of cell lines from 17 different cancer types.
ecDNA appears to be especially common in GBM and prostate, breast, lung, and renal carcinoma, as well as melanoma [8].

In the following text, we use eccDNA as the common term for all previously defined classes of eukaryotic, nonmitochondrial, and nonplasmid extrachromosomal circular DNA. ecDNA will be used as the term for the cancer-specific subset of large eccDNA with oncogenes, rather than DMs. Since small eccDNA and ecDNA are generally studied separately, many findings from the literature can only be considered valid for one of these subsets. In the following text, we have therefore chosen to differentiate between what is known about formation, functions and clinical relevance for small eccDNA and ecDNA, respectively.

**Formation and maintenance**

**Formation**

A number of models exist for how eccDNA is formed in human cells. They often involve damage to the chromosomal DNA and erroneous actions by different DNA repair pathways. For example, two double-strand breaks (DSBs) in the same chromosome can result in a stretch of DNA deleted, which could become circularized (Figure 1A), or secondary DNA loop structures formed in several processes, for example, mismatch repair (MMR) (see Glossary), could be excised and circularized [35–37]. Therefore, the mechanisms for eccDNA formation can be different depending on how and where chromatin is subjected to damage and which DNA repair mechanisms are active in a given cell.

In several studies, eccDNA were sequenced and their junctions were examined for evidence of which DNA repair mechanisms were likely to have generated them. Junctions that indicate where an eccDNA has formed can indicate whether they were formed by DNA repair mechanisms dependent on homology or not. eccDNA formed between regions with no homology is likely to have formed through nonhomologous end joining (NHEJ) (Figure 1A) [38], which has been observed in several studies of eccDNA and ecDNA in the eukaryotic model organism Saccharomyces cerevisiae (baker’s yeast) and human cancer cells [6,35,36,39,40]. To directly validate that eccDNA can be generated by DSBs, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 method was used to create two DSBs in the same chromosome. Subsequently, endogenous eccDNAs of various sizes were formed and the formation happened in regions without homology [41]. There are also reports of eccDNA forming between regions with high homology, potentially through homologous recombination (HR) [36,42–45]. The newer reports of this were all studies in S. cerevisiae, but this was also observed in early human cell studies as reviewed by Gaubatz in 1990 [5]. eccDNA formed by HR is expected to be rare, since HR is primarily active in mitosis and most healthy mammalian cells are postmitotic, where NHEJ is the primary repair mechanism for DSBs. The studies in S. cerevisiae indeed suggest that HR contributes only a minority of eccDNA, but that eccDNAs formed by HR form repeatedly from the same loci [36,42–45].

NGS-based studies of eccDNA may underestimate HR effects. This is because sequence reads from repetitive DNA are often filtered out in bioinformatics analysis because they are difficult to accurately map to the human genome. Human eccDNA from repetitive regions has been estimated in a few studies, after removal of all linear DNA. One study indicated that DNA repeat sequences are slightly more common in eccDNA compared with the whole human genome (72% of eccDNA to 52% of the genome) [46], while another indicated that they had occurrences of repeat elements at the same level as the whole genome [35].

A complementary approach to determine how eccDNA forms is to measure it in cells deficient in different DNA repair proteins. In two studies, deficiency of genes involved in MMR has been
shown to result in a decreased amount of eccDNA in chicken lymphoma and human cancer cells [35,37]. In the most recent of these, deficiency of genes involved in damaged DNA resection and microhomology-mediated end joining (MMEJ) also resulted in decreased eccDNA amounts [37]. In cells lacking proteins essential for NHEJ, both decreased and increased amounts of eccDNA have been reported [22,37,47]. These results might be explained by the different methods used for quantification or a context- or cell-type-dependent role of NHEJ. Thus, proteins from the DNA damage repair pathways MMR, NHEJ, and MMEJ, and DNA resection prior to repair are indicated to be involved in eccDNA formation at least in some cell types. In these studies, cells with defective HR, single-strand annealing repair, base excision repair, and nucleotide excision repair were found to have unchanged amounts of eccDNA compared to wild-type cells [35,37].

When profiling eccDNA in cancer cells, it has been observed that sometimes their size distribution displays clear peaks in a periodicity of approximately 200 bp [18,25]. This is reminiscent of the ladder pattern observed in linear DNA from apoptotic cells, which reflects the breakdown of chromosomal DNA into fragments corresponding to one or more nucleosomes. Recently, it was demonstrated that inducing apoptosis in mouse embryonic stem cells indeed massively increased their amounts of eccDNA [48]. When sequenced, the apoptosis-induced eccDNA displays the characteristic size distribution peaks and originates from genetic loci found evenly across the whole genome. The increase is dependent on the apoptotic enzymes DNase I and DNA ligase 3, confirming that apoptotically fragmented DNA is indeed converted to eccDNA.

In a study of telomeric circles, collapse of replication forks in telomeric DNA with single-strand breaks was shown to induce the formation of c circles [49]. In S. cerevisiae, re-replication and oligonucleotide-stimulated DNA amplification have also been suggested as sources of eccDNA [50,51], although none of these mechanisms has been tested in mammalian cells.

DNA damage is also suggested to be an initial step in the formation of ecDNA, while less is known about the pathways that lead to their circularization. Circularization could be mediated by the DNA repair pathways also involved in forming small eccDNA, as described previously. In some cases, ecDNA is suggested to arise by multistep processes, potentially from smaller precursors [32,40]. In others, large-scale DNA damage events have been proposed as the sources of ecDNA in cancer. Chromothripsis is one such single-step catastrophic event involving multiple DSBs, which in essence shatters a whole chromosome into small pieces. Repair of the chromosome is then attempted by error-prone DNA repair mechanisms, leading to many different genetic errors [52,53]. In several studies, chromothripsis was shown, directly or indirectly, to lead to ecDNA formation (Figure 1B). When human chromosomes were pulverized in a process similar to chromothripsis in human–rodent hybrid cells, new stable ecDNAs were generated [54]. Furthermore, analysis of whole-genome sequencing (WGS) data from human cancers and chemotherapy-resistant clonal

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**Figure 1.** Selected mechanisms by which eccDNA and ecDNA form. (A) Two DSBs in the same chromosome can lead to deletion of a stretch of DNA. This can be circularized by DNA repair mechanisms active in the cell. eccDNA formed can undergo further evolution; for example, fusion with other eccDNA to generate chimeric eccDNA (and ecDNA) consisting of noncontiguous DNA segments (compared to the chromosomal DNA). (B) Chromothripsis is the shattering of a whole chromosome into smaller pieces. Cellular DNA repair mechanisms will attempt to repair it, leading to many errors, including potentially forming ecDNA. (C) BFB cycles start with the loss of a telomere (breakage), which leads to aberrant fusion of the sister chromatids. The fused chromosome (bridge) is pulled apart in anaphase resulting in two unevenly sized chromosomes. Multiple rounds of this is known to generate HSRs in chromosomes (1). It is furthermore suggested to lead to formation of ecDNA; however, it is not clarified whether this is dependent on chromothripsis, which can also occur after repeated BFB cycles (2). Abbreviations: BFB, breakage–fusion–bridge; DSB, double-stranded break; eccDNA, extrachromosomal circular DNA; ecDNA, circular extrachromosomal DNA; HSR, homogeneously staining region.
cell lines have suggested that chromothripsis is an important driver of cancer genome rearrangements including ecDNA formation [55,56].

A breakage–fusion–bridge (BFB) cycle is another type of event known to lead to severe genetic aberrations in cancer cells. The BFB cycle starts with a DSB in a chromosome such that it loses a telomere. The chromatid ends lacking telomeres can fuse to form a dicentric chromosome. At anaphase, the dicentric chromosome is pulled apart to form a chromatid bridge that breaks and generates a variety of chromosomal aberrations, including ecDNA (Figure 1C) [2,56]. Although BFB cycles and chromothripsis have historically been considered separate types of events, recent studies have suggested that they are mechanistically linked, in the sense that chromothripsis can be caused by consecutive rounds of BFB cycles [56,57] (Figure 1B,C).

Evolution

Several studies suggest that eccDNA can evolve over time. This was first proposed by Wahl and coauthors [4,31]. This was also observed in several later studies, generally showing that smaller eccDNA can gradually increase in size over time by fusing with, for example, other eccDNA (Figure 1A). In several recent studies chimeric eccDNA, consisting of several noncontiguous chromosomal DNA segments, has been detected, which also supports an evolutionary process after the initial formation event [14–17,48,58].

The gradual fusing of smaller eccDNA has been suggested as a formation mechanism for ecDNA. Later in its evolution, ecDNA has been demonstrated to integrate into chromosomes to create regions of gene amplifications known as homogeneously staining regions (HSRs) [32,59,60].

Maintenance

Maintenance in cells has mainly been described for ecDNA. During mitosis, chromosomes are replicated once and then pulled apart at the centromeres by the mitotic spindle. This process ensures an equal distribution of chromosomes in the two daughter cells. ecDNA molecules have also been shown to replicate once per cell cycle [61], but unlike normal chromosomes and ring chromosomes, these elements lack recognizable centromeres [62], and they are thus not segregated evenly by the mitotic spindle. Their dynamic behavior during the segregation process has been studied in cell models, where it has been shown that they ‘hitch-hike’ during segregation by tethering to the ends of chromosomes furthest away from the mitotic spindle poles [63–65]. Their distribution in daughter cells after cell division is highly variable from cell to cell and has been suggested in separate studies to follow either a binomial random distribution or a Gaussian distribution [65,66].

This uneven segregation can lead to accumulation of ecDNA molecules with oncogenes in subpopulations of cells, accelerating cancer progression, and increasing tumor heterogeneity with daughter cells that contain increasingly amplified copy numbers of the oncogene [64,66] (Figure 2). Thus, some daughter cells can achieve survival and proliferation advantages as discussed further in Box 1. ecDNA amplification elevates oncogene copy number faster than chromosomal amplification [8], which can promote the further propagation of ecDNA. Similar observations have been made for S. cerevisiae, where maintenance of, for example, nutrient transporter genes on eccDNA provides selective advantages in nutrient limiting conditions [45]. Loss of ecDNA has been observed as a result of DNA damage, induced by, for example, hydroxyurea. They have been observed to aggregate in micronuclei and eventually be extruded from cells [67,68].

The mechanisms of replication, segregation, and loss of small eccDNA from cells are not well described. In one recent study, it was observed that cells had reduced eccDNA levels immediately
Methods for descriptive and functional studies on eccDNA

Methods from the pre-NGS era

In the first report describing eccDNA from 1965, eccDNA was isolated as a fraction of DNA using sedimentation in a sucrose gradient and the circular structure was visualized by electron microscopy [10]. This launched a new field and led to investigation in other mammals, including humans. Larger DMs were discovered using light microscopy by Cox et al. using human tumor biopsies [12]. Southern blotting showed that eccDNA was homologous to genomic DNA [5,69,70]. In situ hybridization and later fluorescence in situ hybridization (FISH) enabled scientists to distinguish extrachromosomally amplified oncogenes from those on chromosomal DNA in preparations of metaphase chromosomes from cancer cells [71,72]. This use of FISH is exemplified by a study of cells from a transplanted tumor, showing how the oncogenes MYC and EGFR were amplified on circular elements early in tumor development and later integrated into chromosomes to form HSRs [3]. In the 1990s, 2D gel electrophoresis was used to separate eccDNA from chromosomal DNA based on their different physical properties, such as supercoiled and relaxed states [73].
NGS methods

NGS-based methods have undergone technological advances in the recent years and have been the primary drivers of the recent research into eccDNA. These methods allow for the analysis of the sequence content of these DNA elements in-depth and to map their structure relative to the reference genome (Figure 3).

When a certain eccDNA is highly amplified in a cell population, it will be apparent as a copy number amplification in WGS data. The presence of soft-clipped reads spanning the circle junctions as well as discordant read pairs are features that can be analyzed by bioinformatics algorithms to determine whether amplifications are circular (Figure 3A) [74,75]. In a series of papers, WGS data has been analyzed for these features to identify amplified ecDNA and

**Box 1. eccDNA and ecDNA functions in cancer**

**Oncogene amplification on ecDNA**

Amplification of oncogenes is one of the central genetic alterations in cancer pathogenesis. Oncogenes can be amplified on both chromosomal DNA, that is, HSRs, and extrachromosomally, that is, ecDNA [2].

The dynamics of ecDNA segregation during mitosis are different from chromosomal segregation and allow for rapid accumulation of ecDNA molecules, and oncogene copy number increases (see Figure 2 in main text). Examples of oncogenes found frequently on ecDNA include EGFR, MYC, and MYCN [6,97,98].

There is significant correlation between gene copy number and oncogene transcription from both ecDNA and chromosomal DNA [16,17,76]. However, oncogenes on ecDNA show even higher expression than can be explained by copy number amplification [17,77]. This copy number-independent transcription increase can be partially explained by enhanced chromatin accessibility on ecDNA compared to linear amplicons [17,76].

ecDNA furthermore has altered organization of oncogenes and enhancers. Two papers have described how ecDNA can incorporate normally distal enhancers that stimulate the expression of MYC, MYCN, and EGFR [14,15]. Analysis of the 3D topology of ecDNA also showed novel contacts between oncogenes and regulatory elements compared with genes localized on chromosomes [14,15]. ecDNA-bound promoters and enhancers can also make contact with chromosomal DNA and other ecDNA in trans to stimulate transcription [77,99]. ecDNA can reinsert in chromosomes, creating HSRs and preserving the amplification of the oncogene in a more stable form [8,16,34,100,101]. Several studies also find indications that HSRs can give rise to ecDNA again [8,101].

**Drug resistance**

ecDNA has been demonstrated to contribute to drug resistance in cancer cells through upregulation of drug target genes and transporters [95,96,102–104]. Furthermore, resistance to epidermal growth factor receptor inhibitors in a GBM xenograft model overexpressing a constitutively active mutant of EGFR, EGFRvIII, was shown to occur by reduction of the cell population positive for ecDNAs carrying the gene. After drug withdrawal, cells positive for EGFRvIII-carrying ecDNAs reaccumulated. This indicates a dynamic mechanism for cancers to adapt to their conditions and evade targeted therapy by rapidly switching between dominant subpopulations with and without genes amplified on ecDNA [101].

**Small eccDNA functions**

For the most part, only ecDNA has been described to have a functional role in cancer cells. However, eccDNAs of smaller sizes have been observed to stimulate innate immune responses in two studies. Transfection of eccDNA to the cytosol of dendritic cells and accumulation of telomeric circles in fibroblasts have been shown to potently activate the cytosolic sensor of double-stranded DNA, cyclic GMP–AMP synthase, leading to upregulation of immune stimulatory proteins [48,105]. This effect of eccDNAs has not been studied in the context of cancer, but could be speculated to impact cancer cells’ interaction with their surrounding tissue and immune cells.

Furthermore, eccDNA in the size range below ecDNA still sometimes carry and express whole genes or miRNA that could have cancer-promoting effects [9,24].

In summary, ecDNA and potentially eccDNA drives cancer in several ways. The segregation dynamics accelerate oncogene copy number gain and the accessible chromatin structure and altered enhancer and promoter landscape all contribute to overexpression of oncogenes, tumor cell plasticity and ultimately an aggressive cancer phenotype. As a result, patients with ecDNA-positive cancers show significantly shorter survival than patients without ecDNA-based oncogene amplification [16,17].
(A) EcDNA detection in WGS data

- 2 mb ecDNA segment
- 1 mb ecDNA segment

(B) EccDNA detection in Circle-Seq data

- 500 bp eccDNA
- 1000 bp eccDNA
- >10,000 bp eccDNA

(C) Chimeric eccDNA detection

- with paired-end short reads
- with long reads

(See figure legend at the bottom of the next page.)
techniques such as FISH and electron microscopy used to confirm their extrachromosomal status and circular structure [8,19,76]. Further features of ecDNA have been studied by overlapping the genomic coordinates of circular amplicons with whole-genome assays of chromatin accessibility, methylation and 3D contacts and comparing to the features of linear amplicons [14,15,76,77].

WGS data will generally only reveal eccDNA and ecDNA that are highly amplified (Figure 3A). As a different approach, a method called Circle-Seq enables genome-scale screening for eccDNA of all sizes and is not dependent on their copy numbers [36,78] (Figure 3B). In this method, circular DNA is first separated from linear DNA by denaturation under alkaline conditions and renaturation. Next, any remaining linear DNA is removed using an exonuclease to degrade DNA from free ends. Finally, the remaining circular DNA undergoes rolling-circle DNA amplification to increase the signal before NGS. The method is highly sensitive and has uncovered thousands of eccDNAs in S. cerevisiae, pigeon, healthy human tissue, and neuroblastoma [9,16,36,46]. Although it can reveal more individual eccDNAs in tumors than WGS can, the Circle-Seq method is less accurate at determining copy numbers of unique eccDNAs, since the rolling-circle amplification method has a bias towards amplification of smaller eccDNA molecules [9,79]. In some studies, better quantification has been achieved by foregoing rolling-circle amplification for transposase-based tagmentation during NGS library preparation, which achieves more linear amplification [26,48].

The aforementioned strategies for eccDNA detection have mainly been applied to short-read, paired-end sequencing datasets. However, short reads have limitations in accurately identifying complete eccDNA and ecDNA structures. In several studies, long read sequencing methods have been applied to perform de novo assembly of eccDNA structures. This allows for more confident identification of repeat-containing and chimeric eccDNA; that is, those consisting of several noncontiguous genomic sequences [15,16,19,48,58] (Figure 3C).

Sequencing of DNA from cells and tissues in bulk provide an average of eccDNA content across a population of cells and does not reflect cell-to-cell heterogeneity, even though this is expected to be high (Figure 2). Single-cell sequencing has the potential to address questions on this in great detail, although the likely low amount of eccDNA in single cells could be technically challenging, as it has been for other variations such as copy number variations and single-nucleotide variations [80].

**Imaging**

In addition to FISH, which is based on sequence-specific probes and can only be applied to dead cells, other microscopy techniques were developed for imaging ecDNA. Epstein–Barr virus vectors and plasmids have been used to specifically recombine with ecDNA to introduce lac operator repeats that are specifically bound by a lac repressor–green fluorescent protein (GFP) fusion protein.
This method enables visualizing ecDNA independently of sequence content and imaging ecDNA in live cells.

Recently, a technique using catalytically dead Cas9 and guide RNAs with binding sites for fluorescent reporter molecules was used to image specific ecDNA in live cells based on guide RNAs targeted towards their breakpoint sequences [66]. This technique thus enables sequence-specific imaging of ecDNA in live cells and contributed to the discovery of transcriptionally active hubs of eccDNA in cancer cells [66].

Imaging of eccDNA independently of sequence has historically been done by electron microscopy [10,11,82]. However, the recent advances in microscopic methods allows for high resolution microscopy of fluorescently labeled eccDNA with structured illumination microscopy [58] and eccDNA in its native form with atomic force microscopy [83].

Cell model systems

Many cancer cell lines with defined oncogenes amplified on ecDNA exist [8,17]. The isogenic colorectal cancer cell lines COLO320-DM and COLO320-HSR each carry an MYC amplification in ecDNA and HSR-form, respectively. These cell lines are frequently used for comparative studies of ecDNA behavior in cancer, as they allow for comparison between chromosomally and ecDNA amplified oncogenes [77,84]. However, to further understand and investigate the functional impact of small eccDNA in cells and tissues, it is also important to develop cell and animal models that mimic these.

A major challenge in the development of models for eccDNA research beyond ecDNA in cancer cells is producing specific eccDNA in vivo and in vitro. eccDNA cannot be produced massively in vitro via typical Escherichia coli-dependent DNA cloning workflows, because eccDNAs do not possess prokaryotic replication origins or selection markers. Instead, eccDNA can be directly synthesized. For this, the ligase-assisted minicircle accumulation (LAMA) approach, invented in 2007, can be used to produce covalently closed DNA minicircles [85]. The LAMA method relies on cycles of denaturation, annealing, and ligation, and has been used to synthesize microDNAs that encode small regulatory RNAs with gene-suppression effects [24].

A recent advance has been the application of CRISPR-Cas9 genome editing to generate eccDNA [41]. As described, evidence suggests that endogenous eccDNAs can be produced after DSBs occur in chromosomes [36,37,43]. This event can be modeled by CRISPR/Cas9-mediated introduction of DSBs. Targeting the Cas9 endonuclease to specific genetic sites with guide RNAs is widely used for gene deletions by simultaneous cleavage at two sites in the same chromosome [86]. This gives rise to an extrachromosomal fragment of deleted DNA, which has the potential to be circularized [36]. Based on this principle, the CRISPR-C strategy was successfully implemented in cell culture to produce endogenous eccDNAs ranging in size from a few hundred to millions of base pairs and even ring chromosomes in human cells [9,87] (Figure 4). CRISPR-C is adaptable and efficient, but the long-term stability of CRISPR-C-induced eccDNA in cells is not well characterized [9]. Nonetheless, CRISPR-C is a promising tool for functional investigations of endogenous eccDNA in a broad range of sizes.

Perspectives for eccDNA in clinical applications for cancer

Small eccDNA elements have been identified in human plasma in several studies [25,26,88]. Cell-free eccDNA has been detected in plasma and serum from both humans and mice and human lung tumors have been shown to release eccDNA to the circulation [25]. Plasma samples collected prior to tumor resection contained more eccDNA compared with samples from the same patient weeks
after surgery, suggesting that the eccDNA load in plasma can serve as a marker for cancer. Although the idea is attractive, designing a test for eccDNA in plasma samples to identify tumors and follow their progression is still challenging [89]. This is mainly due to the low quantity of eccDNA in plasma and that methods for their quantification in patients have yet to be developed.

eccDNA has a more significant and functional role in cancer compared with small eccDNA. Since the presence of certain ecDNA in tumors is correlated with an aggressive phenotype and poor prognosis [15–17], it is reasonable to propose that targeting these DNA elements would be a good therapeutic strategy. Several studies have shown that the amount of oncogene-bearing ecDNA molecules can be reduced by drug treatment in cells [90–93]. This was also demonstrated in a study of 16 patients with advanced ovarian cancer, who were prescribed a noncytotoxic oral dose of hydroxyurea, suggesting that this might be a way to target ecDNA and slow cancer progression in patients [94].

In addition to drug treatment, radiotherapy is commonly used to treat certain cancers. The number of ecDNA molecules carrying MYC and MDR1 genes has been shown to be significantly reduced by ionizing radiation in human epidermoid and colon carcinoma cell lines [95,96].

Concluding remarks
ecDNA can be formed in human cancer cells by several different mechanisms and can result in amplification of cancer-associated genes. In particular, the uneven segregation of large ecDNA

Figure 4. CRISPR/Cas9-mediated generation of custom eccDNA in cells. The CRISPR/Cas9 genome-editing method can be used to introduce DSBs at selected positions in a chromosome, resulting in excision of a stretch of DNA, which can be circularized by the cell’s active DNA repair mechanisms (e.g., NHEJ). This makes it possible to generate any desired, specific eccDNA in cell culture in a manner resembling their normal biogenesis. Abbreviations: CRISPR-CAS9, clustered regularly interspaced short palindromic repeats; DSB, double-strand break; eccDNA, extrachromosomal circular DNA; NHEJ, nonhomologous end joining.

Outstanding questions
Is small eccDNA important for cancer phenotype? It is well known that large ecDNA can affect cancer phenotype by amplifying oncogenes and further contributing to their high expression via accessible chromatin and novel enhancer contacts. Small eccDNA has been shown to stimulate innate immunity and to serve as a template for transcription of small regulatory RNA, which could be speculated to impact cancer phenotype as well.

Can ecDNA be specifically targeted for cancer patients? If there is clinical benefit to ecDNA as a cancer treatment target, methods like CRISPR/Cas9 could be used to directly target, for example, breakpoint sequences unique to ecDNA.

What is the value of eccDNA as a component of liquid biopsies from cancer patients? eccDNA has been measured in the plasma of human subjects, including cancer patients, but its clinical value in relation to cancer diagnosis and prognosis is not established yet.

What can single-cell sequencing tell us about the heterogeneity of eccDNA and ecDNA in cell populations? This is an important future direction for the field, but methods for single-cell eccDNA sequencing will need to be carefully benchmarked since amplification of low amounts of material can easily lead to artifacts.

Can CRISPR-Cas9 be used to develop cell and animal models that allow for more functional studies? Functional studies of ecDNA and eccDNA in vitro and in vivo are still limited and CRISPR/Cas9 has been demonstrated to be efficient in generating specific eccDNAs.
that happens at mitosis, can lead to a rapid increase in the DNA copy number and protein products of oncogenes. ecDNA promotes greater tumor heterogeneity, malignant phenotype, and development of drug resistance; all of which contribute to poor patient prognosis.

Research in eccDNA has advanced in recent years, relying greatly on different NGS-based technologies. However, model systems to study different kinds of eccDNA functionally in vitro and in vivo still need to be developed to a more mature stage. For this purpose, CRISPR/Cas9-based methods show great promise (see Outstanding questions).

Due to their apparent role in cancer progression, targeting of ecDNA in tumors is a promising therapeutic strategy, but methods to target them specifically need to be developed. eccDNA of all sizes may prove to be an important biomarker for cancer, both for diagnostics and for targeted treatment. Cell-free eccDNA is found in the blood, and although eccDNA can also originate from healthy cells, preliminary evidence indicates that eccDNA in plasma can serve as a cancer-treatment. Cell-free eccDNA is found in the blood, and although eccDNA can also originate from healthy cells, preliminary evidence indicates that eccDNA in plasma can serve as a cancer-specific biomarker. The research results so far create an opportunity to develop technologies for sensitive, specific detection of eccDNA in both tumor biopsies and plasma samples for directing diagnosis, treatment, and surveillance of cancer.

There has been great progress in our understanding of eccDNA in recent years, but many findings are still novel. Studies of eccDNA in cancer are often based on one or few specific cancer types or a small number of human samples. In the years to come, more extensive research on eccDNA could advance development of novel, eccDNA-based therapeutic strategies to target the most aggressive cancer cell populations, monitor cancer progression via blood tests, and overcome the issue of drug resistance.

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Declarations of interest
The authors declare no conflict of interest.

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