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A unifying model for extrachromosomal circular DNA load in eukaryotic cells

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ABSTRACT

Extrachromosomal circular DNA (eccDNA) with exons and whole genes are common features of eukaryotic cells. Work from especially tumours and the yeast Saccharomyces cerevisiae has revealed that eccDNA can provide large selective advantages and disadvantages. Besides the phenotypic effect due to expression of an eccDNA fragment, eccDNA is different from other mutations in that it is released from 1:1 segregation during cell division. This means that eccDNA can quickly change copy number, pickup secondary mutations and reintegrate into a chromosome to establish substantial genetic variation that could not have evolved via canonical mechanisms. We propose a unifying 5-factor model for conceptualizing the eccDNA load of a eukaryotic cell, emphasizing formation, replication, segregation, selection and elimination. We suggest that the magnitude of these sequential events and their interactions determine the copy number of eccDNA in mitotically dividing cells. We believe that our model will provide a coherent framework for eccDNA research, to understand its biology and the factors that can be manipulated to modulate eccDNA load in eukaryotic cells.

1. Introduction

There is growing evidence that eukaryotic cells can obtain rapid evolutionary change from circular DNA of chromosomal origin. Their lack of centromeres releases eccDNA from the 1:1 segregation constraint imposed by linear chromosomes, allowing them to segregate unevenly. Such uncontrolled segregation might have large implications, because compared to chromosome amplifications, genes released from the chromosomal constraint on circular DNA can increase more rapidly in number. If higher gene-copy number also provides a selective advantage at the cell level, cells with eccDNA amplifications outgrow other cells. So far, hard evidence for this phenomenon has been limited to a few oncogenes and yeast cells with nutrient-transporter genes on eccDNA. However, during the past couple of years it has become clear that eccDNA can form from all parts of the genome of tested eukaryotic organisms (humans, pigeons, worms and yeasts). This suggests that any locus can become ‘abducted’ on an extrachromosomal circle, increase in copy number and thereby alter gene expression level and the ultimate fitness of host cells. Eventually, this means that subpopulations with eccDNA amplifications can outgrow populations with canonical chromosomal gene amplification. While eccDNA may thus create transient advantages, some studies suggest that eccDNA can become reinserted in one of the chromosomes, thereby returning the short-term poorly organized selective advantage of extrachromosomal copy number variations (CNV) to standard long-term propagation. The dynamics of eccDNA fragment generation and demise, relative to standard mitosis, may therefore have significant implications for our understanding of adaptive evolution and diseases that are related to cell division. In this review, we propose a unifying model covering five main factors that determine eccDNA copy number and the evolutionary consequences of their persistence. The model builds on the theories from the 1980s where studies of plasmids in yeast led Murray and Szostak to suggest that the frequency of a circle is a function of its formation rate, its replication efficiency and its segregation bias [75]. Wahl and Schimke, who simultaneously worked on tumours and cancer cell lines, further suggested that oncogenes on extrachromosomal elements can provide a selective advantage to host cells and thereby lead to tumourigenesis [47, 92,118,121]. Recently, we have shown that these factors are also essential for circle load in yeast [87] and Mischel and coworkers have confirmed that that oncogenes on extrachromosomal elements can provide a selective advantage to host cell in the context of cancer [127].

In concrete terms, we hypothesize that eccDNA load within a mitotic cell is determined by (i) the rate by which eccDNA species form, (ii) whether they replicate and (iii) the efficiency by which they are...
Degraded. The eccDNA load is further defined by (iv) how they segregate during cell division and (v) what selective (dis)advantage they offer to the cell as a whole (Fig. 1). We also discuss the implications circular DNA might have for long-term genome evolution when they integrate in chromosomes to become permanent heritable variation accompanied by change in synteny (gene order).

We present empirical evidence for the importance of each of the five key factors, and argue that the model should apply to eukaryotes in general, even though we restrict our review to eccDNA in tumours and the yeast S. cerevisiae, where eccDNA dynamics are best understood. In somatic cancers the effects of eccDNA are unambiguously deleterious for general, even though we restrict our review to eccDNA in tumours and key factors, and argue that the model should apply to eukaryotes in other organisms and cell types and therefore given many different names (BOX 1). To establish consistency in nomenclature, we will use the umbrella term eccDNA (extrachromosomal circular DNA) following the criteria provided by James Gaubatz [27]. This term encompasses all chromosome-derived circular DNAs present inside the eukaryotic cell, regardless of size, complexity or content including eccDNA (extrachromosomal DNA), which is the term for mega-base sized eccDNA in tumours with one or multiple genes and visible under the light microscope [116].

Nomenclature: Apart from specific names for eccDNA we occasionally need names to denote discreet eccDNA. We have so far described the genotypes of circled genes according to their locus of origin, such as [CUP1]* [HXT6]* for circular DNA in yeast and [ITT]* for circular DNA in human [69,70]. Square brackets are used to indicate the non-chromosomal inherited material, similar to what is used for prions [108]. Alternatively, if nucleotide resolution is needed, circles may be indicated with chromosomal coordinates such as [1:200000–1:230000] [CUP1]$.Ø$ is the symbol for diameter, a defining characteristic of any circle.

3. Mechanisms governing the formation and evolution of eccDNA

eccDNAs appear to generate from random positions in the genome of mitotically dividing cells [69,70,95] (Fig. 1A). DNA damage followed by DNA repair appears to be an important cause of circularisation, since hypoxia [15] and chromosome shattering (chromothripsis) can increase the number of eccDNA in eukaryotic cells [100].

In addition, errors during DNA replication have also been suggested as a mean for DNA circularisation [5,117]. Thus, other mechanisms beyond DNA repair are thought to play a role. Within DNA repair, the relative contributions of specific pathways to this process are less obvious. This is partly overcome by knowing the sequence of DNA surrounding the junction point of a circle, from which a particular DNA repair mechanism can be inferred. A high degree of homology between the recombined ends indicates the involvement of a homology-based repair mechanism such as microhomology-mediated end joining (MMEJ), homologous recombination (HR) or mismatch repair (MMR) [21,70]. On the other hand, little or no homology indicates that non-homologous end joining (NHEJ) played a role in the circularisation process. While mechanistic studies have implicated each of these pathways in eccDNA formation, the fractions of eccDNA formed by the different pathways probably depend on cell type, organism and where in the cell cycle damage takes place.

3.1. Two ends coming together

An early study of eccDNA in Chinese hamster ovary cells demonstrated that recombination through homologous pairing does not sufficiently explain the nucleotide sequence present at the recombination junction of eccDNA [104]. Similarly, the characterisation of a small subset of eccDNA purified from HeLa S3 cells revealed a lack of obvious homology or repeat sequences at recombination junctions, indicating that they were circularised by non-homologous end joining [115]. This interpretation is further supported by linear intermediates and the absence of homology at junction points in eccDNA purified from murine melanoma cells [14]. The situation in S. cerevisiae is similar, as an unbiased analysis of S. cerevisiae revealed that approximately 75% of the detected eccDNA show no homology at the chromosomal loci that led to their formation [70].

The strongest evidence yet for the involvement of NHEJ in eccDNA formation comes from studies that investigate the importance of specific components of this pathway in DNA circularisation. In the NHEJ pathway, the DNA ends associate with Ku70/Ku80 heterodimers and are held within close proximity by DNA-PKcs before being ligated by ligase IV. Knockdown of Ligase IV inhibited the circularisation of mouse major satellite DNA [14]. A recent study of U2OS and 293T human cells

![Fig. 1. Factors that control eccDNA load inside a mitotically dividing cell. (A) Formation rates determine how frequently a particular eccDNA forms at cell level and how these rates add up at the population level. (B) Replication. The presence of replication origins allows an eccDNA to replicate during S-phase and increase their number. EccDNAs without replication origins are diluted out. (C) Segregation. Ranges from random distribution to variably skewed distributions depending on degree of segregation bias. (D) Elimination. Either because of integration events into the chromosome or degradation in micronuclei. (E) Selection. Increase because eccDNA provides phenotypic benefits to the cell because of its genotype per se or because of changes in copy number that affect that genotype’s expression. Inspired and adapted from [87].](image-url)
knocked out in different NHEJ components and reported increased levels of microDNAs, suggesting that the NHEJ machinery has an inhibitory role in eccDNA generation [85]. An alternative explanation to this counterintuitive result is that the NHEJ mutants have higher levels of eccDNA because the level of damaged DNA is generally higher in these mutants.

Further evidence for NHEJ involvement in mammalian DNA circularisation comes from studies of chromothripsis – the catastrophic shattering of one or more chromosomes by a sudden genomic instability event [105]. The repair of locally shattered chromosome fragments occurs in a random order and orientation, leading to a broad range of possible outcomes, including highly rearranged chromosomes and DNA circularisation [100,113,128]. Sequence analysis of the junctions of rearranged chromosomes revealed little or no microhomology, suggesting that NHEJ is a major process of DNA repair in chromothripsis [105]. Pharmacological inhibition of Ligase IV or DNA-PKcs, but not components of HR or MMEJ, prevented the reassembly of chromosomal fragments in an inducible model of chromothripsis [66]. Support of the role of NHEJ in chromothripsis came from [100] who demonstrated that chromothripsis is a major driving mechanism for NHEJ-mediated eccDNA formation in different human tumours.

Fig. 2. Model for change in cell line composition when a gene becomes eccDNA. (A) A gene located in a chromosome (bound to a centromere) segregates symmetrically through cell divisions, both in the presence or absence of selection, but the outcome for cells with genes on eccDNA is different and depends on selection (B, C and D). Dividing cells are represented as spheres in pedigrees. Spheres with dots are non-dividing or dead cells while spheres with colour represent cells with different gene copy numbers of eccDNA. Each cell division is shown as a fork and the length of the branch represents the time between cell divisions. The model assumes replication efficiency of the eccDNA of 100% at every cell division, no degradation and segregation frequency (SF) of 50%, meaning that the segregation is random with no preference for either daughter cells. In the absence of selection for cells carrying the gene (B), the number of cells carrying a gene on eccDNA is likely to decrease in the population (outcome: 16 cells of which 9 cells carry 16 gene copies). In the presence of positive selection on the gene itself (C), the gene is likely to be maintained in the population, although its propagation efficiency remains rather low (outcome: 8 cells with 16 gene copies). If not only the gene itself but also a higher gene copy number provides a positive selective advantage (here shown as faster growth, D), the asymmetric segregation of eccDNA allows for the most rapid accumulation during subsequent cell divisions (outcome: 22 cells with a total of 64 gene copies) when compared to a gene on a chromosome with 1:1 segregation (outcome: 16 cells with 16 gene copies, A).
3.2. A bit of homology helps

Sequencing of EGFR DM, a highly frequent eccDNA in gliomas, revealed microhomology at the joined ends, suggesting that MMEJ was the mode of formation [117]. Later studies in healthy and tumour mammalian cells found microhomologies at circular breakpoints, supporting MMEJ involvement and suggesting replication errors as a source for DSB leading to eccDNA [21,55,99]. Knock out of MMEJ protein PolQ and addition of MMEJ PARP1 inhibitor, lead to reduction in microDNA levels in human U2OS cells [85]. In S. cerevisiae 12% of all detected eccDNA is found to form between regions with microhomology [70]. Also loss of the MMEJ components Sae2p and Mus81p, led to a reduction of the transcriptionally active CUP1 circle in S. cerevisiae [38], supporting that MMEJ is indeed important for formation of eccDNA.

Another pathway relying on sequence homology is the MMR. Loss of MSH3 in DT40 chicken cells, a key component of MMR, led to a reduction of 80% in small (<400 bp) eccDNA [21]. Later studies also show how knock out of MSH1, MSH2 and MSH3 lead to reductions in eccDNA levels [85]. How MMR is involved in eccDNA generation is not clear. One possibility is that DNA loops are generated between the point of mismatch and the recognition signal for template DNA strand, leading to DNA bends susceptible rearrangements [21,84]. Overall, these mutant studies show that replication errors, resolved by either MMEJ or MMR, are involved in eccDNA generation.

3.3. A lot of homology helps a lot

HR is predominately active in mitosis and meiosis. After DSB, the chances of HR to occur increase with the level of homology and the distance for homology search [1]. Therefore, tandem repeats in the genome are highly susceptible to homologous recombination via illegitimate intrachromatid recombination in the parts of the cell cycle where HR is active. Numerous examples of tandem repeats have been suggested to circularise via homologous recombination in different eukaryotic species [12,13]. In S. cerevisiae, these include: the ribosomal region [101], the copper metallothionein paralogue array of CUP1 genes [70], adjacent hexose transporter genes, HXT6-HXT7 [70] and the general amino-acid transporter GAPI, assisted by flanking LTR elements [30]. Interestingly, low levels of circularisation between HXT6 and the less similar yet adjacent HXT3 are also detected [70], supporting that close vicinity and the level of homology are critical factors for circularization.

In humans, the evidence of eccDNA and ecDNA forming from repetitive regions is less evident despite the large proportion of repetitive regions in the human genome. This is partly due to the current technology for eccDNA detection, where eccDNAs are sequenced with short reads that do not suggest a circle junction point [69]. Long sequence reads resolve this problem and give better information about circle junctions and the potential impact of homologous recombination [35]. Additionally, inhibition of RAD51 in 293T human cells lead to lower levels of eccDNA, suggesting their partial role in eccDNA formation [85].

3.4. Tuning circularisation rates

In yeast, a number of recurrent eccDNA are consistently found in independent experiments and conditions, suggesting deviations from random generation of eccDNA. In addition to homology length and distance described above, specific locus-level characteristics can modulate the frequency of circularisation. Active transcription induces circularisation rates of the copper metallothionein gene CUP1 [38], directly linking changes in environmental conditions (increase in copper concentrations) to eccDNA profiles. Although the mechanistic details remain to be elucidated, this observation resembles the way that
transcription regulates recombination [110]. Transcription stress makes DNA susceptible to DNA damage [62], which may generate genetic variability, and thereby eccDNA. Also, the correlation between gene frequency in human chromosomes and their circularisation frequency [69] suggests that this is a genome-wide phenomenon, rather than a locus-specific characteristic.

Another important factor influencing circularisation is localised genomic instability. The amplification of DHFR, R2 and CCND1 in double minute eccDNA can be dependent on c-Myc-induced genomic instability at their respective loci [59–61]. While the current understanding of the underlying mechanism is incomplete, circularisation of these loci could be linked with multiple roles of c-Myc, in replication stress, decreasing DSB repair capacity, the accumulation of reactive oxygen species, as well as illegitimate recombination licensing [58,61].

With these examples in mind, it is tempting to speculate that the ability for a locus to circularise is a selectable trait in itself, potentially modulated by the presence of nearby homology, transcription and genomic instability. If this hypothesis is true, it would at least partly explain the existence of recurrent circles and confirm one aspect of the adaptive potential of eccDNA [37].

3.5. Reshaping eccDNA

Besides eccDNA circularised from a single piece of DNA, complex structures made of two or more loci are also observed [17,29,116,125]. Two models can explain this phenomenon. In the first, a simple circle is subject to further mutational processes. These mutational processes may include intra- or inter-eccDNA fusions, insertions, deletions and point mutations [125]. The biological implication is that once an eccDNA forms and increases its copy number within a cell, the likelihood of generating gain of function mutations that confer a higher adaptability on the cell is inherently increased. In HL-60 promyelocytic leukemia cells, early amplification of MYC occurs in submicroscopic (episomal) eccDNA [4,10,78]. However, extensive passage in culture led to those eccDNAs shifting to larger (DM). (D. D. [119]). Similarly, the human multidrug resistance (MDR) genes MDR1 and MDR2 amplify in one submicroscopic eccDNA in early stages of colchicine selection. After multiple stepwise increases in colchicine, eccDNA dimerize into a much larger, cytologically detectable double minute DM [92,94]. These observations strongly indicate a fusion mechanism for the generation of large extrachromosomal elements.

In the second model, complex circles initially form from multiple linear fragments, though evidence for this model is only supported by a high percentage of complex circles in human sperm that are likely formed during spermatogenesis [35].

Perhaps the best evidence of coexisting formation mechanisms and their continuous evolution are likely to be the cause of the adaptive potential eccDNA confers on tumour cells [116]. For example, longitudinal studies revealed that successive rounds of chromothripsis can drive the structural evolution of eccDNA to support the rapid acquisition of drug tolerance in HeLa cells [90,100]. Furthermore, the extensive selection process that eccDNA undergoes could explain some of its characteristics in terms of size, content, and transcriptional activity [34,74,124].

Enhancer elements, which are located distal to their target gene, regulate temporal and cell type specific gene expression by interacting with the gene promoter via looping of the chromatin within topologically associated domains (TADs). Distal enhancer elements are distinguishable from proximal promoters by the enrichment of H3 histones monomethylated at lysine 4 (H3K4me1), and H3 histones acetylated at lysine 27 (H3K27ac), when activated. Tumour cells frequently exhibit abnormal locus-specific enhancer activity, in some cases driven by the repositioning of enhancers near proto-oncogenes by genomic rearrangements [5,31,79]. Scacheri and colleagues observed in glioblastoma that EGFR invariably co-amplifies on eccDNA with neighbouring enhancers to maintain the topological contacts present on the linear chromosomes [74]. However, due to random incorporation of fragments during the circularisation of these amplics, repositioning of enhancer elements that are normally insulated from EGFR were found on some eccDNA to have formed new regulatory connections with the oncogene. The creation of new regulatory connections within eccDNA was similarly observed in neuroblastoma, where two classes of circularised amplics containing MYCN were characterised by (i) co-amplification with an endogenous local enhancer, or alternatively, (ii) the presence of distal chromosomal fragments harbouring ectopic enhancers that compensate for disrupted endogenous interactions by forming neo-TADs [34]. It is suspected that this expanded pool of topologically rewired loci provides a selective advantage to the effect of these oncogenes on the fitness of a tumour cell [34,74].

4. Propagation of eccDNA – Replication and Segregation

In mitotically dividing cells, a necessary feature for eccDNA to propagate in a population is replication. In addition to the selective pressures that determine the utility of eccDNA to the cell, the stability of a particular eccDNA molecule in a cell population is primarily determined by factors affecting its replication and heritability (Fig. 1B and C).

4.1. Replication

The replication of DNA initiates at specific loci called replication origins, where DNA helicases and polymerases coordinate DNA synthesis. Like chromosomal DNA, the replication of eccDNA should be necessary for their maintenance. Therefore, it is presumed that recurrent eccDNA harbour replication origins.

The strongest evidence in support of this comes from studies of yeast, where specific circles that carry replication origins are long known to replicate and propagate in populations of yeast cells [30,65,101], whereas eccDNA lacking replication origins would be expected to be lost during cell growth. We addressed this question for eccDNA in S. cerevisiae, by measuring the population of eccDNA through 15 cell division. S. cerevisiae has well annotated replication origins and it was therefore possible to establish that most eccDNA are lost from the cell population in the absence of replication origins [87]. The S. cerevisiae genome contains ~400 active replication origins spread across its 12 Mb genome. This suggests that a large fraction of randomly formed eccDNA will not contain a replication origin and therefore will not replicate in a canonical fashion. Some small circles without a replication origin, such as t-circles, multiply by rolling circle amplification [80], which might also be a mean for other eccDNA to replicate.

Evidence of replication of mammalian eccDNA was first given by the classical Meselson-Stahl procedure where hamster cell line with a CAD episome were passed through labelled nucleotides for one generation [121]. The resultant eccDNA all changed density through the canonical semi-conservative replication, suggesting that eccDNA replicate once per cell cycle and obey replication similar to that known for chromosomal sequences [121]. Many other studies confirm that large eccDNA replicate in cancer cell lines [41,92,107], but it is still questionable if small eccDNA without replication origins are able to replicate in mammalian cells. This is especially relevant for human eccDNA, where no canonical replication origins exist, but rather discreet regions in the genome have this role.

4.2. Heritability and segregation

During cell division, euakryotic genomes ensure the correct partitioning of homologous chromosomes via the centromere, which acts as a recognition site for the assembly of the kinetochore. As described in Section 2, the majority of eccDNA do not accommodate a complete, canonical centromere [87]. Absence of a centromere frees eccDNA from the regular even segregation in mitosis and allows for repeated accumulation or loss of eccDNA.
In humans, MYCN-carrying circles were shown to segregate by a binomial random distribution in neuroblastoma cells, suggesting that eccDNA segregate randomly (Lundberg et al., 2008). This in terms led to some cells accumulating eccDNA. Recent studies in human tumours and cancer cells confirm this observation as a general characteristic of eccDNA that is randomly inherited between daughter cells in mitosis in an uneven fashion allowing rapid accumulation [112,127]. Other studies of acenitic circular DNA molecules show that in mammalian cells, circles can associate with chromosomes ends by a phenomenon called “chromosome tethering” [45,46,63], also observed in plants [56]. The association occurs in clusters of eccDNA that are pulled together when the chromosome segregates. Fluorescent histoane labelling (H2B-GFP) revealed that DM associate with histones and aggregate in clusters at chromosome arms, sometimes creating “chromosomal bridges”. During cytokinesis these bridges of eccDNA are asymmetrically cleaved leading to uneven distribution [46]. However, the molecular mechanisms supporting this association remain unresolved.

The mechanisms behind segregation in S. cerevisiae are better understood. eccDNA in yeast are retained in the mother cell to varying degrees, thereby reducing the number of eccDNA passed on to the new daughter cell [75]. Unlike that of humans, the nuclear envelope of yeast is maintained through mitosis, preventing the nuclear content being mixed with the cytoplasm. Two mechanisms have been proposed to influence segregation of eccDNA during mitosis. One is an active retention mechanism where ERC and non-ERC are attached at the nuclear envelope of the mother cell by binding to the nuclear pore complexes (NPC). During cell division, circle-bound NPC are retained in the mother cell, and thus creating a segregation bias [20] and BOX 2. The second mechanism suggests that non-ERC circles are passed from the mother nucleus to the daughter nucleus by passive diffusion [28]. This leads to a segregation bias in the fast-dividing yeast cells because all eccDNA will not distribute evenly between mother and daughter cells at cytokinesis. Proof of the passive mechanism is based on experiments where GFP-labelled plasmids were observed to distribute randomly across the nucleus rather than attaching to the nuclear envelope [28]. A recent unbiased study of eccDNA in replicative aging cells supports both models [87], since ERC circles are retained with a strong propensity to mother cells, while most other eccDNA, with the ability to replicate, have a weaker retention rate.

5. Elimination of eccDNA

A key question concerns if and how cells handle the agglomeration of eccDNA. Data from yeast suggest that ERCs accumulate in cells as they replicate and that this accumulation eventually cause cells to cease growth [73]. It is reasonable to speculate that a cell would take measures to avoid the potential deleterious effects of the accumulation of genetically benign eccDNA (discussed in section 6.2). On the other hand, there are clear benefits to retaining the genetic content of specific circles that can confer a selective advantage (Figs. 1E and 2).

5.1. eccDNA is cleared by micronuclei entrapment and extrusion

Yeast cells eliminate ERCs during meiosis [49]. After replication of the DNA content, two subsequent meiotic divisions are required to obtain haploid genomes. During the first meiotic division, two important events occur: (i) pairing and recombination between homologous chromosomes and (ii) separation of homologs into two daughter cells. In the second mitosis-like division, the sister centromeres separate and each sister chromatid migrates into one daughter cells. This process also allows the rejuvenation of gametes and eliminate age-induced cellular damage. In yeast, during the second division and the generation of the four gametes, a fifth vesicle is formed containing ERCs and other aging factors [49]. Whether this process is similar for non-ERC circles remains unknown. Interestingly, studies of the plant A. palmieri, suggest that at least some non-ERC eccDNA are transmitted through meiosis by a mechanism involving chromosome tethering [56].

Studies of mitotic cancer cells have shown that eccDNA can be eliminated from human cancer cells by entrapment within a micronucleus particle (Fig. 1D) [96,99,120]. This process is enhanced by low doses of DNA replication inhibitor hydroxyurea [9,97,103] because it promotes eccDNA detachment from chromosomes during mitosis [99]. Micronucleus structures containing eccDNA are different from chromosome-type micronuclei, arising independently and being

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**BOX 2**

The ribosomal DNA (rDNA) locus is generally organised in arrays of tandem repeats in eukaryotic genomes [23,88]. This organisation makes rDNA repeats prone to circularisation and formation of extrachromosomal ribosomal circles known as ERCs. This phenomenon has been studied intensively for the yeast S. cerevisiae, where accumulation of ERCs appear to be the main cause of replicative aging [101].

**What phenotypic effect do ERCs confer?** Yeast cells divide mitotically with the nuclear envelope intact into a mother and daughter cell. ERCs segregation is biased towards the mother cell and ERC accumulation is regarded as a hallmark of replicative aging in yeast [101] since it reduces cell fitness. ERCs aging theory states that accumulation of ERCs eventually leads to a highjack of the transcriptional machinery leading to deregulation of homeostasis, which ultimate triggers cell senescence [73].

**How are ERCs formed?** A high levels of transcription in the rDNA locus leads to increased DNA damage due to the collapse of the replication and transcription forks [7,51,53]. To avoid such collisions, Fob1 proteins transiently create replication fork barriers, in which occasional DSB occur [18,43,51]. At this point, activation of the bidirectional E-pro by the Sir2 protein leads to transcription of non-coding RNAs and will determine if the DSB is repaired by unequal sister-chromatid recombination (leading to ERC) or equal sister-chromatid recombination [44,52,54]. Iida and Kobayashi [40] elegantly described how this process is sensed and controlled with a musical chairs model [40].

**How do ERCs segregate during cell division?** When S. cerevisiae cells divide, almost all ERCs remain within the mother cell and do not segregate to the daughter cell [101]. The maintenance of the nuclear membrane during cell division appear to allow the mother cells to retain the ERC. This accumulation is mediated by an active mechanism of retention involving components of the nuclear pore complexes TREX-2 and a more general transcription complex, SAGA [20]. During division, these complexes promote interaction between ERCs and nuclear pore complexes that leads to specific retention of the ERC in the mother nucleus.

**Are ERCs degraded?** Mother yeast cells accumulate ERCs with no apparent degradation during replicative life span [73,101], and their asymmetric segregation ensures that mitotic daughter cells are formed without ERCs. This is different in mitotically dividing cells, where, King et al. [49] described a mechanism during meiosis in which ERCs are specifically entrapped together with protein aggregates within a confined space between the meiotic cells, thereby excluding ERCs from the gamete nuclei [49,50].
Elaborated on the underlying mechanisms remain obscure, these observations indicate that the induction of DNA damage is important for eccDNA elimination by micronuclei capture. In support of an explanation as to why DSB-inducing agents cause the selective elimination of eccDNA, the response to hydroxyurea-induced DSBs sustained by eccDNA is different from the response sustained by chromosomal loci [97]. While most chromosomal $\gamma$H2AX foci were resolved by metaphase, persistent $\gamma$H2AX signals were associated with eccDNA, which remained behind when the chromatids separated, aggregating in micronuclei. Exploitation of eccDNA capture and clearance could represent a promising therapeutic strategy to eliminate eccDNA harbouring oncogenes in cancer. Moreover, the recent observation that targeting DSBs in eccDNA using CRISPR-Cas9 triggers their aggregation in micronuclei [83] indicates that specific eccDNA could be targeted for clearance from the cell by this approach in the near future.

5.2. Closing the loop: the reintegration of eccDNA

An alternative fate of eccDNA is incorporation in the chromosomes (Fig. 1D). Thus, the fate of some eccDNA follows the journey of formation from chromosomal DNA (Section 3), to reshaping of its genetic content (Section 3.5), to stable genomic reintegration. The integration of an eccDNA molecule can cause changes in gene synteny at the landing site [112], reversion to faithful 1:1 segregation, and changes in gene expression at the landing site [77].

Several examples demonstrate or propose eccDNA integration as a causal mechanism for adaptation to a new conditions, phenotypic diversity or genomic evolution [19,22,25,26]. An example of this is found in the European wine yeast strains where a fragment of the yeast *Zygosaccharomyces bailii* genome was identified in the genome of *S. cerevisiae* strain EC1118 [81]. Structural analysis of the introrgression breakpoint revealed a reorganization and expansion of the segment, most likely through a eccDNA intermediate [26]. This suggests a cross-species transfer can occur through eccDNA that will further integrate the material, thereby fixing it in the general population.

Similar reversion of the unfaithful transmission of eccDNA is found in cancer cells when amplified eccDNA reintegrates into chromosomes in arrays of homologously stained regions [6,15,76,112]. This has been shown for oncogenes *MDR1* and also later *MYC*, among others [15,106]. Integration of an optimised eccDNA allows the new genotype to segregate faithfully through mitosis, thereby improving the heritability of the new trait. Furthermore, the process of integration can also allow oncogenes on eccDNA to escape chemotherapy. While epidermal growth factor receptor variant III (EGFRVIII) amplification on eccDNA allows cells to increase growth signaling through the EGFR pathway, it also sensitises cells to specific chemotherapy such as EGFR tyrosine kinase inhibitors or TKIs. In the presence of EGFR inhibitors, integration events of the [EGFR<sup>circle</sup>] into the linear genome are selected for [76], underpinning the genomic plasticity associated with eccDNA.

6. The phenotypic consequences of eccDNA

As proposed in the introduction, the eccDNA load within a cell is determined by the relative rates of formation, replication, clearance and segregation. These factors create a copy number gradient for each eccDNA molecule in a given cell population. Individual eccDNA lacking any selective advantage will gradually drift out of a growing population. On the other hand, selective pressures are likely to maintain eccDNA with advantageous growth effects and further shape them by (i) increasing their copy number, (ii) supporting the formation of chimeric circles, and (iii) introducing point mutations. In addition to enhancing the evolvability of a locus (Section 5.2), increased eccDNA copy number can lead to an elevated level of gene expression. Although eccDNA has been reported to be present in the cytoplasm [102], eccDNA is predominantly nuclear, allowing for the expression to be reflected in the phenotype. While this can increase the fitness of a cell, for example by acting as a vehicle for oncogene amplification, the accumulation of eccDNA can also have deleterious effects (see Box 2) and potentially induce adverse immunological responses.

6.1. Selection

When circularisation encompasses the promoter and the open reading frame, all of the elements necessary for expression of a gene are present in eccDNA. Therefore, eccDNA copy number changes can have a profound impact on expression. In yeast, adaptation to limited nitrogen include amplification of the amino acid transporter gene *GAP1* on eccDNA, allowing the cells a selective advantage in this environments [30,91]. Similarly, in glucose limited conditions, yeast cells with a glucose transporter genes in a [HXT6<sup>circle</sup>] have a selective advantage. Cells that carry a stable chromosomal amplification of [HXT6-HXT7<sup>circle</sup>] eventually take over the population, suggesting that circularisation allows for a transient amplification that is followed by a stable insertion and amplification [87]. The environment itself can also impact the rate by which eccDNA that provide a selective advantage form. In presence of copper, yeast cells can upregulate the expression of *CUP1* leading to higher mutation rates of *CUP1* and eventually more [CUP1<sup>circle</sup>] by circularising the gene [38].

In humans, the phenotypic consequences of eccDNA is arguably best exemplified in situations when cancer cells upregulate the expression of key oncogenes on eccDNA [46,116]. More than one third of tumours are estimated to contain large Mb eccDNA amplification with oncogenes [112], and these tumours are typically more aggressive and drive poorer outcomes [48]. Similarly, tumour cells can develop drug resistance through eccDNA-amplification of genes such as *DHFR*, which confers methotrexate resistance to numerous cell lines [32]. However, it is not only the increase of copy number driving the high expression of circularised oncogenes. Epigenetic rewiring also appear to play a key role in the transcriptional deregulation of genes encoded by eccDNA. In cancer cell lines and clinical samples, the expression of oncogenes amplified on eccDNA is higher than the expression of non-circularised copies of the...
same genes [112,124]. Analysis of the chromatin revealed that although eccDNA is packaged into chromatin with intact domain structure, it lacks higher-order compaction that is typical of chromosomes and displays significantly enhanced chromatin accessibility.

We previously discussed the formation of new connections between gene-regulatory elements on eccDNA (Section 3.5). Recently, Zhu et al. [129] revealed that enhancers on eccDNA can traverse the nucleus and serve as mobile regulatory elements that interact with chromosomal oncogenes to stimulate high-level gene expression [129]. Additionally, enhancer recruitment and interactions in trans between eccDNA allow a hub-type aggregation that boosts transcription [39]. These pleiotropic effects of eccDNA-encoded mobile enhancers have so far been shown to confer adaptive advantages of cancer cells and contribute to tumour heterogeneity. Understanding how eccDNA-directed interactions disrupt normal TAD regulation could lead to new therapeutic strategies for reducing eccDNA-chromosomal contacts that promote cancer progression or drug resistance.

Even the smallest eccDNA or microDNA have been reported to be transcribed, suggesting a role in regulating gene expression. eccDNA containing miRNA coding sequences are able to produce functional miRNAs leading to gene silencing, even in the absence of promoters [86].

We summarized the eccDNAs dynamics that different selective advantages provide (Fig. 2). In the absence of selection, eccDNA is continuously lost due to drift (Fig. 2B). On the other hand, if an eccDNAs provides a selective advantage, not all daughter cells will benefit from the advantage due to uneven segregation of eccDNAs (Fig. 2 C). Finally, if a higher copy number of an eccDNA fragment translates into faster cell division or any other cell-fitness advantage, the impact of uneven segregation of eccDNAs will be amplified because eccDNA will accumulate in fewer cells that will replace cells with fewer copies leading to faster increase in eccDNA load (Fig. 2D).

7. Discussion

After eukaryotic genomes have long been considered to essentially only generate novel variation by chromosomal or mitochondrial mutation, eccDNA release has emerged as an additional structural mechanism for increasing cell-level genetic variation. Besides the phenotypic changes a circularised locus and its corresponding deletion can provide, the eccDNA is also relieved from centromeric control, escaping 1:1 segregation. From here, genetic diversity arises at multiple levels even in the absence of selection through (i) random copy number changes (ii) gain of function mutations leading to novel isoforms, (iii) integration of eccDNA into the linear genomes.

We believe that our model is relevant because normal wild type cells contain thousands of eccDNA in population samples of just 10⁶ cells taken from birds, mice, yeast and humans [57,69,70,72,95]. A high number of eccDNA therefore means that there is a large phenotypic variation for selection to work on when the environment changes. The same might occur in metazoan bodies when cell lineages have mutated to become cancerous. This also implies that some eccDNA mutations, such as [HXT6 HXT7] and [CUP1] are always present in one or a few members of a breeding population of yeast [71]. Maintaining mutants that are not favourable in one selection regime and favourable in another is known as bet hedging, a strategy of dividing cells that ultimately incurs higher fitness in varying and unpredictable environments [64,92]. Frequently occurring eccDNA mutations with conditional selective advantages, such as the [GAPF] [38], are good candidates for bet-hedging, because the phenotypic traits mediated by these circles allow populations of S. cerevisiae to quickly adapt to limited amino acid availability in the environment, and vice versa [68]. Studies from cancer have shown that tumours often respond to chemotherapy because they harbour an enormous amount of genetic variation through eccDNA and eccDNA [48] allowing susceptible cell lines to be quickly replaced by resistant cell lines.

In humans, eccDNA has mainly been studied in the context of cancer and not in relation to other diseases. This raises the question on whether eccDNA may be involved in other complex and degenerative diseases or is a cancer-specific characteristic. Certainly, the evolutionary road that tumour cells undertake provides the perfect set up to select large and evolved eccDNA, which might not happen in other non-tumour diseases. On the other hand, several degenerative diseases are characterised by increased levels of DNA damage. This occurs in non-mitotic cells where eccDNA are unlikely to replicate and amplify. In these cases, eccDNA could generate new gene isoforms via circularisation with different deleterious effects [2]. The fact that eccDNA has a potent immunostimulatory effect [123] suggests that the phenotypic implications of eccDNA are not restricted to their cell of origin but have systemic effects, contributing to persistent inflammatory conditions.

In conclusion, we propose a model in which formation, replication, segregation, elimination and selection of eccDNA determine eccDNA load inside eukaryotic cells. The model is intended to offer a structural framework for future studies on the genetic characteristics and phenotypic effects of eccDNAs, not only in yeast where targeted experimental evolution is possible and in treatment of metazoan cancer, but also in other animal models. Perhaps most interesting, the interplay between all these factors can shed light into how evolution shaped eukaryotic genome architecture. This includes far-reaching questions regarding the evolution of gene families, how genes change position in the genome and how loci evolve.

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Conflict of interests

The authors declare no conflict of interests.

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