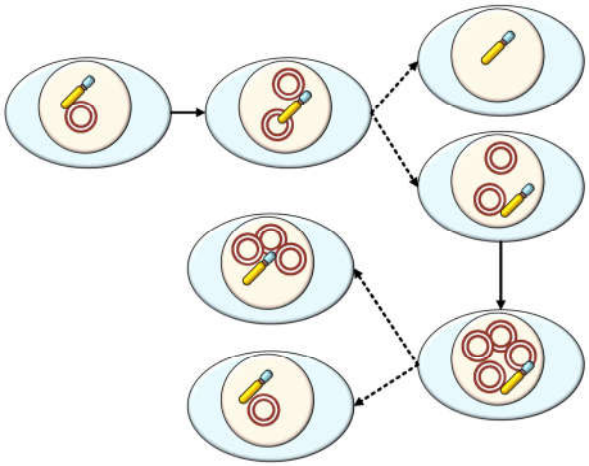

EXTRACHROMOSOMAL DNA AND CANCERS

Curiouser and Curiouser

ABSTRACT

Extrachromosomal DNA are circular DNA segments located in the nucleus. They are generally greater than 1 million bp and also often act as copy number variants of oncogenes or proto-oncogenes. We examine these in the context of the current literature.

Terrence McGarty

TGL 198 March 2023

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Extrachromosomal DNA

Contents

1 INTRODUCTION

There is a continuing understanding of the complexity of the underlying elements facilitating and enhancing various cancers. The old paradigm of here being some genetic change in a linear DNA sequence is now of limited use. Besides such changes as the multiple epigenetic changes, the conformational changes, the micro environmental changes, and the like we have the impact of extrachromosomal DNA. These are generally small and often circular DNA sequences located in the nucleus. They piggyback on the on promoters and other enzymes used by chromosomes to express RNA and subsequently proteins. However their presence is often seen only in malignant cells and they become a prodrome for an aggressive malignancy.

1.1 ECDNA AND INTRODUCTION

In this report we examine ecDNA and consider it from the perspective a one of several drivers of malignancies. There are several entities attempting to develop therapeutics for cells presenting with ecDNA. Thus from Boundless Bio[1]:

ecDNA are a primary site for activating oncogene amplifications in cancer. ***ecDNA are large units of circular DNA that reside within the nuclei of cells yet are physically distinct from chromosomal DNA****. They often range in size from 1-3 mega base pairs in length and can encode one or more full-length genes and regulatory regions. ecDNA have accessible chromatin and are highly transcribed, meaning they are fully functional and often more active than chromosomally located genes. ecDNA are one of the primary locations for high copy number focal oncogene amplifications in cancer cells; in fact, more than half of all high copy number amplifications in cancer occur on ecDNA.*

As NCI notes[2]:

Extrachromosomal circular DNAs (ecDNAs), or particles of DNA existing outside the autosomal genome, were discovered in the 1960s and more recently have been implicated in cancer development. EcDNAs frequently occur across many cancer types and often in high copy numbers. The oncogenes they carry are thought to be highly expressed compared to copy number-matched linear DNA. Cancers carrying ecDNAs are also associated with shorter survival for patients….

What are extrachromosomal circular DNAs (ecDNAs)? Do they have any known "normal" functions?...Extrachromosomal circular DNA elements are pieces of DNA that have broken off the linear chromosomes and circularized. There are two types: small 100 bp – 10 kb elements that can be found in many different cell types in the body, with unknown function. And then there are larger (50 kb – 5 Mb) oncogenic elements, which are only detected in cancer cells and carry

[1] https://boundlessbio.com/what-we-do/

[2] https://www.cancer.gov/about-nci/organization/ccg/blog/2022/interview-ecdna

genes known to activate cancer cells. These oncogenic ecDNAs are found in ~15% of newly diagnosed cancer.

…describe the different types of chromatin interaction assays you've used to study ecDNAs, such as ChIA-PET and ChIA-Drop? What kind of different information do they provide? …These are methods used to map genome-wide, long-range chromatin interactions between regulatory elements. ChIA-PET (Chromatin Interaction Analysis by Paired-End Tag sequencing) is a method we developed to reveal the general spatial chromatin organization and to identify chromatin interactions associated with specific proteins. The resulting paired sequences from ChiA-PET tell us about the connectivity between different genomic regions and the 3D organization of the chromatin.

While informative, ChIA-PET is limited in that it is only telling us about pairwise interactions aggregated from bulk cells—we don't know whether or not those pairs are occurring together within a single complex. So we have developed the ChIA-Drop (Chromatin Interaction Analysis by Droplet sequencing) chromatin interaction method to identify the combinations of chromatin interactions that occur within a single complex. …

…With its mobility and potentially high copy numbers, ecDNAs can potentially transverse the nucleus, and function as trans-acting, mobile transcriptional enhancers, establishing extensive chromosomal interactions and driving transcription of specific chromosomal genes. Thus ecDNAs may be a very powerful mechanism to promote cellular fitness.

… It is really challenging to accurately assign chromatin interactions to ecDNAs or chromosomes, given their nearly identical sequences. However, since ecDNA copy numbers far exceed chromosomal copy numbers, and chromosomal interactions are known to be greatly physically limited by their organization and structure, we believe that the majority of interactions we found are contributed by ecDNAs. In our study, we also perform imaging-based validation of interactions between ecDNAs and specific chromosomal sites.
Extracellular DNA may congregate as gene expression hubs.

…In the normal chromosomal context, to turn on a protein coding gene, the cell needs enhancers and promoters to control when the gene turns on and off. And those control elements are nearly always located on the same chromosome….

We discovered that inside the ecDNA hub there's very promiscuous sharing of DNA regulatory elements—one ecDNA can actually use enhancers from other ecDNAs, even if they derive from originally different chromosomes! For example, a gastric cancer we studied contains an ecDNA containing the MYC oncogene coming from chromosome 8 and also a separate ecDNA with FGFR2 from chromosome 10. When these ecDNAs intermix, an ecDNA oncogene can get all this input from other molecules, resulting in a very strong level of transcription. Using an enhancer from one ecDNA to activate an oncogene on another ecDNA is what we mean by "intermolecular enhancer–gene interactions."

…It seems so easy for an ecDNA to just ramp up oncogene expression (through either model)! Should we be terrified?... It really is kind of terrifying—we often think about the protein products

of oncogenes as what is helping the cell. But here, we're talking about the oncogenic DNA itself affecting each other's gene expression. Going back to this phenomenon of intermolecular activation that I mentioned earlier with gastric cancer example: once both MYC and FGFR2 become ecDNAs, they intermix, and now DNA elements from one is turning on the oncogene on the other one and vice versa. So normally those two sets of genes don't talk to each other or see each other, but now they have a chance to get together and have this very nefarious mode of gene activation.

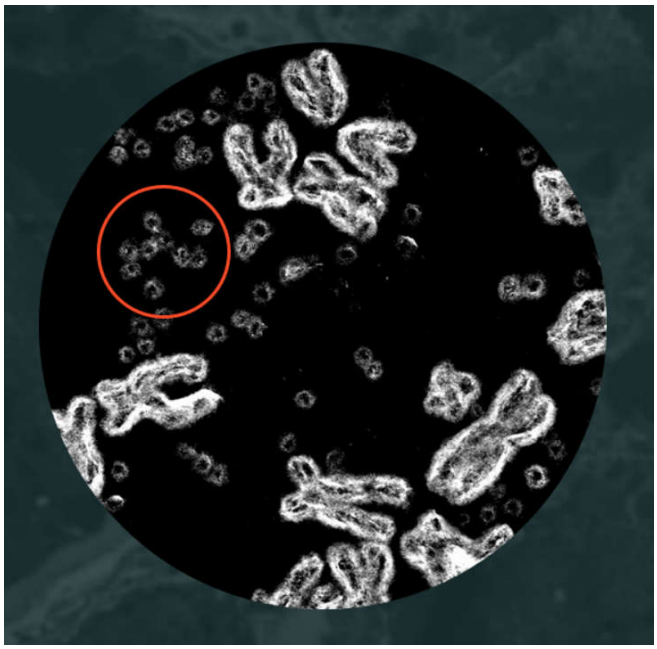
…EcDNAs can be detected across a wide range of cancer types, including but not limited to breast cancer, lung cancer, and ovarian cancer. Given the consistency of our observations between our glioblastoma and prostate cancer models, we anticipate our findings of the functioning of ecDNA elements as mobile enhancers will translate to many other cancer types in which ecDNAs can be detected….

Oncogenic ecDNAs are exclusively found in cancer cells and contribute to intratumoral heterogeneity and therapy resistance. Patients with ecDNA positive cancers have worse outcomes compared to those whose cancers do not contain ecDNA. We think ecDNA is demonstrating unique biological behavior, such as uneven segregation and functioning as mobile enhancers, which provide substantial rationale for the development of ecDNA-specific therapies.

As Zhu et al have noted:

Extrachromosomal, circular DNA (ecDNA) is emerging as a prevalent, yet less characterized oncogenic alteration in cancer genomes. We leverage ChIA-PET and ChIA-Drop chromatin interaction assays to characterize genome-wide ecDNA-mediated chromatin contacts that impact transcriptional programs in cancers. EcDNAs in glioblastoma patient-derived neurosphere and prostate cancer cell cultures are marked by widespread intra-ecDNA and genome-wide chromosomal interactions.

EcDNA-chromatin contact foci are characterized by broad and high level H3K27ac signals converging predominantly on chromosomal genes of increased expression levels. Prostate cancer cells harboring synthetic ecDNA circles comprised of characterized enhancers result in the genome-wide activation of chromosomal gene transcription. Deciphering the chromosomal targets of ecDNAs at single-molecule resolution reveals an association with actively expressed oncogenes spatially clustered within ecDNA-directed interaction networks. Our results suggest that ecDNA can function as mobile transcriptional enhancers to promote tumor progression and manifest a potential synthetic aneuploidy mechanism of transcription control in cancer.



Absent in normal healthy tissue, ecDNA are found in 14% of primary cancers and >40% of metastatic cancers.

Driving high copy number gene amplifications and non-Mendelian genomic adaptation, ecDNA enable tumors to rapidly evolve and switch their oncogene dependency when under therapeutic pressure, thereby rendering current targeted and immunotherapy approaches largely ineffective in patients with gene amplified cancers.

1.2 QUESTIONS

There are a set of questions we seek to explain. They are:

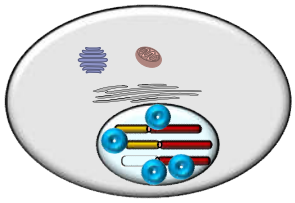
1. What are extrachromosomal DNAs?
2. What are the different types of extrachromosomal DNA
3. What are eccDNA vs ecDNA, and does eccDNA include ecDNA as well as others
4. Where are the ecDNA found in a cell
5. How are ecDNA created
6. What are ecDNA comprised of
7. How do ecDNA function in a cell
8. How do ecDNA replicate
9. What does ecDNA do to promote cancers
10. What therapeutics are there that can mitigate ecDNA adverse effects

11. What specific cancers are influenced by ecDNA and how are they so influenced

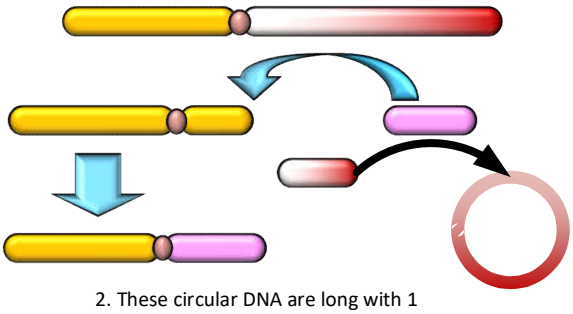
These are other related questions will be focused on in this report.

1.3 Some Answers

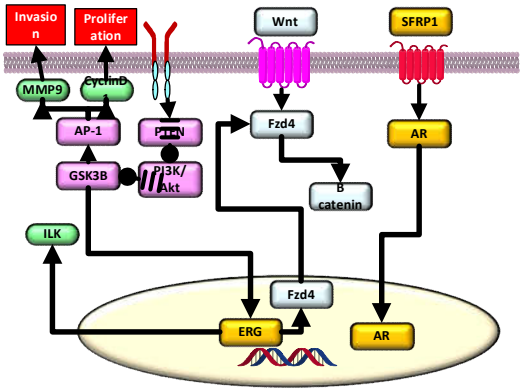
The following graphic is a summary of much of what we discuss herein. Namely the ecDNA infiltrates cells and results in metastatic behavior. The ecDNA can be produced in a variety of ways. It is a long strand of 1 million + bases and can provide all necessary for replication. Since the DNA has no centromere its replications is simpler and does not need a cell cycle.



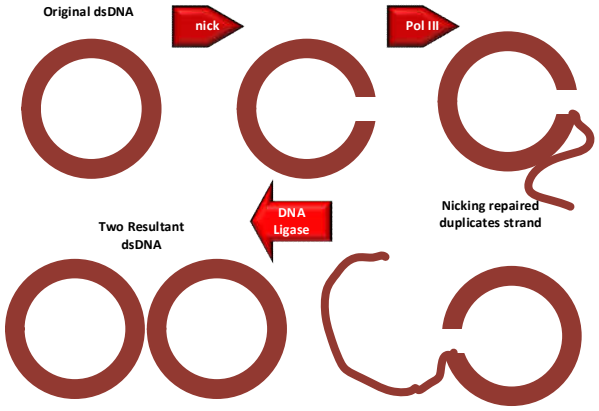
1. Many cancer cells have circular DNA separate from the chromosomes in the nucleus



2. These circular DNA are long with 1 million + bases and are generated from the parent DNA in many ways



4. dsDNA, ecDNA, produces multiple mRNA for cancer pathways driving metastatic result



3. dsDNS reproduces in nucleus generating large numbers

Thus some simple answers are:

1. ecDNA is a circular DNA located in the nucleus but not part of the chromosomal DNA
2. ecDNA is created by some alteration of the normal chromosome

3. ecDNA contains genes that are often oncogenes or proto-oncogenes and thus they tend to act as a copy number variation, CNV, causing the increase of proliferation or suppression of control elements.
4. Just how this process occurs is not fully known
5. ecDNA proliferates independent of the normal cell cycle.
6. Just what the drivers are for ecDNA proliferation are not fully known.
7. ecDNA can cause rapid metastasis and result in large amounts of ecDNA in the malignant cells
8. ecDNA may be a biomarker for cancers
9. Therapeutics for cancers containing ecDNA are being examined but none are currently acceptable

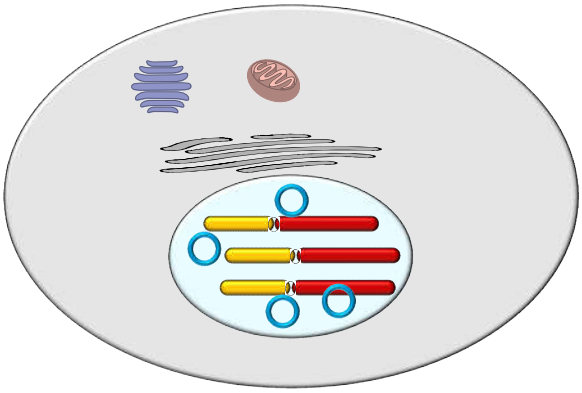
We examine these items based upon a growing amount of current literature.

2 TYPES OF EXTRA CHROMOSOMAL DNA

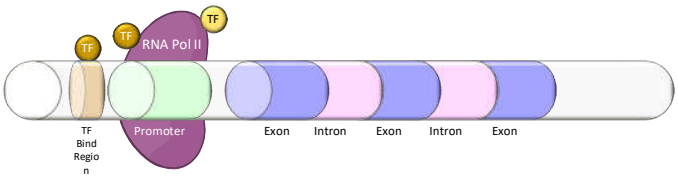
We now begin to describe at a high level the extrachromosomal DNA. The focus is on circular DNA.

2.1 BASIC ELEMENTS

We first review some basic elements. A prototypical cell is shown below. In the diagram we show the chromosomes in the nucleus but we also show circular DNA, namely it is eccDNA or extrachromosomal circular DNA a catchall phrase for any circular extrachromosomal DNA. As we noted earlier we desire to classify these and then determine how they are created, proliferate, and then effect malignant changes.

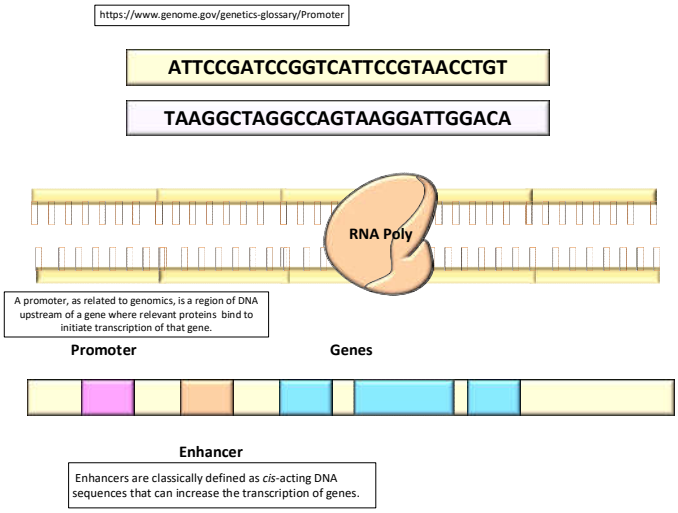


The figure below depicts the classic understanding of gene processing and the elements related thereto. These classic steps may not apply to eccDNA, and in fact, the steps and their understanding is still a work in progress.



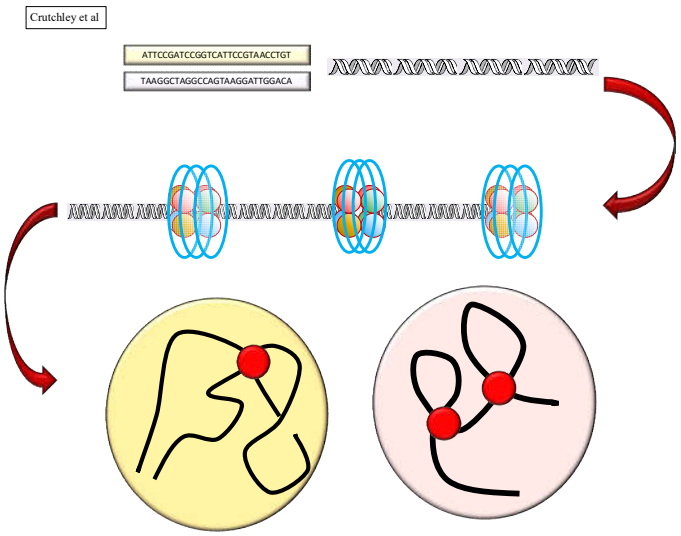
- Transcription Factor (TF): A protein that binds to a specific site on the DNA and which can exert activation or repression.
- Promoter: A DNA sequence which binds RNA Pol
- Operator: Site where a Repressor binds.
- Repressor: A protein that can bind to the Operator and inhibit transcription
- Activator: A protein that binds to the Activator site on the DNA and enhances transcription.
- Activator Binding Site: The site of binding of the Activator protein.
- RNA Polymerase: One of three types of polymerases that produce the actual transcription.

We further depict this below. The process is a well-controlled one controlled by the cell cycle mechanism. But we shall see that the eccDNA, especially the ecDNA, can over-ride the process that we neatly show below. We have previously discussed the complexities of chromatin conformation, namely the non-linear influences of DNA chromosome interactions[3]. In the case of ecDNA, that set of interactions become more complex.

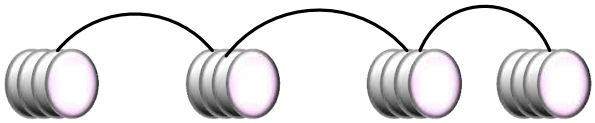


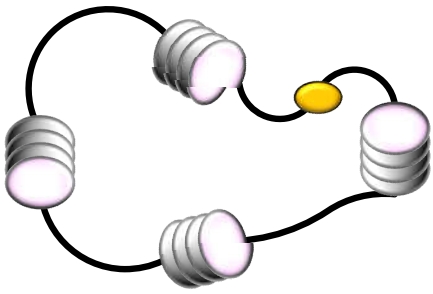
An example of some of the existing complexities is shown below.

[3] https://www.researchgate.net/publication/368661141_Prostate_Cancer_and_Chromatin_Conformation



Finally we can show below the purported construct differences between out classic DNA structures and that of an ecDNA. The ecDNA is circular, bonded together, with genes, promoters, enhancers and the ability to hijack polymerases to read the genes contained and produce RNA and thus proteins. The top example below is the classic model and the bottom is the ecDNA prototype.





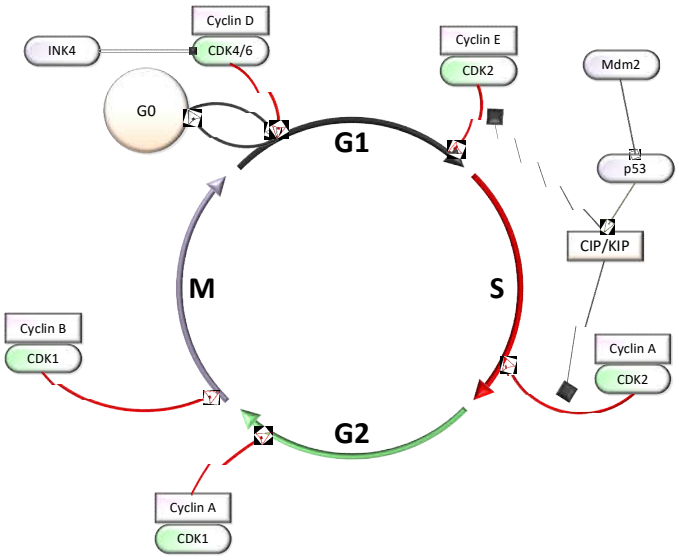
See Bafna and Mischel

We shall now proceed to fill in the details of these extrachromosomal DNA segments in the following sections.

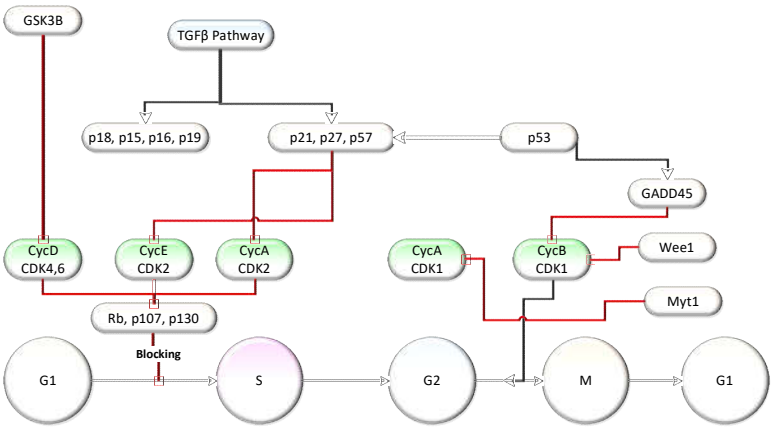
2.2 Cell Cycle

It is worth a brief review of the classic cell cycle. The reason is to be able to understand the similarities and differences between classic DNA in a chromosome and that of eccDNA. They act fundamentally differently. ecDNA does not follow the normal cell cycle.

The classic cell cycle is detailed below. It is driven by CDKs and cyclins. It allows for the splitting and duplication not only of the chromosomes but the cells themselves.



The DNA replications occurs in the S phase. That is for the chromosomes. The details of the pathways controlling the above cycle are shown below.



From Solimini et al: "*Known pathways with multiple shRNAs in the validation screen. Genes that validated in the secondary screen by a factor of ≥4 with multiple shRNAs are denoted with circles corresponding to three to five shRNAs (orange) and six or more shRNAs (red).*"

Now, all the above details do not necessarily apply to eccDNA. As we shall discuss they can reproduce or replicate themselves in an aggressive and more complex manner. They lack centromeres and thus do not have the ability to create new cells but do have the ability to

proliferate. The proliferation or replication process most likely parallels the S phase but it appears that it can be a continual process independent of the cell cycle. The question posed however is; what are the controlling elements in the cell and its environment that initiate the replication processes of eccDNA.

2.3 ECCDNA

Extrachromosomal circular DNA, eccDNA, is a term which encompasses a large set of circular sections of DNA found in the nucleus. Extrachromosomal DNA, ecDNA, is a specific type of eccDNA. Our focus here is ecDNA.

As Yang et al note:

Extrachromosomal circular DNA (eccDNA), ranging in size from tens to millions of base pairs, is independent of conventional chromosomes. Recently, eccDNAs have been considered an unanticipated major source of somatic rearrangements, contributing to genomic remodeling through chimeric circularization and reintegration of circular DNA into the linear genome. In addition, the origin of eccDNA is considered to be associated with essential chromatin-related events, including the formation of superenhancers and DNA repair machineries.

Moreover, our understanding of the properties and functions of eccDNA has continuously and greatly expanded. Emerging investigations demonstrate that eccDNAs serve as multifunctional molecules in various organisms during diversified biological processes, such as epigenetic remodeling, telomere trimming, and the regulation of canonical signaling pathways. Importantly, its special distribution potentiates eccDNA as a measurable biomarker in many diseases, especially cancers.

The loss of eccDNA homeostasis facilitates initiation, malignant progression, and heterogeneous evolution in many cancers. An in-depth understanding of eccDNA provides novel insights for precision cancer treatment. In this review, we summarized the discovery history of eccDNA, discussed the biogenesis, characteristics, and functions of eccDNA. Moreover, we emphasized the role of eccDNA during pathogenesis and malignant evolution. Therapeutically, we summarized potential clinical applications that target aberrant eccDNA in multiple diseases

2.4 TYPES

As Noer et al have indicated:

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 …*

Covalently 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, but the term is now mostly used in the field of virology.

***Small polydisperse circular DNA (spcDNA)**: 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 … spcDNA was used to describe eccDNA at the smaller end of the size spectrum (<100–10 000 bp) …. The name comes from their heterogeneous size distribution and sequence content. spcDNA was described as mainly containing repetitive genome sequences, although this could reflect the limited DNA sequence analysis methods available at the time rather than the true frequency of repeat sequences on spcDNA. …*

***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…*

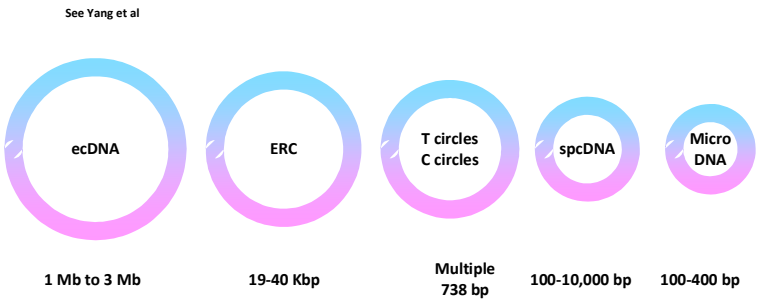
***Telomeric circles:** Telomeric circles are a specialized group of eccDNA that have been found to be important in immortalization of telomerase-negative cancers though the alternative lengthening of telomeres (ALT) mechanism. Telomeric circles serve as templates for telomere elongation and the ALT mechanism is reported to be responsible for telomere maintenance in 10–15% of all cancers. …*

***DMs**: Large eccDNA in the megabase range … DMs are a DNA species without recognizable telomeres and centromeres 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.*

***Episomes:** …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. … 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.*

***ecDNA**: …. sequences of circular DNA from tumors …. ecDNA with oncogenes is reported in a broad variety of tumors …especially common in GBM and prostate, breast, lung, and renal carcinoma, as well as melanoma.*

We show graphically the types of circular DNA that make up eccDNA below based upon Yang et al:



Yang et al use the following definitions:

Small polydispersed DNA (spcDNA)*: SpcDNA is an obsolete concept to commonly characterize small eccDNAs that are between hundreds of bp to a few thousand bp and measure 0.05 to 2.00 μm. SpcDNA was first observed in the electron microscope examination of the closed DNA from unfractionated HeLa cells in 1967. From the 1980s to the 1990s, repetitive sequences were widely detected in spcDNAs, and therefore, it is speculated that spcDNA mainly originates from repetitive regions in the genome*

MicroDNA*: MicroDNA, with an average length of 100 to 400 bp, is derived from unique non-repetitive genomic regions with high gene density. It is enriched in the 5'-untranslated regions of genes, exons, and CpG islands.82 In terms of distribution, microDNAs are ubiquitous in normal cells of every species, from yeast to humans.98 Studies have shown that microDNA levels are dependent on microhomology-mediated end-joining (MMEJ), inhibited by the c-NHEJ pathway, and stimulated by DNA damage*

Telomeric circle (t-circle/c-circle) *Telomeric circles, as a special type of eccDNA, are duplex (t-circle) or single-stranded (c-circle), consisting only of telomeric repeats. They are integral multiples of 738 bp sequences. T-circles occur in a wide range of organisms, including yeasts, plants, and animals. Various DNA damage-associated proteins may regulate the production of t-circles.*

Extrachromosomal rDNA circle (ERC) *ERCs have an average size of 19.3 to 40.4 kb. ERCs can be produced by intramolecular HR of chromosomes and function as templates for ribosomal RNA transcription. They are much more abundant in healthy tissue. Additionally, ERCs can self-replicate due to their autonomously replicating sequences.*

Extrachromosomal DNA (ecDNA) *EcDNA was first discovered as paired small chromatin bodies in 1964 and was referred to as* ***DMs****.35 DMs were first found in metaphase neuroblastoma cells and subsequently found in numerous types of cancers. With the combined applications of WGS, structural modeling, and computational and cytogenetic analysis, Turner et al. analyzed 17 different cancer types and showed that only 30% of ecDNA in tumor cells presents with DMs-like features. As this group of eccDNAs can either be detected in a double-body form or a single-body form, the definition of these extrachromosomal particles needs to be broadened.*

Therefore, the term ecDNA refers to those gene-containing extrachromosomal particles of DNA with a size range from 1 to 3 Mb, including both DMs and single-body forms.

EcDNA lacks centromeres and segregates randomly or asymmetrically during cell division. Gene sequences on ecDNA are highly rearranged and amplified. It integrates multiple regions scattered throughout different chromosomes in tumors. In addition, breakpoints were found to be randomly distributed around oncogenes. Hence, ecDNA is unlikely to have a unified genome template

Note the difference between Noer et al and Yang et al. They are subtle but do tend to indicate the ongoing developmental stage of understanding. Our interest is primarily in ecDNA.

2.5 FORMATION

The formation or creation of eccDNA in general has been examined. Again from Noer et al we have the following:

A number of models exist for how eccDNA is formed in human cells.

It is critical to note that these are still works in progress. As we shall note later for ecDNA the uniqueness and selectivity have yet to be adequately addressed. They continue:

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, or secondary DNA loop structures formed in several processes, for example, mismatch repair (MMR), could be excised and circularized.*

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), 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.

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.

There are also reports of eccDNA forming between regions with high homology, potentially through homologous recombination (HR). The newer reports of this were all studies in S. cerevisiae, but this was also observed in early human cell studies …

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. 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), while another indicated that they had occurrences of repeat elements at the same level as the whole genome.

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. 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 amount. In cells lacking proteins essential for NHEJ, both decreased and increased amounts of eccDNA have been reported.

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…

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. 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.

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 γ 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.

In S. cerevisiae, re-replication and oligonucleotide-stimulated DNA amplification have also been suggested as sources of eccDNA, 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. 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. …chromothripsis is an important driver of cancer genome rearrangements including ecDNA formation.*

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.*

3 ecDNA

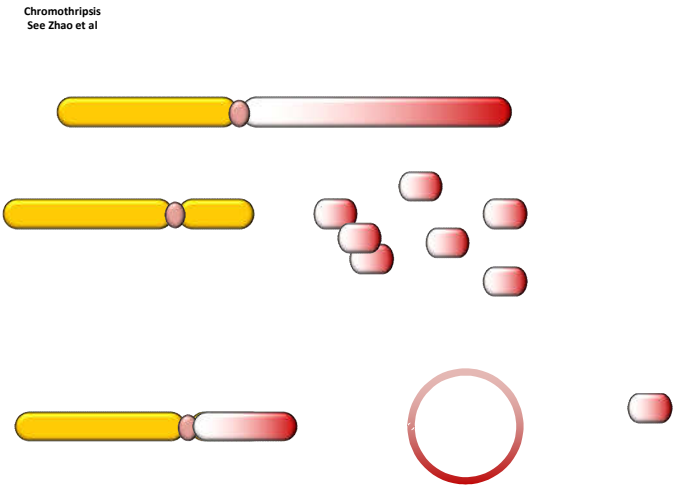
We now focus on ecDNA, the larger set of extrachromosomal DNA elements that appear to have a driving influence on the development and progression of cancers.

3.1 Creation of ecDNA

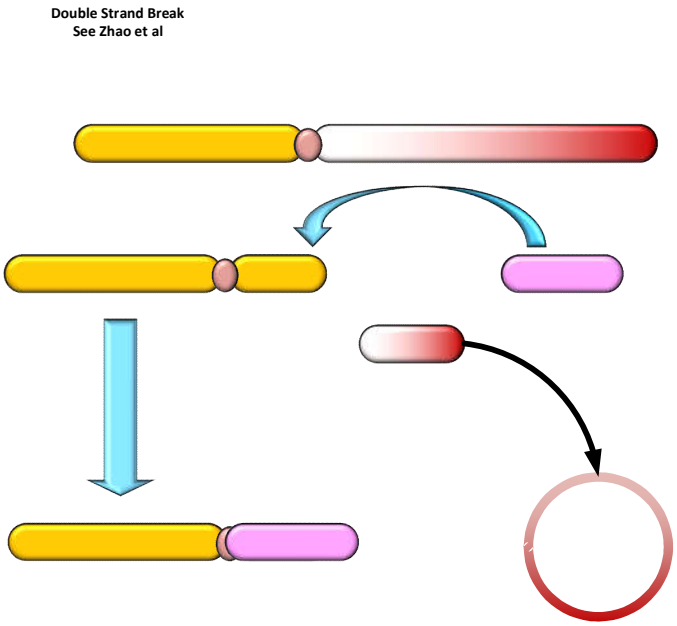
The next question we address is; how are ecDNA formed? We began a general discussion above and new seek to provide some specifics on ecDNA. We show three possible variations below as they are generally presented in the literature.

First is chromothripsis. This is a result of a "smashing" a part of the DNA into small sections and the assembly into a ring, an ecDNA, and a mutated chromosome. As Shorokhova et al note:

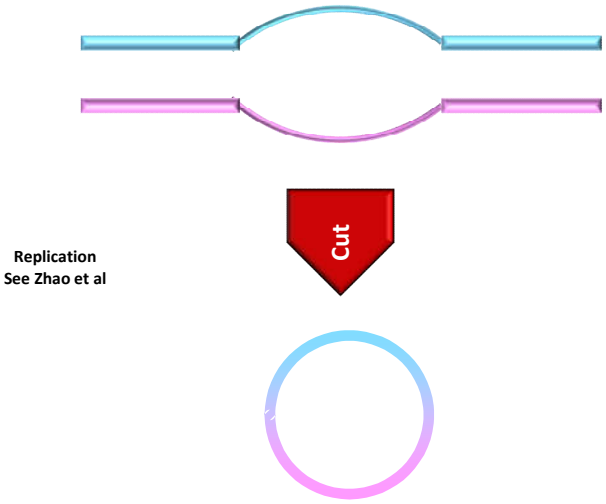
Chromothripsis is a complex chromosomal rearrangement and is characterized by up to thousands of cluster chromosomal rearrangements that occur simultaneously and are localized in limited regions of the genome in one or several chromosomes. Thripsis in translation from Greek means destruction into small parts. Stephens with colleagues first described chromothripsis in 2011 when massive genome rearrangements were detected in patients with lymphocytic leukemia. In this work, the authors were faced with an unusual case in which one of the patients had 42 genomic rearrangements localized in the long arm of chromosome 4, associated with chromosomal breaks at several points with the subsequent random assembly of fragments.



The second type is a double strand break. We show that graphically below. It is the cutting out of a strand, the creation of the circular DNA and the bonding of the remaining chromosome elements.



The third is a replication where two parts are reproduced and then cuts as shown below.



There also are a variety of other ways to create this circular DNA and they are discussed in the literature. What we have been discussing thus far is all occurring in the nucleus and interacting with the area of a chromosome or even inter-chromosome. But recent work has demonstrated that extrachromosomal DNA, ecDNA, has a controlling impact as well[4]. As NCI has noted[5]:

[4] https://news.cancerresearchuk.org/2023/02/20/how-ecdna-drives-cancer-evolution/

[5] https://www.cancer.gov/about-nci/organization/ccg/blog/2022/interview-ecdna

Extrachromosomal circular DNAs (ecDNAs), or particles of DNA existing outside the autosomal genome, were discovered in the 1960s and more recently have been implicated in cancer development. EcDNAs frequently occur across many cancer types and often in high copy numbers. The oncogenes they carry are thought to be highly expressed compared to copy number-matched linear DNA.

Cancers carrying ecDNAs are also associated with shorter survival for patients. …

Extrachromosomal circular DNA elements are pieces of DNA that have broken off the linear chromosomes and circularized. There are two types: small 100 bp – 10 kb elements that can be found in many different cell types in the body, with unknown function. And then there are larger (50 kb – 5 Mb) oncogenic elements, which are only detected in cancer cells and carry genes known to activate cancer cells. These oncogenic ecDNAs are found in ~15% of newly diagnosed cancer. …

These are methods used to map genome-wide, long-range chromatin interactions between regulatory elements. ChIA-PET (Chromatin Interaction Analysis by Paired-End Tag sequencing) is a method we developed to reveal the general spatial chromatin organization and to identify chromatin interactions associated with specific proteins. The resulting paired sequences from ChiA-PET tell us about the connectivity between different genomic regions and the 3D organization of the chromatin.

While informative, ChIA-PET is limited in that it is only telling us about pairwise interactions aggregated from bulk cells—we don't know whether or not those pairs are occurring together within a single complex. So we have developed the ChIA-Drop (Chromatin Interaction Analysis by Droplet sequencing) chromatin interaction method to identify the combinations of chromatin interactions that occur within a single complex. …

Our goal was to determine the spatial chromatin organization and chromatin interactions of ecDNAs in general. Given the circular structure of ecDNAs, we anticipated that they would exhibit unique spatial patterns. We uncovered very high levels of chromosome connectivity and transcriptional activity: the ecDNAs exhibit a pattern of dense and widespread chromatin interactions with actively transcribed genes and regulatory elements that reside both within the ecDNA (in cis) and on the chromosomes (in trans).

We reasoned that this is because the small size of ecDNAs allow them to move freely amongst the chromosomes. Such mobility could enable ecDNAs to interact with genes residing on chromosomes. Moreover, the interaction sites on the ecDNAs exhibited key characteristics of super-enhancers (SEs), which are known to exert a unique regulatory influence that could promote tumorigenesis. …

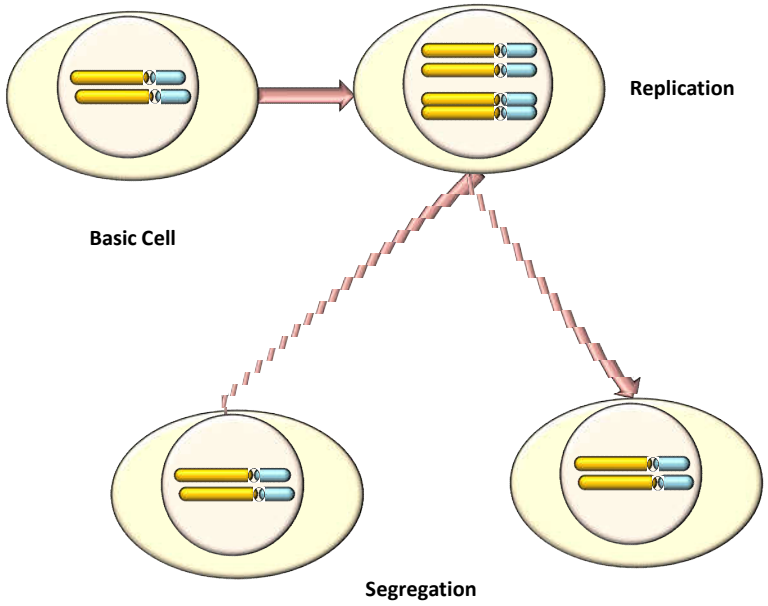
With its mobility and potentially high copy numbers, ecDNAs can potentially transverse the nucleus, and function as trans-acting, mobile transcriptional enhancers, establishing extensive chromosomal interactions and driving transcription of specific chromosomal genes. Thus ecDNAs may be a very powerful mechanism to promote cellular fitness*. …*

We observed a sharp elevation of nTIFs in ecDNA regions compared to the interaction frequencies of their corresponding native chromosomal regions in cells without ecDNAs. These interactions were specifically enriched with chromosomal promoters. Chromosomal genes whose promoters interact with ecDNAs were expressed at significantly higher levels.

There has recently been a great deal of investigation of this area[6]. The major issue with the above paradigms is that ecDNA in cancer seem to neatly extract oncogenes and protooncogenes along with necessary sequences for proliferation. Just how this is accomplished in not at all certain. One would expect that this is a purely random process yet it seems to be quite directed. The literature appears to not address this issue at all. Thus, a further set of investigations combining these disparate areas appears to present a significant opportunity.

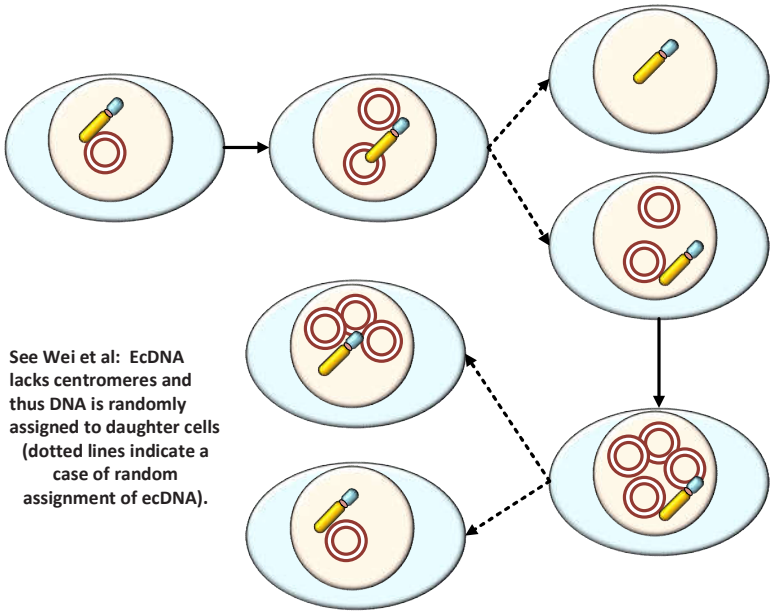
3.2 Dynamics and Replication of ecDNA

We understand how the cell cycle works and reproduced identical offspring, subject to normal processes. The general construct is shown below. Namely the chromosome duplicate and the process is generally flawless. The centromere is critical to this process. The general understanding is shown below.



Now we have a different issue with ecDNA. With no centromere to anchor the process down there is a random like reproduction of the ecDNA. Conceptually we show the Wei understanding below.

[6] See Wang et al, Zhao et al, Zuo et al, Kim et al, and Li et al



There are multiple views of this process. Recall that DNA replication of circular DNA is somewhat straight forward. As noted in Nature[7]:

Replication is the process by which a double-stranded DNA molecule is copied to produce two identical DNA molecules. DNA replication is one of the most basic processes that occurs within a cell. Each time a cell divides, the two resulting daughter cells must contain exactly the same genetic information, or DNA, as the parent cell. To accomplish this, each strand of existing DNA acts as a template for replication.

Replication occurs in three major steps: ***the opening of the double helix and separation of the DNA strands, the priming of the template strand, and the assembly of the new DNA segment.***

During separation, the two strands of the DNA double helix uncoil at a specific location called the origin. Several enzymes and proteins then work together to prepare, or prime, the strands for duplication. Finally, a special enzyme called DNA polymerase organizes the assembly of the new DNA strands. The following description of this three-stage process applies generally to all cells, but specific variations within the process may occur depending on organism and cell type.
What triggers replication?

The initiation of DNA replication occurs in two steps.

First, a so-called initiator protein unwinds a short stretch of the DNA double helix.

[7] https://www.nature.com/scitable/topicpage/cells-can-replicate-their-dna-precisely-6524830/

Then, a protein known as helicase attaches to and breaks apart the hydrogen bonds between the bases on the DNA strands, thereby pulling apart the two strands.

As the helicase moves along the DNA molecule, it continues breaking these hydrogen bonds and separating the two polynucleotide chains.

Meanwhile, as the helicase separates the strands, another enzyme called primase briefly attaches to each strand and assembles a foundation at which replication can begin.

This foundation is a short stretch of nucleotides called a primer….

In the prokaryotic bacterium E. coli, replication can occur at a rate of 1,000 nucleotides per second. In comparison, eukaryotic human DNA replicates at a rate of 50 nucleotides per second. In both cases, replication occurs so quickly because multiple polymerases can synthesize two new strands at the same time by using each unwound strand from the original DNA double helix as a template. One of these original strands is called the leading strand, whereas the other is called the lagging strand.

Now as Yang et al note:

Advances in nextgeneration sequencing technologies and computational analysis technologies have revealed several key structural features of eccDNA as follows:

First, eccDNAs are circular and independently replicate outside of chromosomes.

A head-to-tail configuration in the nucleotide sequence was detected through polymerase chain reaction and mapping by restriction enzyme digestion of eccDNA in a human neuroblastoma cell line.

A recent study from late 2019, which combined DNA sequencing and high-resolution imaging, obtained definitive evidence of the circular shape of eccDNA.

Second, eccDNAs vary widely in size, from a few dozen base pairs to hundreds of thousands of base pairs.

The sizes and features of eccDNAs vary in different life stages and tissues. Fetal derived eccDNAs are shorter and hypomethylated compared with maternal eccDNAs.25,81 The methylation density of eccDNA is positively correlated with its size. Recent studies have shown that most eccDNAs in normal cells are less than 1000 bp in length.

Cancer cells have larger eccDNAs than normal cells (usually greater than 1 kb), and these eccDNAs are long enough to carry the full-length region for the amplification of oncogenes.

Third, eccDNAs have different genetic contents, which constitute the structural diversity of eccDNAs.

According to their genomic origins and genetic contents, eccDNAs can be categorized into the following eight types: full-gene eccDNA, exon eccDNA, intron eccDNA, repeat eccDNA, repeat-intergenic eccDNA, intergenic eccDNA, TE eccDNA, and promoter/enhancer eccDNA.

The structural diversity and topological structure of eccDNA contribute to the versatility of its functions by possibly driving the expression of coding RNAs, noncoding RNAs, and other RNAs. …

Recent evidence suggests that eccDNA is also associated with aging.

First, eccDNA accumulates dramatically as cells age in yeast and mammals. Kunisada T et al. analyzed the aging process of rat lymphocytes and human lung fibroblasts and found that the size and copy number of eccDNA increased. Sinclair DA et al. showed that the accumulation of ERCs is a general phenomenon that occurs in aging yeast cells.

These ERCs are able to replicate with an autonomously replicating sequence (ARS), and they are preferentially segregated to mother cells in each cell division. *Such asymmetrical segregation results in a marked increase in ERCs in aging mother cells and limits the amount of ERCs in daughter cells. It has been detected that aging yeast mother cells typically exhibit progressive enlargement and fragmentation of the nucleolus due to the accumulation of ERCs. Furthermore, such accumulation is more obvious after Sgs1 mutations, but the lack of Fob1 and Bud6 will reduce the accumulation of ERCs and extend life.*

Episome model *The episome model is one of the classic models for the biogenesis of eccDNA, where eccDNAs are produced by DNA slippage and R-loops during the DNA synthesis process. These eccDNAs are also named episomes.*

Episomes are able to self-replicate and can be expanded by incorporating other DNA components, such as transposable elements (TEs) and enhancers/promoters …

MYC-containing DMs in leukemia cases are triggered by excision and amplification, which underpins the episome model. Additionally, they also investigated ten cell lines from solid tumors and demonstrated that the MYC-containing ecDNAs are derived from excision and amplification as well, which expands the applicability of the episome model to solid tumors.

Furthermore, the formation of EGFR-containing ecDNAs results in the generation of cancer-associated circular EGFR amplicons, contributing to the oncogenic activation of EGFR. As the production of ERCs depends on DSB formation at the replication fork barrier (RFB), it is possible that ERCs are released from combined fork breakage at two neighboring replication forks.

3.3 FUNCTIONS OF ECDNA

ecDNA, especially those related to cancers, are copy number variations, CNVs. The specific CNVs are often from oncogenes and thus promote or enhance carcinogenic changes. The timing of the appearance of these specific ecDNA are yet to be fully understood. But principally their

existence facilitates the proliferation of cancer cells. We shall examine what genes are often found and what their functions are.

As Wang et al have noted:

Recent studies have demonstrated fundamental roles of ecDNA in cancer in modulating cell growth, metastasis/invasion, autophagy, DNA damage repair, drug response and clinical outcome. In addition, ecDNA contributes to intra-tumoral heterogeneity through genetic, epigenetic and microenvironmental factors. …

Oncogene amplification is one of the driving factors of tumorigenesis and can occur at either the HSR structures on chromosomes or ecDNA.

Studies have reported significantly elevated copy numbers of oncogenes encoded in ecDNA (e.g. EGFR, MYC, CDK4, and MDM2).

This set of genes raises the question; why, given the putative creation of ecDNA, do we find such a specific selection process so neatly performed? They continue:

The amplification of oncogenes in ecDNA markedly increases overall oncogene expression, which can be found in both primary and metastatic tumors regardless of treatment. In addition to elevating oncogene levels by copy number amplification, ecDNA may re-integrate into HSRs of chromosome and/or affect DNA accessibility to further "stabilize" the expression of oncogenes (e.g. EGFR in glioblastoma)

The distinct inheritance pattern of ecDNA differs from the traditional Mendel's law of inheritance and raises the question of whether and how the location of amplified oncogenes impacts tumorigenesis. In this regard, Lobachev et al. found that the breaking sites of yeast chromosomes determine the consequences of gene amplification. EcDNA is often observed to be produced from oncogene amplification, if the breaking sites locate between the hairpin break and the telomere. In contrast, when the break occurs between the oncogene and telomere, the amplification of oncogenes will generate HSR.

Importantly, a positive feedback regulatory loop between the elevated expression of ecDNA-encoded genes and the accumulation of ecDNA has emerged.

Hull et al. found that yeast cells obtain high levels of ecDNA containing the copper resistance gene CUP1 under copper exposure, and CUP1 expression may cause further accumulation of CUP1-bearing ecDNA. Moreover, Ji et al. showed that down-regulation of genes in ecDNA may result in the integration of ecDNA into cytoplasmic micronuclei and the subsequent reduction of ecDNA.

These results reveal a mechanistic link between the accumulation of ecDNA and oncogene hyper-activity.

As Li et al have noted:

Through the analysis of allele-specific RNA sequencing, it was found that ecDNA can be used as a template for gene transcription.

More intriguingly, oncogenes encoded on ecDNA generally have high expression levels. The abundance of ecDNA-derived transcripts was the highest in tumours. Generally, ecDNA facilitates oncogene overexpression in two modes.

First, ecDNA with a high copy number level was detected in tumours, where its number can reach the hundreds.

The ability of ecDNA to embrace a high copy number is probably related to the uneven separation of ecDNA. Although ecDNA segregates unevenly, ecDNA is not lost during mitosis, such as moving into micronuclei. EcDNAs might tether themselves to chromosomes during cell division to avoid the loss. In fact, a high copy number is only one explanation for the high expression of oncogenes.

Second, ecDNA containing highly accessible chromatin has been confirmed by assays of accessible chromatin (ATAC-seq, ATAC-see, etc.).

Given that the circular topological structure endows ecDNAs with higher chromatin accessibility, ecDNA boasts higher transcriptional activity. Consistently, recent studies have reported that even chromosomal DNA and ecDNA have similar copy numbers, ecDNA exhibits a huge advantage in transcribing oncogenes. Cis-regulation function of ecDNA.

The information encoded in DNA is usually determined by its physical appearance. As long as the DNA forms a circular structure, the fragments of ecDNA will develop a novel chromatin domain that is different from their linear chromosome structure. Interestingly, because DNA forms a circular conformation, it is possible to bring distant DNA elements (enhancers) nearby, forming a new cis-regulatory configuration that is impossible for chromosomal DNA.

Thus, ecDNA acts as a stronger enhancer hijacking vector to participate in the evolution of tumours. Enhancer hijacking that occurs on ecDNA typically exhibits two patterns: the local enhancer hijacking model and the distal enhancer hijacking model.

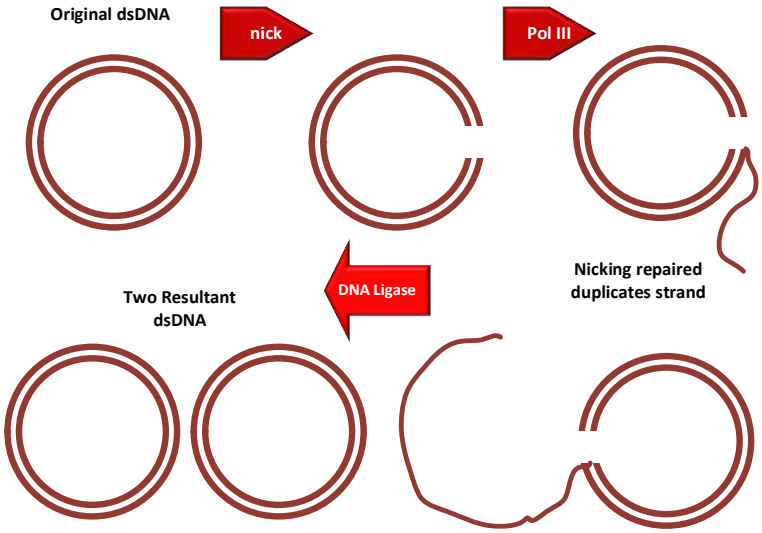
In the local enhancer hijacking model, the ecDNA circularizes and hijacks the enhancers at the distal end of the oncogene to bring them into the vicinity. Insulators insulate enhancers from oncogenes, which can prevent enhancers from participating in oncogene regulation in the chromosomal DNA.

As long as the enhancers and the oncogene coexist in an ecDNA circular domain, the enhancers can cross the insulator and participate in the transcriptional regulation of the oncogene.

It has been found that the oncogene EGFR often co-amplifies with upstream enhancers and forms ecDNA in glioblastoma, generating new enhancer-oncogene contacts and promoting tumour progression.

If we intervene in the ecDNA-specific domain, it is possible to weaken the transcriptional regulatory function of the enhancer, thereby affecting oncogene expression and delaying tumour progression.

The putative process for proliferation is a rolling-circle replication as shown below and discussed by Wawrzyniak et al.



Wawrzyniak et al note:

Horizontal gene transfer (HGT) contributes greatly to the plasticity and evolution of prokaryotic and eukaryotic genomes. The main carriers of foreign DNA in HGT are mobile genetic elements (MGEs) that have extremely diverse genetic structures and properties.

Various strategies are used for the maintenance and spread of MGEs, including

(i) vegetative replication,

(ii) transposition (and other types of recombination), and

(iii) conjugal transfer.

In many MGEs, all of these processes are dependent on rolling-circle replication (RCR). RCR is one of the most well characterized models of DNA replication.

Although many studies have focused on describing its mechanism, the role of replication initiator proteins has only recently been subject to in-depth analysis, which indicates their involvement in multiple biological process associated with RCR. In this review, we present a general overview of RCR and its impact in HGT. We focus on the molecular characteristics of RCR initiator proteins belonging to the HUH and Rep_trans protein families. Despite analogous mechanisms of action these are distinct groups of proteins with different catalytic domain structures. This is the first review describing the multifunctional character of various types of RCR initiator proteins, including the latest discoveries in the field.

Recent reports provide evidence that (i) proteins initiating vegetative replication (Rep) or mobilization for conjugal transfer (Mob) may also have integrase (Int) activity, (ii) some Mob proteins are capable of initiating vegetative replication (Rep activity), and (iii) some Rep proteins can act like Mob proteins to mobilize plasmid DNA for conjugal transfer. These findings have significant consequences for our understanding of the role of RCR, not only in DNA metabolism but also in the biology of many MGEs.

Namely we can see the process as follows:

It starts with an initiator protein which nicks the circle DNA opening it up. That protein is bound to a 5' end and the 3' end becomes a primer for the extension which is created. The un-nicked portion becomes a template and a helicase is used for the extension. Various polymerases are used such as Pol III shown above and the single strand developed and then matched to create a fully second stranded copy. The net result is that one obtains an identical copy of the original strand. This process occurs independent of any cell cycle we have reviewed above.

In the cell cycle the circular DNA are then sorted in a random fashion to the two identical cells wherein the may again replicate freely ultimately filling up the nucleus.

As we shall note later, however, the details of how this is temporally initiated is not fully understood at this time.

4 ecDNA AND CANCER

We now examine the impact of ecDNA on various cancers. The primary driver is the proliferation of oncogene and protooncogene transcription factors such as MYCN and others. As Bafna and Mischel have noted:

In cancer, complex genome rearrangements and other structural alterations, including the amplification of oncogenes on circular extrachromosomal DNA (ecDNA) elements, drive the formation and progression of tumors.

ecDNA is a particularly challenging structural alteration. By untethering oncogenes from chromosomal constraints, it elevates oncogene copy number, drives intratumoral genetic heterogeneity, promotes rapid tumor evolution, and results in treatment resistance. The profound changes in DNA shape and nuclear architecture generated by ecDNA alter the transcriptional landscape of tumors by catalyzing new types of regulatory interactions that do not occur on chromosomes.

The current suite of tools for interrogating cancer genomes is well suited for deciphering sequence but has limited ability to resolve the complex changes in DNA structure and dynamics that ecDNA generates. Here, we review the challenges of resolving ecDNA form and function and discuss the emerging tool kit for deciphering ecDNA architecture and spatial organization, including what has been learned to date about how this dramatic change in shape alters tumor development, progression, and drug resistance

As is well known, the CNV[8] presence is an accelerator of the malignant process:

Refers to the genetic trait involving the number of copies of a particular gene present in the genome of an individual. Genetic variants, including insertions, deletions, and duplications of segments of DNA, are also collectively referred to as copy number variants. Copy number variants account for a significant proportion of the genetic variation between individuals. Also called CNV.

From Hastings et al:

Copy number variants (CNVs) arise by homologous recombination (HR) between repeated sequences (recurrent CNVs). Or by non-homologous mechanisms that occur throughout the genome (non-recurrent CNVs). Non-recurrent CNVs frequently show microhomology at their end-points, and can have complex structure. Locus-specific mutation frequency for CNV and other structural changes are 2 to 4 orders of magnitude greater than for point mutations. HR mechanisms generally achieve accurate repair of DNA damage.

Double-strand breaks are repaired by HR or by end-joining mechanisms, which are generally non-homologous. Broken replication forks with single double-strand ends are also repaired by

[8] https://www.cancer.gov/publications/dictionaries/genetics-dictionary/def/copy-number-variant

HR. There is evidence that repair of broken replication forks underlies some non-homologous recombination. Repair of broken forks in stressed cells could cause non-homologous repair because of stress-induced down-regulation of HR Proteins

As Noer et al note:

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, but unlike normal chromosomes and ring chromosomes, these elements lack recognizable centromeres, 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. 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.

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. Thus, some daughter cells can achieve survival and proliferation advantages …

ecDNA amplification elevates oncogene copy number faster than chromosomal amplification, 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

The above basically notes two factors. First the ecDNA replicate independently of the cell cycle itself. The replicate at random times. Second, the ecDNA during a cell cycle can segregate at random to the daughter cells and then commence replication there as well. What we do not seem to know is the elimination of the ecDNA.

From Wei et al we have the following table of CNV oncogene drivers found typically in ecDNA:

Name of gene	Function related to tumor
ATM	Promote tumor cell cycle progression, proliferation, and tumor growth
CAD	Promote multidrug resistance in tumors
CCND1	Promote tumor cell cycle progression and proliferation
CCND3	Promote tumor cell cycle progression and proliferation
CCNE1	Promote tumor cell cycle progression and proliferation
CDK4-MDM2 gene cluster	Promote drug resistance in tumors
CyclinD2	Promote tumor cell cycle progression and proliferation
DHFR	Promote tumor cell resistance to methotrexate
EGFR	Promote tumor growth, inhibit apoptosis, lead to tumor drug resistance
ERBB2	Promote tumor growth and proliferation
GBAs	Maintain the malignant features of the tumor
LANCL2	Promote multidrug resistance in tumors
MDR1	Promote multidrug resistance in tumors
Mecom-PIK3CA-SOX2 gene cluster	Promote tumor metastasis and recurrence
MET	Promote the proliferation of tumor cells
MRPS17	By encoding mitochondrial ribosomal proteins, promoting tumor growth
MYC	Promote cell proliferation and inhibit apoptosis
MYCN	Promote Gl/S phase progression and tumor invasion
PEGFRA	Promote tumor proliferation and increases the adaptive potential of tumor cells
REL	Promote tumor cell development and proliferation
SEPT14	Regulate the proliferation or apoptosis of tumor cells
V0PP1	Promote tumor cell survival
ZNF713	Involved in transcriptional regulation and tumorigenesis

As Hong et al note regarding specific cancers and oncogenes:

Traces of ecDNA activity and mutation can be found in a variety of tumor cells, including thyroid cancer, ovarian cancer, hepatic carcinoma, gastric carcinoma, neuroblastoma, neuroepithelioma, colon cancer and prostate carcinoma. In a sort of sense, ecDNA remodels the epigenomic landscape phenotype of chromosomal genome and affects chromosomal gene expression and tumorigenesis.

Oncogenes on ecDNA include epidermal growth factor receptor (EGFR), MYC, c-MYC, HER2, platelet derived growth factor receptor (PDGFRA), mesenchymal-epithelial transition factor (MET), MECOM/ PIK3CA/SOX2 gene cluster and CDK4/Murine Double Minute 2 (MDM2) gene cluster. The improved chromatin accessibility of ecDNA brings a higher amplification level to oncogenes. And the presence of these oncogenes creates the necessary conditions for malignant progression.

For instance, EGFR signal pathway can activate the RAS/ MAPK/ERK, PI3K/AKT, p38 and STATS pathways to promote tumorigenesis.

Furthermore, the over-expression of MYC can affect many cells functions including cell cycle, self-renewal, survival, growth, metabolism, protein and ribosomal biogenesis, differentiation and canceration.

Tumorigenesis, tumor progression and cancer immunosuppression in various carcinoma types can be promoted by an over-activation of the MET axis. The highly expressive nature of oncogenes encoded in ecDNA are also identified by the relative high copies of oncogenes on ecDNA compared to any other gene expression … taking EGFR/p38 pathway as example, the presence of ecDNA structure drove the amplification of oncogenes and thus to elevate the tumorigenesis transcription level directly

4.1 MYCN EXAMPLE

We present a simple MYCN example to demonstrate. The MYC family is an essential set of proto-oncogenes seen in many cancers[9] acting as tr. From Liu et al we have the following discussion. The key observation is that MYCN if overexpressed, say by the eDNA, results in aggressive malignancies. They state:

Gene amplification is a frequent mechanism that can cause proto-oncogene overexpression. It is a process that involves unscheduled DNA replication, recombination and/or formation of extrachromosomal DNA, leading to a selective increase of gene copy number up to several hundred. The occurrence of proto-oncogene amplification can be detected by the presence of "double minutes" or "homogeneously staining chromosomal regions".

MYCN was the first discovered paradigm of proto-oncogene amplification and is an important bio-marker to stratify clinical risk. It was initially detected in about 20% to 25% of neuroblastoma, then at a much lower incidence in small cell lung cancer, retinoblastoma, hepatocellular carcinoma, malignant gliomas, and peripheral neuroectodermal tumors. Amplification of MYCN has been recognized as a consequence of genomic instability and occurs sporadically.

[9] See NCBI https://www.ncbi.nlm.nih.gov/gene/4609 : *This gene is a proto-oncogene and encodes a nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. The encoded protein forms a heterodimer with the related transcription factor MAX. This complex binds to the E box DNA consensus sequence and regulates the transcription of specific target genes. Amplification of this gene is frequently observed in numerous human cancers. Translocations involving this gene are associated with Burkitt lymphoma and multiple myeloma in human patients. There is evidence to show that translation initiates both from an upstream, in-frame non-AUG (CUG) and a downstream AUG start site, resulting in the production of two isoforms with distinct N-termini.* MYCN also https://www.ncbi.nlm.nih.gov/gene/4613 *This gene is a member of the MYC family and encodes a protein with a basic helix-loop-helix (bHLH) domain. This protein is located in the nucleus and must dimerize with another bHLH protein in order to bind DNA. Amplification of this gene is associated with a variety of tumors, most notably neuroblastomas. Multiple alternatively spliced transcript variants encoding different isoforms have been found for this gene.*

Overexpression of N-MYC initiates tumorigenesis by preventing the normal physiological process of neural crest cell death in TH-MYCN transgenic mice in which human MYCN is under the control of a tyrosine hydroxylase (TH) promoter, and the formation of neuroblastoma involves further changes of the persisting embryonal neural crest cells, including MYCN amplification.

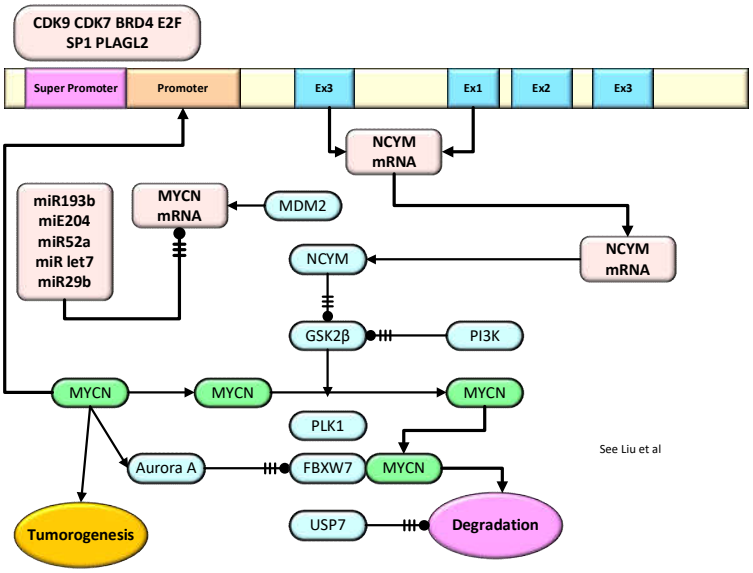
In addition, MYCN amplification is associated with advanced neuroblastomas, suggesting that the amplification is a late event during the tumorigenesis ….

This observation is one of the timeliness of the development of ecDNA. Namely it begs the question: which comes first, the ecDNA or the proliferation?

Efficient translation guarantees the oncogenic level of N-MYC protein. N-MYC has been shown to promote the expression of many genes involved in ribosome biogenesis and protein synthesis, suggesting N-MYC contributes to its own overexpression by enhancing the capacity of translation. The N-MYC protein level is decreased as a result of ribosome biogenesis inhibition.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that controls initiation of protein translation. mTOR directly phosphorylates and inactivates eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1), which leads to activation of eIF4E and thus promotes capdependent translation of mRNAs including MYC family. Pharmacological inhibition of the AKT/mTOR pathway reduces NMYC level and exhibits therapeutic efficacy in MYCN-amplified Neuroblastoma.

We depict an example from Liu et al below:



4.2 PROSTATE CANCER

We have examined a multiplicity of factors regarding prostate cancer, PCa. As Dolgin notes:

Circular tracks of DNA found inside cancer cells commonly contain large numbers of replicate oncogenes, and the uneven inheritance pattern of this DNA, located outside of chromosomes, enables tumor cells to rapidly adapt to selective pressures such as chemotherapy*.*

However, the impact of this so-called extrachromosomal DNA (ecDNA) goes beyond genomic amplifications, regulatory flexibility, and accelerated tumor evolution. Two new studies reveal how the topology of ecDNA affects gene regulation to promote cancer growth. A third report details how ecDNA can reintegrate itself into chromosomes, disrupting cancer-related genes. And a preprint article demonstrates the broad clinical relevance of the phenomenon by linking ecDNA-based amplifications to shorter survival times across a range of tumor types. The findings collectively fill in key details about how oncogene-containing ecDNA boosts tumor aggressiveness and fuels resistance to therapies—insights that have helped jump-start at least one new company, Boundless Bio.

The company, which launched in September 2019, is focused on "understanding and finding the vulnerabilities that are created by having this really different kind of DNA," says cofounder Paul Mischel, MD, of the University of California, San Diego (UCSD). With the growing appreciation of ecDNA's critical role in cancer development, Mischel adds, "I think we're at the dawning of a new field." Late in January, researchers gathered in Berlin, Germany, for the first-ever meeting dedicated to circular DNA's role in disease and normal development.

Scientists first noticed ecDNA under the microscope more than a half-century ago, but it was only in the past 5 years or so that the research community began to appreciate the contribution of these loops to cancer cell behavior.

A study from Mischel and UCSD's Vineet Bafna, PhD, also a Boundless Bio cofounder, demonstrated that ecDNA amplifications can rapidly increase oncogene copy number and accelerate intratumor heterogeneity, enabling cancers to adapt more effectively to variable environmental conditions. Yet questions loomed about how the structure and spatial architecture of ecDNA contributed to pathogenesis. In their recent study, …—showed that the genomic packaging of ecDNA in several tumor types is unlike that of normal.

Although ecDNA is wound into condensed complexes, it is not as compact as typical chromatin. This structure makes the encoded genes more accessible for active transcription, which, along with the increased copy number, helps explain why oncogenes encoded on ecDNA are among the most highly expressed genes in tumors. The circularity of ecDNA also creates more long distance interactions within the active chromatin that could further promote oncogene expression.

We have examined several PCa biopsies and the presence of ecDNA does not seem to be universal.

4.3 Other Cancers

We now examine other cancers and their relationship to ecDNA. From Zhao et al we have the following summary table:

Name	Disease	Function
eccDNA (EGFR)	Glioblastoma	Endogenous enhancer elements
ecDNA (ecEGFRxl, ecCCATI, ecEGFR, and ecCCDC26)	Glioblastoma	Uneven segregation of ecDNA during mitosis
eccDNA (PDGFRA, CDK4)	Radiation-induced high-grade glioma	eccDNA-mediated amplification of oncogenes
eccDNA (TRPS1)	Breast cancer	TRPS1-driven genome deletions
ecDNA (ecMYC)	Prostate cancer	Mobile transcriptional enhancers
ecDNA/eccDNA (cyclin-E1, ERBB2, CDK12, EGFR, MYC)	Gastric cardia adenocarcinoma	Focal amplifications of oncogene prognostic molecular markers
eccDNA (RAB3B)	Hypopharyngeal squamous cell carcinoma	Promote cisplatin resistance
eccDNA (MYCN, CDK4, MDM2)	Neuroblastoma	Seismic amplification model
eccDNA (entire genome)	Immune system	Trigger immune response
TTN^{cirde}	Musculoskeletal system	Function of transcription
MI-related eccDNA (MIRECD)	Myocardial infarction (Ml)	Ml prognosis prediction and risk stratification

Now as Li et al have noted for the following cancers:

ecDNA in glioblastoma *ecDNA initiates a great number of carcinogenic amplifications and mutations and has been identified in 10–40% of glioblastomas.*

In glioblastoma, a variety of oncogenes were amplified on ecDNA, such as EGFR, MYC, CDK4, MDM2 and PDGFRA …both ecDNA-mediated oncogene amplification and somatic single-nucleotide variants are involved in the dynamic evolution of glioblastoma

ecDNA in gastric cardia adenocarcinoma *Oncogenic focal amplification plays a pivotal role in the progression of gastric cardia adenocarcinoma and is associated with poor prognosis. … Moreover, they explored the correlations between the focal amplifications (including ecDNA-derived circular amplicons) and prognosis based on an immunohistochemistry analysis from 1,688 gastric cardia adenocarcinoma patients.*

The results show that ***ERBB2-positive patients have worse prognosis than ERBB2-negative patients when their survival time is less than two years****.*

ecDNA in colon cancer *As one of the oncogenic genomic features, gene amplification can markedly prompt tumour evolution and drug resistance. MTX, an inhibitor of dihydrofolate reductase (DHFR), plays an antitumor role in a variety of cancers by interfering with the synthesis of cellular DNA. However, colon cancer often develops resistance to MTX due to DHFR gene amplification. Treating HT29 cells with MTX significantly increased DHFR gene expression via ecDNA-mediated amplification.*

Additionally, withdrawing MTX treatment decreased ecDNA-mediated DHFR amplification in MTX-resistant cells. The loss of the DHFR amplicon in MTX-resistant cells can suppress their capacity to generate resistance. As expected, when these MTX-resistant cells that lost the DHFR amplicon were re-exposed to MTX, the cells may become responsive to the second round of MTX treatment. These observations provide a promising treatment strategy for overcoming drug resistance induced by ecDNA-mediated amplification …

depleting DNA-PKc (an NHEJ-related protein) reduces ecDNA-mediated DHFR amplification and increases MTX sensitivity. … NHEJ is presented as a promising target for overcoming MTX-resistant colon cancers. …also demonstrated that homologous recombination activity was upregulated in MTX-resistant cells… the silencing of the BRCA1 gene (a major player in homologous recombination) decreased the amount of ecDNA and downregulated the expression of ecDNA-amplified oncogenes.

Furthermore, silencing BRCA1 makes MTX-resistant cells containing ecDNA more sensitive to MTX but has no discernible effect on MTX-resistant cells containing HSRs. Therefore, the homologous recombination pathway may also serve as a target to advance current therapies by decreasing ecDNA-mediated oncogenic amplification

ecDNA in ovarian cancer *… noncoding regions on ecDNAs perform a considerable function in regulating gene expression. They discovered several matrix attachment regions (MARs) within an ecDNA derived from UACC-1598 cell line using sequence analysis and bioinformatics analysis. Moreover, they have identified the interaction between the MARs and the nuclear matrix, which results in a significant enhancement of gene expression. Transfecting the MAR construct into 293 T cells could also enhance the expression of oncogenes located near the MARs, including MYCN and EIF5A2. Accordingly, ecDNAs might play an important role in the regulation of gene expression in ovarian cancer.*

ecDNA in neuroblastoma *The first appearance of ecDNA was observed in the metaphase of neuroblastoma cells in 1965 … Alt et al. confirmed the presence of a new oncogene, MYCN, in ecDNA in the neuroblastoma cell line. This was the first report that confirmed that oncogenes were located on ecDNA. MYCN amplification drives one in five cases of neuroblastoma, and the MYCN gene is mainly amplified on ecDNA and HSRs. Recently, Helmsauer et al. examined the structure of MYCN amplicons in neuroblastoma ecDNA and revealed the mechanism of ecDNA-mediated MYCN amplification.*

There are two main aspects to the mechanism: 1) local enhancer-induced MYCN amplification in neuroblastoma ecDNA. 2) Distal enhancer-induced MYCN amplification in

neuroblastoma ecDNA. These findings may provide promising therapeutic targets for MYCN-amplified tumours.

5 THERAPEUTIC OPTIONS

Therapeutic approaches directly targeting ecDNA are limited. We present a few recent contributions here. As Wei et al note:

Understanding the underlying molecular mechanisms of tumor evolution can help identify more effective therapies to eradicate tumors.

ecDNA is an important mechanism driving the copy number variation of oncogenes, which is closely related to the curative effects and the prognosis of tumors. For example, in patients with non-small-cell lung cancer (NSCLC), there is no significant correlation between the number of mutations in tumor cells and prognosis, but patients with more changes in oncogene copy number have worse tumor efficacy and prognosis.

Moreover, ecDNA also plays an important biological function in the development of tumors, which can drive the development, invasion and metastasis of tumors and the formation of multidrug resistance. Therefore, the elimination of ecDNA or oncogenes amplified in ecDNA can effectively reduce the malignancy of tumor cells and can be the basis of tumor therapy.

The discovery of ecDNA may lead to a fourth revolution in cancer treatment, according to Boundless Bio, which believes that the replication mechanism of ecDNA is slightly different from that of normal chromosomal DNA, leading to a way to specifically inhibit ecDNA replication directly.

Another approach Boundless Bio is exploring involves the inhibition of the metabolic pathways required for ecDNA replication.

The frequency and amplitude of oncogene amplification can be changed with various stimuli, and radiotherapy can destroy all cancer cells as much as possible. Compared with those in unirradiated cells, the gene copy number and expression level of amplified genes in the extrachromosomal DNA of irradiated cells were significantly reduced after exposure to relatively low doses of radiation.

Unfortunately, these treatments are limited to the area of exposure, rarely leading to cures, with most tumors locally recrudescing within a few months. In addition, some drugs can effectively reduce the copy number of ecDNA and thus achieve a certain therapeutic effect. In the process of drug-induced differentiation of HL-60 cells into granulocytes, c-myc expression was decreased and c-myc copies were lost in double minutes.

Low doses of hydroxyurea and etoposide led to the degradation of extrachromosomal circular DNA molecules into smaller DNA fragments, accelerating the loss of oncogene amplification (such as EGFR and c-myc) in ecDNA in a dose- and time-dependent manner. Low concentrations of hydroxyurea showed preclinical activity by eliminating ecDNA, reducing the tumorigenicity of human tumor cells, and inducing apoptosis in some cases, without including cytotoxic effects or overall toxicity. In addition, hydroxyure (HU) combined with cisplatin has shown enhanced cytotoxicity in tumor cells.

Gemcitabine was able to decrease the number of ecDNA in cells at a 7500-fold lower concentration than that used for the common cancer drug hydroxyurea, and it also inhibited the growth, colony formation and invasion of tumor cells. Intricate pathways in cells play different roles in gene amplification and may be new targets to improve the effect of tumor chemotherapy by reduced amplification. CRISPRi can also be used to eliminate ecDNA, but whether the CRISPR technology used in research can be used in the clinic remains to be discerned.

A second investigation by Li et al note:

Cancer cells gain survival advantages by continually altering their genomes. Oncogene amplification is a classic form of genome alterations. Cancer cells are addicted to ecDNA because ecDNA is a carrier that maintains oncogene amplification.

Therefore, limiting the survival of cancer cells by eliminating ecDNA may be an effective therapeutic approach.

However, there are currently few targeted drugs related to ecDNA.

Most notably, since the formation of micronuclei contributes to ecDNA elimination, it has been confirmed that the antimetabolite ***hydroxyurea (HU)*** *can enhance this process. It has been proven that HU does not show good clinical antitumor activity, so HU is not used in the treatment of ecDNA-positive tumours.*

Although HU did not achieve the desired effect, existing observations might provide the research basis for further drug screening. Additionally, we believe that the ecTag might uncover the mechanisms contributing to ecDNA elimination by tracing the spatiotemporal dynamics of ecDNA moving into micronuclei.

Tumour heterogeneity is an important cause of drug resistance, and the increased frequency of ecDNA reintegration may reduce heterogeneity among cancer cells. Recently, PARP has been shown to reduce the frequency of ecDNA reintegration and thus may be a candidate therapeutic target. However, ecDNA reintegration also inevitably exerts some adverse effects, such as affecting the expression of adjacent oncogenes, increasing genomic instability, and destroying the sequence structure of tumour suppressor genes.

Therefore, further study is required to avoid the side effects of ecDNA as much as possible to improve clinical therapies….

the spatially abnormal distribution of ecDNA, which eventually forms ecDNA hubs, can cause trans-interactions between ecDNA and between ecDNA and chromosomal DNA. Therefore, the spatially abnormal distribution of ecDNA may represent a treatment-related vulnerability.

Proteins involved in the formation of ecDNA hubs have been considered as emerging potential therapeutic targets. The stability of ecDNA hubs is inseparable from the existence of the ***extraterminal domain (BET) protein BRD4****. However, further research is needed to determine*

whether BRD4 plays a decisive role in maintaining ecDNA hubs stability. If we want to treat cancer by interfering with the formation of ecDNA hubs, there are still some aspects that need to be elucidated.

For example, are the segregation of ecDNA in the form of singletons or smaller hubs? Whether the composition of ecDNA hubs changes with cell passage? Whether the spatial distribution of ecDNA hubs in the nucleus is random or directed? Are there differences in the composition of ecDNA hubs in different cancer species?

Basically therapeutic targets for ecDNA remediation are at best a work in progress. It may be more efficacious to target pathways related thereto.

6 OBSERVATIONS

ecDNA has become a focal point for recent studies in various cancers. Recent observations in AACR reflect on ecDNA in PCa[10]:

*Twenty-three pairs of chromosomes make up the human genome of normal cells. But in 1965, researchers observed that in some cancer cells, DNA was present outside of these chromosomes—**in circular, acentromeric structures that we now call extrachromosomal DNA** (ecDNA).*

About one-third of ecDNAs are thought to form through chromothripsis, a process in which catastrophic chromosomal damage leads to shattered DNA fragments that can ligate to form circles. The remaining ecDNAs likely form through replication errors or faulty DNA repair.

Despite the discovery of ecDNA in cancer over 50 years ago, it is only in recent years with the advent of new technologies that scientists have come to appreciate its prevalence and role as a major cancer driver. …

EcDNAs are present in approximately 14 percent of all cancers, including in 60 percent of primary glioblastomas, one of the deadliest forms of cancer. The presence of ecDNA has been linked to shorter patient survival, even after normalizing for the fact that these structures are most common among advanced, aggressive cancers.

As explained by Verhaak, ecDNAs may contribute to cancer development and progression by increasing oncogene expression. This occurs, in part, because ecDNAs often contain multiple copies of known oncogenes and are hubs of high transcriptional activity. Compounding this is the recent observation from Verhaak and colleagues that ecDNAs segregate unevenly during mitosis, leading to rapid accumulation of these ecDNAs—and consequently more oncogene copies to serve as transcriptional templates—with each cell division. These results were published in the AACR journal Cancer Discovery and were presented by Verhaak during the session.

In this study, Verhaak and colleagues developed a novel CRISPR-based technique called ecTag to label and visualize ecDNAs in real time. The technique utilizes guide RNAs targeted to ecDNA-specific DNA sequences that arise from the ligation of DNA fragments when ecDNAs are formed. Binding of a guide RNA to these ecDNA-specific sequences leads to the recruitment of fluorescent proteins, thus labeling ecDNAs. Tracking the fluorescently labeled ecDNAs over time demonstrated that daughter cells inherited different numbers of ecDNAs from the parental cell instead of the 1:1 inheritance associated with normal chromosomal segregation. The researchers also found that ecDNAs formed clusters and colocalized with RNA polymerase II, consistent with

[10] https://www.aacr.org/blog/2022/05/23/annual-meeting-2022-extra-extra-a-role-for-extrachromosomal-dna-in-cancer/

the previously observed high transcriptional activity of ecDNAs. Features of ecDNAs themselves also contribute to greater oncogene expression, according to data presented by Mischel. He and colleagues found that the chromatin of ecDNAs is enriched for transcription activating marks and deficient in repressive marks, accounting for the high transcriptional activity of these structures. Furthermore, the circular structure of ecDNAs allows transcriptional machinery to access the chromatin more easily. Due to the high transcriptional activity of ecDNAs, they can undergo rapid genetic change that may lead to treatment resistance and worse outcomes for patients with ecDNA-positive cancers, Mischel added.

We now make several observations which may indicate areas for further investigation.

6.1 DO WE NOW HAVE TOO MANY CANCER RELATED FACTORS TO PROVIDE A REASONABLE UNDERSTANDING?

There now is a long list of cancer causing and related genetic issues as well as such issue as the tumor microenvironment and tumor associated immune cells. ecDNA is just another element at play. We have a wide variety of epigenetic factors, mutations, pathway aberrant actions and the like. Ranking ecDNA in the totality is just one more factor. We have miRNA and other factors as well. How they get integrated into a total profile of the malignancy is an open question.

6.2 ECDNA CREATION REQUIRES A MORE DISPOSITIVE UNDERSTANDING

How are the ecDNA created? They do not appear to be random sets of sequences. There is a strong selective effect where the genes included enhance the malignancy and furthermore a well-structured to include self-sustaining elements.

6.3 ECDNA REPLICATION LACKS AN UNDERSTANDING OF INITIATING AND CONTROLLING PATHWAYS

With many cancer causing factors we have an understanding of precipitating and supporting pathway elements. These are generally lacking for ecDNA.

6.4 THE DYNAMICS OF ECDNA HAVE SOME CURRENT BASES BUT MORE EXPERIMENTAL UNDERSTANDING IS DEMANDED

How do ecDNA develop and under what influences.

6.5 IS THERE ANY CHANCE THAT THERAPEUTICS FOCUSED ON ECDNA CAN BE EFFECTIVE

Some discussions have focused on this area as we have noted albeit a limited amount. Perhaps the focus is on the CNV that results rather than the ecDNA. Moreover one wonders if an ecDNA is found is it a single oncogene or a mix. Are the oncogenes tissue specific and why?

6.6 ARE ecDNA CAUSES OR RESULTS OF A MALIGNANT CHANGE

The temporal characteristics of ecDNA are of import. Namely when do they occur and why.

6.7 WHAT IS THE PROCESS WHEREBY THE ecDNA SELECT ONCOGENES AND WHY ARE THEY NOT JUST RANDOM SEQUENCES

As we noted above, there must be some selection process that enables the selection of oncogenes and proto-oncogenes in the development of ecDNA. The literature does not appear to have addressed this issue.

6.8 SHOULD ecDNA BECOME A PART OF A PATHOLOGY REPORT AND IF SO WHAT IS THE SIGNIFICANCE

Path reports are slowly catching up to the genomic world. There may often be genomic profiling of lesions but in most cases that is quite limited. Breast cancers are common for ER and HER presence but not much beyond that. Thyroid cancers may get genomic profiles at high level teaching hospitals but not anywhere else. As to the presence of ecDNA, it seems to be grossly overlooked other than at a research facility. It appears to have prognostic value yet no therapeutic advantage may present itself. Its inclusion we believe would be beneficial.

6.9 SUMMARY

The following Table present a summary of our observations herein.

Issue	Questions	Observations
Initiation	What is the process that initiates the development of ecDNA?	The current literature seems to have little to address here.
Cause or Effect	Are ecDNA the "result" of other malignant processes or the cause of them? If so which ones.	This is a critical issue. It does appear that they occur after the fact as in the case of MYCN ecDNA
Selection	Why do we see ecDNA primarily as well known transcription factors which are oncogenes? What is the process of the selection?	This is the most critical question. The ecDNA are highly selective and complete oncogene transcription structures.
Replication	How are the ecDNA actually replicated? Does the existing cell facilitate this? Is the facilitation unique in cancer cells?	There are many possible means.
Dynamics	What are the temporal as well as spatial dynamics of ecDNA? Are they totally random or are they influenced by related cancer cell factors?	This is a complex issue to address but perhaps it may add to therapeutic options.
Impacts	Are the impact of ecDNA primarily via oncogene transcription factors?	Transcription factor CNV impacts seem to be the sole effect.
Reporting	Should part of a path report include ecDNA analyses and if so how detailed. Also what impact would it have on patient care?	This may be a costly effort with as of yet limited diagnostic or treatment value.
Therapeutics	How should ecDNA be attacked? If its creation is known then can that be a controlling path. Should the transcription factors be the principal approach?	Give the circular DNA structures, perhaps a double stranded viral approach may be useful
Interactions	Do the ecDNAs interact with other cancer related effects such as epigenetic factors?	Currently one sees ecDNA as single threaded effectors on enhanced malignant cells.

Overall, there is some understanding of ecDNA. However much of the details regarding their oncogenic nature are yet to be elucidated.

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