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Q&A

What Is Extrachromosomal Circular DNA and What Does It Do?

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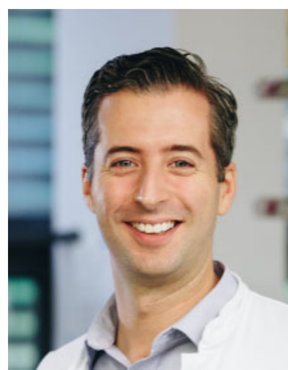
It is known that DNA in the cell nucleus is packed in the form of linear chromosomes. For many years, researchers have observed smaller lengths of DNA alongside the chromosomes that are organized in circular forms. Some of these particles, which have been referred to as extrachromosomal circular DNA (eccDNAs) or microDNAs, are typically small (<1 kb), gene-sparse, and non-amplified. Total eccDNA abundance in cells can be up to a few hundred per cell. eccDNA molecules are also present in the circulation in cell-free form and offer the possibility to serve as blood-based biomarkers. In tumors, another type of extrachromosomal circular DNA can be detected, which appears to be exclusive to cancerous cells. Previously referred to as double minutes, but now referred to as extrachromosomal DNA (ecDNA) because they are usually not doublets, these ecDNA particles are often very large (mean size 1.3 Mb), highly amplified with many copies per cell, and contain many genes and regulatory regions, with a marked enrichment for oncogenes. Importantly, in cancer cells, ecDNAs seem to be more transcriptionally active than their chromosomal counterparts and have been suspected to confer growth and survival advantage to cancer cells. At present, the relationship between eccDNA in normal cells, and ecDNA in cancer, if there is one, is not understood. Being such an enigmatic form of genetic material, we posed questions about eccDNAs and ecDNAs to a panel of experts in the field who have studied facets of eccDNAs and ecDNAs, ranging from their biophysical properties, production mechanisms, physiological roles, roles in cancer biology, and diagnostic potential.

What do eccDNAs do? What has been the biggest surprise to you about eccDNA so far?

Anton Hensson: ecDNA is a vehicle for proto-oncogene amplifications in cancer. This has been known for some time. What was surprising is the frequency of ecDNA in cancer and the ability of ecDNA to form very complex structures, including parts from different chromosomes, as well as their ability to reinsert into the genome.



Paul Mischel: I will focus my comments on my area of research, ecDNA in cancer. The biggest surprise, to me, is what a critical role ecDNA plays in human cancer. Our data suggest that ecDNA plays a critical role in driving the aggressive behavior of some of the most malignant forms of cancers through at least three interlacing mechanisms: 1) because ecDNAs lack centromeres, they are subject to non-Mendelian (i.e., nonchromosomal) inheritance, which enables tumors to achieve very high oncogene copy number while maintaining intratumoral genetic heterogeneity; 2) the intratumoral genetic heterogeneity generated by this mechanism of inheritance enables tumors to evolve

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rapidly in response to changing conditions, including treatments, accounting for the remarkable ability for some cancers to change their genomes at rates that cannot be explained by chromosomal inheritance; 3) the high DNA template level achieved by non-Mendelian inheritance and selection, coupled with the altered chromatin organization generated by the circular architecture that we demonstrated (identified in work done closely with Dr. Howard Chang), results in massive transcription of oncogenes. Taken together, these features begin to explain why some cancers seem to genomically explode and change, why they do not undergo pure selective sweeps, and why any cell in the tumor seems to be able to recapitulate the entire tumor, with its full spectrum of heterogeneity, in some cancer types. It also provides some insight into why targeted therapies against oncogenes amplified on ecDNA have not been as successful as anticipated.

Anindya Dutta: Long eccDNAs visible in cancers by karyotyping, also termed double minutes or ecDNAs, have been known to carry oncogenes that are amplified to promote cancers. Recent results suggest that there is a large population of smaller eccDNAs, <1000 bp long that constitute 90% of the eccDNA in normal cells and cancer cell lines. Cancer cells also contain longer ecDNAs, not always visible by karyotyping, which range in size from 1 kb to that of double minutes. The circles that are long enough to contain full genes can overexpress the genes and amplify them. This is very important for cancers containing the long ecDNAs. The function of the small circles is unclear, but we have shown that they can express RNAs in a deregulated manner, and that the RNAs are processed into microRNAs and small interfering RNAs to repress cellular genes.

For me, the biggest surprise remains our original discovery of how ubiquitous the eccDNAs are, even in normal tissues and the fact that most of them are somatically mosaic (different between different cells) even in cancers. It is only when they give a selective advantage to cells, as eccDNAs carrying oncogenes do in cancers, that the same eccDNA is seen in many cells in a cancer.

Birgitte Regenberg: To find that: 1) eccDNA is a common genetic element in eukaryotic cells, 2) eccDNA can arise from all parts of eukaryotic genomes, 3) selection can lead to co-amplification of enhancers and oncogenes on complex eccDNA in tumors, 4) certain loci appear to form eccDNA recurrently and at high rate in yeast (*CUP1* and *HXT6 HXT7*). The latter result is really interesting, because it suggests that eccDNA can play an important role in evolution by providing rapid adaptation to changes in the environment (high cupper, *CUP1*, and low glucose, *HXT6 HXT7*).

Dennis Lo: My group first became interested in eccDNA when we started looking for circular DNA molecules in human plasma. Our journey started with the investigation of mitochondrial DNA (mtDNA), which exists inside a mitochondrion as a circular piece of DNA molecule of approximately 16 kb. Our results demonstrated that both circular and linear mtDNA molecules exist in human plasma. One surprise from this work is our demonstration that the circular mtDNA molecules and linear mtDNA molecules have different tissues of origin. Hence, the circular mtDNA molecules are predominantly from the hematopoietic system, while the linear mtDNA molecules are predominantly from the liver.

We have since extended our work to look for eccDNA in plasma. In particular, we have demonstrated

that fetal eccDNA molecules are detectable in the plasma of pregnant women. Our group has been interested for many years in the size distribution of circulating DNA. It is interesting to note that eccDNA molecules in maternal plasma (with prominent size peaks at 202 bp and 338 bp) have a longer size distribution than linear DNA molecules (modal size at 166 bp). Our previous work on linear DNA molecules in plasma demonstrated that linear fetal DNA molecules in maternal plasma have a slightly shorter size distribution than the linear DNA molecules of maternal origin. Another surprise of our work is that we have observed a similar shortness of circulating fetal eccDNA molecules when compared with those of maternal origin.

What is your preferred hypothesis regarding the production mechanism of eccDNAs within cells? What evidence is there to support this hypothesis?

Anindya Dutta: I think the eccDNAs are produced as a byproduct of DNA repair. The main evidence for this is that they are increased by agents that increase DNA damage, and we have reported that certain DNA repair genes such as *MSH3* (involved in mismatch repair) are required to produce eccDNAs.

Birgitte Regenberg: I favor a model in which any form of DNA damage can potentially lead to DNA circularization through the known DNA-repair mechanisms. This involves their formation through homologous recombination, microhomology, and nonhomologous end joining together with other DNA repair pathways. Most of our evidence is based on homology around the chromosomal breakpoint that led to eccDNA, and I think mutant studies are still required to establish causality. Re-replication might also produce circular DNA, as explained in the Origin-Dependent Inverted-Repeat Amplification model (from Maitreya Dunham), but we still need to explore how important this mechanism is. Besides the random processes, a few circles form through directed recombination (the T-cell receptor excision circles) and retro-transposition when long terminal repeat eccDNA arise from the circularization of extrachromosomal linear DNA during the transpositional life cycle of retrotransposons.

Anton Henssen: Based on published literature and our own observations, I believe that there might be many different mechanisms contributing to eccDNA generation. EccDNA can be created through catastrophic genome rearrangement processes such as chromothripsis, but there are also other processes of genomic instability that may contribute to their formation.

Paul Mischel: Again, I will focus my responses on ecDNA in cancer. There is a historical view of ecDNA formation, or at that time called double minute formation, in which something happens that results in removal of a stretch of DNA from its native chromosomal location, followed by replication and amplification as ecDNA. Researchers, including Robert Schimke, Geoff Wahl, Nicholas Vogt, and Bernard Malfoy, amongst others, contributed to this knowledge. The precise molecular mechanisms, their relationship to possible abnormalities in the DNA damage and response system, remain incompletely understood. It is an area of active research, including in our laboratory. In addition, David Pellman and others had suggested that chromothripsis, which occurs when a lagging chromosome gets "stuck," placed into a micronucleus, and effectively chopped up, could potentially form ecDNA. Recent experimental work from Peter Ly and Don Cleveland, in which they engineered a chromothriptic Y chromosome, suggests that chromothripsis can result in ecDNA formation as a mechanism of gene amplification. Therefore, it is quite possible that multiple mechanisms could lead to ecDNA formation, which is then acted upon by selection. It will be important to develop a deeper mechanistic understanding of the processes that contribute to ecDNA formation.

Dennis Lo: In our size distribution analysis work involving eccDNA molecules in maternal plasma, we have observed a number of telltale evidence of nucleosomal signatures. For example, we have observed a 10 bp periodicity in the size distribution in the vicinity of the prominent size peaks of 202 bp and 338 bp. Our conjecture is that the size of 202 bp is approximately that of a nucleosome core plus two linkers, while the size of 338 bp is approximately that of two nucleosome cores plus two linkers. Another notable observation is that amongst the most frequently observed eccDNA molecules in maternal plasma, we have observed four sets of trinucleotide motifs at the junctional site of an eccDNA molecule. At such a site, the first and the third motifs are direct repeats, while the second and fourth are another set of direct repeats. We hope that these observations will contribute towards an improved understanding of the production mechanism of eccDNA. We fully understand that we do not have all the information to build a complete model but we believe that the field as a whole is advancing towards that.

What is known about eccDNAs and cancer? In what ways do eccDNAs contribute to the malignant properties of cancer cells?

Paul Mischel: We have learned the following: 1) ecDNAs appear to be exclusive to cancer, or at least, we have yet to see it in normal cells, 2) ecDNAs drive high oncogene copy number and maintain intratumoral genetic heterogeneity through their mechanism of

non-Mendelian, nonchromosomal inheritance; 3) ecDNAs, because of this inheritance mechanism, can change their genomes rapidly, including to evade therapies; 4) the high DNA template level of ecDNA, coupled with the altered chromatin architecture, drives massive oncogene transcription, and may remodel the epigenome in ways that contribute to tumorigenesis.

Anton Henssen: ecDNA is not only a vehicle for oncogene amplification but can also contribute to genome remodeling through its re-integration into the linear genome. We have shown that circular DNA re-integration leads to disruption of functionally important genomic regions and that this disruption could contribute to many malignant features of cancer cells.

Birgitte Regenberg: We know that amplification of a number of oncogenes on eccDNA correlates with cancer, and cancer patients with certain eccDNA amplifications have poor prognosis. Overexpression of oncogenes such as *MYC* and *EGFR* on eccDNA is likely to reprogram cells and induce the tumorigenic state.

Anindya Dutta: The circles in cancers are longer than that in normal cells, and it has been suggested that they get a different name: ecDNAs. We now know that they are present in nearly all cancers but are not large enough to be detectable as double minutes by cytogenetics. The long ecDNAs carry complete genes, and when these genes are oncogenes or cancer-driver genes, the ecDNAs enable their overexpression and amplification. For example, ecDNAs carry the following oncogenes: the *MDM2* oncogene (originally discovered in a double minute) inactivates the p53 tumor suppressor, while the *EGFR* oncogene makes glioma and glioblastoma cells hyper-responsive to EGF. Because ecDNAs are not segregated equally between daughter cells, the random distribution of the circles between daughter cells makes it easier for some of the daughters to get more copies of the circles and thus gain a growth advantage by expressing more of an encoded oncogene. Thus, the non-Mendelian inheritance of the circles of DNA makes it easier for the cancer cell to amplify circles that give the cancer a growth advantage.

Do you think eccDNAs could serve as biomarkers for disease assessment, in what ways and how?

Dennis Lo: I think that eccDNA molecules in plasma would be an interesting direction for biomarker research. One challenge is that their overall concentration appears to be substantially lower than that of linear DNA molecules in plasma. The large size distribution of eccDNA molecules in plasma has one advantage that longer molecules would potentially carry more genetic and epigenetic information from the tissue of origin.

Anindya Dutta: We have already shown that eccDNAs are 1) released into the blood from tumors and from the fetus and 2) can be detected and quantified in the pool of cell-free circulating DNA. Because they are longer (mean: 250 bases) than linear cell free circulating DNA (mean: 150 bases) and more stable, circulating cell-free eccDNAs could be useful for detecting mutations in oncogenes (in cancers) or for detecting mutations in developmentally important genes (for non-invasive-prenatal testing). The longer size of the circles seen in cancers relative to normal tissue may also be useful as a screening tool in liquid biopsy of cancers.

Birgitte Regenberg: Yes, I think that eccDNA can potentially serve as a biomarker for a number of diseases that are linked to mutation and genomic rearranging. EccDNA from the T-cell receptor gene is already used for detection of Severe Combined Immunodeficiency Disease and recent data have shown that eccDNA from a fetus can be detected in the plasma of the mother. It seems likely that other eccDNA in the plasma can serve as markers for monitoring of cancers, though the concentration of eccDNA in plasma is likely to be limiting.

Anton Henssen: In pediatric oncology, ecDNA in the form of MYCN-containing double minute chromosomes is already an established biomarker for clinical risk assessment in patients suffering from neuroblastoma. I believe that similarly, other ecDNAs could serve as biomarkers for various disease properties in many tumor entities.

Paul Mischel: Yes, there are considerable data to suggest that ecDNA may be a biomarker of more aggressive cancer types and may provide new insight about the ability of some cancers to evolve so rapidly, including in response to therapies. There are also compelling reasons to think that patients whose cancers are driven by ecDNA may need to be treated in a different way.

What is your favorite approach to analyze eccDNA and what are the advantages?

Birgitte Regenberg: Most eccDNA exists in low copy numbers and is not captured by whole genome sequencing. To measure both high and low copy eccDNA, my laboratory has developed methods to isolate, sequence, and assemble eccDNA (Circle-Seq and Circle-Map, in collaboration with L. Maretty, D. Botstein, and M. Mohiyuddin). These methods allow us to profile eccDNA across genomes in any given cell and condition. We can thereby gain insight into how eccDNA

correlates with age and disease (collaborations with J.S. Johansen and Y. Lou), and at the basic level understand how they form, evolve, and perish in a population of cells.

Anindya Dutta: My laboratory has mostly used rolling circle amplification of exonuclease-resistant circles with random hexamers followed by paired-end sequencing (Circle-Finder) to identify the junctions that are characteristic of circles. Since most genomics experiments do not include rolling circle amplification, we cannot re-analyze genomics data generated by other groups to identify circles. However, recently we have shown that Assay for Transposase-Accessible Chromatin using sequencing (cheaper) or whole genome sequencing (much more expensive) can detect circles of DNA. We are hoping that these more widely used techniques will allow the identification of circles in already existing datasets. In addition, a recent paper from Dennis Lo and co-workers, shows that circles can be detected by digestion with common cutting restriction enzymes and sequencing the fragments for junctions.

Dennis Lo: We would first treat plasma DNA with an exonuclease that would remove much of the linear DNA molecules in the sample. Then, we would cut open the circles, using either restriction enzymes or a transposase, to form linear DNA molecules for further analysis (e.g., DNA sequencing). We think that the transposase-based method has an advantage that, unlike the restriction enzyme-based approach that requires the existence of restriction enzyme recognition site within the eccDNA molecule, the transposase method can potentially act on any eccDNA molecule.

Paul Mischel: My colleague Vineet Bafna has developed a powerful tool kit, including Amplicon Architect and Amplicon Reconstructor to analyze ecDNA structure. In fact, ongoing work with Dr. Bafna and Dr. Verhaak is being conducted to better analyze ecDNA in publicly available whole genome sequencing databases. Also, working close with colleagues Drs. Howard Chang and Bing Ren, we are using aspects of the "epigenetic" tool kit to characterize ecDNA in cancer.

Anton Henssen: We like to specifically isolate and sequence ecDNA with long-read sequencing, which offers the opportunity to accurately map the structure of ecDNA.

What are the research questions related to eccDNA you are most eager to explore?

Paul Mischel: We are very interested in understanding a number of critical issues related to ecDNA, not listed in order of importance. First, how does ecDNA form and what are the key molecular "players" involved in its formation? Second, what are the molecular mechanisms involved in ecDNA maintenance and function? Are different components used? Are the same components used differently? Third, what are the clinical implications for patients? Can ecDNA be used to tell us something important about clinical course? Fourth, can we find points of intervention that can be used to develop new treatments that will help patients whose cancers are driven by ecDNA?

Anton Henssen: As a physician scientist, I am most eager to explore the possibilities to use our understanding about ecDNA to find new diagnostic and therapeutic approaches for patients suffering from ecDNA-driven cancers.

Dennis Lo: I would like to explore the ability of eccDNA in maternal plasma for detecting or monitoring pregnancy-associated disorders (e.g., preeclampsia). I am also interested in developing newer and potentially more comprehensive approaches for eccDNA analysis. I am aware that currently available methodologies might have certain bias in selected subsets of circulating eccDNA molecules.

Anindya Dutta: I want to find the functions of the eccDNAs present in normal cells. Because they are so ubiquitous and somatically mosaic, it will be very exciting if they contribute to inter-cellular heterogeneity in normal tissues or contribute to some form of pathology. I also want to delineate what pathways are involved in the formation of the circles in normal and cancer cells, in the hope that interfering with these pathways in cancers will allow us to help clear cancers of potential loci of gene amplification and thus help in therapy. Finally, I want to see the adoption of eccDNA sequencing in the liquid biopsies of cancers and in non-invasive prenatal testing of genetic diseases in the fetus.

Birgitte Regenberg: Besides understanding how eccDNA might contribute to genetic variation and evolution of eukaryotic genomes, I am eager to understand how eccDNA is formed and maintained in a genome. Four factors are likely to determine the turnover of a circular DNA in a cell lineage: the rate by which it is formed from its chromosomal locus, its ability to replicate, its mode of segregation, as well as the growth advantage or disadvantage it provides to its hosting cell. Furthermore, eukaryotic cells can potentially have mechanisms to degrade or secrete eccDNA. This is particularly important for meiotic cells in multicellular organisms, since eccDNA in the germline might have large negative effects in the next generation. Recent data from yeast suggest that meiotic cells have indeed evolved

mechanisms to sequester a subset of eccDNA (from Ünal's laboratory at Berkley), and it seems likely that the same will be true for germline cells in multicellular organisms such as humans.

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