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Gene regulation on extrachromosomal DNA

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Oncogene amplification on extrachromosomal DNA (ecDNA) is prevalent in human cancer and is associated with poor outcomes. Clonal, megabase-sized circular ecDNAs in cancer are distinct from nonclonal, small sub-kilobase-sized DNAs that may arise during normal tissue homeostasis. ecDNAs enable profound changes in gene regulation beyond copy-number gains. An emerging principle of ecDNA regulation is the formation of ecDNA hubs: micrometer-sized nuclear structures of numerous copies of ecDNAs tethered by proteins in spatial proximity. ecDNA hubs enable cooperative and intermolecular sharing of DNA regulatory elements for potent and combinatorial gene activation. The 3D context of ecDNA shapes its gene expression potential, selection for clonal heterogeneity among ecDNAs, distribution through cell division, and reintegration into chromosomes. Technologies for studying gene regulation and structure of ecDNA are starting to answer long-held questions on the distinct rules that govern cancer genes beyond chromosomes.

The cancer genome undergoes extensive genetic alterations, often leading to dysregulation of gene expression. One important mechanism is amplification of oncogenes and drug-resistance genes; this process increases the copies of genes that provide a selective advantage to cancer cells. Although it has long been known that gene amplification can occur within or outside chromosomes, it is increasingly appreciated that the context and spatial architecture of gene amplification has an enormous impact on changes in gene expression, which cannot solely be explained by gene copy number.

Major recent advances have been made regarding ecDNA, a common mode of oncogene amplification. ecDNA was first observed in 1962 and described in detail by Cox et al. in 1965 (refs. [1,2]). Since then, ecDNA has been detected in nearly half of human cancer types, carrying oncogenes such as *MYC*, *MYCN*, *EGFR*, and *ERBB2* in tumor cell lines, cell cultures derived from people with cancer and clinical tumor samples[3–13]. ecDNA can integrate into chromosomes and therefore may act as an intermediate step toward stable chromosomal gene amplification in a subset of cases[9,14–16]. ecDNA is associated with poor survival of patients, even when compared with other forms of gene amplification[5]. These observations suggest that gene amplification in the context of ecDNA may have unique impacts on cellular programs that profoundly contribute to tumor pathogenesis and progression.

ecDNA refers to circular DNA molecules that are self-replicating, clonally selected and amplified, and range from 100 kilobases (kb) to several megabases (Mb) in size. They are selected for in cancer-cell populations as they provide a fitness advantage, typically by carrying oncogenes and drug-resistance genes. ecDNA was classically termed 'double minutes,' which referred to paired, extrachromosomal chromatin bodies that could be microscopically observed on metaphase spreads. However, some ecDNA molecules are submicroscopic, and the majority of ecDNAs (~70–80%) appear as singletons, rather than paired bodies, on metaphase chromosome spreads[3,4,16–18]. Therefore, the classic double-minute structure describes only a fraction of ecDNA, leading to a shift toward the more inclusive term 'ecDNA.' The large clonal ecDNAs found in cancer cells should not be confused with another class of smaller, nonclonal, extrachromosomal elements termed eccDNAs, or extrachromosomal circular DNA elements. Although eccDNA is sometimes used as a broad umbrella term for circular DNA, it typically refers to DNA circles that are found in normal tissues or as byproducts of programmed cell death[19,20], are not amplified or selected, are only up to ~10 kb in size, usually do not carry genic or regulatory sequences, and can span any part of the genome[20–22]. Given the similarity in nomenclature, despite stark differences in sequence, function, behavior, and the cell types in which the DNA elements are observed, we have constructed a summary table to clarify the main distinctions (Table 1). This Review focuses on large clonal ecDNAs observed across many cancer types.

Gene copies located on ecDNA are expressed at much higher levels than are those located in the native chromosomal locus and linear gene amplicons, even when normalized for copy number[4,5,23]. In addition to copy-number amplification, ecDNA shows unique structural, genetic, and epigenetic features that are conducive to gene activation, suggesting that there are fundamental differences in how amplified genes are regulated on ecDNA. Given the observation that ecDNA is prevalent in cancer, is a key form of oncogene amplification, and is linked to poor clinical outcomes, there is a need for a better understanding of how genes are regulated on ecDNA. In this Review, we highlight aspects of the regulation of gene expression on ecDNA that differ from chromosomal DNA. These unique ecDNA features are linked to alterations in structure, sequence, chromatin composition, and contacts with DNA regulatory elements.

ecDNA enables high levels of oncogene transcription

Copy-number amplification. ecDNA is associated with increased oncogene expression compared with linear amplicons as well as the native chromosomal locus[4,5,23,24]. This is partly driven by gene copy-number amplification[4,5]. As ecDNAs lack centromeres, they are distributed randomly among daughter nuclei during cell division[25–27] (Fig. 1a). This random segregation results in copy-number heterogeneity and selection of cells carrying ecDNAs that provide a fitness advantage (Fig. 1b). This characteristic of extrachromosomal oncogene amplification can lead to up to several hundreds of ecDNAs in a single cell[4,15,24] and has been linked to rapid adaptation to selective pressures and development of therapeutic resistance[4,27–29].

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Table 1 | Differences between ecDNA and eccDNA

ecDNA	eccDNA
100 kb to several megabases	Several hundred to thousand bases[19,22]
Circular	Circular
Self-replicating	Not known to replicate
Clonally selected	Typically not amplified or selected
Contains oncogenes, drug-resistance genes, or other genes that provide a selective advantage[3–13,28,101]	Sequences cover the entire genome with some hotspots; typically does not contain genic sequences[19]
Contains regulatory sequences such as oncogene enhancers[24,46,48,53]	Usually does not contain regulatory sequences
Found in cancer cells	Found in many cell lines and tissues, including healthy samples[20,22,102–104]
Can contain heavily recombined genomic sequences and specific mutations[49,87]	Not known to carry mutations

However, copy number alone does not fully explain the high level of oncogene expression observed in ecDNA$^+$ cancer cells; tumors containing circular amplicons express amplified genes more highly than do those containing linear or other types of rearranged amplicons, even with copy-number normalization[5]. This observation suggests that there are additional mechanisms that overexpress oncogenes on ecDNA in ways distinct from chromosomal regulation of gene expression.

Oncogene overexpression. In addition to increased copy number, ecDNAs are associated with increased transcriptional activity and highly accessible chromatin[5,23,24] (Fig. 1c). ecDNAs lack higher-order compaction through depletion of large nucleosome arrays, which may allow binding by transcription factors and access to gene loci by the transcriptional machinery. There seem to be multiple mechanisms causing this oncogene overexpression. First, ecDNA molecules physically cluster with one another in the nucleus and engage in intermolecular, combinatorial enhancer-promoter interactions. Second, the circular structure of ecDNA is a stable, covalently closed structure, allowing increased chromatin *cis* interactions compared with chromosomes. Third, extensive genome sequence rearrangements alter the regulatory context of gene loci. This is an area of active research, and there are likely additional mechanisms driving oncogene overexpression. It remains an open question whether the ecDNA characteristics that enable oncogene overexpression are consequences of selection for transcriptionally active molecules or inherent differences between circular extrachromosomal chromatin and native chromosomes. These mechanisms driving oncogene overexpression are described in detail in the following sections of this Review.

ecDNA hubs: a new nuclear structure for intermolecular gene activation

The three-dimensional structure of mammalian chromosomes is organized at various length scales: chromosome territories, compartments A and B, topologically associating domains (TADs), and long-range enhancer-promoter interactions spanning tens to hundreds of kilobases[30,31]. On a finer scale, chromatin interactions, such as those occurring between enhancers and target genes, are most often found within TADs on the same DNA molecule (*cis* interactions). Although interchromosomal interactions have been documented, they represent exceptions rather than the norm[32,33] (reviewed by Maass et al.[34]). In contrast, a cancer cell can have up to hundreds of ecDNA copies in the nucleus, raising the possibility that multiple ecDNA copies can interact with one another, fostering new cooperative interactions.

Rather than being randomly scattered around, ecDNA molecules cluster with one another to form micrometer-sized hubs in the interphase nucleus[24,35]. ecDNA hubs, or 'extrasomes,' represent a counterpart to chromosomes as units of genetic information and organization (Table 2). Chromosomes have linear arrays of genes and permit gene activation by DNA regulatory elements on the same DNA molecule. In contrast, ecDNA hubs permit intermolecular gene activation of combinatorial enhancers and promoter elements in spatial proximity[24]. Chromosomes are dispersed during interphase of the cell cycle and condense by ~10,000-fold during mitosis. ecDNA hubs coalesce during interphase but disperse during mitosis[24]. These fundamental differences distinguish DNA in the form of chromosomes versus ecDNA hubs in the same nucleus.

Previous studies have also reported preferential localization of these ecDNA clusters at the nuclear periphery during G1 phase and M phase, although the significance and mechanism of this localization pattern is not well understood[36]. As the nuclear periphery is a transcriptionally repressive environment, whereas ecDNAs are highly transcriptionally active[37], their peripheral localization is counterintuitive and warrants further investigation into its generalizability and potential functional significance. ecDNA hubs are observed during mitosis with dynamic changes in size, suggesting that these clusters are not stable during DNA partitioning[24,25,38]. Finally, double-strand DNA breaks in ecDNA have been associated with aggregation of ecDNA molecules and formation of chromosomal tandem amplicons termed homogeneously staining regions (HSRs)[39], suggesting that ecDNA clustering may explain the formation of some chromosomal amplicons as well.

ecDNA hubs drive intermolecular oncogene activation. Formation of nuclear ecDNA hubs is linked proportionally to the rate of oncogene transcription from each ecDNA molecule[24,35] (Fig. 2a). ecDNA hubs bring 10–100 ecDNAs into proximity and enable intermolecular enhancer-promoter interactions, increasing the level of combinatorial enhancer input to oncogenes[24] (Fig. 2b). Whereas genes on chromosomes are activated by enhancer and regulatory DNA elements on the same chromosome, ecDNA molecules can promiscuously engage enhancers on other ecDNAs within the spatial proximity of an ecDNA hub. Even two distinct ecDNAs derived originally from two chromosomes can cross-activate each other through enhancer-promoter contacts[24]. The ability to enact intermolecular gene activation appears to be a bright dividing line between normal cellular physiology and cancer cells harboring ecDNA.

Potential mechanisms of ecDNA hub formation. Small transient transcriptional hubs are necessary for gene transcription[40], and we speculate that ecDNA hubs are a kind of transcriptional hub mediated by protein-protein interactions. The specific proteins involved in these interactions may differ depending on the amplified genetic elements on ecDNA. This is supported by dispersal of *MYC* ecDNA hubs via inhibition of the bromodomain and extraterminal (BET) proteins in a colorectal cancer cell line[24]. *MYC* ecDNA hubs are not disrupted by transcriptional inhibition with alpha-amanitin or by 1,6-hexanediol[24], suggesting that ecDNA hubs do not depend on active transcription by RNA polymerase II or specific interactions between intrinsically disordered regions that are sensitive to hexanediol, such as those of Mediator 1 (ref. [41]). As BET proteins can normally concentrate accessible DNA, exclude heterochromatin, and mediate long-range enhancer-promoter communication[42,43], it is possible that ecDNA hubs may co-opt endogenous mechanisms of long-range gene looping within chromosomes to promote intermolecular chromatin interactions in ecDNA ensembles. Other

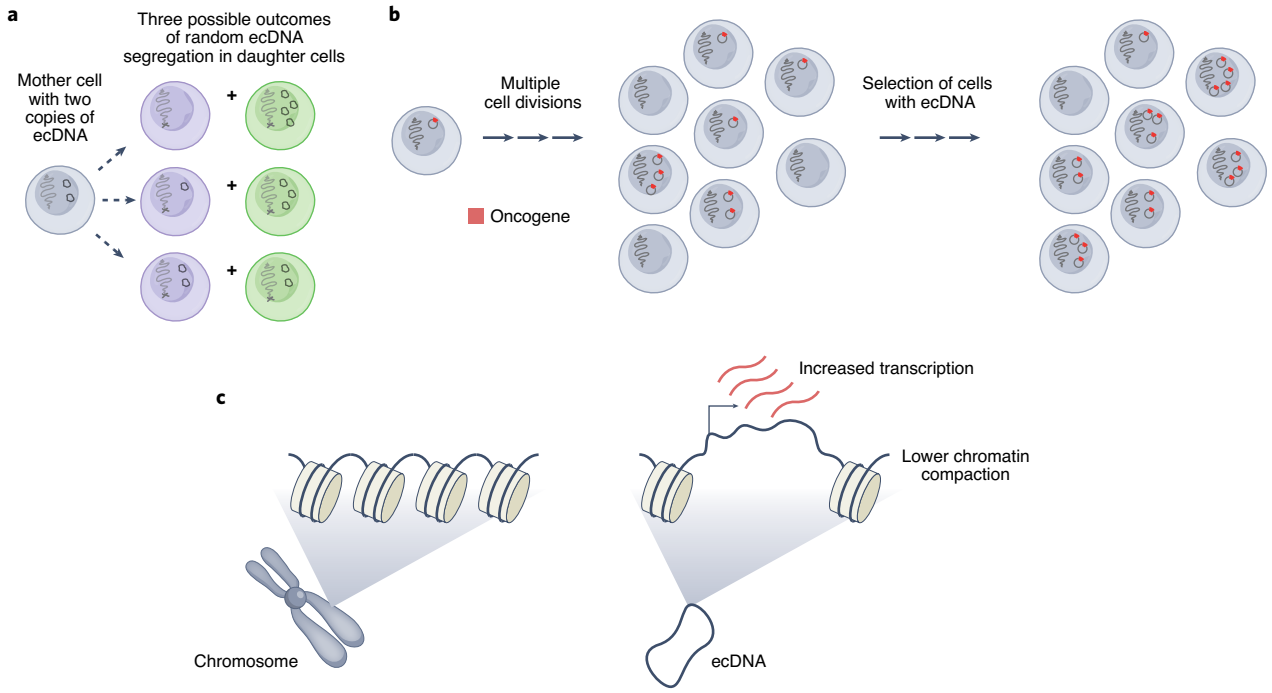

Fig. 1 | Unique characteristics of ecDNA. a, Random segregation of ecDNA molecules during cell division allows several possible outcomes in the number of gene copies inherited. **b**, Cancer cells containing ecDNAs, after undergoing multiple cell divisions with random ecDNA segregation, can have a wide range of copy numbers. ecDNAs that provide a fitness advantage to cancer cells can be selected for. **c**, ecDNAs have lower chromatin compaction and increased transcription.

Table 2 | Organization of ecDNA hubs and chromosomes

ecDNA hubs ('extrasomes')	Chromosomes
Tens to hundreds of ecDNAs in proximity that interact with one another; tethered by proteins	Endogenous gene loci are covalently linked; one long piece of DNA per chromosome
Regulatory elements, such as enhancers, interact with target genes in *cis* and in *trans*	Regulatory elements primarily interact with genes in *cis* within the same TAD
ecDNA hubs coalesce during interphase but can dynamically break into smaller clusters of ecDNAs during mitosis	Chromosomes are dispersed during interphase and condense during mitosis

than *MYC*, which is regulated by BET proteins[44,45], this model predicts that other oncogenes amplified on ecDNA may exploit their endogenous enhancer mechanisms to operate in ecDNA hubs. As functional enhancers are co-selected with *EGFR* on ecDNAs in glioblastoma[46], we speculate that proteins that mediate endogenous enhancer-*EGFR* interactions could be involved in ecDNA hub maintenance as well.

Dispersal of ecDNA hubs was associated with reduced oncogene expression in ecDNA-containing cells, suggesting that ecDNA hubs may be a vulnerability of these oncogene-addicted cells. This observation also implies that ecDNAs may depend on unique transcriptional regulators, warranting further investigation of distinct or common regulators of ecDNA hub formation and transcription across cancer cells with various ecDNA amplicons. As long non-coding RNAs (lncRNAs) are involved in the formation of interchromosomal interactions[34], additional studies may address whether lncRNAs play a role in ecDNA hub formation. Finally, it is still an open question whether ecDNA hubs inhabit specific territories in the nucleus in relation to other chromosomes. Chromosome territories are non-randomly distributed in the nucleus and can even be conserved across different species, suggesting functional importance of the radial organization of chromosomes in the nucleus[31,47]. The radial, sub-nuclear arrangement of ecDNA hubs in relation to chromosome territories may provide novel insights into ecDNA functions.

Implications of ecDNA hubs for evolution of oncogene diversification, cooperation, and ecDNA reintegration

Given that ecDNA has been separated from the 3D genomic context of its chromosomal origin, it has been proposed that the co-selection of oncogenes and enhancers shapes ecDNA amplicon structures[46,48]. With the observation of intermolecular interactions among ecDNAs carrying distinct enhancer elements, we propose a two-level model for oncogene-enhancer co-selection (Fig. 2c). The first level of co-selection occurs at individual ecDNAs: molecules that possess functional enhancers can promote oncogene expression and provide better fitness to cancer cells than can ones that do not. The second level of oncogene-enhancer co-selection occurs at the repertoire of ecDNAs in hubs. We predict that each ecDNA molecule does not need to contain the full set of enhancer elements for oncogene activation; rather, they exist as part of an ecDNA hub that facilitates chromatin interactions among a diverse repertoire of regulatory elements and promotes interactions between the target oncogene and functional enhancers, which may be located on distinct molecules. This model raises the intriguing concept that winning the clonal competition among cancer cells occurs through clonal cooperation among ecDNA molecules. Furthermore, the presence of functional regulatory elements in a cooperative ecDNA hub may increase tolerance of mutations on individual molecules. Others have previously reported ecDNA mutational diversity and rapid response to environmental changes[49], though further investigation is needed to measure mutational diversity in functional enhancers on ecDNAs.

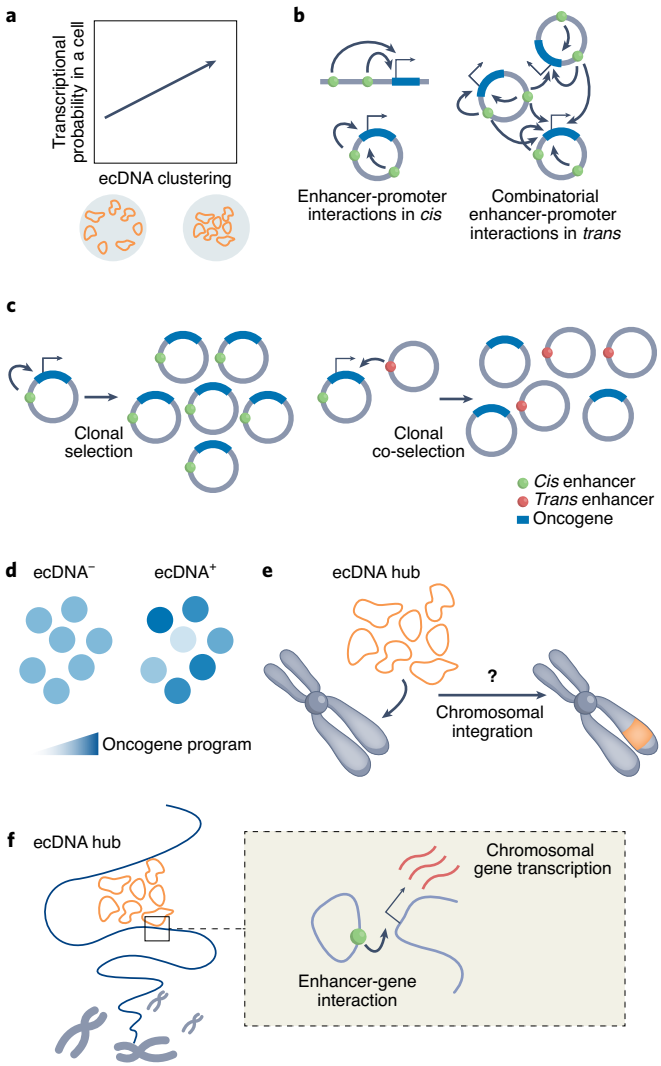

Fig. 2 | ecDNA hubs drive oncogene expression and may shape cancer-cell evolution. a, ecDNA clustering in hubs is associated with oncogene transcription. **b**, Enhancer-promoter contacts in *trans* within ecDNA hubs enable combinatorial interactions. **c**, Enhancers and oncogenes may be co-selected at two levels. First, enhancer-oncogene pairs on the same molecule, which drive oncogene expression, can be selected together. Second, distinct ecDNA molecules containing enhancers and oncogenes that interact intermolecularly may be co-selected. **d**, Dynamic ecDNA hub transcriptional activity is linked to heterogeneous oncogene expression levels in a cell population. **e**, ecDNA hubs may allow correlated integration of multiple amplicon copies into chromosomes to form HSRs. **f**, ecDNA-chromosome contacts allow enhancers on ecDNAs to interact with chromosomal genes and activate transcription.

ecDNA hubs are also associated with variable enhancer usage and heterogeneity in oncogene activity among cancer cells[24] (Fig. 2d). This may be attributed to differential enhancer-promoter interactions that occur in the context of ecDNA hubs. As dozens of ecDNA molecules can cluster together in many possible spatial configurations, this may provide an opportunity for ectopic enhancer-promoter interactions that do not normally occur on linear chromosomes. We speculate that these differential interactions contribute to highly variable enhancer activities and enhancer rewiring on ecDNAs. Furthermore, we speculate that ecDNAs markedly extend the concept of cancer genetic heterogeneity, as tumor cells can contain ecDNAs with diverse sequences and different oncogenes and regulatory sequences, which can interact with each other and even potentially combine into larger, single circular elements. The potential for driving diversity and accelerated evolution is remarkable.

ecDNA-chromosome interactions. The formation of ecDNA hubs may provide a palatable explanation for the well-known tendency of a subset of ecDNA$^+$ cancer cells to develop homogeneously staining regions (HSRs, a type of tandem amplifications on chromosomes). Double-strand breaks in ecDNAs can trigger aggregation, micronucleus formation, and reintegration into chromosomal HSRs[39]. ecDNAs that are spatially proximal in hubs could enable correlated DNA breaks[50] and concentrated DNA cargo, creating a potential set up for HSR formation (Fig. 2e). Hub formation may also impact ecDNA segregation. Previous work suggested that ecDNAs are transmitted into daughter cells in clusters during mitosis[38]. Future studies may address whether an ecDNA hub serves as a unit of inheritance or merely as a transient congregation. Other cellular processes, such as DNA repair and replication, are also regulated by genome organization[51,52]. For example, replication units, or replicons, form clusters in which replication origins fire synchronously[51]. Furthermore, genomic loci located in the nuclear interior contain early-replicating domains, while the nuclear periphery is associated with late-replicating domains[51]. Therefore, the observation of ecDNA hubs warrants further investigation into whether this unusual 3D organization of DNA molecules impacts these cellular processes.

In addition to regulatory interactions among ecDNAs, ecDNAs also interact with specific sites within chromosomes[53] (Fig. 2f). These sites of ecDNA-chromosome interactions are associated with increased transcriptional activity and active histone H3 lysine 27 acetylation (H3K27ac) marks, suggesting that these may be functional regulatory interactions that involve ecDNA enhancers and transcriptionally active regions in chromosomes[53]. Therefore, in addition to amplifying oncogenes, ecDNAs may also act as 'mobile enhancers' that modulate chromosomal gene expression. Further investigation may address how these ecDNA-chromosome interactions affect cancer-cell fitness.

Genetic basis of regulatory rewiring on ecDNA

Genetic alterations and rearrangements on ecDNAs are frequently observed[5,24,48,54–58]. As enhancer-promoter interactions are sensitive to physical distance between regulatory elements, sequence rearrangements can alter this physical distance and subsequently influence enhancer-promoter circuitry, leading to dysregulation of gene expression[59–62]. As a platform for genomic rearrangements, ecDNAs frequently contain sequences originating from the same locus as the amplified oncogene, as well as sequences originating from distal chromosomal regions or other chromosomes[24,48,54–58]. These rearrangements enable co-amplification of cognate enhancers, as well as ectopic enhancers with oncogenes[46,48]. Sequence rearrangements can result in fusion-gene transcripts, leading to dysregulation of gene expression through promoter hijacking[24,63]. As ecDNA is associated with accelerated adaptation and selection, molecules with increased transcriptional activity due to regulatory rewiring can be selected by providing a fitness advantage to cancer cells harboring them.

Enhancer hijacking. ecDNAs contain sequences that originate from chromosomes, but they are uncoupled from their chromosomal loci of origin and the regulatory context thereof. Importantly, regulation of gene expression is tightly connected to non-coding regulatory elements, such as enhancers, which physically interact with target genes. Consistent with this idea, ecDNAs containing oncogenes typically co-amplify enhancer elements that upregulate oncogene expression[46,48] (Fig. 2c). Unlike the native chromosomal locus,

ecDNAs containing sequence rearrangements can create regulatory circuitry between ectopic enhancers and oncogene promoters[46,48] (Fig. 3a). These new regulatory interactions include enhancers that are normally insulated from the target oncogene on the linear chromosome and those that are normally located in distal chromosomal regions[46,48]. This adoption of novel enhancers is termed enhancer hijacking[48,64–66]. As ecDNA amplicon structures can vary greatly among tumor samples, including different amplified sequences and different structural rearrangements, there is likely a high level of diversity in enhancer-promoter circuitry among different tumors with ecDNA amplification of a given oncogene.

Promoter hijacking. Sequence rearrangements on ecDNA can also lead to gene fusions, resulting in upregulation of oncogenes via hijacking of highly active promoters (Fig. 3b). An example of promoter hijacking was observed for the *MYC* oncogene on ecDNAs[24,57,67]. *MYC* is located ~50 kb upstream of the lncRNA gene *PVT1*, which negatively regulates *MYC* expression through enhancer competition with the *MYC* promoter normally[68]. Fusion of the *PVT1* promoter with the coding sequence of *MYC* is proposed to overcome this negative regulatory feedback loop by direct linkage of the *PVT1* promoter activation with *MYC* transcription[69]. *PVT1-MYC* fusion has been reported in multiple cancer types, including breast, colon, ovary, and esophagus cancers and medulloblastomas[57,69–72]. This rearrangement can be observed on ecDNAs[24,67] and is generally associated with focal copy-number amplifications[57,69–72]. *PVT1-MYC* fusion was observed in 60% of select *MYC*-amplified cases of Group 3 medulloblastomas[69], a cancer subtype that is 18% *MYC* ecDNA$^+$ (ref. [73]). Therefore, *PVT1-MYC* fusion potentially accounts for a significant portion of medulloblastoma cases with ecDNA amplifications, although further systematic analyses are needed to provide more insight into how frequently promoter hijacking events are observed in the context of ecDNAs in various tumor types. A recent study showed viral-human hybrid ecDNAs containing sequences of human and human papillomaviral (HPV) origins in ~20% (6 out of 28) of HPV oropharyngeal cancer samples, including amplicons that enable high levels of viral-human fusion-gene transcription driven by HPV promoters[63]. These observations hint at promoter hijacking as a powerful mechanism for upregulating gene expression on ecDNAs.

Ectopic topologically associating chromatin domain. Most of the genome is organized into TADs, which are self-associating chromatin domains on the scale of 100 kb to over 1 Mb[74–78]. DNA elements within a chromosomal TAD are in contact with each other at much higher frequencies than with elements outside of the TAD, enabling *cis* interactions between regulatory elements, including enhancer elements and gene promoters. These enhancer-promoter *cis* interactions activate gene transcription and control cellular programs. Typically several hundred kilobases to several megabases, ecDNAs and chromosomal TADs are on similar length scales. Nevertheless, ecDNAs differ from chromosomal TADs in two major ways. First, ecDNAs are circular, as demonstrated by electron microscopy and pulsed field gel electrophoresis[23,79–82]. Therefore, In contrast to chromosomal TADs, ecDNAs are covalently closed, rather than stabilized by looping proteins such as cohesin and CTCF. Thus, although TADs vary between cells[83–85], the covalent circular structure of ecDNA is stable (Fig. 3c). Consistent with this idea, gene loci and regulatory elements on a circular ecDNA interact with each other much more often than do corresponding loci on chromosomes[23,53]. Second, chromosomal TADs are relatively conserved across cell types[74,86], but ecDNA amplicons can have variable boundaries and sequence arrangements in individual tumors, allowing non-interacting loci in the native chromosomal locus to interact with each other ectopically on the circle[23,46,48] (Fig. 3a). Therefore, by bringing regulatory elements into proximity on the circular structure, ecDNAs effectively serve as a type of covalently closed, ectopic 'TAD.' This circular structure also serves as the basis for enhancer hijacking through incorporation of distal enhancer elements into the same DNA circle as the target gene, as described above.

Methods for studying gene regulation on ecDNA

ecDNAs containing oncogene amplifications arise from chromosomes originally and therefore comprise sequences that heavily overlap with chromosomal DNA. Thus, bulk sequencing data typically cannot separate signal derived from ecDNA from that derived from the native chromosomal loci containing the corresponding genes and must therefore be carefully interpreted. Imaging-based methods can provide good separation of chromosomal and ecDNA signals depending on the approach, but suffer from limited throughput and DNA sequence resolution. We have recently demonstrated a strategy for isolating ecDNAs and separating amplicons by size, providing empirical evidence for ecDNA structures and enabling targeted profiling of their genetic sequences as well as epigenomic landscapes[87].

Imaging-based methods. ecDNAs were originally discovered by imaging of metaphase chromosome spreads from cancer cells[1]. Cells arrested in metaphase are fixed, and condensed mitotic chromosomes are physically spread out on a microscope slide, providing excellent separation of chromosomes and ecDNAs. Metaphase spreading followed by DNA fluorescence in situ hybridization (FISH) further allows hybridization of a sequence-specific probe and detection of oncogenes on ecDNA (Fig. 4a). This method typically requires cell culturing followed by metaphase arrest. DNA FISH can also be performed on cells in interphase without arrest (Fig. 4a), allowing detection of oncogene amplifications in clinical tumor sections. Interphase DNA FISH provides information about the spatial distribution of ecDNAs in intact cancer-cell nuclei and led to the discovery of ecDNA hubs[24]. Interphase DNA FISH combined with nascent RNA FISH (using probes that target intronic sequences on pre-messenger RNA) also allows single-molecule assessment of transcriptional activity on ecDNAs[24]. However, interphase FISH alone does not identify ecDNA presence definitively and, therefore, should be used in combination with other methods, such as metaphase FISH. Finally, live cell imaging has been used to visualize ecDNA dynamics in live cancer cells during interphase and mitosis[24,27,35] (Fig. 4a).

Bulk-sequencing-based methods. High-throughput sequencing has enabled detailed characterization of the cancer genome and epigenome and has provided novel insights into the spatial and structural bases of the regulation of gene expression on ecDNA. Some examples are (1) the mapping of DNA regulatory elements using assays for transposase accessible chromatin with sequencing (ATAC-seq)[88] and chromatin immunoprecipitation with sequencing (ChIP–seq) targeting markers of regulatory elements, such as H3K27ac[89]; (2) the identification of chromatin interactions associated with spatial organization involving regulatory elements using HiChIP[90,91], chromatin interaction analysis by paired-end tag sequencing (ChIA-PET)[92], or chromatin interaction analysis with droplet sequencing (ChIA-Drop)[93]; and (3) the fine-scale assessment of variable regulatory element activities at the single-cell level using single-cell multiomics, allowing simultaneous measurements of RNA expression and chromatin accessibility[94–96] (Fig. 4b). These various methods have been applied to studying gene regulation on ecDNAs[23,24,46,48,53]. Sequencing signals represent all DNA material in the samples, including both chromosomal DNA and ecDNA. In cancer cells containing high copy numbers of ecDNA, the majority of sequencing signals is assumed to originate from ecDNA molecules. However, ecDNA-focused interpretations of bulk sequencing data are more challenging when ecDNA molecules

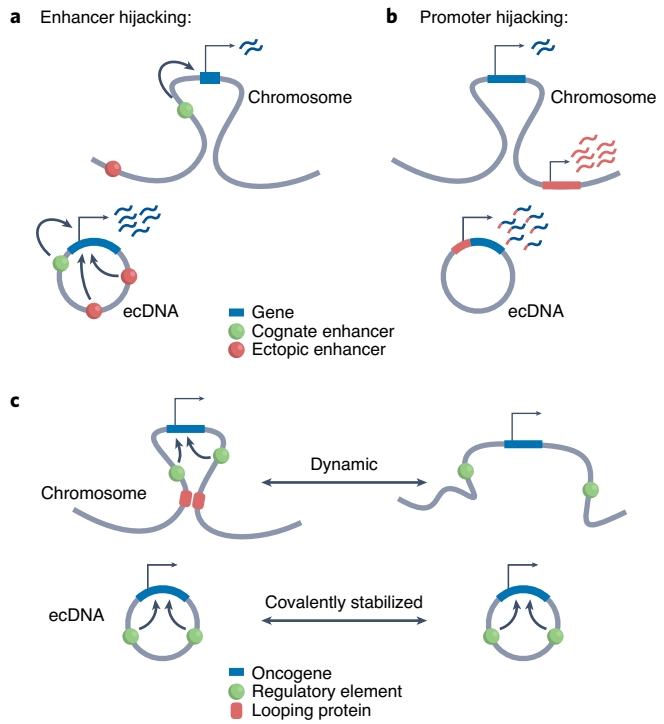

Fig. 3 | Genetic and structural basis of the regulatory circuitry on ecDNA. **a**, Enhancer hijacking on ecDNA leads to ectopic enhancer-gene interactions by bringing distal enhancers into proximity via sequence rearrangement. **b**, Promoter hijacking drives gene expression by structural rearrangement on ecDNA. **c**, The circular structure of ecDNA brings regulatory elements into spatial proximity and increases their interactions, as compared with the more variable and dynamic loop structure of a chromosomal TAD.

are present at lower copy numbers or in a small subset of cells in a heterogeneous cell population. Furthermore, ecDNAs typically contain extensive structural rearrangements and can be a mixture of heterogeneous amplicons containing various sequence elements within a cancer-cell population[5,24,48,54–58,87], thus altering the two-dimensional distances between loci as compared with the chromosomal reference sequence. Therefore, interpretations of chromatin regulatory interactions in relation to gene loci can benefit from construction of custom rearranged ecDNA sequence maps in addition to alignment to the reference genome.

Isolation of ecDNA. To better profile the genetic and epigenetic landscapes of ecDNAs, there is a need for molecular methods to isolate ecDNAs from cancer cells for targeted profiling and comparisons between ecDNAs and chromosomes. A technique for unbiased isolation of DNA circles, termed Circle-seq, was previously developed for small eccDNA molecules[19–21] and has recently been applied to oncogenic ecDNAs[97] (Fig. 4c). Circle-seq involves magnetic-bead-based genomic DNA isolation and exonuclease digestion of linear DNA fragments, followed by multiple displacement amplification (MDA) of remaining DNA. Application of this method to neuroblastoma samples enabled analysis of structural rearrangements on ecDNAs[97]. However, as megabase-sized ecDNAs are extremely fragile in solution and prone to breakage, Circle-seq favors small ecDNA and eccDNA species. In-solution DNA isolation is not recommended for DNA molecules that are above 100 kb in size.

To isolate ecDNAs that are megabases in size, which are commonly observed in cancer cells, a method termed CRISPR–CATCH (CRISPR–Cas9-assisted targeting of chromosome segments) can be used[87,98] (Fig. 4c). In this method, ultra-high-molecular-weight genomic DNA is embedded in agarose to maintain DNA integrity[87,99]. Following agarose entrapment of genomic DNA, CRISPR–Cas9 ribonucleoprotein is used to cleave ecDNA circles in vitro, and the resulting DNA is separated in pulsed field gel electrophoresis (PFGE). As linearized ecDNAs represent specific amplicon sizes, they are separated by size in PFGE and extracted from the gel. CRISPR–CATCH allows targeted profiling of the ecDNA genetic sequence and epigenomic landscape. This method serves as an empirical approach for heterogeneous amplicon separation and sequence reconstruction. It also enables physical separation of chromosomal DNA and ecDNA from the same cell sample, allowing direct comparisons. However, this targeted approach can enrich only for ecDNAs containing the CRISPR–Cas9 target sequence (for example, a known oncogene) and therefore cannot be used as a method for unbiased ecDNA detection.

Computational inference of ecDNA structure. In parallel to molecular techniques for ecDNA isolation, computational tools can infer ecDNA amplicon structures from bulk sequencing data (Fig. 4d). Whole-genome short-read sequencing data can be analyzed by AmpliconArchitect, which constructs breakpoint graphs on the basis of structural rearrangements detected, and infers amplicon structures[54]. Sequence information from long DNA molecules can be used to provide more accurately phased structural arrangements. Such information can be collected using long-read sequencing or optical mapping (OM)[100], both of which have been applied to ecDNA amplicons to resolve complex structures[23,24,48]. AmpliconReconstructor integrates short-read sequencing data and OM data for accurate reconstruction of ecDNA amplicons[55]. Finally, ecDNA sequence maps can be orthogonally constructed using sequence data from CRISPR–CATCH[87] (Fig. 4d). Analysis of ecDNA amplicon structures in the context of regulatory elements may provide a better picture of how regulatory circuitry can be rewired by structural rearrangements on ecDNA, including the enhancer and promoter hijacking events described earlier.

Functional interrogation via CRISPR interference. To interrogate the regulation of gene expression by perturbation, CRISPR interference (CRISPRi) has been used to both directly target gene promoters on ecDNA and target non-coding regulatory DNA elements like enhancers[23,24,46] (Fig. 4e). Owing to elevated ecDNA copy numbers, Cas9-mediated DNA cleavage can lead to many double-strand breaks and is likely to come with unintended effects caused by the DNA-damage response as well as chromosomal integration of ecDNAs[58]. However, CRISPRi can effectively silence ecDNA promoters despite high copy numbers, though effects of enhancer targeting appear diminished, potentially owing to combinatorial enhancer-gene interactions and compensation by other enhancers within ecDNA hubs[24]. Nonetheless, enhancer effects on cell survival and oncogene expression can be detected in more sensitive, pooled assays[24,46]. Pooled perturbation of enhancers by CRISPRi has identified cognate as well as ectopic oncogene enhancers that upregulate oncogene expression on ecDNAs and increase ecDNA$^+$ cancer-cell survival. These studies demonstrated an enhancer hijacking mechanism as well as intermolecular cooperativity in ecDNA hubs, two driving forces of ecDNA evolution and oncogene upregulation[24,46].

Conclusions

While ecDNAs have been long known to be an important mechanism of oncogene amplification in cancer, their impacts on cancer development, progression, evolution, and drug resistance are only beginning to be appreciated. With recent advances in sequencing technologies, we are now able to obtain detailed information about alterations in the cancer genome, including oncogene amplifications, as well as consequences in gene expression programs. While mapping sequencing data of cancer cells to the reference sequence shows amplified oncogene

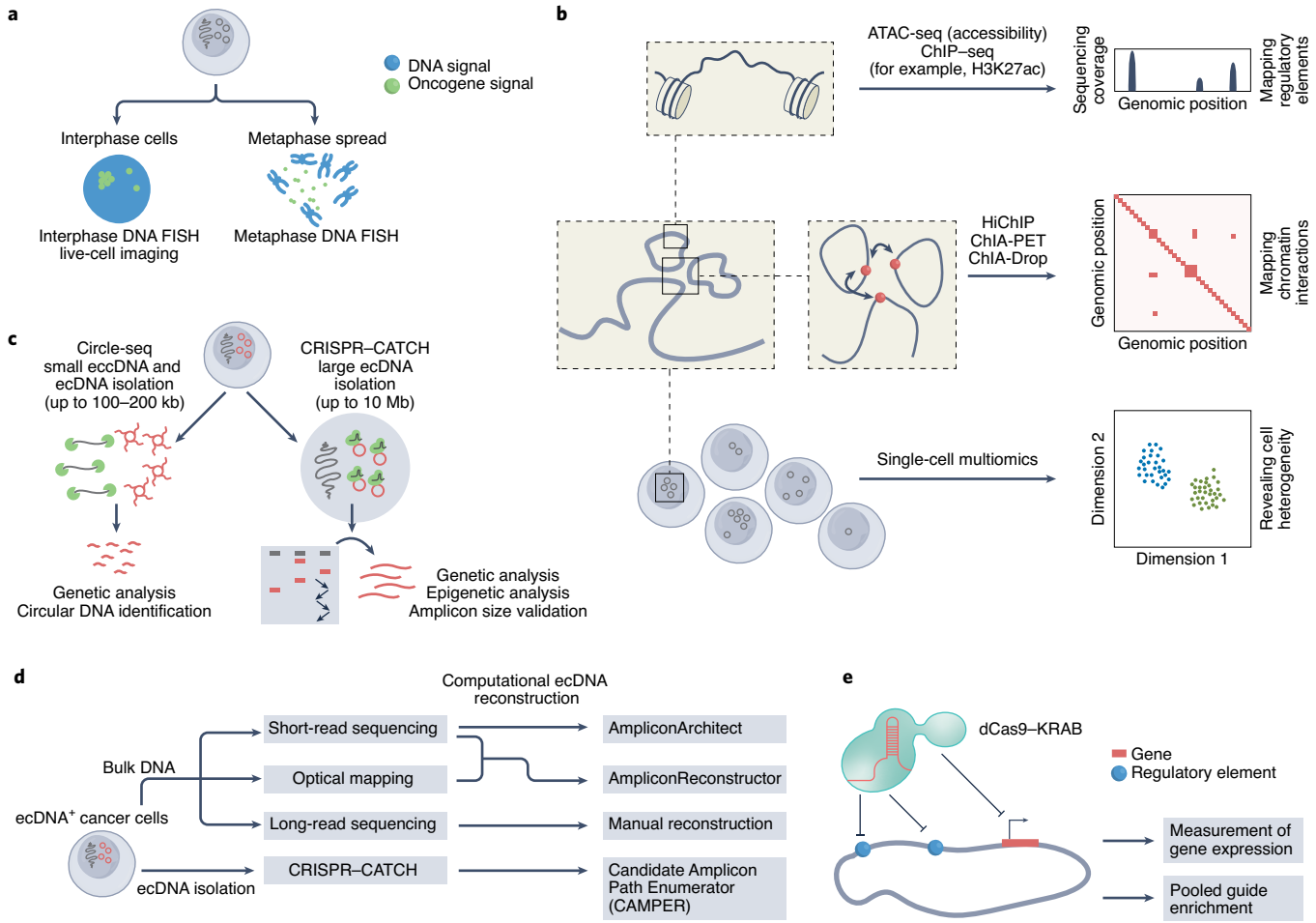

Fig. 4 | Technologies used to reveal ecDNA gene regulation and structure. a, Imaging of ecDNA in interphase cells or metaphase spreads. **b**, Bulk and single-cell sequencing approaches to identify regulatory elements, map chromatin interactions, and reveal cell heterogeneity in the context of ecDNA. **c**, Isolation of ecDNA and targeted analyses. **d**, Computational methods and tools for reconstruction of ecDNA sequence. **e**, CRISPR interference of gene promoters and regulatory elements on ecDNA.

sequences and structural rearrangements, it obscures differences in the spatial distribution of these oncogene amplifications. With the renewed interest in extrachromosomal oncogene amplification, we are now equipped to study a wide range of cancer-biology questions in the context of ecDNA, including how gene expression is regulated.

As collective attention shifts from a sequence-oriented view to interrogating molecular topology, architecture, and structural organization in cancer, ecDNA has emerged as a challenge at the interface of cancer genetics and epigenetics. ecDNA also poses exciting challenges for our understanding of tumor evolution. As described, the concept of ecDNA hubs and intermolecular combinatorial interactions opens a new paradigm in understanding how physical architecture regulates gene regulation, as the fundamental unit of transcription shifts from the gene to the hub.

Future development. The discoveries of ecDNA hubs and regulatory rewiring on ecDNAs have provided a strong basis for the identification of unique aspects of transcriptional regulation of oncogenes not previously appreciated. These unique regulatory mechanisms on ecDNAs may point to oncogene transcriptional dependencies of ecDNA$^+$ cancer cells. Future studies dissecting these regulatory mechanisms may provide insights into potential vulnerabilities of ecDNA-driven and oncogene-addicted cancers. Genome-wide genetic screens, as well as small-molecule screens with a focus on ecDNA$^+$ cancer models, may identify these vulnerabilities. The observation that ecDNA hubs drive extrachromosomal and chromosomal gene expression warrants further investigation of their significance in in vivo models, as well as models of various cancer types. These future investigations will address whether ecDNA hubs are universal or cancer-type- or oncogene-specific. In addition, large-scale studies tracking extrachromosomal oncogene content within primary tumors, as well as metastases in people with cancer during chemotherapy and immunotherapy, may address the long-standing question of whether ecDNAs provide an additional advantage to cancer cells undergoing selective pressure.

Ongoing efforts in method development allow ecDNA characterization with ever increasing resolution. As molecular and computational tools now enable isolation of ecDNAs as well as prediction and reconstruction from bulk cancer samples, these techniques may provide important insights into the prevalence and evolution of ecDNAs in clinical tumor samples. Finally, future development of empirical ecDNA-detection methods in clinical samples is urgently needed. Although oncogene copy-number amplifications can be detected by interphase DNA FISH and whole-genome sequencing, it is still challenging to empirically and definitively attribute these amplifications to ecDNAs in clinical tumor samples. Development of such techniques will enable systematic examination of clinical outcomes associated with ecDNAs, as well as sequence and

structural features of ecDNAs in tumors. Together, these upcoming advances will more precisely pinpoint the role of ecDNA-based oncogene amplification in cancer and identify ways to target oncogene-addicted tumors therapeutically.

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Competing interests

The authors declare no competing interests.

Additional information

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