Extrachromosomal Circular DNAs, Amplified Oncogenes, and CRISPR-Cas9 System

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ABSTRACT

Structurally rearranged extrachromosomal circular DNAs (eccDNAs) have been identified in tumor cells, many of which carry regions related to recurrent cancer driver oncogenes (e.g., CCND1, EGFR, and MYC). In a tumor cell, eccDNAs are carrying regions associated with oncogene amplification (>10-fold amplified-copy numbers in human tumors) and poor outcome across multiple cancers. Even though dual-delivery of pairs of CRISPR and CRISPR-associated protein 9 (Cas9) guiding RNAs into normal human cells was reported to induce circularization of genes and chromosomes, in bacteria, the CRISPR-Cas9 system primarily targets extrachromosomal rearranged elements. Likewise, in cancer cells, it is expected that a designed CRISPR-Cas9 system would be able to target extrachromosomal copy number amplifications and produce double-strand breaks detrimental to cellular fitness by dictating gene-independent copy number loss-offitness effects and antiproliferative responses. A system designed against amplified amplicons may provide a novel approach for cancer therapy and propose a practical implication for CRISPR-Cas9 pairs as a pathway in therapeutic strategies of cancer.

SIGNIFICANCE STATEMENT

Structurally rearranged extrachromosomal circular DNAs (eccDNAs) have been identified in tumor cells. Many eccDNAs are carrying regions related to recurrent cancer driver oncogenes (e.g. CCND1, EGFR and MYC). It is expected that a designed CRISPR-Cas9 system would be able to target extrachromosomal recurrent oncogenes.

Introduction

Extrachromosomal circular DNAs (eccDNAs), which are present in both prokaryotes and eukaryotes, are anticipated to be the major source of somatic rearrangements. Circular DNA elements act as supplements for chromosomal genetic material that deviate from normal rules of Mendelian inheritance with accelerated mutation (Hull and Houseley, 2020; Koche et al., 2020). In human cancers, however, eccDNAs are associated with copy number amplification of proto-oncogenes and transposable element circularization, inversion of genes, and mutagenesis (Møller et al., 2018; Koche et al., 2020). Whole-genome sequencing analysis of cancer patients has demonstrated that eccDNA-based oncogene amplification is common in cancers. EccDNAs and extrachromosomal rearrangements are different from chromosomal amplicons, which derive multiple copy number genes across cancer types, and are associated with poor outcomes in patients. Compared with chromosomal amplifications, patients with eccDNA-based oncogene amplifications have shown shorter survival. Cancer types contain certain eccDNAs (Table 1) with high oncogene amplification and recurrence in individuals. The most common and recurrent oncogenes or related regulatory sequences are highly amplified in eccDNAs (Table 1). Additionally, compared with copy number–matched chromosomal oncogenes, eccDNA-related amplicons exhibit enhanced accessibility, which can lead to higher levels of oncogene transcription and frequent fusions of the transcripts (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020).

Circular and chimeric DNA elements can reintegrate into the genome and cause chromatin remodeling and oncogenic rearrangements (Koche et al., 2020). Thereby, eccDNAs can derive complex chromatin rearrangements and an ongoing mutagenic process, which leads to further cancer lesions and poor outcomes (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020).

Although eccDNAs most likely arise by mutational processes in human somatic cells, they can cause genome-wide mutagenic processes on their own. EccDNAs become highly amplified and derive extrachromosomal oncogene amplification by carrying a large copy number of oncogenes and accumulating in somatic cells. They contribute to elevated levels of oncogene transcripts through chimeric circularization and enhanced amplicon accessibility, which is coupled with

ABBREVIATIONS: Cas9, CRISPR-associated protein 9; CNV, copy number variation; DSB, double-strand break; eccDNA, extrachromosomal circular DNA; ESCC, esophageal squamous cell carcinoma; LOF, loss of fitness; sgRNA, single-guide RNA.
transcript fusions. Even more, in the nuclei, eccDNAs are able to amplify the expression of chromosomal genes through the regulatory regions presented on eccDNAs and associated with gene expression. They represent a major source of oncogenic remodeling and complex DNA rearrangements through circularization and reintegration into linear chromosomes (Möller et al., 2018; Hull and Houseley, 2020; Kim et al., 2020; Koch et al., 2020).

The CRISPR-Cas9–based system is attending as a genetic tool to tractably edit genomes (Kovač et al., 2010; Molenda et al., 2019; Halpin-Healy et al., 2020). By providing a single-guide RNA (sgRNA) and Cas9 endonuclease, the CRISPR-Cas9 technology has evolved into an ideal tool for genetic therapy and is expected to be enabled for efficient gene targeting at predetermined sites in the human genome (Fig. 1). It provides a powerful approach to generate genetic models both for fundamental and preclinical research. According to the literature, the CRISPR-Cas9 system would specifically target copy number–amplified regions outside the chromosomes, where it would promote antiproliferative and antisurvival effects on cancerous cells and be detrimental to tumor cellular fitness (Aguirre et al., 2016; Munoz et al., 2016; Song et al., 2016; Gonçalves et al., 2019).

### EccDNA and Copy Number Variations in Somatic Tissues

EccDNAs can be found in a tumor at the preamplification stage, where they are transposase-sensitive and represent a resistance marker to therapy. In an analysis of various tumor types, high copy numbers of eccDNAs were identified that carried recurrent regions related to cancer driver genes and amplifications. For example, in glioma cells, hundreds of eccDNAs were identified that carried recurrent regions related to EGFR oncogene (which is located on chr7) (Möller et al., 2018; Kim et al., 2020; Kumar et al., 2020).

There are reports on patients with lung cancer showing that eccDNAs can migrate from malignant tissue into the healthy tissues. EccDNAs have been also detected in healthy muscle and blood samples from normal individuals, where they exhibit nuclear origin and exosome secretion (Möller et al., 2018; Kim et al., 2020). It must be noted that eccDNAs may exist in normal cells and tissues such as muscle and blood, where they carry genes or gene-related fragments as a result of mutations or aging in the cells (with the size of <25 kb) (Hull et al., 2017; Hull and Houseley, 2018, 2020; Kim et al., 2020). Deletions have been found to produce eccDNAs in yeast and in cattle, however, eccDNAs were found to include one or several full-length genes and be associated with mutations or aging process in human aged tissues (Möller et al., 2018; Hull and Houseley, 2020).

Three characteristic properties have been leveraged to eccDNAs: 1) circular elements, 2) lack of a centromere, and 3) high amplification (Kim et al., 2020). EccDNAs of chromosomal origin are believed to exist in aged tissues to provide accelerated adaptation through stimulated copy number variations (CNV) of driver genes in response to environmental change (Hull et al., 2017; Lanciano et al., 2017; Möller et al., 2018; Hull and Houseley, 2020). Thus, somatic aged cells are rich in environment-driven eccDNAs that may influence phenotypes through amplified gene copy numbers and transcription of full-length or truncated genes (Hull et al., 2017; Möller et al., 2018). Transcriptional activity of eccDNAs and their contents has helped to formalize this idea of adaptive function in response to particular environmental conditions by simply connecting eccDNA formation to transcriptional induction and amplification of specific genes. By this clever strategy to upregulate specific genes by accumulating eccDNA, cells could gain the maximum chance of adaptation and thereby stability. Therefore, eccDNAs produce benefits for cells by providing gene copy numbers and the potential for a high mutation rate without the associated risk of generating deleterious chromosomal mutations (Hull et al., 2017; Hull and Houseley, 2020).

### TABLE 1

Some human cancers and the most-known driver genes amplified in eccDNA/gene duplications

<table>
<thead>
<tr>
<th>Cancer Types</th>
<th>Associated Driver Genes</th>
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<tbody>
<tr>
<td>Adrenocortical carcinoma</td>
<td>EGFR, FGFR2, KIT, FOXA1, NFE2L2, SOS1, H3F3A, PCBP1, PMS1, SF3B1, AKT1, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
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<tr>
<td>Breast invasive carcinoma</td>
<td>EGFR, FGFR2, KIT, FOXA1, MYC, CG, AKT1, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Brain Lower Grade Glioma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, SOX17</td>
</tr>
<tr>
<td>Glioblastoma multiforme</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Kidney renal papillary cell carcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Liver hepatocellular carcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Kidney renal clear cell carcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Stomach adenocarcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Skin Cutaneous Melanoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Thyroid carcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Uterine Corpus Endometrial Carcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
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<tr>
<td>Prostate adenocarcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
</tr>
<tr>
<td>Lung squamous cell carcinoma</td>
<td>EGFR, FGFR2, CDK4, FOXA1, KRAS, WT1, H3F3A, CCND1, PIK3CG, PIK3CA, PIK3R2, MTOR, SF1, TP53, ERCC2, ERBB2, ERBB3, SOX17</td>
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For more details, see references (Aguirre et al., 2016; Munoz et al., 2016; Hull et al., 2017; Lanciano et al., 2017; Gonçalves et al., 2019; Kumar et al., 2020).
Data propose a general mechanism for CNV formation and define the key mechanistic elements underlying this selectivity. Quantification of eccDNAs in individual cells revealed remarkable allele selectivity and recurrence of certain eccDNAs in several individuals in the rate at which specific circular amplicons were highly nonrandom (Hull et al., 2017; Kim et al., 2020). The chromosomal origin of the circular amplicons was found to be highly nonrandom. In tumors with amplified oncogenes, actively transcribed DNAs, such as DNA repeats and 5S ribosomal DNA, are also found on circular DNAs. In human cells under certain conditions, circular DNAs can thereby form and make up a fraction of the genome that would be expressed (Hull et al., 2017; Lanciano et al., 2017; Møller et al., 2018). In budding yeast, for example, under certain conditions environmental copper stress induces eccDNAs that contain the ribosomal DNA (ERCs) and copper-resistance gene CUP1. Formed eccDNAs highly accumulate in stressed cells, where they drive premature aging and shortened life span. ERCs and CUP1 carry active replication origins that account for the massive abundance of eccDNAs (Hull et al., 2017; Hull and Houseley, 2020). In other words, as the cell ages or experiences stressful conditions, a subset of eccDNAs forms and reaches high copy numbers while the diversity of circular DNA decreases (Hull and Houseley, 2020; Møller et al., 2018).

In replicating cells, oncogenes tend to be amplified by circular structures. EccDNA-based amplifications that occur in cancers are different from chromosomal-oncogene amplifications and are associated with poor patient prognosis (Møller et al., 2018; Kim et al., 2020).

Under certain conditions, highly repetitive DNAs can also contribute to circular DNA formation. For example, epigenetic mechanisms strictly silence retrotransposons, whereby epigenetic erasing during development or upon stressful conditions leads to retroelements activation and the ability to form eccDNAs (Hull et al., 2017; Lanciano et al., 2017). To limit the number of active elements and their insertions into the genome, repeat end-joining mechanisms of nonhomologous/homologous recombination by DNA repair processes are involved that form eccDNAs (Lanciano et al., 2017; Møller et al., 2018). Other genomic repeats, such as transposon elements, tandemly repeated genes (the ribosomal DNA), and telomeres can contribute to eccDNA formation (Lanciano et al., 2017; Mølenda et al., 2019; Hull and Houseley, 2020).

The genome sequencing of pediatric medulloblastoma exhibited a catastrophic event of eccDNA formation that was linked with p53 mutation. Findings indicate that there is a strong association between somatic p53 mutations and chromothripsis in medulloblastoma and acute myeloid leukemia where massive chromosome rearrangements and eccDNA formation were unavoidable (Rausch et al., 2012).
Recurrent Oncogenes in Highly Amplified eccDNAs

CNV or high copy number amplifications (recurrence of certain eccDNAs in human somatic tissues, originating from certain DNA circularization hot spots) occur in cancers and are associated with tumor progression and resistance to chemotherapy, as well as human age-related disorders (Hull et al., 2017; Hull and Houseley, 2018, 2020; Kim et al., 2020). It has been found that the highly amplified and recurrent oncogenes (with copy numbers >10) are present on circular amplicons (Fig. 2; Table 1). Data demonstrate that eccDNA-based amplifications and CNVs are present and common in cancer types, for example glioma, sarcoma, and esophageal carcinoma, where they can drive poor outcomes for patients (Hull et al., 2017; Möller et al., 2018; Kim et al., 2020; Sun et al., 2021). More than 18,000 eccDNAs have been identified in tumor types, particularly in gliomas and glioblastomas, many of which carry known cancer driver genes, including the well-known EGFR gene amplon from chr7 (Rausch et al., 2012; Kim et al., 2020; Kumar et al., 2020). Despite providing high copy number amplifications and diversity, eccDNAs are maintaining a mechanism by which oncogenes are easily accessible and highly transcribed (Fig. 2). EccDNA mechanisms of amplification are evident in various human tumor types, independent of cancer lineage, and negatively affect patient prognosis (Aguirre et al., 2016; Munoz et al., 2016; Möller et al., 2018; Kim et al., 2020).

Oncogene CNVs can enhance cell growth, bestow drug resistance, and complement genetic defects, particularly in challenging environments. Oncogene CNVs drive tumor growth (e.g., of AKT4, MYC, FGFR2, or CDK4) or mediate drug resistance (e.g., DHFR, KRAS, or BRAF) (Table 1) (Munoz et al., 2016; Hull et al., 2017; Lanciano et al., 2017). CNVs or high copy number amplifications of recurrent oncogenes (e.g., CCND1, EGFR, and MYC), are the most common types of genomic alterations that occur in cancers where they make the main contents of circular amplifications in tumor samples (Gonçalves et al., 2019; Aguirre et al., 2016; Munoz et al., 2016).

Whole-genome sequencing approaches have characterized 85% of amplicons in tumor cells as eccDNAs, with a median count of 16.6 eccDNAs per cell. Circular amplicons have a tendency for high copy numbers and reintegration into the chromatin, where they induce worse outcomes in patients whose tumors contain at least one circular amplicon. During cancer development, there is a selection for higher copies of growth-promoting genes, which leads to primary eccDNA formation and rapid oncogene amplification. Formed eccDNA tends to be amplified and unevenly inherited, which results in intratumoral genetic heterogeneity. Onco-eccDNAs that predict patient resistance to chemotherapy, poor prognosis, and shorter survival are detectable in the primary stages of a tumor. In the nuclei of cells, eccDNAs are able to amplify the expression of chromosomal genes through the regulatory regions presented on eccDNAs and associated with gene expression (Kim et al., 2020).

As mentioned above, well-known oncogenes are enriched on the recurrent eccDNAs, where they are highly amplified and actively transcribed. Besides exhibiting higher levels of oncotranscriptional activity, onco-eccDNAs can contribute chromosomal DNA accessibility and oncotranscript fusions (Møller et al., 2018; Kim et al., 2020).

EccDNAs in Tumor Metastasis and Patient Outcomes

There is a close association between high levels of eccDNAs and poorer cancer prognosis and outcomes. In a study, whole-genome sequencing of about 3,212 cancer patients was recorded. Data analysis demonstrated that oncogenes were highly enriched on amplified eccDNAs, and the most common

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**Fig. 2.** Somatic gain-of-function alterations in cell growth driver genes, CRISPR-Cas9 system against copy-number amplifications and its association with LOF effects on tumor cells. The illustration represents different genomic rearrangements and somatic gain-of-function alterations in cell growth driver genes by copy number amplifications that can be a potential target for CRISPR-Cas9. Targeting copy number amplifications (copy number ratio > 8) with the designed CRISPR-Cas9 system elicits a gene-independent antiproliferative/DNA damage response in an sgRNA-directed sequence-specific manner and through the induction of DSBs in DNA by the Cas9. Targeting cell growth driver genes that are scored as essential in tumor growth, such as AKT2, MYC, or CDK4, also decreases proliferation effects. G, copy number amplified genes or oncogene-related sequences.
recurrent oncogene amplifications arose on eccDNAs. Patients whose cancers carried eccDNAs had significantly shorter survival, even when controlled for tissue type, than patients whose cancers were not driven by eccDNA-based oncogene amplification (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020). Another study on the samples from pediatric high-grade glioma and adult glioblastoma patients, showed that there existed some large signals for EGFR, CDK6, and MYC with eccDNAs in the relapse but not in diagnosis (Noer et al., 2022).

In a genome-wide mapping of neuroblastoma, eccDNAs were associated with massive oncogene amplification and expression. Additionally, cancer-causing lesions and ongoing mutagenic processes in neuroblastoma were also found to emerge out of circle-derived rearrangements and associated with adverse clinical outcomes (Koche et al., 2020).

Another whole-genome sequencing of pediatric medulloblastoma brain tumors and samples from myeloid leukemia patients revealed highly complex chromosome rearrangements, massive oncogene amplifications, and chromothripsis, all of which could be linked to eccDNAs. The clinical follow-up data for patients also uncovered an association of chromothripsis and complex DNA rearrangements with poor survival and prognosis. With aforementioned evidence, these events could be linked to onco-eccDNAs formation (Rausch et al., 2012).

In a study, the level of eccDNAs was markedly elevated in patients with metastatic colorectal cancer. The patients who were carrying at least one circular amplicon of eccDNA exhibited poorer survival and prognosis than those patients without eccDNA-associated amplicons (Spindler et al., 2017; Wang et al., 2021). There was also a genome-wide presence of eccDNAs in samples from esophageal squamous cell carcinoma (ESCC) patients. ESCC is a leading cause of cancer-related mortality with high incidence and poor prognosis. The pathway analysis of genes associated with the massively expressed eccDNAs showed enrichment in cancer-related pathways, all of which have been shown to play key roles in ESCC progression and mortality (Sun et al., 2021).

In respect to poor outcome, recent studies put fundamental roles for cancer-cell exosomes and eccDNAs in the bases and origins of tumor growth, metastasis, and invasion. OncoeccDNAs could be in the origins of tumors propagating neoplastic stem-like cells in the body and may also explain their relationships to the bulk population of tumor cells. Propagating tumors and primary cancer cells export eccDNAs to the extracellular space by exosomes as a messenger to transmit oncogenic information to other cell types. Messenger eccDNAs cause cancer progenitor cells develop the potential to dedifferentiate and acquire a stem-like phenotype. These findings have linked eccDNAs to cancer metastasis and poor patient outcomes.

In the description of the role of eccDNAs in tumor stem-like cell generation and stemness maintenance, cancer cells use exosomes and eccDNAs to transmit oncogenic information to other cell types. The eccDNAs mediate amplification of oncogenes (e.g., EGFR, MYC, CDK4, and MDM2) in target cells as a driving force for the acquisition of the cancer stem cell–like phenotype and signaling of invasiveness and chemoresistance. Results of human studies have linked onco-eccDNAs to increased cancer metastasis and poor patient outcomes. According to the findings, eccDNAs can induce dedifferentiation and reprogramming of progenitor cells to acquire a stem-like phenotype in response to genetic manipulation (Li and Laterra, 2012; Turner et al., 2017; Xu et al., 2019; Wang et al., 2021; Noer et al., 2022).

**CRISPR-Cas9 as a Tool for Gene Targeting**

There are several protocols developed by different research groups to investigate the CRISPR-Cas9 system as a novel technology and an ideal tool for editing genomes (Halpin-Healy et al., 2020; Koche et al., 2020; Kovac et al., 2020). This molecular system represents a defense immune mechanism type II in bacteria and is a candidate to become an efficient technology for targeted gene editing in tractable organisms. An sgRNA (usually about 20 nucleotides complementary to the target gene or locus) is designed to target a specific sequence site and is anchored by a spacer motif to the system (Fig. 1). Cas9 nuclease then cleaves DNA at the specific sequence site, where it is targeted by the guide RNA and generates double-strand breaks (DSBs). DNA damage responses are subsequently activated and arrest cell growth (Fig. 1) (Aguirre et al., 2016; Song et al., 2016; Gonçalves et al., 2019). Even though dual delivery of pairs of CRISPR-Cas9 guide RNAs into normal human cells was reported to induce circularization of genes and chromosomes, CRISPR-Cas9 systems primarily target extrachromosomal rearranged elements in bacteria. Likewise, in cancer cells, it is expected that a CRISPR-Cas9 system is able to target extrachromosomal copy number amplifications and produces DSBs that are detrimental to cellular fitness by dictating gene-independent copy number loss-of-fit (LOF) effects and antiproliferative responses. A system designed against amplified amplicons may provide a novel approach for cancer therapy and propose a practical implication for CRISPR-Cas9 as a path in therapeutic strategies of cancer (Møller et al., 2018). When targeting CNVs or high-copy number amplifications by CRISPR-Cas9 systems, the nuclease can introduce multiple strand breaks and deletions in targeted DNAs where DNA damage response is highly activated and leads to cell cycle arrest and death (Fig. 2) (Aguirre et al., 2016; Munoz et al., 2016; Gonçalves et al., 2019). By targeting copy number amplifications on eccDNAs formed mostly from proto-oncogenes and repeats in the human genome, CRISPR-Cas9 mediates LOF effects on cancerous cells that are associated with genomic instability signals and deleterious responses (Koche et al., 2020; Gonçalves et al., 2019; Aguirre et al., 2016; Munoz et al., 2016).

**Gene-Independent Copy Number LOF Effects by CRISPR-Cas9**

Data demonstrate that somatic gain-of-function alterations in cell-growth driver genes play a central role in cell transformation and cancer development. Deriver genes gain-of-function alterations enable transformed cells to evade the checks and balances in the cell cycle to maintain homeostasis. However, eccDNA-based oncogene amplifications seem to make up most gain-of-function alterations occurring in cancers (Aguirre et al., 2016; Gonçalves et al., 2019; Kim et al., 2020). In normal cells, oncogene copy number amplifications are
actually rare and detrimental, but in tumor cells, they are the most common genomic events. In fact, tumor cells show lethal dependencies on oncogene gain-of-function alterations that exhibit great impacts on cellular fitness (Koche et al., 2020; Gonçalves et al., 2019).

Data represent a high association between oncogene copy numbers and eccDNA amplifications in all types of cancers (Møller et al., 2018; Kim et al., 2020; Kumar et al., 2020). Onco-eccDNA amplifications enable a proto-oncogene to reach gain-of-function alterations and high copy numbers (>8) of tumor-deriving genes. Inheritance of onco-eccDNAs does not obey the chromosomal mechanism and leads to genetic heterogeneity throughout a tumor. Oncogenes amplified by onco-eccDNAs, attain higher copy numbers than the same genes amplified on noncircular structures and is associated with much more aggressive cancers (Hull et al., 2017; Kim et al., 2020).

Herein, CRISPR-Cas9 is a natural defense system in bacteria targets extrachromosomal elements to protect the genome from invading mobile elements. This system was reported to primarily target extrachromosomal circular elements in bacteria species to prevent gene-clustering or tandem-duplications reintegration (Mølend et al., 2019; Petassi et al., 2020). On the other side, utilizing CRISPR-Cas9 to knock out or target tandem or interspersed, structurally rearranged amplifications exhibited highly detrimental effects on different cancer cell lines (Fig. 2). In this case, gene-independent copy number LOF effects by CRISPR-Cas9 would be robustly expected, since they target high copy number genes commonly found in eccDNAs and amplified in tumors (e.g., MYC, PI3K, CCND1, and EGFR) (Fig. 2, Table 1). LOF effects induced by targeting copy number amplifications could also be associated with other structural rearrangements by eccDNAs, such as tandem duplications (Aguirre et al., 2016; Munoz et al., 2016; Gonçalves et al., 2019). EccDNAs are among the most frequent structural variables found with tandem duplications or gene amplifications and are associated with CRISPR-Cas9–mediated deleterious effects (Gonçalves et al., 2019).

Compared with the heavily rearranged and linear chromatin, the amplics of eccDNA type are significantly more accessible for CRISPR-Cas9 systems (Hull et al., 2017). The most frequent and recurrent genomic alterations in cancers were found to be on circular amplicons, which contain amplified oncogenes, such as PI3K, MYC, CCND1, EGFR, PAX8, and CDK4 (onco-eccDNAs) (Møller et al., 2018; Kim et al., 2020; Koche et al., 2020; Kumar et al., 2020). Genomic copy numbers on circular amplicons dictate a cell LOF response to CRISPR-Cas9 targeting (Gonçalves et al., 2019).

Recently, gene-independent copy number LOF effects by CRISPR-Cas9 were observed in blood cancer cell lines. CRISPR-Cas9 was inducing antisuicide effects associated with increased levels of DNA damage markers by targeting amplified regions in the BCR-ABL rearrangement and JAK2 amplification. Targeting amplified oncogenes (>20 sites) also showed similar deleterious effects on cancer cell lines of differing lineages. These findings may provide researchers great approaches to designing sgRNAs that target amplified onco-eccDNAs and induce cell cycle arrest, LOF, and antisuicide effects (Aguirre et al., 2016; Munoz et al., 2016; Gonçalves et al., 2019).

By designing sgRNAs that map to multiple genomic sites, Cas9 nuclease introduces DSBs in genomic DNAs (Figs. 1 and 2) by inducing disastrous effects on the cells: cell cycle arrest (mostly at the G2 checkpoints) and cell deaths (Aguirre et al., 2016; Gonçalves et al., 2019).

**Discussion**

The most frequent and recurrent genomic gain-of-function alterations in aggressive cancers are amplified cell-growth driver genes, such as PI3K, MYC, CCND1, EGFR, PAX8, and CDK4 (CNV > 8), which are associated with circular DNA amplifications. Herein, CRISPR-Cas9–targeted amplicons with more than eight CNVs would put cancer cells in a DNA DSB shock, resulting in DNA repair responses and cell cycle arrest. There would be a strong correlation between extra-chromosomal elements/gene copy number amplifications and decreased cell viability after genome targeting by CRISPR-Cas9. The numbers of target loci and DSBs by CRISPR-Cas9 correlate strongly with regions of copy number gain, expressed and unexpressed genes, and intergenic loci. By designing an sgRNA that maps to these multiple genomic sites, DSBs in these regions eventually leads to antiproliferative effects through induction of a G2 cell cycle arrest. Cell response to CRISPR-Cas9 DSBs correlates with the number of target loci. CRISPR-Cas9 targeting of onco-eccDNAs elicits gene-independent copy number LOF effects. By induction of multiple DSBs in DNA by Cas9 in an sgRNA-directed sequence-specific manner, DNA damage response is activated and counters antiproliferative/antisuicide effects.

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**Authorship Contributions**

*Participated in research design: Pourrajab, Zare-Khormizi.*

*Performed data analysis: Zare-Khormizi.*

*Wrote or contributed to the writing of the manuscript: Pourrajab, Zare-Khormizi.*

**References**


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