

Translational relevance of Telomeric C-circles in Cancer: from Experimental Research to Potential Clinical Applications

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Abstract

Alternative lengthening of telomeres (ALT) is a recombination-based telomere maintenance mechanism that sustains replicative immortality in approximately 10–15% of human cancers, particularly those of mesenchymal and neuroepithelial origin. As precision oncology increasingly demands biomarkers that accurately define telomere maintenance mechanisms, extrachromosomal telomeric C-circles - partially single-stranded, C-rich telomeric circular DNAs - have emerged as robust, quantifiable indicators of ALT activity with growing diagnostic, prognostic, and therapeutic relevance. Here, we summarize recent mechanistic and translational advances that establish C-circles as both functional readouts of ALT biology and clinically actionable biomarkers.

We integrate emerging evidence into a unified model in which replication stress drives C-circle biogenesis through two complementary replication-coupled pathways that are dynamically shaped by telomeric DNA structures and DNA repair factors, linking the molecular origins of C-circle formation to specific vulnerabilities with direct therapeutic implications. We further critically evaluate current detection technologies, spanning the gold-standard C-circle assay and its quantitative PCR and high-throughput adaptations and single-cell native FISH, which collectively enable sensitive and scalable detection across tissue specimens and liquid biopsies, including exosome-protected C-circles. Clinically, C-circles outperform surrogate genomic markers such as ATRX/DAXX loss for defining ALT status, improve prognostic stratification across pediatric brain tumors, diffuse gliomas, sarcomas, and other ALT-associated malignancies, and show considerable promise as dynamic pharmacodynamic biomarkers for treatment monitoring, minimal residual disease assessment, and longitudinal disease surveillance. Therapeutically, the molecular circuitry underlying C-circle biogenesis exposes selective ALT dependencies on replication stress and DNA repair regulators, including the BLM/BTR and FANCM pathways. Perturbation of these nodes reshapes C-circle dynamics while revealing synthetic lethal vulnerabilities that can be exploited through helicase-, nuclease-, chromatin-, replication stress-, and G-quadruplex-targeted strategies. We also discuss the important caveat that telomerase-positive tumors with hyperextended telomeres can generate C-circles, emphasizing the need for cautious interpretation of C-circle measurements.

Collectively, C-circles have evolved from experimental hallmarks of ALT into mechanistically informative and clinically actionable biomarkers. Continued assay standardization and integration into tissue and liquid biopsy workflows will facilitate their application in diagnosis, patient stratification, therapeutic monitoring, and biomarker-driven clinical studies.

1. Telomere maintenance mechanisms and cancer

Telomeres are nucleoprotein complexes located at the ends of linear eukaryotic chromosomes (1). They are composed of double-stranded DNA (Fig. 1) consisting, in humans, of tandem repeats of the sequence 5'-TTAGGG-3', typically spanning ~ 5–15 kb in normal cells. The G-rich strand extends beyond its complementary C-rich strand, forming a 3' single-stranded overhang of approximately 50–200

nucleotides (1). This overhang can fold back and invade the adjacent double-stranded telomeric DNA, generating a lasso-like structure known as the telomeric T-loop (Fig. 1), which functions as a protective cap at chromosome ends (2). The T-loop is stabilized by the shelterin complex (Fig. 1), a multiprotein assembly comprising six (TRF1, TRF2, POT1, TIN2, TPP1, and RAP1) core components (1, 3). Together, these factors play essential roles in telomere protection and replication (3). In addition, the CTC1-STN1-TEN1 (CST) complex contributes to telomere replication and maintenance (1).

Telomeres are essential for maintaining genome integrity by preventing chromosome ends from being recognized as DNA double-strand breaks (DSBs), thereby avoiding inappropriate activation of DNA damage signaling and aberrant repair processes (3). Functional telomeric complexes suppress the DNA damage response (DDR) by inhibiting key signaling pathways mediated by ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), and poly-(ADP-ribose) polymerase 1 (PARP1). In doing so, telomeres block the engagement of DSB repair mechanisms, including classical non-homologous end joining (c-NHEJ), alternative NHEJ (alt-NHEJ), and homology-directed repair (HDR) (3).

In addition to their protective role, telomeres regulate the proliferative lifespan of cells (4). Due to the end-replication problem - namely, the incomplete synthesis of the lagging DNA strand - telomeres progressively shorten with each cell division in normal or premalignant (mortal) cells, at an estimated rate of approximately 50–100 base pairs per year in humans (5). Progressive telomere erosion ultimately leads to telomere dysfunction and loss of end protection, triggering activation of the DDR and inducing cellular senescence and/or apoptosis in a cell type-dependent manner (4). In this context, telomere attrition functions as a tumor-suppressive mechanism by limiting unchecked cellular proliferation. However, in cells with compromised cell cycle checkpoints, dysfunctional telomeres can instead promote genomic instability, thereby facilitating tumorigenesis (6).

The preservation of telomere length is a non-negotiable requirement for the genomic stability and indefinite proliferative capacity of malignant cells (7). While the majority of human cancers achieve this through the reactivation of telomerase (7), approximately 10–15% of human tumors, especially those of mesenchymal and neuroepithelial origin, utilizes the Alternative Lengthening of Telomeres (ALT) mechanism (8). ALT is primarily driven by Break-Induced Replication (BIR), a recombination-based pathway that restores telomeric DNA through aberrant replication fork dynamics (9).

The molecular landscape of ALT is characterized by unique hallmarks (10–14). ALT occurs in the absence of telomerase activity and is associated with marked heterogeneity in telomere length, ranging from undetectable to abnormally long telomeres within the same cell population (10). Another hallmark is the presence of ALT-associated promyelocytic leukemia (PML) bodies (APBs), a subset of PML bodies enriched in telomeric chromatin, shelterin factors, and proteins involved in DNA replication and recombination (10, 11). APBs are thought to function as a “recombinogenic microenvironment” that enables the spatial clustering of telomeres with DNA repair and replication factors. Consistent with this model, the recently developed ALT telomere DNA synthesis in APBs (ATSA) assay demonstrated that telomeric DNA synthesis occurs almost exclusively within these structures (15). ALT mechanism is also

characterized by increased DNA damage at telomeres, which may result from replication stress and the presence of telomeric variant repeats, such as TCAGGG, TGAGGG, and TTGGGG. These sequence variants may impair the binding of shelterin factors, thus further contributing to telomere dysfunction (11, 14).

A distinctive hallmark of ALT is the accumulation of extrachromosomal telomeric DNA (ECTD) species (11, 12, 16). These include double-stranded telomeric circles (t-circles), partially single-stranded circular DNA (G- and C-circles), linear double-stranded telomeric DNA, and high-molecular-weight “t-complexes” (16). Importantly, ECTD species are not merely inert by-products of ALT. Rather, they represent a critical feature of the ALT phenotype and may provide a mechanistic window into the replication stress and recombination processes underlying ALT-mediated telomere maintenance (11, 12, 16).

Moreover, a significant correlation between ALT activity and mutations or abnormal expression of ATRX chromatin remodeler (*ATRX*) and/or its binding partner death domain-associated protein (*DAXX*) gene has been observed (17). In particular, loss of ATRX was found to be associated with ALT activity in different subtypes of complex karyotype sarcomas, including dedifferentiated liposarcoma, angiosarcoma, and uterine smooth muscle tumors (17). Moreover, loss of *ATRX* or *DAXX* has been associated with poor prognosis in smooth muscle tumors of uncertain malignant potential and early-stage uterine leiomyosarcoma (17). However, loss of *ATRX/DAXX* should not be considered synonymous with ALT activity. The association between *ATRX/DAXX* loss and ALT appears to be context-dependent, occurring preferentially in specific tumor types and under particular genomic or epigenetic conditions. (18). Moreover, even if the loss of ATRX creates a permissive background for ALT, it is insufficient *per se* to activate this pathway (19). In pluripotent cells, ALT hallmarks and immortalization emerge only after differentiation, which induces telomeric stress and a recombination-prone state. When combined with disruption of key tumor suppressor pathways (e.g., loss of cyclin dependent kinase inhibitor 2A, *CDKN2A*), ATRX-deficient cells acquire full ALT activity and can become immortalized (19). This evidence establishes that ATRX loss primes cells for ALT, but cellular differentiation acts as the critical switch that enables full ALT activation, with p53 serving as a gatekeeper of this process (19). Additionally, ALT activation has been shown to occur independently of telomere crisis and can arise even in the presence of telomerase (19). Indeed, telomerase-positive cells depleted for factors, such as histone chaperone ASF1 or SNF2 related chromatin remodeling annealing helicase 1 (SMARCAL1) (20, 21), or with “hyper-elongated telomeres” (22) showed features of ALT even in the presence of telomerase activity.

While both telomerase and ALT are capable of sustaining the unlimited proliferative potential of tumor cells, they have been associated with distinct effects on tumor progression and patient outcomes (17). In this context, tumors of mesenchymal origin provide a particularly intriguing example. In osteosarcoma, the absence of any detectable mechanism for telomere maintenance has been associated with improved overall survival (23). However, though TA or ALT did not differ significantly from each other in clinical outcome, (23) TERT-positive lesions has been associated with an unfavorable prognosis compared to TERT-negative lesions (24). Moreover, telomerase activity has been reported to predict patient outcomes in diffuse malignant peritoneal mesothelioma (25) and malignant peripheral

nerve sheath tumors (26). In addition, telomerase reactivation represents the predominant telomere maintenance mechanism (TMM) in myxoid liposarcoma, a soft-tissue sarcoma (STS) histology characterized by a high frequency of telomerase reverse transcriptase (TERT) promoter mutations (27). Conversely, ALT has been detected in a significant fraction of STS histologies (28, 29), including leiomyosarcoma, where it has been associated with adverse prognostic features, such as high mitotic activity (28), elevated genomic instability (29), and poor prognosis (29, 30). Furthermore, ALT was identified as a prognostic factor for reduced disease-free and overall survival in uterine sarcomas (31) and was similarly associated with poorer survival in malignant fibrous histiocytoma (32, 33). Likewise, ALT has been observed in approximately 30–40% of liposarcomas (34, 35), where it correlates with unfavorable prognosis (35). On the other hand, it has been recently reported that an “ALT-like state”, defined based on a 100-gene signature, may be associated with a favorable prognosis in chondrosarcomas, contrasting the frequent adverse implication of ALT in other sarcomas (36).

Collectively, these findings indicate that the clinical relevance of TMM is strongly dependent on tumor types, with ALT appearing to confer an unfavorable prognosis in several STS histologies and subtypes. This scenario is further complicated by evidence showing that telomerase and ALT may coexist within the same tumor lesion. However, it remains unclear whether the two mechanisms are simultaneously active within individual tumor cells or instead define distinct cellular subpopulations within the tumor mass (17). This observation has recently been supported by evidence from a screen of more than 900 cell lines, which identified a subset of cells exhibiting either a mixture of ALT and telomerase activity in single cells or comprising individual subclones with both TMM activated (37). In addition, TMM may also dynamically change during disease progression, with primary tumors and their matched metastatic lesions having distinct telomere maintenance profiles (17). These observations suggest that cellular subpopulations relying on distinct TMM may contribute differently to the biological aggressiveness of the bulk tumor, thereby exerting divergent effects on disease progression and patient outcomes. In this context, telomere maintenance heterogeneity and plasticity may represent additional drivers of tumor evolution, metastatic potential, and therapeutic resistance. Consequently, accurate identification and characterization of the operating TMM within individual tumors become essential to better define tumor biology, improve prognostic stratification, and guide the development of targeted therapeutic approaches.

In this context, while TA may be readily detectable by measuring its enzymatic activity, reliable biomarkers capable of accurately identifying ALT-positive tumors are of particular importance. Among these, telomeric C-circles have emerged as highly specific markers of ALT activity and are now considered one of the most robust tools for ALT detection. In this review, we summarize current evidence on the origin, regulation, and biological functions of C-circles and discuss their translational potential. We argue that C-circles extend beyond serving as functional readouts of ALT biology, as they may provide mechanistic insights into tumor evolution while offering new opportunities as clinically actionable biomarkers.

2. C-Circles: detection methods and molecular mechanisms of their biogenesis

This section outlines current methods for the detection of C-circles and reviews the molecular mechanisms driving their biogenesis in light of recent literature. Given the rapid evolution and complexity of the specific field, this overview does not aim to be exhaustive; rather, it highlights key concepts and emerging insights central to understanding C-circle biology.

2.1 C-circle detection methods

C-circles (CC) are defined as a type of extrachromosomal circular DNA (eccDNA) (Fig. 2) consisting of a circularized intact C-rich telomeric strand ($[5'\text{-CCCTAA-3'}]_n$) that is non-covalently associated with a fragmented, discontinuous complementary G-rich telomeric strand ($5'\text{-TTAGGG-3'}$). CC are heterogeneous in size, typically ranging from several hundred base pairs to kilobases (38), and are entirely composed of telomeric DNA. Biochemically, CC are defined by their unique capacity to serve as a self-priming substrate for Rolling Circle Amplification (RCA) (Fig. 2). The fragmented G-strand, complementary to the continuous C-strand template, provides multiple exposed 3'-hydroxyl termini that serve as initiation sites for $\phi 29$ DNA polymerase. This enables highly processive strand-displacement DNA synthesis under isothermal conditions, resulting in robust amplification of CC templates and the generation of long G-rich telomeric concatamers (Fig. 2).

A comparative overview of CC detection methods is provided in Table 1.

The CC assay (CCA), developed by Henson et al. (12) is a specific method for the detection and quantitative assessment of CC levels by RCA in different type of experimental and clinical samples (12, 39). As CCA is highly sensitive to DNA concentration, accurate fluorometric quantification is essential upon DNA extraction (12, 39). To enrich circular substrates, samples may be treated with exonucleases λ , I, and V, which digest linear DNA while sparing circular molecules. Genomic DNA is also commonly digested with *HinfI* and *RsaI* to reduce viscosity and improve polymerase accessibility (12, 39).

In the original protocol, amplified products are detected by dot-blot hybridization (12). RCA products are transferred onto a nylon membrane, UV cross-linked, and hybridized with an end-labeled $(\text{CCCTAA})_3$ probe that specifically recognizes the amplified telomeric G-strand concatamers (12). CC levels are quantified following subtraction of background controls lacking either DNA or $\phi 29$ polymerase, and results are expressed in arbitrary units. Filters may be stripped and re-probed with an end-labelled Alu probe to ensure for equal DNA loading (39). This assay is highly sensitive, detecting CC from as little as 1 ng of genomic DNA (~ 100 cells), and maintains linearity across an input range of approximately 1–32 ng (12).

Alternatively, RCA products may be quantified by qPCR (39). In this modified approach, CC levels are determined by measuring the increase in total telomeric DNA following RCA. qPCR-based CCA is particularly advantageous for clinical samples in which insufficient DNA is available for dot-blot analysis,

although it requires careful management of background signals and reaction inhibitors (39). A critical methodological consideration may be the presence of both ϕ 29 DNA polymerase and inorganic pyrophosphate carried over from the RCA, that can significantly impair qPCR performance. To minimize inhibition, RCA products are typically diluted such that they comprise no more than 5% of the final qPCR reaction volume, corresponding to approximately 2 ng of original input DNA (39). Moreover, yeast inorganic pyrophosphatase may be used to hydrolyze pyrophosphates prior to qPCR (39).

Quantification in qPCR-based CCA is generally performed using a relative standard curve approach. Serial dilutions of DNA from a known ALT-positive cell line (e.g., U-2 Os osteosarcoma cells) are used to generate standard curves for both telomeric DNA and a single-copy reference gene, such as hemoglobin subunit beta (*HBB*) or ribosomal protein lateral stalk subunit P0 (*RPLP0/36B4*), used to normalize for input DNA (39). Because qPCR includes a denaturation step that detects all telomeric DNA, not solely RCA-derived products, each sample must also be analyzed in a corresponding “No Phi” control lacking ϕ 29 polymerase (12, 39). This control accounts for baseline telomeric background, including endogenous single-stranded G-rich DNA and double-stranded telomeric sequences. Final CCA values are calculated by subtracting the normalized telomeric content (NTC) of the “No Phi” control from that of the corresponding RCA-amplified sample (39).

Fronney et al. (40) have described recently an automated adaptation of the original CCA. The high-throughput CCA (HT-CCA) has been designed for 384-well plate formats to support large-scale compound screening for potential modulators of ALT activity with improved scalability and reproducibility (40). In this workflow, cells are seeded at optimized densities per well to ensure that DNA input remains within the linear range required for downstream amplification. Following compound treatment, cells are lysed directly in plate using a heat-assisted lysis step (40). C-circle amplification is performed using EquiPhi29 DNA polymerase, a thermally enhanced variant of ϕ 29 with improved speed and reduced amplification bias (40). Amplification products are quantified by qPCR (39) after a controlled dilution (typically 1:20) to ensure compatibility with the dynamic range of detection (40). Relative CC abundance is calculated using the $2^{-\Delta Ct}$ method (39, 40). For high-throughput analysis, compound effects are evaluated using the Strictly Standardized Mean Difference metric (40). Hits are defined as compounds deviating beyond ± 3 median absolute deviations from solvent-treated controls. The assay has been reported to reliably detect $\geq 40\%$ changes in CC levels in neuroblastoma and $\geq 50\%$ in osteosarcoma cells. It was validated across ALT-positive and ALT-negative cell lines, demonstrating strong sensitivity and robustness (40).

The Strand-Specific Southern-blot for Single-stranded Extrachromosomal Telomeres (4SET) assay described by Lee et al. (38) is a modified Southern blot-based method to detect and quantify small single-stranded ECTD. Unlike the traditional CCA, which requires enzymatic amplification, 4SET allows for the direct detection of both linear and circular extrachromosomal telomeric single-strand DNA (ssDNA), providing a clearer picture of their size distribution and strand origin (38). The assay has been optimized to preserve short telomeric fragments that are frequently lost during conventional genomic DNA extraction and blotting procedures. The protocol begins with cellular fractionation to separate

soluble extrachromosomal DNA from chromatin-associated genomic DNA. To preserve small telomeric fragments, the assay avoids silica column-based purification methods (38). DNA samples are handled under low-temperature dissolution and minimal freeze-thaw cycling, to prevent degradation and destabilization of circular telomeric structures (38). For analysis, DNA is resolved on low-percentage native agarose gels and transferred onto positively charged nylon membranes using standard transfer procedures optimized to retain small nucleic acids. Detection is performed using highly sensitive strand-specific G-rich or C-rich telomeric probes to detect C-rich or G-rich telomeric ssDNA, respectively (38). The assay also enables structural characterization of the detected molecules. Fragment sizes are estimated using nucleotide ladders, revealing a typical size range of approximately 200-1,500 nucleotides (38). Treatment with S1 nuclease confirms the single-stranded nature of the detected species, while resistance to exonucleases λ , I and V distinguishes CC from linear ssDNA (38).

The native telomeric fluorescence in situ hybridization (N-FISH), also referred as to ss-TeloC assay, is a single-cell imaging technique developed to detect C-rich single-stranded telomeric DNA (41, 42). Unlike conventional FISH, the assay is performed under non-denaturing conditions, allowing selective detection of naïve single-stranded telomeric DNA regions (41, 42) and correlates closely with CC abundance measured by conventional CCA (41). Briefly, cells are cultured either on coverslips for low-throughput analysis or in 384-well plates for high-throughput imaging applications (41, 42). Hybridization is carried out using a telomeric PNA probe complementary to the C-rich strand, typically a Cy3-labeled [TTAGGG]₃ probe. Critically, hybridization is performed at room temperature without heat denaturation, ensuring that only endogenous single-stranded telomeric DNA is detected (41, 42). Prior to hybridization, samples are treated with RNase A to eliminate false-positive signals arising from telomeric RNA species (41, 42). Following hybridization, excess probe is removed by washes and nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI) prior to fluorescence imaging. For conventional analysis, at least 100 nuclei per condition are examined in a blinded manner (41). In high-throughput imaging workflows, automated confocal microscopy platforms are used to acquire multiple fields per well, enabling analysis of approximately 1,000 cells per condition (42). To allow quantitative single-cell measurement of ss-TeloC fluorescence intensity, automated nuclear segmentation and spot detection may be performed using image analysis softwares (42). The application of the assay to pediatric tumor samples enabled the reliable identification of CC-positive tumors, whose status was confirmed in parallel using the gold-standard CCA, where the intensity of CCA signals appeared to correlate better with the abundance than with the fluorescence intensity of ss-TeloC foci (43). These findings indicate that, although the conventional CCA remains a highly robust method for CC detection in research settings, its implementation in routine diagnostic pathology may be limited by practical constraints. By contrast, N-FISH may offer a more accessible and clinically adaptable alternative (43).

Table 1
Comparative overview of C-circle detection methods

Method	Principle	Sample type	Strengths	Limitations	Ref.
CCA	RCA followed by dot-blot and detection of probe hybridization	Cell lines Tumor Tissues Blood/Plasma	Highly sensitive and specific for CC Gold-standard assay Linear quantification at low DNA input	Labor-intensive Requires radioactive or chemiluminescence detection Limited clinical scalability	(12, 39)
	RCA followed by qPCR		More compatible with clinical samples; higher throughput than blotting	Sensitive to inhibitors (pyrophosphate, polymerase carryover) Requires strict normalization	(39)
HT-CCA	Automated RCA-based screening platform using EquiPhi29 polymerase	Cell lines	High-throughput drug screening Reproducible and scalable	Requires specialized automation; reduced mechanistic resolution compared with blot-based methods	(40)
4SET	Strand-specific Southern blot detection of extrachromosomal telomeric ssDNA (no enzymatic amplification)	Cell lines Tumor samples	Direct visualization of ssDNA and CC Provides size and strand information Distinguishes linear vs. circular DNA	No amplification (lower sensitivity than CCA) Labor-intensive Limited quantification precision	(38)
N-FISH/ss-TeloC	Non-denaturing single-cell FISH detecting endogenous C-rich single-stranded telomeric DNA	Fixed cells Tumor tissue sections	Single-cell resolution Compatible with histology Clinically adaptable	Indirect measure of CC Not structurally specific Requires imaging infrastructure	(41, 42)

2.2. C-circle biogenesis

CC are generated through the machinery governing ALT (15, 38) as a consequence of the high levels of telomere replication stress (13). The primary mechanism for CC generation is linked to BIR during telomere elongation (44, 45). CC represent circularized lagging strand of telomeric DNA and their formation is tightly linked to endogenous DNA break occurring on C-rich telomeric strand leading to the collapse of replication forks (46). Although replication fork regression and homologous recombination (HR)-mediated replication restart can rescue replication fork collapse thus likely contributing to telomere elongation, unresolved replication fork are converted into CC at lagging synthesized strand (46).

ALT utilizes a bifurcated framework consisting of two distinct BIR pathways (15). The primary axis (BIR1) is DNA repair protein RAD52 homolog (RAD52)-dependent and is the principal driver of long-term telomere maintenance. In this pathway, RAD52 plays a unique role by catalyzing the annealing of ssDNA and promotes telomeric D-loop formation even when the ssDNA is coated by Replication Protein A (RPA) (15). This ability to overcome RPA-mediated inhibition allows telomere ends to engage double-stranded templates, facilitating DNA synthesis (15). However, the production of CC is nested within a parallel, RAD52-independent axis (BIR2) (15) (Fig. 3). The molecular drivers of these axes include RecQ-like DNA helicase BLM (BLM), which is essential for the spatial organization of APBs. Depletion of BLM abrogates the formation of APBs and significantly reduces CC abundance, indicating it as a fundamental factor in CC biogenesis (41, 47). While RAD52 is indispensable for the strand invasion step and D-loop formation required for telomere maintenance by BIR1 its engagement in BIR2 is attenuated, indicating that CC are definitive hallmark of the RAD52-independent axis. Moreover, within the BIR2, the subunits 3 (POLD3) and 4 (POLD4) of the DNA polymerase δ (Pol δ) are strictly necessary for the DNA synthesis yielding CC, whereas their role in the RAD52-dependent pathway remains a subject of ongoing investigation (15). Finally, in contrast to their roles in classical recombination, DNA repair protein RAD51 homolog 1 (RAD51) and double strand break repair nuclease MRE11 (MRE11) act as negative regulators or "gatekeepers" of CC formation. In the bifurcated model, RAD51 and MRE11 appear to block the divergence toward circle formation, likely by stabilizing recombination intermediates that would otherwise be excised or processed into extrachromosomal DNA (15).

Yadav et al. (48) have provided additional evidence showing that CC production driven by BIR2 can be orchestrated by the long non-coding RNA transcribed from telomeres known as TERRA (49) and RAD51-associated protein 1 (RAD51AP1). In this regard, RAD51AP1 depletion strongly impairs the recruitment of POLD3 and RAD52 to telomeric DSBs, thus leading to progressive telomere shortening and consequent telomere dysfunction and fragmentation (50). In addition, RAD51AP1 directly binds to TERRA, thus favoring the formation of R- and D-loops, which are essential intermediates for telomere elongation (51). This mechanism involves a dynamic R-to-D-loop switch (Fig. 3) that allows telomere synthesis to proceed even in the absence of the canonical recombination factor RAD52 (48, 51). In this context, RAD51AP1 acts as the primary factor driving the formation of telomeric R-loops (DNA:RNA hybrids) by enabling TERRA to hybridize with the C-rich telomeric strand. While RAD52 also has some ability to form these hybrids, RAD51AP1 is significantly more potent in generating TERRA-mediated R-loops (48). By enabling the R-to-D-loop switch and recruiting the POLD3-containing replisome, RAD51AP1 creates the necessary molecular environment for BIR events that culminate in CC biogenesis

(51). In particular, the formation of telomeric R-loops displaces the G-rich DNA strand as ssDNA, resulting in the increased formation of G-quadruplexes (G4) at telomeres (48). Both TERRA and RAD51AP1 are essential for maintaining G4 levels as shown by a drastic reduction in telomeric G4 signals upon their depletion (51). Additionally, telomeric R-loops are dynamic, as they are constantly assembled and disassembled (48). For CC production and telomere synthesis to occur, the R-loops must be resolved, a process facilitated by enzymes like RNase H1 (52). This results in the persistence of G4 structures on the G-rich strand (48) which keep the telomeric DNA in an open configuration, allowing the invasion of G-strand DNA ends to form D-loops (Fig. 3), the necessary intermediates for initiating BIR by POLD3/POLD4, leading to CC generation (48). In this context, depletion of TERRA or RAD51AP1 significantly reduces CC production (50, 51) because the initial R-loops cannot form to trigger the G4-mediated switch (48). By analogy, persistent R-loops caused by the loss of RNase H1 actually inhibit CC production, thus confirming that the transient removal of the RNA is a necessary step to allow the R-to-D-loop transition (48). Finally, while RAD51AP1 is essential for CC production, the depletion of its vicinal chromatin remodeling factors, such as SWI/SNF related BAF chromatin remodeling complex subunit ATPase 4 (SMARCA4) or MYND-type zinc finger-containing 8 chromatin reader (ZMYND8), did not significantly alter CC levels, suggesting that the core RAD51AP1 recombinase activity may be central to circle formation (51). Similarly, it has been shown that the DNA mismatch repair MutS β complex acts as a key guardian of telomere integrity in ALT cancer cells (53). MutS β is specifically enriched at telomeres, where it binds to and destabilizes G4 structures and TERRA-derived R-loops, thereby preventing their accumulation and protecting telomeres from excessive processing (53). Functionally, MutS β serves as a potent suppressor of CC formation. Loss of MutS β (e.g., through MSH3 depletion) leads to a marked increase in CC in ALT cells, driven by the accumulation of G4 structures and R-loops (53). These structures generate aberrant DNA intermediates that are actively processed by structure-specific endonucleases such as Structure-specific endonuclease subunit MUS81 and ERCC excision repair 4, endonuclease catalytic subunit (XPF), resulting in the excision of telomeric DNA in the form of CC. This effect is further exacerbated by G4-stabilizing agents (53). Interestingly, MutS β exhibits an opposing role to the related mismatch repair complex MutS α , which instead appears to promote CC formation, underscoring a functional divergence within the mismatch repair pathway (53). Overall, MutS β maintains telomere stability by limiting the accumulation of deleterious DNA secondary structures and restraining nucleolytic processing; its loss leads to increased CC production, telomere shortening, and compromised telomere integrity in ALT cells (53).

More recently, it has been reported that the generation of CC is a direct consequence of dysregulated telomere lagging-strand synthesis (38, 54). Through their recently developed 4SET assay, Lee et al. (38) demonstrated that CC are nascently generated from the lagging daughter strand of telomeres during DNA replication, according to a specific sequence of molecular events, collectively referred to as "Excessive Strand Displacement" (38). Briefly, telomeres in ALT cells often expose G-rich ssDNA or form higher-order structures like G4. The CST-PP complex (comprising the CST complex and DNA Polymerase α (POLA)-Primase) recognizes these gaps and initiates the synthesis of new C-rich Okazaki fragments (*Priming*, Fig. 3). These primed fragments are extended by Pol δ . In the unique environment of ALT

telomeres, Pol δ works in conjunction with BLM and exhibits "excessive" strand displacement activity (*Extension and Displacement*, Fig. 3). As Pol δ and BLM extend the new C-strand, they displace the downstream Okazaki fragments, creating long 5'-flap structures (38, 54). If these flaps are completely displaced, they are released as extrachromosomal C-rich ssDNA (*Flap Formation and Release*, Fig. 3). These molecules, ranging from 200 to 1,500 nucleotides in size, are abundant in both the cytoplasm and nucleoplasm and exist either in linear and circular forms (38).

This process is finely tuned by several key proteins that ensure a rigorous regulatory circuit to titrate the levels of telomeric C-rich extrachromosomal DNA. In particular, the loss of ATRX is a primary driver of CC production. ATRX normally prevents the accumulation of G4 and R-loops (55); its absence leaves telomeres permissive for the CST-PP complex to prime excessive synthesis. MRE11 and DNA replication ATP-dependent helicase/nuclease 2 (DNA2) act as suppressors of CC formation. MRE11 likely resolves the G4 structures that trigger priming, while DNA2, together with RPA, suppress ECTD formation by trimming the long 5'-flaps of the displaced fragment (38, 54). In this regard, inhibiting MRE11 or depleting DNA2 leads to a significant increase in CC levels. Furthermore, SMARCAL1 acts as a vital gatekeeper; its ability to suppress C-rich ssDNA generation is strictly dependent on its ATPase activity, as demonstrated by the failure of the ATPase-dead R764Q mutant to recapitulate its suppressive function (54). Additionally, the flap structure-specific endonuclease 1 (FEN1) partially contributes to CC generation by cleaving the middle of 5'-flap structures when long-flap structures are not adequately processed by DNA2 (38), whereas the lack of the APB component PML results in a dramatic reduction in both C-rich ssDNA and CC in ALT-positive osteosarcoma cells (38).

The discovery of abundant G-circle production under specific genetic co-deficiencies has recently redefined the ALT paradigm, demonstrating that ECTD biogenesis is not restricted to the lagging strand (54) (Table 2). The generation of G-rich ssDNA is triggered by the co-deficiency of SMARCAL1 and Fanconi anemia complementation group M (FANCM), including its interaction with Fanconi anemia core complex-associated protein 24 (FAAP24). This loss of translocase-mediated fork protection allows for rampant G-strand displacement (54). Mechanistically, G-rich species originate from the leading telomeric (G-rich) daughter strand and it is initiated by RAD51 (54). Once synthesized, these strands are displaced by the same BLM/POLD3 engine utilized in CC biogenesis. Crucially, the displaced G-rich ssDNA is sequestered and stabilized by the shelterin factor POT1, which coats the product to prevent premature degradation (54). However, the leading-strand displacement pathway is actively suppressed by PARP1 binding protein (PARI) and DNA annealing helicase and endonuclease ZRANB3. These factors function as essential regulators that inhibit the RAD51-mediated synthesis of the species that eventually form G-circles (54). The shift from lagging-to leading-strand production of ECTD demonstrates that ALT cancers can utilize multiple replication forks and templates to maintain genomic integrity (54). Such a dual CST- and RAD51-dependent pathway model confirms that ECTD production is a regulated consequence of replication stress, not a stochastic error (54).

Table 2
Comparative Molecular Profiles of C-rich and G-rich extrachromosomal telomeric DNA

Nucleotide composition	C-circles	G-circles
	Cytosine-rich (5'-CCCTAA-3')	Guanosine-rich (5'-GGGTTA-3')
Strand Origin	Lagging daughter strand	Leading daughter strand
Initiation Complex	CST-PP	RAD51
Stabilizing/Binding Factor	RPA	POT1
Suppression Machinery	DNA2 (5' flap nuclease) / RPA	PARI / ZRANB3
Physical Structure	Predominantly circular	Predominantly linear
Size Range	200–1500 nt	500–3000 nt

Additionally, the telomeric recruitment of orphan nuclear receptors (ONRs) has been identified as a primary event in the *de novo* activation of the ALT pathway (14, 56, 57). The tethering of ONRs to telomeric variant TCAGGG repeats, which are enriched in ALT telomeres but absent in normal or telomerase-positive cells, triggers the recruitment of the zinc finger protein 827 (ZNF827) (56). This interaction is thought to facilitate alterations in chromatin structure, making telomeres permissive to the homologous recombination events generating CC. The ability of ONRs to induce CC generation is strictly dependent on the integrity of their AF2 domain, which is essential for the formation of APBs where telomeric recombination occurs (56). Deletion of this domain completely abolishes the formation of CC and other ALT features, even when the receptors are successfully targeted to telomeres (56). Consistent with a role for ONRs in ALT regulation, depletion of nuclear receptor subfamily 2 group F member 2 (COUP-TF2) or nuclear receptor subfamily 2 group C member 2 (TR4) markedly reduced CC levels in ALT-positive cells (14). In addition, the transcription intermediary factor 1-beta (TRIM28) was found to be essential for ONR-driven H3K9 methylation and the induction of ALT-associated phenotypes (57). Accordingly, both TRIM28 knockdown and ectopic expression of an RBCC-deficient TRIM28 mutant impaired ALT induction, as evidenced by reduced CC levels detected by N-FISH (57).

In addition, it has been reported that lamina-associated polypeptide 2 alpha (LAP2α) acts as a key suppressor of the ALT pathway by maintaining telomeric heterochromatin (58). Indeed, it has been shown that LAP2α interacts with histone deacetylase 1 (HDAC1) and TRF1/TRF2 to preserve chromatin compaction at telomeres, thereby limiting their accessibility to recombination machinery (58). In particular, loss of LAP2α in ALT-positive cells leads to chromatin decondensation, reduced three-methylation of H3K9, and increased recruitment of homologous recombination factors (e.g., RPA, RAD52), resulting in enhanced ALT activity, including elevated APBs and CC (58). Notably, LAP2α has been reported to be downregulated in ALT-positive osteosarcoma tissue specimens and correlates with more aggressive disease and poorer patient outcomes compared to non-ALT tumors (58).

A critical reassessment of CC as markers specifically associated with ALT has been recently provided by Jones et al. (22). Their key finding challenges the notion that telomerase activity and CC are mutually exclusive, as long-telomere, telomerase-positive (LTT+) cells - including cen3tel, HeLa1.3, and HeLaE1 - displayed robust CC signals (22). Specifically, in long-term cultured cen3tel cells, which are TERT-immortalized primary skin fibroblasts derived from a centenarian female, telomeres reached approximately 100 kb at 1000 population doublings, exceeding by more than tenfold (i.e., hyperextended telomeres) the typical telomere length observed in most human cells (22). In these LTT+ cells, CC levels were comparable to those observed in ALT osteosarcoma cells, despite the absence of other ALT-associated hallmarks (22). Importantly, analyses across population doublings showed that CC signals in LTT+ cells increased progressively with telomere elongation. Moreover, telomerase knockout led to a gradual decrease in CC levels, which could be attributed to telomere shortening rather than a direct effect of telomerase on CC formation (22). Conversely, short-term restoration of telomerase activity did not immediately elevate CC levels, supporting the conclusion that telomerase does not directly contribute to CC biogenesis, thus indicating that telomere length itself acts as the primary driver of CC production in these cells (22).

Consistent with this interpretation, the molecular mechanisms regulating CC in LTT+ cells seem to differ from those in ALT cells. For example, depletion of FANCM did not restrain CC production in LTT+ cells (22). Moreover, LTT+ cells were significantly less sensitive to replication stress induced by hydroxyurea than ALT cells (22). Together, these findings suggest that, although long telomeres may increase endogenous replication stress, ALT status is a stronger determinant of the replication stress typically associated with CC formation (22). Altogether these findings caution that the use of the CCA as a stand-alone method to identify ALT activity may lead to tumor misclassification (22). Specifically, telomerase-positive tumors with hyper-elongated telomeres (22) or high telomeric DNA content (37) could be incorrectly classified as ALT-positive, as it has also been suggested for patient-derived dedifferentiated liposarcoma cells (59).

In line with these observations is the evidence that the deregulated expression or function of some specific factors (e.g., PML, BLM, ATRX) has also been implicated in CC production and the onset of ALT in cancer cells that use telomerase activity for telomere maintenance (41, 60). For instance, in telomerase-positive cell lines, reduced amounts of the replication fork remodeling enzyme SMARCAL1 or ASF1, which induces replication stress, promotes CC formation (20, 21).

3. Translational relevance of C-circles in oncology

Beyond their utility as mechanistic readouts of ALT activity (12, 39), emerging evidence suggests that the identification of factors involved in CC formation may reveal potential therapeutic vulnerabilities in cancer (9, 61). Moreover, the close relationship between CC levels and ALT activity supports their potential application as biomarkers for tumor characterization and patient stratification according to the operative TMM (12, 39). Emerging evidence further suggests that CC may have prognostic value, while treatment-induced changes in CC production may provide a sensitive means of monitoring ALT activity

and evaluating therapeutic response. These findings also raise the possibility that CC could serve as biomarkers for disease monitoring and the early detection of relapse. Their potential clinical utility is further supported by the feasibility of detecting CC in liquid biopsy specimens (12) and by the use of isothermal RCA, which may facilitate implementation of the CCA in routine pathology laboratories. Altogether, these observations highlight the translational potential of CC as both a source of novel therapeutic targets and potential biomarkers in cancer, as discussed in the following sections.

3.1 Factors involved in C-circle production as possible therapeutic vulnerabilities

Although the precise mechanism of CC biogenesis remains under investigation, some factors involved in APB assembly or HR-mediated DNA repair (61, 62) have been shown to regulate CC levels in cancer cells. Factors that have been identified to govern CC production to date and that may be associated with a therapeutic potential are schematically represented in Fig. 4.

Pioneering studies have established FANCM as a critical factor in restraining CC production (63, 64). Depletion of FANCM in ALT-positive cancer cells leads to a dramatic increase (~ 20-fold to over 50-fold) in CC levels, whereas this effect is not observed in telomerase-positive cells, in which FANCM loss does not induce CC formation (63, 64). Mechanistically, Pan et al. (63) showed that this suppressive function requires the interaction between FANCM and FAAP24, while Lu et al. (64) demonstrated that FANCM-dependent inhibition of CC production relies on both its translocase activity and its MM2 domain, which mediates binding to the BLM–TOP3A–RMI (BTR) complex. The molecular trigger underlying excessive CC production in the absence of FANCM appears to be the accumulation of R-loops (63). In particular, FANCM actively resolves TERRA-derived R-loops at ALT telomeres. In the absence of FANCM, these structures accumulate, obstruct replication fork progression, and induce replication stress (63). Consistently, overexpression of RNase H1 in FANCM-depleted cells markedly reduces CC levels, confirming that R-loop accumulation is a primary driver of their formation (63). Moreover, unresolved replication stress is thought to promote the collapse of stalled replication forks into DSBs, which then serve as substrates for ALT-mediated repair (64). These findings have contributed to clarify that CC are generated during the repair phase of collapsed forks, through the BIR pathway (63, 64). Although the robust increase in CC following FANCM depletion has been reported to be strictly dependent on POLD3 and BLM (64), a broader network of factors has been implicated in promoting CC formation in this context, including checkpoint kinases (ATR), homologous recombination proteins (RAD51, BRCA2, PALB2), and the translesion DNA polymerase Pol η (63). Interestingly, Lu et al. (64) reported a stronger dependence on RAD52 than on RAD51, whereas Pan et al. (63) proposed that BRCA2 and PALB2 may facilitate RAD51-dependent strand invasion to initiate BIR.

Collectively, these findings suggest that the complex machinery underlying CC production represents a potential vulnerability in ALT-positive cancers. In this regard, it has been reported that FANCM is essential for the viability of ALT cancer cells (64) and that disrupting its interaction with the BTR complex, either by a synthetic peptide mimicking the conserved MM2 domain or a small-molecule inhibitor (PIP-199),

phenocopies FANCM depletion (64). This results in hyperactivation of ALT, increased CC formation, and selective killing of ALT-positive cancer cells (64). On the other hand, it has been shown that in the absence of key repair factors such as BLM or BRCA1, stalled/collapsed replication forks at telomeres cannot be properly resolved in FANCM-deficient ALT cells, ultimately leading to cell death (63). Moreover, additional factors required for ALT cells to tolerate telomeric replication stress have been proposed, many of which are enzymes, including TOP3A, Pol δ and Pol η , whose inhibition may represent a promising therapeutic strategy in ALT-driven cancers (63).

In line with these studies, work by Loe et al. (41) further clarifies the crucial role of PML protein and the BTR complex in CC production, delineating a molecular cascade required for activation of the ALT pathway. Specifically, PML is essential for maintaining key ALT hallmarks. PML-null cells exhibit an approximately five-fold reduction in CC levels compared to parental ALT-positive osteosarcoma cells (41). Notably, loss of PML leads to a uniform depletion of CC across the entire cell population, as evidenced by ss-TeloC assay, suggesting that PML plays an active role in their generation rather than merely stabilizing them. Consistently, exogenous expression of PML restores CC formation in PML-null cells (41). Mechanistically, it has been shown that the primary function of PML is to recruit the BTR complex to telomeres through the formation of APBs (41). This finding implies that interfering with APB assembly could represent a therapeutic strategy to selectively target ALT-positive cells (65). However, depletion of factors belonging to the BTR complex, such as BLM or the scaffold protein RecQ-mediated genome instability protein 1 (RMI1), phenocopies PML loss, leading to a marked reduction in CC levels (41). Importantly, while PML overexpression increases CC formation in wild-type cells, it has no effect in the absence of the BTR complex, indicating that BTR acts downstream of PML as the enzymatic machinery driving CC production (41).

A key finding of the study is that the mere physical recruitment of the BTR complex to telomeres is sufficient to trigger CC formation, bypassing the requirement for other ALT-associated structures (41). Using the tethering system RMI1-TebDB, a fusion protein consisting of RMI1 linked to the telomere-binding domain of Telomere End-Binding protein 1 (Teb1), to artificially localize the BTR complex to telomeres, the authors were indeed able to induce CC production in PML-null cells, at levels comparable to those observed in parental ALT cells (41). Strikingly, this mechanism proved sufficiently potent to induce CC formation even in telomerase-positive HeLa cells, suggesting that BTR activity at telomeres constitutes a fundamental driving force for the generation of C-circles (41).

The crucial role of BLM in CC production has been further underscored by studies showing that ALT activity is governed by a dynamic balance between two opposing DNA repair pathways (47, 66, 67). On one side, the BTR complex driven by BLM promotes recombination-based telomere elongation, while on the other, the SLX4–SMX complex, a multi-nuclease DNA repair assembly composed of SLX1–SLX4, MUS81–EME1, and XPF–ERCC1, counteracts BTR activity by facilitating telomeric resolution (47). In this context, recent evidence has shown that polyubiquitinated proliferating cell nuclear antigen (polyUb-PCNA) acts as a platform for SLX4 recruitment to telomeres (68). Failure to remove polyUb-PCNA leads

to unscheduled telomeric recombination and increased CC levels, indicating that CC formation can be directly influenced by PCNA loading onto chromatin and its ubiquitination state (68).

Moreover, SLX4 interacting protein (SLX4IP) has emerged as a critical modulator of the balance between BTR and SLX complexes (66, 67). This scaffolding protein uniquely interacts with both complexes, functioning as a molecular brake on BLM and restraining its potentially aberrant or hyperactive activity at ALT telomeres (66). Notably, SLX4IP operates in a pathway that is distinct from, yet parallel to, that of the translocase FANCM in limiting BLM-driven replication stress (67). Consistent with this role, depletion of SLX4IP results in uncontrolled BLM activity and a consequent increase in CC production (66).

Mechanistically, it has recently been shown that, in the absence of SLX4IP, BLM aberrantly unwinds non-ligated Okazaki fragments, generating 5'-flaps and gaps that favors the circularization of telomeric DNA into CC (67).

The dependence of ALT-positive cancers on these tightly regulated pathways creates several potential vulnerabilities for therapeutic intervention. In this context, co-depletion of FANCM and other repair factors (e.g., BLM or SLX4IP) can give rise to synthetic lethal interactions that are selectively toxic to ALT-positive cells (64, 67). For example, while ALT cells can tolerate the loss of either FANCM or SLX4IP individually, their combined loss results in unrestrained BLM activity, severe replication stress, and ultimately cell death (67).

Notably, SLX4IP has been reported to be inactivated in a subset of ALT-positive osteosarcomas (66), suggesting that these tumors may be particularly vulnerable to emerging therapeutic strategies targeting FANCM (67). More broadly, because productive ALT relies on a finely tuned balance between telomeric resolution (mediated by SLX4-SMX) and dissolution (mediated by BLM), pharmacological approaches that disrupt this equilibrium could selectively induce senescence or apoptosis in ALT-positive tumor cells (66).

As ALT-mediated telomere synthesis and the associated production of CC are critically dependent on the BTR complex, disruption of this complex or hindering its recruitment to telomeres may effectively impair telomere maintenance (41, 47, 64, 67). In this context, BLM has recently emerged as a promising therapeutic target in ALT-positive cancers. BLM silencing or pharmacological inhibition of its helicase activity using the small-molecule inhibitor ML216 has been shown to significantly reduce C-circle levels in ALT-positive osteosarcoma cells and to exert a synergistic effect when combined with G4-stabilizing agents (69). More broadly, BLM has been proposed as a key determinant of cellular responses to DNA-damaging agents. Its inhibition enhances the cytotoxic effects of alkylating agents in multiple myeloma and prostate cancer preclinical models, and may offer therapeutic benefit in tumors that are intrinsically resistant to PARP inhibitors or radiation therapy (69).

It has been reported that SMARCAL1 associates with telomeres within APBs, where it remodels stalled replication forks and promotes their restart, directly influencing CC production (21, 70). Loss of SMARCAL1 leads to a marked increase in CC, although the consequences vary depending on the cellular context. In ALT-positive cells, SMARCAL1 is significantly enriched at telomeres to resolve chronic

replication stress. Its depletion results in an approximately three-fold increase in CC abundance, indicating that SMARCAL1 normally limits the hyperactivation of the ALT pathway (21, 70). However, in telomerase-positive cells, SMARCAL1 deficiency can induce CC formation, although at lower levels compared to ALT cells (21, 70). This evidence suggests that CC may represent a broader marker of telomere instability that can arise when replication stress is not properly resolved. Mechanistically, suppression of CC formation depends on specific features of SMARCAL1 activity at telomeres that distinguish it from its role elsewhere in the genome (21). Mutant forms of SMARCAL1 lacking ATPase activity (e.g., the R764Q mutant) fail to prevent CC accumulation (21, 54). Moreover, unlike its function during bulk chromosomal replication, SMARCAL1-mediated prevention of CC at telomeres occurs independently of RPA, as mutants defective in RPA binding can still rescue telomere instability (21). Overall, these findings identify SMARCAL1 as a unique and essential factor in managing the intrinsic replication stress associated with telomeric DNA (21, 70, 71) and it may represent a selective genetic vulnerability and a primary therapeutic target in ALT-positive tumors (71). In particular, large-scale analysis of the DepMap database revealed an inverse correlation between telomerase expression and dependency on SMARCAL1, positioning this protein as a main dependency factor in telomerase-negative cells (71). Specifically, it has been demonstrated that loss of SMARCAL1 in ALT-positive cells exacerbates the ALT phenotype, leading to a massive accumulation of C-rich telomeric ssDNA, both nuclear and extrachromosomal, ultimately resulting in catastrophic telomere instability. In this regard, SMARCAL1 counteracts ssDNA accumulation through its DNA annealing activity, opposing BLM-mediated unwinding, and suppresses re-priming activity driven by the DNA-directed primase/polymerase (PRIMPOL) protein, which would otherwise generate unresolved telomeric ssDNA gaps (71). This study highlights several clinically relevant opportunities arising from the dependence of ALT cells on SMARCAL1. Experimental suppression of ALT activity (e.g., DAXX re-expression) abolishes the requirement for SMARCAL1 in cell survival, suggesting that SMARCAL1 inhibitors would have minimal effects on healthy telomerase-positive cells, thus providing ALT tumor selectivity (71). SMARCAL1 loss not only induces acute cell death but also triggers robust cell senescence in ALT-positive tumors. Consequently, cells driven into senescence by SMARCAL1 depletion become vulnerable to senolytics (e.g., navitoclax) thus creating a potential “one-two punch” therapeutic strategy (71). Combined loss of SMARCAL1 and the translocase FANCM produces a synergistic reduction in cellular fitness, consistent with a synthetic lethal interaction (71). Overall, these findings suggest that small-molecule inhibitors targeting SMARCAL1, such as the recently identified aza-anthracenedione, pixantrone (72), may represent a promising and actionable therapeutic strategy.

In this regard, it has previously been reported that SMARCAL1 deficiency enhances the activity of DNA-damaging agents or DNA-replication inhibitors (73). Additionally, SMARCAL1 has been shown to be essential in ATRX-deficient gliomas and osteosarcomas (71, 74), and to exert oncogenic functions in small cell lung cancer (72, 75), highlighting its relevance in aggressive tumors with limited treatment options (71, 72, 74, 75). More recently, the systematic integration of a tabular foundation model-based approach for inferring ALT status from whole-genome sequencing data, combined with CRISPR-Cas9 screening, has identified a selective dependency of ALT-positive cell lines on SMARCAL1 (76). These

findings further establish SMARCAL1 as a therapeutically relevant target in ALT-positive malignancies (76).

It has been reported that XPF is essential for telomere maintenance and proliferation in ALT cells, while being largely dispensable in non-ALT cells (77). In this context, TERRA-derived R-loops have been proposed to act as scaffolds to recruit XPF to telomeres. Once localized, XPF, together with the DNA excision repair protein ERCC-5 (XPG), promotes a local DNA damage response (77). These XPF-mediated DNA breaks trigger BIR, facilitating the recruitment of POLD3, as well as recombination factors such as BRCA1 and RAD51, which are essential for telomere elongation (77). Importantly, ALT cells exhibit sensitivity to XPF inhibitors (e.g., NSC130813), which disrupts telomere maintenance and suppresses cancer cell proliferation. This evidence supports the therapeutic potential of targeting XPF, even if the impact of its inhibition on CC production remains to be evaluated (77).

Moreover, it has been reported that the overexpression of the Sp100 protein suppresses ALT activity by sequestering the MRN complex, a key mediator of telomeric recombination. This leads to more than 80% reduction in CC levels with the magnitude of the effect scaling linearly with Sp100 expression (12). Similarly, depletion of the SMC5/6 complex, which is required for telomere maintenance in ALT cells, results in an approximately 50% reduction in CC production (12), while the stable expression of TERT in certain ALT-positive clones attenuates ALT activity, leading to an approximate 50% decrease in CC abundance (12). Finally, it has been reported that the restoration of the chromatin remodeler ATRX, or its interacting partner DAXX, may inhibit ALT activity resulting in impaired production of CC (78, 79).

Broderick et al. (80) show that the exonuclease 3'-5' domain-containing protein 2 (EXD2) regulates replication-fork processing at ALT telomeres and thereby controls the choice of telomere-maintenance pathway. Under normal conditions, EXD2 is recruited to telomeres and ALT-associated PML bodies, where its nuclease activity promotes a productive, RAD52-dependent BIR1 pathway (80). When EXD2 is lost, telomeric replication becomes highly unstable, with increased fork stalling (80). EXD2-deficient ALT cells display a "hyper-ALT" phenotype characterized by increased CC abundance (80). Mechanistically, the authors propose that EXD2 normally processes stalled telomeric replication forks in a way that favors RAD52-dependent BIR1. In the absence of EXD2, aberrant fork processing promotes a switch toward a RAD52-independent BIR2 pathway, which requires the fork remodeler SMARCAL1 and the structure-specific nuclease MUS81. This pathway is associated with increased formation of extrachromosomal telomeric DNA, including CC (80). Thus, EXD2 does not appear to be a direct CC generating nuclease. Rather, it acts as a negative regulator of CC accumulation by controlling replication-fork processing and ALT pathway choice. Its loss shifts telomere repair toward a more aberrant, RAD52-independent mode of BIR, resulting in increased CC but less productive telomere maintenance (80). The study also highlights potential therapeutic vulnerabilities, showing synthetic lethality when EXD2 loss is combined with depletion of other ALT-associated replication factors such as BLM, DNA2, or POLD3 (80).

Recently, analysis of gene dependency data from a genome-wide CRISPR/Cas9 screen identified a set of genes that are selectively essential for ALT-positive cells (37). These included the previously validated

FANCM and *SMARCA1*, as well as additional potential therapeutic targets involved in DNA replication and repair, replication fork restart and progression; G-quadruplex-interacting factors at stalled replication forks and telomere protection through homology-directed repair (37). However, the roles of these factors in regulating CC biogenesis remain to be determined.

3.2. Treatment-mediated modulation of C-circle production: preclinical evidence.

CC are highly sensitive to targeted disruption of telomere maintenance pathways. Indeed, several studies (section 3.1) have reported how the modulation in the expression/function of some ALT-associated factors may result in either the increase or reduction of CC production (Fig. 4). However, there is no substantial body of evidence yet directly demonstrating that standard anticancer therapies may modulate CC levels. However, because chemo- and radio-therapy induces replication stress and DNA damage, and ALT activity is highly stress-responsive, it is reasonable to expect that CC levels may be affected by these therapeutic approaches. In other words, while the link is biologically plausible, it remains insufficiently supported by direct experimental evidence, although some indications from the literature do exist.

In their seminal paper, Henson J et al. (39) reported that the exposure of ALT-positive cell lines to increasing doses of γ -radiation induces a dose-dependent increase in CC levels. Moreover, they showed that CC abundance continues to rise over time, approximately doubling every 24 hours for up to 72 hours dose post-irradiation, following exposure to 15 Gy radiation (39). This increase was actually driven by DNA damage and strictly dependent on ongoing DNA synthesis, as treatment with the DNA polymerase inhibitor aphidicolin immediately after irradiation completely abolishes the accumulation of CC (39).

Methotrexate, an anticancer drug that promotes heterochromatin formation and restores the telomeric chromatin condensation lost following LAP2 α depletion, has been reported to inhibit the proliferation of ALT-positive osteosarcoma tumors and concomitantly reduce CC levels in patient-derived xenograft models (58). In this regard, since CC reflect the open chromatin state of ALT cells, they may serve as useful biomarkers to stratify patients who could benefit from methotrexate treatment (58). This concept is further supported by a preclinical evidence showing that the sensitivity to trabectedin, an anticancer drug that binds the DNA minor groove and it is used to treat certain soft tissue sarcomas and relapsed ovarian cancer (81), inversely correlates with CC levels across different cancer cell lines (81).

Moreover, Kusuma et al. (82) demonstrated that ponatinib, a multi-receptor tyrosine kinase inhibitor approved for the use in different forms of Leukemia (83), can selectively target ALT-positive cancer cells by disrupting a specific signaling circuit. In their study, Kusuma et al. have used telomeric CC as bona fide markers to assess the impact of ponatinib on ALT activity, leading to the identification of the ABL1-JNK-JUN axis as a critical pathway for maintaining telomere stability in ALT cells (82). Mechanistically, ponatinib inhibits ABL1, resulting in reduced phosphorylation of JUN and its subsequent degradation (82). In vitro, ponatinib treatment induced a significant increase in CC levels across all tested ALT-

positive cell lines, consistent with disruption of the ABL1-JUN axis (82). This effect is further supported by the observation that depletion of either ABL1 or JUN phenocopies ponatinib-induced CC accumulation (82). In addition, ponatinib exhibits synergistic interactions with inhibitors of DNA synthesis. For instance, co-treatment with tricitabine further enhances CC formation, underscoring the drug's impact on DNA homeostasis and the engagement of repair pathways attempting to counteract this stress (82). Interestingly, residual tumors from ponatinib-treated mice displayed a marked reduction in CC levels (82). Although this appears to contrast with *in vitro* findings, the authors propose that ponatinib preferentially eliminates cells with the highest ALT activity. Consequently, depletion of these ALT-active subpopulations within the tumor mass may lead to an overall reduction in detectable CC *in vivo* (82). The study reveals that ponatinib disrupts telomeric homeostasis by intercepting a specific signaling circuit, making it a viable candidate for drug repurposing in ALT-positive cancers such as osteosarcoma and glioblastoma (82).

In the same frame, it has been reported that arsenic trioxide, an inorganic compounds used to treat adult patients affected by acute promyelocytic leukemia (84), abolishes CC generation by promoting the degradation of PML protein and disrupting APBs both in cell cultures and *in vivo* mouse xenografts (56).

Recent evidence indicates that elevated reactive oxygen species (ROS) represent a critical secondary event required to trigger and sustain the ALT pathway in cells lacking ATRX. In this context, ROS act as environmental or genetically driven stressors that enable cells to surpass the threshold necessary for telomeric recombination (85, 86). In this regard, it has been shown that in high-grade gliomas, the loss of Histone-lysine N-methyltransferase SETD2 leads to downregulation of antioxidant gene expression and a global reduction in H3K36me3, resulting in elevated ROS accumulation and oxidative DNA damage, including 8-oxoG (8-oxo-7,8-dihydroguanine) lesions (85). In low-grade gliomas, ATRX-associated dysregulation of gene expression reduces the levels of key ROS scavengers such as superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPX1), and scavenger receptor class A member 3 (SCARA3), further exacerbating oxidative stress (85). Consistently, silencing of SOD1 increases ROS levels and acts as a potent trigger for CC production; this effect is markedly amplified when combined with camptothecin (CPT), which stabilizes DNA topoisomerase I (TOP1)-DNA covalent complexes (a form of DNA-protein crosslinks, DPCs), resulting in robust overactivation of the ALT pathway in ATRX-deficient cancer cells (86).

These observations align with the evidence that severely hypoxic tumor microenvironments (< 0.1% O₂) promote ROS generation sufficient to induce ALT-associated features in ATRX-deficient cells (85). Mechanistically, elevated ROS levels drive the accumulation of R-loops and DPCs, including trapped TOP1 cleavage complexes (TOP1cc), which act as physical barriers to replication fork progression. In the absence of ATRX, stalled replication forks are prone to collapse, generating one-ended DSBs (85, 86). These lesions subsequently serve as substrates for BIR, ultimately driving the recombination-based telomere elongation (85, 86). Consistent with this model, combined loss of ATRX and SETD2 induces robust CC formation in HeLa LT models, whereas overexpression of RNase H1, which resolves R-loops, reduces CC levels in SETD2-deficient and hypoxic ATRX-null cells (85). Moreover, direct treatment with

ROS-inducing agents (e.g., hydrogen peroxide or tert-butyl hydroperoxide) is sufficient to trigger CC production in ATRX-null cells (85, 86). Importantly, these ROS-dependent effects on CC formation are largely reversed by the antioxidant N-acetylcysteine, confirming that oxidative stress is a primary driver of ALT-associated phenotypes (85, 86). Collectively, these findings define a “hyper-ALT” state with potential therapeutic implications. Exploiting ROS-generating agents to pre-sensitize ATRX-deficient tumors may enhance the efficacy of lower, less toxic doses of DNA-protein crosslink-inducing agents, such as irinotecan or cisplatin. This strategy may be particularly relevant in pediatric ALT-positive cancers, where minimizing long-term treatment-related toxicity remains a critical priority (85, 86).

Finally, it has been demonstrated that stabilization of G-quadruplex (G4) structures by RHPS4 induces marked perturbations in CC levels in dedifferentiated liposarcoma (DDLPS) (59). In DDLPS cells harboring long, ALT-like telomeres, RHPS4 exposure results in a time-dependent increase in CC levels, suggesting that G4 stabilization exacerbates telomeric replication stress and stimulates recombination-based telomere maintenance (59). In this setting, ALT activity appears to remain functional, potentially supporting cell survival despite increased genomic stress. In contrast, DDLPS cells characterized by shorter telomeres and a phenotype more consistent with telomerase activity exhibit a progressive loss of CC upon treatment with equitoxic doses of RHPS4 (59). This depletion of ALT markers is accompanied by extensive DNA damage and induction of apoptosis, indicating that G4 stabilization can, in this context, effectively disrupt telomere maintenance. Collectively, these findings highlight that while G4 ligands such as RHPS4 are broadly active in DDLPS, their impact on CC biogenesis and, consequently, on cell fate is dictated by the underlying telomeric and genetic landscape (59). Consistent with these observations, RHPS4 has been shown to induce a significant increase in CC abundance in ALT-positive osteosarcoma cell lines, whereas no such effect is observed in telomerase-positive counterparts (87). This ALT-specific response supports a model in which G4 stabilization creates physical impediments to replication fork progression (87). In ALT-permissive cells, replication fork stalling and collapse at telomeres generate DNA damage that activates recombination pathways, with CC arising as byproducts of compensatory telomere synthesis (87). More recent evidence further underscores the context dependency of G4 ligand effects (69). Naphthalene diimide-based G4 stabilizers induce replication stress and a progressive increase in CC levels in *TP53* wild-type osteosarcoma cells, consistent with activation of ALT as an adaptive stress response (69). Conversely, in *TP53*-deficient cells, the same treatment leads to reduced CC levels and increased apoptosis (59). Importantly, under these conditions, CC production is tightly linked to the activity of BLM helicase (69). Pharmacological inhibition of BLM results in a marked reduction in CC and exhibits synergistic effects with G4 stabilization in *TP53* wild-type cells, further underscoring BLM as a critical mediator of CC biogenesis during the response to G4-induced telomeric stress (69). In addition, treatment of osteosarcoma cells with the platinum derivative Tetra-Pt(bpy), a highly specific stabilizer of telomeric G4, has been reported to cause a marked reduction in CC levels (88). This decrease directly correlates with a lower frequency of other ALT-associated hallmarks, including APBs and telomere sister chromatid exchanges (88). Moreover, whereas Tetra-Pt(bpy) effectively suppresses CC formation, conventional cisplatin fails to reduce either CC or APBs in U2 Os cells (88).

A pilot screen of an 85-compound epigenetic library carried out using the HT-CCA (40) identified multiple modulators of CC, highlighting both shared and cell-type-specific responses. In particular, the most consistent hits were compounds that disrupt DNA repair and replication by trapping proteins on DNA, including PARP inhibitors (Olaparib, Niraparib, Talazoparib) and topoisomerase inhibitors (e.g., camptothecin, etoposide), which increased CC levels in a dose-dependent manner proportional to the “trapping strength” (40). The screen also revealed context-dependent epigenetic regulation: HDAC inhibitors increased CC in osteosarcoma cells, while BET inhibitors did so in neuroblastoma cells (40).

At the bottom line, ALT cancers frequently harbor loss of SMARCA1, ATRX, or DAXX (89), creating a cellular environment characterized by persistent replication gaps. Because both C- and G-circle pathways converge on a requirement for BLM and POLD3(47, 89), these factors represent high-value therapeutic targets. Inactivation of BLM or POLD3 in translocase-deficient ALT cells induces synthetic lethality (64), abolishing the capacity of cells to maintain telomeres via strand displacement mechanisms and ultimately compromising tumor proliferative potential. Importantly, the dependence of CC formation on factors such as BLM and the DNA synthesis machinery highlights key vulnerabilities that could be therapeutically exploited. Overall, these findings support a model in which CC are not merely byproducts of ALT, but functional indicators of telomeric stress adaptation.

In conclusion, although direct evidence linking standard anticancer therapies to CC modulation remains limited, multiple studies suggest that agents inducing DNA damage, replication stress, or chromatin remodeling can significantly influence CC production in a context-dependent manner. The relationship between CC dynamics and chemotherapy, radiotherapy, or targeted therapies remains poorly defined and warrants further investigation. Nonetheless, current data support CC as promising candidate biomarkers for monitoring tumor response to therapy and detecting early recurrence, pending validation in dedicated translational studies.

3.3 Relevance of C-circles in patient stratification and prognosis

As telomerase- and ALT-specific inhibitors are developed, CC testing may become increasingly important for stratifying patients according to the active TMM, thereby identifying tumors most likely to benefit from mechanism-specific therapeutic strategies (12).

A summary of studies investigating the relevance of CC measurement in clinical specimens is presented in Table 3. One of the earliest studies demonstrating the presence of telomeric CC in tumor specimens reported that 36.4% of paraffin-embedded uterine sarcomas were positive for CC detected by CCA (31). Importantly, CC levels significantly correlated with the presence of APBs detected by immuno-FISH (31). These findings indicated that both APB detection and CCA are robust and reliable approaches for assessing ALT-associated features in paraffin-embedded tumor tissues (31). This observation is further supported by an early analysis of liposarcoma specimens revealing that ALT-positive tumors, identified by the presence of APBs, generally exhibited abundant CC (90). However, a subset of tumors displaying features of both ALT and telomerase activity lacked detectable CC, suggesting that telomerase may

selectively interfere with CC biogenesis (90). Chudasama et al (91) reported that approximately 78% of leiomyosarcoma (LMS) specimens tested positive for CC, which represents the highest frequency of CC reported to date for any tumor entity (91). The frequency of CC positivity greatly exceeded the incidence of *ATRX* alterations, which were detected in 49% of LMS cases, indicating that additional molecular alterations may converge to activate the ALT pathway in these tumors (91). Moreover, extensive telomere length heterogeneity, including the presence of very long telomeres and generally elevated telomere content, was observed. However, no correlation was found between CC status and telomere content, leading to the conclusion that CC represent the most reliable functional biomarker for confirming ALT activation in LMS, although this cohort was not screened for TA (91).

On the other hand, a screening of 43 malignant pleural mesothelioma (MPM) using the CCA showed uniformly negative results in terms of CC detection across the entire MPM cohort, likely indicating that these tumors predominantly rely on telomerase-based telomere maintenance (92).

Another application of the CCA for the detection of CC in clinical specimens was that reported by Mangerel et al. (93) for a large-scale screening of major histologies of pediatric brain tumors (PBTs). By analyzing 517 tumor samples, the authors demonstrated that the ALT phenotype represents a key molecular feature of specific malignant PBTs and is strongly associated with somatic *TP53* mutations and *ATRX* alterations (93). A major finding of the study was the observation that ALT positivity, defined by CC detection, constitutes a favorable prognostic factor within otherwise highly aggressive *TP53*-mutant PBTs. Although *TP53* mutations are generally linked to poor patient outcomes, the presence of ALT significantly mitigated their adverse prognostic effect (93). Specifically, among children with *TP53*-mutant malignant gliomas, one-year overall survival was significantly higher in CC-positive cases compared with CC-negative cases (93). Likewise, in *TP53*-mutant choroid plexus carcinomas, five-year overall survival reached 67% in CC-positive tumors versus 27% in CC-negative tumors (93). Collectively, these findings establish CC not only as a diagnostic biomarker for ALT, but also as a clinically relevant tool for patient risk stratification across different tumor histologies, enabling the identification of high-risk patient subsets with comparatively improved prognoses (93).

By analyzing a cohort of 104 adult patients affected by diffuse gliomas harboring mutations in isocitrate dehydrogenase (*IDH*) and *ATRX* loss, ALT activity, quantified through the C-circle assay (CCA), has been demonstrated to vary according to both tumor malignancy grade and patient age. In grade III anaplastic astrocytomas, ALT activity showed a significant inverse correlation with age, with younger patients exhibiting markedly higher CC levels (94). In contrast, this association was absent in secondary glioblastomas (grade IV), where ALT intensity remained relatively stable regardless of age (94). In addition, the authors showed that the CC score strongly correlates with ALT-associated ultra-bright telomeric foci detected by Telo-FISH, and established a threshold of 6 arbitrary units to confidently define *true ALT-positive tumors* (94). These findings further reinforce the utility of CC as reliable molecular tools for ALT detection in brain tumors. Moreover, though CC levels were not found to directly correlate with overall or disease-free survival, the study highlights their value for molecular stratification of ALT-positive gliomas (94). Quantification of CC has allowed the identification of biologically distinct

subgroups of ALT-positive tumors and may therefore help refine patient classification (94). Moreover, the detection of high ALT activity through CC could have important therapeutic implications, particularly for identifying patients who may benefit from ALT-targeted approaches (94).

In a previous study, Fogli et al. (95) investigated the clinical implications of ALT in 63 malignant gliomas (grades II–IV). Using the CCA, the authors identified ALT activity in 14.2% of tumors and demonstrated significant associations with the presence of *IDH1/2* mutations, O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation, and loss of *ATRX* expression (95). The authors indicated that the CCA enabled reliable detection of ALT activity and proved particularly advantageous over *ATRX* immunohistochemistry. While ALT in lower-grade gliomas was frequently associated with *ATRX* loss, several ALT-positive glioblastomas retained functional *ATRX* expression and would therefore have escaped detection by conventional *ATRX*-based screening (95). In contrast, the CCA successfully identified ALT activation regardless of *ATRX* status, highlighting its superior diagnostic accuracy (95). Moreover, patients harboring ALT tumors showed significantly longer median survival (41.9 months vs. 15.8 months) and markedly higher five-year survival rates (62% vs 22%) compared to non-ALT cases (95). ALT-positive gliomas were also associated with younger age at diagnosis and lower Ki-67 proliferation indices, both features linked to better prognosis (95). Although multivariate analysis did not identify ALT status as an independent prognostic factor, the strong survival trends observed suggest that CC may still serve as clinically informative biomarkers for patient stratification (95). In addition, the study also highlighted the inverse relationship between ALT and telomerase activation. CC-positive tumors consistently displayed low or undetectable *TERT* expression, whereas elevated *TERT* levels were associated with poorer prognosis (95). Collectively, these findings establish CC not only as robust molecular markers of ALT activity, but also as valuable tools for the biological and clinical characterization of diffuse gliomas (95). More recently, the TeloDIAG was proposed as a telomere-based diagnostic classification system for gliomas (96). This algorithm was introduced to improve the molecular diagnosis of gliomas by integrating TMM status with established molecular and histopathological markers (i.e., *IDH1/2* mutational status, histological grade) (96). This method is centered on the detection of ALT activity using the CCA, which was specifically adapted for frozen tissue, formalin-fixed, paraffin-embedded (FFPE) specimens, and blood-derived liquid biopsies (96). The authors demonstrated that CC detection could reliably identify the operating TMM in gliomas with very high diagnostic performance: 100% sensitivity and 97.3% specificity in their validation cohort (96). One of the key motivations behind TeloDIAG was the observation that approximately 25% of gliomas display atypical combinations of *ATRX* and *TERT* alterations, making conventional molecular classification ambiguous (96). By directly assessing TMM through CC detection and telomere length analysis, TeloDIAG was able to reclassify a substantial fraction of diagnostically discordant tumors (96).

In line with this observation are recent findings showing the clinical utility of combining CC detection with *TERT* expression profiling for the molecular stratification and prognostic assessment of high-risk neuroblastoma (97). By integrating CCA data with *TERT* mRNA analysis, the authors classified tumors into three biologically distinct subgroups: *TERT*-high, ALT-positive (ALT+), and *TERT*-low/non-ALT tumors. Notably, the ALT phenotype, defined by the presence of CC, was detected in 23.4% of high-risk

neuroblastomas (97). As shown in previous studies, CC provided a more definitive indicator of ALT activation compared with conventional APBs detection, which may also occur in non-ALT cells with long telomeres (97). The CCA was successfully applied not only to primary tumor samples, but also to neuroblastoma cell lines and patient-derived xenografts, highlighting its robustness across experimental models. Importantly, CC positivity identified ALT activation even in tumors lacking alterations in canonical ALT-associated genes such as *ATRX*, *DAXX*, *H3.3 histone A*, and *SMARCA1*, emphasizing that functional ALT biomarkers may be more informative than genomic profiling alone (97). The study further revealed the prognostic significance of CC status. Although event-free survival was comparable among the different groups, overall survival differed markedly. ALT-positive tumors exhibited particularly poor outcomes, with a 10-year overall survival of only 24% compared with 75% in TERT-low/non-ALT tumors (97). The prognostic value of ALT status remained significant even after adjustment for established neuroblastoma risk factors, including *MYCN* amplification, *ALK* alterations, and ploidy (97). Interestingly, the TERT-low/non-ALT subgroup displayed an “ever-shorter telomere” (EST) phenotype characterized by progressive telomere erosion and reduced long-term tumor viability, which may explain the improved response to salvage therapies observed in these patients (97). Collectively, these findings may help identify ALT-positive neuroblastomas and refine risk stratification (97). CC may also inform therapeutic decision-making by supporting the selection of patients who may be eligible for potential ALT-targeted treatments, as well as the identification of lower-risk patients who may benefit from reduced treatment intensity(97).

More recently, Burrow et al. (98) systematically evaluated the prevalence of ALT in pediatric sarcomas using the qPCR-based CCA. Analysis of a large cohort of fresh frozen tumor specimens revealed striking differences in ALT prevalence among pediatric sarcomas. ALT activity, defined by CC positivity, was highly prevalent in osteosarcoma, occurring in 71% of cases, whereas it was rare in rhabdomyosarcoma (2.7%) and completely absent in Ewing’s family sarcoma (98). The absence of ALT in Ewing’s sarcoma likely reflects the predominance of telomerase activation through *EWSR1* fusion-driven TERT expression, consistent with the generally mutually exclusive nature of telomerase and ALT mechanisms (98). The study emphasized that defining the operating TMM of a tumor is becoming increasingly important in precision oncology, as ALT-positive cancers may exhibit distinct biological behaviors and therapeutic vulnerabilities (98). In osteosarcoma, where ALT predominates, the CCA could serve as a companion diagnostic to identify patients who may potentially benefit from ALT-targeted therapies, including ATM kinase inhibitors or p53-reactivating agents, which have shown activity against ALT-positive models (98). Collectively, these findings reinforce the central role of CC as functional biomarkers for the detection, classification, and potential therapeutic targeting of ALT-driven pediatric sarcomas (98).

Finally, the study by Bicanova et al. (99) represents the first evaluation of CC levels in canine models. By analyzing 50 archived FFPE specimens from spontaneous occurring therapy naïve appendicular osteosarcoma (OSA), the authors demonstrated the presence of CC in 20% of cases, confirming that their production is conserved across species, although at a lower prevalence than in human osteosarcoma (99). Moreover, CC positivity emerged as a prognostic indicator of aggressive disease and poor clinical outcome, with multivariate analysis confirming CC status as an independent prognostic

factor (99). The study highlights the translational relevance of CC detection in comparative oncology, supporting the use of canine immunocompetent models as valuable platforms for investigating TMM biology. By recapitulating key molecular, cellular, and immune features relevant to CC biology and TMM development, these models may provide clinically relevant insights into tumor evolution, disease mechanisms, host-tumor interactions, therapeutic response, and treatment resistance, thereby facilitating the translation of preclinical findings into human oncology (99).

Table 3
Translational relevance of C-circles in cancer

Tumor type	Specimens & method	% of CC-positive specimens	Observations	Ref.
Uterine sarcoma (n = 22)	FFPE tumor specimens CCA	36.4%	First validation in FFPE samples; Strong correlation with APB (immuno-FISH)	(31)
Liposarcoma (n = 32)	Tumor specimens (not otherwise specified) CCA	36.4% dedifferentiated LPS 80.0% pleomorphic LPS	APBs partially concordant (some ALT-positive tumors lack CC); telomerase activity may suppress C-circles	(90)
Leiomyosarcoma (n = 49)	Fresh frozen tumor specimens CCA	78%	Poor correlation with ATRX alterations (49%); Poor correlation with telomere length/content	(91)
Malignant pleural mesothelioma (n = 43)	Fresh frozen tumor specimens CCA	0%	Consistent telomerase dependence; Strong negative diagnostic control (absence of ALT)	(92)
Pediatric brain tumors (n = 517)	Tumor specimens (not otherwise specified) CCA	22.6% choroid plexus carcinoma 22.0% supratentorial high grade glioma 18.8% diffuse intrinsic pontine glioma 11.6% primitive neuroectodermal tumors 2.19% medulloblastoma 1.67% low grade glioma 0% (ependymomas; atypical teratoid rhabdoid tumor; choroid plexus papilloma)	High concordance with APB and TRF; Defines ALT-positive subset strongly associated with TP53/ATRX mutations; Favorable prognosis in <i>TP53</i> -mutant tumors (↑ survival); Refines risk stratification in aggressive pediatric gliomas	(93)

Tumor type	Specimens & method	% of CC-positive specimens	Observations	Ref.
Adult diffuse gliomas (n = 104)	Tumor specimens (not otherwise specified) CCA	CC quantification provided in arbitrary unit. No threshold for positivity specified	CC quantification varies by grade and age; Strong correlation with ALT-FISH foci; Defines biologically distinct ALT subgroups	(94)
Glioma (n = 63)	Frozen tissue specimens CCA	14.3%	Improves diagnostic accuracy over ATRX-based screening (detects ATRX-independent ALT cases); Inverse relation with TERT expression; Significantly associated with <i>IDH1/2</i> mutation and <i>MGMT</i> promoter methylation; CC-positive patients diagnosed at younger age than CC-negative patients; Median survival: 41.9 months in CC + vs. 15.8 months in CC- patients (p = 0.03, HR = 0.28, 95% CI [0.09; 0.90]); 5-year survival: 62% in CC + vs. 22% months in CC- patients; CC status did not appear to be an independent prognostic factor in a multivariate model	(95)
Glioma (n = 284)	Frozen, FFPE, blood specimens Integrated CCA s (TeloDIAG)	High diagnostic accuracy (100% sensitivity, 97.3% specificity)	Resolves ATRX/TERT discordance; Enables telomere-based molecular classification (TeloDIAG as an effective diagnostic tool)	(96)
High-risk neuroblastoma (n = 145)	Fresh frozen tumor specimens CCA	23.4%	More reliable than APB detection; Enables risk stratification and therapy selection Poor prognostic indicator (10-year overall survival: 24% in CC + vs. 75% in CC- patients)	(97)
Pediatric sarcomas (n = 369)	Fresh frozen tumors qPCR-CCA	71% osteosarcoma (n = 87) 2.7% rhabdomyosarcoma (n = 187) 0% Ewing sarcoma (n = 95)	Matches telomerase-driven biology in Ewing sarcoma; Companion diagnostic for ALT, Hel guiding ALT-targeted therapy selection (e.g., ATM inhibitors); Suggested prognostic value	(98)

Tumor type	Specimens & method	% of CC-positive specimens	Observations	Ref.
Canine appendicular osteosarcoma (n = 50)	FFPE tumor specimens CCA	20%	CC showed an association with male gender and Rottweiler breed; Veterinary prognostic marker: CC+ patients had shorter overall survival times than patients with CC- tumors (156 vs. 364 days); CC status was a significant prognostic factor besides treatment in the Cox proportional hazard model.	(99)

3.3 C-circles in liquid biopsies

Liquid biopsy has emerged as a promising approach in oncology by enabling the minimally invasive analysis of tumor-derived components, including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), extracellular vesicles, and other biomarkers present in body fluids such as blood (100). Unlike conventional tissue biopsy, liquid biopsy allows for serial sampling, providing a dynamic assessment of tumor evolution, treatment response, and the emergence of therapeutic resistance (100). While technical challenges related to sensitivity, specificity, and assay standardization remain, continued technological advances are steadily expanding its clinical utility (100). Within this rapidly evolving field, the identification of disease-specific biomarkers suitable for liquid biopsy has become a major focus. In this regard, the evidence that CC can be detected in liquid biopsy samples substantially strengthens their potential as clinically relevant biomarkers.

The very first evidence showed that the CCA could successfully discriminate between ALT-positive and ALT-negative osteosarcoma patients using archival blood specimens (12). In this study, blood samples from ALT-positive patients generated markedly higher CC signals (~ 102.8 AU) compared with ALT-negative patients (~ 23.4 AU) and healthy controls (12). Importantly, although CC could be detected in whole blood, plasma samples provided a superior signal-to-noise ratio for clinical testing (12). This improvement likely reflects the lower abundance of leukocyte-derived background DNA in plasma, which may otherwise inhibit assay performance at high concentrations. Furthermore, the authors demonstrated that the intensity of CC signal in clinical blood specimens was linearly proportional to the amount of input DNA, confirming that the assay can reliably quantify circulating CC (12). Consistent with this, Billard et al. (96) demonstrated the feasibility of detecting tumor-derived CC in blood for glioma monitoring. Using the TeloDIAG framework, blood-based CC detection achieved sensitivities of approximately 56%-72%, markedly exceeding those reported for TERT promoter mutations in ctDNA (96).

The potential for detecting CC in circulating exosomes - a subtype of extracellular vesicles involved in diverse biological processes and integral to the tumor microenvironment (100) - has also been recently highlighted (98, 101). Chen et al. (101) have provided the first direct evidence that CC are actively secreted by cancer cells through exosomes. They demonstrated that CC are not merely intracellular by-products of ALT activity, but are selectively packaged into extracellular vesicles. Exosomal CC were

detected in all ALT-positive cancer cell lines analyzed, including osteosarcoma, neuroblastoma, and glioblastoma, and their presence was validated using multiple purification approaches, such as differential centrifugation, iodixanol density gradients, and immunopurification with CD81 and Syntenin exosomal markers (101). Importantly, encapsulation within exosomes protected CC from degradation by circulating nucleases such as DNase I, thereby ensuring stability in the bloodstream and supporting their suitability for clinical applications (101). Quantitative analyses further revealed that ALT-positive cells secrete approximately 0.4% of their intracellular CC content into exosomes every 24 hours (101). Similar to intracellular CC, secreted CC were detected exclusively in ALT-positive cells and were absent from telomerase-positive cancer cells and normal mortal cells. The clinical utility of this approach was validated in serum samples from high-risk neuroblastoma patients, where the serum-based CC assay achieved 100% sensitivity and high concordance with conventional tumor-based ALT testing (101). Because circulating exosomes originate from multiple tumor sites, the detection of CC in liquid biopsies may also overcome limitations associated with intra-tumoral heterogeneity and single-site biopsies (101). In addition, beyond their diagnostic value, CC trapped into circulating exosomes may also have biological significance within the tumor microenvironment. In this regard, although ALT-positive cancer cells frequently suppress the cGAS–STING pathway to avoid sensing their own extrachromosomal telomeric DNA, secreted CC could potentially be internalized by neighboring stromal or immune cells, thereby eliciting inflammatory responses that may contribute to immune evasion and tumor progression (101).

Collectively, these findings, though still very limited, have established CC as *bona fide* circulating biomarkers (12, 101). Their circular structure, relative stability, and abundance in the peripheral blood provide a robust platform for assessing tumor burden, tracking disease progression, evaluating treatment response, and ultimately supporting more personalized clinical management of cancer patients (12, 98, 101).

4. Conclusions and future directions

Over the past two decades, CC have evolved from a distinctive molecular feature of ALT into one of the most informative and clinically relevant biomarkers of telomere maintenance in cancer. Their remarkable specificity for ALT-positive tumors, combined with their close association with telomeric replication stress and recombination, has established CC as a robust functional readout of ALT activity. Beyond their diagnostic value, studies on CC biogenesis have provided important insights into the molecular mechanisms that sustain telomere maintenance, revealing the complex interplay between DNA replication, repair, and recombination pathways. Increasing evidence that CC may arise through multiple mechanisms, rather than representing simple byproducts of lagging-strand synthesis, further underscores their biological significance and highlights the dynamic nature of telomere metabolism.

The clinical potential of CC continues to expand. Their application has progressed from tissue-based ALT detection to minimally invasive liquid biopsy approaches, following the demonstration that CC can be released into the circulation, including through exosome-associated pathways. This advance

substantially strengthens their value as biomarkers by enabling longitudinal assessment of tumor burden, disease progression, treatment response, and relapse through serial blood sampling. As liquid biopsy becomes increasingly integrated into precision oncology, circulating CC may provide a unique opportunity to monitor ALT-positive malignancies in real time while reducing the need for repeated tissue biopsies.

Despite these advances, several challenges must be addressed before CC testing can be implemented in routine clinical practice. Current RCA-based assays remain technically demanding and relatively low throughput, emphasizing the need for standardized, highly sensitive, and clinically scalable detection methods. The development of digital droplet PCR-based assays, next-generation technologies such as 4SET, and other quantitative platforms capable of accurately detecting low-abundance circular telomeric DNA will be critical for improving reproducibility, facilitating automation, and enabling widespread clinical adoption.

Equally important is the establishment of the clinical utility of CC across diverse tumor types, which still needs to be widely investigated. Large prospective studies are needed to define diagnostic and prognostic thresholds, determine how CC levels correlate with tumor burden and patient outcome, and evaluate their performance alongside existing ALT biomarkers. Longitudinal analyses performed within clinical trials will be essential to determine whether changes in CC abundance can serve as reliable indicators of therapeutic response, minimal residual disease, or emerging treatment resistance. In parallel, further investigation of CC biology may reveal whether fluctuations in their levels reflect alterations in TMM or identify tumors that are particularly susceptible to therapies targeting replication stress and DNA repair pathways.

Importantly, CC abundance may also be influenced by extrinsic factors that shape telomere homeostasis and genome stability. In this context, the exposome (i.e., the cumulative set of environmental, lifestyle, and endogenous exposures experienced throughout an individual lifetime) represents a potentially relevant but largely unexplored modulator of CC production (102). Exposure-related changes in oxidative stress, inflammation, or cellular stress responses could conceivably affect telomeric replication and recombination and, consequently, the generation or abundance of CC (85, 86). Defining whether and how the exposome contributes to inter-individual and temporal variation in CC levels may therefore provide an additional layer of biological interpretation and could be particularly relevant when CC are evaluated as dynamic biomarkers in longitudinal studies.

In conclusion, CC are poised to become more than biomarkers of ALT. Their unique biological properties position them as potential surrogate markers of tumor evolution, functional indicators of therapeutic vulnerability, and dynamic tools for patient stratification and disease monitoring. Continued integration of mechanistic studies with technological innovation and prospective clinical validation will be essential to fully exploit their diagnostic and translational potential. As liquid biopsy technologies mature and telomere-targeted therapeutic strategies continue to emerge (103), CC analysis has the potential to become an integral component of precision oncology.

Abbreviations

ALT

alternative lengthening of telomere

alt-NHEJ

alternative non-homologous end joining

APB

ALT-associated promyelocytic leukemia (PML) bodies

ASF1

anti-silencing function 1 histone chaperone

ATM

ataxia-telangiectasia mutated

ATR

ataxia-telangiectasia and Rad-3 -related

ATRX

Chromatin remodeler ATRX

ATSA

ALT telomere DNA synthesis in APB assay

BIR

break-induced replication

BLM

RecQ-like DNA helicase BLM

BRCA1

Breast cancer type 1 susceptibility protein

BRCA2

Breast cancer type 2 susceptibility protein

BTR

BLM–TOP3A–RMI complex

CC

C-circles

CCA

C-circle assay

CDKN2A

cyclin dependent kinase inhibitor 2A

c-NHEJ

classical non-homologous end joining

COUP-TF2

nuclear receptor subfamily 2 group F member 2 (NR2F2)

CPT

camptothecin

CTC1
CST telomere replication complex component 1
ctDNA
circulating tumor DNA
DAPI
4',6-diamidino-2-phenylindole
DAXX
Death domain-associated protein 6
DNA2
DNA replication ATP-dependent helicase/nuclease 2
DDR
DNA damage response
DPCs
DNA-protein crosslinks
DSB
double strand break
dsDNA
double-stranded DNA
eccDNA
extrachromosomal circular DNA
ECTD
extrachromosomal telomeric DNA
EXD2
exonuclease 3'-5' domain-containing protein 2
FAAP24
Fanconi anemia core complex-associated protein 24
FANCM
Fanconi anemia complementation group M
FEN1
flap structure-specific endonuclease 1
FFPE
formalin-fixed, paraffin-embedded
FISH
fluorescence in situ hybridization
G4
G-quadruplexes
GPX1
glutathione peroxidase 1
HDAC1
histone deacetylase 1

HDR
homology-directed repair
HR
homologous recombination
IDH1/2
isocitrate dehydrogenase (NADP+) 1/2
LAP2α
lamina-associated polypeptide 2 alpha
LMS
leiomyosarcoma
LTT+
long-telomere, telomerase-positive cells
MGMT
O-6-methylguanine-DNA methyltransferase
MMS21
E3 SUMO protein ligase NSE2, component of SMC5/6 complex (NSMCE2)
MPM
malignant pleural mesothelioma
MRE1
Double strand break repair nuclease MRE11
MRN complex
MRE11–RAD50–NBS1
MSH2
mutS homolog 2
MSH3
mutS homolog 3
MUS81
MUS81 Structure-specific endonuclease subunit
MutSα
DNA mismatch repair complex Msh2–Msh6
MutSβ
DNA mismatch repair protein Msh2-Msh3
NBS1
Nijmegen breakage syndrome 1 (Nibrin)
ONRs
orphan nuclear receptors
OSA
canine appendicular osteosarcoma
PALB2
Partner and localizer of BRCA2

PARI
PARP1 binding protein
PARP1
poly-(ADP-ribose) polymerase 1
PBTs
pediatric brain tumors
PCNA
proliferating cell nuclear antigen
PML
Protein PML (promyelocytic leukemia)
Polδ
DNA polymerase delta
Polη
DNA polymerase eta (Y-family translesion synthesis enzyme)
POLD3
DNA polymerase delta subunit 3
POLD4
DNA polymerase delta subunit 4
POT1
protection of telomeres 1
PRIMPOL
DNA-directed primase/polymerase protein
RAD50
RAD50 double strand break repair protein
RAD51
DNA repair protein RAD51 homolog 1
RAD51AP1
RAD51-associated protein 1
RAD52
DNA repair protein RAD52 homolog
RAP1
Telomeric repeat-binding factor 2-interacting protein 1
RBL2
RB (retinoblastoma) transcriptional corepressor like 2
RCA
Rolling Circle Amplification
RMI1
RecQ-mediated genome instability protein 1
ROS
reactive oxygen species

RPA
Replication Protein A
SCARA3
scavenger receptor class A member 3
SETD2
SET domain containing 2, histone lysine methyltransferase
SLX4
SLX4 structure-specific endonuclease subunit
SLX4IP
SLX4 interacting protein
SMARCA4
SWI/SNF related BAF chromatin remodeling complex subunit ATPase 4 (BRG1)
SMARCAL1
SNF2 related chromatin remodeling annealing helicase 1
SMC5
structural maintenance of chromosomes 5
SMC5/6 complex
SMC5-SMC6-MMS21 complex
SMC6
structural maintenance of chromosomes 6
SMX complex
SLX1-MUS81/EME1-XPF-ERCC1
SOD1
superoxide dismutase 1
ssDNA
single-strand DNA
STN1
CST complex subunit STN1
STS
soft-tissue sarcoma
Teb1
Telomere End-Binding protein 1 (*Tetrahymena thermophila*)
TEN1
CST complex subunit TEN1
TERT
telomerase reverse transcriptase
TIN2
TRF1-interacting nuclear factor 2
TMM
telomere maintenance mechanism

TOP1
DNA topoisomerase I
TOP3A
DNA topoisomerase 3-alpha
TPP1
TIN2-interacting protein 1
TR4
nuclear receptor subfamily 2 group C member 2 (NR2C2)
TRIM28
transcription intermediary factor 1-beta
TRF1
telomeric repeat binding factor 1
TRF2
telomeric repeat binding factor 2
T-SCE
telomere-sister chromatid exchange
XPF
ERCC excision repair 4, endonuclease catalytic subunit (ERCC4)
XPG
ERCC excision repair 5, endonuclease (ERCC5)
ZMYND8
MYND-type zinc finger-containing 8 chromatin reader
ZNF827
zinc finger protein 827
ZRANB3
DNA annealing helicase and endonuclease ZRANB3

Declarations

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Consent for publication

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Competing interests

The authors declare that they have no competing interests

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Author Contribution

M.F. contributed to the conception and design of the review, the search, analysis and interpretation of the literature, the drafting of the manuscript and figures. N.Z. and S.P. contributed to data interpretation and the critical revision of the manuscript for important intellectual content.

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Figures

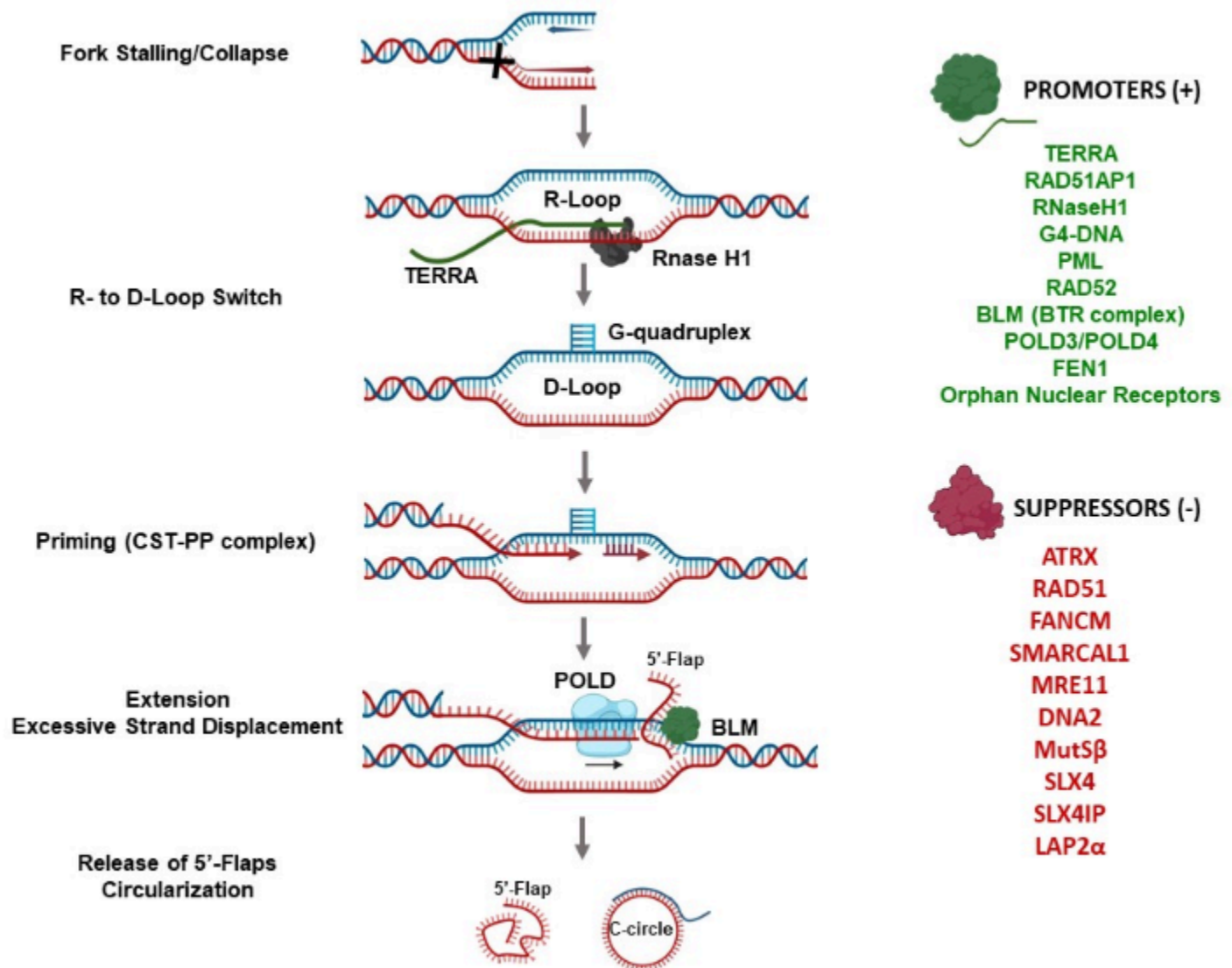


Figure 3

The birth of a C-circle. Schematic representation summarizing the steps leading to C-circle generation during replication of ALT telomeres according to the BIR2 axis. The R-to-D-Loop switch and the Excessive strand displacement models have been included. Factors reported in the main text as promoters (+) or suppressors (-) of C-circle production are listed on the right. (Created with BioRender).

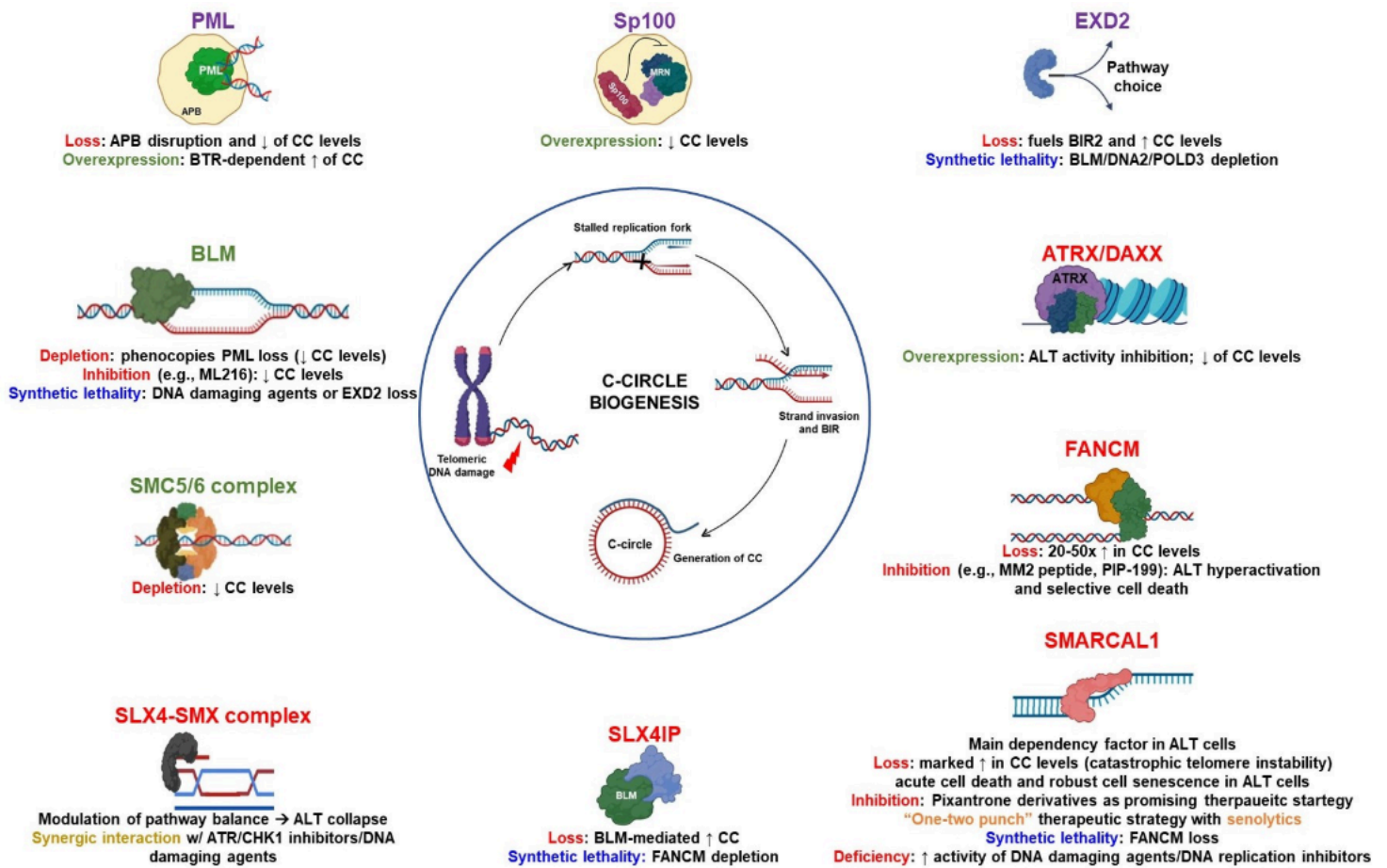


Figure 4

Molecular factors involved in C-circle biogenesis and their potential as therapeutic vulnerabilities. Promoters (green), inhibitors (red) and fine-tuning/context-dependent (purple) factors involved in CC formation have been schematically reported (created with BioRender)