

Review

G-Quadruplexes at the ‘Interface’ of Transcription and Alternative Splicing

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Abstract

DNA and RNA G-quadruplexes (G4s and rG4s, respectively) are well-known modulators of transcription and splicing. G4s are enriched in transcriptionally active chromatin domains that produce transcripts susceptible to alternative splicing (AS), at least in higher vertebrates. These domains are arranged close to nuclear speckles (NSs), where key AS regulators are stored. Recent findings have revealed new aspects of cotranscriptional AS, emphasizing the advantages of transient rG4s and revealing the connection between excessive rG4s and disease. Here, we discuss how (r)G4s’ presumed ability to promote liquid–liquid phase separation at active chromatin and their affinity for dual regulators of transcription and splicing has brought them to the forefront of communication between NSs and their associated chromatin domains. We also discuss how NS components may contribute to maintaining balanced (r)G4 folding, provide examples of imbalance, and analyze the prospects of G4-based AS reprogramming.

Keywords: G-quadruplex; chromatin domain; nuclear speckle; transcription; alternative splicing

1. Introduction

G-quadruplexes are noncanonical structures found in guanine-rich DNA (G4s) and RNA (rG4s) [1–3]. The key sequence requirement for G4 folding is the presence of G₃₊ tracts (four in unimolecular structures), which are separated by reasonably short loops (typically up to 7 or 12 nucleotides). Guanine residues from different tracts form Hoogsteen-bonded G-tetrads that can coordinate metal cations and stack on top of each other. The resulting quadruple helices have grooves and facets that differ from those of canonical structures. Depending on guanosine configurations, tract orientations, and loop arrangement, they adopt various topologies and therefore show a distinctive yet broad interactome [4].

Intramolecular (r)G4 motifs are abundant in the human genome and transcriptome, and their folding is related to the regulation of key biological processes [4,5]. The relevance of G4s to transcription is supported by their enrichment in promoters [6,7], (super)enhancers [8–10], and insulators [11], their ability to block RNA polymerase II (Pol II) or stabilize R-loops [7,12], and their affinity for multiple transcription factors (TFs) [13] and chromatin remodelers [14]. The relevance of rG4s to alternative splicing (AS) is supported by their enrichment at exon/intron boundaries [15] and their affinity for AS regulators, including heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine-arginine-rich splicing factors (SRSFs) [16].



Academic Editors: Keiko Kawauchi and Tamaki Endoh

Received: 25 September 2026

Revised: 19 October 2026

Accepted: 22 October 2026

Published: 24 October 2026

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Most (r)G4s are transient due to the activity of specific helicases [17–19] and competition with canonical secondary structures among other factors. The local impact of G4 folding/unwinding on transcription is position- and context-dependent and can be negative [20] or positive [21]. In general, however, G4s are associated with transcription activation [22,23] and have been detected in active, superenhancer-governed, GC-rich topologically associating domains (TADs) [10]. Recent studies suggest that GC-rich transcripts produced from such TADs are particularly prone to co-transcriptional AS [24,25]. The precise regulation of AS in higher vertebrates [26] requires the timely recruitment of regulatory factors (hnRNPs and SRSFs) from their “depots,” which are membraneless organelles known as nuclear speckles (NSs) [27,28]. Consistently, GC-rich active TADs are physically close to NSs and can therefore be referred to as speckle-associated domains (SPADs) [29,30].

In this review, we summarize the recent evidence of G4/rG4 folding in SPADs and SPAD-derived transcripts, as well as the contributions of (r)G4s to NS-SPAD juxtaposition through interactions with NS-associated proteins. We also discuss the importance of these interactions for transcription and AS, as well as the consequences of excessive (r)G4 formation upon the expansion of G-rich genomic repeats. Most aspects of (r)G4 contributions to transcription and AS have been reviewed previously [31–33]. Here, we contextualize these contributions spatially, focusing on the narrow spatial region (the “interface”) between NSs and SPADs in higher vertebrates. Additionally, we mention key technologies developed in the last decade that gave rise to the concept of NS-SPAD communication and illuminated the role of (r)G4s in this communication.

2. Spatiotemporal Overlaps Between Nuclear Speckles, Active Chromatin and G-Quadruplexes

2.1. Active Chromatin Domains with GC-Rich Isochores Are Associated with Nuclear Speckles

The SPAD concept emerged due to progress in measuring cytological distances [29,34]. This technology is based on labeling DNA proximal to organelles of interest via catalytic tyramide activation (tyramide signal amplification, TSA), which has recently been implemented in proximity ligation assays and in situ hybridization to invigorate spatial molecular biology [35]. In TSA assays, tyramide is typically conjugated to biotin for eventual pull-down and is activated in situ by a peroxidase fused to an antibody to the organelle protein marker. Labeling efficiency reflects proximity to the protein marker because it is determined by the diffusion-dependent local concentration of active tyramide radicals, which decreases exponentially with distance [29]. Initially, TSA was mainly applied in proteomics. A major breakthrough that made it applicable to SPAD mapping was optimizing the conditions for DNA labeling and subsequent sequencing (TSA-seq) [29]. Current gold-standard protocols ensure efficient DNA labeling due to saturating protein labeling (TSA-seq 2.0) [36]. Consequently, the normalized density of sequencing reads can be converted to cytological distances on a micron scale.

Another approach to SPAD mapping relies on chromatin immunoprecipitation and sequencing (ChIP-seq) or cleavage under targets and tagmentation (CUT&Tag) [37] with antibodies to NS-associated proteins. The CUT&Tag technique also uses a protein A/G-fused transposase. When activated by magnesium ions, the transposase cleaves DNA under target proteins (e.g., NS-associated proteins) and adds tags (sequencing adapters). The ChIP-seq/CUT&Tag approach enables SPAD identification based on interactome data rather than actual distances, which might cause some bias. Thus, despite its widespread use in mapping various chromatin compartments, this approach is suboptimal for spatial biology and could not generate the respective concepts. The development of the SPAD concept was only possible with the implementation of TSA-seq. TSA-seq-based SPAD mapping (the red leaf in the shamrock scheme, Figure 1) has been reported for several species and cell

lines, including human embryonic stem cells and immortalized cells (H1 and HEK-293), human cancer cell lines (K562 and HCT116), human neonatal (HFF) and adult (HDFa) fibroblasts, and mouse embryonic fibroblasts (3T3) among others [24,25,29,36]. Pairwise comparisons between human cell lines revealed moderate (~10%) differences in SPAD profiles that followed gene expression differences [36]. CUT&Tag-based SPAD mapping has only been performed on mouse embryonic cells, focusing on different developmental stages [38]. Thus, the profiles obtained by these two approaches cannot be compared directly. However, both have been compared to the profiles of active TADs obtained using conventional chromosome conformation capture methods [29,38,39].

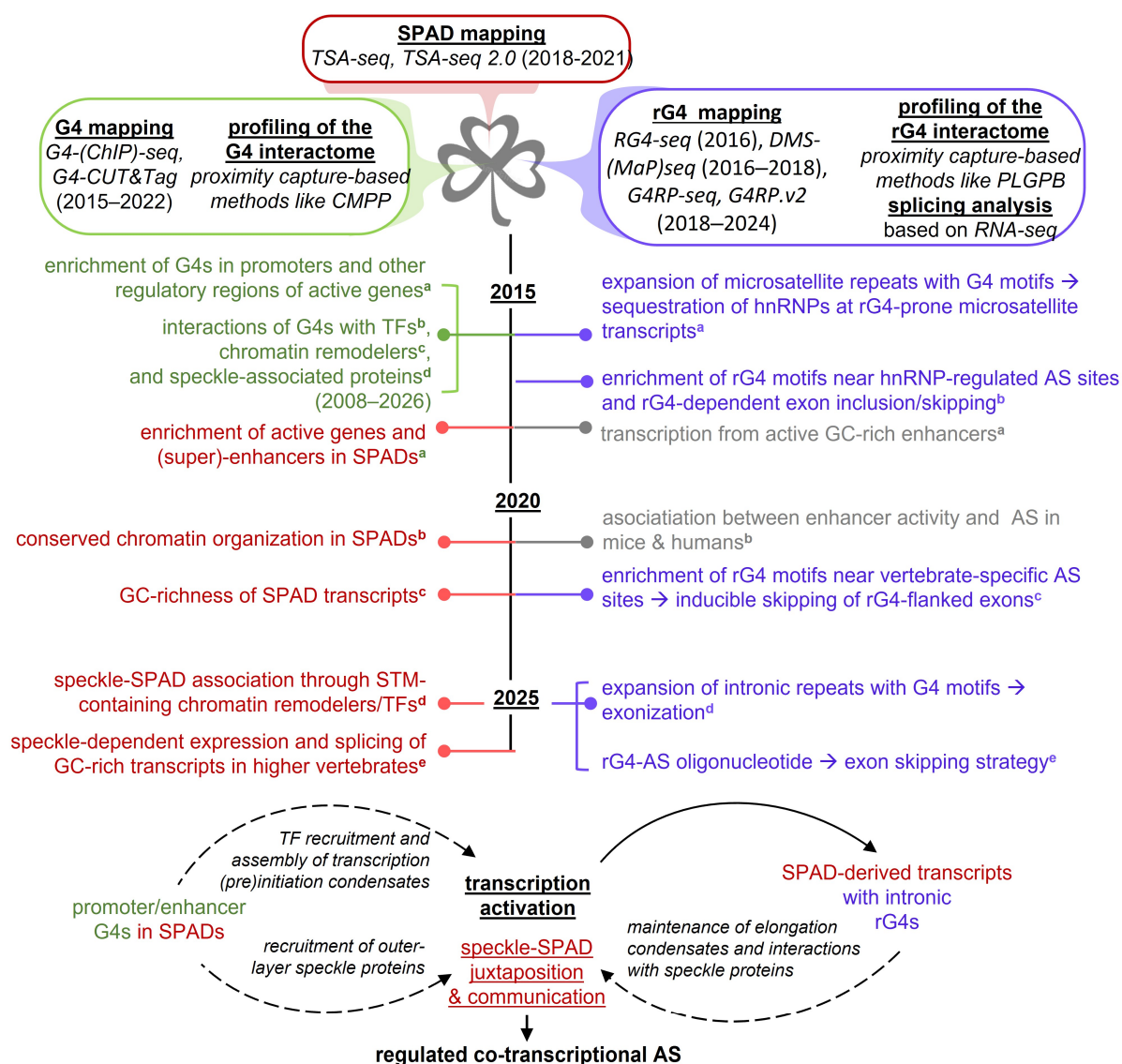


Figure 1. Evidence for the contributions of DNA/RNA G-quadruplexes (G4s/rG4s) to co-transcriptional speckle-dependent alternative splicing (AS). **(Top):** a shamrock-like scheme summarizing three lines of research that converged to support the role of transient rG4s and G4s from speckle-associated domains (SPADs) in AS regulation. The key types of assays are underlined, and the respective methods are italicized. **(Middle):** a timeline of related recent advances in studies of G4s (green), rG4s (blue), and SPADs (red). The two selected advances in enhancer studies (grey) are supposedly relevant to the role of SPAD-associated (r)G4s in AS regulation. Superscripts link the selected advances to references. Green: ^a, [22]; ^b, [13]; ^c, [14]; ^d, [40–47]. Red: ^a, [29]; ^b, [36]; ^c, [24]; ^d, [48]; ^e, [25]. Blue: ^a, [49,50]; ^b, [51]; ^c, [15]; ^d, [52]; ^e, [53]. Grey: ^a, [54]; ^b, [55]. **(Bottom):** schematic summary of the hypothetical contributions (dashed arrows) of G4s and rG4s to the speckle-SPAD communication during co-transcriptional AS. The presumed (r)G4 functions are italicized, and the key outcomes are underlined.

SPADs are very similar to active TADs; however, their boundaries do not fully coincide. As evidenced by CUT&Tag assays, SPADs emerge earlier than TADs during embryogenesis; at least some SPADs have well-defined boundaries while TAD boundaries are still obscure [38]. This implies that NSs do not merely “adjust” to the 3D chromatin structure but rather assist in shaping it. Analysis of SPADs mapped by TSA-seq techniques revealed the prevalence of transcriptionally active regions (>95% euchromatin) with similarly high GC content (GC isochores) [24,25], suggesting the presence of GC-isochores-specific secondary structures, such as G4s in SPADs and rG4s in SPAD-derived transcripts.

2.2. G-Quadruplexes Are Enriched in Active Chromatin Domains and Related Transcripts

The enrichment of G4s in active TADs and rG4s in TAD-derived transcripts has been evidenced using diverse mapping techniques over the past decade (Figure 1: the green and blue leaves in the shamrock scheme). Initial G4 mapping in genomic DNA relied on polymerase stop assays under G4-favoring versus G4-disfavoring conditions, followed by sequencing of primer extension products (G4-seq) [56]. Although the resulting maps were arguably more biologically relevant than sequence-based predictions, they only indicated G4 folding potential in a double-stranded DNA context and did not account for the impact of nucleosome occupancy or other cell-type-specific epigenetic marks. The development of G4-specific antibodies [57,58] has enabled G4 verification in a given epigenetic context using ChIP-seq [59] and CUT&Tag [60] assays. The former assays have been performed on human embryonic stem cells and downstream lineages [61], multiple cancer cell lines, normal fibroblasts [59], etc. The latter assays have been performed on mouse embryonic stem cells and downstream lineages [60]. Both types of assays have evidenced G4 colocalization with active chromatin marks, as well as prevalence in promoters and enhancers, especially in stem cells. Although a subset of G4s was lost during differentiation, colocalization with active chromatin marks remained genome-wide. Thus, the results were mostly consistent with each other and qualitatively agreed with G4-seq data. In contrast, transcriptomic studies raised some controversy.

First-generation high-throughput rG4 mapping methods relied on reverse transcriptase stop assays and sequencing of primer extension products (rG4-seq) [62]. Similar to G4-seq, rG4-seq data can be regarded as maps of potential rather than established structures. Although cell-line-specific RNA processing is intrinsically taken into account in rG4-seq experiments, other specific factors, such as the interplay between helicases and rG4-stabilizing proteins, are not recapitulated. The rG4-seq results reported for the transcriptome of human cancer cells (HeLa) [62] did not align well with classical in-cell dimethyl sulfate (DMS) footprinting [63], and this was attributed to the transient nature of rG4 folding. Despite their generally high thermodynamic stability [64], rG4s appear to be in a more complex equilibrium with other secondary structures than G4s due to RNA's enhanced conformational polymorphism [65]. A conclusive interpretation of rG4-seq awaits verification using modern DMS-based techniques [66,67], such as mutational profiling (MaP-seq) [68], which uses a specific reverse transcriptase to encode DMS modifications as mismatches [69]. These methods have already confirmed several SPAD-derived therapeutically relevant rG4s [70] but they have yet to be applied in transcriptome-wide studies.

In parallel to DMS-MaP-seq, another rG4 mapping technique called G4-RNA-specific precipitation and sequencing (G4RP-seq) [71] was developed to address the aforementioned RNA polymorphism challenge. G4RP-seq inherits from G4-ChIP-seq, but it also requires initial fixation of the transient rG4 state through cross-linking using a functionalized G4-specific small-molecule probe. This probe is eventually recognized by a corresponding antibody [72]. The application of G4RP-seq and its recent update, G4RP.v2 [73], to the transcriptome of MCF-7 cancer cells confirmed that rG4 folding correlates with transcript

G-richness and G4 folding in transcript-encoding DNA. Taken together with G4 mapping results, these findings support the presence of rG4-rich transcripts at active SPADs. However, the transient nature of these rG4s should be noted.

2.3. Transient G-Quadruplexes Are Likely Present near Speckles in G and S Phases

The relatively short lifespan of most (r)G4s hinders their quantification in dynamic processes. Additional limitations stem from the inability of commercially available antibodies, such as BG4 and its derivatives [58], to distinguish between RNA and DNA structures when used in combination with fluorescently labeled secondary antibodies for (r)G4 imaging. RNase treatment and the use of selective G4 and rG4-stabilizing small molecules (such as pyridostatin and its carboxy derivative, respectively) enable partial decoupling of the G4 and rG4 contributions [74]. Nevertheless, the variation in the rG4 fraction with the cell cycle has yet to be fully characterized.

The G4 fraction approaches zero in M phase, increases from G0 to G1, peaks in S phase, and declines in G2 phase [75]. S-phase-specific G4s must be transient because their persistence would impede replication forks [76]. These S-phase-specific G4s differ from G4s in replication origins, which may play a role in replication pre-initiation and origin licensing during the G1 phase [77]. Other G4s are enriched in promoters, enhancers, and untranslated regions (UTRs) [78]. They may be formed in active TADs throughout G-phases to fine-tune transcription [13]. Promoter G4 folding is predetermined by chromatin context and precedes transcription initiation [23], while UTR G4s can precede or follow transcription initiation and are prone to regulation by nascent transcripts through the formation of hybrid DNA/RNA structures, such as R-loops and G-loops [79].

Correlating the overall G4 dynamics with the dynamics of active TADs is challenging. Transcription-related G4s are overshadowed by replication-induced ones [58,75], while TADs simply transform into replication timing domains during the G1-to-S transition [80]. Active TADs form in early G1, achieve maximum insulation from inactive TADs by late G1, and demonstrate enhanced segregation in G2. SPAD dynamics is supposed to be a convolution of TAD and NS dynamics, essentially repeating TAD dynamics because NSs are more or less stable throughout interphase [81]. During M phase, NSs disassemble due to the hyperphosphorylation of their SR-rich core components by SR-specific kinases, Cdc2-like kinases, and casein kinase 2 [82–84]. Internal NS dynamics, i.e., reshaping of subdomains scaffolded by different SR-rich proteins [85,86] cannot be ruled out, though partial verification revealed no such reshaping [87].

In summary, promoter/enhancer G4s, as well as some UTR G4s, appear to be present within SPADs from the beginning of interphase. The spatiotemporal proximity of G4s and NSs is an important prerequisite for the hypothesis that G4s contribute to NS positioning or chromatin organization around NSs (the bottom scheme in Figure 1). Along with rG4s, these G4s are presumed to participate in NS-SPAD communication by recruiting speckle-associated proteins, as discussed in the following sections. Hereafter, we focus on the proposed causative relations illustrated by the dashed arrows in Figure 1, noting that they can be bidirectional. In particular, we emphasize the roles of prefolded G4s in activating transcription but also acknowledge that this activation facilitates G4 folding under certain conditions. Similarly, we emphasize the contributions of G4s and rG4s to the recruiting NS proteins, but we also note that these proteins can modulate G4 formation, at least in vitro. Thus, the proposed causative relations shape the core hypothesis, and its future refinements may contain dynamic feedback networks.

3. G-Quadruplex-Dependent Biocondensates at Speckle-Associated Active Chromatin

3.1. Transcriptional Biocondensates Form at Speckle-Associated Chromatin

The presence of long, multivalent enhancers (superenhancers) enables SPADs to accumulate TFs and chromatin remodelers to the critical concentration required for liquid–liquid phase separation [88]. This process can be visualized as the assembly of dynamic protein hubs (Figure 2a) on hyperacetylated promoters and enhancers that are decorated by the bromodomain-containing chromatin remodeler, BRD4. Prior to transcription initiation, the mediator complex connects BRD4 and accessory activators to the initially non-phosphorylated (inactive) Pol II and major TFs [89]. The mediator, BRD4, and several TFs, including those with zinc-finger domains, engage in transient multivalent interactions through their intrinsically disordered regions (IDRs). A combination of non-specific IDR contacts and specific interactions involving bromodomains, zinc finger domains, or other domains holds the biocondensate together [90].

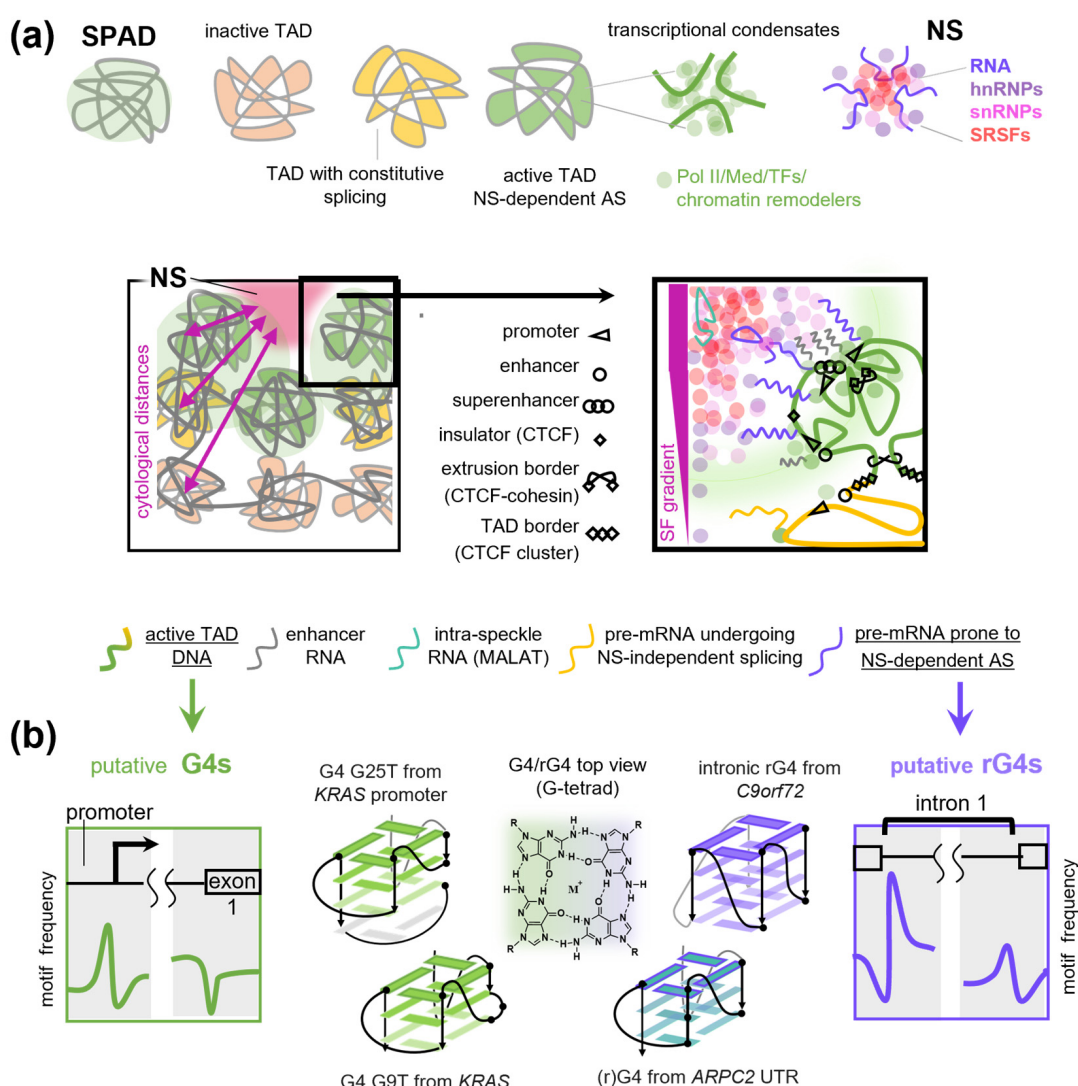


Figure 2. G-quadruplexes at the forefront of communication between speckles (NSs) and their associated chromatin domains (SPADs). **(a) (Left):** Schematic representation of the relative positions of NSs and active versus inactive topologically associating chromatin domains (TADs). **(Right):** Schematic representation of the interface between NSs and transcriptional condensates at SPADs. **(b)** Putative G4s (green) and rG4s (blue) at the NS-SPAD interface: the positions of the key motif clusters and examples of confirmed structures.

The nucleation step of the biocondensate assembly requires the simultaneous recruitment of multiple TFs. This requirement is only met at superenhancers, which can contact several promoters simultaneously [90,91]. Their juxtaposition is enabled by DNA loop extrusion by cohesin, with loop borders marked by the insulator protein CTCF [92]. Subsequent maturation of the condensates during transcription initiation may involve the incorporation of enhancer RNA that transiently interacts with the IDRs of the condensate proteins.

This view of superenhancer-governed transcription activation stems from a comparison of 3D chromatin maps revealed by chromosome conformation capture-based methods and Pol II/TF occupancy revealed by classical ChIP-seq and RNA sequencing. Recent updates to these methods, which supported the specific roles of superenhancers and GC-rich enhancer RNA, include high-sensitivity nascent transcript sequencing [93] and cap analysis of gene expression [54]. Super-resolution imaging of cells expressing biocondensate components with photoconvertible fluorescent tags confirmed the co-separation of these components [89]. Tracking clusters of tagged components after photoconversion or monitoring fluorescence recovery after photobleaching revealed the dynamics of the biocondensate interior, consistent with its presumed viscoelastic state [94]. Combined immunofluorescence and DNA fluorescence in situ hybridization demonstrated the positioning of the biocondensates at superenhancers [89,95]. Thus, several lines of evidence suggest that at least some SPADs serve as scaffolds for biocondensate formation. It should be noted that the key evidence was obtained in mouse embryonic stem cells [89,95]. However, the majority of transient protein clusters—presumed transcriptional biocondensates—persisted during differentiation and resembled those previously observed in differentiated human cells [96,97]. Therefore, the thesis about biocondensate formation likely applies to multiple cell lines and species.

3.2. *G-Quadruplexes Assist in Assembling the Transcriptional Condensates*

Although G4 enrichment in promoters and (super)enhancers is well-documented [6–10], the contribution of these G4s and rG4s to biocondensate formation by increasing the multivalency of promoter DNA and enhancer RNA has only recently been recognized [98,99]. This contribution is partly due to their semi-specific binding affinity toward chromatin remodelers/TFs with bromodomains or zinc-finger domains, including the transcriptional condensate scaffolding proteins BRD4 [100] and CTCF [101,102], as well as the ubiquitous GC-box-targeting TF SP1 [103].

CTCF recruitment to G4s has been confirmed to promote chromatin looping, as evidenced by the formation of new 3D chromatin contacts after a G4 insertion [104]. While not a general prerequisite for biocondensate formation, chromatin looping is crucial at (super)enhancers because it enables enhancer-promoter communication [105]. Whether BRD4 is crucial for G4-mediated enhancer-promoter communication remains to be verified in cells. Recent immunoprecipitation of enhancer quadruplexes revealed the potential for protein-independent, G4-mediated communication [9] through intermolecular folding of G-tracts from promoters and enhancers.

SP1 is not a well-established contributor to enhancer-promoter interactions. Although it facilitates the assembly of the preinitiation complex by interacting with the TATA-binding protein, its ability to recruit transcriptional repressors makes the outcome condition-dependent [106]. Furthermore, SP1 recruitment to canonical double-stranded GC-rich motifs versus G4 motifs is context-dependent. Nevertheless, SP1 is strongly associated with G4 motif-containing oncogene promoters and with superenhancers in several human cancer cell lines, where it activates transcription by promoting biocondensate formation [107]. Whether SP1 is recruited to promoter/superenhancer G4s or double stranded GC-rich motifs awaits clarification. Recently, SP1 was confirmed to colocalize with Pol II and the

mediator and to facilitate biocondensate formation in a G4-dependent manner at least in the case of Bcl3 oncogene promoter [108].

In addition to semi-specific interactions with zinc-finger domains, bromodomains and probably some other domains of TFs and chromatin remodelers, G4s are prone to non-specific contacts with protein IDRs through π - π and cation- π interactions, which are among the most frequent in biomolecular condensates [109].

In summary, G4s could assist in assembling transcriptional biocondensates in multiple ways, but further investigation is needed. The most direct evidence of G4-mediated intracellular phase separation reported thus far is probably the formation of speckle-like puncta following the addition of exogenous G4s. However, this evidence was obtained in artificial cells [98]. Additionally, although G4 enrichment in promoters and superenhancers is well-established for multiple cell lines [10,22], the fact that G4s precede transcription initiation has only been definitively confirmed for the K562 cell line [23], which had been used in most early G4 mapping experiments [78,110].

3.3. G-Quadruplexes Are at Play in Both Initiation- and Elongation-Related Condensates

Liquid–liquid phase separation at superenhancers suggests that the NS-SPAD interface is a two- condensate interface, or a three-condensate interface if transcription (pre)initiation condensates and elongation condensates are distinguished [111]. The assembly of the latter can be considered a maturation of the former. The key component present in both (pre)initiation and elongation condensates is Pol II [112]. Initiation requires phosphorylation of its C-terminal domain by the cyclin-dependent kinase subunit of TFIIH, while elongation requires further phosphorylation by the cyclin-dependent kinase subunit of P-TEFb [113]. Eventual hyperphosphorylation alters co-separation preferences of Pol II [114], which is consistent with changes in the biocondensate content.

According to the “scaffold-client” model [115], the mediator complex, BRD4, and inactive Pol II are scaffolds of (pre)initiation condensates [89], while active (hyperphosphorylated) Pol II and some accessory proteins (e.g., the histone chaperone FACT) are scaffolds of elongation condensates [116]. Most TFs appear to be “clients,” entering condensates due to their affinity for DNA and scaffolds [117]. The defining feature of a scaffold is the presence of long IDRs. These IDRs are typically low-complexity sequences showing specific amino acid biases and repeated patterns, such as the YSPTSPS heptad repeats in the C-terminal tail of Pol II.

DNA is not a canonical scaffold; rather, it has been referred to as a “surface” for condensate assembly [118]. However, it can be described using the sticker-spacer framework proposed for scaffolds. Within this framework, scaffolds are composed of “inert” flexible elements (spacers) interspersed with interaction-prone elements (stickers) [119]. Key SPAD stickers are canonical TF binding sites and G4s within (super)enhancers and promoters [23]. Whether G4s in UTRs and first introns are folded prior to transcription and can act as stickers in (pre)initiation condensates is an open question. There is strong evidence that clustered intronic and intergenic G4s contribute to drug resistance-related gene activation in ovarian cancer cells, where these clusters act similarly to superenhancers [120].

In general, however, clusters of quadruplex motifs downstream of the transcription start site (Figure 2b) [121] may correspond predominantly to rG4s and co-transcriptionally folded G4s in R-loops. Interestingly, the ability of coding strand G4s to stabilize R-loops [12] results in a positive feedback-like relationship. However, R-loop-related DNA damage remains low under normal conditions [79,122], because this positive feedback is balanced by the activity of helicases and NS-associated proteins, as reviewed below. Regarding transient rG4s in intronic motif clusters, one can expect them to support Pol II-scaffolded

biocondensates during transcription elongation, given the tendency of rG4s to promote liquid–liquid phase separation of IDR-containing proteins in artificial models [123].

3.4. Nuclear Speckles and Transcriptional Condensates Are Interdependent

The major scaffolds of NSs are SR-rich proteins. These include the nearly entirely disordered SRRM2 and SON [86,124] as well as the SRSFs [85]. The latter contain RNA recognition motifs (RRMs) in addition to SR motifs. Together with noncoding RNAs (primarily MALAT1), they constitute the inner (core) parts of NSs [125]. Other RNAs and small nuclear ribonucleoproteins (snRNPs) act as clients and are distributed between the inner and outer layers of NSs. The outer layer is enriched with heterogeneous ribonucleoproteins (hnRNPs) [126], which can be regarded as secondary scaffolds. Similar to SRSFs, they contain RRM and IDRs, but these IDRs are more diverse in terms of amino acid bias (e.g., acidic or aromatic IDRs, prion-like domains, and RGG motifs).

The multilayer organization of NSs was initially identified using canonical *in situ* hybridization, immunostaining, and immunoblotting methods [87], and was recently confirmed using TSA-based methods combined with mass spectrometry [127]. Outer layer proteins likely shuttle between NSs and SPADs. Many of these proteins affect transcription elongation (e.g., hnRNP A1 and hnRNP R [128]) and/or termination (e.g., hnRNP K [129]), as well as pre-mRNA polyadenylation [129], splicing [130] and trafficking [131]. Thus, hnRNPs act both as transcription regulators and as RNA processing factors, accounting for the communication between elongation condensates and NSs.

The interdependence of NSs and (pre)initiation condensates is supported by the association between AS and the activity of multiple transcriptional enhancers, at least in mice and humans, as summarized in the Visualizing Enhancer-AS Associations (VENAS) database [55]. This could be attributed to enhancer RNA, which either recruits NS components or enters NSs itself (Figure 2a). Another aspect of the NS-SPAD communication involves the ability of major transcription initiation factors, such as TAF2, and architectural proteins, such as CTCF, to enter NSs under certain conditions, which affects AS [132,133]. For instance, AS reprogramming has been reported in response to SRRM2 interactions with CTCF aggregates in senescent cells [133].

The propensity of TFs and chromatin remodelers to enter NSs has recently been linked to the presence of specific signaling motifs termed “speckle-targeting motifs” (STMs) [48]. Common STMs, identified by aligning the sequences of proteins with confirmed NS localization, are ~30-mer motifs with proline residues at defined positions. Future studies are needed to further elucidate the sequence requirements, and the set of NS-associated proteins may be refined or expanded. Nevertheless, the interdependence of NSs and SPAD biocondensates due to the exchange of the outer-layer proteins is firmly established. Below, we review evidence that many of these proteins are G4- and/or rG4-interactors, suggesting that (r)G4s contribute to NS-SPAD communication.

4. Interactions Between G-Quadruplexes and Speckle-Associated Proteins

4.1. G-Quadruplexes Recruit Speckle Proteins to Transcriptional Condensates

SPAD-associated components of transcriptional condensates, such as TAF2 and CTCF, are recruited to speckles through transient interactions of IDRs [132,133], which may include STMs. In contrast, the recruitment of NS components to transcriptional condensates involves specific interactions between NS components' RRM and RNA/DNA. Importantly, RRM hardly recognize B-form DNA and most unstructured single-stranded DNA, but they can recognize G4s and rG4s. This explains why some NS components, particularly hnRNPs, modulate transcription initiation at G4-harboring promoters [40]. Initially, such G4-hnRNP interactions were revealed by classical, non-high-throughput methods like

mass spectrometry-based analysis of mixtures obtained by pull-down assays using magnetic beads or peptide-based scaffolds decorated with G4s [41,42]. Subsequently, these interactions were confirmed by in situ proximity capture-based methods including high-throughput assays [43] like co-binding-mediated protein profiling (CMPP), a method that employs a functionalized G4-specific ligand to crosslink G4s with protein interactors [44]. Examples of hnRNP-binding G4s shown in Figure 2b are the *KRAS* oncogene promoter structures recognized by hnRNP A1 [45].

Most hnRNPs are best known as rG4 binders [46,47]. Interactions of hnRNPs with UTR rG4s, especially those that overlap with internal ribosome entry sites (IRES), affect translation [134], whereas interactions with rG4s at intron/exon boundaries affect spliceosome assembly and eventually AS [32]. Interestingly, SRSFs rarely interact with G4s in transcription (pre)initiation condensates but likely interact with rG4s in nascent transcripts [135]. This is probably because only the SRSF-poor outer layer of the speckles partially intermixes with transcription (pre)initiation condensates. In contrast, nascent transcripts can enter both layers, according to the “interfacial” splicing model (Figure 3a), hence the accessibility of rG4s to inner NS proteins.

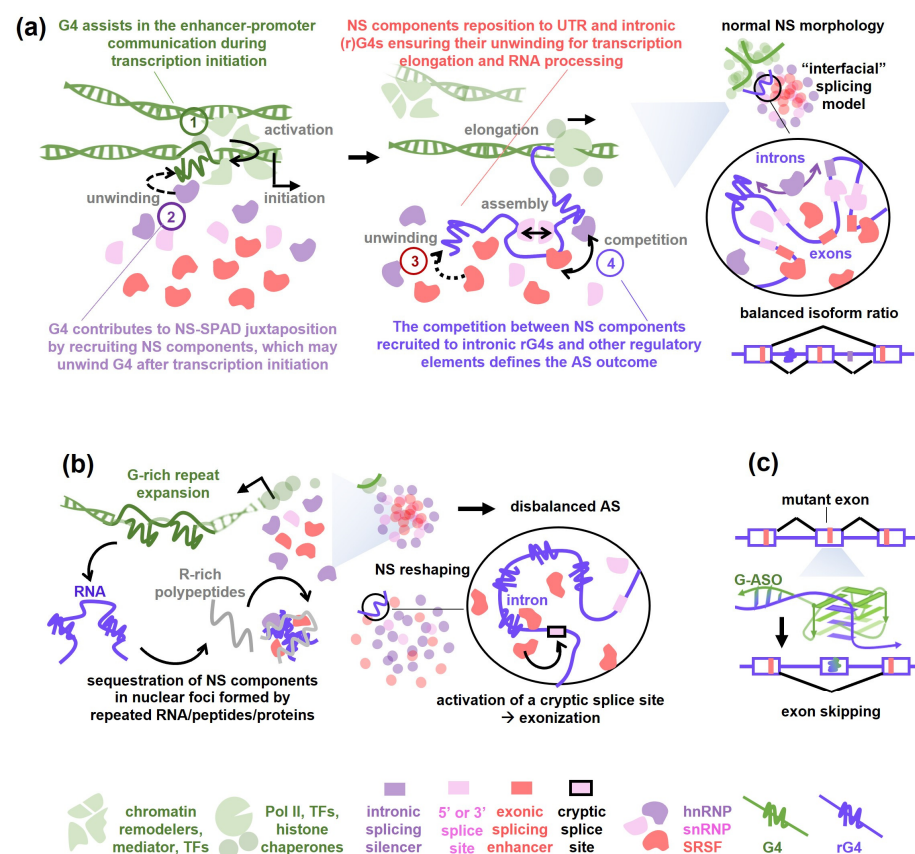


Figure 3. G-quadruplexes in co-transcriptional splicing: regulatory roles, consequences of expansion, and application in splicing reprogramming. (a) Normal contributions of (r)G4s from speckle-associated chromatin (SPAD) to transcription and co-transcriptional alternative splicing (AS) through interactions with the transcriptional machinery and the components of nuclear speckles (NS). Solid arrows indicate relations supported by multiple lines of evidence, including assays on cell cultures. Dotted and dashed arrows indicate hypothetical relations and those supported by in vitro assays in cell-free systems, respectively. (b) The impact of rG4 accumulation due to pathogenic expansion of G4-prone genomic repeats (e.g., G_4C_2 repeats in *C9orf72*) on NS morphology and AS. (c) Reprogramming AS by inducing an rG4 within the exonic splicing enhancer using a G-rich antisense oligonucleotide (G-ASO).

Examples of SRSF1-binding rG4s shown in Figure 2b are the IRES-regulating rG4 from 5'-UTR of the actin-related gene *ARPC2* [136] and the rG4 from the first intron of the *C9orf72* gene [135]. The latter also has affinity to hnRNP A1 [137]. Like G4-interacting proteins from NSs, most rG4-interacting proteins were first identified by pull-down assays [41] and then confirmed by high-throughput, proximity-capture-based methods such as eCLIP (enhanced cross-linking and immunoprecipitation) [138] or PLGPB (G-quadruplex-interacting proteins specific biotin-ligation procedure) [139].

4.2. Speckle Proteins Unwind G-Quadruplexes in Transcriptional Condensates

One underinvestigated issue regarding NS proteins is their (r)G4 helicase-like activity [40,135,140,141]. Unwinding of promoter/(super)enhancer G4s associated with active transcription seems counterintuitive, yet it may facilitate the transition to productive elongation. The details await clarification. The domain structures of the NS proteins, specifically the presence of RRM and IDR, allow for several possible explanations of the helicase-like activity [126,142]. First, RRMs may recognize G-tracts as unstructured single strands (linear motifs), shifting the equilibrium between G4 folding and unfolding towards the latter. Second, RRMs may initially recognize loop sequences; the subsequent engagement of G-tracts could then distort the G4 geometry. Third, together with adjacent IDRs, RRMs could recognize the secondary structure rather than the sequence and destabilize the G4 by disrupting its core with IDR side chains.

The first two hypotheses can be partially verified by comparing G4 motifs to the canonical linear binding motifs of NS proteins. Considering the common tandem RRM arrangement in SRSFs and hnRNPs, these canonical binding motifs can be bipartite. However, tandem RRMs tend to act in concert while adopting different orientations and showing different specificities, depending on the linker between their binding sites [143]. One example is TDP-43. It recognizes sequences that are different from the tandem arrangement of its isolated RRM1 and RRM2 binding motifs [144] and exhibits pronounced affinity for GT-rich single-stranded DNA (ssDNA), as well as parallel-stranded G4s. Another representative example is hnRNP A1. Its RRMs have high affinity for purine-rich sequences. In complex with the intronic splicing silencer of the *SMN2* transcript, they recognize the silencer elements GGAA and GAC [145]. These elements resemble, but do not fully match, the hnRNP A1 consensus binding motifs selected in vitro: 5'-UAGGGA/U-3' in RNA and 5'-TAGGGA/T-3' in DNA [146]. Some (r)G4s recognized by hnRNP A1 comply with the consensus motif; others do not, suggesting both sequence- and structure-specific interactions. Examples of the former include telomeric (r)G4s folded from TTAGG repeats [147]. Examples of the latter include G4s from the nuclease-hypersensitive element of the *KRAS* promoter, 32R (AG₃CG₂TGTG₃AAGAG₃AAGAG₅AG₂) [45], which contains multiple G-tracts and exists as a mixture of G4 conformers, modelled by the G-to-T substitution variants G9T and G25T (Figure 2b). Thus, the recognition of G-tracts or loop sequences as linear motifs can only explain unwinding of a subset of G4s, which indirectly argues for structure-specific binding and unwinding.

Conclusive evidence of structure-specific versus sequence-specific binding can be obtained by comparing the interactomes of G4s and their analogs with guanosine residues substituted with 7-deazaguanosine [148], 8-oxo-guanosine [149], or other derivatives that impair Hoogsteen bonding while largely preserving Watson-Crick bonding. Reported high-throughput assays of this kind lacked focus on NS proteins, and the borderline thermodynamic stability of the analyzed 2-tetrad G4s complicated the interpretation of the results [150]. Additional challenges arose from the conformational polymorphism of (r)G4s. Although most of them are parallel-stranded, i.e., adopt topologies of the same type [151], non-uniform G-tracts (excessive, extended, truncated, or interrupted) enhance

the topological diversity due to register shifts and various core defects, such as bulges or imperfect tetrads. This can be seen in the *KRAS* G4 variants (Figure 2b). With all limitations taken into account, comparative analyses of interactomic data obtained for (r)G4s and their non-G4 analogs with modified nucleobases [150] argue that at least some interactions are structure-specific.

Other recent studies suggest that arginine-rich RRM-neighboring IDRs play a role in both G4 recognition and unwinding [152–154]. These arginine-rich IDRs are SR motifs present in all SRSFs [135,152] and RG/RGG motifs present in multiple hnRNPs, including hnRNP A1 [154], hnRNP A2/B1 [155], FUS [156], TDP-43 [157], hnRNP K [158], hnRNP U [159], etc. The mechanisms have been partially disclosed based on binding assays with SR/RGG mutants [160] and characterization of the wild-type complexes using classical biophysical methods (e.g., NMR spectroscopy) and modern single-molecule techniques [155]. Arginine residues appear to be the principal residues involved in recognition. Cation- π interactions between IDR arginine guanidino groups and the outer (r)G-tetrad appear to promote (r)G4 unwinding by facilitating further electrostatic contacts with the internucleotide phosphate linkages of the (r)G4 backbone, altering the backbone packing.

In summary, multiple NS-associated proteins exhibit an (r)G4 helicase-like activity due to the combined effects of their RRMs and IDRs. G4 unwinding is likely followed by protein dissociation, consistent with the dynamic nature of the NS-SPAD communication. The consequences of the repositioning of NS-associated proteins to nascent transcripts, where rG4s are mostly clustered at intronic boundaries [121], are discussed in the following section.

5. Splicing-Related Consequences of G-Quadruplex Interactions with Speckle Proteins

5.1. G-Quadruplexes Act Alongside Canonical Splicing Enhancers and Silencers

The fact that both SRSFs [152,155] and hnRNPs [46,137,161] bind to rG4s suggests a potential competition between these factors at intronic boundaries (Figure 2b). Although cis-regulatory elements are commonly classified as intronic splicing silencers versus exonic splicing enhancers, the bound AS regulator rather than position alone defines the regulatory outcome. Thus, the clustering of rG4 motifs in introns does not automatically mean they repress splicing. Some cis-regulatory sites have comparable affinities for SRSFs and hnRNPs, and rG4 motifs appear to be among these sites [16]. Moreover, rG4s can exist in equilibrium with alternative secondary structures and unfolded RNA, which results in complex competition between hnRNPs and SRSFs for linear motifs and rG4s (Figure 3a).

The role of intronic rG4s in AS has been demonstrated and partially decoupled from that of linear regulatory sites in human neurons [15]. Enhanced skipping of G4-flanked exons, as evidenced by RNA-seq, was observed after treating neurons with potassium chloride. This treatment reduces membrane potential and triggers intracellular Ca^{2+} influx, supposedly affecting hnRNPs that are phosphorylated by Ca^{2+} -sensitive calmodulin-dependent kinases [162] and their partners or competitors. eCLIP assays revealed the accumulation of RGG-containing hnRNPs, including hnRNP K and hnRNP U at the boundaries of the potassium-sensitive differentially skipped exons [15]. Thus, the skipping could be attributed to the recruitment of splicing inhibitors by exonic rG4s. Incorporating representative differentially skipped exons and their G4-prone or mutant (G-rich non-G4) flanks into artificial minigenes enabled a direct comparison of AS outcomes and confirmed the importance of rG4 folding. This example illustrates the membrane potential-dependent rG4 contribution to AS regulation, which may be of particular importance in excitable cells.

In several immortalized non-cancer cell lines, analysis of RNA binding protein footprints suggests that major clusters of rG4 motifs recruit RGG-free hnRNP H and hnRNP F [51]. Unlike most family members, these hnRNPs promote exon inclusion. Like most

family members, they bind folded rG4s more efficiently than linear G-rich motifs. Consistently, mutations that disrupt intronic rG4 folding while maintaining linear G-rich motifs promoted skipping of G4-flanked exons, as evidenced by minigene reporter assays [51]. Similar exon skipping has been observed upon hnRNP H/F knockdown or cell treatment with hnRNP-displacing ligands [163].

It can be summarized that rG4s act alongside canonical intronic splicing enhancers and silencers, mostly in an hnRNP-mediated manner. The benefits of their unwinding by a subset of hnRNPs are likely linked to translation rather than AS [164]. The role of SRSFs is either underinvestigated or less pronounced, probably because key G4 clusters contact hnRNPs first, as suggested by intron positioning at the periphery of NSs in the “interfacial” splicing model (Figure 3a) [165]. Finally, it should be noted that although intron boundaries are the most common positions of rG4 motifs and motif clusters (Figure 2b), individual motifs and folded rG4s are also present in exons. The effects of such rG4s on AS are similarly context-dependent and defined by the proteins recruited [32].

5.2. Excessive G-Quadruplexes Alter Speckles and Disrupt Alternative Splicing

Transient rG4s contribute to the balanced AS by recruiting NS proteins without disrupting NS morphology. In contrast, excessive rG4s resulting from the expansion of G-rich microsatellites are associated with pronounced NS distortions [27]. Two relevant examples are GGC and G_4C_2 expansions.

GGC expansions occur in diverse genomic loci (UTRs/introns of at least six genes) and cause various pathologies, the most common of which are myopathy and neurological disorders [166,167]. Expansions within open reading frames lead to the accumulation of toxic polyglycine- or polyalanine-containing proteins, which often affect NSs and other biocondensates [167]. For instance, the polyalanine-containing polyadenylate-binding protein nuclear-1 forms toxic aggregates and disrupts NSs by extracting poly-A mRNA [168]. Alternatively, its aggregation may occur within NSs. GGC repeats can theoretically fold into G4s or rG4s [169], but these structures are rarely observed in genomic DNA, probably due to the insufficient GC skew and the destabilizing effects of CpG methylation. The most common structures in GGC repeat-containing RNA are noncanonical loops, which recruit and sequester non-NS proteins, such as the microRNA biogenesis regulator DGCR8 and its partners [170].

The expansion of G_4C_2 repeats in the *C9orf72* intron 1 is the most common genetic cause of several neurodegenerative diseases, including amyotrophic lateral sclerosis and frontotemporal dementia [171]. Similar to GGC expansion, the G_4C_2 expansion triggers pathology through a multi-pathway mechanism, involving both RNA repeats and the products of their translation [172]—poly-GA, poly-GR, poly-GP, poly-PR, and poly-PA—among other factors. The transcripts fold into rG4s, aggregate through multimerization [173], and promote aggregation of recruited proteins [49]. The relevance of the RNA repeats to pathology is supported by the detection of RNA foci [174], which accumulate and sequester RNA processing-related proteins, including NS proteins [49,50,175] (Figure 3b). This explains AS aberrations in patients with amyotrophic lateral sclerosis or frontotemporal dementia caused by repeat expansions [49].

The role of the poly-dipeptides [176] is supported by the fact that preventing translation of respective RNAs by editing the repeat-preceding CUG codon [177] alleviates most symptoms [178]. Among the dipeptide repeats, arginine-rich ones appear particularly toxic because they disrupt NSs and other intracellular biocondensates due to their high propensity for cation- π interactions [179]. The exact mechanism of their accumulation has long been puzzling. Recently, it was demonstrated that multiple rG4s can reshuffle NS layers and recruit SRSF1 to the *C9orf72* intron 1 (Figure 3b). This activates a cryptic splice

site (Figure 3b), resulting in the exonization of the expanded repeats [52]. This finding does not contradict the proposed role of the CUG codon as a target for genome editing [178]. However, it suggests that at least some dipeptide repeats are produced through the canonical translation of the extended exon 1. This is a rare but significant example of rG4-driven AS reprogramming in an SRSF-mediated manner.

5.3. Inducing G4 Formation Within Splicing Enhancers Enables Controlled Exon Skipping

Recently, substantial effort has been devoted to AS reprogramming for preventing the inclusion of exons that introduce premature stop codons [180]. Such stop codons may result from nonsense or frameshift mutations. Well-known examples include DMD mutations causing Duchenne muscular dystrophy through a loss of dystrophin function [181]. Skipping the damaged exon or the adjacent one can restore protein function if the remaining exons align in-frame to restore translation. For instance, skipping mutant exon 23 in the mouse dystrophin transcript and skipping single or multiple exons from the DMD-related deletion hotspot of the human dystrophin transcript (ex. 45–55) [182,183] produce partially functional proteins. This strategy has been implemented using heteroduplex-forming antisense oligonucleotides that target the exonic splicing enhancer, as well as some peptide-oligonucleotide conjugates [183,184].

To date, four heteroduplex-forming antisense oligonucleotides, all with a morpholino phosphoramidate backbone, have been approved for muscular dystrophy treatment in patients with respective exon-skipping-amendable mutations [185]. Limitations of these drugs include relatively narrow patient coverage (below 15% for each drug) and modest therapeutic efficiency. Due to the accelerated FDA approval and the lack of long-term comparisons, it is difficult to make conclusive remarks about the overall clinical benefits. However, dystrophin recovery induced by these drugs is low in most cases, suggesting inefficient exon skipping, probably because the antisense oligonucleotides cannot fully shield the splicing enhancer and/or displace the splicing-activating protein. In search of more reliable ways to distort splicing enhancers, an rG4-based splice switching strategy was developed [53] (Figure 3c).

In this strategy, the antisense oligonucleotide module is combined with a G-rich, rG4-inducing module. The latter is the key functional unit, and the former accounts for specificity. A similar double-module design was recently used to inhibit the translation of monoamine oxidase-encoding RNA. The thermodynamically unstable 2-tetrad unimolecular rG4 in its UTR served as a weak translation inhibitor, but it was rearranged into a more stable and potent 3-tetrad bimolecular rG4 by a G-rich oligonucleotide conjugated to an antisense module [164]. In the case of rG4-based dystrophin splice switching (Figure 3c), the complete four-tract G4 motif does not need to be present in the target sequence. A conjugate with a G-rich module comprising several G-tracts can be directed to a single G-tract located up to 70 nt from the antisense-targeted sequence [53]. A key limitation of rG4-inducing antisense oligonucleotides comprising multiple G-tracts is their propensity for multimerization. This problem can be mitigated by using interrupted G-tracts, which produce G4s with bulges.

The tested bulged G4-inducing antisense oligonucleotides were highly efficient (induced approximately 80% exon skipping) in cultured cells and moderately efficient (induced nearly 15% exon skipping) in a mouse model [53]. Direct comparisons between these oligonucleotides and conventional heteroduplex-forming antisense oligonucleotides [184] are needed to determine if G4-based splice switching therapy significantly improves protein function recovery. The prospects of its translation to clinics also depend on the drug delivery strategy, which in turn depends on the potential optimization of the oligonucleotide backbone structure. The 2'-OMe modification selected for rG4-inducing oligonucleotides

has disadvantages (e.g., reduced penetration through cellular membranes) as well as advantages (e.g., the wider commercial availability) over the morpholino modification. Variations in the backbone structure can profoundly affect the rG4-inducing potential.

In summary, inducing rG4 folding within splicing enhancers is an exciting, albeit under-explored, splice-switching strategy that will hopefully be further developed in upcoming studies. In view of the complex and context-dependent role of rG4s in NS-dependent splicing, elucidating the interactions of induced rG4s with NS-associated proteins—especially splicing-enhancing versus splicing-repressing hnRNPs—could advance progress in the field.

6. Conclusions

In this review, we summarized the potential contributions of (r)G4s to the regulation of transcription and splicing in higher vertebrates. A distinctive aspect of this regulation is the accumulation of regulatory machinery in interdependent biomolecular condensates, namely NSs and transcriptional condensates at SPADs. The latter appear to be rich in G4s and produce G-rich transcripts, which form rG4s. Studies in model systems suggest that (r)G4s facilitate the formation of transcriptional condensates and their communication with NSs. This communication involves incorporating the rG4-rich enhancer RNA and SPAD-derived transcripts into NSs during transcription initiation and elongation, respectively. It also involves the shuttling of some transcription factors or (r)G4-binding outer-layer NS proteins between transcriptional condensates and NSs during transcription initiation or elongation, respectively. Some of these shuttling proteins unwind G4s and rG4s, which may facilitate productive transcription elongation and the eventual translation of processed transcripts. Like many other cis-regulatory elements, G4s and rG4s recruit trans-regulators in a context-dependent manner. However, their key hotspots in promoters and introns, respectively, are generally associated with transcription activation and exon inclusion.

Thus, although (r)G4s are not crucial for transcription and splicing in general, they recruit regulators of these processes, especially dual regulators. This facilitates co-transcriptional alternative splicing (AS) and enables timely AS reprogramming in response to stimuli. While this offers evolutionary benefits, it also predisposes higher vertebrates to pathology if the intricate balance between (r)G4 folding and unwinding is disrupted. Excessive (r)G4s—for example, those formed by expanded G-rich repeats—can sequester NS proteins, alter NS architecture, and cause widespread splicing defects. This has been demonstrated in models of neurodegenerative diseases driven by G_4C_2 repeat expansion. In contrast, controlled (r)G4 folding within canonical exonic splicing enhancers enables splice switching, as demonstrated by the programming of exon skipping in the mouse dystrophin transcript in the Duchenne muscular dystrophy model. Further research on (r)G4s in the context of NS-SPAD communication and AS control could advance splice-switching strategies and related therapeutic approaches. Specifically, elucidating the sequence and structural determinants of NS protein-rG4 interactions is necessary to clarify their targetability. A deeper understanding of how transient versus persistent/excessive rG4s impact NSs and transcriptional condensates, as well as the development of new rG4-destabilizing molecules, may inspire new approaches to mitigating the effects of G-rich repeat expansion.

Author Contributions: Conceptualization, A.V.; writing—original draft preparation, P.R., J.K. and A.V.; writing—review and editing, P.R., J.K. and A.V. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by Russian Science Foundation (22-15-00129-P).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AS	Alternative splicing
ChIP-seq	Chromatin immunoprecipitation and sequencing
cMPP	co-binding-mediated protein profiling
CUT&Tag	Cleavage under targets and tagmentation
DMS	Dimethyl sulfate
MAP-seq	Mutational profiling and sequencing
eCLIP	Enhanced cross-linking and immunoprecipitation
G4	DNA G-quadruplex
G4RP	G4-RNA-specific precipitation
G4-seq	G4 mapping based on polymerase stop assays and sequencing
G-ASO	Antisense oligonucleotide with a G-rich module
hnRNP	Heterogeneous nuclear ribonucleoprotein
IDR	Intrinsically disordered region
NS	Nuclear speckle
PLGPB	G-quadruplex-interacting proteins specific biotin-ligation procedure
Pol II	RNA polymerase II
rG4	RNA G-quadruplex
RG4-seq	rG4 mapping based on reverse transcriptase stop assays and sequencing
RRM	RNA recognition motif
snRNP	Small nuclear ribonucleoprotein
SPAD	Speckle-associated domain
SRSF	Serine-arginine-rich splicing factor
STM	Speckle targeting motif
TAD	Topologically associated domain
TF	Transcription factor
TSA	Tyramide signal amplification
UTR	Untranslated region

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