

Artificially inserted G-quadruplex DNA secondary structures induce long-distance chromatin activation

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Abstract

Although the role of G-quadruplex (G4) DNA structures has been suggested in chromosomal looping this was not tested directly. Here, to test causal function, an array of G4s, or control sequence that does not form G4s, were inserted within chromatin in cells. *In vivo* G4 formation of the inserted G4 sequence array, and not the control sequence, was confirmed using G4-selective antibody. Compared to the control insert, we observed a remarkable increase in the number of 3D chromatin looping interactions from the inserted G4 array. This was evident within the immediate topologically associated domain (TAD) and throughout the genome. Locally, recruitment of enhancer histone marks and the transcriptional coactivator p300/Acetylated-p300 increased in the G4-array, but not in the control insertion. Resulting promoter-enhancer interactions and gene activation were clear up to 5 Mb away from the insertion site. Together, these show the causal role of G4s in enhancer function and long-range chromatin interactions. Mechanisms of 3D topology are primarily based on DNA-bound architectural proteins that induce/stabilize long-range interactions. Involvement of the underlying intrinsic DNA sequence/structure in 3D looping shown here therefore throws new light on how long-range chromosomal interactions might be induced or maintained.

eLife assessment

This **valuable** study demonstrates that genomic insertion of a G4-containing sequence can be sufficient to induce chromosome loops and alter gene expression. The evidence supporting the conclusions is **convincing**. Effects were shown by Hi-C as well as qPCR for chromatin modifications and expression, and the specificity of the effects was controlled by mutating the G4-containing sequence or treating with LNA probes to abolish G4 structure formation. The work will be of interest to researchers working on chromatin organization and gene regulation.

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Introduction

G-quadruplexes (G4s), non-canonical DNA secondary structures with quartets of Guanines bonded by Hoogsteen base pairing, are instrumental in regulating gene expression (Sengupta et al., 2020 [↗](#); Varshney et al., 2020 [↗](#)). G4s were primarily observed to be able to regulate gene expression when present around transcription start sites (TSSs) (Huppert and Balasubramanian, 2007 [↗](#); Rawal et al., 2006 [↗](#); Verma et al., 2008 [↗](#)). G4s can regulate gene expression by directly regulating recruitment of transcription factors and RNA polymerase or via alteration of DNA accessibility by modulating the epigenetic state of the gene promoters (Hussain et al., 2017 [↗](#); Kumar et al., 2011 [↗](#); Lago et al., 2021 [↗](#); Mukherjee et al., 2019 [↗](#); Saha et al., 2017 [↗](#); Sharma et al., 2021 [↗](#); Varshney et al., 2020 [↗](#)). Recent studies have implicated the role of G4s in long-distance gene regulation (Robinson et al., 2021 [↗](#)).

High-throughput chromosome conformation capture techniques reveal that specific regions of the human genome interact in three dimensions (3D) via chromatin looping and formation of topologically associated domains (TADs) (Bonev Boyan and Cavalli Giacomo, 2016 [↗](#); Denker and De Laat, 2016 [↗](#); Roy et al., 2018 [↗](#)). Interestingly, recent computational studies observed enrichment of G4s in TAD boundaries along with higher enrichment of architectural proteins like CTCF and cohesin (Hou et al., 2019 [↗](#)). Further, multiple studies noted the presence of G4s correlated with enhancer histone marks like H3K27Ac and H3K4Me1, and predominantly open chromatin regions (Calo and Wysocka, 2013 [↗](#); Hou et al., 2021 [↗](#); Shlyueva et al., 2014 [↗](#)).

Although these studies implicate the role of G4s in long-range interactions and/or enhancer function, this was not directly tested. Here we asked if G4 structures might directly alter 3D chromatin, and affect long-range interactions including the epigenetic state of chromatin. To address this, we inserted an array of G4s into an isolated locus devoid of G4-forming sequences using CRISPR-Cas9 genome editing. To evaluate the specific function of G4s, a similar sequence of identical length but devoid of G4-forming capability was introduced. Using these pair of cell lines, we observed insertion of G4s specifically led to the recruitment of enhancer histone marks and increased expression of genes in a 10 Mb window. 3C and Hi-C results showed induced long-range interactions throughout the genome affecting topologically associated domains (TADs) that were specifically due to the incorporated G4s, and not found in case of the control insertion.

Results

Insertion of an array of G4s in an isolated locus

First, we sought to insert an array of G4s in a relatively isolated locus. We looked into Hi-C data from Rao et al., 2014 [↗](#) and identified a region that was markedly isolated with little or no interaction with its surrounding regions (as shown by snapshots of Hi-C interaction matrices obtained using the 3D genome browser (Wang et al., 2018 [↗](#)) in **Figure S1** [↗](#)). In addition, this region was devoid of any G4s in the vicinity (no G4 forming motifs in a ± 2.5 kb window). Thereafter we artificially inserted an array of G4 forming sequences (275 bp long) at this region near the 79 millionth position of chromosome 12 (79M in following text, chr12:79872423-79872424, hg19 genome assembly) using CRISPR-Cas9 genome editing (**Figure 1A** [↗](#), **S2** [↗](#)). To study specific effects due to G4s, if any, a control sequence of identical length was inserted in HEK293T cells at the same locus where specific G/Cs necessary for G4 formation were substituted so that G4s are not formed by this sequence (G4-mutated control, **Figure 1A** [↗](#), **S2** [↗](#)); we also ensured that the GC content was minimally affected by the substitutions (72.4% from 76.73%). Homozygous insertion was confirmed by PCR using primers adjacent to the insertion site followed by Sanger sequencing

(**Figure 1B** [↗](#), **S3** [↗](#)). The array of G4-forming sequences used for insertion was previously reported to form stable G4s in human cells ([Lim et al., 2010](#) [↗](#); [Monsen et al., 2020](#) [↗](#); [Palumbo et al., 2009](#) [↗](#); [Sharma et al., 2021](#) [↗](#)).

Chromatin epigenetic landscape upon insertion of G4s

To understand how the formation of G4s altered the local chromatin, chromatin immunoprecipitation (ChIP) of different chromatin-modifying histone marks was done followed by qRT-PCR using primers spanning the inserted locus. PCR primers were designed such that none of the primers bind to any site of G/C alteration in the mutated control insert; either the forward/reverse primer is from the adjacent region for specificity; covers adjacent regions for studying any effects on chromatin; and, PCRs optimized keeping in mind the repeats within the inserted sequence. Given these, primer pairs R1-R4 were chosen for further work following optimizations (**Figure 2** [↗](#), top panel). For G4 formation within cells by the G4-array insert sequence we used the reported G4 antibody BG4 ([Hänsel-Hertsch et al., 2016](#) [↗](#)). Using primer pairs R2, covering >100 bases of the inserted G4-array, or the G4-mutated control, BG4 ChIP followed by qPCR was performed. Significant BG4 binding was clear in the G4-array insert, and not in the G4-mutated insert, demonstrating formation of G4s by the inserted G4-array (**Figure S4** [↗](#)).

We observed significant increase in H3K4Me1 and H3K27Ac enhancer marks in the G4-array when compared to the G4-mutated control (**Figures 2A, B** [↗](#)). However, there was no G4-specific change in the presence of chromatin compaction marks, H3K27Me3 and H3K9Me3, or the promoter activation mark H3K4Me3 (**Figures 2C, D, E** [↗](#)). The G4-dependent recruitment of H3K4Me1 (associated with enhancers ([Heintzman et al., 2009](#) [↗](#), [2007](#) [↗](#))) and H3K27Ac (associated with active enhancers and promoters ([Creyghton, 2010](#) [↗](#); [Heintzman et al., 2009](#) [↗](#), [2007](#) [↗](#))) indicated enhancer-like characteristics of the inserted G4s.

Enhancer-like features emerged upon insertion of G4s

We next asked how the insertion of the G4-array influenced the expression of surrounding genes. To understand the distance-dependent gene regulatory impacts of the inserted G4-array, the mRNA expression of the nearest three genes and then some arbitrarily chosen genes further away up to 5 megabases (Mb) both up and downstream from the insertion site was quantified. Notably, the expression of four of the tested genes (*PAWR*, *PPP1R12A*, *NAV3*, and *SLC6A15*) increased in the G4-array insert compared to the mutated insert control cells (**Figure 3A** [↗](#)). Based on this enhanced expression, we further tested and observed a somewhat concomitant increase in the recruitment of Ser5 phosphorylated RNA Pol II in the surrounding gene promoters (**Figure 3B** [↗](#)). Next, we tested if chromosomal looping interactions between the insertion site and the gene promoters were involved in these long-distance effects by using chromosome conformation capture (3C). The 3C assay between the insertion locus and the gene promoters could only be performed till the *NAV3* promoter 1.6 Mb away. Beyond this distance, there was not any significantly detectable PCR amplification of 3C interaction products. The 3C assays revealed that there was a G4-dependent increase in chromosomal looping interactions of the insertion locus with the gene promoters (**Figure 3C** [↗](#)). These results suggested that the inserted G4-array sequence was acting like an enhancer element.

To understand the mechanism behind the enhancer-like property of the inserted G4-array we analyzed the recruitment of transcriptional coactivator p300 ([Kalkhoven, 2004](#) [↗](#)). There was a relatively modest increase in the recruitment of p300, and a more substantial increase in the recruitment of the more functionally active acetylated p300/CBP, was seen within the G4-array when compared against the mutated control (**Figures 3D, E** [↗](#)). Together, these results supported the enhancer-like function of the inserted G4-array.

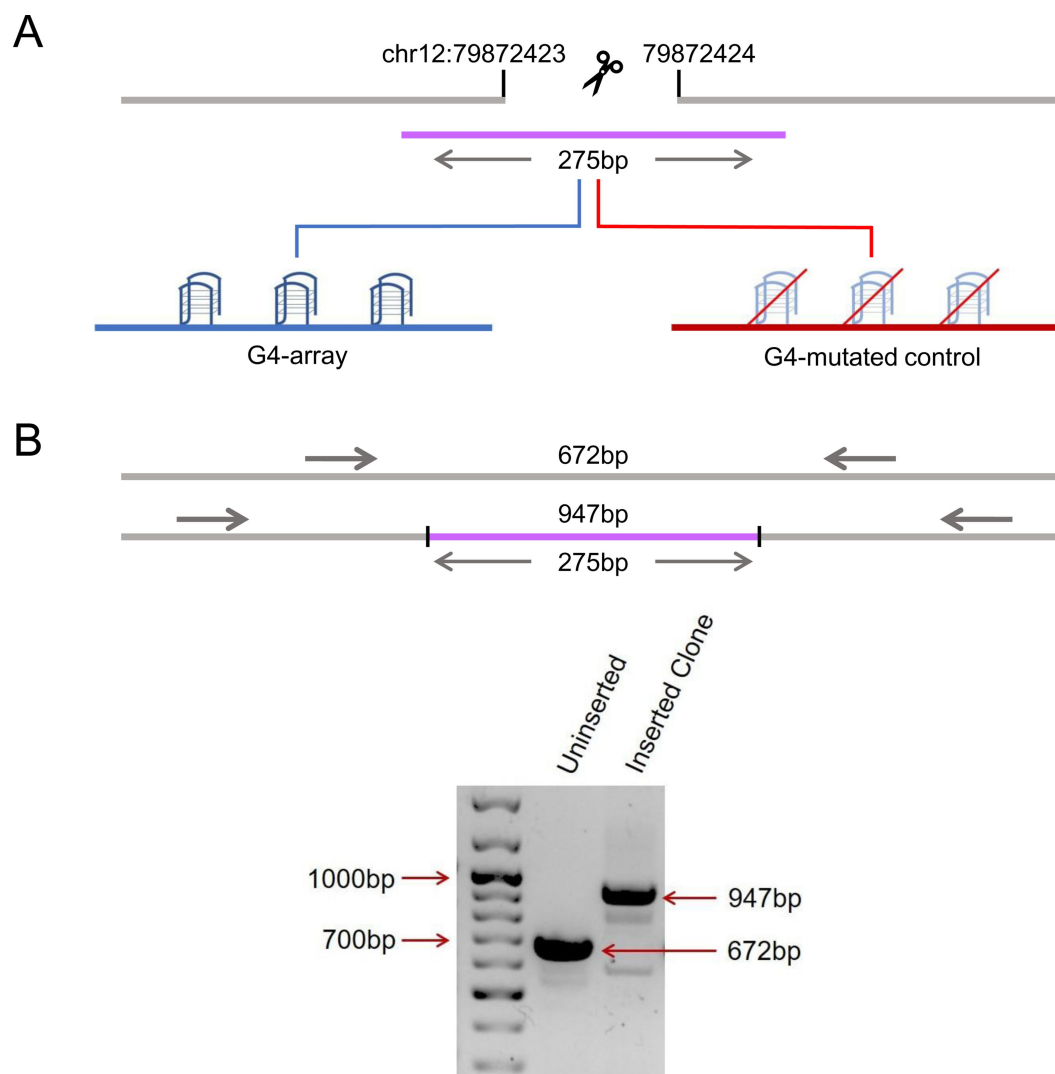


Figure 1

Insertion of an array of G4s in an isolated locus.

(A) Schematic showing the insertion of the G4-array and the G4-mutated control at chr12:79,872,423-79,872,424 (hg19). (B) PCR of the insertion locus showing the successful insertion of the 275bp long insert sequence.

Figure S1

An isolated locus was chosen for insertion.

(A) 3D Genome Browser (Wang et al., 2018) snapshots showing the Hi-C interaction matrices from 4 cell lines (NHEK, IMR90, HUVEC and HMEC) of a section of chromosome 12 with chr12:79,870,000-79,875,000 (hg19) locus in the middle (marked by arrows); the chr12:79,870,000-79,875,000 locus has very low interaction with its surrounding loci, indicated by the lack of red dots or very faint red dots.

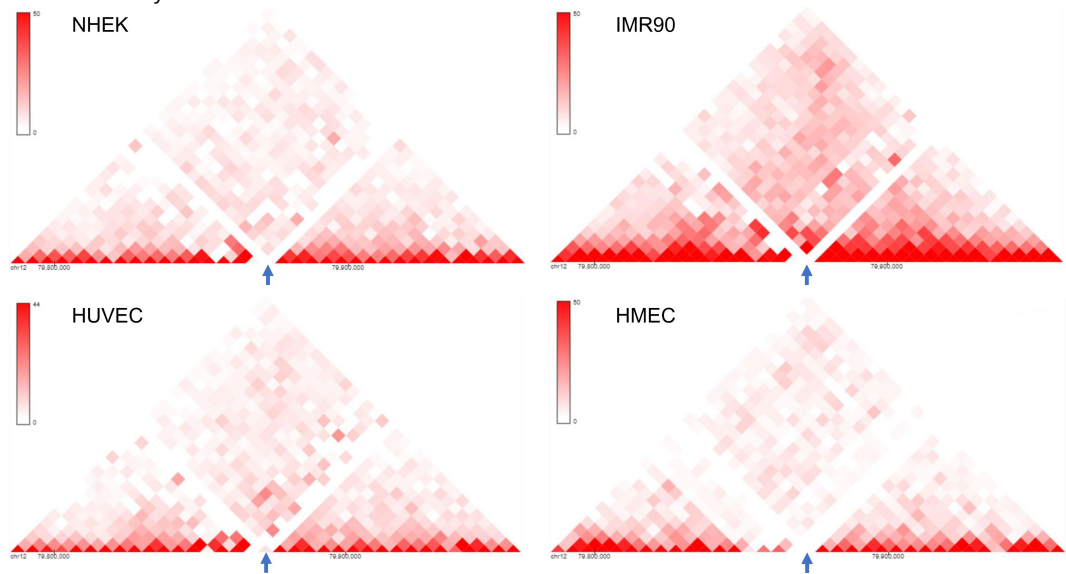


Figure S2

Insert sequences.

Sequences of the G4 array and the G4-mutated array that were inserted; three or more runs of G/Cs (stem of a potential G4) are shown in bold and the G/Cs (marked in blue) that were changed to T/As (marked in red) in the G4-mutated array are marked.

G4-array	G4-mutated control
CCAGGCC GGG CT CCC AGTGGATT CGCGGG CA	CCAGGCC GGG CT CCC AGTGGATT CGCGTG CA
CAGAC GGG CT CCC AGGACCGCGCTT CCC ACGTGGC	CAGAC GGG CT CCC AGGACCGCGCTT CCC ACGTGGC
GGAG GGG ACT GGGG ACCC GGG CAC CC GTCTCT	GGAG GGG ACT GGGG ACCC GGG CAC AC GTCTCT
GGG CTCTACCTTCCAGCTCCGCCTCCTCCG	GGG CTCTACCTTCCAGCTCCGCCTCCTCCG
CGCGGACCCCGCCCGT CCC GAC CCCTCC	CGCGGACCCCGCCCGT CCC GAC ACCTCACG TG
GGG TC CCCGG CCAGCC CCCT CCGGG CCCT	TC ACCGG CA AGCCACCT CCGGG CCCT CCCA
CCC AGC CCCTCC CTTCTTTCCGCGG CCCC	GC ACCTCCAC TTCTTTCCGCGG CCCC CCCC
GGG CTCTCTCGCGGCGGAGTTTCAGGCAG	TCTCTCGCGGCGGAGTTTCAGGCAGCGCT
CGCTGCGTCTGCTGCGCACGT GGGA AGCC	GCGTCTGCTGCGCACGT GGGA AGCC

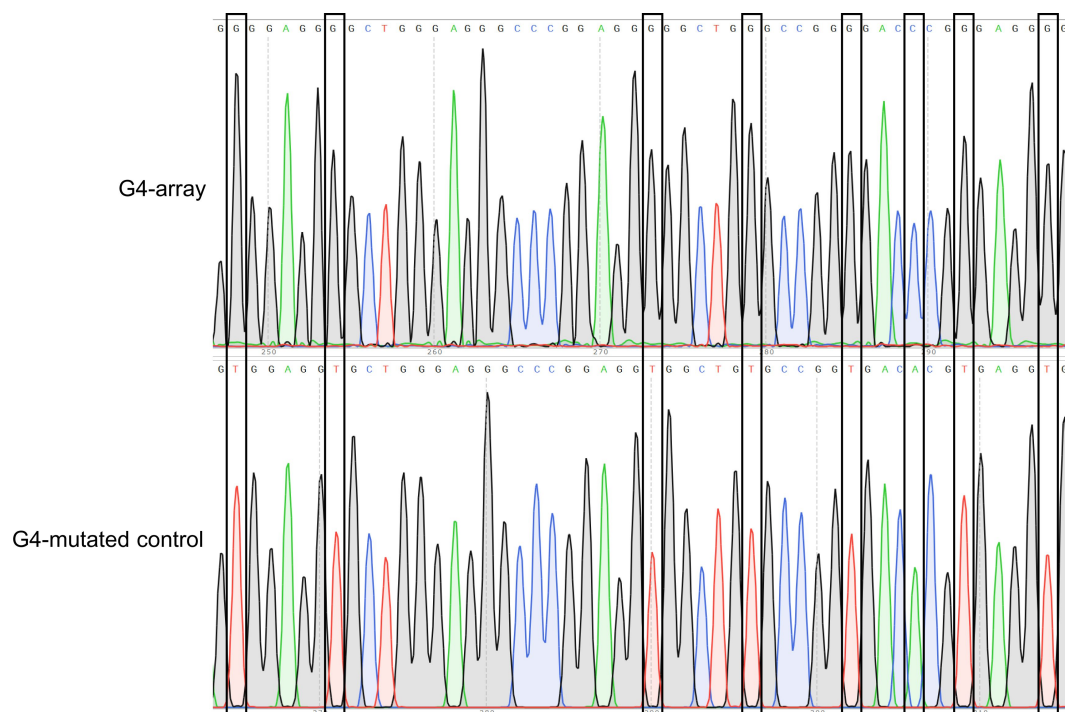


Figure S3

Insertion confirmed by Sanger sequencing.

Representative snapshots of the Sanger sequencing chromatogram of the reverse strand of the insertion locus PCR product showing some of the specific G/C to T/A substitutions in the insert.

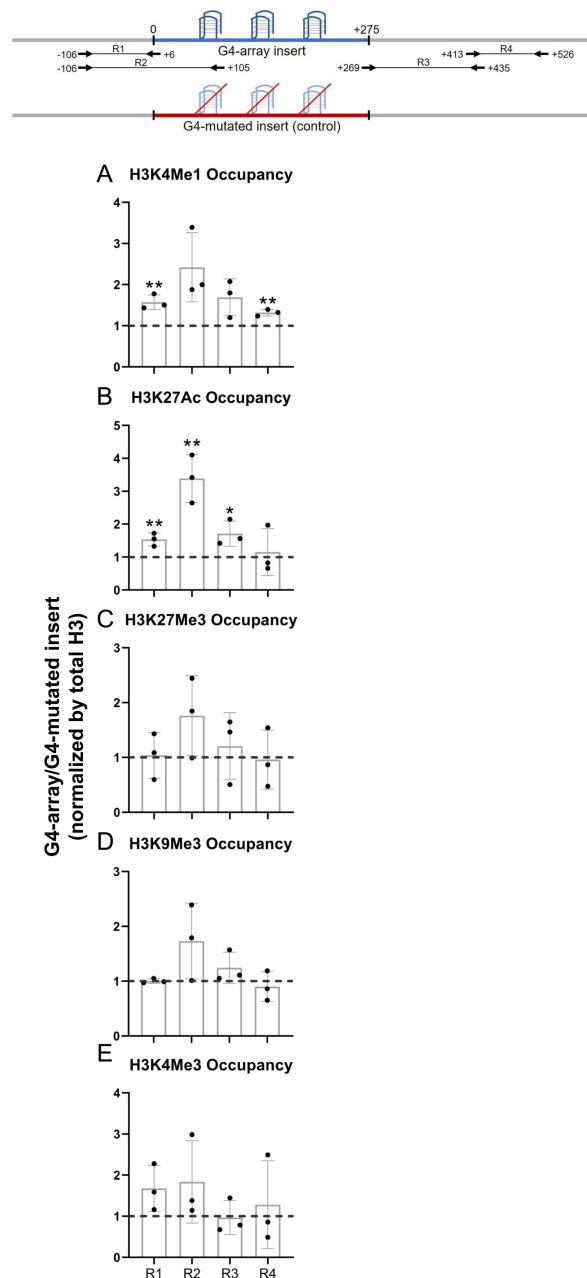


Figure 2

Changes in chromatin upon G4-array insertion.

The top panel shows the positions of the PCR amplicons used in the Histone ChIP experiments. Changes in chromatin-modifying histone modifications in the insert region represented by calculating the ratio of occupancy of different histone marks in the G4-array insert cells over the G4-mutated insert (control) cells- enhancer mark, H3K4Me1 (A); active enhancer/promoter mark, H3K27Ac (B); facultative repressor mark, H3K27Me3 (C); constitutive repressor mark, H3K9Me3 (D) and active promoter mark, H3K4Me3 (E). Mean $\pm$ SD (n=3); unpaired, two-tailed t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

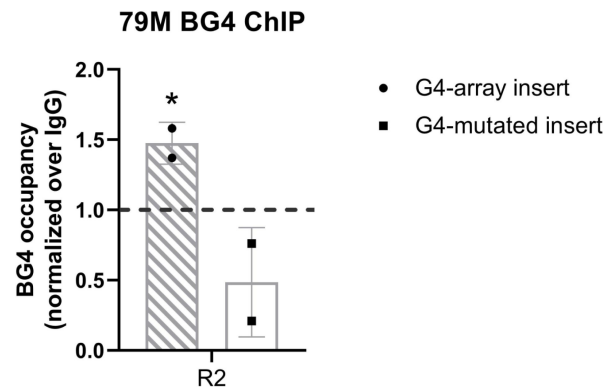


Figure S4

G4 formation analyzed by BG4 ChIP.

BG4 antibody enrichment at the 79M insertion locus in the G4-array and the G4-mutated (control) insert cells. Mean $\pm$ SD (n=2); unpaired, two-tailed t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Primer R2, covers >100 bp of the respective inserted regions, as shown in [Figure 2](#) top panel.

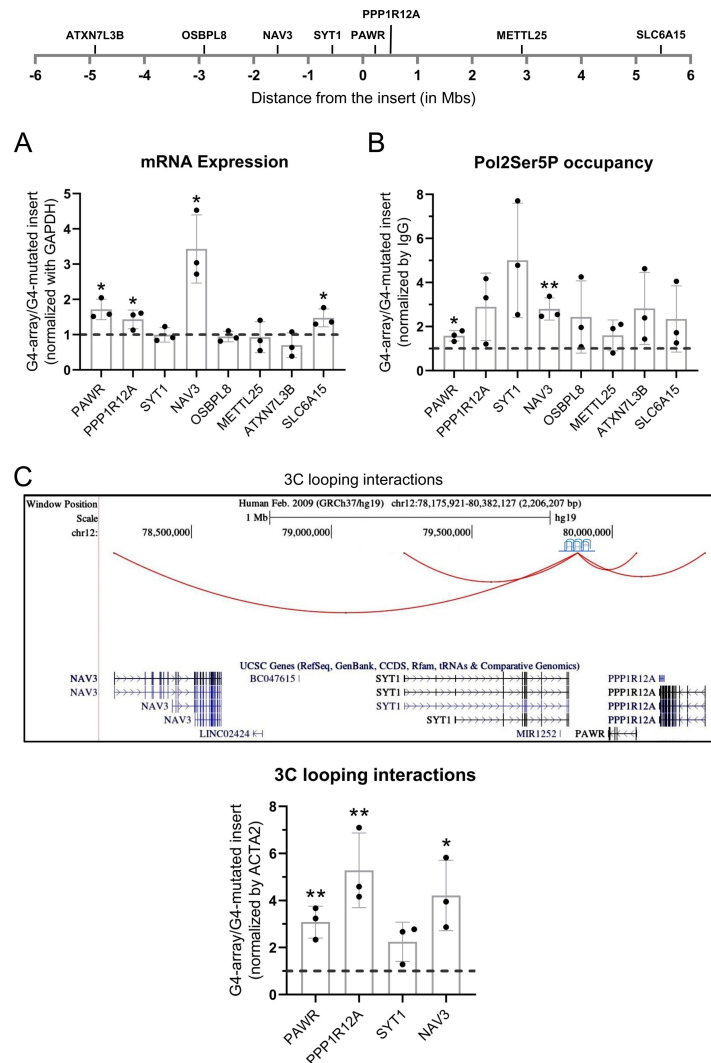


Figure 3

Insertion of the G4-array led to enhancer function.

(A) Long-range G4-dependent changes in mRNA expression are represented by calculating the ratio of expression of surrounding genes in the G4-array insert cells over the G4-mutated insert (control) cells. Top panel shows the positions of the gene promoters with respect to the insertion site. (B) Ratio of Pol2 Phospho-Ser5 Occupancy at the promoters of the surrounding genes in the G4-array insert cells over the G4-mutated insert (control) cells. (C) A UCSC genome browser snapshot showing the 3C looping interactions between the insertion and the surrounding gene promoters. Fold change in 3C looping interactions between the insertion and the surrounding gene promoters in the G4-array insert cells over the G4-mutated insert (control) cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Top panel shows the positions of the PCR amplicons used in the ChIP experiments. The ratio of occupancy of p300 (D) and Ac p300/CBP (E) in the G4-array insert cells over the G4-mutated insert (control) cells. Mean $\pm$ SD ($n=3$); unpaired, two-tailed t -test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

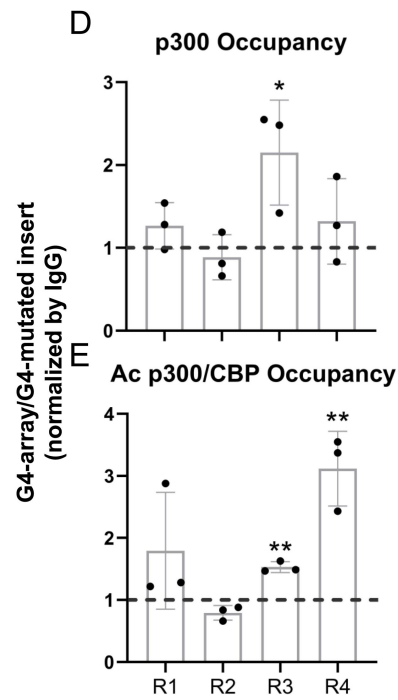
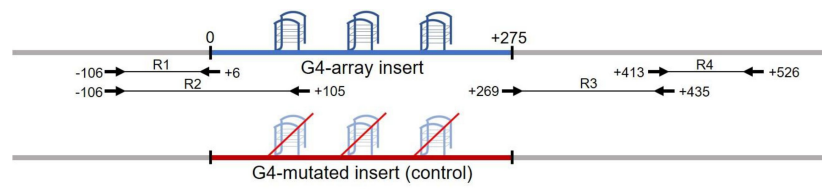


Figure 3 (continued)

LNA-mediated disruption of the inserted G4s reverses enhancer phenotype

To further establish that the enhancer effects upon the G4 array insertion are due to the formation of G4s, we wanted to see if some of the effects observed could be reversed upon disrupting the inserted structures. Specific Locked Nucleic Acid (LNA) probes were designed to target and disrupt the G4 using a similar approach as shown by others (Cadoni et al., 2021 [↗](#); Chowdhury et al., 2022 [↗](#); Kumar et al., 2008 [↗](#)). Three probes were designed with stretches of mostly cytosines (Cs) as LNAs which would hybridize with stretches of guanines (Gs) in the G4-array insert important for the structure formation (Figure 4A [↗](#); see Methods). We observed that there was a significant decrease in the expression of *PPP1R12A* and *NAV3*, two of the genes initially observed to have G4-dependent enhanced expression (Figure 3A [↗](#)), when the G4 array inserted cells were treated with the G4 targeting LNAs (Figure 4B [↗](#)). As expected, although modest, a decrease in the H3K4Me1 and H3K27Ac enhancer histone modifications was evident within the insert upon the LNAs treatment (Figures 4C, D [↗](#)). As a control experiment, we next tested whether the LNA probes affected surrounding gene expression in the G4-mutated insert cells. Changes in the expression of the genes were not significant across replicates in case of G4-mutated insert cells (Figure S5 [↗](#)). Together these confirmed the decrease in the expression of *PPP1R12A* and *NAV3* in the G4-array insert upon LNA treatment was likely specific to G4 disruption. These indicate that the disruption of the inserted G4s can reverse the enhancer functions observed upon G4 insertion, further supporting the role of the G4 structure in enhancer functions.

Domain-wide increase in looping interactions by G4s

For in-depth analysis of the long-range changes in chromatin architecture upon G4 insertion, we performed genome-wide interaction by Hi-C. First, we compared all the Hi-C contacts originating within a ± 10 kb window comprising the G4-array insert, or the G4-mutated control insert. Compared to the mutated control, the G4-inserted locus had more than twice as many genome-wide Hi-C interactions (6390 vs 3133) (Figures 5A, B [↗](#), Supplementary Table 1 [↗](#)). To rule out the possibility of artifacts due to the insertion we independently analyzed Hi-C data in HEK293T cells reported earlier (taken from GSE44267, Zuin et al., 2014 [↗](#)). After normalizing for sequencing depth, the number of Hi-C contacts from the same window in HEK293T was relatively similar to the G4-mutated insert control (3968 and 3133 respectively, Figure 5C [↗](#), Supplementary Table 1 [↗](#)). Together, these showed that a significant number of new long-range interactions were induced throughout the genome due to the inserted G4s, but not from the inserted control sequence.

For closer analysis, we focused on intrachromosomal Hi-C interaction matrices of the G4-array insert, or the mutated control insert. This was centered on the insertion locus on chromosome 12 (chr12:7,80,72,423-8,16,72,423; insertion site marked with arrows in Figures 6A, B [↗](#)). The number of Hi-C interactions in the G4-array insert was clearly enriched compared to the G4-mutated insert control, as expected from the global Hi-C contacts noted above. We noted that while the interactions from the G4-array insert were significantly more, the insertion *per se* did not affect the overall domain architecture, which was largely similar between G4 or G4-mutated inserts as clear from Figures 6A [↗](#) and B [↗](#). Further, we asked if the domain architecture was retained from that seen in HEK293T cells (with no insertion): Comparison using reported HiC data for the same region from HEK293T cells showed this to be the case confirming that the chromatin domain architecture remained relatively unchanged on introducing the G-array or G4-mutated regions (Figure S6 [↗](#)).

To evaluate the effect of G4s in more detail, we plotted a Hi-C heatmap to show the enhanced or reduced (differential) contacts in the G4-array insert compared to the G4-mutated insert control cells (Figure 6C [↗](#); relatively enriched/reduced contacts in the G4-array insert w.r.t. the G4-

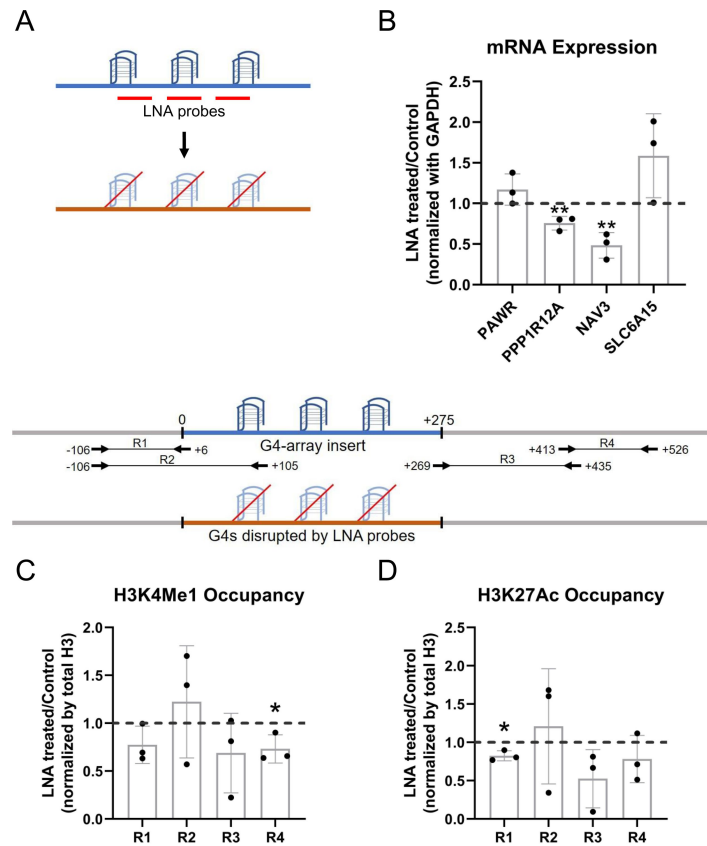


Figure 4

LNA-mediated disruption of the inserted G4s reverses enhancer phenotype.

(A) Schematic showing the inserted G4 structures disrupted using LNA probes (details in methods). (B) Effects of LNA treatment in the G4-array insert cells on the expression of surrounding genes which showed enhanced expression when compared against the G4-mutated insert (control) cells in [Figure 3A](#) – represented by the ratio of expression of surrounding genes in the LNA-treated over the vehicle-treated (control) cells. Effects of LNA treatment in the G4-array insert cells on the levels of H3K4Me1 (C) and H3K27Ac (D) histone modifications at the insert locus represented by the ratio of occupancy of the histone marks in the LNA-treated over the vehicle-treated (control) cells. The top panel shows the positions of the PCR amplicons used in the Histone ChIP experiments. Mean $\pm$ SD (n=3); unpaired, two-tailed t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Figure S5

Effects of LNA treatment in the G4-mutated insert (control) cells

on the expression of surrounding genes represented by the ratio of expression of surrounding genes in the LNA-treated over the vehicle-treated (control) cells. Mean $\pm$ SD (n=3); unpaired, two-tailed t-test showed differences were insignificant.

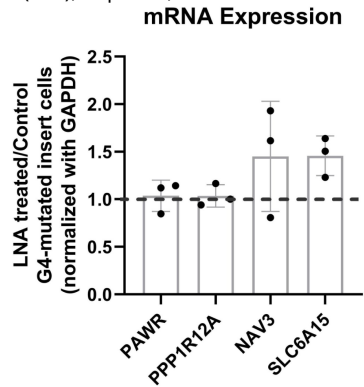
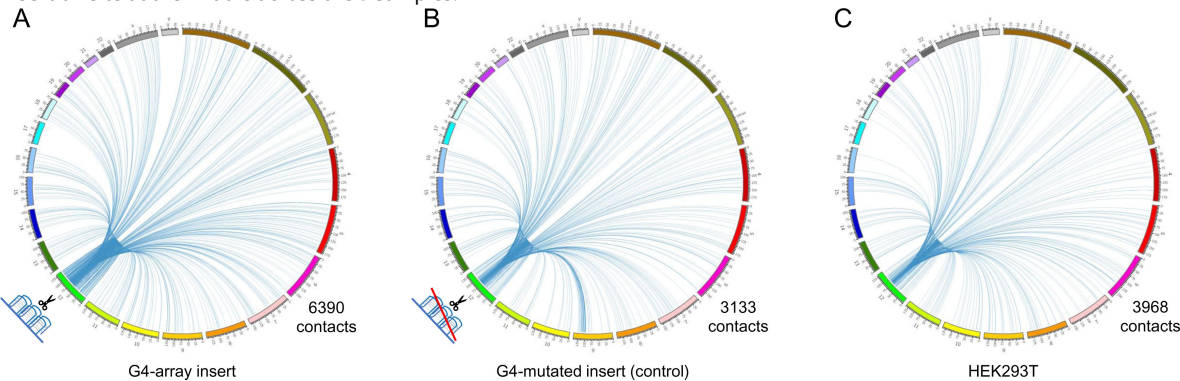


Figure 5

Insertion of the G4-array increased Hi-C interactions.

Circos plots showing raw Hi-C contacts across the genome originating from a ± 10 kb window with the insertion site at the middle across 3 samples- (A) G4-array insert cells, (B) G4-mutated insert (control) cells and (C) HEK293T control cells (taken from GSE44267). (D) Table showing the number of genome-wide raw Hi-C contacts and normalized contacts (normalized against the total raw Hi-C contacts to normalize for the sequencing depth) originating from the ± 10 kb window with the insertion site at the middle across the 3 samples.



D

	G4-array insert	G4-mutated insert (control)	HEK293T
Total Hi-C contacts	649,455,101	692,798,603	366,957,555
Normalized contacts from insertion site	6390	3133	3968

	G4-array insert	G4-mutated insert (control)	HEK293T
Total Hi-C contacts	649,455,101	692,798,603	366,957,555
Normalized contacts from insertion site	6390	3133	3968
Actual contacts from insertion site (± 10 kb, i.e. 20kb window)	5990	3133	2102
Mean Hi-C contacts across 10,000 random 20kb windows across the genome	8910	9490	5021
Standard deviation of Hi-C contacts across 10,000 random 20kb windows across the genome	4078	4716	2806
Z-score for the actual contacts from the insertion site	-0.71	-1.34	-1.04

Supplementary Table 1

Comparative analysis of the G4-dependent increase in Hi-C interactions upon insertion.

Table showing the number of genome-wide raw Hi-C contacts, actual number of contacts originating from the ± 10 kb window with the insertion site at the middle and the comparative analysis (mean, standard deviation and z-score) of these number of contacts with contacts across 10,000 random 20kb windows across the genome across the 3 samples.

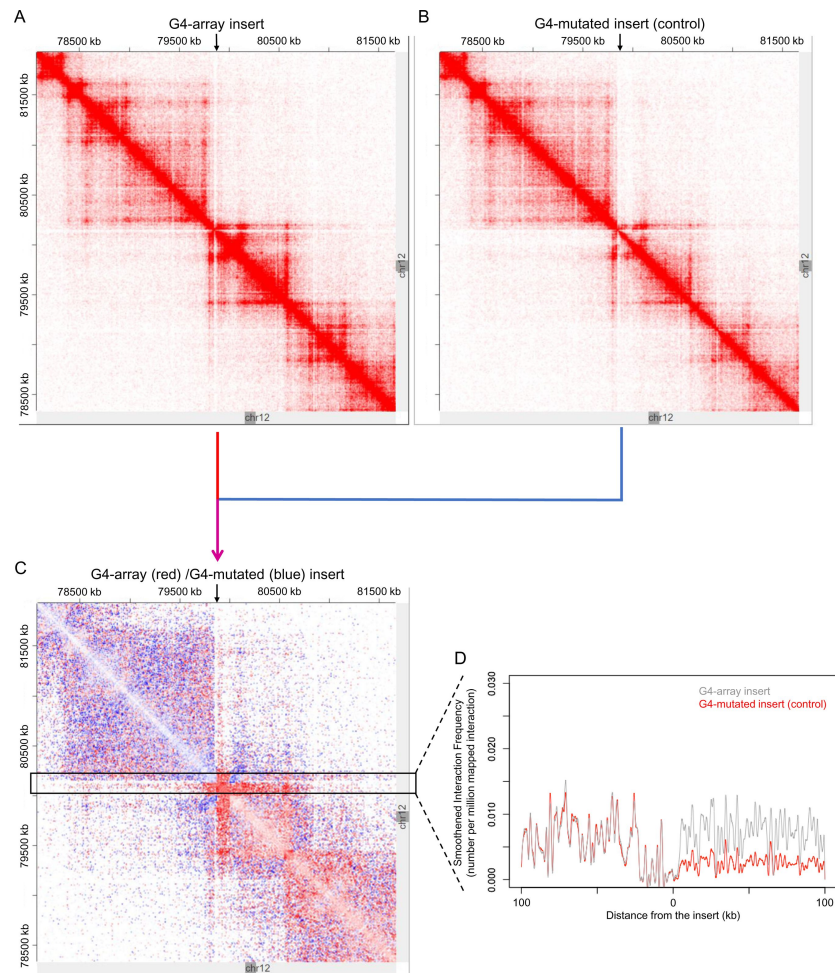


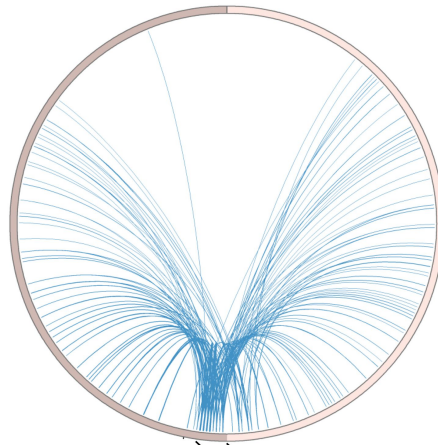
Figure 6

G4-dependent changes in local chromatin architecture.

Juicebox Hi-C matrices showing Hi-C contacts in the (A) G4-array insert cells, (B) G4-mutated insert (control) cells in a 3.6Mb region of chromosome 12 with the insertion site at the middle of the matrices. The arrows at the top of the Hi-C matrices indicate the site of insertion. (C) Juicebox Hi-C matrix showing normalized Hi-C contacts in the G4-array insert cells over the G4-mutated insert (control) cells as a heatmap. The region of interest (i.e., interactions associated with the immediate vicinity of the insert) is marked with a box. The arrow at the top of the Hi-C matrix indicates the site of insertion. (D) A line histogram displaying the differences in interaction frequency across G4-array insert cells and G4-mutated insert (control) cells in regions up to 100 kb away from the insertion site. As seen interactions downstream of the insertion site are more enriched than upstream in the G4-array insert cells as compared to the G4-mutated control. (E) Circos plot showing differential interactions (fold enrichment ≥ 2) originating from a ± 100 kb window with the insertion site at the middle, in the G4-array insert cells over the G4-mutated insert (control) cells. (F) UCSC genome browser snapshot showing the more significant differential interactions (fold enrichment ≥ 2 , interaction reads > 20) originating from a ± 50 kb window with the insertion site at the middle, in the G4-array insert cells over the G4-mutated insert (control) cells. The color intensity of the arcs indicating the interacting bins is proportional to the fold enrichment. Density of potential G4 motifs (per 10 kb) shown in lower panel; G4-forming sequences identified using pqsfinder (Hon et al., 2017); interaction regions marked in red at the bottom of lower panel.

E

Chr12:75872423-83872423



F

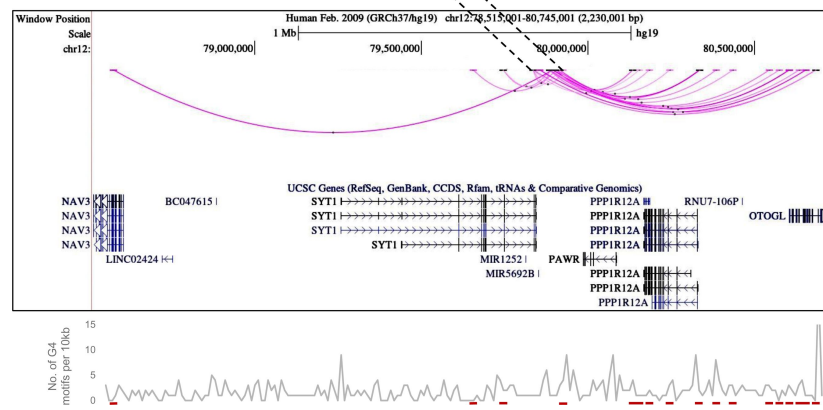


Figure 6 (continued)

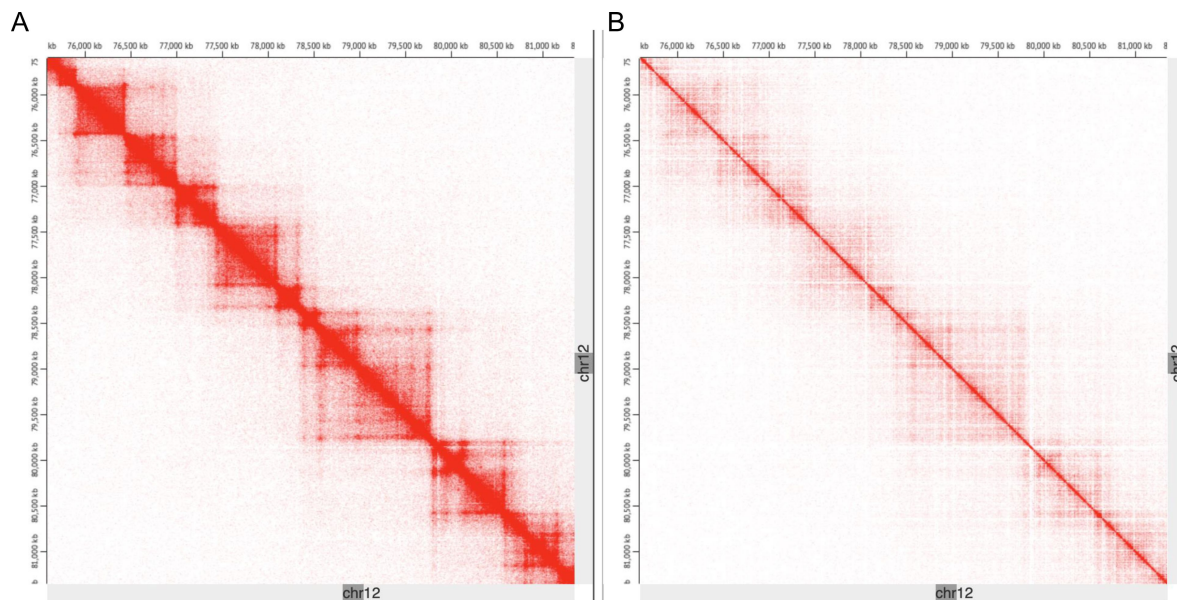


Figure S6

The chromosomal architecture of the insertion locus in the G4-array insert cells is broadly similar to uninserted cells except for the increase in looping interactions.

Comparison of Hi-C contact matrices around the insertion site in the G4-array insert cells (A) and the HEK293T control cells (taken from GSE44267) (B) shows that the broad chromatin organization is conserved. The TADs appear to be otherwise unaltered upon insertion.

mutated insert plotted in red or blue, respectively; using Juicebox for analysis). This clearly showed that the G4-array induced significantly more Hi-C interactions; interestingly this was particularly evident in the downstream regions. For a closer analysis, we mapped the interaction frequency in a ± 100 kb window centered on the insertion site. This clearly showed the difference in the number of interactions between the upstream regions vis-a-vis the region downstream of the insertion (**Figure 6D** [↗](#)).

To further confirm we used an independent HiC analysis method, HOMER (Hypergeometric Optimization of Motif EnRichment, [Heinz et al., 2018](#) [↗](#)) to compute the enhanced/reduced long-range interactions in the G4-array insert, compared to the control G4-mutated insert. Differential analysis using HOMER showed that the inserted locus induced significantly higher number of interactions in the case of G4-array insert relative to the control G4-mutated case (**Figure 6E** [↗](#)). When we plotted the significantly different chromosomal interactions with minimum 20 interaction reads, it was again clear that the number of interactions with the G4-array insertion region was significantly enhanced in the downstream region relative to the upstream (**Figure 6F** [↗](#)).

Together these show a clear role of G4s in inducing long-range interactions. A similar sequence devoid of G4-forming capability did not induce such interactions. Furthermore, the overall nature of the TAD was not disturbed, and largely consistent with what is noted in cells with no insertion. Overall, these support that the insertion of G4s induced long-range interactions with minimal organizational changes in the 3D chromatin domain, underlining the molecular role of G4s in the arrangement of 3D chromatin.

A second significant feature was notable at the insertion locus. The number of induced long-range interactions was more significant downstream of the insertion site, compared to the upstream region (**Figures 6C, D, F** [↗](#)). A close look at the Hi-C contact matrices indicated that the site of insertion was very close and downstream to the TAD boundary (**Figures 6A-C** [↗](#)). We reasoned that the G4-dependent long-range interactions were largely within the TAD, and limited in the upstream region due to the TAD boundary. This is clearly seen in **Figure 6C** [↗](#), akin to an ‘architectural stripe’ displaying that the inserted G4 array had enhanced Hi-C interactions across the domain, thus prominently featured in the downstream regions.

G4-array insertion at a second locus gives enhancer-like functions

Finally, we checked if enhancer-like effects were observed upon insertion of G4 array at another locus. Like the first site of insertion, we first identified an isolated locus devoid of G4s in the vicinity and with low interactions with surrounding regions near the 10 millionth position of chromosome 12 (10M hereafter, chr12:10588429-10588430, hg19; **Figure S7** [↗](#)). The G4-array, or its G4-mutated (control), sequences were inserted at the 10M locus (**Figures 7A, B** [↗](#), **S8** [↗](#)).

As for the 79M locus, to validate intracellular G4 formation and study the chromatin state at the inserted locus, PCR primers were designed keeping multiple points in mind (as described above). Here, for testing formation of G4 at the 10M insertion, we used primer pairs R2 (scheme for 10M shown in **Figure 7** [↗](#) top panel), covering >100 bases of the inserted G4-array, or the G4-mutated control. BG4 ChIP-qPCR validated formation of intracellular G4s within the G4 array, and not the G4-mutated control sequence (**Figure S8** [↗](#)). Next, we checked for changes in chromatin and the surrounding gene expression due to G4 formation. A relative increase in the H3K4Me1 and H3K27Ac enhancer marks in the G4-array was evident compared to the G4-mutated control (**Figures 7C, D** [↗](#)), consistent with earlier observations following G4 insertion at the 79M locus (**Figures 2A, B** [↗](#)). We noticed, however, that the enhanced levels of H3K27Ac were not as marked as the 79M locus. On the other hand, interestingly, relative increase in the H3K27Me3 repressor mark compared to the control mutated-G4 insert, particularly at the downstream end of the insertion locus was seen (**Figure 7E** [↗](#)). There was no G4-specific change in the presence of the chromatin compaction mark H3K9Me3, or the promoter activation mark H3K4Me3 (**Figures 7F,**

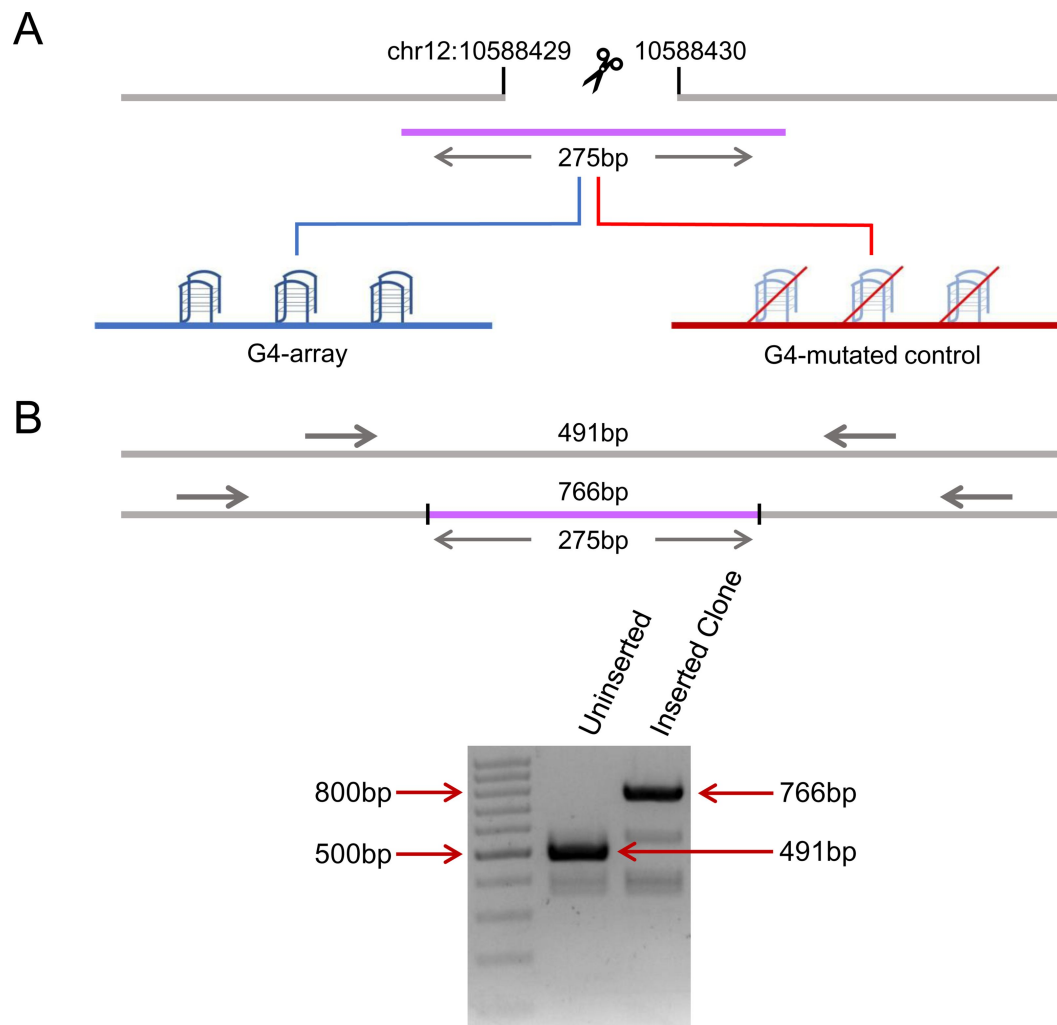


Figure 7

Insertion of the G4-array in another isolated locus and subsequent changes in chromatin and surrounding gene expression.

(A) Schematic showing the insertion of the G4-array and the G4-mutated control at chr12:10,588,429-10,588,430 (hg19). (B) PCR of the insertion locus showing the successful insertion of the 275bp long insert sequence. The top panel shows the positions of the PCR amplicons used in the Histone ChIP experiments. Changes in chromatin-modifying histone modifications in the insert region represented by calculating the ratio of occupancy of different histone marks in the G4-array insert cells over the G4-mutated insert (control) cells- enhancer mark, H3K4Me1 (C); active enhancer/promoter mark, H3K27Ac (D); facultative repressor mark, H3K27Me3 (E); constitutive repressor mark, H3K9Me3 (F) and active promoter mark, H3K4Me3 (G). (H) Long-range G4-dependent changes in mRNA expression are represented by calculating the ratio of expression of surrounding genes in the G4-array insert cells over the G4-mutated insert (control) cells. The panel above shows the positions of the gene promoters with respect to the insertion site. Mean $\pm$ SD (n=3); unpaired, two-tailed *t*-test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

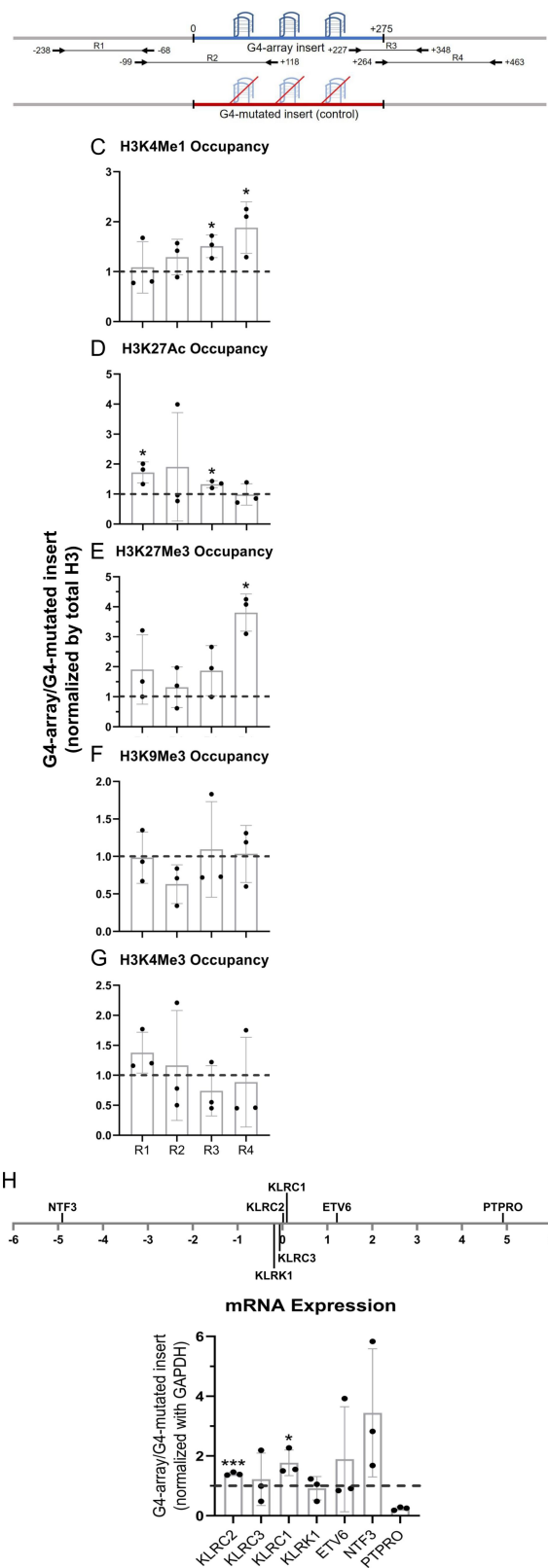


Figure 7 (continued)

Figure S7

Another isolated locus was chosen for insertion.

(A) 3D Genome Browser (Wang et al., 2018) snapshots showing the Hi-C interaction matrices from 4 cell lines (NHEK, IMR90, HUVEC and HMEC) of a section of chromosome 12 with chr12:10585000-10590000 (hg19) locus in the middle (marked by arrows); the chr12:10585000-10590000 locus has low interaction with its surrounding loci, indicated by the lack of red dots or very faint red dots.

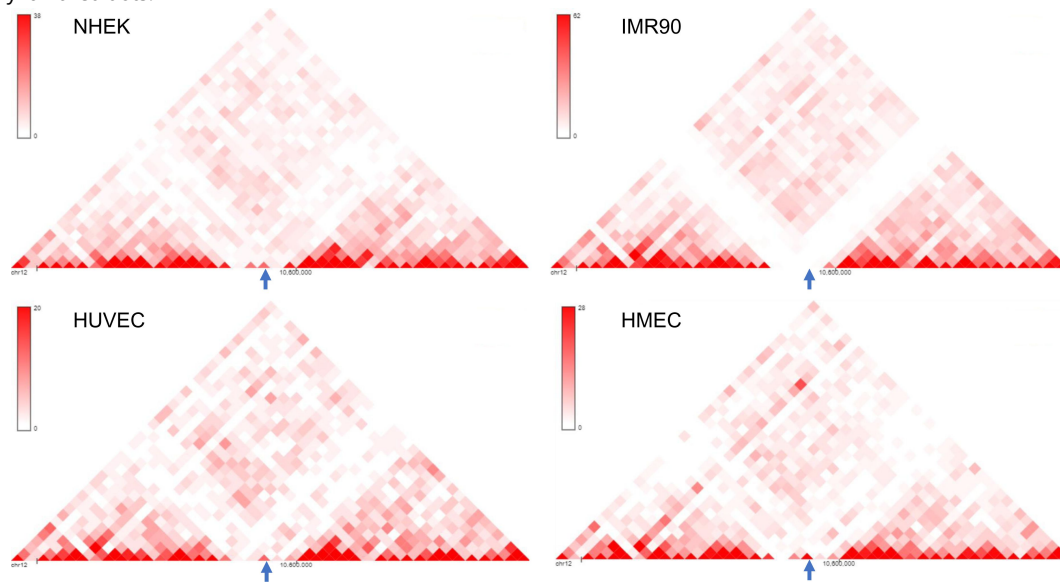
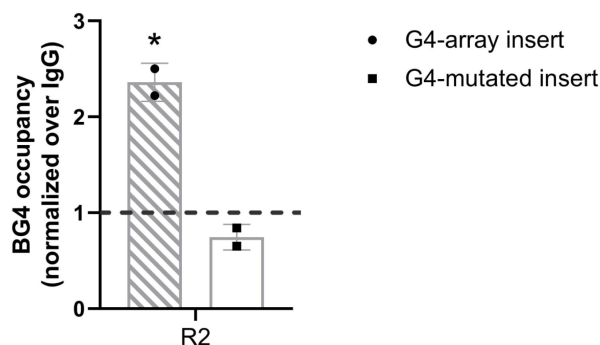


Figure S8

G4 formation analyzed by BG4 ChIP.

BG4 antibody enrichment at the 10M insertion locus in the G4-array and the G4-mutated (control) insert cells. Mean $\pm$ SD (n=2); unpaired, two-tailed t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Primer R2, covers >100 bp of the respective inserted regions, as shown in [Figure 7](#) top panel.

10M BG4 ChIP



G (Figure 7H). As expected from earlier observations and the enhancer histone marks, there was a G4-dependent increase in the expression of surrounding genes *KLRC2*, *KLRC1* and *NTF3*; except for *PTPRO*, which had reduced expression (Figure 7H). Taken together, G4-specific chromatin changes were evident at the 10M locus consistent with the 79M locus. Notable variations however must be pointed out: like the presence of the H3K27Me3 repressor histone mark, along with H3K27Ac/H3K4Me1 enhancer histone marks, indicating a poised enhancer-like state as described earlier (Calo and Wysocka, 2013). These suggest the impact of G4 formation on chromatin is likely context-specific, that is, dependent on the chromatin state of the adjacent regions.

Discussion

To directly test if G4s affect long-range chromatin organization we artificially inserted an array of G4s in the chromatin. Hi-C experiments clearly showed an enhanced number of cis- and trans-chromosomal long-range interactions emanating from the introduced G4s. This was G4-specific because a similar sequence devoid of G4-forming capability introduced at the same site did not result in enhanced interactions. Furthermore, interestingly, most new long-range interactions following G4 incorporation were downstream from the site of insertion. This is likely because the G4 insertion locus was proximal to the upstream TAD boundary thereby restricting most new interactions to the downstream regions within the TAD (Figures 5, 6).

The insertion of the G4 array led to enhanced expression of genes up to 5 Mb away compared to cells with the G4 mutated control insertion. Furthermore, there was enrichment in the H3K4Me1 and H3K27Ac enhancer histone marks, along with recruitment of transcriptional coactivator p300 and more prominently the functionally active acetylated p300/CBP. This was clearly due to the introduction of G4s and not found upon the introduction of the G4-mutated control sequence. The enhancer marks were relatively reduced, although not markedly, when the inserted G4s were specifically disrupted (Figure 4).

The enhancer-like effects observed upon G4 insertion at the 79M locus were largely replicated when the same G4-array was inserted in another locus (10M). Interestingly, the G4-dependent increase in H3K27Me3 at the 10M locus, in addition to the enhancer mark H3K4Me1, supports formation of a poised enhancer-like state, as described earlier (Calo and Wysocka, 2013). Although both the inserted G4 regions induced enhancer-like chromatin, notable context-specific influence, likely due to the chromatin-state at the regions adjacent to the insertion locus, were evident. Taken together, findings here directly support the function of G4s as enhancer-like elements and as factors that enhance long-range chromatin interactions. It is possible that such interactions are also contextually dependent on the type of G4 structure, in addition to the adjacent sequence context, and further studies will be necessary to elucidate these.

To ensure that the observed effects were from intracellular G4 formation we accounted for the following while designing the experiments. First, we introduced an array of G4s and confirmed *in vivo* G4 formation; the inserted sequence was from the hTERT promoter region with multiple arrayed G4s (Lim et al., 2010; Monsen et al., 2020; Palumbo et al., 2009). Second, we selected an insertion locus that was otherwise devoid of intrinsic G4s in a ± 2.5 kb window. Third, the selected insertion locus was relatively sparse in long-range interactions. Fourth, we independently inserted a sequence of identical length (and similar GC%) which does not form G4s at the same locus (G4-mutated control). All results were compared to the G4-mutated insertion. Although the introduced mutations for the control sequences were minimal, the impacts of such mutations on the binding of specific transcription factors associated with the sequence, particularly SP1, reported to bind G4s (Raiber et al., 2012), cannot be ruled out.

Existing literature shows promoter G4s are involved in regulating gene expression (Huppert and Balasubramanian, 2007 [DOI](#); Rawal et al., 2006 [DOI](#); Verma et al., 2008 [DOI](#)). Additionally, G4s have been reported to regulate chromatin epigenetics through both cytosine methylation and histone modifications (Halder et al., 2010 [DOI](#); Mao et al., 2018 [DOI](#); Sengupta et al., 2020 [DOI](#)). Previous studies by us further show that promoter G4s regulate gene expression by recruiting histone-modifying regulatory complexes (Hussain et al., 2017 [DOI](#); Mukherjee et al., 2019 [DOI](#); Saha et al., 2017 [DOI](#); Sharma et al., 2021 [DOI](#)). Here we aimed to study how G4s affect the expression of genes far from their location, and if this was through G4-induced modifications in long-range 3D chromatin interactions.

Multiple studies have correlated the presence of G4s with long-range associations. CTCF, an architectural protein primarily involved in TAD boundary formation, was observed to bind to G4s and G4 stabilization was noted to enhance CTCF occupancy (Tikhonova et al., 2021 [DOI](#)). In addition, G4s were noted to be enriched in TAD boundaries and associated with the formation of chromatin loops (Hou et al., 2019 [DOI](#)). G4s were also found to coincide with open chromatin regions and H3K27Ac and H3K4Me1 ChIP-Seq peaks, which are markers for transcriptional enhancers (Calo and Wysocka, 2013 [DOI](#); Hou et al., 2021 [DOI](#); Lyu et al., 2022 [DOI](#); Shlyueva et al., 2014 [DOI](#)). Most of these regions were observed to overlap with annotated enhancers and promoters regulated by such enhancers were enriched in G4s (Williams et al., 2020 [DOI](#)). A recent G4 CUT&Tag study further noted G4 formation at both active promoters and active and poised enhancers (Lyu et al., 2022 [DOI](#)).

Further, it was proposed that inter-molecular G4 formation between distant stretches of Gs may lead to DNA looping (Hegyi, 2015 [DOI](#); Liano et al., 2022 [DOI](#)). Consistent with this, we noted with interest promoters of the four genes (*PAWR*, *PPP1R12A*, *NAV3* and *SLC6A15*; **Figure 3A** [DOI](#)), activated on long-range interaction with the inserted loci, harbour potential G4-forming sequences (pG4) (**Figure S9** [DOI](#)). Further, we analysed the long-range contact regions shown in **Figure 6F** [DOI](#), along with the whole locus, for pG4s (Hon et al., 2017 [DOI](#)). Relative enrichment in pG4s was evident, particularly within the significantly enhanced contact points, at times spreading beyond the interacting region (**Figure 6F** [DOI](#), lower panel). Together, these support G4-induced long-range interactions.

The YY1 transcription factor was found to bind to G4s and dimerization of G4-bound YY1 led to chromatin looping interactions and consequent regulation of target gene expression (Li et al., 2020 [DOI](#)). A recent study also showed R-loops and possibly R-loop-associated G4 formation are enriched at CTCF binding sites, and stronger CTCF binding facilitated by G4s promotes chromatin looping (Wulfridge et al., 2023 [DOI](#)). In addition, it was shown that G4s assist in RNA polymerase II-associated chromatin looping (Yuan et al., 2023 [DOI](#)). In this context, further work will be required to understand whether and how formation of R-loops or RNA-DNA hybrid G4s (Fay et al., 2017 [DOI](#)), and/or association of factors like cohesion and CTCF, at the G4-array insertion sites impact chromatin looping.

In summary, our findings here demonstrate a causal role of G4s in inducing both long-range associations and enhancer function. Findings from a G4-forming stretch inserted at two independent loci illustrate the function of G4s in 3D gene regulation. Together these shed new mechanistic light on how DNA secondary structure motifs directly control the state of 3D chromatin and thereby biological function.

Materials and Methods

Cell lines and Cell Culture Conditions

HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium-High Glucose (DMEM-HG) supplemented with 10% FBS and 1X Anti-Anti (Gibco).

CGTTCCTCCGCCCCGGAGTCGGAGGCGCCGGGAGGCCGGACGCCGGAAGCTGCTAGCCAGGAATGTGCCGTCTAACTCGCAGGCCGCGGGCGGAGCGCGG
CGGGCGCGCTGTGTGCTCGCGCGGGAGCGGGGACAGGAGACGCTGGCGCAGGGCAGGCTGCACGCGCGGGCCGGACGCGACGCCGCGACCTGAGCGC
CGGGGGCGGGGCGTCAAGCGGCCACGACCTCTCCACAGCGCGCGCGCCCTCGCGCGCGGCTCGGCCCTTTGCTCGCTGCTGTCGCGCGCTCGGCT
CCCTTCCGCGCCCTTGCTCCCTCCCTCCCTCCTCCTCTCTTCTTCCTCTCCCTCGCTGCTCGGAGTGCAGACCGGAGATTGCCGCGGC
CTTTCGCGCGCGGGAAGACGCGCCGACGTTCTGGGCACACCTGGGAGCCGATCCGACGCTACGCTCTGTCCTCCCAAGCTCCTCAAGTAAGGC

GGGCGGGCGCACCCGGCCGAGCGGACTTCCCTTCTTACCACAGAAGCCCTCCCGCCCCAGCAGCGGCCACCGGTCACCGGCGGCCAATCTAACGTAACCTTCGC
GAGATCCGGAGTCGGGAGGCGCCCTTCGACCAAGTCGCGATTCGCGCCCTGGGCTGTGCGCCGCCACAGGAAGACGCGTCCCGCGAACTCGCGCGGTGTGTGGCTG
GGCCACACAGAGGGAAGCGGGTGTGGCCACAGCAGCGGGGCGCTGGGACAGCACTGGAAAGGAGGAAAGCGGGGAAGGAAGGTGTCTCTGTGCGGCTCGGGG
CTCCCGGGAATTCGGGAGGCGAGTGTGGAACCGCAAGGCTATGACGAGCAGATCTGCTCTCCGCTGTCTGTCACTACGCCGAGTCGTTGGCTCGACGGGCGGA
GGATTTTCTATTGCGCCGCTTATTGAATGGAATGCGAGGCTCGTGAAGTAGGTGCTGCTGGTTGATCTTTCTAAGGAACCTCAGATGAACCT

[illegible]

GAGAATATTAGATGGTGCCAGGTAGCACCATTGCCTATTATTATTCTCACTGAGACAAAAGTCAGCGGCTGGTAAGTGGTGCCATTCCTTCGCTTGAGTGGGTTG
GAGAAAGACGGCATGGCTGAGGAATTTGTTTGTGTTTGCTTGAGCGGGCTTCTCAGTTGCATCCTGCATCGGGCGGGCAGAGCCCGGGCTCTGGGAAG
ACGCCGCTCGGCAGCCGACGCGCTGCATCAATGGGTTTGGGCGAGGAGGTGCGGAGCCAGCCTCGCTCCTGGCTACCCCTCCGCGCCCGCCCGC
CGCGCCACGCTACCGTCTTCTGCTGCAGCGGTAGCGGCCCTTAACACGCGGGCGGCGACGCTGGGCGCCCTCGGTACACCCCTCACATACACACAC
CGCGCGCGCAGCACACATACGCATGTACACGCGCACCCGCAACCCGCGCCTGTATCCCGTGCCTGTTTCCCTGGCAGACACAGG

pG4s in the activated gene promoters.

Shuvra Shekhar Roy *et al.*, 2024 eLife. <https://doi.org/10.7554/eLife.96216.2>

Primary Antibodies

Histone H3 rabbit polyclonal (Abcam ab1791), H3K4Me1 rabbit polyclonal (Abcam ab8895), H3K27Ac rabbit polyclonal (Abcam ab4729), H3K4Me3 mouse monoclonal (Abcam ab1012), H3K27Me3 mouse monoclonal (Abcam ab6002), H3K9Me3 rabbit polyclonal (Abcam ab8848), p300 rabbit monoclonal (CST 54062), Ac-p300/CBP rabbit polyclonal (CST 4771), BG4 antibody (MABE917).

Genomic Insertions using CRISPR-Cas9 genome editing

For the genomic insertions CRISPR-Cas9 genome editing technique was used (Ran et al., 2013 [↗](#)). For the G4 array insertion, 275 bp long *hTERT* promoter region was PCR amplified from HEK 293T genomic DNA. For the insertion of the mutated G4s, a synthetic DNA template was synthesized and cloned into pUC57 vector by Genscript Biotech Corp, where 12 Gs were substituted with Ts (see supplementary methods for detailed sequences). Both the G4 array and the G4 mutated insertion templates were PCR amplified using longer primers where the short homology arms were introduced as overhangs of the primer for the accurate insertion at the 79M locus via homologous recombination (see supplementary methods for primer sequences) (Paix et al., 2017 [↗](#)). For cleavage at the 79M locus (chr12:79,872,423-79,872,424 (hg19)), the gRNA sequence, 5'-ACTATGTATGTACATCCAGG-3', was cloned into the pX459 v2.0, a gift from Feng Zhang, that co-expresses cas9 protein and the gRNA. For cleavage at the 10M locus (chr12:10,588,429-10,588,430 (hg19)), the gRNA sequence, 5'-ATCCTTCCTGAATCATCAA-3', was used. Guide RNAs (gRNAs) were designed using the CRISPOR tool (Haeussler et al., 2016 [↗](#)). Once the gRNA cloned vector and the insertion donor templates were ready, they were transfected into HEK293T cells and the transfected cells were selected using puromycin, whose resistance gene was present in the pX459 vector. Then these selected cells were serially diluted to isolate clones originating from single cells. Many such clones were screened to detect cells with homozygous/heterozygous insertion of the G4 array or mutated G4 insert by performing locus-specific PCR. Either primers adjacent to the insertion site or cross primers, i.e., one primer within the insert and another from the adjacent region, were used to screen and identify insertions. While using adjacent primers, a shift in PCR product with an increase in amplicon size by 275 bp (size of the insert) indicated successful insertion (see supplementary methods for primer sequences).

ChIP (Chromatin Immunoprecipitation)

ChIP assays were performed as per the protocol previously reported in (Mukherjee et al., 2018 [↗](#)). Immunoprecipitation was done using relevant primary antibodies. IgG was used for isotype control. Total histone H3 was used as a control for the histone modifications ChIP. Three million cells were harvested and crosslinked with ~1% formaldehyde for 10 min and lysed. Chromatin was sheared to an average size of ~250-500 bp using Biorupter (Diagenode). 10% of sonicated fraction was processed as input using phenol-chloroform and ethanol precipitation. ChIP was performed using 3 µg of the respective antibody incubated overnight at 4°C. Immune complexes were collected using salmon sperm DNA-saturated magnetic protein G Dynabeads (Anti-FLAG M2 magnetic beads for BG4 ChIP) and washed extensively using a series of low salt, high salt and LiCl Buffers. The Dynabeads were then resuspended in TE (Tris- EDTA pH 8.1) buffer and treated with proteinase K at 65° C for ~5 hrs. Then, phenol-chloroform-isoamyl alcohol was utilized to extract DNA. Extracted DNA was precipitated by centrifugation after incubating overnight at -20 ° C with isopropanol, 0.3M sodium acetate and glycogen. The precipitated pellet was washed with freshly prepared 70% ethanol and resuspended in TE buffer. ChIP DNA was analyzed by qRT-PCR method (see supplementary methods for primer sequences).

Real-time PCR for Gene (mRNA) expression

Total RNA was isolated using TRIzol® Reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. RNA was quantified and cDNA was synthesized using iScript cDNA Synthesis Kits. A relative transcript expression level for genes was measured by quantitative real-time PCR using a SYBR Green based method (see supplementary methods for primer sequences). Average fold change was calculated by the difference in threshold cycles (Ct) between test and control samples. GAPDH gene was used as internal control for normalizing the cDNA concentration of each sample.

Chromosome Conformation Capture (3C)

Chromosome Conformation Capture (3C) assay was done as per the protocol reported in (Cope and Fraser, 2009) with certain modifications. Briefly, about 5-6 million cells were crosslinked using 1% formaldehyde for 10 minutes and then lysed to isolate the nuclei. Nuclei were digested overnight by HindIII and then ligated in a diluted reaction so that intramolecular ligation is favored. After ligation, the reaction mixture was treated with proteinase K at 65° C to de-crosslink the DNA, followed by RNase A treatment. Then, phenol-chloroform-isoamyl alcohol was utilized to extract DNA. Extracted DNA was precipitated by centrifugation after incubating overnight at -80 ° C with 70% ethanol, 0.1M sodium acetate and glycogen. The precipitated pellet was washed with freshly prepared 70% ethanol and resuspended in TE buffer. 3C looping interactions were analyzed by TaqMan qRT-PCR method. For comparison, each interaction frequency was normalized to the interaction between exons 2 and 8 of the human α -actin (ACTA2) (Hadjur et al., 2009). See supplementary methods for primer sequences.

G4 disruption using LNA probes

Probes were designed to specifically bind to regions of genomic DNA containing G repeats which would form the G stems of the G4 structure. The probes containing LNA nucleotides should hybridize with the target with higher stability than the stability of the G4 structure thus destabilizing the G4. The probes used to target the G4 array insert were: 5'- **C*CCGACCCCTCC***C-3', 5'- **C*CAGCCCCCTCC***G-3', 5'- **C*CCCTCCCCTTC***C-3'. Stretches of three or more Cs are shown in bold, LNA nucleotides within the probes are underlined, the ends of the probes were protected using phosphorothioate bonds, shown as *. Approximately 0.8 μ g of LNA probes (all 3 mixed in equimolar amounts) were transfected per million cells. Cells were treated with the LNA probes for 108 hours by transfecting thrice with a gap of 36 hours in between. The schematic below shows the LNA probes designed to disrupt the inserted G4 structures along with the inserted G4 array sequence to show the specific sites of hybridization by the LNA probes.

CCAGGCCGGGCTCCAGTGGATTGCGGGGCACAGACGCCAGGACCGCGCTTCCACGTGGCG
GAGGGACTGGGGACCCGGGCACCCGTCTGCCCCCTTACCTTCCAGCTCCGCCTCCTCCGCGCG
GACCCCGCCCCGTCCCGACCCCTCCGGGTCCCCGGCCAGCCCCCTCCGGCCCTCCAGCC
CCTCCCCTTCCTTTCCGCGGCCCGCCCTCTCCTCGCGCGCGAGTTTCAGGCAGCGCTGCGTC
CTGCTGCGCACGTGGGAAGCC

C*CCGACCCCTCC*C

C*CAGCCCCCTCC*G

C*CCCTCCCCTTC*C

Hi-C

Hi-C was performed using the Arima-HiC Kit as per the manufacturer's protocol. After the proximally-ligated Hi-C templates were generated, sequencing libraries were prepared using NEBNext Ultra II DNA Library Prep Kit as per the Arima-HiC Kit's protocol. The quality of the

sequencing libraries was cross-checked using TapeStation (Agilent Technologies) and the KAPA Library Quantification Kit (Roche) before proceeding with sequencing using NovaSeq 6000 (Illumina).

Hi-C data analysis

Hi-C reads were mapped to the hg19 human genome and processed using default parameters using Juicer (<https://github.com/aidenlab/juicer>). Hi-C count matrices were generated at 5kb, 10kb, 25kb, 50kb, 100kb, and 250kb using Juicer. Hi-C heatmap figures were rendered using Juicebox (<https://github.com/aidenlab/juicebox/wiki/Download>). Hi-C contacts originating in the loci flanking the G4 insertion site were generated using bedtools (<https://bedtools.readthedocs.io/en/latest/>). The circos plots were rendered using Circos (<http://circos.ca>). To identify significant interaction the data was processed using homer (<http://homer.ucsd.edu/homer/>) using analyzeHiC function. The bins showing 2-fold enrichment in G4 WT over G4 Mut and vice-versa were retained for filtering contacts for representation on circos plots.

Data Availability

The sequencing data underlying this article are available in the NCBI Sequence Read Archive, scheduled to be released upon publication and would be accessible using the following link- <https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1048044>. The rest of the data are available in the article and its online supplementary material. Further inquiries can be directed to the corresponding author.

Author Contributions

SSR- Methodology, Resources, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft preparation; SB- Resources, Investigation; SV- Investigation; AS- Visualization; CRT- Investigation; RK- Formal analysis, Visualization; SS- Methodology, Formal analysis, Visualization; SC- Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Editors

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Reviewer #1 (Public Review):

In this manuscript, Chowdhury and co-workers provide interesting data to support the role of G4-structures in promoting chromatin looping and long-range DNA interactions. The authors achieve this by artificially inserting a G4-containing sequence in an isolated region of the genome using CRISPR-Cas9 and comparing it to a control sequence that does not contain G4 structures. Based on the data provided, the authors can conclude that G4-insertion promotes long-range interactions (measured by Hi-C) and affects gene expression (measured by qPCR) as well as chromatin remodelling (measured by ChIP of specific histone markers).

In this revised version of the manuscript, G4 formation of the inserted sequence was validated by ChIP-qPCR, and the same G4-containing sequence was inserted at a second locus, and similar, though not identical, effects on chromatin and gene expression were observed.

Strengths:

This is the first attempt to connect genomics datasets of G4s and HiC with gene expression. The use of Cas9 to artificially insert a G4 is also very elegant.

<https://doi.org/10.7554/eLife.96216.2.sa2>

Reviewer #2 (Public Review):

Roy et al. investigated the role of non-canonical DNA structures called G-quadruplexes (G4s) in long-range chromatin interactions and gene regulation. Introducing a G4 array into chromatin significantly increased the number of long-range interactions, both within the same chromosome (cis) and between different chromosomes (trans). G4s functioned as enhancer elements, recruiting p300 and boosting gene expression even 5 megabases away. The study reveals that G4s directly influence 3D chromatin organization via facilitating communication between regulatory elements and genes.

Strengths:

The authors' findings are valuable for understanding the role of G4-DNA in 3D genome organization and gene transcription. The authors provide convincing evidence to support their claims.

<https://doi.org/10.7554/eLife.96216.2.sa1>

Reviewer #3 (Public Review):

Summary:

This paper aims to demonstrate the role of G-quadruplex DNA structures in the establishment of chromosome loops. The authors introduced an array of G4s spanning 275 bp, naturally found within a very well characterized promoter region of the hTERT promoter, in an ectopic region devoid of G-quadruplex and annotated gene. As a negative control, they used a mutant version of the same sequence in which G4 folding is impaired. Due to the complexity of the region, 3 G4s on the same strand and one on the opposite strand, 12 point mutations were made simultaneously (G to T and C to A). Analysis of the 3D genome organization shows that the WT array establishes more contact within the TAD and throughout the genome than the

control array. Additionally, a slight enrichment of H3K4me1 and p300, both enhancer markers, was observed locally near the insertion site. The authors tested whether the expression of genes located either nearby or away up to 5 Mb were up-regulated based on this observation. They found that four genes were up-regulated from 1.5 to 3 fold. An increased interaction between the G4 array compared to the mutant was confirmed by the 3C assay. For in-depth analysis of the long-range changes, they also performed Hi-C experiments and showed a genome-wide increase in interactions of the WT array versus the mutated form.

Strengths:

The experiments were well-executed and the results indicate a statistical difference between the G4 array inserted cell line and the mutated modified cell line.

Weaknesses:

- (1) It would have been nice to have an internal control corresponding to a region known to be folded in several cell lines to compare the level of pG4 signal within their construct with a well-characterised control (for example, the KRAS promoter region).
- (2) The mutations introduced into the G4 sequence may also affect Sp1 or other transcription factor binding sites present in this region, and some of the observations may depend on these sites rather than G4 structures. While this is acknowledged in the text, the conclusion in the title of the paper seems an overstatement.

<https://doi.org/10.7554/eLife.96216.2.sa0>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

Summary:

In this manuscript, Chowdhury and co-workers provide interesting data to support the role of G4-structures in promoting chromatin looping and long-range DNA interactions. The authors achieve this by artificially inserting a G4-containing sequence in an isolated region of the genome using CRISPR-Cas9 and comparing it to a control sequence that does not contain G4 structures. Based on the data provided, the authors can conclude that G4-insertion promotes long-range interactions (measured by Hi-C) and affects gene expression (measured by qPCR) as well as chromatin remodelling (measured by ChIP of specific histone markers).

Whilst the data presented is promising and partially supports the authors' conclusion, this reviewer feels that some key controls are missing to fully support the narrative. Specifically, validation of actual G4-formation in chromatin by ChIP-qPCR (at least) is essential to support the association between G4-formation and looping. Moreover, this study is limited to a genomic location and an individual G4-sequence used, so the findings reported cannot yet be considered to reflect a general mechanism/effect of G4-formation in chromatin looping.

Strengths:

This is the first attempt to connect genomics datasets of G4s and HiC with gene expression. The use of Cas9 to artificially insert a G4 is also very elegant.

Weaknesses:

Lack of controls, especially to validate G4-formation after insertion with Cas9. The work is limited to a single G4-sequence and a single G4-site, which limits the generalisation of the findings.

In the revised version we validated G4 formation inside cells at the insertion site using the reported G4-selective antibody BG4. Significant BG4 binding (by ChIP-qPCR) was clear in the G4-array insert, and not in the G4-mutated insert, supporting formation of G4s by the inserted G4-array (included as Figure S4).

To directly address the second point, we inserted the G4-sequence, or the mutated control, at a second relatively isolated locus (at the 10 millionth position on Chr12, denoted as 10M site in text). First, BG4 ChIP was done to confirm intracellular G4 formation by the inserted array. BG4 ChIP-qPCR binding was significant within the inserted region, and not in the negative control region (Figure S8), consistent with the 79M locus. Together these demonstrate intracellular G4 formation by inserted sequences at two different loci.

We next checked the state of chromatin of the G4-array inserted at the 10M locus, or its negative control. Histone marks H3K4Me1, H3K27Ac, H3K27Me3, H3K9me3 and H3K4Me3 were tested at the G4-array, or the negative control locus. Relative increase in the enhancer histone marks was evident, relative to the control sequence. This was largely similar to the 79M locus, supporting an enhancer-like state. Interestingly, here we further noted presence of the H3K27me3 histone mark. The presence of the H3K27Me3 repressor histone mark, along with H3K4Me1/H3K27Ac enhancer histone marks, support a poised enhancer-like status of the inserted G4 region, as has been observed earlier in other studies. Together, although data from the two distinct G4 insertion sites support the enhancer-like state, there are contextual differences likely due to the sequence/chromatin of the sites adjacent to the inserted sequence.

Effect of the 10M G4-insertion on activation of surrounding genes (10 Mb window), and not the G4-mutant insert, was evident for most genes. Consistent with the enhancer-like state of the G4-array insert; in line with the 79M G4-array insert.

These results have been added as the final section in the revised version, data is shown in Figure 7.

Reviewer #2 (Public Review):

Summary:

Roy et al. investigated the role of non-canonical DNA structures called G-quadruplexes (G4s) in long-range chromatin interactions and gene regulation. Introducing a G4 array into chromatin significantly increased the number of long-range interactions, both within the same chromosome (cis) and between different chromosomes (trans). G4s functioned as enhancer elements, recruiting p300 and boosting gene expression even 5 megabases away. The study proposes a mechanism where G4s directly influence 3D chromatin organization, facilitating communication between regulatory elements and genes.

Strength:

The findings are valuable for understanding the role of G4-DNA in 3D genome organization and gene transcription.

Weaknesses:

The study would benefit from more robust and comprehensive data, which would add depth and clarity.

(1) Lack of G4 Structure Confirmation: The absence of direct evidence for G4 formation within cells undermines the study's foundation. Relying solely on in vitro data and successful gene insertion is insufficient.

Using the reported G4-specific antibody, BG4, we performed BG4 ChIP-qPCR at the 79M locus. In addition, a second G4-insertion site was created and BG4 ChIP-qPCR was used to validate intracellular G4 formation. Briefed below, more details in the response above.

In the revised version we validated G4 formation inside cells at the insertion site using the reported G4-selective antibody BG4. Significant BG4 binding (by ChIP-qPCR) was clear in the G4-array insert, and not in the G4-mutated insert, supporting formation of G4s by the inserted G4-array (included as Figure S4).

Further, we inserted the G4-sequence, or the mutated control, at a second relatively isolated locus (at the 10 millionth position on Chr12, denoted as 10M site in text). First, BG4 ChIP was done to confirm intracellular G4 formation by the inserted array. BG4-ChIP-qPCR was significant within the G4-array inserted region, and not in the negative control region (Figure S8), consistent with the 79M locus. Together these demonstrate intracellular G4 formation by inserted sequences at two different loci. Added in revised text in the second and the final sections of results, data shown in Figures 7, S4 and S8.

(2) Alternative Explanations: The study does not sufficiently address alternative explanations for the observed results. The inserted sequences may not form G4s or other factors like G4-RNA hybrids may be involved.

As mentioned in response to the previous comment, we confirmed that the inserted sequence indeed forms G4s inside the cells. RNA-DNA hybrid G4s can form within R-loops with two or more tandem G-tracks (G-rich sequences) on the nascent RNA transcript as well as the non-template DNA strand (Fay et al., 2017, 28554731). A recent study has observed that R-loop-associated G4 formation can enhance chromatin looping by strengthening CTCF binding (Wulfridge et al., 2023, 37552993). As pointed out by the reviewer, the possibility of G4-RNA hybrids remains, we have mentioned this possibility for readers in the second last paragraph of the Discussion.

(3) Limited Data Depth and Clarity: ChIP-qPCR offers limited scope and considerable variation in some data makes conclusions difficult.

We noted variation with one of the primers in a few ChIP-qPCR experiments (in Figures 2 and 3D). The changes however were statistically significant across replicates, and consistent with the overall trend of the experiments (Figures 2, 3 and 4). Enhancer function, in addition to ChIP, was also confirmed using complementary assays like 3C and RNA expression.

(4) Statistical Significance and Interpretation: The study could be more careful in evaluating the statistical significance and magnitude of the effects to avoid overinterpreting the results.

We reconfirmed our statistical calculations from biological replicate experiments. We carefully looked at potential overinterpretations, and made appropriate changes in the manuscript (details of the changes given below in response to comment to authors).

Reviewer #3 (Public Review):

Summary:

This paper aims to demonstrate the role of G-quadruplex DNA structures in the establishment of chromosome loops. The authors introduced an array of G4s spanning 275 bp, naturally found within a very well-characterized promoter region of the hTERT promoter, in an ectopic region devoid of G-quadruplex and annotated gene. As a negative control, they used a mutant version of the same sequence in which G4 folding is impaired. Due to the complexity of the region, 3 G4s on the same strand and one on the opposite strand, 12 point mutations were made simultaneously (G to T and C to A). Analysis of the 3D genome organization shows that the WT array establishes more contact within the TAD and throughout the genome than the control array. Additionally, a slight enrichment of H3K4me1 and p300, both enhancer markers, was observed locally near the insertion site. The authors tested whether the expression of genes located either nearby or up to 5 Mb away was up-regulated based on this observation. They found that four genes were up-regulated from 1.5 to 3-fold. An increased interaction between the G4 array compared to the mutant was confirmed by the 3C assay. For in-depth analysis of the long-range changes, they also performed Hi-C experiments and showed a genome-wide increase in interactions of the WT array versus the mutated form.

Strengths:

The experiments were well-executed and the results indicate a statistical difference between the G4 array inserted cell line and the mutated modified cell line.

Weaknesses:

The control non-G4 sequence contains 12 point mutations, making it difficult to draw clear conclusions. These mutations not only alter the formation of G4, but also affect at least three Sp1 binding sites that have been shown to be essential for the function of the hTERT promoter, from which the sequence is derived. The strong intermingling of G4 and Sp1 binding sites makes it impossible to determine whether all the observations made are dependent on G4 or Sp1 binding. As a control, the authors used Locked Nucleic Acid probes to prevent the formation of G4. As for mutations, these probes also interfere with two Sp1 binding sites. Therefore, using this alternative method has the same drawback as point mutations. This major issue should be discussed in the paper. It is also possible that other unidentified transcription factor binding sites are affected in the presented point mutants.

Since the sequence we used to test the effects of G4 structure formation is highly G-rich, we had to introduce at least 12 mutations to be sure that a stable G4 structure would not form in the mutated control sequence. Sp1 has been reported to bind to G4 structures (Raiber et al., 2012). Therefore, Sp1 binding is likely to be associated with the G4-dependent enhancer functions observed here. We also appreciate that apart from Sp1, other unidentified transcription factor binding sites might be affected by the mutations we introduced. We have discussed these possibilities in the fourth paragraph of the Discussion section in the revised manuscript.

Reviewer #1 (Recommendations For The Authors):

Whilst the data presented is promising and partially supports the authors' conclusion, this reviewer feels that some key controls are missing to fully support the narrative used. Below are my main concerns:

(1) The main thing missing in the current manuscript is to validate the actual formation of G4 in chromatin context for the repeat inserted by CRISPR-Cas. Whilst I appreciate this will form promptly a G4 in vitro, to fully support the conclusions proposed the authors would need to demonstrate actual G4-formation in cells after insertion. This could be

done by ChIP-qPCR using the G4-selective antibody BG4 for example. This is an essential piece of evidence to be added to link with confidence G4-formation to chromatin looping.

To address the concern regarding whether the inserted G4 sequence forms G4s in cells, as suggested, we used the G4-selective antibody BG4. PCR primers in the study were designed keeping multiple points in mind: Primers should not bind to any site of G/C alteration in the mutated control insert; either the forward/reverse primer is from the adjacent region for specificity; covers adjacent regions for studying any effects on chromatin; and, PCRs optimized keeping in mind the repeats within the inserted sequence. Given these, primer pairs R1-R4 were chosen for further work following optimizations (Figure 2, top panel). For BG4 ChIP-qPCR we used primer pairs R2, which covered >100 bases of the inserted G4-array, or the G4-mutated control. Significant BG4 binding was clear in the G4-array insert, and not in the G4-mutated insert, demonstrating formation of G4s by the inserted G4-array (Figure S4).

In response to comment #3 below, we inserted the G4-forming sequence (or its mutated control) at a second locus. This insertion was near the 10 millionth position of chromosome 12 (10M insertion locus in text). Here also, BG4 binding was significant within the G4-array inserted region, and not in the negative control region (Figure S8). Together these demonstrate G4 formation by the inserted sequence at two different loci.

(2) I found the LNA experiment very elegant. However, what would be the effect of LNA treatment on the control sequence that does not form G4s? This control is essential to disentangle the effect of LNA pairing to the sequence itself vs disrupting the G4-structure.

As per the reviewer's suggestion, we performed a control experiment where we treated the G4-mutated insert (control) cells with the G4-disrupting LNA probes. The changes in the expression of the surrounding genes in this case were not significant, indicating that the effects observed in the G4-array insert cells were possibly due to disruption of the inserted G4 structures. This data is presented in Figure S5.

(3) The authors describe their work and present its conclusion as if this were a genome-wide study, whilst the work is focused on a specific genomic location, and the looping, along with the effect on histone acetylation and gene expression, is limited to this. The authors cannot conclude, therefore, that this is a generic effect and the discussion should be more focused on the specific G4s used and the genomic location investigated. Ideally, insertion of a different G4-forming sequence or of the same in a different genomic location is recommended to really claim a generic effect.

To address this we inserted the G4-array sequence, or the G4-mutated control sequence, at another relatively isolated locus – at the 10 millionth position of chromosome 12 – denoted as 10M. Using BG4 ChIP-qPCR intracellular G4 formation was confirmed. We observed that the enhancer-like features in terms of enhancer histone marks and increase in the expression of surrounding genes were largely reproduced at the 10M locus on G4 insertion (Figure 7). These results are added as the final section under Results.

Reviewer #2 (Recommendations For The Authors):

The study proposes a mechanism where G4s directly influence 3D chromatin organization, facilitating communication between regulatory elements and genes.

While the present manuscript presents an interesting hypothesis, it would benefit from enhanced novelty and more robust data. The study complements existing G4 research (e.g., PMID: 31177910). While the conclusions hold biological relevance, they largely

reiterate established knowledge. Furthermore, the presented data appear preliminary and still lack depth and clarity.

Hou et al., 2019 (PMID: 31177910) showed presence of potential G4-forming sequences correlated with TAD boundaries, along with enrichment of architectural proteins and transcription factor binding sites. Also, other studies noted enrichment of potential G4-forming sequences at enhancers along with nucleosome depletion and higher transcription factor binding (Hou et al., 2021; Williams et al., 2020). These studies proposed the role of G4s in chromatin/TAD states based on analysis of potential G4-forming sequences using correlative bioinformatics analyses. Here we sought to directly test causality. Insertion of G4 sequence, and formation of intracellular G4s in an isolated, G4-depleted region resulted in altered characteristics of chromatin, and not in the negative control insertion that does not form G4s. These, in contrast to earlier studies, directly demonstrates the causal role of G4s as functional elements that impact local and distant chromatin.

Major concerns:

(1) Lack of G4 Structure Confirmation: Implement G4-specific antibodies or fluorescent probes to verify G4 structures inside the cells.

Detailed response given above. Briefly, in the revised version we validated G4 formation inside cells at the insertion site using the reported G4-selective antibody BG4. Significant BG4 binding (by ChIP-qPCR) was clear in the G4-array insert, and not in the G4-mutated insert, supporting formation of G4s by the inserted G4-array (included as Figure S4).

Further, we inserted the G4-sequence, or the mutated control, at a second relatively isolated locus (at the 10 millionth position on Chr12, denoted as 10M site in text). First, BG4 ChIP was done to confirm intracellular G4 formation by the inserted array. BG4 ChIP-qPCR binding was significant within the G4-array inserted region, and not in the negative control region (Figure S8), consistent with the 79M locus. Together these demonstrate intracellular G4 formation by inserted sequences at two different loci. Added in revised text in the second and the final sections of results, data shown in Figures 7, S4 and S8.

(2) Alternative Explanations: Explore the possibility that the sequences may not form G4s or that other factors like G4-RNA hybrids are involved.

Response provided in the public reviews section.

(3) Limited Data Depth and Clarity: ChIP-qPCR offers limited scope. Consider employing G4 ChIP-seq for genome-wide analysis of G4 association with histone modifications. Address inconsistencies in data like H3K27me3 variation and incomplete H3K9me3 data sets.

A recent study performed G4 CUT&Tag (Lyu et al., 2022, 34792172) and observed G4 formation at both active promoters and active and poised enhancers. We have discussed this in the sixth paragraph of the Discussion. The H3K27Me3 occupancy at the 79M locus insertions did not have any significant G4-dependent changes, however, at the second insertion site at the 10M locus (introduced in the revised manuscript, Figure 7) there was significant G4-dependent increase in H3K27Me3 occupancy along with the H3K4Me1 and H3K27Ac enhancer histone marks, indicating formation of a poised enhancer-like element.

We completed the H3K9me3 data sets for both insertion sites.

(4) Statistical Significance and Interpretation: Re-evaluate the statistical significance of results and interpret them in the context of relevant biological knowledge. Avoid

overstating the impact of minor changes.

We revised several lines to avoid overstating results. Some of the changes are as below (changes underline/strikethrough)

- There was an a relatively modest increase in the recruitment of both p300 and a substantial increase in the recruitment of the more functionally active acetylated p300/CBP to the G4-array when compared against the mutated control.
- As expected, although modest, a decrease in the H3K4Me1 and H3K27Ac enhancer histone modifications was evident within the insert upon the LNAs treatment.
- Moreover, the enhancer marks were relatively reduced, although not markedly, when the inserted G4s were specifically disrupted.

(5) Unexplored Aspects: Investigate the relationship between G4 DNA and R-loops, and consider the role of CTCF and cohesin proteins in mediating long-range interactions. Integrate existing research to build a more comprehensive framework and draw more robust conclusions.

As mentioned in response to one of the earlier comments, a recent publication extensively studied the association between G4s, R-loops, and CTCF binding (Wulfridge et al., 2023). While, here we focused on the primary features of a potential enhancer, further work will be necessary to establish how G4s influence the coordinated action between cohesin and CTCF and consequent chromatin looping. We have described this for readers in the second last paragraph of the Discussion in the revised version.

Minor Concern:

(1) Enhancer Definition: The term "enhancer" requires specific criteria. Modify the section heading or provide evidence demonstrating the G4 sequence fulfills all conditions for being an enhancer, such as position independence and long-range effects.

Although we checked some of the primary features of a potential enhancer: Like expression of surrounding genes, enhancer histone marks, chromosomal looping interactions, and recruitment of transcriptional coactivators, further aspects may need to be validated. As suggested, in the revised manuscript the section heading has been modified to 'Enhancer-like features emerged upon insertion of G4s.'

Reviewer #3 (Recommendations For The Authors):

In addition to the points in my public review, I would like to mention some less significant points.

The authors mention that "the array of G4-forming sequences used for insertion was previously reported to form stable G4s in human cells" (Lim et al., 2010; Monsen et al., 2020; Palumbo et al., 2009). However, upon reading the publications, I found that these observations were made in vitro. I may have missed something, but there are now several mappings of folded-G4 in human cells based on different approaches. It would be beneficial to investigate whether the hTERT promoter is a site of G-quadruplex formation in vivo. If confirmed, a similar analysis should be conducted on the 275 bp region inserted into the ectopic region to determine if it also has the ability to form a structured G4.

We performed BG4 ChIP to confirm in vivo G4 formation by the inserted G4-array as suggested (Figures S4, S8). Detail response given above. Briefly, in the revised version we

validated G4 formation inside cells at the insertion site using the reported G4-selective antibody BG4. Significant BG4 binding (by ChIP-qPCR) was clear in the G4-array insert, and not in the G4-mutated insert, supporting formation of G4s by the inserted G4-array (included as Figure S4).

Further, we inserted the G4-sequence, or the mutated control, at a second relatively isolated locus (at the 10 millionth position on Chr12, denoted as 10M site in text). First, BG4 ChIP was done to confirm intracellular G4 formation by the inserted array. BG4-ChIP-qPCR was significant within the inserted region, and not in the negative control region (Figure S8). Consistent with the 79M locus. Together these demonstrate intracellular G4 formation by inserted sequences at two different loci. Added in revised text in the second and the final sections of results, data shown in Figures 7, S4 and S8.

The inserted sequence originates from a well-characterized promoter. The authors suggest that placing it in an ectopic position creates an enhancer-like region, based on the observation of increased levels of H3K27Ac and H3K4me1 on the WT array. To provide a control that it is not a promoter, it would be useful to also analyze a specific mark of promoter activity, such as H3K4me3.

As suggested by reviewer, we also analysed the H3K4Me3 promoter activation mark at both the 79M and 10M (introduced in the revised manuscript, Figure 7) insertion loci. We did not observe any significant G4-dependent changes in the recruitment of H3K4Me3 (Figures 2, 7).

In the discussion, the authors mention "it was proposed that inter-molecular G4 formation between distant stretches of Gs may lead to DNA looping". To investigate this further, it would be worthwhile to examine whether the promoter regions of activated genes (PAWR, PPP1R12A, NAV3, and SLC6A15) contain potentially forming G-quadruplexes (pG4). Additionally, sites that establish more contact with the G4 array described in Figure 6F could be analyzed for enrichment in pG4.

Thank you for pointing this out. We found promoters of the four genes (PAWR, PPP1R12A, NAV3, and SLC6A15) harbour potential G4-forming sequences (pG4s). Also as suggested, we analysed the contact regions in Fig 6F, along with the whole locus, for pG4s. Relative enrichment in pG4 was seen, particularly within the significantly enhanced interacting regions, which at times spreads beyond the interacting regions also. This is shown in the lower panel of Figure 6F in the revised version. We have described this in Discussion for readers.

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