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Identification of putative G-quadruplex DNA structures in S. pombe genome by quantitative PCR stop assay

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ABSTRACT

In order to understand in which biological processes the four-stranded G-quadruplex (G4) DNA structures play a role, it is important to determine which predicted regions can actually adopt a G4 structure. Here, to identify DNA regions in Schizosaccharomyces pombe that fold into G4 structures, we first optimized a quantitative PCR (qPCR) assay using the G4 stabilizer, PhenDC3. We call this method the qPCR stop assay, and used it to screen for G4 structures in genomic DNA. The presence of G4 stabilizers inhibited DNA amplification in 14/15 unexplored genomic regions in S. pombe that encompassed predicted G4 structures, suggesting that at these sites the stabilized G4 structure formed an obstacle for the DNA polymerase. Furthermore, the formation of G4 structures was confirmed by complementary in vitro assays. In vivo, the S. pombe G unwinder Pif1 helicase, Pfh1, was associated with tested G4 sites, suggesting that the G4 structures also formed in vivo. Thus, we propose that the confirmed G4 structures in S. pombe form an obstacle for replication in vivo, and that the qPCR stop assay is a method that can be used to identify G4 structures. Finally, we suggest that the qPCR stop assay can also be used for identifying G4 structures in other organisms, as well as being adapted to screen for novel G4 stabilizers.

1. Introduction

G-quadruplex (G4) DNA structures are formed in guanine-rich DNA. Sequences that form G4 structures, called G4 motifs, encompass a characteristic sequence with four GGG repeats connected by loop regions with variable length from 1 to 25 nucleotides [1]. DNA with this consensus sequence motif folds into a structure built up by three planar G-tetrads that are stacked upon each other and stabilized by monovalent cations. These planar G-tetrads are formed between four guanines that bind to each other through Hoogsteen hydrogen bonds. Instead of three planar G-tetrads, G4 structures with four or more guanines in the G-tracts are also described [2–4]. Furthermore, G4 structures consisting of only two stacked G-tetrads or with longer loops are also found in different genomes, but these types of G4 structures are relatively unexplored [5,6]. G4 motifs can adopt many diverse G4 structures, and their formation and stability is highly dependent on the orientation of the guanines in the stacked G-tetrads and the length and sequence of the loop regions [7–11]. Folded oligonucleotides with a G4 motif can adopt parallel, antiparallel, or hybrid structures, but the structure formed by a certain G4 motif is not uniform and can vary depending on the experimental conditions [12,13].

In recent years, G4 motifs that fold into G4 structures have been identified in various viral, bacterial, and eukaryotic genomes [11,14–23]. To confirm the formation of G4 structures and to characterize the types of structures that are formed, oligonucleotides with G4 motif sequences predicted to form G4 structures have been analyzed using a wide spectrum of in vitro methods. However, because sometimes some methods show opposing results, it is common to use three to four different approaches to determine if a certain DNA sequence adopts a G4 structure. These methods include detection of G4 structures by light-up probes [24–26], structural and melting temperature determination by circular dichroism (CD), ultraviolet-visible spectroscopy (UV-VIS) or Förster resonance energy transfer (FRET) [27,28], electrophoretic mobility shift assay (EMSA), dimethyl sulfate (DMS) footprint, polymerase stop assay [29], nuclear magnetic resonance (NMR) [30], and G4 sequencing [5,6]. All the methods described above complement each other and give valuable information about analyzed G4 structures. One common in vivo method to determine G4 structures is chromatin immunoprecipitation combined with quantitative PCR (ChIP-qPCR) or with high throughput sequencing (ChIP-seq) to examine binding of

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proteins, especially helicases and DNA polymerases, to G4 motifs [19,31,32]. Another in vivo method is use of a G4-specific antibody that is used for ChIP experiments or immunofluorescence microscopy [33–36].

G4 motifs are not randomly distributed in the genome but rather are enriched in specific genomic regions with biological roles in basic cellular processes and development [37]. They are linked to various neurological and cancer diseases and thus present a potential target for medical intervention [38,39]. G4 sequencing has identified more than 700,000 G4 structures in the human genome, however 450,000 of these do not match the conserved definition of a G4 consensus sequence motif [5]. Using the consensus sequence motif described above, bioinformatics studies have identified approximately 450 G4 motifs in the Schizosaccharomyces pombe genome [19]. This number excludes most of the G4 motifs in ribosomal DNA (rDNA) and telomeric DNA, which are repetitive regions, and most of the repeats are not included in the current S. pombe genome sequence assembly [40] but are highly enriched in G4 motifs [19].

Both the S. pombe rDNA G4 and telomeric G4 motifs adopt stable G4 structures in vitro [20]. Moreover rDNA G4 structures block DNA replication in vitro [41]. In addition to telomeres and rDNA, and similar to other eukaryotes, G4 motifs are also enriched in promoters of RNA-pol II transcribed genes, dubious ORFs, 3' and 5' untranslated regions, nucleosome-depleted regions, meiotic double-strand break hotspots [19], and dormant origins in S. pombe [42]. By ChIP-seq, approximately 20% of the predicted G4 structures are associated with Pfh1 helicase, the sole S. pombe Pif1 family helicase. Pif1 family helicases are evolutionary conserved [43,44] and they unfold G4 structures efficiently [19,20,31,45–48]. In the absence of Pfh1, regions containing G4 motifs cause replication fork pausing and DNA damage, suggesting that these predicted sites form G4 structures and are resolved by the Pfh1 helicase [19]. In fact, in vitro Pfh1 efficiently unfolds G4 structures [20,49].

Here we used KCl and the G4 stabilizing compound PhenDC3, which increased the pausing sensitivity of DNA polymerase at G4 DNA but not at non-G4 DNA, and this allowed us to optimize a qPCR stop assay [16] to study G4 structure formation in genomic DNA (Fig. 1A). By using the qPCR stop assay, we demonstrated the in vitro formation of G4 structures that had been predicted in S. pombe [19] on the genomic level. The qPCR stop assay was also tested with the S. cerevisiae genome, and we could confirm the formation of previously studied G4 structures. Furthermore, we confirmed the consistency of this method by determining the formation of the G4 structures in oligonucleotides by both CD analysis and primer extension assays. Using ChIP-qPCR, we found that the Pfh1 helicase binds to these sites in vivo. Based on these results, we conclude that the examined G4 motifs in S. pombe fold into G4 structures both in vitro and in vivo, suggesting that many of the in silico predicted G4 structures in S. pombe form G4 structures. Our optimized qPCR stop assay is likely to be a very useful method for detecting the formation of predicted G4 structures in different genomes in most labs as it does not need advanced instruments, and thus providing a better understanding of the biological relevance of G4 structures.

2. Material and methods

2.1. Genomic DNA isolation

Total genomic DNA from S. pombe 972h- and S. cerevisiae S288C were isolated using a Yeastar genomic DNA kit (Zymo Research), and the concentration of the DNA was measured using a Nanodrop ND-100 spectrophotometer (Saveen Werner).

2.2. qPCR stop assay

All oligonucleotides were purchased from Eurofins MWG Operon (Germany). QPCR experiments were performed by Light Cycler® 96 using 96-well white plates (Roche Diagnostics) and Cq values were obtained by the analysis of amplification curves in Light Cycler 96® software version 1.1.0.1320 (Roche Diagnostics). Each qPCR experiment was performed in 10 μl reaction mixtures containing 1× Kapa polymerase (Kapa Biosystems), 0.3 μM of each primer pair (Table S1), 20 ng of S. pombe or S. cerevisiae genomic DNA, and 0.5% (v/v) DMSO. Wherever noted, different concentrations of KCl (0.1, 1, 10, 25, and 50 mM KCl) were added to each reaction and/or different concentrations of G4 stabilizing compounds (PhenDC3, TMPyP4, or BRACO-19). PhenDC3 was synthesized based on a published procedure with some modifications ([50], Supporting methods). TMPyP4 and BRACO-19 were purchased from Sigma-Aldrich. PhenDC3 can also be purchased...

Fig. 1. Stabilized G4 structures inhibit G4 DNA amplification. (A) Schematic of the principle of the qPCR stop assay. Stabilization of G4 structures by K+ ions and/or G4-stabilizing ligands causes an obstacle for the DNA polymerase during DNA amplification. (B) At 25 mM and 50 mM, KCl significantly inhibits DNA amplification of a DNA region containing a G4 motif in S. pombe genomic DNA (G4-1) compared to a non-G4 region (non-G4-1). qPCR reactions were either run in the absence of KCl (control conditions) or in the presence of different concentrations of KCl. Each reaction was run in triplicate, and error bars represent the standard deviation. The y-axis of the graph represents relative DNA amplification (ΔΔCq) for each reaction. * Indicates p < 0.05.
from Sigma-Aldrich. The thermocycler program was 95 °C for 5 min (1 cycle) followed by a 2-step reaction of 85 °C for 10 s and 60 °C for 15 s (33 cycles) in one-point acquisition mode. A total of 5 μL of each qPCR reaction was loaded onto an agarose gel to confirm the presence and size of the PCR product. ΔΔCq values [51] were calculated in Microsoft Excel to express the relative DNA amplification of G4 motif-containing DNA to reference non-G4 DNA using the following equation:

\[
\Delta \Delta Cq = \Delta Cq_{\text{sample}} - \Delta Cq_{\text{control}} - \Delta Cq_{\text{Ref}}\]

G4 - region containing selected G4 motif
Ref - reference region, does not contain G4 motif
Control - control conditions without G4 stabilization
Sample - sample conditions with KCl and/or G4 stabilizing ligands

### 2.3. CD measurements

To fold the oligonucleotides, 50 μM of the oligonucleotides (Table 1) were prepared in 10 mM Tris-HCl pH 7.5 and 100 mM KCl, heated to 95 °C for 5 min, and slowly cooled down to room temperature in a buffer containing 10 mM Tris-HCl pH 7.5 and 100 mM KCl. A JASCO-720 spectrometer with Peltier temperature control was used for the measurements. CD spectra were recorded at 25 °C between 205 nm and 350 nm. Each spectrum was the result of four accumulations. The blank sample contained 10 mM Tris-Cl and 100 mM KCl and was used for baseline corrections. The measurements were performed in a quartz cuvette (0.1 cm path length). All data were normalized to molar ellipticity.

In the CD melting experiments, each sample contained 5% DMSO. The concentration of the G4 ligand PhenDC3 was in a 2:1 M ratio to G4 DNA. The CD signals were recorded at 264 nm for the oligonucleotides G4-4 and G4-15 and at 285 nm for the oligonucleotide G4-8 over a temperature increase from 25 °C to 95 °C at a rate of 1 °C per minute. All data were normalized to molar ellipticity.

### 2.4. Taq DNA polymerase stop assay

TET-labeled primer (1 μM) was annealed to 1.25 μM non-G4 or G4 (G4-4, G4-8 or G4-15 DNA) template in the presence of 100 mM KCl and 10 mM Tris-HCl (pH 7.5) by incubating the mixture at 95 °C for 5 min and slowly cooling down overnight to room temperature. To perform the primer extension reaction, annealed DNA at a final concentration of 40 nM was incubated with either 0.5 μM PhenDC3 or 5% (v/v) DMSO for 30 min in a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 7.5), 1.5 mM MgCl₂, and 200 μM dNTPs at room temperature. Each primer extension reaction was performed in 45 μl reactions and started by the addition of 0.025 U of Taq DNA polymerase (Thermo Scientific) and incubated at 50 °C. Each reaction was stopped by transferring 10 μL of the reaction mixture into an equal volume of stop solution (95% formamide, 20 mM EDTA, and 0.1% bromophenol blue) after 0.5, 1, 5, or 10 min. A total of 5 μL of the reaction mixture was loaded onto a denaturing 10% polyacrylamide gel containing 8 M urea, 25% formamide, and 1× TBE buffer (Tris/borate/ethylenediaminetetraacetic acid). The gel was visualized with a Typhoon Scanner 9200 (GE Healthcare) at the Alexa setting of λ = 532 nm and quantified with the ImageQuant TL software (GE Healthcare).

### 2.5. ChIP-qPCR

ChIP was performed as previously described with the strains NS29 (h⁻ ade6-210 leu1-32::pJk148-phi-13MYC-kanmx6 ura4-D18 his3-D1) and NS112 (h⁻ ade6-M210 leu1-32 ura4-D18 his3-D1) [52]. Briefly, 50 ml of S. pombe cells were crosslinked with 1% formaldehyde for 5 min. Pelleted cells were resuspended in ChIP lysis buffer (50 mM Heps/KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 2 mM PMSF, cOmplete Mini EDTA-free protease inhibitor (Roche)), and glass beads were used to lyse the cells in a FastPrep²-24 instrument (MP Biomedicals). Chromatin was isolated and sheared with a Covaris E220 instrument. Immunoprecipitation was performed using the anti-Myc antibody (Clontech Laboratories), and DNA was purified using the ZYMO Clean ChIP purification kit (Zymo).
Research. qPCR was performed using primer pairs that flanked the G4 sites of interest (Table S1).

3. Results

3.1. DNA regions with G4 structures reduce the amount of amplified DNA

To determine whether the presence of K\(^+\) in PCR reactions stabilizes G4 structures in the genomic DNA, and thus reduces the quantity of full-length products during DNA amplifications, we performed qPCR reactions in the absence or presence of different concentrations of K\(^+\) [53] (Fig. 1). We amplified a region in the S. pombe genome containing a G4 motif (G4-1), and as a control we used a non-G4 region (nonG4-1) (Table S1). To perform the qPCR, we used the Kappa HiFidelity HotStart reaction, which does not contain any K\(^+\), and supplemented each reaction with different amounts of KCl. We found that the relative amount of G4-1 product decreased with increasing amounts of KCl in the reaction, showing that K\(^+\) inhibits the amplification of the G4-1 region (Fig. 1B). When the reaction was supplemented with 25 mM KCl or higher, the amplified G4-1 products were significantly reduced compared to nonG4-1 (Fig. 1B). For instance, at 25 mM and 50 mM KCl only 50% and 40%, respectively, of the G4-1 products were detected compared to untreated samples, while 80% of the nonG4-1 qPCR products were detected at both concentrations of KCl. These data suggest that the presence of KCl inhibits amplification of DNA containing a G4 motif more than a non-G4 site.

3.2. PhenDC3 inhibits amplification of G4 DNA but not non-G4 DNA

To determine if we could enhance the selectivity of inhibiting the qPCR reactions when amplifying G4 DNA over non-G4 DNA, we tested three different well-known G4-binding compounds, TMPyP4 [54,55], BRACO-19 [56], and PhenDC3 [50], could inhibit the reaction further. In these experiments, all qPCR reactions were supplemented with 25 mM KCl, and we tested two different G4 motif-containing sites (G4-1 and G4-2) and two non-G4 control sites (nonG4-1 and nonG4-2) (Table S1). The reactions were treated with DMSO (control) or 5, 20, 100, 250, or 500 nM of each compound.

We found low selectivity between G4 DNA and non-G4 DNA with TMPyP4 (Fig. 2A). Already at 100 nM, TMPyP4 reduced amplification of nonG4-1, nonG4-2, and G4-1 to ‘80%’. Although the inhibition of amplification was stronger for G4-2 (‘85%’ and 40% amplification at 5 nM and 20 nM TmPyP4, respectively), we concluded that the selectivity of TmPyP4 between G4 and non-G4 DNA was not good enough for further qPCR analysis (Fig. 2A).

In the presence of BRACO-19, the amplification of both G4 sites was more inhibited than amplification of the non-G4 regions (Fig. 2B). For instance, at 500 nM BRACO-19 the relative amounts of amplified DNA for G4-1 and G4-2 were reduced to 63% and 24%, respectively, while amplification of the nonG4-1 site was reduced to 77% and the amplification of the nonG4-2 was unaffected (Fig. 2B). These data suggest that Braco-19 is more selective in binding to the G4 DNA compared to the non-G4 DNA. These optimized settings in combination with PhenDC3 could adopt G4 motifs and non-G4 DNA regions compared to BRACO-19 and TMPyP4. Amplification of two G4 motif-containing regions (G4-1 and G4-2) and two non-G4 control regions (nonG4-1 and nonG4-2) was compared in the presence of 0, 5, 20, 100, 250, and 500 nM of the G4-stabilizing compounds (A) TMPyP4, (B) BRACO-19, and (C) PhenDC3. Y-axis represents the relative amplification of DNA with the compounds compared to the 1% (v/v) DMSO-treated sample. All reactions were supplemented with 25 mM KCl and run in triplicate. Closed triangles indicate complete inhibition of DNA amplification. * Indicates p < 0.05, ** indicates p < 0.005, and *** indicates p < 0.0005.

**Fig. 2. The G4-stabilizing compound PhenDC3 demonstrates higher potency and selectivity between G4 motifs and non-G4 DNA regions compared to BRACO-19 and TMPyP4.**

compared to G4-1 (Fig. 2).

Furthermore, we also optimized the denaturation and elongation temperatures (85°C and 60°C, instead of 95°C and 68°C, respectively), and time of elongation (15 s instead of 30 s), all of which resulted in increased inhibition of G4 DNA amplification relative to amplification of non-G4 DNA. These optimized settings in combination with PhenDC3 were selected for all our further studies.

3.3. Identification of G4 structures in the genomic DNA of S. pombe by qPCR stop assay

Next, we examined if 15 randomly selected G4 motifs [19] (Table1) could adopt G4 structures and could be detected by the assay described above, hereafter called the qPCR stop assay. These canonical G4 motifs \((G_3N_{15}G_3N_{25}G_3N_{15}G_3N_{25}G_3N_{15})\) were selected from the S. pombe nuclear genome [19]. We also examined four non-G4 sites. Hereafter, in all
described qPCR stop assays we used 25 mM KCl together with 0, 20, 100, or 500 nM PhenDC3. The control reaction contained 1% (v/v) DMSO and no KCl. The length of the amplified DNA was between 90 bp and 140 bp (Table S1). Under these conditions, the relative DNA amplification of nonG4-1 and nonG4-4 were only significantly reduced in the presence of 500 nM PhenDC3 (to 60%), while nonG4-2 and nonG4-3 were not significantly affected at any PhenDC3 concentrations (Table 1, Figs. S1A and S2A).

The amplification of six of the G4 motif-containing DNA regions (G4-1, 2, 3, 7, 14, and 15) was already significantly reduced in the presence of 25 mM KCl (Fig. S2B). The presence of PhenDC3 inhibited the amplification of the qPCR reactions of eight other tested G4 sites as well (G4-4, 5, 6, 9, 10, 11, 12, and 13). In the presence of 500 nM PhenDC3, full inhibition of 14 out of 15 tested G4 sites was detected (the exception was G4-8) (Table 1, Fig. S2B). To examine the amplified products and the effects of PCR amplification on the final PCR product in different G4 stabilizing conditions, we ran all reactions on an agarose gel. In lanes where a band was detected, we only observed one product for each primer pair. This confirmed that the PCR primers were specific to their genomic locations. At 500 nM PhenDC3, only a faint band was detected for all reactions, except for G4-8 (Fig. S1B). These data suggest that 14 of the 15 tested G4 motifs from the S. pombe genome inhibit PCR amplification probably by adopting a G4 structure.

3.4. The newly identified G4 structures form different G4 structure topologies

Next, we performed CD to examine the secondary structure of these DNA sites. Oligonucleotides with the different G4 motif sequences were first heated and folded in 100 mM KCl, and the CD spectra were recorded. Nine of the oligonucleotides (G4-1, G4-2, G4-3, G4-4, G4-5, G4-7, G4-11, G4-14 and G4-15) folded into parallel G4 conformations with characteristic CD spectra minima around 240 nm and maxima at 264 nm (Fig. S3A, Table 1) [57]. Five oligonucleotides (G4-6, G4-9, G4-10, G4-12, and G4-13) showed a double peak at 264 nm and 290 nm, which is a characteristic spectrum for hybrid structures or a mixture of both parallel and antiparallel G4 structures (Fig. S3B, Table 1) [57]. The oligonucleotide G4-8, which did not affect the qPCR stop assay, showed a maximum at 270 nm. This suggests that this motif does not have a typical CD spectrum for G4 structures and might therefore not fold into a G4 structure (Fig. S3B). These results confirmed the results from the qPCR stop assay that 14 of the 15 tested G4 motifs form G4 structures and suggest that the qPCR stop assay might be a suitable method for examining the formation of G4 structures in genomic DNA.

3.5. The Taq polymerase stop assay confirms pausing prior to the G4 structure

To further examine the formation of G4 structures and to reconfirm with additional methods that the qPCR stop assay is an efficient method for studying the formation of G4 structures, we selected two of the G4 motifs (G4-4, G4-15) that were suggested to form G4 structures by both the qPCR stop assay and CD measurements, as well as G4-8 and one nonG4 site (nonG4-ref) as control sites, and performed the Taq-polymerase stop assay (Fig. 3A) [41,58]. In this assay, a fluorescently labeled primer was annealed to a DNA template that contained either the selected G4 motifs (G4-4, G4-15) or control sequences (nonG4-ref, G4-8), and the newly synthesized DNA was visualized on a denaturing sequencing gel. If a G4 structure is formed on the single-stranded DNA template, the Taq polymerase pauses and creates pausing sites one or two nucleotides before the first G-tract [41]. In this assay the reactions were run at 50 °C instead of 60 °C that was used in the qPCR stop assay, and therefore G4 structures with lower thermal stability might also be detected. Each primer extension reaction was run under two conditions, either 0.5% (v/v) DMSO (hereafter referred to as untreated) or treated with 100 nM PhenDC3 for several different time points. First, we examined DNA synthesis of the non-G4 DNA under both conditions. In both cases, the full-length product increased with increasing incubation time, and there was no significant difference between the treated or untreated reactions (Fig. 3B, C). Also, no specific pausing of the DNA polymerase was observed (Fig. 3B). Next, we examined the synthesis of G4 motif-containing DNA. For G4-4, the untreated reactions showed pausing of the Taq DNA polymerase one nucleotide before the G4 motif, while the PhenDC3-treated reactions caused pausing one and two nucleotides before the G4 structure (Fig. 3B). In the PhenDC3-treated reactions, the amount of full-length products after 20 min was 16% of the total signal, compared to 55% in the untreated reaction (Fig. 3C). The primer extension experiments with G4-15 showed similar results as for G4-4. Pausing of Taq polymerase was observed already in the untreated sample, and pausing in front of the first G-tract rapidly increased upon treatment with PhenDC3 (Fig. 3B). These results suggest that both of these motifs form G4 structures and confirm our results from the qPCR stop assay.

With G4-8 no pausing site was detected in the untreated samples, and after 20 min 54% of the primers were extended to full-length products (Fig. 3C). In the PhenDC3-treated samples, pausing sites were visible after 5 min incubation, and the full-length product was reduced to 35% (Fig. 3C). This suggests that G4-8 might form a G4 structure in the presence of PhenDC3; however, if it does then that G4 structure is not very stable, which also explains the very low pausing efficiency that was detected in the qPCR stop assay.

To examine if tested G4 structures are stable in the qPCR condition and how thermal stability is affected by PhenDC3, we performed CD melting measurements of the G4-4, G4-15, and G4-8 structures in the presence or absence of PhenDC3. First, we examined the CD spectra for G4-4 and G4-15 and found that the spectra only changed slightly upon binding of PhenDC3. The peak at 264 nm decreased in both cases, and a small shoulder appeared at 290 nm, suggesting that binding of PhenDC3 converts the G4 topology from a parallel to an antiparallel structure (Fig. S4). This type of conversion of G4 topology towards antiparallel folding upon PhenDC3-binding has also been observed with human telomeric DNA [59]. Next, we recorded melting curves for G4-4 and G4-15 at 264 nm with and without PhenDC3 treatment. In the untreated samples, both G4-4 and G4-15 showed a decrease in the CD signal when the temperature was increased, suggesting that both structures melted at higher temperatures. The CD signal at 264 nm did not decrease even at 95 °C in the PhenDC3-treated G4-4 sample, suggesting that G4-4 was stabilized by PhenDC3 (Fig. S4). We did not detect a decrease in the CD signal at 264 nm for the G4-15 DNA either. Instead, an increase in the signal was detected between 56 °C and 75 °C, perhaps due to a refolding of the antiparallel structure to the parallel structure at higher temperatures. As we observed earlier (Fig. S3), the CD spectrum of G4-8 did not show a typical spectrum for G4 folding, which agrees with its very low activity in the qPCR stop assay and Taq-polymerase stop assay. PhenDC3 treatment of G4-8 shifted the spectra, and an antiparallel peak at 290 nm appeared, suggesting that PhenDC3 induced an antiparallel fold in G4-8 (Fig. S4). However, we did not detect a typical melting spectrum in either the PhenDC3-treated or untreated samples, suggesting that G4-8 does not form a stable G4 structure under the tested CD conditions.

3.6. G4 structures in genomic DNA of S. cerevisiae are detected by the qPCR stop assay

To determine if the qPCR stop assay can be used to examine G4 formation in other organisms, we tested G4 motifs from S. cerevisiae genomic DNA that have already been confirmed to form G4 structures (Table S3; G4-Sc1, G4-Sc2, G4-Sc3, G4-Sc4) [1] and nonG4-Sc1 as control DNA. All reactions with G4 structures showed reduced levels of amplified products already in the presence of KCl (Fig. S5). Amplification of G4-Sc1 was decreased to 11% and G4-Sc2 to 73%. Amplification of G4-Sc3 and G4-Sc4 was completely inhibited by KCl. The
Fig. 3. DNA synthesis by Taq DNA polymerase is paused on *S. pombe* templates that carry putative G4 structures. (A) Sequences of the G4-4, G4-8, G4-15, and nonG4-ref DNA primer-templates used in the Taq-polymerase stop assay. GGG-tracts are in bold, underlined, and numbered with respect to the direction of DNA synthesis. All DNA sequences originated from the *S. pombe* genome. (B) Taq-polymerase stop assay in the presence of 0.5% (v/v) DMSO or 100 nM PhenDC3. Reactions were stopped after 1, 5, 10, or 20 min. All reactions were loaded on a denaturing 10% (v/v) polyacrylamide sequencing gel and visualized with a Typhoon scanner. Open arrows indicate the position of the primer, black arrows indicate fully extended primer, lines on the right side of each gel indicate G-tracts in the G4 motif, and asterisks indicate the pausing sites of Taq DNA polymerase. (C) Quantification of full-length product of DMSO (solid line, squares) and PhenDC3-treated (dashed line, triangles) samples in each reaction. Error bars represent the absolute error calculated from two independent experiments.
effect on G4-Sc1 and G4-Sc-2 was enhanced by stabilization of the G4 structures by PhenDC3. Finally, amplification of nonG4-Sc1 was unaffected (Fig. S5). These results indicate that the qPCR stop assay can also be used to examine the formation of G4 structures in the S. cerevisiae genome.

3.7. G4 structures show high occupancy of Pfh1 helicase in vivo

Unresolved G4 structures cause DNA damage in vivo, and in S. pombe the only helicase confirmed so far that associates with G4 motifs in vivo [60,19] and unwinds G4 structures in vitro is the Pfh1 helicase [20]. To determine if the G4 structures studied above are formed in vivo, we asked whether Pfh1 is enriched at these G4 motifs in vivo by performing ChIP-qPCR. We performed ChIP using a strain that expressed an epitope-tagged Pfh1-13Myc, and as a control we used an otherwise isogenic strain that expressed untagged Pfh1. In both strains, we performed qPCR and compared the amount of pulled down DNA at the G4-4, G4-8, and G4-15 sites to the gat1+ site (nonG4-1). Pfh1 is a replisome component and therefore binds to all DNA regions at a certain time during replication [60]. However, Pfh1 is more enriched at high-to-replicate sites [52,60,61]. As expected, Pfh1 binding (Pfh1-Myc) was significantly higher than the untagged control (No tag) at all four sites. The binding of Pfh1 to G4-4, but not G4-8, was significantly higher than nonG4-1 (Fig. 4). Although, Pfh1 binding to G4-15 was also significantly higher than nonG4-1, Pfh1 occupancy was less strong at G4-15 than G4-4. These data suggest that G4-4 and perhaps G4-15 fold into a G4 structure in vivo and that Pfh1 binds there to unwind them.

4. Discussion

Confirming the formation of predicted G4 structures is an important step for the subsequent in vitro and in vivo understanding of G4 motif-containing sequences. Here, we describe an easy, fast, and reliable qPCR method, which we call the qPCR stop assay, for the identification of G4 structures on a small scale directly from isolated genomic DNA. To confirm the formation of G4 structures, we combined K+ and a commercially available G4 stabilizing ligand, PhenDC3, and optimized a 2-step qPCR method. The principle of the assay is based on DNA amplification of an approximately 100 nucleotide region that contains a G4 motif. If the G4 motif adopts a stable G4 structure, it will form an obstacle for the DNA polymerase during DNA amplification. This obstacle pauses/stalls DNA replication, which leads to reduced amounts of final product compared to a non-G4 region (Fig. 1A). The final product of the qPCR reaction is bound by SYBR® green and quantified by an RT-PCR instrument after each replication cycle.

First, we identified the most suitable G4 stabilizing conditions by testing different amount of KCl and G4 binding ligands. Monovalent cations such as K+ bind in between the G-quartets, and we showed that 25 mM KCl was a sufficient concentration for significant G4 stabilization but did not significantly inhibit amplification of a non-G4 region (Fig. 1B). Next, we tested three different commercially available G4 binding ligands – TMPyp4, Braco19, and PhenDC3 – in the qPCR stop assay. TMPyp4 showed very poor selectivity towards G4 DNA over non-G4 DNA, which confirmed previous studies [62–64]. PhenDC3 and Braco19 had the best selectivity for G4 structures over non-G4 DNA. The efficacy of Braco-19 confirmed previous work showing the detection of G4 structures in HSV-1 DNA by Taq-polymerase stop assay and qPCR [16]. To confirm that the predicted G4 motifs formed G4 structures, we used PhenDC3 because its strong stabilization of G4 structures translated into stronger activity compared to Braco19 in the qPCR stop assay (Fig. 2). These data also suggest that the qPCR stop assay may also be a suitable method when examining the selectivity of different novel G4 stabilizing ligands, and not only novel predicted G4 motif sites, because this assay can easily be scaled up and performed in 96 or 384 well plates.

Furthermore, we focused on the eukaryotic model organism S. pombe in which approximately 450 possible G4 structures were predicted as unique G4 structures [19]. In S. pombe, only G4 structures from ribosomal and telomeric DNA repeats, and the cdc13+ promoter have been confirmed [20,41]. We selected 15 unique G4 motifs of S. pombe [19], and we found that 14 out of 15 G4 motif-containing regions showed significantly less final product under G4-stabilizing conditions, six of them already after addition of KCl. The effect was enhanced by supplementing the reactions with PhenDC3. Amplification of non-G4 sites was slightly affected only in two of four reactions and only at very high PhenDC3 concentration (500 nM), suggesting possible binding of PhenDC3 to non-G4 DNA at such high concentrations. The results of the CD spectra analysis of the selected G4 oligonucleotides was in line with the results from the qPCR stop assay. The addition of the lowest concentration of PhenDC3 affected the amplification of eight G4 motif-containing sites (G4-1, 2, 3, 4, 5, 7, 14, and 15). All eight G4 oligonucleotides showed CD spectra that were characteristic for parallel G4 conformations, suggesting that parallel G4 structures are more stable and that PhenDC3 might be more selective towards parallel G4 topologies. However, by increasing the PhenDC3 concentration in the reactions, G4 structures with other topologies were also detected. Furthermore, the qPCR stop assay can be used to identify G4 structures in other organisms. For example, we showed inhibition of DNA replication of all four tested G4 structures in S. cerevisiae [1].

The only negative result in the qPCR stop assay was obtained for G4-8 DNA amplification, suggesting that the G4-8 sequence does not fold into a G4 structure. In fact, neither the CD spectrum nor the CD melting experiments with G4-8 DNA showed a typical pattern of G4 DNA folding (Figs. S3 and S4) or G4 DNA melting (Fig. S4). However, in the presence of PhenDC3 a CD spectrum for G4-8 with a maximum around 290 nm, characteristic for antiparallel folding, was observed (Fig. S4). Similarly, the examination of G4-8 DNA in the Taq polymerase stop assay showed pausing sites in the reaction supplemented with PhenDC3, and no pausing in the control reaction (Fig. 3). Furthermore, Pfh1 helicase occupancy at the G4-8 site in S. pombe cells was not significantly higher than a Pfh1-independent control site, suggesting that G4-8 might not form a G4 structure in vivo or that G4-8 is not unwound by Pfh1 (Fig. 4). To better discriminate between G4 sites, further analysis of in vivo G4 formation is for the moment ongoing in our laboratory. Taken together, our results suggest that G4-8 might not fold into a G4 structure or if it does that it is an unstable G4 structure, perhaps as a consequence of the longer loops of 15, 10, and 5 nucleotides in the predicted G4 structure. However, it is important to point out that we perform the qPCR-stop assay at much higher temperatures than...
yeast cells commonly grow at. Therefore the qPCR stop assay may show some false negative G4 sites, and thus these sites should also be analyzed with other complementary methods.

In conclusion, using the described qPCR stop assay, we showed that 14/15 and 4/4 predicted G4 structures in S. pombe and S. cerevisiae, respectively, fold into G4 structures, demonstrating that the qPCR stop assay is a reliable method for identifying G4 structures directly in genomic DNA in different organisms.

Author contributions
JJ, IO, AM, and KC performed the experiments. All authors analyzed the results. JJ and NS wrote the manuscript, and all authors read, contributed, and approved the final manuscript.

Declaration of Competing Interest
The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data
Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.dnarep.2019.102678.

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