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Genomic instability at a polypurine/polypyrimidine repeat sequence

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GENOMIC INSTABILITY AT POLYPURINE/POLYPYRIMIDINE REPEAT SEQUENCE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

NATHEN S. ZAVADA
B.S., Wright State University, 2021

2022
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Nathen S. Zavada ENTITLED Genomic Instability at a Polypurine/Polypyrimidine Repeat Sequence BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Zavada, Nathen S. M.S. Department of Biochemistry and Molecular Biology, Wright State University, 2022. Genomic Instability at a Polypurine/polypyrimidine Repeat Sequence

Microsatellite repeat sequences have been shown to induce replication stalling, fork collapse, (DSBs), and possibly stimulate break-induced replication. In this study we use a dual-fluorescent HeLa model that is designed to monitor recombination at an ectopic site through use of flow cytometry and inverse PCR with a microsatellite in the lagging strand for DNA synthesis. To test the stability of the 78 bp polypurine/pyrimidine repeat from the PDK1 locus, we subjected cells to replication stress drugs designed to induce DSBs and measure BIR. The study revealed that polypurine repeat cells undergo endogenous stress contributing to instability at the ectopic site as well as slow cell growth. Further, we show that there is an orientation dependency for instability with the (Pu)$_{78}$ cells being more unstable. Lastly, we present a novel candidate for a protein involved in break-induced replication, COPS2. Preliminary experiments show this protein produces unique recombination patterns when knocked down.
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INTRODUCTION

Microsatellite sequences can form non-B-DNA structures

The first identification of microsatellites was in 1960s experiments looking into DNA equilibrium of sedimentation in CsCl gradients and identifying unique banding patterns for different species (Kit, 1961.) In these experiments, microsatellite sequences produced what was termed a ‘satellite’ band from mouse DNA. The different banding pattern can be explained by the contents of the microsatellite. In GC-rich repeat sequences, due to the decrease in hydration at these base pairs, it would be expected that microsatellite sequences would appear less dense than the bulk of the DNA. However, in AT-rich repeats, the opposite could be expected where the microsatellite is denser than the bulk of the DNA.

The differences in the molecular nature can be accounted partially by the instability seen at these sequences. Also known as tandem repeats, microsatellites have mutation rates between 10^{-3} to 10^{-6} per cell division which is 1 to 10 orders of magnitude greater than point mutations (Gemayel et al., 2012; Verstrepen et al., 2006). These tandem repeats consist of 1-9 nucleotides and the increase in mutation rate can be explained by expansions and contractions from DNA strand-slippage replication and recombination (Gemayel et al., 2012). In the strand-slipping model, mispairing occurs between the
template strand and nascent strand; the template strand can loop out resulting in unit contractions or the nascent strand can loop out resulting in a DNA expansion (Fig. 1). Expansions and contractions can also occur during recombination, in which microsatellite sequences can see unequal crossover events as well as gene conversion, often through DNA translocations (Gemayel et al., 2012).

These DNA ‘loops’ that form in the nascent and template DNA are much more complex. Depending on the microsatellite sequence, several different structures can arise. Khristich and Mirkin (2020) highlight several of these structures (fig. 2); in polypurine or polypyrmididine sequences, a DNA strand (either from the same strand or a different one) can loop back around and hydrogen bond through Hoogsteen base-pairing (Takahashi and Sugimoto, 2021) forming triple stranded DNA. In a sequence that contains a G-quadruplex consensus motif, four sets of triplet guanine residues ($[G]_3[N]_n[G]_3[N]_n[G]_3[N]_n[G]_3[N]_n$), interspersed throughout a localized region can form this quadruple stranded DNA structure. Another structure, like the ‘loops’ shown in figure 2, arise from (CNG)$_n$ repeats where ‘n’ is any nucleotide. When present on a single strand, these sequences form what is known as a harpin. However, due to the complementarity of DNA, these sequences can be present on both strands giving rise to structures known as cruciform DNA. Lastly, repeated sequences of A and T nucleotides can cause DNA to denature mimicking what is known as a DNA unwinding element (DUE). DUEs are present endogenously at replication origins, but with repeat expansions at A-T repeats, new DUE’s can be created at these sequences. The DNA becomes unwound primarily due to a decrease in hydrogen bonding because A-T nucleotides only form two hydrogen bonds and G-C nucleotides form three.
DNA repair of microsatellite sequence-based genomic instability

As highlighted, microsatellites can form atypical DNA structures, other than the double helical strands seen in canonical DNA. When these structures are encountered by an ongoing replication fork, they can cause the fork to stall and collapse leading to DNA double-strand breaks (DSBs) (Leffak, 2017). The cell can repair this broken DNA using one of two mechanisms: nonhomologous end joining (NHEJ) or homologous recombination (HR).

Nonhomologous end-joining

Following a DNA DSB, the break is identified and bound by the Ku70/Ku80 heterodimer complex, which then serves as a binding site for other NHEJ proteins. These other proteins help facilitate the ligation between the broken DNA strands, or, if the strands are not compatible for ligation, may recruit other proteins that serve further DNA end-processing such as trimming, filling-in, or blocking-end removal (Chang et al., 2017; Seol et al., 2018). In this classical method of NHEJ (C-NHEJ), mutations of any form that are acquired during the DSB are not corrected and thus inherited considering this a mutagenetic form of DNA repair. A subset of NHEJ exists termed alternative NHEJ (A-NHEJ) that is independent of the Ku70/Ku80 complex and other NHEJ proteins. The prime characteristic of this model is microhomology-mediated end joining (MMEJ) where homology-based regions of 2-20 nucleotides (nt) are exposed and allowed to anneal by an unknown mechanism. This pathway is known to be dependent on the MRX complex to facilitate the formation of the short overhangs [MRX complex: Mre11-Rad50-Xrs2]. This pathway can also have interference from the ssDNA binding protein
complex RPA, which may prevent the annealing and ligation steps, resulting in further DNA damage (Chang et al., 2017).

Homologous recombination

Homologous recombination is a DNA repair pathway that typically relies on large regions of resected DNA and invasion into a sister chromatid. This pathway is considered high-fidelity given that DSBs are repaired using an unbroken chromosome as template to correct mutations that may have been incurred. All sub-processes of HR involve the initial 3’ ssDNA overhang generation, a step called pre-synapsis. Like NHEJ pathways, this is carried out by the MRX complex as well as the 5’-3’ exonuclease Exo1 acting in concert (Li and Heyer, 2008; Wright et al., 2018). Generating 3’ overhangs are important to create a strand that can search for homology and strand invasion, which is largely facilitated by Rad51. Nuclear ssDNA is coated by RPA which has a much higher affinity for ssDNA than Rad51, making it a potent competitor. The loading of Rad51 is accomplished by mediator proteins, whose exact mechanism is unknown, but have been identified as Rad55-Rad57 and Rad52 in budding yeast (Li and Heyer, 2008). This does not mean that RPA is an inhibitor of HR, however; contrarily, it is key in eliminating secondary structures that arise in ssDNA and stimulates HR.

There are several sub-processes of HR but of note to this study is the pathway known as break-induced replication. This sub-process has been largely described in budding yeast has the same preceding steps as standard HR, where extensive 5’ to 3’ resections create 3’ ssDNA overhangs, followed by Rad51-dependent nucleofilament formation. BIR begins to differ at the DNA synthesis step following strand invasion which can be delayed due to the disassociation and reassociation of the 3’ end to the template. This delay in synthesis can cause G2/M cell cycle arrest meaning most BIR events occur
during G2/M phase (Kramara et al., 2018). There are also key differences during the synthesis phase of BIR; synthesis proceeds by a migrating D-loop pushed by an unresolved Holliday junction that displaces the newly synthesized ssDNA. The lagging strand is then used as template for the leading strand resulting in conservative replication (Fig. 3.). This process is also likely to cause DNA translocations and template switching (Malkova and Ira, 2013). It has been shown that at sites of repetitive elements (a.k.a microsatellites), which are regularly dispersed throughout the genome, that a cell undergoing BIR can find homology at these repetitive sites, even if on a different chromosome. The different chromosome would thus be used as template resulting in gross chromosomal rearrangements. Template switching can occur close to the BIR initiation site, most likely due to the dissociation-reassociation during the homology search phase, and this can also lead to DNA translocations.

**Microsatellites and Disease**

When large regions of DNA are being expanded, contracted, and mutated, there are implications that can occur downstream and can result in several neurodegenerative diseases (Brouwer et al., 2009). When an expanded or contracted repeat in the coding region is translated, the resulting protein can have a gain of function, a loss of function, or cause the entire protein to be misfolded. Mental retardation seen in FRAXA which is caused by (CGG)_N repeat expansion in the FMR1 gene is an example of this, where expansion causes hypermethylation at the promoter therefore silencing its transcription in neurons. Repeat expansion and contraction not only can affect protein messages but can also interrupt mRNA information. Repeats that occur in non-coding regions (promoter, UTR, intron/exon boundaries) can interfere which the production of the mRNA itself or can cause misfolding of the mRNA inhibiting the downstream translation step. Figure 4
shows a list of microsatellite associated diseases and their genetic location.
Figure 1. A model of DNA strand-slippage leading to expansions and contractions.

During replication of microsatellite sequences, loops can form on nascent and template strand DNA. Depending on which strand the loop forms will lead to contraction or expansion of microsatellite units in the nascent DNA.
Figure 2. **Microsatellite sequences give rise to non-B-DNA structures.** (A)

Polypurine/polypyrimidine repeat sequences can give rise to Hoogsteen hydrogen bonding creating a triple-stranded DNA structure. (B) Sequences that contain a G-quadruplex consensus motif ($([G]_3[N]_n[G]_3[N]_n[G]_3[N]_n[G]_3[N]_n)$) can form this non-B-DNA structure. (C) (CNG)$_n$ repeats, where N is any nucleotide can form a hairpin structure. (D) A-T repeats, because of their low hydrogen bonding content, can mimic DNA unwinding elements which causing unwound DNA structures.
4. Inherited mutations

Figure 3. **A model of break-induced replication.** During BIR, strand invasion can occur in the sister chromatid, or a non-allelic chromosome and synthesis will occur via a migrating D-loop. The Holliday junction is not resolved, and the nascent strand is displaced before mutations can be corrected. The nascent strand is used as template inheriting any mutations in a conversative replication fashion. *Modified from Malkova and Ira (2013) Curr. Opin. Genet. Dev.*
Figure 4. **Microsatellite associated diseases.** Microsatellite expansion and contractions can occur anywhere throughout a gene and contribute to disease.

Microsatellites can interfere with replication, transcription, mRNA folding, translation, and protein folding.
MATERIALS AND METHODS

Cell lines and cell culture

The two primary cell lines used are termed TTR and TTF. TTR contains the 78 bp polypurine (Pu) repeat sequence from the polycystic kidney disease type 1 (PKD1) locus, that is placed in the lagging strand for DNA synthesis directly next to C-myc in a multiple-cloning site (MCS) region. TTF contains the mirror of this, where a 78 bp polypyrimidine (Py) sequence is placed in the lagging strand for DNA synthesis directly next to C-myc. The ectopic sites also contain the Alu elements, eGFP, dTOM, a thymidine kinase (TK) selection marker, and neomycin & hygromycin selection markers (Gadgil et al., 2020) The full name of these cell lines is termed DF2MycTTR/TTF.

Figures 6 and 7 show a schematic of the ectopic site and the mirror repeat. Cell lines were made using a HeLa acceptor which contains a single FRT site and were co-transfected with a plasmid expressing FLP recombinase & the plasmid containing either the Pu or Py repeat sequence (Fig. 5). Thus, the ectopic site can only be integrated once per HeLa genome.

All cells are maintained in homemade Dulbeco’s Modified Eagle’s Medium (DMEM) with 10% newborn calf-serum (NCS) (R&D Systems, S11520), 5% penicillin/streptomycin (P/S), (Corning®, 30-002-CI) and 5% CO2.
Flow cytometry

Following drug/siRNA treatment or for analysis of a new cell line, cells were washed with PBS, trypsinized, harvested, and centrifuged at 300 g x 3 min. They were then washed 2x in 300 μL of PBS and passed through a 35 μM filter (Falcon®, 352235) to separate clumped cells. Cells were then passed through the C-Flow® Plus Accuri Cytometer and analyzed using the CFlow Plus software. Limits were set at 20,000 cells, and a FSC vs SSC plot was generated. Gates excluded cells on the outer limits of the plots to exclude possible cellular debris. Figure 9 shows an example of flow cytometry analysis and where cells will appear in each quadrant following certain recombination patterns.
Figure 5. Construction of the ectopic site. The HeLa genome was transfected with a plasmid containing a single FRT site and two selection markers. This site could only be integrated in one location within the genome. The modified HeLa cells are then transfected with another plasmid carrying the c-myc origin of replication, non-B-DNA, another FRT site, and the neomycin resistance gene. The finished product will contain three selection markers and two FRT sites.
Figure 6. **A schematic of the ectopic site.** The ectopic site contains three selection markers: hygromycin (Hyg), neomycin (Neo), and thymidine kinase (TK). There also three Alu elements that are frequent hotspots for recombination, two FRT sites, a high-firing C-myc origin of replication, two fluorescent reporters, eGFP & dTOM, and the polypurine or polypyrimidine repeat (Pu\textsubscript{78}/Py\textsubscript{78}).
Figure 7. A schematic of the 78 bp polypurine/polypyrimidine repeat from the PDK1 locus. The TTR and TTF cell lines were derived from the PDK1 gene intron 21 where the 78 bp mirrored repeat is found. Black triangles show the mirrored repeat. The TTR cell line contains the purine repeat in the lagging strand for synthesis while the TTF cell lines contains the pyrimidine repeat in the lagging strand for synthesis.
Figure 8: A schematic of flow cytometry analysis. Cells that contain two fluorescent markers appear in the ‘double-positive’ quadrant. Cells that lose the red fluorescent markers appear in the ‘green’ quadrant and cells that lose the green fluorescent marker appear in the ‘red’ quadrant. Finally, cells that lose both fluorescent markers appear in the ‘double-negative’ quadrant.
Translocation PCR

To detect DNA translocations in vivo, first had to identify what possible translocations can occur. This was done previously by next-generation sequencing to which there were translocations detected from the ectopic site to: DENND3, DDX10, Pote Ankryin, Rad51, Chromosome 16, Chromosome 21, and Chromosome 4. A forward primer was designed inside the ectopic site and a reverse primer was designed within the translocated gene, or vice versa. If there was truly a translocation, then a PCR product would be obtained. 30-100 ng of DNA template was used for PCR reactions and were ran using the Q5® High-Fidelity 2x Master Mix (NEB, M0492S) with a touchdown protocol. Specific conditions are listed below with each primer set. Following PCR, samples were run on a 1% agarose gel and imaged for product formation using the Azure Biosystems C300 imager.

Key: T_D = denaturation temperature; T_A = annealing temperature; T_E = extension temperature

DENND3 primer set 1

Forward: (5’ – AAGAACAGAGGAGGCCAGCA – 3’)
Reverse: (5’ – TTCTCGTTGGGTCTTTGCTC – 3’)

Conditions: (T_D: 98°C, T_A: 68°C, T_D: 75°C, 10 cycles; T_A down to 63°C for 25 cycles)

DENND3 primer set 2

Forward: (5’ – TGTGCCCGTCTGTCTTGTG – 3’)
Reverse: (5’ – AGGGACAGACAGGCCATTTT – 3’)

Conditions: (T_D: 98°C, T_A: 68°C, T_D: 75°C, 10 cycles; T_A down to 63°C
for 25 cycles)

**DDX10 primer set**

Forward (5’ – AGAAGGGTTGCAATAGGAGAAAAAGA – 3’)

Reverse (5’ – AGAGATCTCTGCTTACCCGCA – 3’)

Conditions: (T<sub>D</sub>: 98°C, T<sub>A</sub>: 68°C, T<sub>D</sub>: 75°C, 10 cycles; T<sub>A</sub> down to 63°C for 25 cycles)

**Rad 51 primer set**

Forward (5’ – GCCGTITTTTAGGTTTTGATGG – 3’)

Reverse (5’ – AATTGTCCCCAAGTTGATCTG – 3’)

Conditions: (T<sub>D</sub>: 98°C, T<sub>A</sub>: 68°C, T<sub>D</sub>: 75°C, 10 cycles; T<sub>A</sub> down to 63°C for 25 cycles)

**Chromosome 16 primer set**

Forward (5’ – ACCGGTAATGGCAAAAAACGTGA – 3’)

Reverse (5’ – TTGGGGTTGGCCATTGGTAA – 3’)

Conditions: (T<sub>D</sub>: 98°C, T<sub>A</sub>: 63°C, T<sub>E</sub>: 75°C, 10 cycles; T<sub>A</sub> down to 58°C for 25 cycles)

**Chromosome 21 primer set**

Forward (5’ – CACGTTTGACCATTACCGTTTC – 3’)

Reverse (5’ – GTCCTTTCCACATTGTTTGT – 3’)

Conditions: (T<sub>D</sub>: 98°C, T<sub>A</sub>: 63°C, T<sub>E</sub>: 75°C, 10 cycles; T<sub>A</sub> down to 58°C for 25 cycles)

**Pote Ankryin primer set**

Forward: (5’ – AACGTACACAGCAGCATACTCA – 3’)
Reverse: (5’ – TCCATGCCGAGAGTGATCCC – 3’)

Conditions: (TD: 98°C, TA: 63°C, TE: 75°C, 10 cycles; TA down to 58°C for 25 cycles)

**Inverse PCR**

Inverse PCR (iPCR) is a technique that can give information about recombination with the ectopic site, the sister chromatic, or a non-allelic chromosome following a DNA double-strand break. To begin, 500-1500 ng of DNA of interest is digested with 10 μL XbaI (20,000 units/mL), a frequently cutting restriction enzyme (R.E.), 20 μL of 10x CutSmart® Buffer, and allowed to digest at 37°C overnight (O/N). The R.E. is heat killed at 65°C for 20 min. Then the DNA is diluted to ~0.4 ng/μL and mixed with 1 μL of T4 DNA Ligase (NEB, M0202S) and 500 μL of 10x T4 DNA Ligase Buffer (NEB, B0202A), forming circular DNA products. Ligations were done at 4°C O/N.

Approximately 30-100 ng of DNA is used as template for PCR using Q5® High-Fidelity 2x Master Mix (NEB, M0492S) with a touchdown protocol. The PCR products were run on a 1% agarose gel and imaged using Azure Biosystems C300 imager. These samples were mixed with many other iPCR samples from other lab members and sent out for next-generation sequencing. Because of this, primers were barcoded to identify which reads belong to each specific reaction. Primers and conditions are listed below.

**Barcodes are italicized. Figure 9 shows a schematic of inverse PCR.**

**Key:** TD = denaturation temperature; TA = annealing temperature; TE = extension temperature

**siCOPS2 TTR primer set:**

Forward: (5’ –GTCCGCCGCTACCCCCGACCACA – 3’ )
Reverse: (3’ – atgccagCCGCCCTTGATCTGAATTT – 3’)

siCTL TTR primer set:

Forward: (5’ – GTCGCCGGCTACCCCGACCACA – 3’)

Reverse: (3’ – cagcaggeCCGCCCTTGATCTGAATTT - 3’)

siCOPS2 TTF primer set:

Forward: (5’ – GTCGCCGGCTACCCCGACCACA – 3’)

Reverse: (3’ – cggccttGATCTGAATTTCCCGAAT – 3’)

siCTL TTF primer set:

Forward: (5’ – GTCGCCGGCTACCCCGACCACA – 3’)

Reverse: (3’ – ctgctatCCGCCCTTGATCTGAATTT – 3’)

20.
Figure 9. **A schematic of inverse PCR.** Following a DNA double-strand break, the cell can recombine with the sister chromatid or at a non-allelic chromosome. To be able to detect this, the DNA is cut with a frequently cutting restriction enzyme (R.E.) and then diluted to a very low concentration. This allows the DNA to self-ligate, forming circular DNA. Primers are designed within the ectopic site facing away from each other allowing them to capture any recombination events that may have occurred. These DNAs are then sent for long-read next-generation sequencing.
siRNA transfection

Cells of interest are grown in a 6-well dish until 80-90% confluence. To begin the transfection, one tube containing 250 μL Opti-MEM™ + GlutaMAX™ Reduced Serum Media (Gibco®, 51985-034) is mixed with 10 μL of Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen™, 13778-150). In a separate tube, 250 μL of OptiMEM™ is mixed with 50-100 nM siRNA. Both tubes are incubated at 37°C for 5 min. The tube containing the siRNA is then added to the tube containing the Lipofectamine™ and incubated at 37°C for 20 min. Next, 500 μL of the transfection solution is added to 1.5 mL Dulbecco’s Modified Eagle Medium (DMEM) containing 10% newborn calf serum (NCS) with no antibiotics. The cells are washed with phosphate-buffered saline (PBS) before adding media or transfection solution. The transfecting cells are incubated at 37°C for 48 h. They are then moved to a 10 cm² culture plate containing 10% NCS, 5% P/S and allowed to recover for 4 days before analysis via flow cytometry.

VE-821, pyridostatin, and telomestatin drug treatment assays

Several different drugs were used to induce replication stress. VE-821, an ATR kinase inhibitor (Selleckchem, S8007) was used at concentrations of 1 μM, 5 μM, and 10 μM to assay for dose response. 1 μM was found to be the optimal dosage (see Fig. 13. Pyridostatin and telomestatin, courtesy of Dr. Kazuo Shin-ya (Biomedical Information Research Center, National Institute of Advanced Industrial Science and Technology, Tokyo, Japan) are both G-quadruplex stabilizing drugs but telomestatin is a more potent stabilizer. Based on previous experiments from other lab members, 0.5 μM telomestatin was the recommended dosage. A dose response assay was performed with pyridostatin using 2 μM, 10 μM, and 20 μM concentrations to which 2 μM was found to be most...
optimal. Figure 10 shows a schematic of the drug and siRNA treatments and their interference in the DNA damage response pathways.
Figure 10. A model of drug & siRNA treatments and their inhibition of the DNA damage response. Telomestatin and pyridostatin are both G-quadruplex stabilizing drugs that can induce DSBs. siRad9A is targeting the 9-1-1 complex, a signaling protein that detects DNA damage. VE-821 inhibits ATR signaling, a master regulator of DNA damage signaling. COPS2, a subunit of the COP9 signalosome, may be an inhibitor of break-induced replication so siCOPS2 is aimed at stimulating BIR.
Bioinformatics

DNA was collected from cells that were treated with siCOPS2 and iPCR was performed with barcoded primers. Following PCR, samples were pooled together with other DNAs from members in the laboratory, processed, and sent to Azenta Life Sciences for long-read next-generation sequencing. Reads were obtained for each treatment that were processed including removal of duplicated reads and reads that did not map to the ectopic site. Analysis primarily included looking at translocations and the neighboring areas using Integrated Genomics Viewer (Robinson et al., 2011), BLAST (Madden, 2002) and SnapGene® software (from Insightful Science; available at snapgene.com)

Gel electrophoresis and imaging

DNAs were run on a 1% agarose gel with TAE + ethidium bromide at 90 – 110 volts. Gels were imaged on the Azure Biosystems C300 imager using the ‘gel’ function and auto exposed to UV fluorescence.

DNA Isolation

Cells were washed with PBS, trypsinized, and harvested. Cells were centrifuged at 300 x g for 3 min and resuspended in 300 μL PBS. The Wizard® Genomic DNA Purification Kit (Promega, A1120) was used to collect genomic DNA. A NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher, ND-ONE-W) was used for DNA quantification.
RESULTS

I. Engineered TTR and TTF cell lines

After engineering the TTR and TTF cell lines, it was important to monitor the expression of the dual-fluorescent markers, eGFP and dTOM, as well as to ensure the insert sequence was the correct length. Previous experiments had already indicated that the TTR cell line tends to be more unstable than the TTF counterpart. To test this, the TTR cell line was monitored by flow cytometry over the course of 14 days. Over this time, the number of yellow cells had decreased by 20%. This exact assay was not performed on the TTF cell line, however, flow profiles were gathered over the course of >100 days to compare the loss of yellow cells. Figure 11 shows that over the 100 days, there was about a 10% decrease in the number of yellow cells compared to 20% loss in yellow cells of the TTR cell line in 14 days.

As mentioned, it’s also important to check that the insert is the correct size. For this, PCR primers were designed that would go across the insert sequence with a predicted product size of 992 bp. Figure 12 shows the gel taken following PCR with the correct product size. These bands were excised and sent for sequencing to ensure that the insert has not been mutated. Sequencing results showed the polymerase was able to obtain the first 20 or so nucleotides before the sequencing falls off (data not shown). This is likely due
to the polymerase not being able to go around the G-quadruplex or triplex DNA that these sequences are prone to form.
Figure 11: **TTR cells show endogenous instability over time.** HeLa cells were transfected with the TTR or TTF sequence and visually selected for the appearance of yellow cells. Flow cytometry analysis was performed following selection on day 1, day 6, and day 13 following transfection to monitor the stability of the ectopic site.

(A) Flow cytometry results for TTR cells. (B) Flow cytometry results for TTF cells.
Figure 12: **Verification of TTR and TTF insert into DF2Myc cell line.** PCR primers were designed across the insert site. PCR products have a predicted size of 992 bp if they contain the polypurine/polypyrimidine repeat sequence. Bands were excised and sent for standard sequencing.
II. **Diagnosing an appropriate VE-821 dosage**

To determine if there is any orientation dependent instability, drugs were selected that can induce replication stress. One of the drugs chosen was VE-821, an ATR inhibitor. By inhibiting ATR signaling, the cell cannot properly respond to DNA damage that is typically intrinsic at these microsatellite sequences and will thus result in a double strand break. Before treating the cells however, dosage assay was designed to determine at which dosage provides a desirable phenotype but does not prove toxic to the cells. TTR cells were used as the model as these cells were predicted to be more sensitive than the TTF cell line. TTR cells were dosed at 1, 5, and 10 µM VE-821 for 24h and let recover for four days and then pictures were taken to observe morphology. We noted that at a 10 µM dosage, TTR cell morphology was greatly impacted, and many cells had died. This was true of the 5 µM dosage but to a much lesser extent (Fig. 13) We decided to move forth using both the 1 µM and 5 µM dosage to investigate at any variability in instability.

III. **VE-821 shows orientation dependent instability**

After finding an appropriate VE-821 dosage, TTR and TTF cells were dosed with 1 and 5 µM VE-821 and flow cytometry was performed to see how the ATR inhibitor affected the ectopic site. We dosed with 10 µM VE-821 as positive control knowing that most cells would die. Following a 4-day recovery, figure 14 shows a 2% decrease in yellow cells at 1 µM VE-821 and a 4% decrease in yellow cells at 5 µM VE-821 in the TTR cell line. We noted a 5% decrease in yellow cells when dosing at 10 µM VE-821 but this result
was attributed to extensive cell death, and the sample size is much smaller.
Contrarily, when treating the TTF cells with 1, 5, and 10 μM VE-821 we noted only a 1% decrease in yellow cells across all treatments.

**IV. VE-821 increases telomestatin sensitivity in green cell populations**

As VE-821 showed an orientation dependent instability in the TTR cell line, we wanted to know if this effect was exacerbated with treating with the potent G-quadruplex stabilizing drug, telomestatin (TMS). The TTR cell line is more prone to forming these G-quadruplex structures and so by stabilizing them in the presence of an ATRi, we expected to see a greater amount of instability in the TTR cell line.

TTR and TTF cells were treated with 1 or 5 μM VE-821 with or without the presence of 0.5 μM TMS. Interestingly, flow cytometry analysis revealed an increase in yellow cells during each progressive treatment in the TTR cell line while TTF cells only showed an increase in yellow cells with treated with 5 μM VE-821 and TMS (Fig. 15). The was the opposite expected result so further theories were postulated (See Discussion)
Figure 13: **VE-821 dosage assay on TTR cells.** TTR cells were treated with 1 uM, 5 uM, or 10 uM VE-821 and photos were taken following four-day recovery to observe morphological changes.
Figure 14: **VE-821 shows orientation dependent instability.** TTR and TTF transfected cells were treated with 1 uM, 5 uM, or 10uM VE-821 for 24h and left to recovery for four days. Flow cytometry analysis was performed following recovery. (A) Flow cytometry analysis for TTR cells following variable VE-821 treatment. (B) Flow cytometry analysis for TTF cells following variable VE-821 treatment.
Figure 15. **VE-821 does not increase TMS sensitivity in TTR and TTF cells.** TTR and TTF cells were treated with either 1 μM or 5 μM VE-821 and were treated with or without 0.5 mM TMS for 24 h and left to recover for four days. Flow cytometry analysis was performed following recovery. (A) Flow profile from TTR cells. (B) Flow profile from TTF cells.
V. **RAD9 knockdown causes slight orientation dependent instability**

We noted that inhibiting ATR signaling could cause instability in the TTR/TTF cell line in an orientation dependent manner, we wanted to see if knocking down another DNA damage response protein could produce similar results. For this we chose Rad9 which plays a critical role in cell cycle phase specific DNA damage repair (Pandita, 2006) and is upstream of ATR signaling. Like ATR inhibition, we predicted that knocking down a DNA damage repair protein would lead to double-strand breaks at the microsatellite sequence. TTR and TTF cells were treated with siRAD9 or siCTL for 48 h and then left to recover for 4-days. Figure 16 shows that following siRAD9 treatment there is a 16.1% decrease in the number of yellow cells compared to control in the TTR cell line. This is compared to a 2% decrease in the number of yellow cells in the TTF cell line. Curiously, when we looked at the siCTL TTR cells, we saw a 24.4% decrease in the number of yellow cells. Initially it was assumed that the siCTL was mis-labeled but this same result was observed in several trials as well as by other lab members. It is very likely that the process of transfection itself is presenting as replication stressor as this result is also noted in the TTF cells which show a 1% decrease in siCTL yellow cells.
Figure 16: RAD9 knockdown causes orientation dependent instability. TTR and TTF cells were transfected with siRAD9 and siCTL and allowed to recover for 4 days. Flow cytometry analysis was performed following recovery.
VI. Determining an appropriate pyridostatin dosage

It was hypothesized that G-quadruplex stabilizing drugs should increase microsatellite instability. Since TMS is a highly expensive drug only made by a collaborator in Japan, we chose to use the commercially available G-quadruplex stabilizer, pyridostatin (PDS). We first needed to see what an appropriate dosage would be. Figure 17 shows a dosage assay where TTR and TTF cells were dosed with either 10 or 20 \( \mu \text{M} \) PDS for 24 h and left to recover for 4-days. Following these treatments there did not appear to be any trend in instability. After communicating with another researcher that uses pyridostatin, a 10-fold decrease to 2 \( \mu \text{M} \) was recommended for further treatments.

VII. Pyridostatin shows orientation dependent instability

TTR and TTF cells were treated with 2 \( \mu \text{M} \) pyridostatin for 24h and left to recover for either 1 day or 5 days to observe any differences in recombination. Following 1 day recovery there were not any significant differences in flow profiles, most likely due to high background levels. Allowing for longer recovery times allows fluorescent proteins to fully degrade in dead or dying cells.

After a 5-day recovery, the TTR cell line showed a decrease in the percentage of yellow cells from 83% to 76% compared to the control, while the TTF cell line only observed a 1% decrease compared to the control (Fig. 18). The TTR cells also had a notable 6% decrease in the percentage of green cells, which
was also observed in the telomestatin treated cells. Since these clones did not have a large sub population of green cells, the extreme effects of G-quadruplex stabilizing drugs was only minimally observed and we were able to see the orientation dependent instability in yellow cells.
Figure 17: Pyridostatin dosage assay. Cells were treated with 10 μM and 20 μM pyridostatin for 24 h and left to recover for 4 days. There were no notable differences in flow profiles, but gating (data not shown) revealed high amounts of cell death.
Figure 18. *Pyrdiostatin shows orientation dependent instability.* TTR and TTF cells were treated with 2 μM PDS and left to recover for 1 or 5 days. Flow cytometry analysis was then performed. (A) Flow cytometry analysis for TTR. (B) Flow cytometry analysis for TTF.
VIII. The polypurine insert alone causes slowed cell growth.

After these cell lines were created, clones were sent to collaborators that were interested in studying these sequences. They noticed that TTR cells seemed to grow slower than TTF and this observation was also noted in our work. To see if this observation had any validity, untreated cells were plated and counted after 24, 48, and 96 hours of growth. Table 1 shows the raw counts and figure 19 shows a graph following the trends. After 96 h of growth, in two separate trials, the TTF cells had higher counts than TTR confirming that in fact the TTR cell line shows slower growth kinetics compared to TTF. Following this experiment, we hypothesized that the microsatellite sequence alone might cause differences in cell cycle progression, and in fact the TTR cell line might show cell cycle stalling.
Table 1. **Raw data counts for cell growth assay.**

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Figure 19: TTR and TTF cell show different rates of cell division. Untreated TTR and TTF cells were plated on day 0 (not shown) and counts were taken in duplicate at 24h, 48h, and 96h of growth.
IX. **Designing experiments to test for ectopic site DNA translocations**

Previous bioinformatics data have revealed translocations from the ectopic site to non-allelic chromosomes in TMS treated TTR cells. We were interested to see if we could detect these translocations via PCR in the DNA samples.

We took sequence reads that had contained translocated sequences and aligned them against each other. In the regions of alignment, we identified the ectopic site and the translocation junctions to which we designed a primer set where one primer is in the ectopic site and the other primer is complementary to the endogenous translocated gene. We predicted that if there is indeed a translocation in the ectopic site, we will be able to obtain a PCR product at a predicted size. Figure 20 shows a model of the aligned regions, translocation junctions, and PCR binding site using DENND3 as an example of the translocated genes.

X. **DNA translocations are detectable in telomestatin treated TTR cells**

Three telomestatin TTR genomic DNA samples were analyzed for translocations using the method mentioned above. Figure 21 panels A, B, and C show the products obtained after assaying for the DENND3 translocation. The predicted product size was ~750 bp, which can be seen in panel A. Additionally there is a 1 kb product in treatment 1 as well as a smear in treatment 3. This PCR reaction was repeated with the same samples in panel B, of which there are 1 kb products in each lane. This PCR was repeated a third time with a negative control in panel C, this time showing 800 bp fragments. A similar phenomenon was observed to a lesser extent when assaying
for the DDX10 translocation (panels D and E). Panel D shows a 3 kb fragment in the treatment 3 lane and in panel E, each treatment showed 700 bp fragments.

This raised a major question about why each PCR using the same samples with the same primers were producing different sized products. Figure 23 shows a possible model for why this is happening. Following a DSB and 5’ resection, if the cell opts for homologous recombination, the next step is to find sequence homology. We know that break-induced replication is a possible repair mechanism at microsatellite sequences and one aspect of BIR is homology can be found with very small amount of sequence complementarity (Sakofsky et al., 2012), termed microhomology-mediated BIR (MMBIR). Based on this model, the cell can find homology at multiple instances in a single gene, thus creating different size translocation fragments.

Of the six translocations assayed (see methods), we were able to detect DDX10 and DENND3 via PCR.
Figure 20: A model to assay for DNA translocations via PCR. The black line represents any number of reads showing a DNA translocation to DENND3 that have been aligned together. The yellow region represents regions where those reads have almost perfect alignment. Purple and green represent ectopic site sequence, and cyan is the translocated fragment. For this example, there are two primer sets designed. The first set (DENND3 forward/reverse) contain a forward primer in the translocated gene and a reverse primer in the ectopic site. The second set (DENND3 forward/reverse 2.0) contain the opposite of this: a forward primer in the ectopic site and a reverse primer in the translocated gene. iPCR binding sites (gray) are negligible for this assay.
Figure 21: Translocations are detectable in TMS treated TTR cells. TMS cells were subject to primers designed to detect translocations (see methods) and analysis via gel electrophoresis. (A-C) show PCR products that were testing for the DENND3 translocation. (D-E) show PCR products that were testing for the DDX10 translocation. Translocations were previously identified by bioinformatics. Treatments 1, 2, and 3 are from biological replicates.
Figure 22: **A model for variable translocation fragments.** Following a DSB, if the cell goes through HR, 5’ resection will over. If BIR is the repair mechanism at play, the cell can find homology with very little complementarity with only several nucleotides. This can cause the invading strand to find homology at several instances throughout a translocated gene, producing different sized fragments.
XI. COPS2 as a suppressor of break-induced replication

A shRNA screen targeting suppressors of break-induced replication was performed previously on three microsatellite containing cell lines. These cells were subjected to treatment with hydroxyurea (Hu), doxycycline (Dox), both (Hu + Dox), or no treatment control and then placed in ganciclovir containing medium, a selection marker for cells undergoing break-induced replication in our model system. DNAs from these cells were collected and sequenced to analyze the fold change in transcripts and expression of shRNA. COPS2 came back as a major hit from this screen with greater than 100-fold change in all treatments (Fig. 23).

XII. siCOPS2 produces unique recombination patterns

Given that COPS2 might be a suppressor of BIR, we hypothesized that knocking down this protein would stimulate BIR and lead to potential translocations and mutations. TTR and TTF cells were treated with siCOPS2 and had their DNAs extracted for iPCR. Fig. 24 shows the resulting gel. The second and fourth lanes are the siCOPS2 treated cells which show unique bands that are not present in the control lanes. Moreover, TTR and TTF produced similar recombinations under the same siRNA treatment suggesting siCOPS2 may have a unique pattern of recombination. Products seen in the 250 bp range are likely short amplifications of the ectopic site and not
informative. The predicted product size for these PCRs is 4 kb based on XbaI digestion.
Figure 23. **COPS2 as a suppressor of BIR.** An shRNA screen was performed targeting suppressors of break-induced replication following four different treatments. In all treatments, the shRNA for COPS2 was enriched greater than a 100-fold. Hydroxyurea (Hu); Doxycycine (Dox).
Figure 24: **siCOPS2 produces unique recombination patterns via iPCR.**

(A) TTR and TTF siCOPS2 treated cells were subject to iPCR to look at recombination patterns. Lane 1 is a 1 kb marker. Lanes 2 and 4 are siCOPS2 treated TTR/TTF cells. Lanes 3 and 5 are control cells. Arrows indicate unique bands in siRNA treatments.

(B) The cells were cut with XbaI restriction enzyme and ligated together to carry out iPCR (see methods). The resulting PCR product, without recombinations, is seen in cyan.
XIII. **siCOPS2 treated cells show similar translocation patterns**

Thousands of reads were generated for each treatment that were processed and put into UseGalaxay.org.au software that allows users to visualize where their reads begin and end in a circos plot, visualizing translocations. Reads were grouped based on either siControl treatment or siCOPS2 for both cell lines. Treatments shared several chromosomal translocations for both groups: the control group saw shared translocations at Chr. 1, 15 and 22, while the siCOPS2 group had shared translocations at Chr. 1, 5, 7, 10, 16, 17, 19, and 22 (Fig. 25). This suggests that despite the difference in the microsatellite, the absence in COPS2 causes the cell to undergo a similar mechanism of translocation.

XIV. **Individual cell lines do not share translocation sites among different treatments**

Given that translocation sites were shared among treatment groups, we wanted to see if the opposite was true, if there were shared translocations among the individual cell lines. Reads were grouped based on either the TTR or TTF cell line and a circos plot was generated (Fig. 26). There are several shared sites between the treatments in the individual cell lines but the number of these translocations is not comparable when grouped based on treatment, further suggesting that siCOPS2 produces similar effects in cell lines.
regardless of the microsatellite and we see an increase in the number of translocations with this knockdown.
Figure 25: **Groups reads based on treatment shows similar translocations.** Reads were grouped based on either siCOPS2 or control treatments and fed into a circus plot generator (UseGalaxy.org.au). (Left) TTR and TTF control groups. (Right) TTR and TTF siCOPS2 groups.
Figure 26: **Groups based on cell line do not share fewer translocations.** Reads were grouped based on cell lines and fed into a circus plot generator. (Left) TTF cell line treatments. (Right) TTR cell line treatments.
XV. **Cell lines show partial loss of the ectopic site**

To visualize how the reads compare to a reference sequence, alignments from Integrated Genome Viewer (IGV) were generated. Figure 27 shows the alignments that came out. In all three treatments (TTR/F control and TTR siCOPS2) there were similar patterns of deletions that were observed (white space). The deleted regions mapped to an Alu element and the beginning portion of c-myc, partial deletion in eGFP, and deletion of the microsatellite. Similar patterns are deleted regardless of the treatment suggesting that these cells may have undergone recombination in a similar fashion.
Figure 27: **Partial loss of the ectopic site in all treatments.** Reads for all treatments were aligned against a reference genome, the unmodified ectopic site. Gray bars represent aligned sequences, white spaces represent missing sequence, and colored markings represent mutations, deletions, insertions etc. (A) TTF control. (B) TTR control. (C) TTR siCOPS2. TTF siCOPS2 not shown due to low quality reads.
DISCUSSION

The polypurine repeat is unstable under endogenous conditions

When the TTR cell line was being created, flow cytometry was performed over the next two weeks after its inception to monitor the quality of the fluorescent reporters as the cell line recovered. As the days progressed, there was a dramatic shift in the flow profile from double-positive cells to green and double negative cells. While the same exact monitoring was not done on the TTF cell line, control TTF flow profiles did not change over the course of more than 100 days (Fig. 11). Further, these cell lines were sent to collaborators who noticed that TTR cells seemed to grow slower than TTF. This same observation was noted in our laboratory and when the division rates of these cell lines compared, it was evident that TTR grower more slowly than TTF (Table 1, Figure 19). This would suggest that the polypurine insert alone is enough to cause ectopic instability and down regulate whole-cell kinetics. In fact, it was shown in Liu et al. (2012) that TTR cells had consituative activation of the ATR-Ck1 DNA damage pathway but when the polypurine repeat was excised out, the constitutive activation was reversed. Based on the experiments in this study and the previous work in our lab, it is likely that the 78 bp polypurine repeat contributes to ectopic instability and is likely to stall cell division under endogenous conditions.
Instability is orientation dependent

Previous work had suggested that instability in the TTR/TTF cell line is dependent on the orientation of the mirrored-repeat (Gadgil et al., 2020; Liu et al., 2012). In the TTR cell lines, the polypurine strand is in the lagging strand for DNA synthesis while in the TTF cell line it is the polypurimidine repeat. It has been shown that the (Pu/Py)\textsubscript{78} repeat can form both triplex DNA and Q-quadruplex DNA, and these structures have been observed \textit{in vitro} via atomic force spectroscopy (Gadgil et al., 2020; Rider Jr. et al., 2022; Tiner Sr. et al., 2001.) To further understand this orientation dependency, we wanted to put the (Pu/Py)\textsubscript{78} under the context of different replication stressors. Given that the (Pu)\textsubscript{78} in the lagging strand is more prone to forming G-quadruplex structures (Gadgil et al., 2020) and triplex DNA, and has higher ATR-Ck1 signaling (Liu et al., 2012), it was expected that the TTR cell line would consequently be more sensitive to replication stressors.

To see how the different cell lines reacted when placed under ATR signaling inhibition, TTR and TTF cells were dosed with VE-821. After exposure for 24h and a recovery time there was a decrease in the number of double-positive cells followed by an increase in double-negative in the TTR cell line (Fig. 14). This same effect was not observed in the TTF cell line. This was followed by knocking down Rad9, a DNA damage response protein that is upstream of ATR signaling. During these experiments we saw a dramatic decrease in the number of yellow cells in the TTR cell line with only a miniscual decrease in yellow cells of the TTF cell line. Lastly, since (Pu)\textsubscript{78} is more prone to forming
G-quadruplex (G4) structures, we expected that a G-4 stabilizer would exacerbate the orientation dependency effects. Treating cells with pyridostatin (PDS) confirmed this expectation. In the TTR cell line there was more than 10% decrease in the number of yellow cells compared to a 1% decrease in the TTF cell line (Fig. 18).

Experiments from this study and from previous works in the lab can conclude that a polypurine repeat in the lagging strand for DNA synthesis promotes instability to a greater extent than the polypyrimidine repeat.

**Green cell populations present extreme amounts of instability in TTR cells**

As the TTR cell lines doubled over the course of time, a population of green cells began to diverge. Objectively, it would appear that there was some form of recombination at the ectopic site that removed the red color marker, presumably because of instability at (Pu)$_{78}$. TTR cells that have these green cell populations were treated with VE-821 and TMS to see if the G4 stabilizing effects would exacerbate the orientation dependency, similar to PDS. What was observed however was a 25% decrease in the green quadrant in VE-821 treated cells which increased to 31% when adding the TMS (Fig. 15). This suggests that a large portion of the green cells are dying when under replication stress, and further suggests that there were other forms of erraneous mutations or recombinations when these cells lost the (Pu)$_{78}$ repeat, making the cells hypersensitive.

One such reason for the erraneous mutations could be BIR, which has been known to introduce mutations and gross chromosomal rearrangements. Similar results were seen to a lesser extent in the PDS treated TTR cells, where there was a small portion of the green cells dying when exposed to this drug (Fig. 18).
**TTR cells may exhibit cell cycle stalling**

Given that the (Pu)$_{78}$ repeat alone is enough to slow the cell division of the cell line (Fig. 19) and there was shown to be an increase in the ATR-Ck1 activation in these cells, it is likely that they are experiencing cell cycle stalling via BIR. If the purine repeat causes a DSB and initiates BIR then the abundance of breaks and mutations could be a greater number than the cell has the capacity to resolve. Additionally, if there are unresolved Holliday junctions from BIR, this may serve as reasonable criteria to prevent the cell cycle checkpoints from being activated. Hypothetically, a future researcher could treat the microsatellite containing cells with a replication stressor, fix the cells in ethanol, and stain their DNA contents (propidium iodide/DAPI/DRAQ7™). If there was cell cycle stalling, there would be an doubling in the DNA content at G2, as the DNA will have been replicated but not yet split into daughter cells. It could then be confirmed that the difference in cell division is in fact due to cell cycle stalling in the TTR cells.

**TTR cells have predictable translocation junctions**

TTR cells were dosed with telomestatin which is a potent stabilizer of G4 structures and induced replication stress. When these cells had their DNAs sequenced, there were a number of translocations that had been identified via bioinformatics. We expected that if we could identify the translocation junction, we could PCR across it and obtain a predictable size PCR product (see methods and Fig. 20). We were able to obtain PCR products from a DENND3 and DDX10 translocation, two of the higher hits that the ectopic site translocated to, but the PCR products were of variable size (Fig. 21). Since
we were able to see any product at all, this confirmed that what we see via
bioinformatics is detectable \( \textit{in vivo} \) which could be paramount for future clinical studies.
Moreover, we speculated that the reason for variable PCR product size was due to the
nature of BIR: BIR events often occur at tandem repeat sites and since the search for
homology can be inefficient, the invading chromatid might find homology at multiple
instances in one gene (allelic or non-allelic). This would cause several PCR products to
appear, even within the same sample of DNA (Fig. 22).

**COPS2 is a suppressor of break-induced replication and produces unique
recombination patterns**

The COP9 signalosome has recently been implemented in the DNA damage response but
the specific mechanisms are still at large. When the shRNA screen targeting supressors of
BIR was performed, there were a couple prime candidates that came back, one of which
was COPS2. While there were many proteins that came back from the screen, many who
are involved in the DDR, very few were prevalent in all four treatments (Rider Jr. et al.,
2022, \textit{unpublished}). COPS2 was enriched greater than 100-fold in treatments suggesting
that this protein is a significant player in the BIR pathway (Fig 23).

If COPS2 is a suppressor of BIR, knocking it down should theoretically be a stimulator of
BIR. This is detectable via our ectopic site and iPCR which can monitor recombination
patterns. The gel obtained after iPCR shows that both the siRNA treated lanes contain
PCR products that are not seen in the control lanes (Fig. 24). Further, the bands seen in
the siRNA lanes are similar to each other which might suggest that knocking down
COPS2 has similar effects on recombination, regardless of the cell line.
These iPCR products have a predicted size of 4kb, of which there are no PCR products this size visible. Due to the low yield of iPCR, the intensity of the marker lane can overshadow the fainter bands. When other iPCR gels were ran with the same primers, there are products seen at the 4 kb region and the band patterns were similar between the siCOPS2 treatments (not shown).

The iPCR products were sent for sequencing to further investigate effects of recombination and mutation. The reads were extracted, deduplicated, and processed to look into where translocations, if any, were occurring. When the data grouped so that the control groups were together and the siCOPS2 groups were together, the cells appeared to be translocating to similar regions regardless of the microsatellite. Taking a look at the individual translocations, there were many reads that translocated via Alu element recombination and they all had G4 forming sequencing less than 1 kb away (not shown). Taken together, these experiments suggest that COPS2 may be a master regular of BIR. Since all of the siCOPS2 treatments procuded similar results, it is likely not involved in resolving BIR triggered by certain types of secondary structures, rather it’s much more upstream in the pathway’s activation/deactivation stage.

There may be a more complex story to COPS2 role as well. These PCR products that were sent for sequencing were re-amplified in the barcoding process. By nature of PCR, the primers seem to bind to the low length products and begin amplifying those capturing only one portion of the story. The longer length products that did not get amplified may show similar translocations or may give more information into how the microsatellite affects BIR in the context of siCOPS2.
(Pu/Py)$_{78}$ repeats cause deletions in the ectopic site

The reads obtained from sequencing the siCOPS2 treated cells were aligned against a reference which contained the predicted sequence of the iPCR product. When the reads were aligned against the reference, those that matched all showed deletions (Fig. 27) in the TTR/TTF siCTL/siRNA treatments. These deletions all occurred in the Alu element/c-myc area, eGFP, and the microsatellite insert. Previous work has shown that reads with the (Pu/Py)$_{78}$ repeat showed mutations in eGFP and dTOM after undergoing BIR (Damewood IV., 2021). This could result in loss of the color marker, the microsatellite, and the Alu element where recombination might have taken place. This is only true for the reads that in the 1 kb – 3 kb region, however, due to the reamplification process aforementioned, where sequences <1kb seemed to be preferentially amplified. Sequences from iPCR products in the 4 kb region may show intact ectopic sites and different recombination patterns but as for these reads, they all showed similar deletions.
FUTURE DIRECTIONS

Given the data that was shown that TTR cells show slower growth rate, it was alluded to the experiments measuring DNA content. If the (Pu)$_{78}$ repeat alone seems to cause cell cycle stalling, possibly by inhibition of COPS2 or other stressors, there would be a new link between a microsatellite sequence and the cell cycle.

iPCR results showed similar recombination patterns from siCOPS via gel electrophoresis and sequencing. There has always been a question about certain microsatellite sequences causing predictable patterns of instability. Treating cells with other replication stress drugs targeting the BIR pathway & performing iPCR may give rise to specific recombination patterns and further elucidate the mechanism of BIR.

The information in the sequencing data only scratched the surface of the reads that came back. There are many other ways to group the reads and patterns that are available for analysis. Further dissecting those reads could provide more insight into how COPS2 affects the (Pu/Pu)$_{78}$ cell line.
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joining: good, bad and ugly. *Mutation Research.* 809: 81-97

18. SnapGene® software (from Insightful Science; available at snapgene.com)


