Cellular Effects of Replicating a Polypurine-Polypyrimidine Sequence and the Interactions of DUE-B with Replication Proteins

Shere Lynne Myers
Wright State University

Follow this and additional works at: http://corescholar.libraries.wright.edu/etd_all
Part of the Biomedical Engineering and Bioengineering Commons

Repository Citation
CELLULAR EFFECTS OF REPLICATING A POLYPURINE-POLYPYRIMIDINE SEQUENCE AND THE INTERACTIONS OF DUE-B WITH REPLICAION PROTEINS

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

By
SHERE’ L MYERS
B.S., Cedarville University, 2004

2010
Wright State University

____________________________
Michael Leffak, Ph.D.
Dissertation Director

____________________________
Gerald Alter, Ph.D.
Director, Biomedical Sciences Ph.D. Program

____________________________
Andrew Hsu, Ph.D.
Dean, School of Graduate Studies

Committee on Final Examination

____________________________
Steven Berberich, Ph.D.

____________________________
Paula Bubulya, Ph.D.

____________________________
Madhavi Kadakia, Ph.D.

____________________________
Courtney Sulentic, Ph.D.
Abstract

Myers, Shere’ L., Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2010. Cellular Effects of Replicating a Polypurine-Polypyrimididine Sequence and the Interactions of DUE-B with Replication Proteins.

This work investigates two questions regarding DNA replication. The first aim examines the interactions of DUE-B with replication proteins and the second explores the cellular effects of replicating a polypurine polypyrimididine sequence in human cells.

DUE-B siRNA decreases the chromatin binding of essential replication proteins Cdc45, PCNA and RPA. DUE-B also co-immunoprecipitates with Cdc45 and TopBP1. In vitro kinase assays suggest that the checkpoint protein ATR may phosphorylate DUE-B. These experiments lend further evidence that DUE-B plays an important role in the initiation of eukaryotic DNA replication.

To investigate the effects of replicating a polypurine-polypyrimididine sequence prone to secondary structure (PKD1), our laboratory constructed four cell lines. In TTR and TTF cells, the PKD1 sequence is inserted in opposite orientations in place of the triplex-prone region of the c-myc replicator (which is stably integrated into the same genomic locus in all four cell lines). In DTR and DTF cells, the PKD1 sequence is inserted in opposite orientations in place of the DNA Unwinding Element of the c-myc replicator. The probability of secondary structure formation by the PKD1 sequence varies based on the orientation due to changes in direction of replication.

In these cells, the orientation of the insert affects the cells’ growth rate, overall health, dependence on a functional checkpoint, and ability to replicate DNA after
synchronization with aphidicolin or mimosine. A nearby origin is not required for these effects.

When the PKD1 insert was removed from TTR cell lines, the normal phenotype was partially restored. These cells showed an intermediate response to aphidicolin and mimosine synchronization and to checkpoint inhibition.

These conclusions argue for the efficiency of replication origins and suggest that the differences between the two cellular phenotypes are caused by the direction of replication fork movement through the polypurine-polypyrrimidine sequence. Importantly, these data indicate that a single genomic lesion may have the ability to alter cell growth and recovery from DNA damage.
Contents
Chapter 1: Introduction .................................................................................................................. 1

1.1 Replication Initiation ............................................................................................................. 2

Replication Initiation Proteins ................................................................................................. 2

Origin Characteristics ............................................................................................................. 4

1.2 Non-B form DNA .................................................................................................................. 5

Effects of Stalled Replication Forks ......................................................................................... 10

Chapter 2: The role of DUE-B in mammalian replication .......................................................... 12

2.1 Introduction to DUE-B ......................................................................................................... 12

Hypothesis ................................................................................................................................. 14

2.2 DUE-B Results .................................................................................................................... 15

DUE-B is essential for cell cycle progression ......................................................................... 15

DUE-B is required for the loading of Cdc45, PCNA and RPA onto chromatin .................. 16

DUE-B interacts with the replication fork proteins Cdc45 and TopBP1 ............................. 20

DUE-B may be phosphorylated by ATR Kinase .................................................................. 22

2.3 DUE-B Conclusions ........................................................................................................... 29

Chapter 3 The Orientation-Dependent Effects of Inserting a Sequence Prone to
Secondary Structure Formation ............................................................................................... 32

3.1 Genome Instability and Mutation at the PKD1 Locus ....................................................... 32
3.2 Experimental System ................................................................................................................. 33
Hypothesis ........................................................................................................................................ 36
3.3 Results ......................................................................................................................................... 38
TTR and TTF cells synthesize DNA at the same rate ................................................................. 38
Size Difference Between TTF and TTR Cells ............................................................................ 50
Growth Rate Differences Between TTR and TTF Cells ............................................................. 61
Mortality Rate Between TTR and TTF Cells .................................................................................... 66
Instability of the TTR Cell Line ...................................................................................................... 67
Inhibiting the S-phase checkpoint affects TTR cells more than TTF cells......................... 76
Genetic Differences Between TTR and TTF Cells ...................................................................... 101
The orientation effects of a triplex-prone sequence do not require a nearby origin 102
Growth Rates of DTR and DTF Cells ......................................................................................... 103
Release from Synchronization of DTR and DTF Cells ............................................................. 106
Inhibiting the Checkpoint Response in DTR and DTF Cells ..................................................... 110
3.4 Removal of the mirror repeat insert makes TTR cells more like TTF cells .......... 113
Creation of Flp’d TTR Cell Lines .............................................................................................. 115
Flp’d TTR Cells Released from Synchronization ......................................................................... 117
Treating Flp’d TTR Cells with Checkpoint Inhibitors ............................................................... 117
3.5 Replication Fork Barrier Conclusions ..................................................................................... 125
Effects of DNA damage-inducing synchronization on TTR and TTF Cells .............. 125
Effects of Checkpoint Inhibition in TTR and TTF Cells................................. 125

Implications of the DTR and DTF Cell Line Results ................................... 128

Orientation-dependent effects of inserting a sequence prone to secondary structure formation........................................................................................................ 132

Chapter 4: Methods............................................................................................ 133

DUE-B siRNA........................................................................................................ 133

Flow cytometry and BrdU Incorporation........................................................... 134

Synchronization Methods .................................................................................. 134

Cell Migration Assay.......................................................................................... 135

MTT Assay........................................................................................................... 135

Immunoprecipitations.......................................................................................... 137

ATR Kinase Assay............................................................................................... 138

DUE-B Rescue...................................................................................................... 138

Cell Death ELISA............................................................................................... 139

Southern Blots..................................................................................................... 140

Western Blots...................................................................................................... 140

PCR....................................................................................................................... 141

Immunofluorescence........................................................................................... 141

References.......................................................................................................... 143
## Table of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Unusual DNA Structures and the types of repeats that form them</td>
<td>7</td>
</tr>
<tr>
<td>2. A model of triplex-caused DNA polymerization arrest in vitro</td>
<td>9</td>
</tr>
<tr>
<td>3. DUE-B siRNA decreases the binding of replication-associated proteins on chromatin</td>
<td>17</td>
</tr>
<tr>
<td>4. DUE-B siRNA decreases TopBP1 loading onto chromatin</td>
<td>19</td>
</tr>
<tr>
<td>5. DUE-B, Cdc45 and TopBP1 co-immunoprecipitate in HeLa extracts</td>
<td>23</td>
</tr>
<tr>
<td>6. DUE-B, TopBP1 and Cdc45 co-immunoprecipitate in Xenopus egg extracts</td>
<td>24</td>
</tr>
<tr>
<td>7. DUE-B siRNA resistant plasmid increases DUE-B levels after DUE-B siRNA more than a non-siRNA-resistant DUE-B plasmid</td>
<td>25</td>
</tr>
<tr>
<td>8. ATR Kinase Dependent Phosphorylation of DUE-B</td>
<td>27</td>
</tr>
<tr>
<td>9. Model of suspected role of DUE-B in replication initiation</td>
<td>31</td>
</tr>
<tr>
<td>10. The c-myc ectopic replicator system with the PKD1 insert</td>
<td>35</td>
</tr>
<tr>
<td>11. Southern blots demonstrate correct integration</td>
<td>37</td>
</tr>
<tr>
<td>12. TTR cells encounter a replication fork block in the c-myc insert more frequently than TTR or acceptor cells</td>
<td>39</td>
</tr>
<tr>
<td>13. The orientation of the triplex-prone region does not affect the time required for replication</td>
<td>41</td>
</tr>
<tr>
<td>14. Diagram of cell synchronization methods</td>
<td>44</td>
</tr>
<tr>
<td>15. TTR and TTF cells release identically from a double thymidine block</td>
<td>46</td>
</tr>
<tr>
<td>16. TTR cells release from Aphidicolin and Mimosine more slowly than TTF cells</td>
<td>47</td>
</tr>
</tbody>
</table>
17. Comparison of cell synchronization methods...........................................48
18. Dose response of HeLa cells to UV irradiation......................................51
19. Damaging TTR and TTF cells with UV radiation before releasing from a
double thymidine block may make TTR cells release more slowly than TTF
cells............................................................................................................53
20. TTR cells are smaller and rounder than TTF cells..............................54
21. The size distribution of TTR cells changes more after release from a double
thymidine block than that of TTF cells......................................................57
22. TTF cells migrate to cover a scratch faster than TTR cells....................62
23. TTR cells increase in number more slowly than TTF cells....................64
24. TTF cells increase in number more rapidly than TTR cells....................65
25. The orientation of the PKD1 insert does not measurably affect viability.....68
26. As the cells age, the characteristics of TTR and TTF populations become more
similar...........................................................................................................70
27. The insert size in TTR cells is not changing over time..............................73
28. TTR cells may accumulate large deletions when cultured for longer periods of
time.............................................................................................................75
29. No difference in Rad9 foci is observed between TTR and TTF cells.........78
30. Caffeine increases the differences between TTR and TTF......................79
31. Caffeine increases the differences between TTR and TTF cells after release
from mimosine..............................................................................................81
32. ATR siRNA increases the differences between TTR and TTF cells.........82
33. UCN-01 increases the differences between TTR and TTF cells..............85
34. Chk1 is not being degraded in a proteosome-dependent manner in TTR cells…88
35. TTR cells swell 11 hours after release from aphidicolin into caffeine or
      UCN-01……………………………………………………………………………90
36. TTR cells swell 11 hours after release into checkpoint inhibitors…………………91
37. TTR cells show no decrease in viability 11 hours after release from aphidicolin
      into UCN-01……………………………………………………………………..92
38. TTR cells show nuclear fragmentation 11 hours after release from aphidicolin
      into checkpoint inhibitors………………………………………………..93
39. TTR cells demonstrate loss of membrane integrity after long treatments with
      caffeine or UCN-01………………………………………………………………95
40. TTR cells die 13 hours after aphidicolin release into UCN-01…………………………….96
41. Treatment with UCN-01 causes more cell death in TTR cells than TTF cells….98
42. DTF cells appear smaller and rounder than DTR cells………………………..104
43. DTR cells migrate to fill a “wound” faster than DTF cells…………………..105
44. DTF cells have a slower population doubling rate than DTR cells…………….107
45. DTR and DTF cells release the same from a double thymidine block………108
46. DTF cells release from aphidicolin more slowly than DTR cells………………109
47. DTF cells release from mimosine more slowly than DTR cells………………..111
48. Treating DTR and DTF cells with checkpoint inhibitors does not increase the
      differences in release from aphidicolin……………………………………..112
49. Checkpoint inhibition by UCN-01 causes more cell death in DTF and TTR
      cells than DTR cells……………………………………………………………..114
50. PCR showing deletion of the PKD1 insert in “Flp’d” TTR cells………………116
51. B9 Flp’d TTR cells release from mimosine faster than TTR cells..................118
52. B9 Flp’d TTR cells release from mimosine almost like TTF cells.................119
53. B9 Flp’d TTR cells release from aphidicolin faster than TTR cells.............120
54. Flp’d TTR cells release from aphidicolin similarly to TTF cells...............121
55. TTR cells lacking the PKD1 insert (Flp’d TTR) are less affected by caffeine
treatment than TTR cells but more affected than TTF cells....................122
56. TTR cells lacking the PKD1 insert (Flp’d TTR) are less affected by UCN-01
treatment than TTR cells but more affected than TTF cells....................124
Chapter 1: Introduction

In each round of the cell cycle, cells must completely and accurately replicate their entire genome. In only a few hours, mammalian cells must copy approximately six billion base-pairs of DNA \(^1\), and a single error or omission can lead to the death of the cell, or possibly even of the entire organism. As such an essential and complex process, replication involves scores of different proteins, each with specific roles, many of which are not yet understood.

Even the very initiation of replication is still questioned. What exact proteins are required to trigger replication initiation and what roles do they play? How are the origins selected and are they always the same? Why do only some of the MCM (Mini Chromosome Maintenance) complexes that are loaded onto chromatin begin unwinding DNA to form a replication fork origin? Since there are more potential origins than functional origins\(^2\), how are these different? The first aim of this work will explore the role of a recently discovered protein, DUE-B (DNA Unwinding Element Binding Protein) in replication initiation. Its interaction with other replication proteins will be examined and the effects of depleting it will be observed.

The initiation of replication is only the beginning of the challenge, however. As replication progresses, a number of obstacles may arise. In addition to shortages of nucleotides or proteins, the replication machinery may encounter double or single-stranded breaks, interstrand crosslinks, base dimers or secondary structures such as
hairpins, triplexes or quadruplexes that retard or even arrest its progress. And yet, the cell must find a way to replicate every base.

The second aim of this work will examine the results of inserting a DNA sequence prone to secondary structure formation in human cells. Comparing two cell lines with different probabilities of secondary structure formation at a specific location (but identical everywhere else) reveals some of the effects stable secondary DNA structures have on human cells. This aim will also provide evidence for the efficiency of the c-myc replication origin (i.e., it fires in a high percentage of cell cycles) and will argue for the cellular benefits of having many highly efficient replication origins.

1.1 Replication Initiation

Replication Initiation Proteins

DNA replication is a highly complex process involving a large number of proteins. It is regulated temporally by the cyclin dependent kinases (CDKs) to ensure that each segment of DNA is replicated exactly one time during each cell cycle. To prevent replication of any region more than once, origins are licensed by the loading of pre-RC proteins only when CDK activity is low, and replicated only when CDK activity is high. The “license” is removed when each sequence is replicated, and since it cannot be re-licensed until the next cell cycle, it cannot be replicated until the next cell cycle. In late mitosis and G1 phase, when the CDK activity is low, potential replication origins are licensed by the loading Cdc6 and Cdt1 where ORC is bound. These proteins promote the loading of Mcm2-7, a heterohexameric ring that is believed to be the replicative helicase. This group of proteins is referred to as a pre-replication complex (pre-RC), and increased
CDK levels prevent its formation at any other cell cycle stage, which protects against re-
replication (unless Cdt1 is stabilized) 3,4.

The loading of the MCM activating co-factor Cdc45 is considered the transitional event leading to replication initiation. After the loading of Cdc45, unwinding of the DNA strands begins, which promotes the loading of Replication Protein A (RPA; the mammalian single-strand binding protein). DNA polymerases ε, δ and α are then loaded and polymerization begins.

Cdc45 is an essential replication protein that is involved in the initiation and elongation phases of replication. It has been shown to interact with Mcm2, 5, 7 and 10 as well as Polymerase α, δ and ε, so it may function to bind the helicase (MCM complex) to the replicative polymerases to synchronize their movement. It also binds to GINS, TopBP1 and RPA and is essential for DNA unwinding. Cdc45 binding may be a regulatory mechanism that determines which potential origins will be used during each round of replication 5.

The proteins required for the loading of Cdc45 are better characterized in yeast than in higher eukaryotes. In S-phase of yeast cells, Cdk1 and Dbf4/Cdc7 phosphorylate Sld2 and Sld3. This phosphorylation allows Sld2 and Sld3 to form a trimeric complex with Dpb11 (TopBP1/Mus101), which binds to a potential origin (already having ORC and MCM). The binding of this complex recruits Cdc45, which binds MCM and the polymerase. Origin unwinding and RPA loading follow. Although Cdc45 must remain bound at the replication fork, after it is loaded, Sld2/Sld3/Dpb11 leave the origin 6-8,3.

The homologue of Dpb11 in humans is TopBP1 (Topoisomerase IIβ Binding Protein 1). It plays a role in the initiation of DNA synthesis (loading Cdc45), in
preserving replication fork integrity when stalls occur, and in DNA damage signaling.

TopBP1 is essential for the initiation of DNA replication, but does not move with the replication fork. It is not required for the binding of pre-RC proteins (ORC, Cdc6, Cdt1 and MCM) but is required for the conversion of pre-RCs to pre-initiation complexes (pre-ICs). During S-phase in Xenopus, Xmus101 (TopBP1) binds chromatin in two ways. The first way is independent of S-phase CDK activity, and may be independent of origin location. This is a weak interaction that allows S-phase CDK activity and converts pre-RCs to pre-ICs. This type of binding is necessary for replication.

The second type of Xmus101 (TopBP1) binding is not required for replication, but is dependent on S-phase CDK activity. This Xmus101 binds to chromatin as replication begins and likely plays a role in checkpoint activation and/or signaling. TopBP1 co-immunoprecipitates with Rad9 and has been shown to bind DNA ends in vitro, confirming its role in DNA damage detection/signaling. In vitro assays suggest that TopBP1 is necessary for the ATR-ATRIP dependent phosphorylation of Chk1.

Studies have also shown that TopBP1 foci can form in G1 phase after double-stranded break induction, so it apparently can signal for damage that is not associated with replication as well.

**Origin Characteristics**

The sequence of protein loading just described occurs at each replication origin. Although the defining factors for origin selection are not well understood in mammalian cells, a few origins have been characterized. One essential characteristic of an origin appears to be an AT-rich sequence that is thermodynamically unstable, termed a DNA Unwinding Element (DUE). When the DUE from the c-myc replicator was deleted,
replication at this origin ceased. However, when the DUE was replaced with 27 or 48 repeats of ATTCT (but not 8 or 13 repeats), a thermodynamically unstable sequence whose expansion is associated with Spinocerebellar ataxia type 10, replicator activity was restored \(^\text{12}\). This suggests that the presence of a DUE is a requirement for replication in metazoans.

Although a DUE is required for origin activity, it is not sufficient. Inserting large numbers of ATTCT repeats in chromosomal locations that do not normally function as replicators does not form replication origins \(^\text{12}\). Clearly, there are other factors determining origin selection that have yet to be elucidated. A few origins have been identified in mammalian cells, however, including the c-myc origin.

Our laboratory has studied the c-myc origin, both at its endogenous location and at an ectopic location \(^\text{13,14}\). When the DUE was deleted or truncated, initiation events could no longer be detected in this region by nascent strand abundance assays \(^\text{11}\). We therefore performed a yeast-1-hybrid screen for proteins that bound to this region. One unique protein was identified and named the DNA Unwinding Element Binding Protein (DUE-B) \(^\text{15}\).

1.2 Non-B form DNA

Although replication initiation requires the temporal and spatial cooperation of many factors, the challenges of replication have only begun. Once replication is initiated, replication forks proceed bidirectionally from each origin. As replication forks progress, they can encounter a number of hazards, including strand crosslinks, base dimers, DNA breaks, or stable secondary structures \(^\text{16}\).
The genome contains an abundance of microsatellites, tandem repeats of 1-10 nucleotides. These sequences have been identified as sites of increased genome instability, promoting chromosomal deletions, rearrangements and breaks. Repeated sequences such as these are prone to forming atypical base-pairs (that is, base-pairs other than those that stabilize canonical, duplex DNA) that may promote this instability. Repetitive DNA sequences preferentially form a number of different secondary structures (Fig. 1). Each type is preferred based on the sequence, but some sequences are prone to forming several types of structures. Inverted repeats (having the repeated sequence on opposite strands) promote cruciform structures, while mirror repeats (having the repeated sequence on the same strand) frequently form H-DNA, or triplex structures. Direct tandem repeats may form G-quartets, Z-DNA or slipped DNA (S-DNA), depending on the repeat sequence.\textsuperscript{17}

One class of microsatellites is trinucleotide repeats (TNRs), which cause at least twelve types of neurodegenerative diseases. Large numbers of CGG repeats cause Fragile X syndrome, and have been shown to form G-quadruplexes (as do runs of guanines). Large numbers of GAA repeats are responsible for Friedreich’s ataxia, and are prone to forming triplex structures (as are mirror-repeat sequences). Both G-quadruplexes and triplexes are favored in polypurine-polypyrimidine-rich sequences (sequences rich in purines on one strand and pyrimidines on the other).\textsuperscript{18}

G-quadruplexes (i.e. G-quartets) are four-stranded structures with guanines at the corners (Fig. 1). Initially identified in telomere repeats, G-quadruplexes have also been characterized in genes and frequently in the non-coding regions of the genome.
Figure 1 Unusual DNA structures and the types of repeats that form them.

Structures that can be formed in double-stranded DNA (left) and the types of repetitive sequences that can form them (right) are shown. R, purines; Y, pyrimidines. Identical repetitive units are in black, and the cDNA strands are in white.

Sequences prone to this structure contain four tracts of three guanines with any number of other nucleotides in between\textsuperscript{19}. These have been characterized at the locations of frequent chromosomal translocations, indicating that they may promote genomic instability\textsuperscript{20}. G-quadruplexes are prone to occur when the DNA is unwound for transcription or replication and are believed to block replication fork progress\textsuperscript{19,17}.

Triplexes, occurring when single-stranded DNA base-pairs with duplex DNA, are also believed to hinder replication fork progress (Fig. 2). Mirror repeat structures are especially prone to this phenomenon, since each half can base pair to the complement of its mirror image (e.g. when it becomes single-stranded during transcription or replication). Sequences rich in purines on one strand and pyrimidines on the other (polypurine-polypyrimidine sequences) are also prone to triplex formation\textsuperscript{21}. Triplexes may be formed with two pyrimidine strands and one purine strand, or with two purine strands and one pyrimidine strand. Because a pyrimidine strand has to be protonated to form stable base-pairs with duplex DNA in vitro, triplexes containing two purine strands and one pyrimidine strand are believed to be the predominant form in vivo.

In addition, triplex structures can be artificially formed by the introduction of oligonucleotides that are complementary to endogenous DNA sequences. These are currently under investigation as vectors for gene therapy. One study reported that in CHO cells, transfection with a triplex-forming oligonucleotide (chemically modified to increase binding affinity) increased intermolecular recombination by 5-10 fold at an integrated genomic locus\textsuperscript{22}. Current research is investigating ways to increase delivery of triplex forming oligonucleotides, strengthen their binding with DNA, and conjugate them to DNA damaging agents such as nucleases or psoralen that would better recruit
Figure 2 A model of triplex-caused DNA polymerization arrest in vitro. The lagging strand template folds on the duplex in front of the leading strand polymerase when unwound. Arrows indicate the direction of DNA synthesis. A triplex is formed within a homopurine/homopyrimidine mirror repeat; the pyrimidine strand is white, and the purine strand is black.

DNA repair machinery. Since over 97% of identified human genes have at least one predicted high affinity binding site for triplex-forming oligonucleotides, this could become a powerful tool for genetic manipulation.

**Effects of Stalled Replication Forks**

When a triplex or other non-B form DNA is encountered, the replication fork is thought to stall until the structure can be resolved and replication can continue. Depending on the nature of the aberration and the cellular context, stalled forks may be processed in several ways. The fork may resume polymerization through error-prone translesion synthesis, requiring different replication machinery to take over synthesis in a less-specific and accurate manner. Alternatively, the fork may collapse, allowing the replication machinery to dissociate from the DNA, leaving free, unprotected ends, which must be processed to prevent loss of information. Finally, the fork may remain stalled, being stabilized and restarted through checkpoint activation and the subsequent recruitment of appropriate proteins.

Checkpoints are mechanisms that monitor cellular activities and ensure that critical steps are completed properly before allowing progression to other cell cycle phases. S phase checkpoint proteins sense stalled forks and other genomic lesions and respond to protect the genome. In response to DNA damage, checkpoints may activate cell cycle arrest, DNA repair or apoptosis. If the amount of damage is relatively small, the checkpoint will most likely recruit repair proteins and prevent entry into the next phase of the cell cycle. The S-phase checkpoint has four main functions: 1) Delay entry into mitosis until the damage can be repaired, 2) Prevent firing of new replicative origins
by inhibiting initiation at already licensed origins, 3) Slow the speed of fork progression on a damaged template, and 4) Stabilize a stalled fork

When a replicative polymerase encounters an aberrant DNA structure such as a triplex or quadruplex, it frequently stalls. In some cases, both the helicase and the polymerase are stalled, while under other conditions, the helicase is allowed to proceed while only the polymerase stalls. The latter is likely to occur when a helicase moves through a polypurine-polypyrimidine rich region, leaving it single stranded so that it can form a non-B structure that prevents polymerase movement. Although the polymerase is incapable of synthesizing more DNA, the helicase may uncouple from it and continue unwinding DNA. This results in an abnormally large quantity of single-stranded DNA, which is bound by RPA. This RPA-bound DNA recruits the clamp loader Rad17-RFC2-5, which loads the 9-1-1 complex (Rad9-Hus1-Rad1). The RPA-bound DNA also recruits ATR (Ataxia Telangiectasia and Rad3 Related) and ATRIP (ATR Interacting Protein). TopBP1 binds to ATRIP and the 9-1-1 complex and promotes the activation of ATR, which then phosphorylates the 9-1-1 complex. ATR substrates, including Chk1 and Claspin, are then activated and work to stabilize and restart the stalled replication fork. In the absence of these stabilizing factors, stalled replication forks may collapse, allowing disassociation of the replication proteins and possible digestion of the unprotected DNA ends.
Chapter 2: The role of DUE-B in mammalian replication

2.1 Introduction to DUE-B

Unlike Dpb11, the homologue of Sld3 (identified as a gene that was synthetically lethal with Dpb11) has not been definitively identified in higher eukaryotes. Our laboratory has speculated that DUE-B may be the functional homologue of Sld3. However, two other candidate proteins, Treslin and GEMC1, have been recently identified. All three proteins share characteristics with Sld3; they all are found in complex with TopBP1, are required for Cdc45 loading, and are suspected to require CDK activity for binding to TopBP1 and/or chromatin. None of these was initially found to have sequence homology with Sld3, however, Treslin has one domain that may exhibit slight similarity. Whether these three proteins function redundantly, or whether they divide the roles of Sld3, or whether one or more of them function as Sld2 is yet to be determined. This work will focus on the characterization of DUE-B.

DUE-B is a 24 kDa protein that is abundant through all stages of the cell cycle and is involved in cell proliferation. DUE-B expression was found to decrease in serum-starved cells and to increase in ovarian cancer cells. Additionally, when DUE-B was decreased by siRNA, after four days, only half as many cells remained in the DUE-B siRNA samples as in the control samples. This effect was believed to be the combined result of dying and non-dividing cells. When cells were treated with scrambled or DUE-B siRNA and then released from a mimosine block, cells treated with DUE-B siRNA
accumulated in G1 phase\textsuperscript{15,29}. Therefore, we believe that DUE-B plays an important role in cell proliferation.

When DUE-B is depleted, HeLa cells slow entry into S-phase, and Xenopus egg extracts are unable to replicate DNA. We have shown that DUE-B and Cdc45 bind to chromatin in Xenopus egg extracts at the same points of the cell cycle, indicating that they may be binding together. In support of this, immunodepleting DUE-B from Xenopus egg extracts prevented Cdc45 from binding to sperm chromatin\textsuperscript{30}.

Mammalian DUE-B contains a protease-resistant core and a disordered C-terminal tail that allows it to bind DNA\textsuperscript{29}. This 58 amino acid C-terminal tail is susceptible to digestion by proteases, but becomes protease-resistant in the presence of double-stranded (but not single-stranded) DNA. This suggests that the function of this tail is to bind DNA near the replication origin before it is unwound. DUE-B in lower eukaryotes lacks this C-terminal tail and functions as a D-Tyr-tRNA deacylase (a proof-reading enzyme that prevents D instead of L isomers of amino acids from being incorporated into proteins) rather than having a replication function\textsuperscript{29}. DUE-B binding to chromatin is dependent on the presence of pre-RC proteins.

Our laboratory has expressed recombinant DUE-B (rDUE-B) in HeLa cells and Sf9 insect cells. Interestingly, DUE-B expressed in HeLa cells can restore replication activity to DUE-B-immunodepleted Xenopus egg extracts, but Sf9-expressed DUE-B cannot. Mass spectrometry experiments and analysis of $^{32}$P incorporation indicate that rDUE-B expressed in HeLa cells has three more phosphorylated residues in the C-terminal tail than the same protein expressed in Sf9 cells\textsuperscript{15}. The two types of DUE-B are also localized differently, with HeLa rDUE-B being found mostly in the chromatin
fraction, while Sf9 rDUE-B was not (N. Katrangi, unpublished data). The phosphorylation sites expected to be modified differently in HeLa or Sf9 expressed rDUE-B are targets for Casein Kinase II, which can promote localization of viral (and possibly other) proteins to the nucleus. Therefore, we believe that the ability to be phosphorylated at these sites is an important characteristic affecting DUE-B function.

DUE-B also contains two S/TQ consensus sequences for ATR phosphorylation. ATR is a damage-sensing protein associated with the S-phase checkpoint response. When the Rad9-Rad1-Hus1 (9-1-1) complex senses DNA damage, it recruits TopBP1, which activates ATR. When activated, ATR pauses the cell cycle until the DNA damage can be repaired. If ATR does indeed phosphorylate DUE-B, it could indicate that DUE-B is a link between origin licensing or initiation and cell cycle progression.

Because DUE-B binds at the DUE, which is essential for replication activity at an origin, and since it seems to be involved in cell proliferation, we decided to investigate its role in mammalian replication. We first looked at the DUE-B dependence of Cdc45, RPA and PCNA loading onto chromatin then examined the interaction between DUE-B, TopBP1 and Cdc45, and finally, examined the ATR-dependent phosphorylation of DUE-B.

**Hypothesis**

DUE-B is involved in the loading of Cdc45, RPA and PCNA onto chromatin and interacts with TopBP1 and Cdc45.
2.2 DUE-B Results

DUE-B is essential for cell cycle progression

Previous data has indicated that DUE-B is important for replication \(^{29}\). Immunodepleting DUE-B from Xenopus egg extracts inhibits replication, and this effect can be rescued by adding back recombinant DUE-B produced in HeLa cells. Similarly, HeLa cells treated with DUE-B siRNA show decreased proliferation and increased cell death. When these siRNA–treated cells are synchronized with mimosine and then released into aphidicolin, approximately 10% of the cells are unable to enter S phase. However, adding back recombinant HeLa DUE-B has been unable to rescue this phenotype (Mike Kemp, thesis).

To rule out the possibility that the observed changes after siRNA are a result of DUE-B knock-down and not off-target effects, additional siRNA duplexes were used. If several siRNAs of different DUE-B specific sequences cause the same changes, we can conclude that the observed phenotype is not the result of off-target effects. Three additional DUE-B siRNAs were designed, each targeting a different region of DUE-B. HeLa cells were transfected with the individual siRNAs at 0 and 48 hours and harvested at 96 hours. They were separated into two samples, with one being lysed to yield whole cell extract, and the other fractionated to yield a chromatin-enriched population \(^{33}\). Western blots were performed to determine the level of total and chromatin-bound DUE-B decrease yielded by each siRNA.

All four DUE-B siRNAs decrease chromatin-bound and total DUE-B, although with different efficiencies (Fig. 3). One of the new siRNAs (#3) achieves more complete knock-down than the one used previously (#2) so it is anticipated that this siRNA will
yield even more dramatic effects than previously seen. Since very good knockdown (>99%) is required for obvious changes, a more effective siRNA could improve experimental results.

**DUE-B is required for the loading of Cdc45, PCNA and RPA onto chromatin**

Because DUE-B has a higher affinity for double-stranded DNA than single-stranded DNA, and binds at the DNA Unwinding Element, it is believed to promote DNA unwinding. To investigate the role of DUE-B in DNA unwinding and chromatin binding of other proteins, we used siRNA to knock it down. Preliminary experiments using DUE-B siRNA #2 have indicated a decrease in Cdc45, PCNA and RPA binding to the chromatin, but these had not been validated using the other siRNA constructs.

To confirm that other DUE-B siRNAs reduced the chromatin loading of Cdc45, PCNA and RPA, these experiments were repeated using four different DUE-B siRNAs. HeLa cells were transfected with one of our four DUE-B siRNAs or mock transfected as described previously. I prepared a chromatin enriched fraction according to the Mendez and Stillman method 33, and loaded this on Western blots, probing for DUE-B, Cdc45, PCNA, RPA and TopBP1.

The results obtained were variable, and seemed to depend on the degree of DUE-B knockdown. When a very high level of knockdown was achieved with siRNA, a reduction in the chromatin-bound levels of Cdc45, PCNA and TopBP1 as compared to the mock transfected sample was observed (Fig. 3). These results were obtained with all
Figure 3  DUE-B siRNA decreases the binding of replication-associated proteins on chromatin. HeLa cells were transfected with DUE-B siRNA at 0 and 48 hours, then harvested at 96 hours. A chromatin-enriched fraction was prepared and blotted for replication proteins. M, Mock transfected. 1-5, DUE-B siRNA used. Experiment shown is representative of at least three replicates for each protein. Bottom panel shows quantitation of bands normalized to Orc2 (as a loading control) and plotted as percent of protein binding as compared to Mock transfected sample.
of the siRNAs, but were the clearest for siRNA #3, which frequently gave the greatest level of DUE-B knockdown. Orc2, which binds to chromatin independently of DUE-B, was used as a loading control.

It was very difficult to observe a decrease in RPA70, which may be due to lack of sensitivity in the antibody. When this experiment was performed with siRNA #2 previously, the cells were synchronized, which showed a more dramatic decrease in RPA70 binding. Since DUE-B is anticipated to act before chromatin unwinding, we believe that using any of the DUE-B siRNAs decreases RPA binding, although an extremely high level of knock-down is necessary to see this result.

The decrease in TopBP1 loading was subtle, but was reproducible (Fig. 3, 4). In Figure 3, a much larger amount of protein was loaded in the siRNA #5 lane (see Orc2 band), which makes it appear that TopBP1 was not decreased in this sample. Xenopus Cut5, the homologue of TopBP1, has been shown to interact with chromatin in both replication-dependent and replication-independent manners. If this is also true of human TopBP1, replication-independent loading may be responsible for some of the TopBP1 seen on chromatin after DUE-B siRNA treatment.

These results may have been more robust if the cells were synchronized and then released to increase the percent of the population in S phase. However, because synchronization methods involve disturbing replication, the results may not have been as reliable as those obtained from asynchronous cells. Synchronizing with aphidicolin would stop the cells too late in the cell cycle to observe early protein loading events. Synchronizing with mimosine would probably be ineffective for the same reason, and
**Figure 4** DUE-B siRNA decreases TopBP1 loading onto chromatin. HeLa cells were mock transfected or transfected with one of four DUEB siRNAs at 0 and 48 hours. At 96 hours, the cells were harvested and a chromatin enriched fraction was prepared according to the Mendez and Stillman method. Lower panel shows quantitation of protein binding (bands are normalized to Orc2 loading control). Results shown are representative of four experiments.
would also interfere with the normal loading sequence of proteins, and therefore would be difficult to interpret. Future work should attempt to synchronize cells with nocodozole (an inhibitor of microtubule polymerization), release them for long enough to allow most cells to enter S phase, and then probe for chromatin-bound replication proteins.

Although my results with asynchronous cells were not as dramatic as those observed in synchronized cells, they corroborate the events seen in Xenopus egg extracts\textsuperscript{30}. DUE-B is seen to bind to chromatin at the same time as Cdc45 but after Orc and MCM. Some TopBP1 binding precedes DUE-B binding, which can be explained by the non-Cdk2-dependent pathway for TopBP1 binding. RPA binding follows DUE-B/Cdc45/TopBP1 binding\textsuperscript{30}. Depletion of DUE-B decreases the chromatin binding of Cdc45 and TopBP1.

**DUE-B interacts with the replication fork proteins Cdc45 and TopBP1**

It was anticipated that a replication protein might interact with Orc or MCM, but DUE-B does not pull down either of these in Xenopus or HeLa extracts (Michael Kemp, thesis). However, DUE-B does bind to chromatin in an ORC-dependent fashion\textsuperscript{30}. The C-terminus of DUE-B is required for binding oligonucleotides in vitro, but DUE-B lacking this disordered C-terminus (Δ-CT DUE-B) associates with chromatin. This indicates that the C-terminus is required for direct binding to DNA, but that DUE-B can interact with chromatin through another protein. It shows transient chromatin binding during S-phase, similar to the pattern of Cdc45\textsuperscript{30}.

When His-tagged DUE-B was overexpressed in HeLa cells and pulled down with Ni-NTA, a 180 kDa protein was also pulled down. Mass spectrometry analysis indicated
that this protein was TopBP1. Immunoprecipitations in Xenopus egg extracts using recombinant DUE-B produced in Sf9 insect cells (Sf9 rDUE-B) identified a 70 kDa binding partner, which we suspected to be Cdc45. DUE-B siRNA decreases Cdc45 and TopBP1 binding to DNA, and DUE-B appears to bind to chromatin through another protein, so we decided to explore interactions among these three proteins.

To investigate an interaction between DUE-B and Cdc45 or TopBP1, immunoprecipitations in HeLa extracts or Xenopus egg extracts were performed. Initial experiments were confounded by the heavy chain of the antibody co-migrating with Cdc45. Differing percents of acrylamide gels were used, but clear resolution was not possible. A native IgG reagent (Pierce) that should not interact with denatured antibody was ordered and used for the westerns, but it was unclear whether or not the heavy chain was still visible. I then attempted to use DSS (Disuccinimidyl suberate) to crosslink the antibodies to the beads so that they would not be in the samples. The antibodies did, in fact, still come free from the beads, but ran 20 kDa higher as a result of the heavy and light chains being crosslinked together. A combination of these methods yielded some success, and the resulting data suggests that DUE-B does interact with both Cdc45 and TopBP1 in vivo in both HeLa cell lysate (Fig. 5) and Xenopus egg extracts (Fig. 6). In comparing the amount of DUE-B immunoprecipitated with the TopBP1 antibody and the DUE-B antibody, it is important to remember that the efficiencies of antibodies for immunoprecipitation vary widely. The TopBP1 antibody is a commercial, purified antibody, whereas the DUE-B “antibody” is antiserum. There was more total protein immunoprecipitated with the TopBP1 antibody than with the DUE-B antibody, and
therefore more DUE-B immunoprecipitated with the TopBP1 antibody than with its own antiserum.

Future work should examine the cell cycle dependence of the interactions between DUE-B and Cdc45 or TopBP1. It should also characterize the phosphorylation sites of DUE-B, determining which kinase is responsible for the phosphorylation and at what times they are phosphorylated in a mammalian replication system. A DUE-B siRNA rescue plasmid that produces the same amino acid sequence but is resistant to DUE-B siRNA #3 was constructed. When cells were transfected with DUE-B siRNA #3 and this rescue plasmid, the DUE-B knock down was less efficient than when cells were transfected with the siRNA alone or the siRNA and a non-resistant DUE-B plasmid (Fig. 7). The wildtype plasmid also restored some amount of DUE-B to the siRNA treated sample, indicating that more DUE-B was produced from the plasmid than could be destroyed by the siRNA. Future work should additionally modify the siRNA resistant rescue plasmid at the phosphorylation sites suspected to dictate its interactions between Cdc45 and TopBP1. Cells can then be treated with DUE-B siRNA to knock down endogenous DUE-B and have mutant DUE-B added back to examine the effects of losing one or more of the phosphorylation sites. This would provide valuable information, not only about the binding of these proteins, but also about whether or not DUE-B is indeed the Sld3 homologue in higher eukaryotes.

**DUE-B may be phosphorylated by ATR Kinase**

In examining the potential phosphorylation sites of DUE-B, we discovered that it contains ATR target motifs (SQ or TQ) 35. To test whether ATR can phosphorylate
Figure 5 DUE-B, Cdc45 and TopBP1 co-immunoprecipitate in HeLa extracts.

Immunoprecipitations were performed with antibodies indicated along the bottom of the figure, and the membrane was probed with antibodies indicated on the right. The second western is a darker exposure of the top western showing that TopBP1 is immunoprecipitated with the Cdc45 antibody. The lower band in all lanes of the DUE-B blot is light chain antibody. Results shown are representative of over six experiments.
Figure 6 DUE-B, TopBP1 and Cdc45 co-immunoprecipitate in Xenopus egg extracts. IPs were performed with antibodies indicated at the bottom of the figure, the samples run on a gel, and different portions of the membrane probed with the antibodies listed on the right. Two different exposures of the TopBP1 western blot are shown (top two rows). Results shown are representative of at least six replicates.
Figure 7 DUE-B siRNA resistant plasmid increases DUE-B levels after DUE-B siRNA more than a non-siRNA-resistant DUE-B plasmid. Western blot showing DUE-B levels after HeLa cells were transfected with the following: Lane 1: Lipofectamine 2000 alone. Lane 2: DUE-B siRNA #3. Lane 3: DUE-B siRNA #3 and a DUE-B plasmid that was not resistant to siRNA #3. Lane 4: DUE-B siRNA #3 and a DUE-B plasmid mutated to give the same amino acid sequence but be resistant to siRNA #3. A Coomassie stained band is shown below as a loading control. Results shown are representative of two experiments.
DUE-B, I carried out in vitro kinase assays (Fig. 8). Wildtype or kinase dead Flag-ATR was over-expressed in 406-myc cells (HeLa derivatives). Cell extracts were prepared and some were exposed to single-stranded DNA to activate the checkpoint pathways. The ATR was pulled out by immunoprecipitating with anti-Flag and then recombinant DUE-B produced in Sf9 cells and γ-32P-ATP were added.

Wild-type ATR appears to be phosphorylating DUE-B, showing two clear bands that correspond to the molecular weights of full length and delta-CT DUE-B (both potential phosphorylation sites are present in ΔCT-DUE-B). These bands do not show up in the sample lacking DUE-B, so they are not another protein that was pulled out of the extract during the ATR-IP. In addition, they are dependent upon a high concentration of ATR (compare lanes 2 and 4 with 3 and 5). Interestingly, the phosphorylation of DUE-B does not require activation of ATR by single-stranded DNA. It is assumed that during the preparation, ATR becomes activated even in the absence of artificial stimulation. The kinase dead ATR does not appreciably phosphorylate DUE-B, indicating that the phosphorylation that is occurring is dependent on a functional ATR protein.

To ensure that the differences between wild-type and kinase dead ATR were due to differences in activity rather than different levels of expression, I loaded equal amounts of cell extract from the various samples on a western and probed with ATR antibody (Fig. 8, bottom). There was apparently some problem with the loading of lane 2 of this gel (or a problem with that part of the Western blot), but nonetheless, it demonstrates that the expression of kinase dead ATR was approximately equal to the expression of wildtype ATR.
Figure 8 ATR Kinase Dependent Phosphorylation of DUE-B. HeLa cells were transfected with wildtype (wt) Flag-ATR or kinase dead (Kd) Flag-ATR. Cell extracts were then prepared, some of which were activated by adding cut lambda DNA (which contains DNA ends that should activate the checkpoint pathway; designated “DNA +” in table). An immunoprecipitation with Flag antibody was performed to purify the ATR complexes. Either a “high” (H) or “low” (L, 10% as much ATR as high samples) was aliquoted for each reaction and then Sf9 DUE-B and $^{32}$P-ATP were added. Top: Radiograph showing labeled (phosphorylated) DUE-B. The two bands shown
correspond to the sizes of DUE-B and delta-CT DUE-B. Bottom: To normalize for the expression levels of wild-type and kinase dead ATR, equal amounts of each of the cell extracts used in kinase assay were run on western and probed for ATR expression. Similar results were obtained in three experiments.
A collaborator of ours (Mustapha Buhassi, Cincinnati, OH) performed kinase assays using Chk1, and found that it can phosphorylate DUE-B (unpublished data). It is possible that in my reactions, Chk1 is pulling down with ATR and is actually phosphorylating DUE-B instead of ATR. If this is occurring, however, it must be functioning in an ATR-dependent manner, or the pull-downs with kinase-dead ATR would also lead to DUE-B phosphorylation. It is also possible, although unlikely, that Chk1 co-IPs with wildtype ATR but not with kinase-dead ATR, which could also yield the observed results.

Since we believe that DUE-B plays a role in replication initiation, interaction with either ATR or Chk1 could be an important mechanism for preventing initiation when a checkpoint is activated. At this point, we are not certain how the phosphorylation status of DUE-B affects its activity, but it is possible that when a checkpoint is activated, DUE-B is phosphorylated by ATR or Chk1, which interrupts its interactions with Cdc45 and/or TopBP1, preventing replication initiation.

2.3 DUE-B Conclusions

The experiments described here have investigated the role of the DNA Unwinding Element Binding Protein (DUE-B) in eukaryotic replication. They have shown that DUE-B is essential for cell cycle progression and for the chromatin-loading of Cdc45, PCNA and RPA. They have also demonstrated that DUE-B interacts with Cdc45 and TopBP1, and that it may be phosphorylated by ATR kinase.

As a result of these and other experiments, our lab has proposed a model for DUE-B’s role in replication initiation (Fig. 9, M. Leffak). During G1 phase, ORC binds to origins, followed by Cdc6 and Cdt1. These proteins lead to the loading of Mcm2-7,
which then recruits Cdc7 and Mcm10. At the same time, DUE-B forms a complex with TopBP1 and Cdc45, which leads to the stable loading of Cdc45 in a Cdk2-dependent manner. The origin is then able to unwind, which allows DUE-B release, RPA binding and DNA synthesis.

Our laboratory has recently begun to suspect that DUE-B is the mammalian functional homologue of Sld3. Along with Sld2, the phosphorylation of Sld3 by CDKs is sufficient to promote replication in budding yeast \(^36\). In yeast, Sld3 is phosphorylated by CDKs, allowing it to interact with Dpb11 (yeast homologue of Xenopus Mus101 or human TopBP1). This interaction, along with a phospho-mimetic of Sld2, is sufficient to promote replication \(^37,36\). Therefore, Sld3 plays a crucial role in the regulation of replication initiation.

Our laboratory has shown that DUE-B interacts with both Cdc45 and TopBP1 in mammalian cells and in Xenopus extracts \(^30\). In addition, using DUE-B siRNA reduces the loading of TopBP1 and Cdc45 onto chromatin (this work). We have also demonstrated that immunodepleting DUE-B from Xenopus extracts prevents replication, and that using DUE-B siRNA causes an accumulation of human cells in G\(_1\) phase \(^15\). For these reasons, we believe that DUE-B, like Sld3, is involved in loading Cdc45 onto chromatin. Unlike Sld3, however, DUE-B does not seem to require phosphorylation to bind Cdc45, but only at a later step. Despite this difference, we suspect that DUE-B may be the functional homologue of Sld3 in higher eukaryotes and function in loading Cdc45 onto replication origins.
Figure 9 Model of the suspected role of DUE-B in replication initiation. Figure courtesy of Michael Leffak, taken from Chowdhury et al, 2010.
Chapter 3 The Orientation-Dependent Effects of Inserting a Sequence Prone to Secondary Structure Formation

3.1 Genome Instability and Mutation at the PKD1 Locus

DNA triplex structures, whether endogenous or induced by an exogenous triplex-forming oligonucleotide, are sufficient to cause DNA damage and recombination. In confirmation of this, genetic loci prone to triplex formation (containing mirror repeats and polypurine-polypyrimidine tracts) are unstable, demonstrating frequent mutations, insertions and deletions. The largest identified polypurine-polypyrimidine tract in the human genome is within intron 21 of the PKD1 gene, consisting of a 2.5 kb tract that has a sense strand that is 97% C+T.

The Polycystic Kidney Disease (PKD1) genetic locus contains many large mirror repeat sequences. When studied in vitro, these mirror repeat sequences form triplex structures (as visualized by atomic force microscopy) that inhibit primer extension on the purine-rich template. This is anticipated, since the purine strand is able to form a much more stable bond with the duplex at physiological pH than the pyrimidine strand. Due to its sequence, the PKD1 intron 21 is also highly prone to G-quadruplex or other secondary structure formation. In vivo, this locus has an extremely high rate of mutations, including point mutations, insertions and deletions.

The PKD1 locus (chromosomal location 16p13.3) codes for Polycystin 1, a 460 kDa transmembrane protein that is important for cell adhesion and signaling. Polycystin 1 is found in several types of cell junctions and in the primary cilium of
mammalian cells. This is a non-motile cilium that may serve as a sensory antennae that plays a role in cell signaling and controlling cell growth. Defects in Polycystin 1 result in 85 percent of the cases of Autosomal Dominant Polycystic Kidney Disease (ADPKD), a frequent cause of end stage renal disease. Most individuals affected by this disease inherit one mutant copy of the PKD1 gene, and undergo a somatic mutation of the other. Occasionally, a non-predisposed person will accumulate mutations in the Polycystin gene of both copies of chromosome 16 in the same cell, resulting in a large, fluid-filled renal cyst. The disease can also cause cysts to develop in the liver, pancreas and other organs.

When the largest mirror repeat from the PKD1 locus was studied in vitro, it inhibited primer extension in one direction. Due to its genomic instability in vivo, and its apparent block to replication in vitro, we decided to investigate the effects of replicating it from either direction in HeLa cell derivatives.

3.2 Experimental System

In order to study the behavior of these triplex-prone sequences, we created four stable human cell lines (Fig. 10). We used the ectopic c-myc replicator system, which contains an origin that has been shown to fire efficiently in its endogenous location, in a plasmid or at an ectopic chromosomal location. The replicator contains a DNA Unwinding Element and a triplex-prone region. The donor plasmid flanking the replicator contains selectable markers which enable us to easily screen for cells which have the replicator inserted.

For each cell line, a modification of the replicator was inserted into the same Flp Recombinase Target on the HeLa derived acceptor cell line. Each of the four cell lines
has the longest mirror repeat sequence from the PKD1 locus (88 bp) inserted in it, but the location and orientation differ. To make DTF and DTR cell lines, the DUE of the replicator was replaced with the PKD1 sequence (the inherent triplex-prone region remained in the c-myc replicator). In DTF cells, the PKD1 sequence was in the forward orientation (the polypurine strand is the template for leading strand synthesis when replicated from the c-myc origin), while in DTR cells, the PKD1 sequence was in the reverse orientation (the polypurine strand is the template for lagging strand synthesis when replicated from the c-myc origin). It should be noted that replacing the DUE of the replicator with the PKD1 insert abolished origin activity so it is uncertain what origin will replicate this sequence and therefore which strand will be used as the lagging strand template. The forward and reverse designations were maintained, however, for consistence in comparing these cell lines to TTR and TTF cells.

To make TTF and TTR cells, the PKD1 sequence was inserted in place of the endogenous triplex-prone sequence of the replicator (the DUE was left intact). For TTF cells, the triplex-prone sequence was placed in the forward orientation (the polypurine strand is the template for leading strand synthesis). For TTR cells, the triplex-prone region in the reverse orientation (the polypurine strand is the template for lagging strand synthesis).

To confirm the correct construction of the TTR and TTF cell lines, I performed Southern blots. A probe inside the inserted sequence (Neo probe) produced a single product, indicating that the insert was only placed in one location in the cells (Fig. 11, left). (A minor, larger product occurs in TTR and TTF cell lines, indicative of a partially
**Figure 10 The c-myc ectopic replicator system with the PKD1 insert.** The ectopic replicator was specifically integrated into HeLa-derived acceptor cells using the Flp Recombinase system. The black bar represents the c-myc replicator, and the brackets above it demonstrate where the PKD1 mirror repeat sequence was integrated in each cell line and its orientation. FRT: Flp Recombinase Target Sites. Hyg, Neo and TK: Selectable markers for Hygromycin, Neomycin and Thymidine Kinase that allow for confirmation of integration and selection of cells. 1,2 and 3: Primer sites that allow for confirmation of integration. DF and DR: Show the purine (R) and pyrimidine (Y) orientation of the PKD1 sequence inserted in place of the c-myc DUE to produce DTF and DTR cells. TF and TR: Show the purine and pyrimidine orientation of the PKD1 sequence inserted in place of the triplex-prone sequence of the c-myc replicator in TTF and TTR cells. In TTF and TTR cells, replication initiation occurs primarily to the left of the PKD1 insert. In DTR and DTF cells, replication activity of the c-myc replicator is abolished by the deletion of the DUE, and therefore initiation occurs outside of the ectopic c-myc. The inserted PKD1 mirror repeat sequence is:

```
CCCCCCTCCTCCTCCCCCTCCTCCCCCTCCTCCTCCCCCT
```

(/ indicates the center of mirror symmetry).
cut product. If this were a cross-reacting DNA sequence, it would also appear in HeLa cells. If it were the Neo probe integrated into another chromosomal location, it is virtually impossible that it would have occurred in a position in both TTR and TTF cells that gave the same size of fragment when cut with either enzyme. The stoichiometry of the bands also argues that the minor band is a partially cut product.)

In addition, Southern blots using a probe near the insertion site (TK probe) indicate that the insert was placed into the intended location (Fig. 11, right). Since TTR and TTF cells originated from the same acceptor cell line and we know that the engineered changes occurred as planned, any differences between the two cell lines must be a result of the insertion of the PKD1 locus in the ectopic c-myc location.

The polypurine strand forms a much more stable bond with the duplex at physiological pH than the polypyrimidine strand. Because the lagging strand template is single-stranded longer than the leading strand template, a stable triplex structure is more likely to form when the polypurine strand is replicated as the lagging strand template. When the purine-rich strand is single-stranded longer, it also increases the probability of G-quadruplex formation. Since the polypurine template strand will be single-stranded longer in TTR than in TTF cells, it is anticipated that a stable secondary structure will occur at this site in TTR cells more often than in TTF cells.

**Hypothesis**

Inserting the PKD1 mirror repeat into the c-myc replicator will create an orientation-dependent replication fork barrier that is sufficient to affect the population doubling rate, ability to recover from synchronization and checkpoint dependence of cells.
Figure 11 Southern blots demonstrate correct integration. Left: Probing with Neo (inside the insert) demonstrates that the c-myc replicator inserted only once. TTR, TTF, HeLa (negative control) or 8A DNA (negative control) was digested with BamH1 (B) or HindIII (H) and then used for a Southern Blot. Right: Digesting with BamHI (producing a 6500 base product) and then probing with TK (outside the insert) demonstrates that the c-myc replicator inserted in the correct location. If insertion had not occurred at the FRT site, the TK probe-containing fragment would have been smaller. Experiments were repeated twice.
3.3 Results

**TTR and TTF cells synthesize DNA at the same rate**

TTR and TTF cells differ only in the orientation of the PKD1 insert, 88-base pair sequence placed into the ectopic c-myc replicator site (Fig. 10). This insert has been shown to form a replication fork barrier more frequently in TTR cells than in TTF cells (Fig. 12) (Guoqi Liu, unpublished data). However, this was not expected to cause a measurable difference in the time required for replication. The endogenous inserted sequence is a non-essential gene (so even if it was disrupted, it should not affect the cells), and is a tiny fraction of the genome that must be replicated. In addition, cells must deal with multiple instances of both endogenous and exogenous damage in every cell cycle, so it was unlikely that the addition of one more of such sites would cause a global difference in the time required to complete replication. The cell is equipped with mechanisms to deal with stalled replication forks, and should be capable of resolving the structure without sacrificing genomic integrity or hindering replication. Because this insert is so small in comparison with the rest of the genome, and effects are believed to be confined to a single replication origin, it was not expected that any difficulties encountered at the triplex-prone region would cause a measurable difference in net DNA synthesis. However, we were not certain whether *exogenous* DNA damage would affect one cell line more than the other.

If TTR cells encounter problems during replication that extend the time required to complete S-phase, examining an asynchronous population should reveal a greater percentage of TTR cells in S-phase than TTF cells. This was examined by flow
Figure 12 TTR cells encounter a replication fork block in the c-myc insert more frequently than TTF or acceptor (WT) cells. Nascent strands were quantified upstream or downstream of the c-myc replicator using primers pBr and pML, respectively. Replication originates in the c-myc replicator upstream (to the left) of the PKD1 insert (denoted by three horizontal lines above the map). Four sizes of replication intermediates were collected, ranging from 0.2 kb to 4.0 kb. Very few nascent strands were detected in the two shortest categories, indicating that most replication initiates more than 500 bp from either primer set. A. At pBr, all three cell lines showed the same amount of nascent strands. B. At pML, WT and TTF cells showed nascent strand abundances that were comparable with the pBr results (which is expected since replication forks proceed bidirectionally from an origin). However, TTR cells showed a dramatic decrease in the number of nascent strands detected at pML, indicating that many replication forks were blocked at some point between the origin and pML (which includes the region of the PKD1 insert). Nascent strand abundances were normalized to the beta-globin locus. C. Map of the chromosome region containing the c-myc replicator. Primer sets pBr and pML are shown. Data courtesy of Guoqi Liu.
cytometry in two ways: propidium iodide staining for cells with DNA content between 2n and 4n, and BrdU staining to reveal which cells were actively incorporating nucleotides. Utilizing both techniques provides information that neither technique alone could. If cells begin replicating and then activate a checkpoint and pause replication, propidium iodide staining would count them as S phase cells, but they would not be incorporating BrdU (or any other nucleotides). Therefore, if a significantly larger percentage of cells were found in S phase (by propidium iodide staining) than actively synthesizing DNA (by BrdU incorporation), it would be hypothesized that these “extra” S-phase cells were pausing part way through replication.

If the replication forks of TTR cells moved more slowly than those of TTF cells, TTR cells would spend more time actively incorporating nucleotides, and therefore would have a higher percentage of BrdU positive cells. These cells would also have a lower amount of BrdU incorporated during the pulse period than TTF cells (because they were incorporating it at a slower rate).

As expected, TTR and TTF cells synthesize DNA at the same rate overall. They had indistinguishable asynchronous flow cytometry profiles (Fig. 13a), indicating that they had the same percent of cells in each phase of the cell cycle, or that each stage of the cell cycle took the same amount of time for TTR cells as for TTF cells.

The number of actively replicating cells was confirmed by pulse-labeling with BrdU. In two of the three experiments, TTR cells had a slightly higher number of BrdU positive cells than TTF (Fig. 13b). (The TTR sample in the third experiment exhibited unusual behavior that is not consistent with other experiments.) If this were confirmed, it
Figure 13 The orientation of the triplex-prone region does not affect the time required for replication. A. TTR (pink overlay) and TTF (solid purple) cells show identical asynchronous flow cytometry profiles, indicating that they spend the same amount of time in each phase of the cell cycle. B. Percent of TTR or TTF cells that incorporated BrdU during a 2, 4 or 6 hour pulse. TTR and TTF cells incorporate the same amount of BrdU. TTR or TTF cells were pulsed with BrdU for 2 hours (first 2 lanes), 4 hours (middle 2 lanes) or 6 hours (last 2 lanes). They were then harvested and processed.
by flow cytometry. C. The average amount of BrdU in the BrdU positive part of the population was determined. Results shown are from a single experiment.
would indicate that there are slightly more TTR cells in S phase at any time than TTF cells, or that TTR cells require more time to complete S phase. Of the cells that were actively incorporating nucleotides during the BrdU pulse, TTR and TTF cells showed approximately the same amount of BrdU incorporation (Fig. 13c).

Since TTR and TTF asynchronous cultures displayed approximately the same number of BrdU positive cells, and these labeled cells had the same average amount of incorporation (Fig. 13c), this assay confirms that TTR and TTF cells spend the same amount of time replicating DNA. The orientation of the insert is therefore not measurably changing the length of S-phase. It is still possible that some cells have paused replication, but not enough of them to show a difference between cell lines. Stalling of a single replication fork would not be visible without utilizing single molecule analysis (fiber labeling). So, unless damage induced by replicating the PKD1 insert resulted in global replication slowing or pausing, this assay would not detect differences between the cell lines.

Because global effects of replicating the PKD1 insert in TTR cells have been observed in other experiments described below (cell morphology, population doubling rate, release from aphidicolin and mimosine and response to checkpoint inhibitors), it is probable that some degree of replication slowing takes place in TTR cells. However, this is difficult to see, especially since some of the BrdU positive cells began replication long before the pulse started, some at the beginning of the pulse, and some at the end. So unless the effect was very dramatic, it would escape notice in this assay.

To examine the movement of TTR and TTF cells through S-phase, several synchronization methods were utilized (Fig. 14). Cells were treated with aphidicolin, a
Figure 14 Diagram of cell synchronization methods. HeLa cells, TTR cells or TTF cells were routinely plated on appropriately sized plates, allowed to attach overnight, and then synchronized the following day. Aphidicolin was used at a concentration of 2 µg/mL, Mimosine at a concentration of 400 µM, and Thymidine at a concentration of 2mM. Before releasing into drug free media, plates were rinsed three times with room temperature PBS.
DNA polymerase inhibitor, mimosine, an iron chelator (that pauses the cell cycle before the formation of active replication forks), or thymidine, a nucleotide whose presence in high amounts pauses cells in late G₁/early S phase.

Releasing TTR and TTF cells from a double thymidine block showed predictable results. Both cell lines appeared to move through S-phase at an equal pace after release, their flow profiles matching at all measured timepoints (Fig. 15). Surprisingly, differences were observed when the cells were synchronized with mimosine or aphidicolin. By eight to ten hours after release, a significant number of TTR cells lagged behind TTF cells (Fig. 16). A portion of the TTR cells were requiring longer than TTF cells to replicate their DNA after treatment with these agents. This result contrasted with their identical release from a double thymidine block (Fig. 15).

This effect could also be explained by the precise point in the cell cycle at which the different synchronization methods pause the cells. However, this is somewhat unlikely, since a double thymidine block stops cells at a point intermediate to that of mimosine or aphidicolin arrest (Fig. 17).

Therefore, I wondered if the difference between thymidine and mimosine or aphidicolin was caused not by the time in the cell cycle that the cells were synchronized, but by DNA damage induced by the synchronization. Treatment with mimosine or aphidicolin causes substantially more γ-H2AX phosphorylation and double-stranded DNA breaks than treatment with thymidine 46-48. Perhaps the PKD1 insert is similar to endogenous fragile sites (which are also believed to form stable secondary structures and impede replication fork progression). Although common fragile sites may initiate
Figure 15 TTR and TTF cells release identically from a double thymidine block.

TTR (pink overlay) and TTF (solid purple) cells were synchronized with a double thymidine block, then released into normal media for the times indicated before being analyzed by flow cytometry. Results shown are representative of over ten experiments.
Figure 16 TTR cells release from Aphidicolin and Mimosine more slowly than TTF cells. Flow profiles of TTF (solid purple), TTR (pink outline) or asynchronous cells (dotted blue outline) after release from aphidicolin or mimosine for the indicated times. Results shown are representative of over ten experiments.
**Figure 17 Comparison of cell synchronization methods.** TTF cells were synchronized with a double thymidine block (solid purple), aphidicolin (pink overlay) or mimosine (blue overlay) and analyzed by flow cytometry. Asynchronous cells (dotted green line) are shown for comparison.
replication in early S phase, they demonstrate slow fork progression and do not complete replication until late S phase. In S. cerevisiae, these sites cause DNA breaks in late S phase. Fragile sites are normally stable in cultured human cells, but cause chromosomal breaks more frequently under folate or thymidylate stress and are specifically induced to breakage by treatment with aphidicolin.  

Thymidine is a gentler way to synchronize cells than aphidicolin or mimosine, requiring two rounds to achieve adequate synchronization. Perhaps the increased damage induced by aphidicolin or mimosine affects the already sensitive TTR cells more than TTF. The additional damage may pull repair and damage-sensing proteins away from the PKD1 insert some proportion of the time, leading to double-stranded breaks as a result of fork collapse. For example, if all of the 9-1-1 complex is used to stabilize the forks stalled by the drugs, there may not be as much available to assist the cell in replicating the triplex-prone region. Because this intervention is required more frequently in TTR than TTF cells, the lack of it may also be differentially challenging.

To test the theory that TTR and TTF cells released differently from aphidicolin synchronization but the same from thymidine synchronization because of the additional damage caused by aphidicolin, I synchronized TTR and TTF cells with a double thymidine block, UV irradiated them, and then released them into normal media. The damage induced by UV irradiation is different than that induced by aphidicolin, so some of the proteins required for a response and the mechanisms of overcoming it are different. However, many of the proteins are the same (RPA, ATR, ATRIP, the 9-1-1 complex, Chk1 etc.), so the cell cycle progression effects may be similar.
I began with a UV dose of 2 J/m^2, applied to cells covered with a thin layer of PBS (media removed temporarily). This dose was sufficient to cause a slight increase in the number of sub-G_1 cells in HeLa cells tested (Fig. 18) and has been shown to decrease the amount of Cdc45 bound to chromatin in HeLa cells^{50} (however, our lab was not able to reproduce this result). The cells that were irradiated and released for four hours showed the TTR cells (Fig. 19, solid purple) lagging behind the TTF cells (green overlay). While many of the TTF cells had moved into G_2, the majority of the TTR cells were still in S phase. At eight, nine and ten hours after release, it appeared that the TTR were still progressing slightly more slowly than the TTF. Note that the G_2 peak of TTF gets smaller, while the G_2 peak of TTR gets larger, indicating that the TTR cells are still moving into G_2 at these timepoints, while the TTF cells are moving out of G_2 and dividing, producing more G_1 cells.

**Size Difference Between TTF and TTR Cells**

When I first began culturing these cells (which had been frozen shortly after the cell lines were developed), I noticed that TTF cells appeared like normal HeLa cells, while TTR cells did not. TTR cells were more round than triangular and appeared smaller (Fig. 20a). The differences in size were also apparent in suspension (Fig. 20b), although they were not quite as obvious. To determine which cells of which phase had the largest difference in size between TTR and TTF, asynchronous samples of each cell line were gated based on DNA content to separate G_1, S and G_2 cells, and then the size of each phase of cells was plotted (Fig. 20 b left: G_1 cells, middle: S phase cells, right: G_2 phase cells). In all phases, TTF cells were larger, but the size difference was most dramatic in the G_1 sample.
Figure 18 Dose response of HeLa cells to UV irradiation. Cell media was removed and cells were covered with a thin layer of PBS. They were then exposed to the described levels of UV radiation in a Strata-linker (dosages are shown in $\mu$J/cm$^2$). The media was replaced and the cells were incubated for 4 hours before being harvested and
analyzed by flow cytometry. Flow plots are quantitated in the lower panel. Results shown are from a single experiment.
Figure 19 Damaging TTR and TTF cells with UV radiation before releasing from a double thymidine block may make TTR cells release more slowly than TTF cells. TTR (solid purple) and TTF (green outline) cells were synchronized with a double thymidine block, irradiated with 2 J/m² of UV radiation, and then released into normal media for the indicated times. Results shown are from a single experiment.
Figure 20 TTR cells are smaller and rounder than TTF. A. Pictures of asynchronous TTR (left) and TTF (right) cells. B. Forward scatter parameter used to measure size of TTR (solid) and TTF (outline) cells in G1 (left panel), S (center panel) or G2 (right panel) phase. Bottom shows gating for cell cycle phase. C. TTR cells are smaller than TTF in asynchronous cultures, the same size in thymidine-arrested cultures, but are smaller than TTF seven hours after release. Forward scatter of flow cytometry used to measure the size of TTF (pink outline) and TTR (solid) cells. Dotted line (black)
indicates size of TTF asynchronous cultures. Results shown were observed in numerous cultures.
Intriguingly, the sizes of TTR and TTF cultures arrested with a double-thymidine block (early S-phase) were identical (Fig. 20c). However, seven hours after release, the size differences were apparent again. It seems that the size distribution of the TTF cells did not change substantially after release, while a population of small TTR cells accumulated. It is speculated that during the block, TTR cells have time to reach their normal size, which is the same size as TTF cells. It is plausible that an unidentified metabolic effect is responsible for this effect. It is conceivable that in asynchronous cultures, TTR cells spend more time in S-phase because of replication difficulties, and therefore have a shorter G2 phase and decreased cell growth. (Previously discussed experiments demonstrated that TTR cells do not spend measurably more time replicating their DNA than TTF cells, but these were based on the observation of nucleotide incorporation and not indicators of cell cycle phase. Therefore, they do not eliminate the possibility that TTR cells spend longer in S phase than TTF cells, since late S phase and G2 cells look identical by flow cytometry.) Although no direct evidence reveals the mechanism for this size difference, it must in some way be related to the orientation of the insert, since the cell lines were identical until the insert was added. It is, however, possible that the orientation of the insert led to secondary genotypic or phenotypic changes in TTR or TTF cells.

To examine this change in size, the percent of cells over an arbitrary size (forward scatter = 400) was plotted for TTR and TTF cells versus time (Fig. 21, top). The mean size of cells in each culture was also plotted (Fig. 21, bottom). These plots again reveal that the size distribution of TTF cells is not greatly changing in the first seven hours after
Figure 21 The size distribution of TTR cells changes more after release from a double thymidine block than that of TTF cells. Cells were synchronized with a double thymidine block, released, and samples were harvested at the timepoints shown above. The top panel shows the percent of TTR and TTF cells that were larger than 400 on the X-axis of a flow cytometry histogram, an arbitrary point chosen to demonstrate the decrease in the number of “large” TTR cells after release. The bottom plot shows the mean size of TTR and TTF cells after release from a double thymidine block. Notice that
they begin close together, but by 6 hours after release, TTR has a much lower mean size,
which is explained by a smaller or slow growing population of TTR cells accumulating.
Results shown are from a single experiment.
release. This may indicate that the cells are moving through S and G₂ phases at the same rate, that is, cells that are becoming smaller due to cell division are being replaced by cells growing larger, so that the number of “large” and “small” cells remains relatively constant. In contrast, TTR cells seem to be accumulating more “small” cells after release, which may indicate that they are not moving through all phases of the cell cycle at equal rates.

Recall that these cells were released from a double thymidine block, and by flow cytometry, they appear to be progressing through the cell cycle at equal rates. However, because this assay looks only at DNA content, late S-phase cells appear identical to early or late G₂ cells. If TTR cells were encountering difficulties during late S-phase that slowed their entrance into G₂ and therefore their growth in size, their DNA content would appear the same as TTF cells (giving identical flow profiles), but their size would be decreased. The difference in size between TTR and TTF cells seems to appear around six hours after release, at which point most cells are in late S or G₂. Incidentally, at fragile sites in the genome, this is the stage of the cell cycle in which double-stranded breaks are observed ⁴⁹.

The relatively constant cell size in TTF cells after release was initially surprising. In synchronized cells, the average cell size is not expected to remain constant, but at this point in the cell cycle, enough are growing in G₂ to average out the ones that are dividing and becoming smaller. The observed timepoints look at cells progressing from the end of G₁ phase to the beginning of G₂, during which time there is DNA synthesis, but little change in cell size. Therefore, if we observed average cell size at longer timepoints, we
would anticipate a decrease, since the majority of cells would be dividing and becoming smaller.

In TTR cells, there is a population that made it to G₂ phase and is dividing, but there is apparently also a population that is unable to finish S phase. Therefore, the cells that complete G₂ phase are dividing and becoming smaller, while other cells remain in S phase and do not grow in size to maintain the same average size.

These results highlight one weakness of flow cytometry assays. Although they are helpful in gauging movement through S-phase, they are insensitive to other phases of the cell cycle. In the future, it would be useful to use more accurate indicators of G₂ and mitotic cells to examine the differences in cell cycle progression between TTR and TTF cells.

Because the size difference between TTF and TTR appeared more dramatic on plates than in suspension, it was suggested to me that perhaps the TTR cells were less able to adhere to the plate. This could cause them to remain more spherical rather than flattening out on the culture plate. One way to approximate this is to conduct a “wound healing” assay. A plate of nearly confluent cells is scratched and then observed to monitor the migration of cells to fill the bare spot. If cells are less able to adhere to the plate or less motile, they will likely take longer to migrate into the cleared area of the plate. TTR cells did indeed fill in less of the gap in 24 hours than TTF cells (Fig. 22), indicating that they may adhere less tightly to the plates than TTF cells. If this were true, it would explain why TTF cells spread out more on the plate, while TTR cells round up and appear smaller. However, it is difficult to control the exact width of the scratch and factors such as a subtle difference in cell division rate or cell size could blur results. A
formal cell migration assay, using plates specifically designed to allow the measured migration of cells from one compartment to another, is a much more definitive way to investigate this. However, since the significance and implications of such a finding are unknown at this point, it does not seem worthwhile to invest resources into investigating it further.

**Growth Rate Differences Between TTR and TTF Cells**

In addition to appearing smaller than TTF cells, plates of TTR cells became confluent more slowly than plates of TTF cells. To study the difference in population doubling rate, I took pictures of the cells each day after plating and counted the number of cells per colony in TTR and TTF cells (Fig. 23b). When the cells were plated, there was one cell per colony in both TTR and TTF. By day 2, the average number of cells per colony was already greater in TTF than TTR, and by day 4, the difference was more visible. An increased number of cells per colony indicated that the population doubling rate of TTF cells was faster than that of TTR cells, leading to the faster rate of reaching confluence.

To measure this quantitatively, I conducted an MTT assay. Cells were counted and plated at a specific density in 12 or 24 well plates. Each day, I used an MTT assay in 3 wells of each cell line, using the amount of blue dye produced by the metabolism of the MTT as a measure of the number of cells present. Because all of the wells had been plated with the same number of cells, I could construct a growth curve. Although the number of TTR and TTF cells was identical at plating, after a couple of days, there were more TTF cells than TTR cells (Fig. 23a). Although these results were not significantly
Figure 22 TTF Cells migrate to cover a scratch faster than TTR cells. TTF cells (top left) or TTR cells (top right) were plated at 90% confluency, allowed to attach to the culture plate, and then scratched with a pipet tip to yield a bare area. Twenty four hours later, the same scratched area was observed (lower panels: TTF on left and TTR on right). The TTF cells had better covered the scratched area than the TTR, indicating that they may be better able to adhere to the plate and migrate to fill the empty space better than TTR cells. Note that TTF cells also appear to adhere to the plate under normal conditions, spreading out instead of “rounding up” like TTR cells (see figure 18). Results shown are representative of three separate experiments.
different, the trend was repeated in over ten separate assays (each including three to six samples of each cell line at each timepoint) in both our laboratory and an independent laboratory (John Bissler, Cincinnati Children’s Hospital).

To infer a population doubling rate from the MTT data, I plotted the daily MTT absorbances of each cell line on a log plot and determined the slope of each plot (Fig. 24). This slope is directly related to the rate of population doubling. This is important for two reasons. The first is that comparing growth rates rather than numbers of cells at a certain point removes the assumption that the exact same number of TTR and TTF cells were plated. For example, if a slightly higher number of TTF cells were plated than TTR cells, the difference might not become apparent until it was amplified by several rounds of cell division, leading us to falsely conclude that TTF cells were doubling faster. Therefore, calculating a doubling rate for TTF and for TTR and then comparing these rates is a more reliable measure of differences or similarities. The second benefit of determining a growth rate is that it allows us to develop a time scale. With the assistance of the Wright State Department of Mathematics, we were able to determine that the population doubling rate of TTR cells is approximately two hours slower than the population doubling rate of TTF cells. Although the mechanism responsible for this remains a mystery, a reproducible difference clearly exists.

The population doubling rates of TTR and TTF were always different, but the differences appeared rather subtle. For this reason, I repeated this experiment over 10 times, and Dr. John Bissler (Cincinnati Children’s Hospital Medical Center), a collaborator of ours, also repeated it in his laboratory to ensure that I was not
Figure 23 TTR cells increase in number more slowly than TTF cells. A. MTT assay showing the number of TTF (pink) and TTR (Blue) cells for several days after plating at an equal number. Results shown are representative of ten separate experiments. B. Number of TTR and TTF cells per colony.
**Figure 24 TTF cells increase in number more rapidly than TTR cells.** An MTT assay was performed and the logarithm of the absorbances was plotted. The slope of the TTF cells is .0092 and the slope of the TTR cells is .0090, indicating that the population doubling rate of TTF cells is faster than that of TTR cells. The extremely high $R^2$ values indicate that the data points fit the slopes very well. Results shown are representative of ten experiments.
inadvertently skewing the results. All of the assays agreed: TTR cells have a slower population doubling rate than TTF cells. Cells possess a vast array of damage sensing and repair proteins, which makes them quite proficient at handling genomic stress. Therefore, their unperturbed failure rate should be quite low, making any differences between TTR and TTF cells quite subtle.

**Mortality Rate Between TTR and TTF Cells**

If a population of cells doubles more slowly than another population of cells, then the cells must either be dying more often, or individual cells must be completing the cell cycle more slowly. Asynchronous flow cytometry profiles of TTR and TTF looked identical and the cells moved through the cell cycle identically after release from a double thymidine block (Fig. 15), indicating that the same percentage of the cell cycle was spent in G₁, S and G₂ phases (asynchronous comparison) and that the total length of the cell cycles were the same (thymidine release). Therefore, we wanted to investigate the mortality rate of TTR and TTF cells.

Two methods were used to investigate the rate of cell death in TTR and TTF cells. The first was a trypan blue based assay utilizing a ViCell machine. For each sample, 100 cells are counted and logged as alive or dead, based on their membrane permeability to trypan blue. Multiple samples of each cell line were assayed, and the results averaged and displayed in figure 25. The ViCell data revealed no difference in the viability of TTR and TTF cells. The ViCell assay requires that cells be harvested, pelleted and resuspended in PBS. I wondered if some of the dead cells were being lost during these steps (for example, not pelleting well) and therefore not being observed in the assay. In a separate experiment, I collected the cells from a plate in which approximately half of the
cells had detached from the plate and were floating in the media. I spun down all of the cells (floating and attached) and analyzed the sample on the ViCell machine. It still calculated a high percent of viability, making me suspect that the (dead) floating cells had been lost during sample preparation (data not shown).

To overcome the risk of losing cells when harvesting, we then conducted a cell death ELISA, which measures apoptosis of cells in the culture plate. Again, however, no viability differences were observed between TTR and TTF cells (Fig. 25b). It should be noted that this assay includes steps to remove cells dying by necrosis, which may be the predominant method of cell death in these HeLa derived cells lacking a functional p53. If necrotic cells were included, the results could be quite different.

This data is puzzling, because TTR cells must either be dying more often (by apoptosis) or growing more slowly for the population to double more slowly than TTF cells. My best explanation is that one or both of these are occurring, but the differences are so subtle that they are not revealed in these assays. Even small differences become amplified when large numbers of cells are compared over a large number of population doublings.

Instability of the TTR Cell Line

One of the difficulties I have faced in these experiments is the apparent adaptation of the TTR cell line. As previously mentioned, when I first began culturing TTR and TTF cells, there was an obvious difference in the amount of time required for plates to become confluent. However, over time, this difference became less and less noticeable. To quantify this phenomenon, I conducted a series of MTT assays on “young” (newly
Figure 25 The orientation of the PKD1 insert does not measurably affect viability.
Top: The trypan-blue based ViCell assay was conducted on five separate TTR and TTF samples and the percent of viable cells was recorded. Bottom: Relative amounts of apoptosis in TTF and TTR as measured by a Cell Death Elisa (K. Kulkarni). Results of Cell Death Elisa represent a single experiment. Y-axis shows absorbance of dye produced by each cell line.
produced) or “old” (passaged) TTR and TTF cells (Fig. 26). Young cells were used for the 5/5/2005 assay, and these same cells were assayed a month later (6/5/2005). The difference in growth rate between TTR and TTF was much greater when the cells were first thawed than a month later. I thawed new, young TTR and TTF cells and conducted MTT assays with them on 6/7/2005 and 7/5/2005. Again, the difference in growth rates declined over a month. I again thawed new, young TTR and TTF and conducted an MTT assay on 7/12/2005 and again on 4/5/2006. Again, the difference in growth rates between the two cell lines decreased, that is, TTR and TTF cells began to grow much more similarly.

We wondered if, over time, a population of TTR cells that was resistant to forming a secondary structure in the PKD1 insert was accumulating (we postulate that some TTR cells adapt through genetic or epigenetic changes). Experiments in yeast and Xenopus egg extracts demonstrate that these cells, after a significant interphase delay caused by DNA damage, can “adapt” the checkpoint and continue with mitosis even in the presence of unrepaired or unreplicated DNA. If this were occurring in TTR cells at or near the PKD1 sequence, a wide variety of genetic alterations would be anticipated. Based on the apparent growth and/or survival handicap imposed by the PKD1 sequence, these mutant TTR cells would have a selective advantage and could eventually take over the population. To investigate this, we wanted to look for point mutations, or small insertions or deletions that would decrease the propensity of secondary structure formation in the PKD1 insert. Since stabilizing factors for secondary structure of this sequence are the mirror repeat and runs of guanine residues, mutations which interrupted
Figure 26 As the cells age, the characteristics of TTR and TTF populations become more similar. The plotted data points were derived as follows:

1. Daily MTT absorbances of TTR and TTF cells were measured.
2. $\log_{10}$ (daily MTT absorbances) vs hours after plating was plotted, producing a straight line that represented growth rate.
3. Slope of this line was determined (using Excel), which is $\log_{10}$ (growth rate).
4. $\log_{10}$ (growth rate) of TTF cells was divided by the $\log_{10}$ (growth rate) of TTR cells.
5. Anti-log of this fraction was calculated to yield growth rate difference as plotted above. Note that if TTR and TTF cells had the same growth rate, the slopes would have been equal and dividing them would have yielded 1. The anti-log of 1 is 10, so equal growth rates would be plotted as 10 on this graph.

The arrows indicate when new samples of cells were thawed. Thawed cells were cultured for approximately one week after thawing before beginning the MTT assay. The cells used for the 5/5/2005 assay were newly produced TTR and TTF cells. The same cells were used on 6/5/2005, but the TTR cells were growing more like TTF cells (since they had been cultured an additional month). Another set of TTR and TTF cells were thawed and used for the 6/7/05 assay and the 7/5/2005 assay, and again, the TTR cells became
more like TTF cells after being cultured for a month. It should be noted that the cells used for the 6/7/05 and 7/5/05 assays had not been frozen immediately after creation, but had been cultured for a period before being preserved. Therefore, even in the initial assay, the TTF and TTR cells were not growing dramatically differently. Finally, cells that had been frozen very soon after developing the cell lines were thawed and used for the 7/12/2005 assay and the 4/5/2006, at which time the TTR and TTF cells grew at a much more similar rate.
either of these would decrease its tendency to form structures such as triplexes and quadruplexes.

We attempted to sequence the PKD1 insert from TTR cells that had been growing for some time, but the structure of the sequence inhibits successful sequencing reactions (this has been observed by several sequencing facilities). Therefore, we are unable to know definitively whether or not point mutations are occurring within the sequence that decrease its susceptibility to secondary structure formation. To look for small insertions or deletions in the PKD1 insert, I used PCR primers just outside of the inserted sequence and ran the products on an acrylamide gel to look for small variations in insert size (Fig. 27). I expected to see multiple PCR products from the TTR cells that had been cultured for some time, but each of the PCR reactions yielded only one product. The DNA from TTF cells, old TTR cells and young TTR cells all resulted in the same length of PCR product, indicating that the insert had not undergone insertions or deletions in any of these cell lines.

This left the possibility that a larger deletion had occurred in some percentage of the cells, removing one or both of the PCR primer sites. If this were the case, those cells would not be detected in my PCR assay, giving the impression that all of the cells in the population still had the same length of insert, when in reality, a population of them may have deleted the entire insert.

To examine this possibility, quantitative PCR was performed on “old” TTR cells and TTF cells at two sites, one in the PKD1 insert, and one at an endogenous site on another chromosome (the β-globin locus) to quantify the cells. The ratio of PKD1 sites
Figure 27 The insert size in TTR cells is not changing over time. PCR was performed with primers just outside of the PKD1 insert on the following cells: Lane 1: Newly developed TTR cells. Lane 2: Another clone of newly developed TTR cells. Lane 3: TTF cells that have been cultured for some time. Lane 4: “Old” TTR cells that have been cultured a long time. Lane 5: “Medium” age TTR cells. Acrylamide gel demonstrates unchanged insert size in all cell lines tested. Similar results were observed both times the experiment was repeated.
to β-globin sites was then calculated (Fig. 28). When the cells were designed, one PKD1 sequence was inserted into each cell. Because HeLa cells are tetraploid, there are four copies of the endogenous β-globin sequence in each cell. For the purposes of this experiment, the number of endogenous sites was divided by four, so that a 1:1 ratio of endogenous to exogenous sequence indicates one insert sequence per cell.

TTF cells, as expected, have a 1:1 ratio of inserted to endogenous sequence, indicating that on average, each TTF cell has one inserted PKD1 sequence. Surprisingly, even “young” TTR cells (both tested cell lines) have fewer than one PKD1 site per cell, indicating that on average, TTR cells have less than one inserted sequence per cell, or that a percentage of TTR cells have deleted at least part of the inserted sequence. “Old” TTR cells, which had been cultured even longer, had an even lower percent of cells still retaining the inserted sequence.

In considering these results, it is essential to remember that TTR and TTF cells were created as clonal cell lines. Therefore, if any of the cells had the insert after creation, all of the cells had the insert (and Southern blots and PCR confirmed the presence of the insert in the cell lines, therefore, all cells began with the insert). This fact removes the possibility that some of the TTR cells never had the insert.

It is interesting that even “young” TTR cells have lost the PKD1 insert, which indicates that none of my experiments have been performed with a pure population of true TTR cells. One can only speculate how dramatic the differences between TTF and TTR cells would be if such experiments were possible. The fast rate of deletion in TTR cells implies that the PKD1 sequence does indeed present a substantial barrier to
Figure 28 TTR cells may accumulate large deletions when cultured for longer periods of time. Quantitative PCR was used to compare the number of PKD1 insert sites per cell with the number of β-Globin sites per cell. A number of one indicates that each cell has on average one PKD1 inserted site, while a number less than one indicates that each cell averages less than one PKD1 insert site, meaning that some of the cells in the population have deleted the PKD1 insert. Two different populations of TTR that had been cultured a short period of time after creation (“young TTR”, see first and last lanes) were compared to TTF cells and to “old TTR”, that had been cultured a substantially longer period of time since creation. Results shown are from a single experiment.
reproduction and/or survival, and therefore is capable of creating the types of differences we see between TTR and TTF cells. It also suggests a mechanism for the time-course MTT assays: TTR and TTF cells are not only becoming more alike in behavior, but are also becoming more alike genetically (that is, in their tendency to form a stable secondary structure at the PKD1 insert site- TTF are not deleting this site but are less likely to form secondary structure, so they are more similar to TTR cells that have deleted the sequence than the initially created TTR cells).

**Inhibiting the S-phase checkpoint affects TTR cells more than TTF cells**

After determining that the PKD1 insert was a detriment to the survival of a population of TTR cells and that there is a selective advantage to deleting it, we sought to identify its effects on the cells. Since the PKD1 insert is likely to form a stable secondary structure when the DNA is unwound for replication, we wondered whether the S-phase checkpoint was required to resolve these structures more frequently in TTR cells than TTF cells. The PKD1 insert is known to form replication fork barriers in bacteria and mammalian cells and to inhibit primer extension on plasmids \(^{41,53,42}\).

Previous work has demonstrated that this sequence forms a replication fork barrier much more frequently in TTR cells than TTF cells (measured by nascent strand abundance upstream or downstream of the inserted sequence) (Fig. 12). In addition, chromatin immunoprecipitation assays have shown higher levels of the damage sensing and response proteins ATR, RPA and Rad9 bound near the triplex insert in TTR cells than TTF (Maloy Ghosh, unpublished data). Surprisingly, when immunofluorescence was performed to look for Rad9 foci in TTR and TTF cells, no differences were observable (Fig. 29). Since the TTR cells seem to experience global effects of replicating
the PKD1 sequence, we anticipated seeing global markers of checkpoint activation. However, since the “additional” stress (as compared with TTF cells) is confined to one genetic locus, perhaps the checkpoint proteins are only visible at that locus.

Because of the observed replication fork block and the enrichment of checkpoint proteins at the PKD1 site in TTR cells, it seems that the checkpoint and its accessory proteins are more frequently involved in resolving the structure formed at the PKD1 insert in TTR cells than TTF cells. Thus, TTR cells might respond more dramatically to S-phase checkpoint inhibition than TTF cells. ATR is required for maintaining the stability of fragile sites under normal conditions (i.e. without the addition of DNA damaging agents) \(^\text{49}\), so we suspected that it might be important for replication of the PKD1 site in TTR cells. For this experiment, the ATR-Chk1 checkpoint pathway was inhibited in three ways: treatment with caffeine (a broad action ATM/ATR inhibitor), ATR siRNA, or UCN-01 (a Chk1 inhibitor).

To examine the effects of caffeine on TTR and TTF cells, cells were first synchronized for 20 hours with aphidicolin. They were then released into normal media or normal media supplemented with 5 mM caffeine (Fig. 30) and harvested at the indicated timepoints for flow cytometry. At three and six hours after release, TTR and TTF cells show very similar flow profiles (compare the solid purple profile with the pink overlay on each plot) whether or not caffeine was present. By nine hours after release (when the cells are in late S phase) even in normal media, TTF cells are progressing more rapidly than TTR cells. This is consistent with previous results (Fig. 16). However, the difference between TTR and TTF cells nine hours after release into caffeine is even more
Figure 29 No difference in Rad9 foci is observed between TTR and TTF cells. Left: TTF cells. Right: TTR Cells. Blue: DAPI. Green: Microtubule antibody. Red: Rad9 antibody. Results shown are from a single experiment.
Figure 30 Caffeine increases the differences between TTR and TTF. Flow profiles of TTF (solid purple) and TTR (pink outline) after release from aphidicolin into normal media or caffeine for the indicated times. Cells shown are gated by size to remove sub-G₁ and clumped cells. Comparable results were obtained in several similar experiments.
dramatic. A close examination reveals that TTR cells barely progress at all from six to nine hours, while TTF continue at a substantially faster rate. This suggests that the proteins inhibited by caffeine may be more necessary in TTR cells than TTF cells. (The unusual results of the 11 hour timepoints will be discussed shortly.)

Caffeine also increased the differences between TTR and TTF cells that were released from mimosine synchronization (Fig. 31). Cells were synchronized with mimosine and then released into normal media for six hours, or media containing caffeine for two hours and then normal media for four hours (total of six hour recovery in both samples). This caused the difference between TTR and TTF cells to be very dramatic (compare to figure 16, bottom panel).

Because caffeine is a general kinase inhibitor, a more direct checkpoint inhibitor was desired. Since ATR siRNA targets a single protein, instead of multiple kinases like caffeine, it is easier to identify the reason for any changes that are observed. To achieve effective knock-down, cells were transfected with ATR siRNA at 0 and 48 hours after plating. At approximately 52 hours, the transfection mixture was removed, and cells were treated with aphidicolin to arrest them in late G1/early S phase. At 72 hours, cells were released into normal media and a sample was prepared for western blotting to gauge knock-down efficiency (Fig. 32). At early timepoints after release from aphidicolin into normal media, TTR and TTF cells exhibited identical cell cycle progression (the differences between the cell lines do not normally appear until later timepoints in untreated cells). However, in the ATR siRNA treated cells, TTR cells were proceeding through S phase more slowly than TTF cells by three hours after release from
Figure 31 Caffeine increases the differences between TTR and TTF cells after release from mimosine. TTR (solid purple) and TTF (pink overlay) cells were synchronized with mimosine and then released into normal media for 6 hours (left) or into media containing caffeine for 2 hours and then normal media for 4 hours (total of 6 hours release). Not only did the cells released into caffeine exhibit slower movement through S-phase, but the differences between TTR and TTF cells were accentuated. (Note that the solid and overlay designation is changed in this figure.) Results were confirmed in two separate experiments.
Figure 32 ATR siRNA increases the differences between TTR and TTF. A. ATR western blot of TTR and TTF before and after ATR siRNA treatment. B. Flow profiles of TTR (pink outline) and TTF (purple solid) after mock transfection (top) or transfection with ATR siRNA and release from mimosine. The light blue trace shows an asynchronous sample for cell cycle phase comparison. Results shown are from a single experiment.
aphidicolin. Without functional ATR, then, the problems with TTR cells develop earlier in the cell cycle. This data also reinforces the theory that TTR cells are more dependent upon a functional checkpoint to complete S-phase than TTF cells.

Although ATR siRNA is expected to have more specific effects than caffeine, it still inhibits a major component of multiple pathways and thus can have additional effects (such as activation of dormant origins). In addition, caffeine is unable to inactivate the S-phase checkpoint in HeLa cells. Similar to nontransformed cell lines, HeLa cells have a caffeine-insensitive checkpoint. That is, their checkpoint still functions even when ATM and ATR are inhibited by caffeine (i.e. these kinases are non-essential for some checkpoint functions). When HeLa cells were treated with hydroxyurea (to prevent DNA replication) and caffeine, cells did not enter mitosis, indicating that the checkpoint response still functioned without ATM or ATR activity. In contrast, HeLa cells treated with hydroxyurea and UCN-01, a Chk1 inhibitor, entered mitosis with unreplicated and damaged DNA, demonstrating the necessity of Chk1 for S-phase arrest. These experiments suggest that in the caffeine-insensitive checkpoint pathway, Claspin leads to activation of Chk1 by facilitating phosphorylation at sites other than Ser\(^{345}\) and Ser\(^{317}\). This demonstrates that caffeine does not inactivate the checkpoint function preventing entry into mitosis in the presence of DNA damage, but that caffeine does inactivate ATM and ATR, as well as preventing phosphorylation of Chk1 at its normal activation sites. Although TTR and TTF cells are still unable to enter mitosis in the presence of caffeine, the differences evoked by caffeine treatment can be attributed to the lack of ATM and ATR activity (i.e. presumably, TTR cells suffer more in the absence of ATR activity than TTF cells). In addition, Chk1 that is activated by Claspin is likely incapable
of many of the DNA stabilizing functions it has when activated by ATR. If this is true, treating cells with caffeine abolishes at least some of the Chk1 activities and may therefore be comparable to UCN-01 treatment.

To examine the true effects of completely inactivating the checkpoint, and allowing cells to progress through the cell cycle even in the presence of DNA damage or unreplicated DNA, the Chk1 inhibitor UCN-01 was utilized. Chk1 inhibition is sufficient to allow increased replication origin firing \(^{57}\), but is expected to have fewer off-target effects since it acts further downstream in the ATR checkpoint pathway than caffeine or ATR siRNA.

Cells were synchronized with aphidicolin and then released into media containing 300 µM UCN-01. After release, cells were harvested at intervals and fixed for flow cytometry (Fig. 33). At three hours post release, the TTR and TTF cells appeared to progress identically. At six hours post release, the TTF UCN-01 cells had progressed like the untreated TTR and TTF cells, but the TTR UCN-01 treated cells had moved very little from the position they were in at three hours. At nine hours after release, when most of the TTF cells had progressed into G2 or divided already (note the increasing G1 peak), a population of TTR cells remained trapped in S-phase. Treatment with UCN-01 appears to speed the progress of TTF cells through S-phase but delay the progress of TTR cells, again confirming that TTR cells are less able to replicate their DNA without a functional checkpoint.

It is unknown why the effects of ATR siRNA appear at three hours after release from aphidicolin, while the effects of UCN-01 are seen most dramatically at six hours,
Figure 33 UCN-01 increases the differences between TTR and TTF. Flow profiles of TTF (solid purple) and TTR (pink outline) after release from aphidicolin into normal media or UCN-01 for the indicated times. Cells shown are gated by size to remove sub-G1 and clumped cells. Results were confirmed in several separate experiments.
and those of caffeine at nine hours. This phenomenon is likely the sum of activating and inhibitory off-target effects. For example, lack of functional Chk1 leads to activation of additional replication origins \(^58\) as well as decreased rate of replication fork movement \(^59\). The differing effectiveness of the methods in completely inactivating the ATR-Chk1 pathway could also contribute to the observed results. Although each method of checkpoint interruption has the possibility of off-target effects, since the results of all three agree, it can be inferred that the primary observed effects are due to inhibition of the ATR-Chk1 pathway. Since treatment with caffeine and UCN-01 are much simpler and less costly than treatment with ATR siRNA, these methods will be used to further investigate the effects of checkpoint inhibition.

It would be interesting to synchronize TTR and TTF cells with a double thymidine block (from which they normally release identically) into caffeine or UCN-01. Synchronization with thymidine would perturb the cells less than treatment with aphidicolin, which would provide more insight into checkpoint function under normal circumstances. Ideally, we could examine the cell cycle progression of asynchronous cells after checkpoint inhibition, but without synchronization, it becomes very difficult to observe differences. Even in the presence of checkpoint inhibitors, TTR and TTF cells would probably still release identically from a thymidine block, since there would be sufficient residual checkpoint activity to deal with the PKD1 sequence (and there would not be additional damage inflicted from the synchronization method).

Whether treated with caffeine (Fig. 31), ATR siRNA (Fig. 32), or UCN-01 (Fig. 33), the TTR cells began to lag behind TTF cells more dramatically and at earlier timepoints after release from aphidicolin than when the checkpoint was intact. This was
predictable, based on the increased binding of Rad9 and ATR near the insert in TTR cells. These observations do not deny a role for the checkpoint in TTF cells, as all cells encounter endogenous and exogenous damage during replication that causes some degree of checkpoint dependence.

Because the ATR-Chk1 pathway was expected to play a role in resolving the structure formed at the PKD1 insert in TTR cells especially, we performed westerns to look for phosphorylated-Chk1. Experiments revealed that TTR cells exhibit a higher level of Chk1 phosphorylation than TTF cells (Guoqi Liu, unpublished data), indicating that TTR cells are activating the checkpoint pathway more frequently than TTF cells.

As Chk1 is phosphorylated by ATR to activate it, it is simultaneously marked for degradation by the proteosome. If a significantly higher amount of Chk1 is being phosphorylated and subsequently degraded in TTR cells, then treating TTR and TTF cells with a proteosomal inhibitor should increase the total amount of Chk1 in TTR cells. I treated cells with MG132 (a proteosome inhibitor) and looked at the levels of total Chk1 (Fig. 34). Cells were synchronized with a double-thymidine block and then released into normal media, MG132, or hydroxyurea for two hours before being prepared for western blotting. The total amount of Chk1 in TTR cells released for two hours appeared greater than the Chk1 in TTF cells (the Chk1 in lanes 5 and 6 looks identical, while lane 6 clearly has much less protein loaded). However, in the MG132 samples, TTF and TTR appear to have the same amount of Chk1 (and in fact, it is less than in the untreated samples, perhaps because Chk1 is primarily expressed in S and G2, and most of these cells are just

87
Figure 34 Chk1 is not being degraded in a proteosome-dependent manner in TTR cells. Western blot performed with Chk1 antibody. Cells were either untreated (lanes 1 and 2), synchronized with a double thymidine block (lanes 3 and 4), or released from a double thymidine block for 2 hours into normal media (lanes 5 and 6), 5 uM MG132 (lanes 7 and 8), or 10 uM hydroxyurea. Coomassie stained membrane is shown for loading control. Lower panel shows flow cytometry profiles of untreated cells, cells synchronized with a double-thymidine block, or cells released for 1 or 2 hours. TTF cells: solid purple. TTR cells: pink outline. Asynchronous cells: blue outline. Results shown are from a single experiment.
entering S phase). These experiments reveal no dramatic differences in Chk1 levels between TTR and TTF cells. This indicates that the Chk1 in TTR cells is not being degraded by the proteosome due to constant replicative stress.

My checkpoint experiments also revealed unanticipated differences at long times after release from synchronization into a checkpoint inhibitor. When the cells released into caffeine or UCN-01 for 11 hours were gated based on size (which is routinely done to eliminate clumps of cells and debris and normally removes less than five percent of the total cells), very few TTR cells remained (see Fig. 30 and 33, bottom panels). I plotted the DNA content of these cells (x-axis) versus their size (y-axis) (Fig. 35). I then colored the cells with DNA content between 2n and 4n and with a normal size red, while the cells with DNA content below 2n (dead cells) were colored green (both of these are in the lower left quadrant). Cells with larger than normal size were colored blue (G1 and S phase cells) or pink (G2/M cells) (upper left quadrant). Cells with greater than 2n DNA content represent clumped cells (upper and lower right quadrants). Both TTR and TTF cells appeared normal at nine hours after release from aphidicolin into UCN-01 or caffeine (Fig. 35). However, at 11 hours after release into either caffeine or UCN-01, approximately one third of the TTR cells were swollen to twice their size, while TTF cells appeared normal (Fig. 35, quantified in Fig. 36). I used the ViCell machine (trypan blue based assay) to measure the viability of cells released into the checkpoint inhibitors for 11 hours, but found no change whether they were released into normal media, caffeine, or UCN-01 (Fig. 37). To understand better what was occurring in the cells, I released TTR and TTF cells into UCN-01 for 11 hours and then stained them with DAPI to visualize the nuclei (representative cells shown in Fig. 38). Almost all of the TTF cell
Figure 35 TTR cells swell 11 hours after release from aphidicolin into caffeine or UCN-01. Cells are plotted by DNA content (x-axis) vs. size (y-axis). Normally, about 95% of cells appear in the lower left quadrant, which contains viable cells (colored red) and sub-G1 cells (green). Cells in the upper and lower left quadrants are clumped cells (having more than 2n DNA content, and most being larger than a normal cell). There typically are very few cells in the upper left quadrant, which represents cells having DNA content between 2n and 4n, but a size larger than a normal cell. These “large” cells are divided by color into G1-S cells (blue) and G2/M cells (purple).
Figure 36 TTR cells swell 11 hours after release into checkpoint inhibitors. Cells were synchronized with aphidicolin and then released into normal media, or media containing caffeine or UCN-01 for 9 (top graph) or 11 hours. They were then gated based on size to determine what percent were in the normal size, sub-G1, or swollen cells (“big G1/S” and “big G2/M”). Results were confirmed in multiple experiments.
Figure 37 TTR cells show no decrease in viability 11 hours after release from aphidicolin into UCN-01. Cells were synchronized with aphidicolin and then released into normal media, media containing UCN-01, or media containing UCN-01 and Nocodazole. Percent of viable cells was determined using the Trypan Blue based ViCell machine. Results shown are from a single experiment.
Figure 38 TTR cells show nuclear fragmentation 11 hours after release from aphidicin into checkpoint inhibitors. DAPI staining of three representative TTF cells (top) or TTR cells (middle and lower panels are the same cells shown in black and white or color) after aphidicin synchronization then 11 hour release in UCN-01. At least 100 cells of each cell line were examined.
nuclei (top pictures) appeared normal. However, the nuclei of over half of the TTR cells were divided into multiple smaller nuclei, an event known as nuclear fragmentation, which is a precursor to cell death\textsuperscript{61,62}. In contrast, less than ten percent of TTF cells exhibited this phenomenon. Just as nuclear fragmentation is an early step in programmed cell death, cell swelling is also observed in cells soon to undergo necrosis\textsuperscript{63}.

To determine if these cells were in the process of dying, I decided to look at longer timepoints. Visually examining TTR cells released into caffeine or UCN-01 for 13 hours revealed many cells with membrane blebbing and loss of membrane integrity, while cells released into normal media or TTF cells released into checkpoint inhibitors did not (Fig. 39). By 13 hours, about one third of the TTR cells appeared as a sub-G\textsubscript{1} population (many of the S/G\textsubscript{2} cells have become sub-G\textsubscript{1}), while the viability of TTF cells had not changed (Fig. 40). There are virtually no “swollen” cells remaining (although a few appear to be present in the TTF normal and TTF UCN-01 samples, these are likely the result of clumped cells and would disappear if these samples had been gated for DNA content as well- occasionally samples are not thoroughly vortexed during preparation leading to an increased number of clumped cells). This suggests that the cells which appeared swollen at 11 hours have died by 13 hours.

In comparing the TTR and TTF cells released into normal media, it appears that many of the TTF cells have already completed the cell cycle, divided and become G\textsubscript{1} cells, while a greater percentage of TTR cells remain in S/G\textsubscript{2}. This is consistent with previous experiments. Two of the samples were treated with Nocodazole, a microtubule polymerization inhibitor that pauses the cell cycle just before cell division. Interestingly,
Figure 39 TTR cells demonstrate loss of membrane integrity after long treatments with caffeine or UCN-01. Cells were synchronized with aphidicolin and then released into normal media or media containing caffeine or UCN-01. After 13 hours, they were observed visually. Insets are increased magnification. At least 100 cells from each sample were observed.
Figure 40  TTR cells die 13 hours after aphidicolin release into UCN-01. Cells were synchronized with aphidicolin and then released for 13 hours into normal media, UCN-01, or UCN-01 and nocodazole. The percentage of cells in each size category (as determined by flow cytometry) is plotted. Results shown are from a single experiment.
the TTR and TTF cells treated with UCN-01 and Nocodazole look almost identical, with
the exception of a slightly increased sub-G₁ population in the TTR cells. This reveals
several things. First, it shows that the TTF-UCN-01 sample has more G₁/S cells than the
TTR-UCN-01 sample because the TTF cells are dividing and entering G₁ again, rather
than because the cells are trapped in G₁. Second, it demonstrates that if the population of
TTF cells is paused in its progression, TTR cells will eventually complete S-phase to the
same degree and “catch up.” Third, it suggests that the majority of TTR cell death may be
occurring as the cells divide. The TTR cells treated with UCN-01 and Nocodazole have
lower mortality than the TTR cells treated with UCN-01 alone. It seems that the damage
leading to death begins during replication, and some of the cells are losing membrane
integrity and dying during S/G₂, but the fatal event for most (indicated by sub-G₁ DNA
content) does not occur until cell division. This is logical since HeLa cells are unable to
activate an S-phase checkpoint response in the presence of UCN-01 and are therefore
unable to detect DNA damage until mitosis. Recent evidence in yeast indicates that
even with an intact checkpoint response, cells may be able to enter mitosis before DNA
replication is complete, especially when the replication of some regions is delayed.
This could then lead to cell death.

To investigate the timing of cell death further, a timecourse experiment was
performed, measuring the percent of sub-G₁ cells at 8, 10, 12 and 14 hours after release
from aphidicolin into normal media or UCN-01 (Fig. 41). This data shows that treatment
with UCN-01 affects the viability of TTF cells very little. However, treatment of TTR
with UCN-01 drastically increases the amount of cell death (as measured by sub-G₁ DNA
content).
Figure 41  Treatment with UCN-01 causes more cell death in TTR cells than TTF cells.  Cells were synchronized with aphidicolin and released into normal media or media containing UCN-01 for 8, 10, 12 or 14 hours before being prepared for flow cytometry.  The percentage of sub-G1 cells in each sample at the release times indicated was plotted.  Data shown are representative of four experiments.
content). These results are slightly different than the results of the previous experiment (Fig. 40) in two ways. First, the death of TTR cells appears to gradually increase over time instead of occurring suddenly at one timepoint. Second, by 14 hours, only ten percent of the TTR cells are dead. I believe that these discrepancies can be attributed to differences in synchronization rather than flawed data. In every experiment, the amount of time it takes cells to reach a certain point in the cell cycle after release is variable. Although care has been taken to standardize every aspect of synchronization (use only fresh aliquots of aphidicolin, use the exact same procedure etc.), this variability exists, which is why all of the experiments showing differences in release between TTR and TTF cells have been repeated many, many times. I believe that in the timecourse experiment (Fig. 41), the cells have not released long enough to reach the critical point where many TTR cells die, thus explaining the lower number of dead cells and the lack of a dramatic increase in cell death. If this experiment had been carried out for 16 or 18 hours, this event likely would have been observed. Regardless of these differences, this data again confirms that inhibition of the checkpoint pathway affects TTR cells more than TTF cells.

It should also be noted that this data appears slightly different than the ViCell data, which shows no measurable difference in TTR cell viability at 11 hours, whether or not they have been treated with UCN-01. I believe that the variation comes from the number of cells counted. The ViCell machine only counts 100 cells of each sample, while flow cytometry is counting 10,000 cells per sample. The differences shown in this plot are relatively small (with even the most affected cells only showing approximately 7% sub-G1 cells at 11 hours after release), so it is probable that these differences didn’t
show up in the ViCell assays. Another point to consider is that the two assays measure different parameters- flow cytometry is looking at DNA degradation, which occurs at some point before loss of membrane integrity (which is what the ViCell measures). So, at 11 hours, it is likely that only a small percentage of TTR cells treated with UCN-01 have progressed far enough in the death process to be picked up by the ViCell assay.

Taken together, these results indicate that in the absence of a functional checkpoint, TTR cells undergo cell death more frequently than TTF cells. Future work should examine these effects more closely, and attempt to determine whether TTR cells are dying by apoptosis or necrosis. It is puzzling that signs of necrosis (cell swelling) and apoptosis (membrane blebbing and nuclear fragmentation) are both observed. Recent investigations have questioned whether the two types of cell death are indeed distinct, and have reported signs of both types of cell death in the same cells 63.

Some investigators are hesitant to perform any DNA damage or checkpoint related studies in HeLa cells because of their altered p53 status (although they express p53, it is quickly degraded due to the activity of HPV protein E6) 65. Nonetheless, HeLa cells are able to induce apoptosis as a result of replication licensing defects 66 and other replication defects. In addition, the S-phase checkpoint pathway upstream of p53 seems to function normally, since HeLa cells are able to stop replication in a Chk1-dependent manner when DNA is damaged or unreplicated 55,56. In the assays performed here, the checkpoint is interrupted upstream of p53 (at ATR or Chk1), where the checkpoint should be functioning as in non-transformed cells. I observed cell death as a result of checkpoint inhibition in TTR cells, therefore, even without a functional p53, the checkpoint must be performing a protective function in these HeLa-derived cells. For
these reasons, although the entire checkpoint pathway is not normal in transformed HeLa cells, I believe that the assays performed here are a reliable indication of checkpoint function as it relates to replication fork stalling.

**Genetic Differences Between TTR and TTF Cells**

Extreme care has been taken to ensure that the orientation of the insert is the only difference between TTR and TTF cell lines. Southern blots have confirmed that the PKD1 sequence was inserted exactly one time in each cell line and that this insertion occurred at the intended location (Fig. 11). Probes inside and outside the inserted sequence yield a single band of the same size in both cell lines. It is important to note that this result does not contradict the data suggesting that the PKD1 insert has been deleted in some percent of even young TTR cells. The Southern blots examined a relatively large section of DNA surrounding the insert site and would have displayed a shift if the entire 1.2 kb c-myc construct had not been inserted, but not if an 88 bp section (the PKD1 mirror repeat) had been deleted from that construct.

To further confirm that the observed differences in TTF and TTR cells were the result of the PKD1 insert and not a random event occurring in a parent cell, four separately isolated clonal TTR and TTF cell lines have been examined. The morphology and growth characteristics of the additional cell lines were consistent with the results previously described.

This information is especially important because it is not currently believed that a single genomic site can affect the health or cell cycle progression of a mammalian cell. The inserted sequence is such a miniscule part of the entire genome, and cells are equipped to deal with genomic aberrations, so it seems counter-intuitive that these effects
occur. Nevertheless, there appears to be no other explanation for my data. TTR cells are smaller, reproduce more slowly and are more sensitive to drug treatment and checkpoint inhibition than TTF cells, all due to the orientation of the PKD1 insert.

The orientation effects of a triplex-prone sequence do not require a nearby origin

Because of little understood replication fork dynamics, the distance from an origin of replication may affect the genomic instability of DNA sequences. Some types of DNA sequences may form stable secondary structures when they are near an origin, but not when farther away. Possibly, the DNA near an origin is unwound a greater percent of the time (since it is near an easily unwound region), which would lend greater opportunity for secondary structure formation. Because the inserted PKD1 sequence forms what seems to be a stable replication fork barrier in vitro and in TTR cells, we believe that secondary structure will form irrespective of proximity to an origin.

To test whether a nearby origin was required to cause an orientation-dependent replication fork barrier, we created two additional cell lines (Liu et al, in preparation). The same PKD1 insert used for the TTR and TTF cell lines was put in place of the DNA Unwinding Element (DUE) of the c-myc replicator, abolishing origin activity. This modified insert was placed into the genome of HeLa-derived acceptor cells using the FLP recombinase system for specific integration, creating stable cell lines. The PKD1 insert was again placed in either the forward or the reverse orientation in relation to the c-myc replicator, yielding DTF or DTR cells, respectively (Fig. 10).

When I began culturing these cells, I immediately noticed a difference in them similar to the difference observed in TTF and TTR cells. Surprisingly, DTR (reverse)
cells have a phenotype similar to TTF (forward), while DTF (forward) have a phenotype similar to TTR (reverse). This suggests that in the absence of c-myc origin activity, the fork that predominantly replicates the triplex sequence in DTR and DTF comes from the opposite direction. This would reverse which strand was leading or lagging and therefore which was more likely to form a secondary structure that would block replication. In TTF and TTR cells, the PKD1 insert is being replicated from the exogenous c-myc origin, but this origin is inactive in DTR and DTF cells. Size-fractionated nascent strand analysis indicated that the nearest endogenous origin was greater than 4-8 kb from the FRT acceptor site. This confirms that the sequence is being replicated from farther away when the replicator activity of the c-myc insert is abolished. However, it does not reveal from which direction the sequence is being replicated.

If the direction of replication were switched (that is, if the PKD1 insert were replicated by a downstream origin rather than an upstream origin), the polypurine strand would then be the lagging strand in DTF cells, increasing the chance of stable secondary structure formation. The polypurine strand would be the leading strand template in DTR cells, decreasing the chance of stable secondary structure formation. This would explain why DTF cells appear smaller and rounder (like TTR cells) than DTR cells, which have typical HeLa morphology (Fig. 42).

**Growth Rates of DTR and DTF Cells**

In a “wound healing” assay, DTF cells migrated to cover an exposed area of a culture plate more slowly than DTR cells (Fig. 43). As with TTF and TTR cells, this may indicate that DTF cells have a reduced ability for substratum attachment. An MTT assay
**Figure 42** DTF cells (left panel) appear smaller and rounder than DTR cells (right panel).

This trend was observed in all DTR and DTF cells cultured.
Figure 43 DTR cells migrate to fill in a “wound” faster than DTF cells. Left top panel: DTR cells immediately after plate was scratched. Left lower panel: DTR cells 24 hours after the plate was scratched. Right top panel: DTF cells immediately after plate was scratched. Right lower panel: DTF cells 24 hours after the plate was scratched. Images shown are representative of several experiments.
investigated their population doubling times as described previously (Fig. 44). When the log of the absorbances was plotted, the slope of the growth rate line of the DTR cells was greater than that of the DTF cells, indicating that the population doubling rate of DTR was greater than that of DTF. That is, DTF cells were dividing more slowly or dying more frequently.

While culturing these cells, there was always an obviously larger number of floating cells in DTF samples than DTR samples, and the plates of DTF cells became confluent much more slowly than the plates of DTR cells. A preliminary apoptosis assay (Roche Cell Death ELISA) indicated a two-fold difference in apoptosis rate between DTR and DTF. If this were confirmed, it might suggest that the difference in population doubling rate between TTF and TTR might also be due to a difference in cell death rate, although only detectable with more sensitive assays.

**Release from Synchronization of DTR and DTF Cells**

DTR and DTF cells were then synchronized with a double thymidine block and released for two to eight hours and then analyzed by flow cytometry. As with TTF and TTR cells, DTF and DTR cell lines released identically from this method of synchronization (Fig. 45). However, DTF cells released from aphidicolin synchronization more slowly than DTR cells (Fig. 46). By six hours after release, the population of DTR cells moving through S-phase was slightly ahead of the DTF cells. By nine hours, this difference was more apparent, and at eleven hours it was the most obvious. These differences are subtle but repeatable.

DTF cells also require longer to replicate their DNA than DTR cells after release
Figure 44 DTF cells have a slower population doubling rate than DTR cells. The log of net absorbance for each cell line was plotted according to hours after plating. Note that three samples were harvested at each time point. The slopes of the lines were determined, with DTF having a slope of .0094, and DTR having a slope of .0109, indicating that DTR cells are increasing in number more rapidly than DTF cells. If the difference were caused purely by a difference in the rate of growth (and not through greater numbers of cell death), this would translate into approximately a 10 hour difference in cell cycle. Results shown are representative of at least three separate experiments.
Figure 45 DTR (solid) and DTF (overlay) cells release the same from a double thymidine block. Cells were synchronized with a double-thymidine block, released for the time periods indicated, and then harvested and analyzed by flow cytometry. Results shown are representative of three experiments.
Figure 46 DTF (solid) releases from aphidicolin more slowly than DTR (outline). DTF and DTR cells were synchronized with aphidicolin and then released for the times indicated before being processed for flow cytometry. Results shown are representative of four experiments.
from mimosine synchronization (Fig. 47). At eight hours after release, there are substantially more DTF cells still in G\textsubscript{1}/early S phase than DTR cells. By eleven hours, most of the G\textsubscript{1}/early S phase cells have progressed in the DTR sample, while a large percent of the DTF cells have still not moved out of early S phase. (Because the G\textsubscript{1} peak decreases in size from eight to eleven hours, it is likely to be primarily composed of cells remaining in early S phase from the synchronization and not from G\textsubscript{2} cells dividing to produce more G\textsubscript{1} cells.)

**Inhibiting the Checkpoint Response in DTR and DTF Cells**

The effect of checkpoint interruption on DTR and DTF cells was then examined. DTR and DTF cells were synchronized with aphidicolin and then released into normal media, media containing UCN-01 (the Chk-1 inhibitor), or media containing caffeine (the ATR/ATM inhibitor) (Fig. 48). Unlike the results observed with TTR and TTF cells, treating DTR and DTF cells did not increase the differences between the cell lines.

It is possible that the reason DTR and DTF cells respond to checkpoint inhibitors differently than TTR and TTF cells lies in the proximity of the origin of replication to the PKD1 sequence. In TTF and TTR cells, the PKD1 insert is replicated by an origin very close to it, leaving little opportunity for a dormant origin (i.e., an origin that does not fire in most cell cycles) that is closer to fire and replicate it from the opposite direction. DTR and DTF replicate the PKD1 insert from an origin far away, which makes it more likely that a dormant origin closer to the PKD1 insert than the normal origin will replicate the inserted sequence. This dormant origin may be upstream in some cells and downstream in some cells, obscuring direction-dependent replication effects.
Figure 47 DTF (outline) releases from mimosine more slowly than DTR (solid).

DTR and DTF cells were synchronized with mimosine for 20 hours and then released into normal media for 8 or 11 hours. Asynchronous samples are shown for comparison. Results shown are representative of three experiments.
Figure 48 Treating DTR and DTF cells with checkpoint inhibitors does not increase the differences in release from aphidicolin. DTR (solid) and DTF (dark outline) cells were synchronized with aphidicolin and then released into normal media (top panel), media containing the Chk1 inhibitor UCN-01 (middle panel) or media containing the checkpoint kinase inhibitor caffeine (bottom panel). Plots were gated to remove sub-G₁ and clumped cells. Each experiment was repeated at least twice.
Although DTR and DTF did not appear to respond differently to treatment with caffeine or UCN-01, we decided to examine the effects of longer treatments with UCN-01. DTR and DTF cells were released from aphidicolin into UCN-01 for 13 hours and then analyzed by flow cytometry. There were more sub-G₁ cells in the DTF sample than the DTR sample, although the numbers of dead cells were not as dramatic as in the TTR sample. I then plotted the fold-change of sub-G₁ cells upon treatment with UCN-01 (Fig. 49). The percentage of sub-G₁ DTR cells doubled, while the percentage of sub-G₁ DTF cells nearly quadrupled. This increase in cell death was equivalent to the increase observed in TTR cells. Therefore, although DTR and DTF cells do not appear to respond dramatically differently to UCN-01 in the first nine hours of treatment, at longer timepoints, DTF cells experienced more cell death.

3.4 Removal of the mirror repeat insert makes TTR cells more like TTF cells

A number of steps have been taken to ensure that the differences between TTR and TTF cells and DTR and DTF cells were caused by the orientation of the PKD1 sequence instead of random mutations that occurred in the cell lines (this does not exclude the possibility that the orientation of the PKD1 insert promoted other mutations). For this reason, we tested three separately isolated TTR cell lines and three separately isolated TTF cell lines. Southern blots and PCR were performed to ensure that the inserted sequence was inserted only once, and that it inserted in the correct location. In addition, DTR and DTF cell lines were created using different plasmids than TTR and
Figure 49  Checkpoint inhibition by UCN-01 causes more cell death in DTF and TTR cells than DTR cells. Cells were synchronized with aphidicolin and then released into normal media or media containing the Chk1 inhibitor UCN-01. The fold increase in sub-G₁ cells with UCN-01 treatment is plotted (for each cell line, the number of sub-G₁ cells in the UCN-01 sample is divided by those in the untreated sample to yield the fold-increase caused by UCN-01 treatment). Results shown are representative of two experiments.
TTF cell lines, and their differences in phenotype confirm that the orientation of the insert is causing the differences.

**Creation of Flp’d TTR Cell Lines**

Nonetheless, to demonstrate definitively that the inserted sequence is causing TTR cells to behave differently from TTF cells, we removed the ectopic c-myc replicator (containing the PKD1 insert). We treated TTR cells with Flp Recombinase to remove the inserted sequence (creating Flp’d TTR cells). I diluted the treated cells to 1/10th cell per well and cultured them until I had sufficient cells for PCR, then screened the cell lines for ones that lacked the PKD1 insert (Fig. 50). Primers 1 and 2 are adjacent in the empty acceptor site, while primers 1 and 3 are adjacent in cells having the c-myc/PKD1 insert (see Fig. 10). I chose three of the cell lines lacking the insert to study further. Interestingly, one of these showed only a product with the 1/2 primer set (indicating that the c-myc insert had been removed), while the other two showed a major product with the 1/2 primer set, and a small amount of product with the 1/3 primer set (suggesting that the insert might be present in a subpopulation of cells). Because the cells were diluted enough to only have one cell per ten wells, it is unlikely that the cell lines are not clonal (although this is possible if trypsanization was not complete and two cells remained clumped together), which means that they all should either have or lack the insert. If two cells (one having the insert and the other lacking it) had adhered together and been plated in the same well, the PCR products from the 1/2 and 1/3 primer sets should be approximately equal. Therefore, the small amount of 1/3 product could be the result of minor contamination. Since the no template control shows no contamination, it is
Figure 50  PCR showing deletion of the PKD1 insert in “Flp’d” TTR cells. TTR cells were treated with the Flp Recombinase plasmid, diluted to 1 cell per 10 wells, and then screened by PCR for cell lines that had lost the insert. Primers 1/2 produce a product in cells without the insert, while primers 1/3 produce a product in cells still possessing the inserted sequence. NTC: No template control. TTR Norm: TTR cells that were not treated with Flp Recombinase (still have PKD1 insert). TTF Norm: TTF cells that were not treated with Flp Recombinase (still have PKD1 insert). TTR B9, TTR B6 and TTR E10: Clonal cell lines created by treating TTR cells with Flp Recombinase to remove the PKD1 insert.
unlikely, but possible that the other samples are contaminated. To confirm this, I collected new DNA from the cell cultures and repeated the PCR, but received the same results.

Even if the Flp’d cell lines are not pure (in lacking the insert), the percentage of cells lacking the insert is sufficient to show a difference if one exists. As I was culturing these Flp’d TTR cells, I could see a visible difference - they resembled TTF cells rather than TTR cells. I selected three representative cell lines to use in the following experiments (B6, B9 and B10).

**Flp’d TTR Cells Released from Synchronization**

I then decided to examine how these cells released from mimosine and aphidicolin. The Flp’d TTR cells released from mimosine faster than TTR cells (Fig. 51), so I decided to compare them to TTF cells. When released from mimosine, the Flp’d TTR cells released almost like TTF cells (Fig. 52). They exhibited a slight delay, but not nearly as much as regular TTR cells. The Flp’d cells were then released from aphidicolin. Again, they released from aphidicolin faster than TTR cells (Fig. 53), but a bit slower than TTF cells (Fig. 54).

**Treating Flp’d TTR Cells with Checkpoint Inhibitors**

Because it was believed that the inserted PKD1 sequence was the cause for increased cell death in TTR cells treated with checkpoint inhibitors, I then examined the effects of caffeine and UCN-01 on Flp’d TTR cells. TTF, TTR and Flp’d TTR cells were synchronized with aphidicolin and then released into normal media or media containing caffeine. At 11 and 14 hours, samples were harvested and prepared for analysis by flow cytometry (Fig. 55). TTF cells demonstrated the least increase in cell death due to
Figure 5: B9 Flp’d TTR (solid purple) release from mimosine faster than TTR cells (green outline). TTR cells and Flp’d TTR cells (with the PKD1 insert removed) were synchronized with mimosine and then released for the times indicated. Results shown are representative of four experiments.
Figure 52  B9 Flp’d TTR cells (solid purple) release from mimosine almost like TTF cells (green outline). B9 Flp’d TTR cells (which have the PKD1 insert removed) and TTF cells were synchronized with mimosine and then released for the times indicated before being harvested and prepared for flow cytometry. Results shown are representative of three separate experiments.
Figure 53 B9 Flp’d TTR cells release from aphidicolin faster than TTR cells. TTR cells (pink overlay) and Flp’d TTR cells (solid purple) were synchronized with aphidicolin and released for the times indicated. Results shown are representative of three experiments.
Figure 54 Flp’d TTR cells release from aphidicolin similarly to TTF cells. TTF cells (pink overlay), TTR cells (blue overlay) or Flp’d TTR cells (solid purple) were synchronized with aphidicolin and released for the times indicated. Flp’d TTR cells behave more like TTF cells than TTR cells. Results shown are representative of two experiments.
Figure 55 TTR Cells lacking the PKD1 insert (Flp’d TTR) are less affected by caffeine treatment than TTR cells but more affected than TTF cells. TTF cells, TTR cells, Flp’d TTR were synchronized with aphidicolin and then released into normal media or media containing caffeine for 11 or 14 hours (see labels below bars). Plotted is the fold increase in sub-G₁ cells with caffeine treatment (for each sample and timepoint, the number of sub-G₁ cells in the caffeine-treated sample is divided by the number of sub-G₁ cells in a sample released into normal media). Results shown are representative of both Flp’d TTR cell lines tested.
caffeine treatment, TTR cells showed the most, and Flp’d TTR had an intermediate increase. Both Flp’d cell lines tested demonstrated approximately the same amount of cell death in response to release from into aphidicolin into caffeine. To further validate these results, a time-course of cell death after UCN-01 treatment was constructed for TTF cells, TTR cells, three separate clonal Flp’d TTR cell lines (B6, B9 and E10 cells), and a mixed population of Flp’d TTR cells (Flp cells: B6, B9 and B10 cells that were combined to create another cell line ) (Fig. 56). The TTF cells showed very little increase in cell death as a result of the UCN-01 treatment. The TTR cells showed the greatest increase in cell death as a result of the UCN-01 treatment. All of the individual Flp’d TTR cell lines and the pooled Flp cells showed a greater increase in death than TTF, but a lesser increase in death than TTR cells. These results indicate that disruption of the checkpoint pathway is less deadly to TTR cells that have the insert removed than to normal TTR cells, and therefore that TTR cells without the PKD1 insert are less dependent upon the checkpoint for survival. This fact suggests that the checkpoint proteins are needed to act at the PKD1 insert to prevent cell death in TTR cells.

This again confirms that insertion of the PKD1 polypurine-polypyrimidine sequence caused the differences between TTR and TTF cells, since removing it from TTR cells partially restored the “TTF” phenotype. It is speculated that the full normal phenotype was not restored because of some type of damage that accumulated in the TTR cells as a result of coping with the polypurine-polypyrimidine insert for a period of time. Another possibility is that the “Flp’d TTR” cells do not all lack the PKD1 insert, and thus are a mixture of cells behaving like TTR cells, and cells behaving like TTF cells.
Figure 56 TTR Cells lacking the PKD1 insert (Flp’d TTR) are less affected by UCN-01 treatment than TTR cells but more affected than TTF cells. TTF cells, TTR cells, Flp’d TTR cells (B6, B9 and E10 are clonal cell lines derived by removing the insert from TTR cells, and Flp cells are a non-clonal population of Flp’d TTR cells) were synchronized with aphidicolin and then released into normal media or media containing UCN-01. Plotted is the number of sub-G1 cells in the sample released into UCN-01 divided by the number of sub-G1 cells in an identical sample released into normal media for the same amount of time. Data shown are representative of at least three experiments per cell line.
3.5 Replication Fork Barrier Conclusions

Effects of DNA damage-inducing synchronization on TTR and TTF Cells

The experiments described reveal two types of information about the orientation effects of the triplex prone sequence. They demonstrate that changing the orientation of the triplex-prone sequence does not measurably affect the time cells spend in S phase. The BrdU incorporation of asynchronous cultures of TTR and TTF is similar, indicating that there are the same number of replicating cells between the cell lines, and that those replicating cells are incorporating nucleotides at approximately the same rate. However, damage-inducing synchronization results in a greater delay in S-phase progression in TTR than TTF, and this effect may be simulated by damaging cells with UV radiation and then releasing them from a thymidine block.

While it is tempting to speculate that synchronization with aphidicolin or mimosine causes a difference between TTR and TTF cells, it may be more accurate to say that it accentuates the differences between the cell lines. Studies in unperturbed and thymidine-synchronized cells reveal differences in cell morphology and growth rate. Therefore, even though asynchronous and thymidine-synchronized TTR and TTF cells appear to replicate their DNA at the same rate, TTR cells must be experiencing some difficulty, or they would yield the same results as TTF cells in all of the assays using undamaged cells.

Effects of Checkpoint Inhibition in TTR and TTF Cells

Synchronizing TTR and TTF cells with mimosine or aphidicolin and then inhibiting the checkpoint by releasing them into caffeine (either for a short or long period of time before adding normal media) showed a marked difference between the cell lines
(Compare Fig. 30 and 31 with Fig. 16, bottom panel). One possible explanation for this is that the PKD1 sequence was replicated while the cells were in caffeine (which would be expected since the ectopic c-myc insert contains an early-firing origin). It is speculated that most TTF cells did not encounter a stable secondary structure there, so replication carried on as normal, while many TTR cells formed a stable secondary structure at the PKD1 insert, leading to a stalled fork. The observed challenges faced by TTR cells would predict a distinct advantage to a defined polarity of replication in vivo. That is, if each sequence were preferentially replicated from a specific direction, many potential DNA damaging fork stalling events could be avoided.

When we tested the cells in the presence of checkpoint inhibitors and they encountered stable secondary structures, the lack of checkpoint proteins to stabilize and resolve this structure could have resulted in a collapsed fork and broken DNA. When the caffeine was removed, the checkpoint proteins sensed the damage and paused the cell cycle to repair it, causing TTR cells to progress through the cell cycle more slowly than TTF cells.

The difficulty with this theory is that it supposes that the PKD1 sequence (which is the only difference in TTR and TTF cells) is somehow distinct and more important in determining the fate of cells than any other secondary-structure-prone sequence in the genome. It assumes that adding one more potential secondary structure is sufficient to cause a visible difference in the two cell lines. Although this seems impossible, it is difficult to explain the data any other way. Perhaps cells are more adept at replicating “difficult” DNA sequences in a way that does not trigger a checkpoint response than we realize, and therefore “forcing” them to replicate the PKD1 sequence in a non-favorable
direction does produce a dramatic result. Note also that this theory does not assume that no TTF cells are encountering stable secondary structures at this or other sites- some of them certainly are. The PKD1 insert, then, is only increasing the obstacles to replication in TTR cells above the background difficulties that both TTR and TTF cells experience.

Caffeine is a kinase inhibitor that interrupts the function of the damage-sensing proteins ATM and ATR, preventing the checkpoint response from sensing and repairing replicative damage that occurs. Because it inhibits proteins involved in multiple pathways, some off-target effects are anticipated. ATR is required to control the timing of origin firing and the rate of replication fork progression even without the presence of damage, so without its activity, replication will not proceed normally.

Another known effect of ATR inhibition is an increase in dormant origin firing. During G1 phase, a far greater number of origins are licensed than will actually be used under normal circumstances. Some of these are normally used for replicating the DNA, and the others are called dormant origins. These origins are only used if they are activated by a checkpoint response, often to prevent un-replicated DNA in the case of a stalled fork, or if ATR/ATM is inhibited.

If dormant origins are activated, the PKD1 sequence may be replicated from atypical origins (rather than the c-myc origin as under normal conditions), and therefore may not be replicated from the typical direction in all cells, therefore obscuring the orientation effects. However, this is unlikely because the c-myc origin is near the insert (so there is a small probability of a dormant origin being activated between the two, or on the other side, closer than the usual origin). Because the c-myc repliator is an early origin, it is also unlikely that a dormant origin will be activated early enough to replicate
the triplex region before the c-myc origin does. Although it is unlikely that firing of dormant origins will affect the direction of replication of the PKD1 insert in TTR or TTF cells, it may change the overall rate of replication and alter protein signaling.

The increased cell death in TTR cells following checkpoint inhibition, along with the increased binding of checkpoint proteins at the c-myc insert in TTR cells strongly suggests that checkpoint proteins are more frequently required for stability at the c-myc insert in TTR cells than in TTF. When cells were treated with aphidicolin and then released into caffeine or UCN-01, 10-25% of the cells died. Again, this parallels with studies at common fragile sites. When ATR deficient cells are treated with aphidicolin, chromosomal breaks at fragile sites drastically increase, indicating that ATR is necessary for the successful replication of fragile sites in the presence of aphidicolin

**Implications of the DTR and DTF Cell Line Results**

DTR and DTF cells exhibit differences in cell growth, morphology, release from damage-induced synchronization and response to checkpoint inhibition, indicating that a nearby origin is not required for the orientation of a triplex-prone region to affect cells.

In addition to confirming that the directional effects we have observed are not dependent upon a nearby replication origin, or upon an ectopic origin, the DTR and DTF cell lines strengthen the evidence from TTR and TTF cell lines. If the differences observed between TTR and TTF cell lines were the result of a non-engineered change in one or both of the cell lines, then the same types of differences would not be observed in DTR and DTF cells. The DTR and DTF cell lines were constructed from a common acceptor cell line (the same one used for TTR and TTF), but used different plasmids than
TTR and TTF, so the differences cannot be caused by something in the acceptor cell line, by something in the plasmid, or by a random event that affected one of the cell lines. The only interpretation remaining, then, is that insertion of the PKD1 sequence affects cells globally in an orientation-dependent manner.

As an additional benefit, these experiments provide evidence for the efficiency of eukaryotic replication origins. The fact that orientation-specific effects are still observed indicates that at least the direction of fork movement is usually consistent, and therefore that one or more origins downstream of the insert must be highly efficient. This indicates that the ectopic c-myc replicator is not the only efficient origin of replication. If most eukaryotic replication origins truly were very low efficiency, as some investigators suppose\textsuperscript{69,70}, then there should be little bias for the direction of fork movement through a given region, since there would be nearly equal probabilities of an inefficient origin upstream of the sequence replicating it as of one downstream. If this were the case, population-level directional effects would be obliterated. In either cell line, the sequence would be replicated in the favorable or unfavorable orientation approximately the same percent of the time, so DTR and DTF cells would behave identically. In contrast, a dominant, efficient origin that usually replicates the sequence would cause a replication fork barrier to form in one cell line significantly more often in one cell line than the other. TTR and TTF cells also indicate this, but could be discounted because they are replicated from an ectopic origin. DTR and DTF cells, however, are replicated from an endogenous, non-disturbed origin.

Although demonstrating that one origin is efficient does not imply that others are, there would be a selective advantage for efficient origins, at least in certain areas. The
genome is filled with potential triplex-forming sequences and other sequences prone to secondary structure formation. Routinely replicating these sequences in the favorable orientation would decrease the possibility for replication difficulties and repair requirement.

For example, the PKD1 insert sequence is present in every cell of the body and must be replicated every time a cell divides. If inserting one of these mirror repeat sequences into TTR can cause population level changes and poor cell health, then replication from an unfavorable direction could cause problems in any dividing cell of the body. There are an enormous number of such sequences in the genome, which makes replicating them in a non-secondary-structure-prone direction very important.

Another possible mechanism for promoting replication through these regions in the favorable orientation is preventing fork entry from one direction. In S. cerevisiae, endogenous polar replication fork barriers are located between rRNA genes. The DNA binding protein Fob1 binds two target sequences and wraps the DNA around itself in such a way that it prevents replication forks from entering the region in the 3’-5’ direction, thereby preventing replication forks from colliding with transcription machinery. In budding yeast, multiple loci have been identified that bind proteins to make polar replication fork barriers, including inactive origins (which may be the result of ORC binding)\(^\text{17}\). Disruption of replication termination sites in budding yeast disrupts genome integrity\(^\text{71}\), which may indicate that certain regions must be replicated in a specific direction to prevent mutation. It would not be surprising, therefore, to discover similar mechanisms near repeat sequences in higher eukaryotes that favor replication in the least-error-prone orientation.
Future work should study the direction of replication through endogenous sequences prone to secondary structure formation. If each mirror repeat in the PKD1 locus, for example, is capable of causing the type of difficulties observed in TTR and DTF cells, it is curious that Polycystic Kidney Disease is not more prevalent, especially in individuals already having one mutant copy of the gene. Based on this work, it would be expected that these sequences are normally replicated in a favorable orientation.

We have considered the possibility that these observed effects are associated with transcription or another cellular process. We believe that the direction of replication is the only activity altered by changing the orientation of the 88 base-pair PKD1 mirror repeat insert. Replication is still proceeding in the same direction in reference to the c-myc sequence, and any transcription occurring in that region should not be affected by reversal of the insert. However, it is possible that transcription is occurring in the ectopic c-myc replicator, and the mirror repeat sequence is impeding progression of the transcription fork. If this were causing the observed differences, though, DTR cells should behave like TTR cells, and DTF cells should behave like TTF cells (except that the insert is in a slightly different location so it might affect transcription differently). This also suggests that the phenomenon must be associated with replication, or a change in direction should not cause reversal of the phenotype.

The experiments with DTR and DTF cells provide some clues to the conditions allowing secondary structure to form. The ability of a replication fork block to form in the absence of a nearby origin may indicate that the structure is thermodynamically stable and therefore able to compete with duplex formation even when the duplex is more stable.
than it is near an origin. It also reveals that nothing unique to a replication origin (e.g. protein binding or superhelical tension) is required for a replication fork barrier to form.

These observations also have implications for the consistency of origin firing timing. In TTR and TTF, the insert is apparently flanked by two highly efficient origins (one is the c-myc origin and the other is the origin that replicates the PKD1 insert in DTR and DTF cells). Because the rate of replication fork progression is so fast, the timing of origin firing within S-phase is more likely to determine which origin replicates a section of DNA than the proximity of the origin to the sequence. If the two origins flanking the triplex prone insert fired at varying times, there would not be a directional bias for replication, and orientation-dependence would be obliterated. From this, we infer that in TTR and TTF cells, the majority of the time, the c-myc origin fires before the downstream origin. (It is also possible that the downstream origin fires first, but the replication fork routinely stalls/pauses before reaching the PKD1 sequence, thereby allowing the c-myc origin to replicate the PKD1 insert.)

**Orientation-dependent effects of inserting a sequence prone to secondary structure formation**

The conclusions presented here support an entirely different view of mammalian replication than is currently widely accepted. Not only do they suggest that the direction of replication fork movement through a region can affect its propensity to cause damage, but they demonstrate that the replication of a single site in the genome can cause significant differences in cell health and the ability to cope with checkpoint deficiencies. Further studies using these and other systems will hopefully provide more insight into these phenomena.


Chapter 4: Methods

DUE-B siRNA

Four siRNA duplexes targeting different areas of DUE-B were purchased from Dharmacon. DUE-B siRNA sequences were as follows:

siRNA 1: GGAGAGCAGAUUAGUGCCAUUGGAA
siRNA 2: CACACUCUUCGACCAGUGCUU
siRNA 3: GAGAUUCUGUGUGUCAGCCAGUUUA
siRNA 5: CCUCUGACCCAAAGCAGCUGUCAAA

Cells were plated at 25% confluency and transfected with 40 nM DUE-B siRNA according to the Lipofectamine 2000 instructions. Twenty-four hours later, they were transfected again with 40 nM DUE-B siRNA. Twenty-four hours after that, they were harvested by scraping into PBS. The cells used for whole cell extracts were then spun down, resuspended in sample buffer, sonicated for 2 minutes and then boiled.

The cells used to prepare a chromatin-enriched fraction were pelleted and then resuspended in Buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl$_2$, .34M Sucrose, 10% glycerol, 1 mM DTT, pH 8.0). They were lysed by the addition of 0.05% Triton-X100 and a 10 minute incubation on ice. The nuclei were then pelleted and washed with Buffer A. The nuclei were then resuspended in Buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitors) and incubated on ice for 30 minutes (to lyse the nuclei). The chromatin was then pelleted and washed with Buffer B. Finally, it was
pelleted and resuspended in sample buffer, sonicated, boiled, and forced through a syringe to fragment it.

**Flow cytometry and BrdU Incorporation**

TTR and TTF cells were plated in 6 cm dishes at 15% confluency and then grown for two days to minimize differences in trypsinization yet allow them to reach approximately 60% confluency. They were then trypsinized, washed with PBS and fixed in 70% ethanol at -20°C overnight. The cells were then washed and resuspended in PBS. RNase A was added (50 U/1 mL PBS) and the samples were incubated at 37°C for 20 min. Propidium iodide was added to 50 µg/mL and the samples were incubated at 4°C for 30 minutes, then warmed to room temperature and analyzed on a flow cytometer.

For BrdU incorporation experiments, 50 µM bromodeoxyuridine was added to cells 2-6 hours before harvesting. After fixation, cells were exposed to acidic conditions for 10 minutes to denature the DNA, and then neutralized with sodium borate. Cells were blocked with BSA and treated with FITC-conjugated anti-BrdU antibody. Cells were also treated with RNase A and propidium iodide as above. Cells were gated for the presence of BrdU incorporation and the appropriate statistics were obtained.

**Synchronization Methods**

To observe the effects of different drug treatments, cells were treated with either mimosine or aphidicolin, or synchronized more gently through a double-thymidine block.

TTR and TTF cells were plated at approximately 20% confluence for mimosine or aphidicolin treatment, or 10% confluence for a double-thymidine block. The following
day, the appropriate treatments began. Cells were synchronized with aphidicolin (2 µg/mL), mimosine (400 µM) or thymidine (2 mM: Sigma T1895) as shown in Figure 14. Each time cells were removed from drug-containing media, they were rinsed three times with PBS before release into normal media. After release, samples were harvested at timed intervals, fixed, and analyzed by flow cytometry as previously described.

Cells were observed under normal growth conditions to check for morphological differences indicating general stress. Photographs were taken to record any variations. Cells that appeared rounder than normal (as opposed to triangular), small colony size or increased number of floating cells were interpreted as unhealthy cells.

Any differences in volume were measured using the forward scatter parameter of the flow cytometer. Asynchronous or synchronized and released cells can be observed and gated based on propidium iodide to reveal at which stage of the cell cycle TTR and TTF are different sizes.

**Cell Migration Assay**

A migration assay indicates if there are differences in ability to attach to the culture plate. Cells with stronger substratum attachment can re-distribute to an adjacent bare spot on the plate more quickly than cells with reduced attachment ability. An 80% confluent plate of each cell line was scratched with a thin pipet tip, and the resulting bare area was visually observed immediately and 24 hours later.

**MTT Assay**

An MTT assay was used to quantitate the population doubling rate. Cells were counted and plated at 5 percent confluency in 12-well plates. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to three wells of each
cell line each day. The cells were allowed to metabolize the MTT for 2 hours, producing a blue dye (mitochondrial dehydrogenase enzymes convert the yellow MTT to purple formazan). The media was then removed, the cells washed with PBS, and the dye solubilized in DMSO. The absorbance of the dye indicates the amount of metabolism that occurred during the “labeling” period, which is an indirect measure of the number of cells present. If these absorbances are plotted on a log scale as a function of time, the slope of the line represents the population doubling rate, which can be used to estimate the cell cycle time.

**MTT Calculations**

P: Population (number of cells)

Q: Quantity of dye

A: Absorbance

All variables are functions of time (t) in hours

Q is proportional to P, so $Q = P \times K$ where K is a constant of proportionality

A is proportional to Q, so $A = Q \times L$ where L is a constant of proportionality

P increases exponentially, so $P(t) = P_0 \times e^{(ct)}$ where c is a constant and $P_0$ is the population at time 0

Therefore, $A = A(t) = LQ = LP_0 e^{(ct)}$

And, $\log(A) = \log(LP_0 e^{(ct)})$. Log(A) is the slope plotted on the MTT graphs.

$\log(A) = \log(LP_0) + \log(e^{(ct)})$

$\log(A) = \log(LP_0) + (ct) \times \log(e)$, rearranging,

$\log(A) = c \times (\log(e)) \times t + \log(LP_0)$, which is in the form of $y = mx + b$ with m equaling the slope of the line we have plotted. So,
c=slope/(log(e))

**Doubling time:**

\[ P(t_1) = 2^*P(t) \]
\[ P_0 * e^{c(t)} = 2 * P_0 * e^{c(t)} \]
\[ e^{c(t)} = 2 \]
\[ e^{c(t_1 - c(t))} = 2 \]
\[ \ln(e^{c(t_1 - c(t))}) = \ln(2) \]
\[ c(t_1 - c(t)) = \ln(2) \]
\[ \text{Time to Double} = t_1 - t = (\ln(2))/c \]

Since \( c = \text{slope}/(\log(e)) \),
\[ \text{Time to double} = \ln(2)/(\text{slope}/\log(e)) \]

Calculated population doubling time for TTF: 31.35 hours
Calculated population doubling time for TTR: 33.08 hours

**Immunoprecipitations**

HeLa cells were lysed in M-Per protein extraction buffer (Pierce) with protease inhibitors. After pelleting the cellular debris, the supernatant was pre-cleared for one hour in 1X NET buffer (150 mM NaCl, .5mM EDTA, 50 mM Tris-HCl, 0.5% NP40, pH 7.4) with protein G agarose beads (Upstate) and normal rabbit IgG. These pre-cleared extracts were then added to protein G beads bound with normal rabbit IgG, TopBP1 antibody (gift from Dr. Karnitz, Mayo Clinic), DUE-B antibody (produced by our laboratory), or Cdc45 antibody (Santa Cruz rabbit anti-Cdc45, sc-20685). The samples
rotated for 2 hours at 4°C and then were washed twice with TE and boiled in sample buffer.

Xenopus egg extracts were prepared as previously described (Casper et al., 2005). Immunoprecipitations from these extracts were performed as in HeLa cells but without a lysis step.

**ATR Kinase Assay**

The ATR kinase assay was carried out similarly to previously described 73. 406-myc cells (HeLa derivatives) were transfected with Flag-ATR wildtype or Flag-ATR kinase dead plasmids. 48 hours later, cells were lysed using T-PER (Pierce) and protease inhibitors. The extracts were divided into two samples- one to be activated with denatured, cut lambda DNA and the other to be used without activation. The ATR was then pulled by an immunoprecipitation with anti-Flag and protein G beads. After washing, the bead/ATR complexes were resuspended in 50 µL freshly prepared kinase buffer (25 mM HEPES, pH 7.5, 2 mM MnCl$_2$, 10 mM MgCl$_2$, .05 mg/mL BSA, 1mM DTT). 500 ng of Sf9 DUE-B and 21 µCi of $^{32}$P-ATP, along with 20 µM unlabeled ATP was added to the appropriate reactions (final reaction volume approximately 100 µL). These were incubated at 37°C for 30 minutes and then 6x sample buffer was added and the reactions were boiled.

**DUE-B Rescue**

A rescue plasmid for DUE-B siRNA #3 was constructed from a previous construct (courtesy of Michael Leffak) that contained the DUE-B cDNA in the pCDNA3.1 plasmid. Mutagenesis to confer resistance to siRNA #3 was accomplished using the Stratagene Quikchange kit and the following primers: Upper Primer: GGT
CGA AGA GTG TGA TGG ACA AAC AGT ACG AAA TAC TAT GCG TTA GTC
AAT TCA CCC TCC AGT GTG TCC. Lower Primer: GGA CAC ACT GGA GGG
TGA ATT GAC TAA CGC ATA GTA TTT CGT ACT GTT TGT CCA TCA CAC TCT
TCG ACC. The resulting plasmid was sequenced to verify that the mutations were
successful and accurate.

HeLa cells were plated at 20% confluence and then transfected 5 hours later with
either Lipofectamine 2000 alone (mock transfection), DUE-B siRNA #3, DUE-B siRNA
#3 and 20 ug of wildtype DUE-B plasmid (the transcripts from which should be
destroyed by the siRNA unless it is too abundant) or DUE-B siRNA #3 and 20 ug of the
DUE-B rescue plasmid (the transcripts from this should be resistant to destruction by
DUE-B siRNA #3). Cells were transfected again the same way 24 hours later and then
harvested 24 hours after the second transfection.

**Cell Death ELISA**

Cell Death ELISA was conducted according to kit instructions (Roche). Briefly, cells
were grown in a microplate, then the media was removed and lysis buffer added. Lysate
was discarded while intact nuclei were separated by centrifugation. Supernatant was
transferred to a new microplate coated with streptavidin. Antibodies to histones (biotin-
labeled) and DNA (peroxidase-conjugated) were added (nucleosomes will be bound to
the plate by the biotin-streptavidin). Wells were rinsed three times to remove unbound
components and peroxidase substrate was added, then the amount of dye produced was
measured using a spectrophotometer.
Southern Blots

DNA was harvested from TTR and TTF cells using a DNAezy kit and 20 µg from each cell line was digested with HindIII or BamHI overnight. Digested DNA was concentrated with a PCR-preparation kit and run on a 1% agarose gel. Gel was soaked in depurination buffer for 15 minutes, rinsed with water, soaked in denaturation buffer for 30 minutes, rinsed, and then soaked in neutralization solution for 30 minutes and rinsed. Gel was then placed in 20x SSC for 30 minutes to equilibrate and then transferred to membrane. Probe was made by cutting Hyg-FRT-TK plasmid with NruI and KpnI and gel purified to yield a TK-containing fragment. This was labeled using γ-32P and hybridized to membrane.

Western Blots

Proteins were isolated from cells and resolved on 10% acrylamide gels before being transferred to nitrocellulose membranes. The membranes were blocked in TBST with 5% milk overnight, probed with primary antibody for 2-6 hours at 4°C, washed, probed with secondary antibody for 1 hour at room temperature, washed, and exposed with luminol and peroxidase. Signal was viewed using a Fuji imaging system. Antibodies used were as follows: ATR: purchased from Santa Cruz Biotechnology (sc-1887), Rad9: Purchased from Santa Cruz Biotechnology (sc-8324), Chk1: Purchased from Cell Signaling Technology (#2345), Phospho-Chk1: Purchased from Cell Signaling Technology (#2341, detects Ser345), DUE-B: Produced by the Leffak lab (raised in rabbits against recombinant DUE-B produced in bacteria), TopBP1: Gift from Dr. Lawrence Karnitz (Mayo Clinic), Cdc45: Purchased from Santa Cruz Biotechnology.
PCR

Q-PCR

Q-PCR reactions were performed using Sybr-Green Master Mix (Invitrogen) according to manufacturer’s instructions. Reactions performed on Applied Biosystems 7000 Real-Time PCR System.

pML Upper Primer: GCT CGG TAG GGT TCC CAA AG
pML Lower Primer: GGG CGG AGA TTA GCG AGA G

Standard PCR

To analyze the size of the insert in TTR and TTF cells, standard PCR was conducted using primers just upstream and downstream of the PKD1 insert, yielding a 220 bp product. Products were analyzed on acrylamide gels to achieve maximum resolution.

Lower Primer: CCGAGATTAGCGAGAGAGGA
Upper Primer: AAGGGAGAGGGTTTGAGAGG.

40 Cycles were Performed:
- Denaturation: 95°C 15:00
- Melting: 94°C 0:30
- Annealing: 55°C 0:30
- Extension: 72°C 0:45

Immunofluorescence

Cells were grown on coverslips for 24 hours, then rinsed with PBS and fixed with 2% formaldehyde for 15 minutes at room temperature. After three five-minute washes with PBS, cells were permeabilized with 0.2% Triton X-100, 0.5% normal goat serum in PBS pH 7.4 for 5 minutes on ice. Cells were washed three times with PBS containing 0.5% normal goat serum. Primary antibody was diluted in PBS containing 0.5% normal goat
serum and incubated for 1 hour at room temperature (RPA was diluted 1:250 and Tubulin diluted 1:4000). After three washes with PBS containing 0.5% normal goat serum, cells were incubated with secondary antibody diluted in PBS containing 0.5% normal goat serum for one hour. Coverslips containing cells were sealed to slides with mounting medium and edges covered with nail polish.
References


