Nonhomologous end-joining: TDP1-mediated processing, ATM-mediated signaling

Amy Hawkins

Virginia Commonwealth University

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NONHOMOLOGOUS END-JOINING: TDP1-MEDIATED PROCESSING, ATM-MEDIATED SIGNALING

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

Amy Jane Hawkins
B.S. University of Virginia, 2003

Directors: Lawrence F. Povirk, Professor, Department of Pharmacology and Toxicology and Kristoffer Valerie, Professor, Department of Radiation Oncology

Virginia Commonwealth University
Richmond, Virginia
November, 2009
ACKNOWLEDGEMENTS

My first thoughts of gratitude go straight to both of my advisors: Dr. Kristoffer Valerie and Dr. Lawrence Povirk. I first did a rotation in Dr. Valerie’s laboratory at the end of my third year of graduate school, and he somehow convinced Dr. Povirk to take me on as a joint graduate student. Although their laboratories have been located side by side in open laboratory space, this has still proven to be a rather unusual arrangement. I hope that both Dr. Povirk and Dr. Valerie feel that they have gotten at least half a graduate student’s work and commitment from me, as time and time again I was reminded that I was attempting to thwart the adage that “no man can serve two masters.” In having both of them as my mentors, I certainly reaped the benefits of two different personalities, two sets of advice and perspectives, two projects that have given me varying degrees of success at different points in time, two sets of lab meetings, and two sets of fellow lab mates. Both Dr. Valerie and Dr. Povirk were gracious enough to allow me to make a little fun of each of them to each other as a way of letting off steam when I was frustrated by either my results (or lack thereof) or conflicting opinions, and for this I am extremely appreciative. I have the utmost respect for both of them, and little else fills me with such a sense of glee as seeing Dr. Povirk’s self-restored electric car in the Massey Cancer Center parking garage on the weekends, plugged into the wall.

My second thoughts of thanks go to my lab mates. My desk has been physically located within the Valerie lab space for the past three years, so my exposure has been biased to them. Sarah Golding and Beth Rosenberg have always been available for any question I might have, whether about the lab or how to live in Richmond, and always seem to know who to call to get something accomplished or where a reagent from ten years ago might be. Besides being of profound assistance in the lab, I want to thank them both personally for inviting me into their lives as a friend: for Sarah for inviting me to attend the gym as a workout partner, and to Beth for the invitation to join her book club. I took you both up on the offers and certainly haven’t regretted either one. I also would like to thank Sarah for encouraging me professionally: informing me as to what my first big professional conference would be like, encouraging me to attend courses on potentially becoming a faculty member, and for staying late to help me with conference posters. I couldn’t ask for a more helpful post-doc fellow or lab manager.

I would like to thank Bret Adams for being hilarious and being the most frequent contributor to the wall of quotations I’ve kept above my desk. Even if Bret is having considerable difficulty with an experiment and feigns hopelessness, he reminds me of a comment by Anne Lamott, that she doesn’t mind if a person has no hope if he or she is sufficiently funny about the whole thing, but then, this being able to be funny in itself definitely speaks of a kind of hope, of buoyancy. Bret was also a very considerate conference roommate to me in September, 2008 when we both frugally attended the Radiation Research Society annual meeting in Boston.
I would like to thank Seth Dever for reminding me of the consequences of mixing business and pleasure.

Both Aaron Randolph and Barbara Szomju have been very helpful in both producing the necessary viruses for my experiments, and offering the kind of perspective that can only be found in coworkers who don’t share your same immediate space.

I’d also like to thank Donna Gilford and Laura Thorpe for further the continuing mission of the Valerie Lab, and Rhiannon Morgan, our exchange student from UWE, for continuing to have a good attitude despite having been mugged in from of my apartment during the year she worked with us.

Although I have spent less time with Dr. Povirk’s staff, I would like to thank Konstantin Akopiants for his invaluable work with proteins that I subsequently used, and his equally invaluable moral support as I poured large acrylamide sequencing gels. As for Susovan Mohapatra, it was always nice to see a smile on his face, and I wish him luck fulfilling the destiny that I have foreseen: becoming a tenured faculty member, focusing on DNA repair. I like to thank Tong Zhou for having the patience I could not maintain to continue doing colony forming assays on our MEF cell lines.

I would also like to thank Robert van CAM Waardenburg, a researcher who enthusiastically first met me at the American Association for Cancer Research meeting in 2007, and with whom, since our initial meeting, I’ve kept up an occasional e-mail correspondence. It has been phenomenally encouraging to meet other researchers outside of my immediate environment who have expressed an interest in and so cared about my research results, and Robert is possibly the most enthusiastic. I look forward to reading his TDP1-related research for years to come.

I am thankful to the anonymous reviewers of our TDP1 mouse manuscript who sent feedback with reasonable suggestions about our manuscript, and to the DNA Repair editor who accepted our paper only two days after we resubmitted it with corrections.

I am grateful to my committee, Dr. James Lister, Dr. Michael Grotewiel, and Dr. Sarah Elsea, who were kind enough to accept when I asked them to join my committee as a fourth-year PhD student. I enjoyed hearing seminar lectures from all three of you, and it was a pleasure to have your advice. I am especially grateful to Dr. Jolene Windle, my longest-running committee member who has assisted me with making decisions about graduate school since my first year, and who has collaborated with Dr. Povirk on the TDP1 mouse project for longer than I have been involved with it.

In addition to my committee, I’d also like to thank other faculty members from the Departments of Human and Molecular Genetics, Pathology, Biochemistry, and the Massey Cancer Center: it’s always nice to run into Lindon Eaves, Rick Moran, or Joyce Lloyd. I’d like to thank Charles Chalfant for a good experience while I did a rotation in
his laboratory, and Shawn Holt for assuring me that I would have funding from the Pathology Department during the spring semester of 2006. I’d also like to thank my department chair Paul Fisher for making room in his busy schedule for conversations about where I should next pursue science.

Outside of my lab-related gratitude, I’m grateful for the variety of online social networking media, without which I would have felt lonelier throughout this process and which has allowed me to stay in touch with a lot of wonderful people without whom my life would be a darker and less interesting place. Thus, I would like to thank my friends who have provided long-distance support. Mike Levy, from whom I’m literally borrowing these sentences, has always been a relief to talk to about the process of graduate school, and provided me with a great vacation destination in Boulder, Colorado. Mary Pumphrey, with whom I talk almost weekly, has always encouraged me to try hard, or to at least attempt to be funny when I’m sick of trying. D. Lyle Elder has been extremely emotionally supportive throughout my graduate studies, and has kindly always been available for intellectual discussions of a philosophical bent. He also wrote a cheerful little poem describing the procedure of western blotting that I kept posted above my desk for several years, and helped to keep me from despairing too much while I attempted antibody after antibody. Doug Marcey and Katie Briggs, who kindly regard me as “chosen family,” provided me with emotional support and a place to stay in the Washington, DC area whenever I wanted their company and comfort either in celebration, or in the midst of family tragedy. Mike Rust always has interesting thoughts on the process of scientific research, and I’ve enjoyed hearing what he’s had to say from his own trenches. He and I have shared interesting discussions on one of my favorite topics, the role of narrative in teaching and communicating science.

I would like to thank the roommates I’ve had since I’ve lived in Richmond: Tiffanie Chan and her husband Taylor Barnett, Elizabeth Barron (now Elizabeth Grissom), and Tony Pomicter. You have all been extremely supportive and tolerant of the strange hours I’ve kept, and I value your friendship. In particular I’d like to thank Tony Pomicter for being a supportive partner, not only while I’ve written my dissertation, but also when I want to go into the lab to grind up mouse livers and borrow his lab’s tissue homogenizer and expertise, for six hours, on a Sunday. I’m sure it has enriched my educational experience to be able to talk about my research with Tony, and in turn, to have some of his molecular biology and neuroscience expertise and sympathy as resources. I’d like to thank Matt Osborne for, among other things, cheerfully coming over to my house the very first night I lived in Richmond to accompany me to the hardware store to buy mouse traps. I’m also glad that Sam Davies and Kat Zarfas moved to Richmond two years ago and decided to raise their family here: their home has always been a welcome retreat for me.

I would like to thank one of my earlier teachers, Mr. Fred Lampazzi, who taught me freshman biology, and later DNA Science I and II, at Thomas Jefferson High School for Science and Technology. I know that I’m only one of hundreds of students who Mr. Lampazzi has motivated to continue studying biology on the graduate level, but his classes may have motivated me more than any lectures I sat through at the University of
Virginia. I still have memories of Mr. Lampazzi taking the time to patiently go through diagrams of metabolic pathways with me, one-on-one, when I was thirteen years old and learning about cellular respiration for the first time. Thanks for your patience, Mr. Lampazzi.

At the University of Virginia, I’d like to thank Dr. Emilie Rissman and her laboratory staff from 2001-2002. I had my first laboratory experience in Dr. Rissman’s laboratory, performing behavioral tests on transgenic mice, and it was the first time it occurred to me that I found something that I enjoyed that I might be able to keep doing as a profession. This feeling was cemented when I was given further exposure and opportunities by Dr. Lisa Goehler and Dr. James Garrison, and their respective staff. Without their encouragement I might not have thought to pursue graduate studies in science. Also at the University of Virginia, I’d like to thank my fellow members of the Washington Literary and Debating Society, who taught me that I am comfortable and have fun speaking in front of a crowd of respected friends.

Lastly, I’ve written the majority of my dissertation on a computer purchased by parents, which sat on top of either a desk made by my paternal grandfather or a table given to me by my maternal grandmother. The obvious metaphor doesn’t escape me; I’ve literally been supplied with the tools to accomplish what I have because my family, both immediate and extended, has supported me without question. I certainly wouldn’t have made it this far without the influence of the overriding value that my parents placed on higher education. Thanks especially to my mother and father, Catherine and Timothy Craig Hawkins, and Ga and Grandpa, also known as June and Hank Hession, who each played no small part in raising me. Thanks also to my sister Kristin Hawkins, who has consistently set an example of astonishingly impressive academic excellence.

I would like to dedicate my dissertation to my late grandmother, June Hession, who died in 2007 while I was in graduate school. I admire her for countless reasons, but it feels appropriate to mention here that I am particularly impressed that as an older person, she decided to learn how to use a computer, which she then followed by getting online. It was a joy to receive e-mail from her in college, and to show her how to use Google. I hope that I’ll be able to follow her good example in retaining a sense of wonder about the world and a similar desire to learn new technologies. I would have loved it if she could have read these lines, but she also knew I was going to turn out fine.

To all of you, and those whom I may have neglected, thank you so much for helping me get to where I am today.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>Ad-SceI</td>
<td>adenoviral vector expressing I-SceI</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>apurinic/apyrimidinic</td>
</tr>
<tr>
<td>APE1</td>
<td>apurinic/apyrimidinic endonuclease</td>
</tr>
<tr>
<td>Apex1</td>
<td>murine apurinic/apyrimidinic endonuclease</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>AT</td>
<td>Ataxia Telangiectasia</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia Telangiectasia and RAD3-related</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>Ca</td>
<td>calcium</td>
</tr>
<tr>
<td>Cd</td>
<td>cadmium</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinases</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>Co</td>
<td>cobalt</td>
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<tr>
<td>CPT</td>
<td>camptothecin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
</tr>
<tr>
<td>Dox</td>
<td>doxycycline</td>
</tr>
<tr>
<td>DSBs</td>
<td>double-strand breaks</td>
</tr>
<tr>
<td>DsRed</td>
<td>Discosoma sp. red fluorescent protein</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<tr>
<td>ETC</td>
<td>electron transport chain</td>
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<tr>
<td>fmol</td>
<td>femtomole</td>
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<tr>
<td>FOB</td>
<td>functional observational battery</td>
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<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<tr>
<td>ΔG</td>
<td>Gibbs free energy</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>Gy</td>
<td>Gray</td>
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<td>h</td>
<td>hour(s)</td>
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<tr>
<td>γH2AX</td>
<td>phosphorylated H2AX</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<td>HeLa</td>
<td>cervical cancer cell line</td>
</tr>
<tr>
<td>HKD</td>
<td>histidine x lysine (x)_4 aspartate sequence</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HP1</td>
<td>heterochromatin protein 1</td>
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<tr>
<td>HRR</td>
<td>homologous recombination repair</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>dose at which a drug affects 50% inhibition</td>
</tr>
<tr>
<td>IR</td>
<td>ionizing radiation</td>
</tr>
<tr>
<td>I-SceI</td>
<td>intron-encoded homing endonuclease from <em>Saccharomyces cerevisiae</em></td>
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<tr>
<td>JRL-2</td>
<td>TDP1+/+ lymphoblasts</td>
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<tr>
<td>KAP1</td>
<td>Kruppel-associated box (KRAB) associated protein 1</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<td>potassium chloride</td>
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<td>kilodalton</td>
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<td>KO</td>
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<td>KU-55933</td>
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<td>DNA-PK inhibitor</td>
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<tr>
<td>LET</td>
<td>linear energy transfer</td>
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<td>microliter</td>
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<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>mer</td>
<td>short for “DNA oligomer”</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>magnesium</td>
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<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MLS</td>
<td>mitochondrial localization signal</td>
</tr>
<tr>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>manganese</td>
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<tr>
<td>MO59K</td>
<td>ATM+/+ cell line derived from brain tumor</td>
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<tr>
<td>MRE11</td>
<td>meiotic recombination 11 homolog</td>
</tr>
<tr>
<td>MRN</td>
<td>MRE11-RAD50-NBS1</td>
</tr>
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<td>NBS1</td>
<td>Nijmegen Breakage Syndrome protein</td>
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<td>NHEJ</td>
<td>nonhomologous end joining</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>NO·</td>
<td>nitric oxide</td>
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<td>NSCLC</td>
<td>non-small cell lung cancer</td>
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<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>oxygen</td>
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<td>OD</td>
<td>optical density</td>
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<td>OMIN</td>
<td>Online Mendelian Inheritance in Man</td>
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<tr>
<td>ONOO·</td>
<td>peroxynitrite</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PARP1</td>
<td>poly(ADP-ribose) polymerase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffered saline with Tween</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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PG       phosphoglycolate
PIKK     phosphoinositide 3-kinase related kinase
PLD      phospholipase D
PNKP     polynucleotide kinase/phosphatase
PO<sub>4</sub>CH<sub>2</sub>COOH phosphoglycolate
pTyr     phosphotyrosyl
qPCR     quantitative PCR
RAD      family of RADial sensitive genes originally identified in yeast
RAD50    Rad50 homolog
RNA      ribonucleic acid
ROS      reactive oxygen species
rpm      rotations per minute
RQ       relative quantity
s        second(s)
SCAN1    hereditary Spinocerebellar Ataxia with Axonal Neuropathy
SDS      sodium dodecyl sulfate
SDS 2.2.2 Sequence Detection Software
SE       standard error
SSBs     single-strand DNA breaks
SUMO     small ubiquitin-related modifier
TBE      Tris/borate/EDTA
TDP1     tyrosyl-DNA phosphodiesterase
<table>
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<th>Abbreviation</th>
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<tr>
<td>TDP1 Comp</td>
<td>Tdp1-/- MEFS complemented with human TDP1</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TOP1</td>
<td>Topoisomerase I</td>
</tr>
<tr>
<td>TOP1cc</td>
<td>Topoisomerase I cleavage complex</td>
</tr>
<tr>
<td>TOP2</td>
<td>Topoisomerase 2</td>
</tr>
<tr>
<td>TOPmt</td>
<td>mitochondrial Topoisomerase</td>
</tr>
<tr>
<td>TSA</td>
<td>trichostatin A</td>
</tr>
<tr>
<td>U2OS</td>
<td>human osteosarcoma cell line, p53+</td>
</tr>
<tr>
<td>U87</td>
<td>human glioma cell line, p53+</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
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<tr>
<td>W</td>
<td>Watts</td>
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<td>wild-type</td>
</tr>
<tr>
<td>XLF</td>
<td>XRCC4-like factor</td>
</tr>
<tr>
<td>XRCC1</td>
<td>x-ray repair cross-complementing protein 1</td>
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<td>zinc</td>
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ABSTRACT

NONHOMOLOGOUS END-JOINING: TDP1-MEDIATED PROCESSING, ATM-MEDIATED SIGNALING

By Amy Jane Hawkins, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009.

Major Directors: Lawrence F. Povirk, Professor, Department of Pharmacology and Toxicology
and Kristoffer Valerie, Professor, Department of Radiation Oncology

This thesis investigates two separate features of nonhomologous end-joining (NHEJ) DNA repair: end processing, and DNA repair kinase signaling. DNA end processing was investigated in a mouse model of hereditary spinocerebellar ataxia with axonal neuropathy (SCAN1), a congenital neurodegenerative disease. SCAN1 is caused by a homozygous H493R mutation in the active site of tyrosyl-DNA phosphodiesterase (TDP1). To address how the H493R mutation elicits the specific pathologies of SCAN1 and to further elucidate the role of TDP1 in processing DNA end modifications, we generated a Tdp1 knockout mouse and characterized their behavior and specific repair deficiencies in extracts of embryonic fibroblasts from these animals. While Tdp1(-/-) mice appear phenotypically normal, extracts from Tdp1(-/-) fibroblasts exhibited deficiencies in processing 3'-phosphotyrosyl single-strand breaks and 3'-phosphoglycolate (PG) double-strand breaks (DSBs). Supplementing Tdp1(-/-) extracts with...
with H493R TDP1 partially restored processing of 3'-phosphotyrosyl single-strand breaks, but with evidence of persistent covalent adducts between TDP1 and DNA, consistent with a proposed intermediate-stabilization effect of the SCAN1 mutation. However, H493R TDP1 supplementation had no effect on PG termini on 3' overhangs of DSBs; these remained completely unprocessed. Altogether, these results suggest that for 3'-PG overhang lesions, the SCAN1 mutation confers loss of function, while for 3'-phosphotyrosyl lesions, the mutation uniquely stabilizes a reaction intermediate. Furthermore, there is evidence that TDP1 also localizes to mitochondria, and mitochondrial DNA damage should not be excluded from significantly contributing to SCAN1 pathology.

The effect of ATM signaling on NHEJ was investigated via a novel vector that allows for inducing I-SceI-mediated DNA DSBs that can then be analyzed for NHEJ repair events by fluorescence- and PCR-based methods. Using highly specific DNA kinase inhibitors and the repair cassette, we showed that inhibiting ATM reduced NHEJ by 80% in a U87 glioma model. Analysis of the PCR products from the NHEJ repair vector by PsiI restriction cleavage allowed for assessment of the fidelity of the NHEJ repair: inhibiting ATM reduced high-fidelity NHEJ by 40%. Together, these results suggest that ATM is critical for NHEJ of I-SceI DSBs and for high-fidelity repair, possibly due to ATM’s effects on chromatin architecture surrounding the DSB.
I. GENERAL INTRODUCTION

“In the future, attention undoubtedly will be centered on the genome, with greater appreciation of its significance as a highly sensitive organ of the cell that monitors genomic activities and corrects common errors, senses unusual and unexpected events, and responds to them, often by restructuring the genome. We know about the components of genomes that could be made available for such restructuring. We know nothing, however, about how the cell senses danger and instigates responses to it that often are truly remarkable.”

- Barbara McClintock, Nobel Laureate speech, delivered in Stockholm, Sweden, December 8, 1983 (McClintock, 1984)

1.1 Historical Note

The field of DNA repair predates experimental evidence showing that the hereditary material (of bacteria, at least) is DNA and not protein as the prevailing dogma dictated (Avery et al, 1944) or Watson and Crick’s elucidation of the structure of DNA (Watson & Crick, 1953). Prior to this research however, the concept of the gene was fully entrenched in the scientific community, and was emphasized by the 1933 awarding of the Nobel Prize to Thomas Hunt Morgan, for his discoveries concerning the role played by the chromosome in heredity (Morgan, 1911). Soon after, it was observed that *Drosophila melanogaster* phenotypes, chromosomes, and the genes the chromosomes carried could be mutated by X-rays (Muller, 1927), for which Hermann Muller was awarded the Nobel in 1946. Despite the evidence of genetic mutation, the publication regarded as the earliest mention of DNA repair (albeit an indirect one) is from 1935, discussing a growth delay after irradiation of bacteria (Curtis & Hollaender, 1935). The breeding grounds for radiobiology and subsequently, DNA repair, were to be found in the national laboratories established for the purpose of the Manhattan project, the development of the atomic bomb. Financial support for radiation biology dramatically increased during the 1940s,
during the establishment of Oak Ridge National Laboratories in Tennessee, Los Alamos in New Mexico, and Argonne near Chicago (Friedberg, 1997). From the beginning, radiobiological research was a priority of the U.S. Atomic Energy Commission – health physics research for nuclear workers, for example, was embraced as a necessary practical investment in order to run its facilities appropriately (Rader, 2006). A central set of concerns that drove genetic research after World War II was connected to the entangled military and civilian uses of atomic energy.

And yet, when Freidberg questioned Franklin Stahl as to why the concept of DNA repair was so late in coming, relative to these other discoveries, Stahl replied,

“I suspect because of a widespread belief (unspoken I suspect, but amounting to worship) among geneticists that the genes are so precious that they must (somehow) be protected from biochemical insult, perhaps by being carefully wrapped. The possibility that the genes were dynamically stable, subject to the hurly-burly of both insult and clumsy (i.e. enzymatic) efforts to reverse the insults, was unthinkable.” (Friedberg, 1997)

1.2 Normal structure of DNA

What are the genes?

What is the nature of the elements of heredity that Mendel postulated as purely theoretical units? What are genes? Now that we locate them in the chromosomes are we justified in regarding them as material units; as chemical bodies of a higher order than molecules? Frankly, these are questions with which the working geneticist has not much concern himself, except now and then to speculate as to the nature of the postulated elements. There is no consensus of opinion amongst geneticists as to what the genes are whether they are real or purely fictitious - because at the level at which the genetic experiments lie, it does not make the slightest difference whether the gene is a hypothetical unit, or whether the gene is a material particle. In either case the unit is associated with a specific chromosome, and can be localized there by purely genetic analysis. Hence, if the gene is a material unit, it is a piece of a chromosome; if it is a fictitious unit, it must be referred to a definite location in a chromosome - the same place as on the other hypothesis. Therefore, it makes no difference in the actual work in genetics which point of view is taken.
According to the central dogma of molecular biology, in the majority of all organisms, the genetic hereditary information is stored in DNA (deoxyribonucleic acid), selective regions of which are used as templates for synthesizing RNA (ribonucleic acid) molecules, which are then used as templates to synthesize polypeptides, the basic linear component which folds into proteins. The directional flow of this detailed sequence of information is visualized in Figure 1-1 (Crick, 1970). The organization of this genetic information is complex: the long double helical polymer of DNA is organized into chromosomes, which undergo dramatic differences in compaction depending on the stage of the cell division cycle. DNA in a metaphase chromosome is about 1/10,000 of its stretched-out length (Strachan & Read, 2004). DNA exists in a complex with proteins called histones, small basic proteins with a globular domain and a flexible and charged NH$_2$-terminus (a “histone tail”) that protrudes from each nucleosome, the basic repeating unit of the chromatin, in which DNA exists wrapped around an octamer core of a H3-H4 tetramer and two H2A-H2B dimers. Histone proteins undergo a variety of modifications that can lead to differential compaction and either activation of gene expression in euchromatin, or gene silencing in regions of heterochromatin (Figure 1-2, reviewed in (Jenuwein & Allis, 2001)). For example, a typical chromosome region that remains transcriptionally inactive and condensed as a heterochromatic region is the (largely) inactive X chromosome in female mammalian cells, known as the Barr body (Brown, 1966).
Figure 1-1. The central dogma of molecular biology. The central dogma is concerned with how complex linear information is both transferred and maintained between information macromolecules DNA and RNA, and polypeptides. The solid arrows above show general information transfers thought to occur in most cells; dotted arrows show special transfers observed in specialized cell types or other exceptional scenarios. Adapted from Figure 3 in (Crick, 1970).
1.3 DNA Lesions

The effects of radiation-induced cell killing are largely the result of damage to DNA (Hutchinson, 1966), though damage to membranes may also cause lethal effects via disruption of subcellular compartmentalization (see Chapter IV, (Altman et al, 1970)). Common challenges to genomic integrity come from environmental stress, such as ultraviolet light, or medical imaging\(^1\) (Brenner & Hall, 2007), but more commonly from endogenous events (De Bont & van Larebeke, 2004), such as the intracellular production of reactive oxygen species that result from normal cellular metabolism (an estimated 10,000 oxidative lesions per cell per day (Ames et al, 1993)), recombination during the immune system’s response and stalled DNA replication forks (Paulsen & Cimprich, 2007). These insults lead to base damage (such as thymine glycols and 8-Oxoguanine, reviewed in (Demple & Harrison, 1994)), single-strand DNA breaks (SSBs) and double-strand DNA breaks (DSBs). DSBs are particularly dangerous to cells as their inefficient or inaccurate repair can result in mutations and chromosomal translocations that may induce cancer. The presence of base damage and SSBs can also subsequently lead to DSBs when encountered by the DNA replication or transcriptional machinery (Branzei & Foiani, 2007, Michel et al, 2004).

\(^1\) There has been considerable debate surrounding the risks of computed tomography (CT) scans for routine medical diagnosis. "On the basis of such risk estimates and data on CT use from 1991 through 1996, it has been estimated that about 0.4% of all cancers in the United States may be attributable to the radiation from CT studies. By adjusting this estimate for current CT use, this estimate might now be in the range of 1.5 to 2.0%." (Brenner & Hall, 2007) This publication was later countered by Tubiana et al., who wrote that that those risk models use the linear no-threshold extrapolation model, which assumes that cancer induction is proportional to dose even for the smallest doses. There is considerable evidence that DNA repair mechanisms are much more effective at low doses, and as such the use of the linear no-threshold model greatly overestimates the risk (Tubiana, 2008).
Figure 1-2. Histone proteins modifications contribute to the structure of DNA. A strong correlation exists between increased histone acetylation and transcriptionally active chromatin and, conversely, between histone deacetylation, methylation, and repressive chromatin. Adapted from Figure 1 in (Jenuwein & Allis, 2001).
Damage from radiation occurs in two varieties, direct and indirect, in which the second form results because of consequences from the first. Direct damage (reviewed in (Altman et al, 1970)) results from the interaction of radiation with molecules, which transfers part of the radiation’s kinetic energy to a target molecule, causing the target molecule to either eject an electron (ionization), or raise electrons in the target molecule into a higher energy level (excitation). These excited or ionized molecules are unstable, and often dissociate into fragments with unpaired electrons, known as free radicals. Radiation can directly interact with DNA causing it to fragment, but when considering low linear energy transfer (LET) varieties of radiation such as x-rays and γ-rays, the majority of cell killing is caused indirectly. Radiation directly interacts with water, which typically comprises 80% of the cell, to form hydroxyl radicals (OH•), which are then thought to account for approximately two-thirds of the damage caused by x-rays (Hall & Giaccia, 2006). This may be expressed as follows:

\[
H_2O \rightarrow H_2O^+ + e^- \\
H_2O^+ + H_2O \rightarrow H_3O^+ + OH•
\]

The hydroxyl radicals in aqueous solution then may diffuse and damage other molecules in aqueous solution, such as lipid membranes, proteins, and DNA (Hutchinson & Watts, 1961). These reactions occur in “clusters” or “spurs” that have an average radius of 15 Å and contain an average of 6 radicals (Schwarz, 1969). The nature of these reactions has implications for the DNA damage, in that several radical damages can occur in close proximity on both strands of the DNA, creating complex damage referred to as “locally multiply damaged sites” or “lesion clusters” (Ward, 1990). Other radicals that contribute significantly to cellular damage include the superoxide radical, O2•, generated
from the reduction of \( \text{O}_2 \) (a substantial portion of the respiratory electrons seem to be diverted in this way (Ames et al., 1993)) and peroxynitrite (\( \text{ONOO}^- \)), generated from nitric oxide (\( \text{NO}^- \)) (both reviewed in (Cadenas, 1989)).

SSBs and DSBs are produced in unequal proportions: approximately 1000 SSBs are produced per cell per Gray (Gy) of radiation (Elkind, 1977) and 20-70 DSB per cell per Gy of radiation (Blocher, 1982) (reviewed in (Ward, 1990)). Isolated SSBs without adjacent complex damage are thought to be of minimal consequence, as they can be repaired using the opposite DNA strand as a template. DSBs are therefore thought of as the most relevant lesion for cell killing.

It is important to note that sensitivity to low LET radiation-induced killing is cell cycle specific: in cell types with a short \( G_1 \) cell cycle phase (such as Chinese hamster cells), cells are most sensitive in mitosis and \( G_2 \), less sensitive in \( G_1 \), and least sensitive (most radioresistant) in the latter part of the \( S \) phase (Sinclair, 1968). The radioresistance in \( S \) phase is thought to be due to homologous recombination repair (HRR) between sister chromatids, which is more likely to occur after DNA has replicated. In cells with a longer \( G_1 \) (such as HeLa cells), early \( G_1 \) is more radioresistant than later \( G_1 \). For higher LET radiation such as \( \alpha \)-particles or neutrons, radiation sensitivity is less variable with cell cycle, implicating indirect damage as one of the potential determinants that varies with the cell cycle (Hall et al., 1972). However, reasons for cell cycle dependent sensitivity to x-rays or \( \gamma \)-rays are not well understood due to the complexity of the issue. Even when considering just the DNA and not the variety of checkpoints and signaling that vary over the course of the cell cycle, DNA changes in both amount and form (condensed in mitosis, relatively diffuse in \( S \)-phase) in ways that would be expected to
yield differences in the variety and number of breaks that occur. The variety of DSB repair employed by the cell also varies over the course of the cell cycle (Figure 1-3). Radiation sensitivity is also tumor- and cell-type specific; this may be related directly to what percentage of that class of tumor is dividing, the extent of oxygenation of that particular tumor, or specific mutations that beget tumors with radioresistance.

1.4 DNA damage responses

*Single-strand break repair*

Small deoxynucleotide lesions are likely the most widespread type of damage to the genome (De Bont & van Larebeke, 2004). Some of these lesions are eliminated by various mechanisms by ‘direct reversal’ without excision and re-synthesis of a part of DNA. Other DNA repair processes involve degradation of at least the damaged nucleotide, followed by DNA re-synthesis. Nucleotide excision repair removes bulky helix-distorting lesions, such as antibiotics bound to nucleotides or pyrimidine dimers (lesions caused by UV light). Mismatch repair corrects errors made by DNA polymerases during replication. Base excision repair (BER) excises damaged base(s) or nucleotides via a variety of DNA glycosylases or endonucleases, creating an SSB intermediate. SSBs are primarily detected by poly(ADP-ribose) polymerase 1 (PARP1) (reviewed in (Caldecott, 2008) ), a DNA damage surveillance protein. PARP1 recruits and interacts with x-ray repair cross-complementing protein 1 (XRCC1) (Kubota et al, 1996), which acts as a scaffold protein. After detection and recruitment of SSB repair factors, SSB ends are processed by specific nucleases and factors prior to gap-filling by DNA polymerase β. This end-processing will be the subject of considerable discussion
Figure 1-3. Nonhomologous end joining is available throughout the cell cycle. Model of the relative contributions of HRR (green) and NHEJ (red) to the repair of radiation-induced DSBs in different cell cycle phases, based on mutant phenotypes. Whereas NHEJ predominates in G1/early S, both HRR and NHEJ contribute substantially to DSB repair during late S/G2. Adapted from Figure 7 in (Rothkamm et al, 2003).
in later chapters. Once the damaged base(s) have been replaced, repair sites are ligated by either DNA ligase III (in short patch BER) or DNA ligase I (in long patch BER).

Double-strand break repair

Misrepair of DSBs can lead to mutagenic events such as chromosome translocations (reviewed in Weinstock et al, 2006), deletions, duplications, or DNA loss. If left unrepaired, DSBs result in lethal events (Ward, 1988). The repair of DSBs in mammalian cells primarily occurs by two repair pathways: non-homologous end-joining (NHEJ) or HRR. The two pathways have very distinct mechanisms: HRR requires a homologue, to be found either in a sister chromatid, homologous chromosome, or repeat sequence, from which the HRR machinery can duplicate an exact copy of the damaged gene. HRR is thought to ensure exact DNA repair fidelity (although HRR between Alu elements has been shown to be the cause of intrachromosomal rearrangements associated with cancer and other diseases (Onno et al, 1992)), while NHEJ can be an error-free or error-prone process, rejoining double-stranded ends with no or little sequence homology.

Both NHEJ and HRR are regulated by phosphoinositide 3-kinase related kinases (PIKKs) that act as sensors of the DNA damage response (Bakkenist & Kastan, 2004, Valerie & Povirk, 2003). NHEJ is coordinated by the actions of DNA-dependent protein kinase (DNA-PK), a holoenzyme comprised of the regulatory KU heterodimer, KU70 (70 kDa) and KU80 (86 kDa), and a large catalytic subunit (DNA-PKcs), the PIKK (Figure 1-4).
Figure 1-4. Nonhomologous end-joining repair. The KU70/KU80 heterodimer (blue) forms a hollow ring that binds to DNA ends. Bound KU70/ KU80 recruits DNA-PKcs (pink). The Cernunnos-XLF protein (yellow) forms complexes with XRCC4 (green) and Ligase IV (brown). DNA-PK autophosphorylates, causing a conformational change. Artemis and other factors nucleolytically process DNA ends prior to joining. DNA ends are ligated by Ligase IV in complex with XRCC4. Adapted from Radiobiology lectures delivered by Dr. Lawrence F. Povirk.
DNA-PKcs binds to damaged DNA directly and functions both architecturally and as a kinase to synapse the two broken ends of DNA and target other NHEJ factors to the site of damage (reviewed in (Meek et al, 2004)). These factors include DNA Ligase IV, which directly mediates DNA-strand joining by this pathway (Robins & Lindahl, 1996). Ligase IV exists in a tight complex with the adaptor protein XRCC4 (Critchlow et al, 1997). Artemis also functions in this repair pathway as an endonuclease, processing DNA overhangs at breaks (Povirk et al, 2007b). XLF/ Cernunnos, the most recently discovered NHEJ factor (Ahnesorg et al, 2006, Buck et al, 2006), has been found to stimulate gap-filling (Akopiants et al, 2009).

HRR is known to be regulated by Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and RAD3-related (ATR) (Golding et al, 2004, Wang et al, 2004). Whereas ATM is activated by DSBs, ATR responds to single-stranded DNA both at resected DSBs and at replication fork stalling that compromises genome integrity during the S phase of the cell cycle (Paulsen & Cimprich, 2007). HRR proceeds by nucleolytic resection that yields single stranded DNA, followed by formation of a recombinase filament on the resected single stranded DNA. DNA ends are retained via a heterotrimeric complex of MRE11, RAD50, and NBS1 (meiotic recombination 11 homolog, Rad50 homolog, and Nijmegen Breakage Syndrome protein) that bind the ends of the DNA, perhaps tethering them to each other (Williams et al, 2009). The resected single-stranded DNA becomes the substrate for the HRR machinery to execute strand invasion into a homologous DNA sequence. After locating a region of homology (an enigmatic process (Barzel & Kupiec, 2008)), strand invasion occurs to form a D-loop
structure. DNA synthesis then extends from the 3′-end of the invading strand (reviewed)

Figure 1-5. Homologous recombination repair. After detection of a DSB, HRR proceeds by nucleolytic resection, exposing 3′ single-stranded DNA. The protruding strands are covered by replication protein A, which is then replaced by Rad51, to preclude secondary structure formation (not pictured for simplicity). After locating a
homologous sequence, strand invasion results in the formation of a D-loop, and the invading 3' ends prime DNA synthesis. Resolution of the Holliday junction intermediate can lead to a crossover of arms swapped between the interacting chromatids. Adapted from Figures 1 in (Barzel & Kupiec, 2008, Mimitou & Symington, 2009, San Filippo et al, 2008).

in (Mimitou & Symington, 2009, San Filippo et al, 2008)). The second DSB end then anneals to the extended D loop, and the formation and resolution of two crossed strand or Holliday junctions should yield intact double stranded products (Figure 1-5).

The “choice” of which DSB repair pathway to utilize is influenced by a variety of factors; these include, but are not limited to: a) the stage of the cell cycle, as the homologue that HRR requires is more likely to be present as a sister chromatid during the S and G2 phases of the cell cycle, whereas NHEJ is available throughout the cell cycle and in terminally differentiated cells; b) the extent of DNA end resection (Aylon et al, 2004, Huertas et al, 2008), the first step of HRR, which is further dependent on factors that vary over the cell cycle; c) the severity and type of the DNA damage; and d) signaling events that are initiated both from within the damaged cell (“inside-out” signaling) or from growth factor signaling cascades (Golding et al, 2007). While HRR is considered to be most important in late S and G2 phases of the cell cycle when sister chromatids are available, HRR can also occur between homologous DNA repeats on heterologous chromosomes outside of the S and G2 phases (Golding et al, 2004). A substantial portion of the human genome consists of repetitive DNA sequences, thus providing at least the theoretical opportunity for HRR outside of S and G2. In mammalian cells (specifically, hamster CHO cells), NHEJ has been reported to be 1.7 – 3.8-fold more frequent than HRR (Guirouilh-Barbat et al, 2004). “Choosing” between merely HRR or NHEJ is also a false dichotomy, as alternative NHEJ pathways (which
have been referred to as “backup NHEJ,” “alternative NHEJ,” or “non-canonical NHEJ”)
have also been described. Alternative NHEJ is dependent on PARP1, which binds to
DNA ends in direct competition with KU, and functions with DNA ligase III (Wang et al,
2006) to end-join DNA. This pathway also displays cell cycle dependence, and is
II. INTRODUCTION FOR PART I

2.1 Hereditary Spinocerebellar Ataxia with Axonal Neuropathy

A homozygous mutation in the active site of tyrosyl-DNA phosphodiesterase (TDP1) has been identified as the causative mutation in hereditary spinocerebellar ataxia with axonal neuropathy (SCAN1). SCAN1 (Online Mendelian Inheritance in Man (OMIM) # 607250) is inherited as an autosomal recessive disorder that becomes apparent at adolescence (Figure 2-1); clinical features in these individuals include distal muscle weakness, absence of deep tendon reflexes, gait disturbances, and mild brain atrophy (Takashima et al, 2002). SCAN1 has been indentified in 9 individuals from a single, large Saudi Arabian family, and detailed clinical evaluations including brain imaging data are available on 3 of the affected individuals. Although the cohort of patients is admittedly small, so far there is no indication that the disorder includes an increased predisposition for cancer.

2.2 Tdp1 identification in yeast

Prior to the characterization of a clinical disorder, tdp1 was initially characterized in budding yeast (Saccharomyces cerevisiae) as a novel enzyme with activity against the phosphodiester linkage formed between the conserved tyrosine within the active site of topoisomerase I (Top1) and a 3’ DNA terminus (Pouliot et al, 1999, Yang et al, 1996). The enzyme was characterized as being active at pH 5.0 – 9.5, and required no co-factors: no stimulation of activity was observed when reactions with the enzyme were stimulated
with 10 mM Mg$^{2+}$, Ca$^{2+}$, Co$^{2+}$, Cd$^{2+}$, or Zn$^{2+}$ (Yang et al, 1996) (though a subsequent study did find that TDP1 was stimulated by Mn$^{2+}$ (Cheng et al, 2002)). The enzyme was

Figure 2-1. SCAN1 pedigree reported by Takashima et al, 2002. This pedigree represents the large Saudi Arabian family that contains the 9 known individuals affected with SCAN1. Squares indicate males, circles indicate females, slashed-through symbols indicate individuals known to be deceased, and double horizontal lines indicate consanguinity. Individuals who are known to possess homozygous affected alleles are marked as filled-in symbols. Genotype data and segregating haplotypes are known for the last 2 generations and can be found in the publication (Takashima et al, 2002). More detailed clinical information is available on 3 of the 9 affected individuals and can be found in Table 1 of the publication.
capable of separating a tyrosine from a 3’ DNA linkage, or an entire Top1 joined to DNA via its enzymatic tyrosine residue. Because the DNA product after treatment with the purified enzyme shifted an additional amount after treatment with bacteriophage T4 polynucleotide kinase/phosphatase (PNKP), an enzyme known to remove phosphate groups from the 3’ end of DNA, researchers concluded that tdp1 removed the tyrosine residue and left a phosphate group on the 3’ end of DNA (Figure 2-2) (Yang et al, 1996).

Curiously, the yeast strain identified as genetically disrupted within the tdp1 coding region was not hypersensitive to camptothecin (CPT) treatment; an additional mutation to RAD9 had to be made to reveal hypersensitivity (Pouliot et al, 1999). Deletion of RAD9 severely impairs the DNA damage response in budding yeast by compromising the G2 cell cycle arrest checkpoint (Weinert & Hartwell, 1988). That an additional mutation has to exist for tdp1Δ yeast to exhibit CPT hypersensitivity emphasizes that yeast have differential reliance on repair pathways compared to mammals. In yeast, the predominant DSB-repair pathway is HRR (Critchlow & Jackson, 1998, Moore & Haber, 1996), while in mammals it is NHEJ (Guirouilh-Barbat et al, 2004). Also curious is that deletion of TOP1 in yeast is not incompatible with life (Thrash et al, 1985), and has the effect of removing the target of CPT (discussed below), and simultaneously increased survival 1000-fold when treated with CPT (unpublished observations referenced in (Pouliot et al, 1999). In contrast, TOP1 is required for embryonic development in Drosophila (Lee et al, 1993), and TOP1-/- mice die during embryogenesis between the 4- and 16-cell stage of development (Morham et al, 1996).
Figure 2-2. Characterization of TDP1 enzyme activity with an oligonucleotide-tyrosine substrate. (A) Substrate schematic and (B) diagram of phosphorimage. In A, the jagged line represents the 18-mer oligonucleotide with an asterisk at its left to indicate the radiolabeled 5' end. At the right of the jagged line, the chemical structure of the 3' end of the oligonucleotide is given. Adapted from (Yang et al. 1996). Polynucleotide kinase/phosphatase (PNKP) then removes the resulting 3-phosphate. In B, lane 1 represents a reaction of the substrate depicted in A without enzyme or cell extract; lane 2 indicates bands observed after processing with cell extract.
2.3 Topoisomerases

Topoisomerases are enzymes that act on the topology of DNA. Both RNA transcription and DNA replication, and possibly DNA repair and chromatin remodeling, produce positive and negative DNA supercoiling generated by local areas of DNA melting (Figure 2-3) (Liu & Wang, 1987). TOP1, initially also referred to as ‘the DNA untwisting enzyme’ (Champoux & Dulbecco, 1972) or ‘DNA Swivelase’ (Champoux, 1978), serves to relieve superhelical tension by creating transient breaks in the DNA using a conserved tyrosine residue within its catalytic site (Y 723). In the process of nicking the DNA, TOP1 forms a covalent reaction intermediate referred to as a Topoisomerase I cleavage complex (TOP1cc) (Depew et al, 1978): a phosphodiester bond between the catalytic tyrosine and the 3′ end of DNA. During the lifetime of this intermediate, the ends at the nick are free to rotate relative to the helix axis if the DNA has an excess or deficiency of helix turns (Champoux & Dulbecco, 1972), and then the DNA break is resealed. The TOP1cc intermediate is normally transient, and strand rejoining is rapid relative to strand cleavage (Pourquier et al, 1997). The TOP1cc is likely processed by proteases and cleaved to a smaller peptide bound to DNA prior to processing by TDP1 (Debethune et al, 2002).

However, conditions exist that have the effect of either slowing the rejoining step or enhancing the initial formation of the cleavage complex, causing the protein linked breaks to accumulate; these include when topoisomerase acts on DNA containing lesions such as thymine dimers, abasic sites (Pourquier et al, 1997), oxidative damage (Pourquier et al, 1999), and mismatched base pairs (Pommier et al, 1998, Yeh et al, 1994).
Additionally, TOP1 can be engineered with specific mutations that suppress rejoining via forming extremely stable TOP1ccs that lead to DSBs, and subsequent cell death in yeast (Megonigal et al, 1997, Woo et al, 2003). Stabilized TOP1cc can be further converted into irreversible complexes after colliding with either DNA replication forks or transcriptional complexes (reviewed in (Pommier et al, 2003)). Most important for the broader implications of the study of TDP1 and TOP1, a pharmacological mechanism exists for slowing the rejoining step of the cleavage complexes: treatment with camptothecins, a class of drugs that targets TOP1 (Hsiang et al, 1985).

Several research groups have also published results on Topoisomerase 2 (TOP2)-induced DNA breaks and TDP1. TOP2 relieves superhelical tension in the DNA via a slightly different reaction mechanism than that of TOP1, forming a reaction intermediate containing a 5'-'phosphotyrosyl linkage with DNA (Nelson et al, 1984). Cultured human cells that were transfected to overexpress a GFP-tagged TDP1 were shown by single cell gel electrophoresis (Comet assay) to be resistant to treatment with etoposide, a drug that targets TOP2 (Barthelmes et al, 2004, Chen et al, 1984). In yeast, deletion of TDP1 has also been shown to confer hypersensitivity to agents that pharmacologically target TOP2 (Nitiniss et al, 2006), and overexpressing TOP2 in the presence of *tdp1Δ* heightened that hypersensitivity. The hypersensitivity could not be rationalized as merely an indirect effect of etoposide producing DNA modifications that trap TOP1, as *tdp1Δ top1Δ* double mutants displayed enhanced hypersensitivity to etoposide, rather than a reduction that would be expected if TOP1 were responsible for enhanced toxicity. Additionally, mutating a conserved amino acid within the active site of the enzyme (H182A, the yeast
equivalent to human H263) eliminated activity against a 5′-phosphotyrosyl substrate, demonstrating that the yeast Tdp1 reaction against 5′ DNA

Figure 2-3. Superhelical tension can result from a variety of normal cellular processes and is relieved by Topoisomerases. Local areas of DNA melting generate superhelical tension in areas adjacent to sites of replication, transcription, and chromatin remodeling. As summarized by J.C. Wang in 1987, “Many amateur gardeners have had the experience that the coiling of a long garden hose is virtually impossible without allowing at least one end of the hose to rotate.” (Wang, 1987) Tension in Supercoiled DNA can then be relieved by nicking, rotation, and resealing by TOP1. Figure adapted from (Pommier, 2006).
end modifications likely proceeds via a similar reaction mechanism to that of 3’ substrates (Nitiss et al, 2006). Further research has shown that recombinant human TDP1 can also process a 5’-phosphotyrosyl substrate (Nitiss et al, 2007). Taken together, these results are somewhat controversial: other research groups have tested the ability of TDP1 to remove 5’ phosphotyrosyl linkages (Yang et al, 1996), and whether SCAN1 lymphoblasts are hypersensitive to etoposide (Interthal et al, 2005b), and each reported negative results. This controversy has been somewhat resolved by a recent publication by Caldecott’s group, showing that a putative member of the Mg\(^{2+}/\)Mn\(^{2+}\)-dependent phosphodiesterase superfamily processes 5’-phosphotyrosyl linkages, and have so renamed it tyrosyl-DNA phosphodiesterase 2 (Ledesma et al, 2009).

2.4 Mitochondrial Topoisomerase

In addition to nuclear DNA requiring enzymes to modify its topology, human mitochondrial DNA possesses similar requirements. Human mitochondria contain ~5–10 copies per mitochondrion of a duplex DNA genome (reviewed in (Clayton, 1982)) consisting of 16,569 base pairs (Anderson et al, 1981), in a covalently closed circle packaged into DNA-protein complexes referred to as nucleoids (Garrido et al, 2003). As such, tension is generated in the circular mitochondrial genome during replication and transcription, indicating a requirement for a mitochondrial topoisomerase. Such an enzyme (TOPmt) was characterized in 2001 as nuclear-encoded, and most biochemically similar to nuclear TOP1 (Zhang et al, 2001), in that TOPmt forms a covalent bond to the
3’ end of the DNA break. Also similar to TOP1, the TOPmt is sensitive to CPT (Zhang et al, 2001, Zhang & Pommier, 2008).

2.5 Camptothecins

CPT (camptothecin) is a compound first isolated from a tree indigenous to China, *Camptotheca acuminata*, in 1966 (Wall et al, 1966) by an extremely successful group of chemists who subsequently went on to isolate and characterize Taxol in 1971 (Wani et al, 1971). While its chemotherapeutic potential was apparent since its initial discovery, the mechanism of action for CPT was not determined until 1985 (Hsiang et al, 1985). Prior to that, it was recognized that CPT inhibited both DNA synthesis and RNA transcription (Horwitz et al, 1971) and induced fragmentation of DNA which induced cell lethality, but that CPT by itself did not cleave purified DNA. To search for which other molecule was required as a target for CPT to mediate DNA fragmentation, researchers tested purified TOP1 and TOP2 in DNA cleavage assays, expecting to find that CPT induced or stabilized TOP2-mediated breaks, as this had recently been discovered as the mechanism for other DNA-fragmenting antitumor drugs, such as methane sulfon-m-anisidide (Nelson et al, 1984). Instead, researchers found that CPT inhibited the catalytic activity of TOP1, increasing the half-life of TOP1cc, and proposed that this might be the mechanism by which CPT inhibits nucleic acid synthesis (Hsiang et al, 1985). Further research showed that more specifically, CPT binds to the TOP1cc and interferes with the DNA religation step of the reaction within the complex (Porter & Champoux, 1989), and that it is the interaction of various cellular processes with the CPT-stabilized TOP1cc that induce lethality (Hsiang et al, 1989). For example, in actively replicating CPT-treated cells, replication forks collide with TOP1cc and become arrested. Thus the predominant
Cytotoxic effect of CPT is S-phase dependent, as treatment with aphidicolin, an inhibitor of replicative DNA polymerases, protects cells from the lethal effects of CPT, though not from its ability to induce SSBs (Holm et al, 1989). In actively transcribing cells, RNA polymerases can collide with TOP1cc (Bendixen et al, 1990), explaining a different mode of toxicity. This mechanism of CPT-induced toxicity has been observed in postmitotic rat cortical neurons in vitro (Morris & Geller, 1996). In culture, astrocytes exhibited greater resistance to CPT-induced killing than neurons; the higher sensitivity to CPT in neurons correlated with both a greater amount of TOP1 expression, and higher transcriptional activity compared to astrocytes (Morris & Geller, 1996). Concomitant treatment with both aphidicolin and CPT failed to protect the neurons from CPT-induced toxicity, suggesting that DNA replication does not contribute to CPT-induced neuronal death (Morris & Geller, 1996). Subsequently, it was shown that CPT-induced neuronal death can be abrogated by inhibiting cyclin-dependent kinases (CDKs) or via treatment with other agents that block the G1-S transition in dividing cells, indicating that CPT toxicity may involve deregulated cell cycle signaling (Park et al, 1997).

After treatment with CPT, TOP1 bound within cleavage complexes is ubiquitinated and partially degraded (Desai et al, 1997, Lin et al, 2008), leaving a smaller phosphotyrosyl (pTyr) peptide adduct covalently bound to DNA, which is then removed by TDP1. This pTyr adduct-removal function of TDP1 was highlighted when it was shown that lymphoblasts from SCAN1 patients are hypersensitive to CPT (Interthal et al, 2005b). Cell-cycle analysis of the CPT-treated SCAN1 cells revealed that the majority of apoptotic cells were in S-phase, and that the proportion of S-phase cells increased over time after CPT-treatment (Interthal et al, 2005b). In contrast, normal lymphoblasts
treated with CPT displayed an increased proportion of cells in the G2 phase of the cell cycle. Subsequent work with an immunoblot assay designed to detect TOP1cc directly demonstrated the elevated levels of TOP1cc in SCAN1 lymphoblasts after CPT treatment. In this same study, researchers observed the protective effect of aphidicolin when applied to normal lymphoblasts treated with CPT, but observed only a minimally protective effect of aphidicolin on SCAN1 lymphoblasts treated with CPT (Miao et al, 2006). However, pre-treatment with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, a transcription inhibitor, before treatment with CPT reduced the formation of DNA-protein crosslinks observed in SCAN1 lymphoblasts, but not in normal cells. Therefore, researchers concluded that the CPT hypersensitivity seen in SCAN1 cells was due in greater part to transcriptional, rather than replicative activity (Miao et al, 2006), complementing earlier results seen in postmitotic neurons treated with CPT (Morris & Geller, 1996). The transcription-specific cytotoxic effects of CPT had previously been noted in XRCC1 deficient cells (Plo et al, 2003), which are also deficient in BER.

In addition to mutant TDP1 modifying sensitivity to CPT, a variety of TOP1 mutations have also been classified that render the enzyme resistant to CPT (reviewed in (Gupta et al, 1995)).

### 2.6 Phosphoglycolate end processing

TDP1 can also process protruding 3′-phosphoglycolate (PG) termini on DNA double-strand breaks (DSBs) (Inamdar et al, 2002a, Zhou et al, 2005) that are formed in response to oxidative stress (Bertoncini & Meneghini, 1995), ionizing radiation (Henner et al, 1983), and specific chemotherapeutic agents such as bleomycin (Gilioni, et al 1981,
Povirk, 1996). More specifically, the 3’-PG termini are formed by a radical species initiating an attack on the 4’ carbon of deoxyribose followed by cleavage of the bond between carbons 3’ and 4’ of the sugar ring. The base is then lost as a base-propanal adduct (Giloni et al., 1981) leaving a two-carbon fragment -PO\_4CH\_2COOH (Figure 2-4). The radical species that may initiate the attack includes, but is not limited to, the superoxide radical \( \text{O}_2^- \), generated from the reduction of \( \text{O}_2 \), the hydroxyl radical \( \text{HO}^- \), generated from the decomposition of \( \text{H}_2\text{O}_2 \) (reviewed in (Cadenas, 1989) or a bleomycin-Fe(III)-\( \text{O}_2 \) complex, which may be reactivated by the process of initiating a DNA strand break to commit to a secondary attack on the opposing strand to create another break (Steighner & Povirk, 1990), thereby creating a DSB with 3’-PG termini on both DNA ends.

TDP1 is not known as the primary enzyme responsible for removing 3’-PG; rather this characteristic has long been attributed to apurinic/apyrimidinic endonuclease/redox effector (APE1/REF-1) (Parsons et al., 2004, Suh et al., 1997). The broad specificity of APE1 implicates the enzyme as a focal point in multiple repair pathways: APE1 is a major enzyme in the BER pathway responsible for removing apurinic/apyrimidinic (AP) sites, and can also initiate the repair of oxidized abasic sites and deoxyribose fragments formed by free radical attack (reviewed in (Demple & Harrison, 1994)). Although APE1 does possess 3’-PG diesterase activity, this activity is 200-fold lower than its AP endonuclease activity (Chen et al., 1991). APE1 can remove 3’-PG from SSBs both with gaps and with internal nicks with no missing bases, and from DSBs with either blunt or 2 base recessed 3’-PG (Suh et al., 1997). However, DSBs that possess PG on 1 or 2-base 3’ overhangs
Figure 2-4. Sugar fragmentation by radical oxygen species can result in 3’ phosphoglycolate DNA end modifications. (i) DNA is attacked by a hydroxyl on carbon 4 of deoxyribose ring (4’ carbon), transforming the 4’ carbon into a radical species, (ii) The radical 4’ carbon reacts with oxygen to become an oxidized species, (iii) the deoxyribose sugar spontaneously fragments, creating a DNA break, releasing a base, leaving a phosphoglycolate bound to the 3’ carbon of the previous base in the DNA strand, and a phosphate bound to the 5’ carbon of the next base in the DNA strand.
show no detectable removal of when treated with APE1 (Suh et al, 1997). TDP1 was characterized as being proficient in processing protruding 3′-PG in a study that tested the ability of APE1, PNKP, the Ku subunit of the DNA-PK holoenzyme, and TDP1 to remove 3′-PG from a DSB engineered into a plasmid substrate (Inamdar et al, 2002a). Just prior to that study, the same research group tested the ability of DNAseIII, the predominant 3’ to 5’ exonuclease in mammalian cells, to achieve the same, and found 3′-PG to be resistant (Inamdar et al, 2002b). Of the enzymes tested, only TDP1 was found to be able to remove protruding 3′-PG, and this was an early event in NHEJ that occurred prior to Ku loading or formation of the DNA-PK complex on DNA ends (Chen et al, 2001b, Inamdar et al, 2002a). Removal of the glycolate left behind a 3′ phosphate group, which was then susceptible to removable by PNKP (Jilani et al, 1999, Karimi-Busheri et al, 1999). Furthermore, research performed with whole cell extracts made from lymphoblast cell lines derived from SCAN1 patients showed that these extracts exhibited a deficiency in processing protruding 3′-PG on DSB (Zhou et al, 2005). However, subsequent research failed to show that SCAN1 lymphoblasts were more sensitive to bleomycin than wild-type cells (Interthal et al, 2005b).

TDP1 has also been shown to process 3′ end modifications other than 3′-pTyr and 3′-PG; these include the phosphoamide linkage that is created when TDP1 is itself transiently linked to 3′ DNA ends (Interthal et al, 2005a), part of the reaction mechanism described in further detail below. In addition to natural substrates, TDP1 exhibited 3′-exonuclease activity on a 2 nucleotide 3′ overhang on an RNA-DNA hybrid, and was also able to cleave artificial 3′ adducts, such as a 3′-tetrahydrofuran moiety, and a 3′-biotin tag.
(Interthal et al, 2005a, Nitiss et al, 2007). Activity against these 3’ adducts suggests that TDP1 may also act on pharmacologically relevant 3’ adducts such as those generated by chain terminating nucleoside analogs used for the treatment of human immunodeficiency virus, type 1.

2.7 Reaction Mechanism of TDP1

Tdp1 is a member of the phospholipase D (PLD) superfamily (Interthal et al, 2001), a family of enzymes characterized by two HxK(x)₄D sequences, termed HKD motifs, that come together to provide a single active site (Figure 2-5) (Stuckey & Dixon, 1999). The crystal structure of human TDP1 and initial biochemical experiments have shown that for TDP1, the HKD motifs are composed of histidines 263 and 493 and lysines 265 and 495, and that these residues are required for normal catalytic activity (Davies et al, 2002a, Davies et al, 2002b, Interthal et al, 2001, Raymond et al, 2004). The aspartate residue within the HKD domains is not in fact conserved in human TDP1, and for other PLD enzymes is not located near the active site of the enzyme, and rather than being involved in the reaction mechanism it is likely to be involved in tertiary structure stabilization (Interthal et al, 2001). As for other PLD enzymes, the reaction between TDP1 and its substrate proceeds through two \( S_N^2 \) reactions (Stuckey & Dixon, 1999) and follows a general acid/base catalytic mechanism. First, the histidine residue from one motif (H263) acts as a nucleophile, leading to the formation of a covalently-bound phosphoenzyme intermediate (referred to as the initial transesterification). In the case of TDP1, this takes the form of a phosphoamide bond to the 3’ end of the DNA.
moiety. The second $S_N2$ reaction takes place with the histidine from the other motif (H493) acting as a base, activating a water molecule that hydrolyzes the reaction.

Figure 2-5. Diagram of the TDP1 gene and crystal structure. (A) The TDP1 gene is located at 14q32.11. The crystal structure determined by Davies et al. (B) is colored by domain, with the N-terminal domain (residues 162–350) colored blue and the C-terminal domain (residues 351–608) colored yellow. The active site lies along a pseudo-2-fold axis of symmetry between the two domains. The active site residues His263, Lys265,
His493, and Lys495 are shown as ball-and-stick structures and colored red (Davies et al, 2002a).

Intermediate, leaving DNA with a 3′ phosphate end (Interthal et al, 2001). However, important for the consequences of the SCAN1 mutation, the H493 residue is involved in both steps of the reaction: in the initial cleavage reaction it is positively charged and serves as a general acid to protonate the leaving group, accelerating the nucleophilic attack by H263 (Davies et al, 2002b, He et al, 2007, Interthal et al, 2001). Loss of a proton from H493 generates a lone pair of electrons that serve to activate the water molecule in the second S_N2 reaction.

The SCAN1 mutant form of TDP1, with an arginine substituted for the H493 residue, retains activity, albeit ~25-fold less than wild-type purified TDP1 (Interthal et al, 2005b). The H493R substitution affects both S_N2 reactions; the covalent TDP1-DNA reaction intermediate accumulates, but the initial cleavage of the phosphodiester bond is also slowed (Interthal et al, 2005b). Direct evidence of this was shown when the half-life of TOP1cc in CPT-treated SCAN1 cells was found to be 4X longer than TOP1cc in CPT-treated cells from normal individuals (Miao et al, 2006). The SCAN1 493 arginine may retain a part of the general acid catalytic function required in the first step of the reaction, but perhaps is unable to activate a water molecule for the second step, or possibly for steric reasons excludes a water molecule from the active site (D Davies, personal communication to Interthal et al, 2005a). The effect of this is that attempted repair of covalently-bound TOP1 by TDP1 H493R merely exchanges TOP1 with covalently-bound TDP1 H493R, which has a relatively long half-life of ~13 min, under conditions when the half-life of the wild-type TDP1 is so short it cannot be reliably measured.
(Interthal et al, 2005a). Thus, both persistent covalently-linked TOP1 and covalently-linked TDP1 may contribute to the molecular pathology of SCAN1.

2.8 TDP1 is altered in cancer and may be an appropriate target for therapy

For cancer therapies that induce DNA damage, overexpression of DNA repair genes involved in the targeted pathway may result in drug resistance. TDP1 has been evaluated as a gene potentially involved in the attenuation of CPT-toxicity in mouse fibroblasts. Under certain conditions of CPT-exposure and recovery (24h drug challenge, 5-20 nM CPT, followed by 5 days in drug-free medium), mouse fibroblasts stably transfected with yeast Tdp1, displaying 3X the normal amount of Tdp1-specific activity had a 2-3X survival advantage over mock transfectants (Nivens et al, 2004). Comet assays confirmed that in both mouse fibroblasts and hamster lung cells treated with CPT, cells overexpressing yeast Tdp1 exhibited a 3-fold reduction in DSBs. Subsequently, TDP1 expression has been shown to be elevated in non-small cell lung cancer (NSCLC), a cancer commonly treated with camptothecins (Liu et al, 2007). Researchers at Johns Hopkins compared 30 NSCLC tissue samples to 8 non-neoplastic tissue samples and found that in 50% of the tumor samples tested, TDP1 expression was increased over normal, and in all tumor samples tested, TDP1-specific activity was increased compared to normal tissues. Furthermore, it was determined that the increased TDP1 activity was cancer-tissue specific, rather than cancer-patient specific, upon evaluation of four pairs of cancer versus normal tissues from the same NSCLC patients (Liu et al, 2007).

Although it may seem counterintuitive for tumor cells to possess elevated levels of DNA repair enzymes - enzymes that impede the acquisition of mutations in an already
genetically unstable environment – this circumstance is not unique to TDP1. APE1 has been shown to be elevated in a variety of cancers: In prostate, cervical, and epithelial ovarian cancers, Ape1/Ref-1 protein levels are dramatically elevated compared to normal tissues (Kelley et al, 2001, Moore et al, 2000, Xu et al, 1997).

Given the involvement with CPT and bleomycin-induced DNA damage, it has been proposed that pharmacologic inhibition of TDP1 may be useful in combination with CPT or bleomycin-based therapies (Interthal et al, 2005b). A variety of aminoglycoside and nonaminoglycoside antibiotics have been found to inhibit the activity of TDP1 (Liao et al, 2006), as has furamidine (Antony et al, 2007), an antiparasitic agent (Soeiro et al, 2005). Furamidine and prodrugs of furamidine are currently in clinical and preclinical trials for treatment of African sleeping sickness (Wenzler et al, 2009); such inhibitors have also been provisionally patented for their use as anti-cancer agents (Pommier & Marchand, 2006). Additionally, the TOP1-DNA interaction itself has been hypothesized as a suitable target for further development of chemotherapeutic drugs (Meng et al, 2003, Pommier, 2006).

2.9 The molecular pathology of SCAN1

Since the initial identification of SCAN1, there have been several different theories of causation of the disorder, which remains unresolved. SCAN1 is unusual among hereditary ataxias associated with deficits in DNA repair; these disorders typically include additional symptoms found to be absent in SCAN1, including mental retardation, photosensitivity, immunodeficiency, and an increased predisposition to cancer (Di Donato et al, 2001). Initially, it was proposed that the disease was the result of a loss of
function: that TDP1 was required to repair TOP1-induced SSBs or DSBs associated with transcription in non-dividing neuronal cells (Takashima et al, 2002), and that the accumulated TOP1cc prevented transcription in cell types with a particularly high demand for it, which would lead to neuronal apoptosis and disease. An alternate hypothesis was also considered: that mutation of TDP1 would have a direct effect, causing DNA damage in cell types that are by their nature hypersensitive to DNA damage relative to other cell types. Either scenario points to cell type specificity: considering that patients are usually wheelchair bound by early adulthood, but retain normal cognitive functioning, SCAN1 must arise from degeneration of a precise subset of neurons (Takashima et al, 2002).

Further investigation showed that TDP1 physically interacts with DNA ligase IIIα, and that lymphoblasts from SCAN1 patients had significantly more persistent SSBs (and not DSBs) than lymphoblasts from wild-type relatives after treatment with either CPT or H2O2 (El-Khamisy et al, 2005). Taken together, investigators concluded from these data that SCAN1 is likely the result of deficits in SSB repair caused by catalytically-inactive TDP1. However, this hypothesis was refuted when TDP1 H493R (the SCAN1 mutant) was shown to retain a residual amount of enzymatic activity (Interthal et al, 2005b), that TDP1 H493R “stalled” on DNA ends (Interthal et al, 2005b), and that TOP1cc were found to be sustained in CPT-treated SCAN1 cells (Miao et al, 2006). Researchers do agree that the cytotoxic effect of TDP1 H493R is transcriptional in origin (El-Khamisy et al, 2005, Miao et al, 2006), providing a potential explanation as to why SCAN1 pathology is specific to neurons, given their elevated levels of transcription.
2.10 Specific aims

SCAN1 is caused by a homozygous H493R mutation in the active site of TDP1. To address how the H493R mutation elicits the specific pathologies of SCAN1 and to further elucidate the role of TDP1 in processing DNA end modifications, we propose:

1. **To model the phenotype of the human disease SCAN1 by creating a Tdp1 knockout mouse.** Examining the gross physiology and detailed behavioral analysis of the Tdp1 knockout mice will test the hypothesis that absence or simple loss of function of TDP1 is what causes the human disorder SCAN1.

2. **To determine whether and to what extent Tdp1 is required for processing specific DNA end modifications.** Because the H493R TDP1 mutation associated with SCAN1 (hereafter referred to as SCAN1 TDP1) retains partial activity, SCAN1 cells fail to provide a true null model of TDP1 deficiency. To address this, the Tdp1 knockout mouse will serve as a source from which Tdp1-/- mouse embryonic fibroblast (MEF) cell lines will be derived. DNA substrates possessing end modifications (tyrosyl or PG) mimicking SSBs or DSBs in specified configurations (overhang or blunt-ended) will be incubated in cellular extracts from each MEF cell line, and the extent of DNA substrate processing can be monitored using radioactive labeling and DNA sequencing gels. Clonogenic survival assays will be employed as a second, complementary method to test which end modifications require Tdp1 for processing, as specific chemotherapeutics (bleomycin, calicheamicin) break DNA, leaving specific end modifications in a highly predictable manner. Complementation of cell extracts with wild-type and mutant forms of TDP1
also allows for testing a further hypothesis: that specific amino acid substitutions within
the active site of TDP1 are more toxic than the absence of TDP1 (Figure 2-6).

3. **To further characterize the intracellular, potentially mitochondrial, localization of TDP1.** MEF cell extracts will be fractionated by differential centrifugation to enrich for the mitochondrial components, and then screened via western blotting for various mitochondrial and nuclear markers to test for their purity. Mitochondrial fractions will be tested for their ability to process DNA end modifications that have themselves been characterized to require TDP1 for processing. MEF cell lines will also be examined via confocal fluorescence microscopy in an attempt to colocalize Tdp1 and mitochondrial markers.
Figure 2-6. Hypothesis on the etiology of SCAN1. Certain amino acid substitutions in the HKD motifs of TDP1 will have a greater toxic effect than the complete lack of TDP1 due to the formation of TDP1-DNA complexes that are more stable than their TOP1-DNA predecessors.
III. METHODS FOR PART I

3.1 Generation of *Tdp1* knockout mouse model

To generate both constitutive and conditional *Tdp1* knockout (*Tdp1*−/−) mice, a *Tdp1* targeting vector was constructed in collaboration with the laboratory of Dr. Shirley M. Taylor, PhD using pKO NTKV-1901, which contains both a PGK/neo/BGH cassette for positive selection of homologous recombinants with G418, and an MC1-tk cassette for negative selection of non-homologous recombinants with ganciclovir (Stratagene). This vector was modified by the insertion of loxP sites on either side of the PGK/neo/BGH cassette (Figure 3-1, panel ii). A 4.8-kb region of the *Tdp1* gene containing exons 5-7 (with 3.2 kb of intron 6 deleted) was generated by PCR from a 129/Sv BAC clone, and a third loxP site (with an associated NheI site) was inserted into intron 5 (Figure 3-1, panel ii). This arm was then inserted between the BglII and XhoI sites of NTKV-1901. A 4.7-kb XmaI/SmaI BAC clone fragment containing *Tdp1* exons 8-12 was inserted into the NTKV-1901 SmaI site. The *Tdp1* targeting vector was linearized with SmaI and electroporated into 129/SV embryonic stem (ES) cells. Genomic DNA from ES cell clones resistant to both G418 and ganciclovir was screened for homologous recombination by long-range PCR using intron 4 and BGH primers (for the 5′ arm), and PGK and intron 12 primers (for the 3′ arm). Retention of the intron 5 loxP site in PCR-positive clones was verified by NheI digestion of the 5′ arm PCR products. Southern blot analysis was performed on DNA from homologous recombinants using a PGK/neo probe to verify clone purity and integrity of both genomic arms.
To delete the neo gene in the targeted Tdp1 allele and generate the desired knockout and conditional knockout alleles (as shown in Figure 3-1 panels iv and v), ES

Figure 3-1. Generation of Tdp1-/- mice, Tdp1 targeting strategy. (i) Structure of the mouse Tdp1 gene, exons 5-12. (ii) Targeting vector for generation of both conditional and total knockout of the Tdp1 gene. (iii) Structure of the initial targeted Tdp1 allele
prior to Cre expression. Structure of the knockout (iv) or conditional knockout (v) allele of \textit{Tdp1} following transient expression of Cre in the targeted ES cells.

cells from a correctly targeted clone were subsequently electroporated with an MC1-\textit{cre} expression vector. After initial growth in the absence of antibiotic selection, individual clones were picked, expanded, and then divided into two wells, one with and one without G418. The G418-sensitive clones were then screened by PCR to identify both KO recombinants (creating a knockout allele) and CKO recombinants (creating a conditional knockout allele). Of 15 G418-sensitive clones screened, 10 were found to be knockout recombinants, and 5 were conditional knockout recombinants. One clone of each type was expanded and injected into C57BL/6 blastocysts, which were then implanted into pseudopregnant CD-1 recipients. The resulting male chimeras were bred to C57BL/6 females and tail genomic DNA from agouti offspring screened by PCR for the presence of the targeted \textit{Tdp1} allele. Germline transmission of both the conventional knockout and conditional knockout alleles was obtained. All mouse generation and breeding was performed in collaboration with the laboratory of Dr. Jolene Windle.

\textit{Tdp1}^{+/−} mice were interbred to generate mice of the \textit{Tdp1} genotypes (+/+, +/- and −/−) used in this study. Offspring were screened by PCR using a common exon 8 anti-sense primer (GI:149292731/25190-25168: 5′-TTC TTG GGT ACA AAG GGC TCA AC-3′), an intron 5 sense primer for the knockout allele (16791-16813: 5′-CTC TAG TCA AAC AGC ACA AAT GC-3′), and an intron 7 sense primer for the wild-type allele (24700-24722: 5′-GTG GAA AGG AAT TGA TGA GAT GG-3′). Knockout and wild-type PCR products were 713 bp and 491 bp, respectively (Figure 3-2). Deletion of \textit{Tdp1}
exons 6 and 7 in knockout mice was also verified by probing a Southern blot of XbaI digests of tail DNA from mice of each of the three genotypes with 5′ (15945-17191) and 3′ (24986-25922) genomic fragments (Figure 3-2).

Figure 3-2. Verification of deletion of exons 6 and 7 in Tdp1+/− and Tdp1−/− mice by PCR and Southern blot analysis. (A) PCR analysis of genomic tail DNA from mice of each genotype. DNA was amplified with a common exon 8 anti-sense primer, an intron 5 sense primer for the knockout allele, and an intron 7 sense primer for the wild-type allele. Knockout and wild-type PCR products were 713 bp and 491 bp, respectively. (B) Verification of deletion of exons 6 and 7 in Tdp1+/− and Tdp1−/− mice. Southern blot analysis of genomic tail DNA from wild-type, heterozygous knockout and homozygous knockout mice. DNA was digested with XbaI (X) and probed with 5′ and 3′ genomic probes (indicated in Figure 3-1 panel iv).
TDP1 transcript levels were quantified by real time PCR using cDNA reverse transcribed from RNA isolated from $Tdp1^{+/+}$ and $Tdp1^{-/-}$ brain and liver. RT-PCR primers were designed to amplify part of the region deleted in the TDP1-/- mouse (exons 6 and 7), as well as regions 5' and 3' of the deleted exons, with one primer in each set derived from adjacent exons. The 5' region primers were 5'-AGAAAGTTGATGATGAC-3' (exon 2 sense) and 5'-CAGAGGGGATATATCCTT-3' (exons 3/2 anti-sense), and yielded an RT-PCR product of 203 bp. The deleted region primers were 5'-GGAACACACCACACGAAAATG-3' (exons 6/7 sense) and 5'-TTTGAAACGGGTGCTTGACTC-3' (exon 8 anti-sense), and yielded an RT-PCR product of 183 bp. The 3' region primers were 5'-CTCTCTCTTCTATAGCATCC-3' (exon 12 sense) and 5'-CTTGGACAGATTGCAGTCTT-3' (exons 14/13 anti-sense), and yielded an RT-PCR product of 184 bp. β-actin was used as an internal standard, using primers 5'-TCCTAGCACCATGAAGATCAAGATC-3' and 5'-CTGCTTTGCTGATCCACATCTG-3', yielding a 118 bp product. Real-time PCR was performed in 15µl reactions on the 7900 HT Real-time PCR System (Applied Biosystems) using SYBR Green PCR Mastermix (Applied Biosystems) (Figure 4-1). PCR bands were separated on a 7% non-denaturing polyacrylamide gel, detected by ethidium bromide staining, and imaged on a Typhoon 9410 variable mode scanner (GE Healthcare) (Figure 4-1) to confirm the size of the amplified products.

3.2 $Tdp1$ knockout mouse behavioral assays
Mice of each genotype (\(Tdp1^{+/+}\), \(Tdp1^{+/-}\), and \(Tdp1^{-/-}\)) were subjected to a variety of behavioral tests, performed in the laboratory of Dr. Jenny Wiley and described as follows. The behavioral evaluation involved a number of tests conducted sequentially on the same day. Following a general assessment of overall health and appearance, a functional observational battery (FOB) of behavioral tests (U.S. Environmental Protection Agency, 1991) was undertaken. Adapted from a protocol for evaluation of neurotoxins (Tegeris & Balster, 1994), it contains tests similar or identical to those recommended for evaluation of behavioral phenotypes of transgenic and knockout mice (Crawley & Paylor, 1997, Crawley, 1999, Watase & Zoghbi, 2003). The FOB consisted of observations of mice in an open field, their response to handling when removed from a locomotor activity chamber, and manipulative behavioral measures. Mice were placed in a 40 X 76-cm open field and scored on the following measures for the first 2 min of the test: posture, arousal, rearing, clonic movements, tonic movements, palpebral closure, gait, and gait abnormalities (see definitions in (Tegeris & Balster, 1994)). Piloerection, righting reflex, forelimb grip strength, the inverted screen task, landing foot splay, approach response, click response, touch response, tail pinch response and mobility were evaluated (in the order listed) over the next 4 min. Latency, in seconds, to climb to the top of the screen after inversion, was measured. Subsequently, when mice were tested in the locomotor activity chamber, they were evaluated for ease of removal and handling reactivity. Scoring of the FOB was done by a single trained technician who was blind with respect to the genotype of the mice.

Following the FOB assessment, three additional tests were employed to assess specific differences in motor performance and possible ataxia. First, general locomotor
activity was measured in standard activity chambers interfaced with a computer-operated automated activity monitor. Mice were placed in individual activity chambers and spontaneous activity was measured for 10 min. Activity was expressed as total number of interruptions of 8 photocell beams per chamber for the 10-min session. Second, the mouse was placed on the cylinder of a standard rotarod apparatus. The cylinder was programmed to rotate initially at 4 revolutions per min, gradually increasing to 40 revolutions per min over 5 min. Latency to fall off of the rotarod was recorded. Third, the hindfeet of each mouse were dipped in ink and the mouse was then placed in a paper-lined 30 cm tunnel to assess stride length. Ataxia may be reflected as shorter stride length (Barlow et al, 1996). At the end of the FOB and motor performance testing, sensitivity to a thermal stimulus was assessed in a standard tail-flick apparatus. Latency to remove the tail from the stimulus was measured. A 10-s maximum latency was imposed in order to prevent damage to the tail. All in vivo tests describe above were conducted at 3, 6 and 12 months of age in the same mice, with the exception that additional mice were tested at 12 months in some groups due to low availability of these mice at earlier time points.

For behavioral assays, continuous and count measures were analyzed using separate ANOVAs for each time comparing the behavior of homozygous knockout, heterozygotes and wildtype mice. As appropriate, categorical data was analyzed with separate Chi square tests. Data for male and female mice were analyzed separately. For behavioral assays, separate ANOVAs for each sex were also used to assess motor activity, tail flick latency, rotarod latency, and stride length. For all significant ANOVAs, Tukey post hoc tests ($\alpha = 0.05$) were used to determine differences from wildtypes.
All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University, and were conducted in accordance with the Animal Welfare Act, the PHS Policy on Humane Care and Use of Laboratory Animals, and the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

3.3 Histologic analysis of brains

For histological analysis, 6 $Tdp1^{-/-}$, 2 $Tdp1^{+/-}$ and 3 $Tdp1^{+/-}$ mice were sacrificed at 12 months of age, and an additional 2 $Tdp1^{-/-}$ and 2 $Tdp1^{+/-}$ mice were sacrificed at 22 months of age. Animals were transcardially perfused with formalin, and the brains were sagitally blocked, embedded in paraffin and sliced at 10µm throughout the hemisphere. Twenty-four to twenty-eight uniformly spaced sections for each animal were deparafinized and rehydrated, then stained with 0.05% cresyl violet, dehydrated and coverslipped. The microscopic examiner was blinded to the genotype and scanned through each section for evidence of gliotic scars or overt loss of cells in numerous brain structures including cerebellum (purkinje and granule cells), cortex, hippocampus, basal ganglia structures (subthalamic nucleus, striatum, globus pallidus). These images were not reviewed by the other investigators involved in the study.

3.4 Generation of the mouse embryonic fibroblast cell lines

For generation of $Tdp1^{+/-}$, $Tdp1^{+/-}$, and $Tdp1^{-/-}$ mouse embryonic fibroblasts, mice indicated in the table below were mated, and the females were evaluated for the presence of a vaginal plug each morning. The day a plug was detected was defined as e0.5. A
pregnant mouse of each genotype was sacrificed at e13.5 – e14.5 by cervical dislocation after anesthesia. Uterine horns were removed and dissected to remove embryos, which were then washed and separated from any visceral tissue. Embryos were passed through a syringe and the resulting cellular mixture incubated with trypsin to further digest the connective tissue. The mouse embryonic fibroblasts (MEFs) were then plated on plastic coated with 0.1% gelatin to facilitate attachment. To derive the initial cell lines used for these studies, the following mating pairs were bred and sacrificed as indicated.

<table>
<thead>
<tr>
<th>Genotype of Derived MEFs</th>
<th>Genotype and # of ♂</th>
<th>Genotype and # of ♀</th>
<th>Date of Birth ♂</th>
<th>Date of Birth ♀</th>
<th>Mated</th>
<th>Plugged</th>
<th>Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tdp1 +/-</td>
<td>#25, Tdp1 +/+</td>
<td>#70, Tdp1 +/+</td>
<td>6/23/06</td>
<td>8/1/06</td>
<td>10/19/06</td>
<td>10/20/06</td>
<td>10/31/06</td>
</tr>
<tr>
<td>Tdp1 +/-</td>
<td>#23, Tdp1 +/-</td>
<td>#56, Tdp1 +/-</td>
<td>6/23/06</td>
<td>7/25/06</td>
<td>9/22/06</td>
<td>9/23/06</td>
<td>10/4/06</td>
</tr>
<tr>
<td>Tdp1 +/-</td>
<td>#23, Tdp1 +/-</td>
<td>#30, Tdp1 +/-</td>
<td>6/23/06</td>
<td>6/30/06</td>
<td>10/22/06</td>
<td>10/23/06</td>
<td>11/3/06</td>
</tr>
</tbody>
</table>

As indicated from the graph, this meant that the cell lines were not entirely age-matched, meaning, the Tdp1 +/- MEFs were in culture for an entire month before the Tdp1 +/- MEFs, which could account for some of the earlier differences seen in the cell lines. After initial plating, cells were subsequently passaged on 150 mm plastic. Cells were cultured in DMEM (Cellgro) supplemented with 10% fetal bovine serum (Gibco), and maintained in an incubator at 37 ºC and 5% CO₂.

### 3.5 Immortalizing the MEFs

Rather than continuously re-deriving primary cell cultures from the Tdp1 mouse model and examine their characteristics, we sought to derive and establish immortalized cell lines. Primary cell cultures are known to have limited viability, and after a certain number of population doublings will stop dividing (Hayflick, 1965). Immortalized cell
lines have acquired the ability to proliferate indefinitely, either through deliberate genetic modification or through random mutation. Here, we sought to induce immortality through continuous growth in culture, which would eventually result in spontaneous, random mutation events, and cells immortalized via these mutation events would grow and become the majority of the culture.

Cells were continuously passaged twice a week for 6-8 months. For passage, cells were first washed with Dulbecco’s CaCl- and MgCl-free phosphate-buffered saline (PBS, Invitrogen/Gibco), then trypsinized for 5 to 7 min with 2 mL of 0.05% trypsin until the cell sheet was completely dispersed to single cells. 4 mL of complete serum-containing media was added back to the single-cell suspension, and the cells were re-suspended in a total of 6 mL of trypsin + media. 4.5 mL of that suspension was removed to waste, and the cells were re-suspended in a total of 20 mL on 150 mm plastic. This method of passaging cells (also known as subculture or splitting cells) was referred to as a 1:4 passage.

After 6 months in culture, when it was believed that the MEF cell lines were at least partially-immortalized, it was hypothesized that the Tdp1+/+, Tdp1+/−, and Tdp1−/− MEFs were exhibiting different growth kinetics. This was tested by performing a growth curve. To determine the growth rate of Mouse Embryonic Fibroblast (MEFs) cells, 60-mm Petri dishes were seeded with 2 x 10⁴ cells. At the time intervals indicated, 3 dishes were taken from each group, the cells were trypsinized, and the number of cells was counted using a hemocytometer. This experiment was performed in March 2007, after the cells had been kept continuously in culture for ~ 150-180 days (Figure 4-17).

3.6 Strategy on complementing Tdp1−/− MEFs and confirmation of complementation
To develop a matched control for the Tdp1-/- fibroblasts, we sought to stably express human TDP1 in Tdp1-/- fibroblasts. First, attempts were made to transfect the fibroblasts with a TDP1-FLAG construct that also expressed green fluorescent protein (GFP), but these attempts did not succeed: no FLAG signal could be detected by Western blotting (Figure 4-4A) and no GFP signal could be detected by fluorescence microscope imaging. At the time of the attempted transfection, the MEFs had been kept continuously in culture for approximately 100 days.

A second strategy was developed using lentiviral vectors, which are an improvement over transfection due to the ability of the vector to enter the cell without the use of toxic transfection reagents. First, the Tdp1-/- fibroblasts were plated at very low density, and individual colonies were picked using pipette tips. These clones (labeled Tdp1 KO fibroblast clone W, X, Y, or Z) were expanded, and it was verified that these cells did infect with a lentiviral construct, LVTHM-DsRed (Figure 4-4B). Fluorescent images and their matched brightfield images were acquired using the Olympus 1X70 inverted fluorescence microscope fitted with an Olympus QColor3 camera, using and QCpture software (Olympus 1X70; Olympus America, Inc., Melville, NY). The exposure time on the DsRed images was 5 seconds. After Tdp1 -/- fibroblast clone W infected with LVTHM-DsRed was shown to express DsRed, these cells were retained and passaged as a separate cell line to be used as a positive control in future experiments.

The IRES-DsRed cassette was subcloned into pWPXL (Figure 3-3) from another plasmid, pLV-tTRKRAB-IRES-red, (also made by Didier Trono) by swapping with the GFP fragment. Unique BamHI and SpeI sites were created for insertion of the TDP1 cassette.
Figure 3-3. pWPXL plasmid used to create vector for infecting MEFs.
The LV-FLAGhTDP1-DsRed vector was designed in an attempt to take advantage of the internal ribosome entry site (IRES) to simultaneously express human TDP1 and (Discosoma sp. red fluorescent protein) DsRed protein. In this experimental design, cells that exhibited DsRed fluorescence would also express human TDP1, and these red cells could be selected using a MoFlow Modular Flow Cytometer and passaged as a new Tdp1-/- Complemented (TDP1 Comp) cell line. However, as measured using both traditional flow cytometry and fluorescent microscopy, there was no evidence that the Tdp1-/- MEFs infected with LV-FLAGhTDP1-DsRed vector exhibited more red fluorescence than uninfected cells, indicating that either neither TDP1 or DsRed was expressing, or that DsRed was not being expressed in amounts equal to TDP1. In these experiments, Tdp1-/- MEFs infected with LVTHM DsRed served as a positive control.

Tdp1-/- MEFs infected with LV-FLAGhTDP1-DsRed vector were then screened for expression of FLAG-tagged hTDP1 by western blotting (Figure 4-4C). Following demonstration that the cells did express a FLAG-tagged protein of the approximate molecular weight of hTDP1, this initial mix of cells was expanded and passaged as a separate cell line, TDP1 Comp, from which a whole-cell extract was subsequently isolated and screened for TDP1 activity, as described below. From the heterogeneous mix of complemented cells, 12 individual clones were isolated, expanded, and screened by PCR using primers designed to amplify the leader sequence of the lentiviral vector which is part of LV-FLAGhTDP1-Red: 5′-GGAGCTAGAAGATTCGAGTTATGTTAGCTGTCCAGTATTTGTC-3′ and 5′-GGTTGTAGCTGTCCAGTATTTGTC-3′ (Figure 4-7, Table 4-2). For this experiment, an alternate PCR 2X reaction mix (2X SYBR Green RT PCR reaction mix, BioRad, cat no. 170-8893) was used rather than the 2X Mix used in subsequent
experiments (SYBR Green PCR Master Mix, Applied Biosystems, Part No. 4309155). From these 12 clones, 5 were selected and lysed in Electrophoretic Mobility Shift Assay (EMSA) buffer (10mM HEPES pH 7.5, 60 mM KCl, 1 mM EDTA pH8, 0.5% Nonidet P40), a non-ionic detergent used for lysing cells, while simultaneously allowing the cells’ contents to remain in their native state. The 5 selected clones (arbitrarily assigned numbers 7, 11, 13, 18, and 21) were expanded on 150 mm plastic plates, scraped with a pivoting blade cell scraper into 1 mL PBS, pelleted at 1500xg at 25°C for 3 min, then resuspended and vortexed in 120 uL EMSA lysis buffer. These 5 clone lysates were then screened with Tdp1+/+, Tdp1-/-, TDP1 Comp whole cell extracts according to the protocol described below, Analysis of End-Processing Radiolabeled DNA Substrates. Three 20-fold dilutions were made in the usual extract dilution buffer and were assayed for a wide range of activity (Figure 4-8).

3.7 Preparation of tyrosyl and 3′ PG DNA substrates

In repair studies that employ mammalian cell extracts, repair is often very inefficient, with only a few percent or less of the substrate being processed, due in part to potent 3′-5′-exonuclease activities (Chen et al, 2001a, Daza et al, 1996). As such, it is of particular importance that the repair substrate is well-defined, otherwise, any observed processing and repair may be with an unintentional substrate that may yield better signal than the intended substrate.

The 3′-PG-terminated 17-mer 5′-CGAGGAACGCGAAAACG-3′ was prepared by bleomycin-mediated cleavage of 5′-CGAGGAACGCGAAAACGCCC-3′,
purified by polyacrylamide gel electrophoresis and HPLC (Chen et al, 2001a, Povirk et al, 2007b).

The 3′-PG oligomers and a 3′-pTyr 18-mer (obtained from Midland Certified Reagents) and unmodified marker oligomers were 5′-\(^{32}\)P labeled with 150 µCi, or 25 pmoles [\(\gamma-^{32}\)P]ATP (Perkin Elmer) and T4 polynucleotide kinase in 10X PNK buffer (New England Biolabs) for 1 h at 37ºC. T4 Polynucleotide Kinase (PNK) catalyzes the transfer of the terminal phosphate of ATP to 5' hydroxyl termini of polynucleotides such as DNA and RNA, oligonucleotides and 3' mononucleotides. This enzyme is commonly used to label DNA or RNA with \(^{32}\)P or \(^{33}\)P at 5' ends. Unincorporated label was removed by loading the sample with 40 mM EDTA in formamide on a denaturing 20% polyacrylamide (19:1 acrylamide:bisacrylamide) gel (the preparation of which is described in detail below) and letting the unincorporated [\(\gamma-^{32}\)P]ATP elute off of the gel and into the running buffer. Electrophoresis was for 4 h at 40 W. Gels were exposed to X-ray film for 1–5 min and bands containing labeled products were excised from the gel. Gel slices (~5 x 0.5 cm) were eluted with agitation at 22°C for 16 h in 18-mm-diameter glass culture tubes containing sufficient 1 mM EDTA, pH 8, to suspend the gel slice.

Labeled oligos were then annealed to complementary unlabeled oligos to create SSB substrates (see Figures 4-3F, 4-11A) (Suh et al, 1997). The double-stranded oligos were then examined on non-denaturing 20% polyacrylamide gels in order to determine that subsequently observed processing would indeed be from a double-stranded, rather than single-stranded substrate, as this particular property of a substrate does to a large extent determine the extent of its accessibility and processing.
A plasmid substrate containing a DNA double-strand break (DSB) with PG-terminated ACG 3’ overhang was constructed as described (Chen et al, 2001b). The substrate containing a DNA DSB with one 3’-PG-terminated blunt end and one recessed 3’-PG end (Gu et al, 1996) was constructed similarly (see Figure 4-12).

### 3.8 Site-directed mutagenesis and enzyme purification

These methods were performed by Dr. Konstantin Akopiants, PhD, in the laboratory of Dr. Lawrence Povirk. A pET plasmid expressing His-tagged human TDP1 was kindly provided by Howard Nash, NIMH. Mutants were generated using the QuickChange site directed-mutagenesis kit from Stratagene and primers selected by the QuickChange Primer Design Program. Wild-type or mutant plasmids were freshly transformed into *E. coli* BL21(DE3) strain and cells were grown in 1 L LB medium supplemented with 100 µg/ml ampicillin. When the OD$_{600}$ reached 0.4-0.5, 1 mM IPTG was added for induction. After 4 h, cells were harvested and dry pellets were stored at –80°C.

Cell pellets were re-suspended in 12 ml lysis buffer (50 mM Na$_2$HPO$_4$, pH 8.0, 0.3 M NaCl, 10 mM imidazole, 1 mg/ml lysozyme, 1 mM phenylmethylsulphonyl fluoride). After 30 min on ice, cells were sonicated 6x15s using a sonicator on 30% power from Ultrasonic Inc. Crude extracts were centrifuged at 14000xg at 4°C for 30 min. Soluble cell proteins were mixed with 1.5 ml Ni-NTA Superflow (Qiagen) and gently stirred 1h at 4°C. The suspension was then added to a chromatography column and washed with 25 ml of wash buffer (50 mM Na$_2$HPO$_4$, pH 8.0, 0.3 M NaCl, 20 mM imidazole 1 mM phenylmethylsulphonyl fluoride). Bound proteins were then eluted four
times with the same buffer containing 0.5 M imidazole. All four fractions were analyzed by SDS-PAGE electrophoresis and peak fractions were dialyzed against 2 L 20 mM Tris-HCl pH 8.0 overnight, passed through a 0.22 µm filter, loaded on a Pharmacia MonoQ FPLC column at 22°C and eluted with a 15-min gradient of 0-0.8 M NaCl in 20 mM Tris-HCl pH 8.0. Fractions were collected and analyzed by SDS-PAGE. Protein concentration was determined using a Pierce bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc.). Proteins were stored in 50% glycerol at -20°C.

3.9 Analysis of DNA end processing and repair

Organ Homogenates

Organ homogenates were prepared by sacrificing mice of each genotype and dissecting the liver and brain, which were then flash frozen. Tissues were thawed and minced, then resuspended in lysis buffer (10 mM Hepes pH 7.8, 60 mM KCl, 1 mM EDTA, 0.5% NP 40, 0.5 mM DTT, 100 mM PMSF, and Sigma protease and phosphatase inhibitor cocktails (catalogue numbers P8340 and P5726)) and homogenized with a Polytron tissue homogenizer (Kinematica).

Whole-cell extracts from fibroblast cell lines

Extracts of MEFs were prepared according to procedures described previously (Baumann & West, 1998). Fibroblasts were expanded and grown to confluency on 20 15-cm dishes and allowed to remain confluent for 1-2 days. Cells were harvested by washing 1X with 20 mL room temperature PBS, and then incubated with 3 mL room temperature 0.25% trypsin-EDTA until cells were detached. Cells were collected with serum-containing medium to inactivate the trypsin, and pelleted by centrifugation (1500 rpm, 5
min) in 50-mL tubes. Cell pellets were resuspended in 4°C PBS, and pelleted by centrifugation (1500 rpm, 5 min, 4°C). The cell pellet was resuspended in 4°C PBS and pelleted again in 15-mL tubes to further remove traces of trypsin-EDTA and growth medium. The approximate volume of the packed cell pellet was recorded, and typically was between 1-2 mL for fibroblast cell lines. The packed cell pellet was then resuspended in 5 x the volume of the packed cell pellet in 4°C hypotonic buffer with protease inhibitors (10 mM Tris-HCl pH 8, 1 mM EDTA, 5 mM DTT, 1 ug/mL Pepstatin, 1 ug/mL Chymostatin, 2 ug/mL Aprotinin, 1 ug/mL Leupeptin, 1 mM PMSF). This should cause the cells to swell, but remain intact. The 5x hypotonic buffer: 1x cell pellet slurry was quickly pelleted, and the cells resuspended in 2 x the volume of the original packed cell pellet of hypotonic buffer and allowed to remain at 4°C for 20 min. Cells were subjected to mechanical shearing by the use of a Dounce homogenizer: the swollen cell/hypotonic buffer slurry was homogenized with 20 pestle strokes, and then allowed to sit on ice for an additional 20 min. Then ¼ of the current volume of cell lysate /hypotonic buffer slurry was added of 5X hypertonic buffer (83.5 mM Tris pH 7.5, 1.65 M KCl, 3.3 mM EDTA, 1 mM DTT). This solution was aliquoted into Beckman polycarbonate thick-walled centrifuge tubes (cat. no. 349622) and matched for their mass before being inserted into a pre-chilled SW Ti55 centrifuge rotor (Beckman). Samples were centrifuged for 3h, 4°C, 42,000 rpm in an Optima L-80 Ultracentrifuge (Beckman Coulter). Once removed from the Ultracentrifuge, multiple fractions of homogenate would be visible; a 22G needle would be used to pass through the top fraction into the middle cytosolic fraction, which was removed for dialysis. Sample was placed into tubing and securely tied off; the dialysis tubing was then placed into 0.5 L storage buffer (20 mM Tris pH 8, 0.1 M KOAc, 1 mM DTT, 0.5 mM...
EDTA, 20% glycerol, 1 ug/mL Pepstatin, 1 ug/mL Chymostatin, 2 ug/mL Aprotinin, 1 ug/mL Leupeptin, 1 mM PMSF), while stirring for 3h at 4ºC. Extract was removed from dialysis tubing, aliquoted in Eppendorf Safe-lock tubes, flash-frozen with liquid N\textsubscript{2}, and stored at -80ºC.

Protein concentrations of both tissue lysates and cell extracts were assessed with a Pierce BCA Assay. The BCA Assay is a colorimetric protein assay and is particularly useful because it is compatible with various detergents that may be found in either whole cell extracts or detergent lysates (Thermo Fisher Scientific Inc.). The appearance of a purple-colored reaction product is dependent on two separate reactions: the reduction of Cu\textsuperscript{2+} to Cu\textsuperscript{1+} by protein (either in an experimental sample or in the supplied bovine serum albumin (BSA) standard) in an alkaline medium, followed by the reaction of two molecules of BCA with the reduced Cu\textsuperscript{1+} ion. After allowing known concentrations of BSA standards and experimental samples to react with the BCA reagents for 30 min, the BCA/copper complex can then be detected with a Perkin Elmer UV/VIS Lambda 25 Spectrometer with absorbance set at 562 nm. By plotting the concentrations of the known standards, concentrations of experimental samples can be calculated via linear regression (Figure 3-4).

Analysis of End-Processing Radiolabeled DNA Substrates

Organ homogenates were diluted in buffer containing 50 mM Tris pH 8.0, 5 mM DTT, 100 mM NaCl, 5 mM EDTA, 10% glycerol, and 500 µg/mL BSA. A 0.5 µL aliquot of each dilution was allowed to react with 50 fmol of a radioactive 3′-pTyr oligomer substrate (see Figure 4-3A), synthesized by Midland Certified Reagent Co. by a procedure described previously (Van Houten et al, 1998), in 5 µL of reaction buffer.
Figure 3-4. Protein concentrations calculated from Pierce BCA protein assay. Protein concentrations of both tissue lysates and cell extracts were assessed with a Pierce BCA Assay. Known concentrations of BSA standards and unknown concentrations of experimental samples react with BCA reagents for 30 min, then the BCA/copper complex indicative of the presence of protein can be linearly detected with a spectrometer, absorbance = 562 nm. By plotting the concentrations of the known standards, concentrations of experimental samples (shown in blue) can then be calculated via linear regression. The graph and table display an example of a single BCA assay for two whole cell extracts of unknown concentration: Tdp1-/- clone W, and Tdp1+/+ MEFs.
containing 50 mM triethanolamine pH 7.5, 1 mM DTT, 5 mM EDTA, 100 µM dNTPs, and 1 mM ATP. Following incubation at 37°C for 1 h, samples were diluted in an equal volume of formamide containing 20 mM EDTA and denatured at 90°C for 5 min. For determining activity toward a pTyr nick substrate (see Figure 4-3F), whole-cell extracts were serially diluted and incubated with 50 fmol of the substrate in the same reaction buffer described above, with the exception of 10 mM Mg(OAc)₂ substituting for 5 mM EDTA. Typically, 3 - 5 µL of extract was added to a total reaction volume of 5 -10 µL, for 1 h unless otherwise noted. In some cases, serial dilutions of FLAG-tagged TDP1 (WT TDP1 or various mutant TDP1s) purified from 293T cells was added to Tdp1⁻/⁻ extracts (Zhou et al, 2005). To test the temporal requirements for pTyr processing, 50 ng of human TDP1 produced in E. coli was added to Tdp1⁻/⁻ extracts in 5 µL reaction buffer and incubated with the radiolabeled substrate from 1 – 60 min, as indicated (Figure 4-9).

To assess activity toward PG nick and gap substrates, 20 µg of Tdp1⁺/+ and Tdp1⁻/⁻ whole cell extract was incubated with 50 fmol of either substrate in 5 µL of reaction buffer containing 50 mM triethanolamine pH 7.5, 1 mM DTT, 100 µM dNTPs, and 1 mM ATP, and either 5 mM EDTA, 2, or 10 mM Mg(OAc)₂. For the DSB substrates, extracts were incubated in 32 µL reaction buffer containing 50 mM triethanolamine pH 7.5, 1 mM DTT, 100 µM dNTPs, and 2 mM ATP, and 1.3 mM Mg(OAc)₂ with substrate for 6 h, unless otherwise noted. Some Tdp1⁻/⁻ extracts were supplemented with 25 or 125 ng of wildtype, SCAN1, or H493N TDP1 protein purified from E. coli as noted. Samples were deproteinized with proteinase K, extracted with phenol and chloroform, nucleic acids were precipitated, and then treated with BstXI and TaqI (Povirk et al, 2007a).

Polyacrylamide Gel Preparation
In the case of testing any form of radiolabeled DNA substrate, samples were analyzed on denaturing 20% polyacrylamide gels run at 40-60 W. Both sizes of these polyacrylamide gels (18 lanes and 32 lanes) were assembled in the lab. These composition of these gels is as follows: 40 mL 40% liquid acrylamide (19:1 acrylamide: methylene bisacrylamide), 40 g urea, 8 mL 10X Tris/borate/EDTA (TBE) buffer, and after these components dissolve and the apparatus is ready for the gel solution to be poured and polymerize, 0.1 g ammonium persulfate (APS), and 45uL Tetramethylethylenediamine (TEMED). When doubling the volume of the gel for the 32-lane format, the first three components of the gel are all doubled, and 70 uL TEMED is used. Plates are washed vigorously with hot tap water and Sunlight lemon dish soap until the sponge squeaks on the glass surface and then immediately rinsed with 100% ethanol. To facilitate dissolving of the urea, the beaker containing the urea-acrylamide slurry was nested into a second large beaker (1L) containing hot tap water, resulting in a room-temperature solution of acrylamide. To prevent the assembly of glass plates and spacers from leaking, the bottom of the gel was sealed with one layer of cloth duct tape (3M), which was secured with a second overlapping strip of tape encircling the bottom end of the plates. The side edges were also sealed and then large 2” binder clips were placed along both edges, directly over the center of the spacers and duct tape seal. Clamping the bottom of the glass plates results in bowing of the plates and uneven migration rates across the gel, and is to be avoided.

When pouring acrylamide gels, it is imperative to avoid bubbles that may form due in part to imperfections or impurities on the glass plates, a solution which is polymerizing too quickly, or pouring at an unsteady pace. When pouring these large gels
it is helpful to keep in mind the advice of Julia Child on the flipping of large pancakes, which is directly transferable to pouring large acrylamide gels: “When you flip anything, you just have to have the courage of your conviction,” and too, “The only way you learn to flip things is just to flip them!” Immediately after pouring, insert the well-comb of appropriate width and thickness, and clamp into place using woodworking clamps, placing the clamp directly on the teeth of the comb. If any bubbles have arising over the course of pouring the gel, at this time one may attempt to tap them loose and allow them to rise out of the gel. The gel should take 30 min to 1 h to polymerize. While polymerizing, samples can be thawed and denatured for 5 min at 90°C. Gels were run in 1X TBE buffer for 4 h at 40 W (18 lanes) and 6 h at 60W (32 lanes). After running, glass plates were pried apart, and wet gels were covered with polyethylene film, frozen and clipped to PhosphorImager screens for 1-2 days exposure at -20 °C. Phosphor images were developed with the Typhoon 9410 (General Electric) and analyzed with ImageQuant 3.3 software (Molecular Dynamics).

3.10 Western blotting

Extracts used in the DNA repair assays were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels consisted of two layers: a top, stacking gel layer and a bottom, resolving gel layer. The 15 mL resolving gel was poured first and was composed of 0.375M Tris, pH 8.8, 0.1% SDS, 0.1% APS, 20 uL TEMED, and 10-15% 30:1 acrylamide: methylene bisacrylamide, the percentage of which was dependent on the size of the proteins to be separated. The ratio of acrylamide to methylene bisacrylamide varies depending on the intended pore size of the sieve of the
polymerized gel matrix; pores are larger for the separation of proteins than for DNA. The 8 mL stacking gel was poured after the resolving gel polymerized and was composed of 0.375 M Tris, pH 6.8, 0.1% SDS, 0.1% APS, 10 uL TEMED, and 4% 30:1 acrylamide: methylene bisacrylamide. Both the different pH of the stacking gel and the SDS running buffer, and the glycine content of the running buffer used in the chambers of the electrophoresis system allow for a charge boundary to form. This concentrates the proteins into a tight band prior to entering the resolving gel (Hockfield et al, 1993).

Cell extracts were diluted 1:1 in Laemmli Sample Buffer (4% SDS, 20% glycerol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH ~ 6.8) (Biorad, cat no. 161-0737) 10% β-mercaptoethanol (Laemmli, 1970), boiled for 10 min, and centrifuged for 15 min at 15,000g to pellet cell membranes. The SDS in both the gel and the sample buffer acts as a negatively-charged denaturing detergent that binds to proteins in proportion to their mass. When boiled, a protein would normally precipitate, but the presence of the SDS with β-mercaptoethanol, a disulfide-reducing agent, causes protein to be denatured and coated with negative charge. This allows protein to remain in solution; the protein can then be separated by SDS-PAGE solely dependent on the basis of molecular mass. Cell extracts were loaded in gel wells in serial dilutions to ensure that antibodies had a range of protein concentrations for linear detection. A prestained protein marker (BioRad, cat no. 161-0374) was also loaded and used to visually monitor the separation of proteins during electrophoresis. Gels were electrophoresed for 30 min at 110V, by which time proteins had entered the resolving gel. The voltage was then increased to 140V and electrophoresed for an additional ~ 2h, until the dye front had completely migrated off the gel.
Gels were transferred to polyvinylidene difluoride (PVDF) membranes for 2h at 0.6 amps, at room temperature. Presence of the prestained protein marker on the membrane was noted as an indicator of a successful transfer. Membranes were incubated with 0.1% casein in PBS for 1h at room temperature, with shaking, to block non-specific antibody interactions. Membranes were exposed to primary antibody diluted in 0.1% casein in PBS, overnight, at 4ºC, with shaking. Primary antibodies included anti-FLAG M antibody (Sigma, mouse monoclonal, 1:1000), anti-human TDP1 (AbNova, mouse polyclonal; cat no. H00055775-A01; 1:2500), anti-COX4 (Cell Signaling, rabbit polyclonal, cat no. 4844; 1:1000), or anti-β-actin (Santa Cruz Biotechnologies, goat polyclonal, cat no. sc-1615; 1:1000). Membranes were washed for 5 x 20 min in PBS with 0.02% Tween 20 (PBST) at room temperature. Membranes were incubated with secondary antibodies conjugated to infrared-emitting fluorophores diluted 1:5000 in 0.1% casein in PBS, for 90 min, with shaking, at room temperature. Secondary antibodies included anti-mouse 680 Alexa (Invitrogen), and anti-goat IRDYE 800 (Rockland Immunochemicals). After washing membranes 3 x 20 min with PBST, specific protein bands were detected using the Odyssey infrared imaging system (LI-COR Biosciences). Both primary and secondary antibody solutions in 0.1% casein in PBS were saved at 4ºC and typically reused 3 times prior to discarding.

In the process of attempting to establish which of the commercially-available antibodies would be an effective and specific antibody against TDP1 that would also detect mouse Tdp1, multiple TDP1 antibodies were screened and found to be inadequate. These included Abcam rabbit polyclonal anti-human TDP1 (cat no. ab4166, used at the recommended dilution 1:1000), TopoGEN rabbit anti-human TDP1 (cat no. 2016, used at
the recommended dilution 1:2500), Santa Cruz Biotechnologies goat polyclonal TDP1 C-15 (cat no. sc-26040, used at the recommended dilution 1:1000), Santa Cruz rabbit polyclonal TDP1 H-300 (cat no. sc-32924, used at the recommended dilution 1:1000), and a TDP1 antibody generously donated by Howard Nash (rabbit polyclonal, used at the recommended dilution 1:1000). Personal communication between myself and Sachin Katyal (Katyal et al, 2007) in May 2009 confirmed that multiple commercially-available antibodies against TDP1 are inadequate for research purposes.

3.11 Drug and radiation hypersensitization assays

Alamar Blue Assay for Cell Growth and Viability

Initially, MEFs that had been in culture for ~90–130 days (referred to as “early-passage MEFs”) were screened for either radiation or chemotherapeutic drug hypersensitivity using 96-well plate assays that exploit differences in cell growth as an endpoint that can be measured using Alamar Blue (AbD Serotec, cat no. BUF012B), a non-toxic fluorogenic redox indicator (Ahmed et al, 1994, Fields & Lancaster, 1993, Nakayama et al, 1997). The oxidized form of Alamar Blue is a dark blue color with little intrinsic fluorescence. When taken into metabolically active cells, the dye becomes reduced and turns red. This reduced form of Alamar Blue is highly fluorescent. The extent of this conversion is a reflection of cell viability and can be quantified by either optical density or fluorescence.

MEFs of all three genotypes were plated in serial dilutions, in quadruplicate, on 96-well plates, the last row remaining free of cells. For radiation testing, all three MEF genotypes were plated in quadruplicate on the same plate, and 18 h post-seeding,
different plates were delivered 0, 1, 2, 5, or 10 Gy radiation using a $^{60}$Co irradiator or MDS Nordion Gammacell 40 (ON, Canada) research irradiator with a Cs-137 source delivering a dose of 1.05 Gy/min. For drug hypersensitivity testing, MEFs were plated in triplicate as shown (Figure 3-5) and 18 h post-seeding, wells were given varying doses of drug in cell media. CPT (camptothecin) (Sigma, cat no. C9911), a drug known to target topoisomerase I complexes (Hsiang et al, 1985), was dissolved in DMSO at 0, 0.01, 0.1, or 1 µM, and calicheamicin, a drug that induces DSBs terminating in 3'-PG, was dissolved in 50% ethanol. After 1 h drug exposure, cells were washed with media and allowed to remain on the plate. Approximately 10 days post-irradiation or drug treatment, cell proliferation was assessed by the addition of Alamar Blue in a 1:4 dilution in cell growth media. The Alamar Blue media solution incubated on the cells for 3–4 h prior to reading with a FluoroCount with Filter Wheels plate reader (Packard Instruments) at $\lambda_{ex}=530$ nm and $\lambda_{em}=590$ nm. Gains typically ranged between 5-7, and were automatically determined for each experiment using the PlateReader 3.0 for Windows software, after assessing which well was emitting the maximum relative fluorescent units (RFU).

**Colony-Forming Assay for Clonogenic Survival**

MEFs exhibiting logarithmic growth were trypsinized to single-cell suspension and counted using a hemocytometer. Serial dilutions of known concentrations of cells were made in sterile conical tubes prior to addition to 100 mm petri dishes. After the plating efficiency of each genotype of MEFs was ascertained, for subsequent experiments cells were plated such that untreated plates would contain ~ 100 colonies. To ensure
Figure 3-5. Alamar Blue Assays to test drug sensitivity in MEFs. MEFs in culture for ~90–130 days (referred to as “early-passage MEFs”) were screened for either radiation or chemotherapeutic drug hypersensitivity using 96-well plate assays. MEFs were plated in serial dilutions, in triplicate, and treated with indicated concentrations of camptothecin (CPT) or calicheamicin.
repeatability in plating efficiencies, MEF clones were expanded and aliquots of known cell number frozen in 5% DMSO at -80°C. A fresh aliquot of cells was thawed for each independent experiment. For drug-treated plates, multiple cell doses for each assay were selected in anticipation that a sufficient number of cells would survive to achieve a convenient number for counting, which for the MEF clones achieved maximum at about 250 colonies/100 mm dish. A large number of colonies either significantly overlap or become confluent and thus cannot be accurately counted.

Cells were allowed to attach to the dish for ~12-16 h prior to drug treatment. Cells were incubated with 1, 3, or 10 µg/mL bleomycin-containing (LKB) media for 4h, though the 10 µg/mL dose was eliminated from repeated experiments due to high cytotoxicity. The medium was changed to non-drug containing medium and the cells were allowed to incubate an additional 10-12 days. Cells were fixed in 3.7% formaldehyde and stained with crystal violet. Stained colonies were visualized with a light box (Laboratory Supplies Company, Hicksville, NY). Colonies larger than 25 cells were counted by eye.

Confocal Microscopy and Immunohistochemistry

MEFs were seeded onto CC2-treated sterile chamber slides (cat no. 154917, LAB-TEK, Nalge Nunc International, Naperville, IL) and allowed to attach overnight. For experiments testing the DSB repair response to radiation, chamber slides were then irradiated using either a ⁶⁰Co irradiator or a MDS Nordion Gammacell 40 research irradiator at doses of 0, 2, or 10 Gy and fixed 30 min, 2h, or 24h later with 3% Paraformaldehyde for 15 min. Cells were then permeabilized by incubation with 0.5% Triton X-100 for 10 min, then incubated with a solution of 10% non-fat dry milk/0.1%
goat serum/PBS for 2h at room temperature, while rocking, prior to exposure to antibodies to block non-specific antibody interactions. Slides were then immunostained using anti-γH2AX antibody (specific for phosphorylation on serine 139, mouse monoclonal antibody, Upstate, cat no. 05-636) at a 1:500 dilution in 10% non-fat dry milk/0.1% goat serum/PBS overnight, at 4ºC, with shaking. For TDP1 cellular localization studies, AbNova anti-human TDP1 was used at a dilution of 1:1000. Slides were subsequently washed 5 x 20 min in PBS with at room temperature, then stained with Alexa Fluor 546 anti-mouse IgG Fab fragment antibody (Molecular Probes) at 1:500 dilution for 3 h at room temperature, protected from light, with shaking. After 2 x 5 min washes with PBS, the chamber slide hardware was removed using the tools provided with the slides, and the slides allowed to dry for ~ 5 min. 15 uL of Vectashield Mounting Media with 4’,6-diamidino-2-phenylindole (DAPI) (1.5 µg/mL) was applied to each well, and coverslips were adhered to the slides using clear nail polish (Maybelline). Samples were imaged using the 63X or 100X objective of a Zeiss LSM 510 Meta confocal microscope imaging system in the Massey Cancer Center Flow Cytometry and Imaging Core Facility. The Core Facility is supported in part by NIH Grant P30 CA16059.

3.12 Statistics

Unpaired two-tailed t tests were done on triplicate (or greater multiples) data sets using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA). P values are indicated as follows: *, <0.05; **, <0.01; ***, <0.001. All error bars depict standard error (SE) for triplicate (or greater multiples) data sets.
IV. RESULTS FOR PART I

4.1 Generation of Tdp1-deficient mice

To assess the function of Tdp1 in vivo and to provide a source of Tdp1-deficient cell lines, we disrupted the endogenous mouse Tdp1 gene in ES cells by deleting exons 6 and 7, which also causes a frame-shift in translation of sequences encoded in the downstream exons (Figure 4-1). The Tdp1 protein contains two HKD sequence motifs, encoded in exons 6-7 and 13-14, which together comprise the single active site of the enzyme (Davies et al, 2002a). Thus, the predicted truncated protein product of the targeted allele, which should contain only the first 253 amino acids of Tdp1 plus 10 out-of-frame amino acids, completely lacks the catalytic site. Our initial targeting vector also allowed for the generation of conditional Tdp1 knockout mice in which a loxP site has been inserted into both introns 5 and 7 (Figure 3-1, panel v), allowing for cell type-specific deletion of exons 6 and 7 upon interbreeding to lines of mice expressing Cre recombinase in specific tissues. However, only the conventional knockout mice (hereafter referred to as Tdp1^-/- mice) were used in the studies described below. Tdp1^-/- mice were born in the expected Mendelian ratio upon interbreeding of Tdp1^+/- mice, indicating that Tdp1 deficiency does not result in embryonic or neonatal lethality. In addition, Tdp1^-/- mice were fertile and had a normal lifespan. Reverse transcriptase-real-time PCR confirmed the presence of Tdp1 mRNA encoding exons 3-5 and exons 13-14, but not exons 6-7, in brain and liver tissue from Tdp1^-/- mice (Figure 4-1).
Figure 4-1. *Tdp1<sup>−/−</sup>* mice produce aberrant transcript. (A) TDP1 transcript levels were assessed by real-time PCR using cDNA reverse transcribed from RNA isolated from *Tdp1<sup>+/+</sup>* and *Tdp1<sup>−/−</sup>* brain and liver. The transcript encoding the first HKD motif (exons 6-8) is absent in *Tdp1<sup>−/−</sup>* tissues. Transcripts encoding exons 2-3 and 12-14 were found to be reduced by approximately half in the *Tdp1<sup>−/−</sup>* tissues compared to their wildtype littermates based on real-time data (see Table 4-1). The 7% acrylamide gel above depicts products from the real-time PCR reaction, confirming the size of the respective amplicons. Continued on the following page.
**Figure 4-1.** *Tdp1*+/− mice produce aberrant transcript. (B) Real-Time PCR amplification curves from TDP1 transcripts encoding exons 2-3 and are reduced by approximately half in the *Tdp1*−/− tissues compared to their wildtype littermates. β-actin transcript was amplified as a control and is also depicted above. This graph was created using SDS 2.2.2 software for Real-Time PCR data analysis. The threshold in this analysis was set to 0.1, approximately halfway through the logarithmic amplification of all samples tested.
Table 4-1. *Tdp1*<sup>−/−</sup> mice produce aberrant transcript. Transcripts encoding exons 2-3 and 12-14 were found to be reduced by approximately half in the *Tdp1*<sup>−/−</sup> tissues compared to their wildtype littermates. No transcript was detected from exons 6-8 in *Tdp1*<sup>−/−</sup> tissues. β-actin transcript was amplified as a control and is also depicted above. All Relative Quantity (RQ) values above are measured relative to the *Tdp1*<sup>+/+</sup> liver sample, which was set equal to 1. RQ values were derived from the CT values using SDS 2.2.2. CT values indicate the cycle number at which the fluorescence amplification curves crossed a set threshold (in this data set = 0.1) of fluorescence.

<table>
<thead>
<tr>
<th>sample</th>
<th>Tdp1 exon 2/3</th>
<th>Tdp1 exon 6-8</th>
<th>Tdp1 exon 12-14</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tdp1</em>&lt;sup&gt;+/+&lt;/sup&gt; brain</td>
<td>27.13</td>
<td>7.35</td>
<td>26.41</td>
<td>10.8</td>
</tr>
<tr>
<td><em>Tdp1</em>&lt;sup&gt;−/−&lt;/sup&gt; brain</td>
<td>28.40</td>
<td>3.26</td>
<td>not detected</td>
<td>27.26</td>
</tr>
<tr>
<td><em>Tdp1</em>&lt;sup&gt;+/+&lt;/sup&gt; liver</td>
<td>32.53</td>
<td>1</td>
<td>32.36</td>
<td>1</td>
</tr>
<tr>
<td><em>Tdp1</em>&lt;sup&gt;−/−&lt;/sup&gt; liver</td>
<td>33.42</td>
<td>0.4</td>
<td>not detected</td>
<td>31.38</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>not detected</td>
<td>not detected</td>
<td>not detected</td>
<td>not detected</td>
</tr>
</tbody>
</table>
4.2 *Tdp1*<sup>−/−</sup> mice are phenotypically and behaviorally indistinguishable from *Tdp1*<sup>+/+</sup> littermates

Visual inspection of the mice did not reveal any notable differences between genotypes. At each age, average weights were similar across genotype for each sex, as were average body temperature and reflex sensitivity to a thermal stimulus (Table 4-2). Similarly, phenotypic differences were not seen in average stride length or in any measure within the five domains of the functional observation battery, including CNS excitability, CNS activity, muscle tone/equilibrium, autonomic effects or sensorimotor effects (data not shown). Figure 4-2 shows motor activity and latency to fall from the rotarod in male and female mice of each genotype and at each age. Statistical analyses of these data revealed that *Tdp1*<sup>−/−</sup> female mice (but not male mice) were significantly less active than *Tdp1*<sup>+/+</sup> mice at the 3-month assessment [F(2,12)=4.1, p<0.05]. However, this difference was transient, as a difference in motor activity across genotype was not observed at later assessment times. In addition, although rotarod performance declined markedly with age, rotarod latencies were not significantly different across genotype at any age in either sex. Microscopic examination of brains sections did not reveal any overt differences between mice of any genotype. In particular, both granule cells and Purkinje cells in the cerebellum were present in equal numbers and appeared morphologically similar in brains of wild-type and *Tdp1* knockout mice, both at 12 and 22 months of age (not shown). Altogether, under normal conditions, *Tdp1*<sup>−/−</sup> mice had no detectable behavioral phenotype.
Table 4-2. Phenotypic measurements of Tdp1^/- mice. Body weight, temperature and pain sensitivity in male and female mice of each genotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Weight (g)</th>
<th>Temperature (°C)</th>
<th>Tail flick latency (s)</th>
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</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype</td>
<td>28 (1.4)</td>
<td>38.6 (0.27)</td>
<td>7.4 (1.1)</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDP1^/-</td>
<td>29 (2.0)</td>
<td>38.7 (0.28)</td>
<td>6.7 (1.1)</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDP1^+/-</td>
<td>30 (1.7)</td>
<td>38.2 (0.30)</td>
<td>6.7 (0.7)</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype</td>
<td>22 (1.5)</td>
<td>38.1 (0.15)</td>
<td>5.0 (1.1)</td>
</tr>
<tr>
<td>(n=5)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TDP1^/-</td>
<td>24 (0.7)</td>
<td>38.3 (0.15)</td>
<td>7.4 (1.1)</td>
</tr>
<tr>
<td>(n=8)</td>
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<td></td>
<td></td>
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<tr>
<td>TDP1^+/-</td>
<td>25 (1.1)</td>
<td>38.4 (0.17)</td>
<td>7.2 (0.7)</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
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<td></td>
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<tr>
<td><strong>Age: 3 months</strong></td>
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<td></td>
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</tr>
<tr>
<td>Wildtype</td>
<td>34 (1.8)</td>
<td>38.4 (0.32)</td>
<td>6.2 (1.0)</td>
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<tr>
<td>(n=5)</td>
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<td></td>
</tr>
<tr>
<td>TDP1^/-</td>
<td>36 (2.2)</td>
<td>38.5 (0.16)</td>
<td>5.6 (0.3)</td>
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<tr>
<td>(n=6)</td>
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<tr>
<td>TDP1^+/-</td>
<td>38 (2.3)</td>
<td>38.2 (0.36)</td>
<td>7.1 (1.0)</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age: 6 months</strong></td>
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</tr>
<tr>
<td>Wildtype</td>
<td>34 (5.2)</td>
<td>38.1 (0.37)</td>
<td>5.4 (0.7)</td>
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<tr>
<td>(n=5)</td>
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<tr>
<td>TDP1^/-</td>
<td>30 (0.95)</td>
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<td>6.4 (0.8)</td>
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<tr>
<td>TDP1^+/-</td>
<td>34 (2.7)</td>
<td>37.9 (0.14)</td>
<td>5.4 (1.2)</td>
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<td>(n=5)</td>
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<td></td>
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<tr>
<td><strong>Age: 12 months</strong></td>
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<tr>
<td>Wildtype</td>
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<td>2.9 (0.4)</td>
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<td>(n=7)</td>
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<tr>
<td>TDP1^/-</td>
<td>42 (2.7)</td>
<td>38.3 (0.19)</td>
<td>2.8 (0.2)</td>
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<td>(n=5)</td>
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<tr>
<td>TDP1^+/-</td>
<td>41 (3.5)</td>
<td>38.2 (0.33)</td>
<td>4.3 (0.3)</td>
</tr>
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<td>(n=5)</td>
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<table>
<thead>
<tr>
<th>Genotype</th>
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<th>Temperature (°C)</th>
<th>Tail flick latency (s)</th>
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<td><strong>Age: 3 months</strong></td>
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<tr>
<td>Wildtype</td>
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<td>37.7 (0.16)</td>
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<tr>
<td>TDP1^+/-</td>
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<td>37.5 (0.21)</td>
<td>2.7 (0.5)</td>
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<tr>
<td>(n=7)</td>
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</table>
Figure 4-2. Behavior of Tdp1-/- mice. Motor activity and latency to fall from the rotarod was determined for male and female mice of each genotype and at each age: 3, 6, and 12 months in top, middle and bottom panels, respectively. Asterisk indicates that at the 3 month assessment, Tdp1-/- female mice were significantly less active than Tdp1+/-+ mice, p < 0.05.
4.3 Tdp1−/− cell extracts are deficient in processing 3′-pTyr linkages

To confirm that Tdp1 activity was abolished by disruption of the Tdp1 allele, Tdp1 enzymatic activity was measured in tissue homogenates from Tdp1−/− mice (Figure 4-3). Brain (Figure 4-3B) and liver (Figure 4-3C) tissue homogenates from a Tdp1+/+ and Tdp1+/− mouse efficiently converted a 3′-pTyr terminus on an 18 nucleotide oligomer (Figure 4-3A) to a 3′-phosphate, while liver and brain homogenates from a Tdp1−/− mouse failed to process the oligomers at any detectable level (quantified in Figure 4-3D, E). This 18-base 5′-labeled 3′-pTyr substrate mimics the covalent DNA-protein linkage that would be formed between a tyrosine in TOP1 and DNA at sites of torsional stress; these linkages must be resolved and effectively rejoined by DNA ligase to prevent these sites from converting to DSBs. All the above reactions were performed in the presence of 5 mM EDTA, which chelates magnesium, a required cofactor for PNKP activity, a magnesium-requiring enzyme that removes 3′ phosphate groups.

To assess 3′-pTyr processing in the context of SSB repair, the nicked duplex shown in Figure 4-3F was constructed and incubated in whole-cell extracts of MEFs derived from mice of each genotype. Accurate repair of this break would require cleaving the tyrosyl-DNA linkage, removing a phosphate group, and ligating the reformed 3′ hydroxyl group to the 5′ phosphate. Ligated repair products can be seen in samples treated with +/+ and +/- extracts, and these products display several distinct mobilities, possibly due to slight 3′ resection at the end of the duplex (Figure 4-3G). No such repair products were generated in −/− extracts; instead these samples showed only a single band corresponding to the unprocessed 3′-pTyr 18-mer. Reactions with the SSB substrate were performed in buffer containing 10 mM Mg++, sufficient for Apex1 (the
Figure 4-3. Tdp1<sup>±±</sup> mice and embryonic fibroblasts are deficient in 3' tyrosyl processing. A radiolabeled (*) 3'-pTyr oligomeric substrate was treated with tissue homogenates from a Tdp1<sup>+++</sup>, Tdp1<sup>++</sup>, or Tdp1<sup>-/-</sup> mouse for 1 h, subjected to denaturing gel electrophoresis, and phosphorimaged. (A) Diagram of 3'-pTyr 18-nucleotide oligomeric substrate. The substrate was treated with four-fold serial dilutions of brain (B), or five-fold serial dilutions of liver (C) homogenate from a Tdp1<sup>+++</sup>, Tdp1<sup>++</sup>, or Tdp1<sup>-/-</sup> mouse. The homogenate and substrate reactions were conducted in buffer containing EDTA. The percent conversion from the tyrosyl substrate to its phosphate product shown in (B) and (C) was quantified by densitometry and shown for brain (D) and (E) liver homogenates. Continued on the following page.
Figure 4-3. Tdp1<sup>−/−</sup> mice and embryonic fibroblasts are deficient in 3' tyrosyl processing. Whole cell extracts were incubated with a substrate mimicking a SSB with a 3'-pTyr end modification. (F) Diagram of 3'-pTyr SSB substrate; asterisk represents the radioactive 5' tag on the 3'-pTyr 18-mer. There is no gap between the tyrosyl modification and the adjoining 25-mer. The substrate was treated with (G) 40, 20, or 10 µg of whole-cell extracts from Tdp1<sup>+/+</sup>, Tdp1<sup>+/−</sup>, or Tdp1<sup>−/−</sup> MEF cell lines for 1 h in reaction buffer containing Mg<sup>2+</sup>. Processed and unprocessed forms of the substrate are indicated.
murine form of Ape1) and PNKP activity. Thus, the band migrating slightly faster than the untreated substrate in the +/- and +/+ reactions is likely a 3′-hydroxyl 18-mer, generated by the combined action of Tdp1 and PNKP.

To verify that the lack of processing in -/- MEFs was due to Tdp1 deficiency, the MEFs were stably transduced with a lentivirus expressing FLAG-tagged human TDP1, after attempts had been made to transfect the MEFs had proven unsuccessful (Figure 4-4). As expected, only cells infected with the FLAG-hTDP1 vector expressed FLAG at the expected molecular weight of TDP1; uninfected cells or cells infected with control viruses (LVTHM-DsRed, LV-IRES-DsRed) gave no signal (Figure 4-4A), though cells infected with control viruses did successfully express DsRed protein (Figure 4-4B). After identification of a more specific TDP1 antibody, TDP1−/− MEFs infected with LV-FLAGhTDP1-DsRed were shown to express TDP1 (Figure 4-4C). This same mix of FLAG-TDP1 infected cells (subsequently referred to as ‘TDP1comp’), were shown to overexpress TDP1 in comparison to MEFs of other genotypes (lanes ‘−/- comp’ in Figure 4-5A), if one assumes that the anti-TDP1 antibody used for immunoblotting detects mouse and human TDP1 with equal efficacy. Notably, there was no evidence of any expression of a full-length or truncated Tdp1 protein being expressed from the targeted knockout allele in -/- MEFs. The ectopic TDP1 expression rescued both 3′ processing and formation of repair products from the tyrosyl-modified SSB substrate (Figure 4-5B). Lower concentrations of the complemented cell extract (5 and 10 µg of extract in a 10 µL reaction volume) produced bands that migrated just above the pTyr; these are likely the result of displacement synthesis after removal of the pTyr from the SSB substrate. An additional experiment with a wider range of extract dilutions indicated that TDP1comp
cells not only overexpressed TDP1, but extract from TDP1\textsuperscript{comp} cells possessed 10X TDP1 activity compared to Tdp1\textsuperscript{+/+} or Tdp1\textsuperscript{+/-} MEF extracts (Figure 4-6).

Figure 4-4. Tdp1 -/- MEFs can be successfully transduced with lentiviral constructs. (A) Attempts were made to transfect Tdp1 -/- MEFs with a TDP1-FLAG construct. At the time of the attempted transfection, the MEFs had been kept continuously in culture for approximately 100 days. Lanes include (1) a positive control for an unrelated FLAG-tagged 50kDa protein. Lanes 2-6 contain whole cell lysates harvested from 10cm plates of Tdp1 -/- MEFs, 10% of the plate was loaded onto the gel. Tdp1 -/- MEFs were transfected with the following constructs: (2) WPXL-GFP, (3) WPI-lins 4, (4) WPI-NHEJ-DsRed, (5) WPI Flag UTGI-GFP, and (6) untransfected negative control. Lanes 7 – 9 contain whole cell lysates from simultaneously transfected 293T cells: 10% of a 6cm plate. 293T cells were transfected with the following constructs: (7) pcDNA3, (8) pcDNA3-hTDP1, and (9) WPI-TDP1. An intense exposure was taken of the membrane in an attempt to detect any low amounts of signal from TDP1-FLAG. Arrow indicates the TDP1 band visible at the expected size (68 kDa) in lanes 8, 9. Dual-Color Marker standards are as indicated. (B) Continued on the following page.
Figure 4-4. Tdp1 -/- MEFs can be successfully transduced with lentiviral constructs. (B) After Tdp1 -/- MEFs were cloned and expanded, clone W was plated at low density and transduced with the lentiviral construct WPXL-DsRed. After ~ 5 days post infection, clones were screened for incorporation of the lentivirus by inverted fluorescent microscope imaging. Red cells indicate expression from the WPXL-DsRed construct. (C) Continued on following page.
Figure 4-4. Tdp1 -/- MEFs can be successfully transduced with lentiviral constructs. (C) 20 and 40 µL (10% and 20% of whole cell lysates harvested from 15 cm plates) of each lentiviral-infected Tdp1 -/- clone W MEF lysate, harvested and identically prepared, were analyzed via Western blotting with anti-FLAG antibody. Lanes 1-8 contain whole cell lysates of Tdp1 -/- clone W MEFs transduced with the following constructs: (1, 2) uninfected, (3, 4) LVTHM-DsRed, (5, 6) LV-IRES-DsRed, (7, 8) LV-FLAGhTDP1-DsRed. Lane 9 contains a positive control for an unrelated FLAG-tagged 17kDa protein. Increasing lysate volumes are indicated with black triangles. Arrow indicates the TDP1 band visible at the expected size (68 kDa) in lanes 7, 8. Dual-Color Marker standards are as indicated.
Figure 4-5. *Tdp1*−/− MEFs can be genetically or biochemically complemented. (A) Presence of Tdp1 was determined in MEF lysates from all three Tdp1 genotypes, as well as MEFs stably complemented by LV-FLAGTDP1-Red transduction (“−/− comp”), by western blotting with anti-TDP1 antibody. The gel contains 15 and 30µL of each MEF lysate, and an identical membrane was loaded and probed with anti-β-actin as a loading control. (B) Continued on the following page.
Figure 4-5. *Tdp1*-/− MEFs can be genetically or biochemically complemented. The substrate was treated with (B) 40, 20, and 10 µg of whole cell extracts from *Tdp1*+/+ and *Tdp1*−/− cell lines, and 40, 20, 10, and 5 µg of whole cell extract from the complemented *Tdp1*−/− cell line for 1 h. Lanes 7–9 contain reactions with 40 µg of *Tdp1*−/− extract, and 5, 1, and 0.2 ng of affinity-purified FLAG-TDP1, respectively.
Figure 4-6. Complemented Tdp1-/- MEFs possess ~10X more activity against 3' tyrosyl end modifications than Tdp1+/+ or Tdp1+/− MEFs. The single strand 3’-pTyr substrate depicted in Figure 4-3A was incubated with five-fold serial dilutions of whole cell extracts from Tdp1++, Tdp1+-, and Tdp1-/- and Tdp1comp MEF cell lines. Reactions were treated for 1 h in buffer containing EDTA. Whole cell extracts were made after the cells had been kept continuously in culture for ~ 210-240 days. Densitometry was performed on the conversion of the 3’-pTyr band to the 3’ phosphate band and is shown in the graph above.
From the original mix of TDP1<sub>comp</sub> cells, 12 individual clones were isolated and screened by PCR using primers against the lentiviral leader sequence of the LV-FLAGhTDP1-Red vector. Of the 12 clones tested, several displayed a relatively high signal (clones 13 and 21), and several displayed a relatively low signal (clones 7, 11, and 18) when compared to the original heterogeneous mix of cells (Table 4-3 and Figure 4-7). Five of these clones were expanded and lysed for a 3′-pTyr processing activity assay, in which 4 of the 5 clones displayed activity at least equivalent to that of Tdp1<sup>+/+</sup> extract, and clone 18 displayed processing activity similar to that of Tdp1<sup>−/−</sup> extract (Figure 4-8).

Supplementing -/- MEF extracts with purified hTDP1 protein also effectively restored the ability to process and repair a 3′-pTyr SSB (Figure 4-5B, Figure 4-9). Quantitative analysis of a reaction time course for the supplemented extract showed that the hydroxyl intermediate appeared almost immediately, substantial repair product was visible within 10 min, and repair appeared complete at 60 min (Figure 4-9). It is notable that under these conditions, a low level of slow processing and conversion to the repair product occurred in the unsupplemented -/- MEF extracts. This small amount of processing was seen in two independent experiments (Figure 4-9, 4-10), with independent preparations of cell extracts, and may indicate an alternate, though less efficient, pathway of processing tyrosyl modifications in the context of SSBs. In summary, rescuing the TDP1 deficiency either by lentiviral infection or simply adding exogenous protein to cell lysates effectively restores TDP1-dependent 3′-pTyr processing.
Table 4-3. Tdp1\textsuperscript{+/-} MEFs can be successfully transduced with lentiviral constructs. To assess if individual clones derived from the mix of Tdp1\textsuperscript{+/-} clone W MEFs infected with LV-FLAGhTDP1-Red (TDP1 Comp) contained the TDP1 vector, 12 clones were expanded. DNA isolation and genomic real-time PCR were carried out as described in Methods, using primers against the leader sequence of the lentiviral vector. β-actin was amplified as a control in parallel reactions and used in the Relative Quantity (RQ) calculations, which were exported directly from SDS 2.2.2 software for Real-Time PCR data analysis. DNA isolations from U87 glioma and uninfected Tdp1\textsuperscript{+/-} clone W MEFs were included as negative controls. All RQs were calculated relative to the Tdp1\textsuperscript{+/-} clone W MEF complement mix, which was set equal to 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RQ</th>
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<tbody>
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</tr>
<tr>
<td>MEF clone W comp 3</td>
<td>0.5 x 10\textsuperscript{-3}</td>
</tr>
<tr>
<td>MEF clone W comp 5</td>
<td>2.5 x 10\textsuperscript{-4}</td>
</tr>
<tr>
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</tr>
<tr>
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<td>0.1</td>
</tr>
<tr>
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<td>0.1</td>
</tr>
<tr>
<td>MEF clone W comp 11</td>
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</tr>
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<tr>
<td>MEF clone W comp 20</td>
<td>2.0 x 10\textsuperscript{-3}</td>
</tr>
<tr>
<td>MEF clone W comp 21</td>
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<tr>
<td>MEF clone W</td>
<td>9.50 x 10\textsuperscript{-5}</td>
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</table>
Figure 4-7. Tdp1-/- MEFs were screened for complementation using primers against lentiviral sequence. Real-Time PCR amplification curves from 12 clones derived from the mix of Tdp1-/- clone W MEFs infected with LV-FLAGhTDP1-Red (TDP1 Comp). The amplification curves from the lentiviral leader sequence primer and the actin amplification curves are depicted on separate graphs for visual simplicity. Graphs were exported directly from SDS 2.2.2 software for Real-Time PCR data analysis. The threshold in this analysis was set to 3.5, within the logarithmic amplification of all samples tested.
Figure 4-8. Further evidence of Tdp1-/- MEFs complementation. To confirm that the individual clones derived from the Tdp1 -/- MEFs stably complemented by LV-FLAGhTDP1-Red transduction (Tdp1 Comp) contained TDP1 activity, 5 individual clones were expanded on 15 cm plates and lysed in an identical manner with nonionic detergent. Lysates from clones and whole cell extracts from Tdp1 +/-, Tdp1 -/-, and the original Tdp1 Comp mix of cells were incubated with a substrate mimicking a SSB with a 3'-pTyr end modification. Processed and unprocessed forms of the substrate are indicated. The substrate indicated in Figure 4-3F was treated with (E) 3 20-fold dilutions of whole cell extracts from Tdp1 +/-, Tdp1 -/-, and Tdp1 -/- Comp cell lines, (10 µg, 500 ng, and 25 ng protein) and 3 20-fold dilutions of lysate from the 5 complemented clones for 1h in reaction buffer containing Mg²⁺. Black triangles indicate the decreasing amounts of lysate added to the reactions within each genotype or clone. The radioactivity remaining in the wells presumably represents DNA-TDP1 adducts.
Figure 4-9. Time course of 3’ tyrosyl end processing and DNA repair. (A) The SSB 3’-pTyr substrate was treated from 1 to 60 min with 35 µg Tdp1/- MEF extract, with (blue symbols) and without (red symbols) supplementation with 50 ng of purified wild-type His-TDP1. Reactions were treated in buffer containing Mg$^{2+}$. The percent conversion from the tyrosyl substrate to its hydroxyl intermediate and repair products was calculated by densitometry (B).
Figure 4-10. Tdp1-/− MEF extracts contain residual activity against 3′ tyrosyl end modifications. The single strand 3′-pTyr substrate depicted in Figure 4-3A was treated with five-fold serial dilutions of whole cell extract from the initial $Tdp1^{-/-}$ cell line, and whole cell extract from a clone W derived from the initial $Tdp1^{-/-}$ mix of cells. Reactions were treated for 1 h in buffer containing EDTA.
4.4 *Tdp1*-/⁻ fibroblasts are not deficient in processing PG on SSBs

Similar experiments were performed with duplexes that contained model free-radical-mediated SSBs, i.e., bearing 3′-PG and 5′-phosphate termini, either with or without a 1-base gap (Figure 4-11). These experiments were performed with different concentrations of magnesium in an attempt to elucidate the effects of APE1/Apex1. Magnesium is required for the exonuclease activity of APE1, but high amounts of magnesium (10 mM) inhibit this activity. In contrast, the endonuclease activity of APE1 requires a much higher magnesium concentration to reach its optimal activity (10-15 mM) (Chou & Cheng, 2003). It was unknown what concentration of magnesium would be optimal for the PG removal activity of Apex1. When magnesium was present, there was no deficiency in repair of PG-terminated SSBs in *Tdp1*⁻/⁻ cells (Figure 4-10B-C), presumably reflecting processing of these lesions by Apex1 (Parsons et al, 2004). However, results also indicated that Tdp1 could process 3′ termini at these lesions when Apex1 activity was suppressed by Mg²⁺ chelation (Figure 4-11B and C).

In our hands, we observed the electrophoretic mobility of the 17mer oligonucleotide terminating in 3′-PG to be slightly faster than a 17mer oligonucleotide terminating in a hydroxyl group. While it may be counterintuitive that an oligonucleotide with a two-base end modification is more mobile than its unmodified equivalent oligonucleotide, PG ends have a high charge to mass ratio (Weinfeld & Soderlind, 1991), and have been found to be the fastest-migrating product in the majority of published literature (Chaudhry et al, 1999, Inamdar et al, 2002a, Suh et al, 1997), with one notable exception (Parsons et al, 2004).
Figure 4-11. Tdp1/- MEFs are not deficient in processing PG on SSBs. TDP1 can also process 3'-PG termini, and under some conditions appears largely responsible for their removal (Zhou et al, 2005). MEF whole-cell extracts were incubated with radiolabeled substrates that mimic SSBs bearing phosphoglycolate end modifications to assess the removal of this 3’ blocking moiety in the -/- MEFs. (A) Diagram of 3'-PG SSB substrates; asterisk represents the 5′ radioactive tag on the 3'-PG 17-mer modification. Both the 42-base (B) and 43-base (C, shown on following page) substrates were treated with 20 µg of whole-cell extract from Tdp1+/+ and Tdp1/- cell lines for 5, 20, and 60 min in reaction buffer with 0, 2, and 10 mM Mg^{2+}. These conditions are either prohibitive (0 Mg^{2+}) or permissive (2, 10 mM Mg^{2+}) for the activity of Ape1 (Chou & Cheng, 2003) or PNKP. In (B), rightmost lane contains radiolabeled marker of 35 bases. Generation of fully repaired 42- or 43-base products was evident for both substrates in both extract genotypes, and appeared to be greater in 10 mM Mg^{2+}, whereas the 3'-hydroxyl 17-mer and 1-base-elongated intermediates tended to accumulate in the presence of 2 mM Mg++. However, there was significant loss of label in 10 mM Mg^{2+} at longer times, probably reflecting nucleolytic removal of the 5'-labeled nucleotide. Continued on following page.
Figure 4-11. Tdp1/- fibroblasts are not deficient in processing PG on SSBs. For both the nicked (B) and the gapped substrate (C), the efficiency and time course of PG removal from the labeled 17-mer in the presence of 2 mM or 10 mM Mg++ was similar in +/+ and -/- extracts, suggesting that removal was catalyzed primarily by Ape1 rather than Tdp1. In the presence of 5 mM EDTA, there was significant processing of the glycolate end only in the +/+ extracts, suggesting that Tdp1 can process these lesions when other enzymes do not. However, the results suggest that TDPI is not the primary enzyme that processes 3’-PG linkages at SSBs.
4.5 Tdp1 is required for processing PG modifications on a subset of DNA DSBs

To assess whether Tdp1 status affects processing of PG-modified ends on DSBs, model DSBs were constructed (Figure 4-12, A and B). These substrates were designed to mimic breaks that would commonly result from DNA sugar damage induced by either bleomycin (Povirk, 1996), neocarzinostatin, or radiation (Henner et al, 1983), in which the deoxyribose fractures, a base is lost, and a 3′-PG group remains. Accurate repair of a break with a 3′ overhang (Figure 4-12A) would require annealing the complementary CG sequences in each overhang, filling in the 1-base gap using the opposite overhang as a template, removal of the PG, and ligation. Substrates were constructed such that they were labeled with $^{32}$P either 14 (Figure 4-12A) or 11 (Figure 4-12B) bases from the 3′ termini. These substrates were incubated in whole-cell extracts and then digested with restriction enzymes, which released short fragments that were analyzed on sequencing gels. The proposed accurate repair would therefore generate fragments of 42 nucleotides in the case of the 3′ overhang DSB substrate, and 38 nucleotides in the case of the blunt-end DSB substrate. When extracts from +/+, +/-, -/- and -/- comp MEFs were incubated for 6 h with the 3′ overhang substrate, the +/+, +/-, and -/- comp extract all produced shorter products that represented processing and resection of the 3′-PG 14-mer, and longer products of 16 and 34 nucleotides that represent repair of the DSB substrate (Figures 4-13, 4-16), whereas no processing or repair could be observed in the -/- extract.

In contrast, when extracts of all aforementioned genotypes were incubated with a radiolabeled plasmid substrate constructed to mimic a blunt-ended DSB, reactions of all genotypes formed processed intermediates and repair products (Figure 4-14A). However, the -/- extract reactions uniquely exhibited a significant fraction (24.3% ± 1.4, n=11,
Figure 4-12. Radiolabeled 3'PG on plasmid DSBs. Plasmid substrates mimicking DSBs with either partially complementary 3' blunt (A) or overhang (B) DNA ends bearing PG modifications were incubated with MEF extracts. Reactions were deproteinized, nucleic acids were precipitated, digested with BstXI and TaqI, and subjected to denaturing gel electrophoresis.
Figure 4-13. Tdp1-/- MEFs are deficient in processing PG on 3’ overhang DSBs. The plasmid substrate depicted in (4-12B) was incubated with 10 or 5 µg extract per µL reaction volume, extract was of the genotype indicated. Substrate was also incubated with boiled extract, and Tdp1+/ extract supplemented with 1µL purified Artemis protein. Rightmost lane contains radiolabeled 42-base oligonucleotide marker. After a 6 h incubation of the 3’-PG overhang DSB substrate with indicated extract, reactions were deproteinized, nucleic acids were precipitated, digested with BstXI and TaqI, and subjected to denaturing gel electrophoresis on a 20% polyacrylamide gel. The experiment was performed 3 independent times, and a scan of a representative phosphor screen is shown. 34-base reaction products visible in Tdp1+/+ and Tdp1+/- extracts were seen in repeated experiments and are the result of DNA end resection prior to substrate repair.
P<0.0001) of persistent unprocessed PG after 6 h of incubation, indicating a deficiency of DNA blunt-end processing in the +/- MEFs (Figure 4-14B). A time course of repair reactions of the blunt-ended substrate indicates that this difference accumulates over time (Figure 4-14C-D).

The longer repair product shown in both Figures 4-13 and 4-14A was seen in three independent experiments with both the 3′ overhang and 3′ blunt-end substrate and always migrated at approximately 34 bases, 8 bases shorter than the expected accurate repair product that dominates repair reactions in human cell extracts (Chen et al, 2001b). This result suggests that in MEF extracts a resection-based end joining pathway is utilized instead. Both the 3′ overhang and blunt-ended substrate can be converted by 3′ resection to an intermediate with self-complementary CGCG 5′-overhangs, the alignment of which would result in a 34-base repair product (Chen et al, 2001b) containing an MluI cleavage site (ACGCGT). Head-to-head joining by the same mechanism results in a 16-base repair product, which also contains a MluI site. This scenario was confirmed by treating the DNA from 3′ blunt-end repair reactions with MluI, which completely eliminated both the 34-base and 16-base repair products, thus confirming their identity (Figure 4-14E).

4.6 H493R (SCAN1) or H493N TDP1 cannot effectively rescue the deficits in processing either 3′-pTyr SSBs or 3′-PG DSBs

The mutant H493R TDP1 enzyme associated with SCAN1 has at least 25-fold lower activity than wild type, but it can under certain conditions cleave 3′-pTyr linkages
Figure 4-14. Tdp1/- MEFs are deficient in processing PG on blunt-end DSBs. In (A), the blunt ended substrate was incubated with 5 µg (or 10 µg *) extract per µL reaction volume. In (A), rightmost lanes contain radiolabeled markers of 35 and 43 bases. After a 6 h incubation of the 3'-PG blunt end DSB substrate with extract, the percent of PG remaining in the lane was calculated by densitometry (B). Graph on includes data from 4 independent experiments, performed with multiple preparations of cell extract per genotype; error bars indicate standard error and *** indicates p<0.001. Continued on the following page.
Figure 4-14. *Tdp1-*/- fibroblasts are deficient in processing PG on blunt-end DSBs. The blunt-end DSB substrate was also incubated for 15 min, 1 h, 3 h, or 6 h with 7 µg of *Tdp1*+/+ or *Tdp1*-- cell extract per µL reaction volume, then deproteinized, precipitated, and digested (C). Lanes on right side of the gel contain a radiolabeled marker of 11 bases, and 7 µg of boiled *Tdp1*+/+ extract incubated with the substrate. After a 6 h incubation of the 3'-PG blunt end DSB substrate with extract, the percent of PG remaining in the lane was calculated by densitometry. Graph (D) shows quantification of (C), time course of PG processing with *Tdp1*+/+ or *Tdp1*-- cell extract. The 6-h time point, conducted in triplicate, includes error bars that are contained within the symbols. The full gel of the experiment can be seen in (E) on the following page, in which the 16 and 34-base repair products seen at 6 h are highlighted in red; both are no longer visible after treatment with Mlu1. The rightmost lane of the gel contains a (partially degraded) radiolabeled marker of 34 bases.
Figure 4-14. *Tdp1*-/ fibroblasts are deficient in processing PG on blunt-end DSBs. The full gel of the experiment described on the previous page (E).
and form a persistent (t1/2~13 min) TDP1-DNA adduct intermediate (Interthal et al., 2005b). In yeast, it has been shown that under conditions of elevated TOP1, H432N TDP1 (the yeast equivalent of H493N human TDP1 mutation) confers greater cytotoxicity than SCAN1 TDP1 (He et al., 2007). To examine the action of mutant TDP1 in the presence of other DSB/SSB repair proteins, wild-type, SCAN1, and H493N forms of human TDP1 were generated in bacteria and purified. Since deficits had been identified in both 3’-pTyr and 3’ overhang PG processing in Tdp1-/- extracts, these assays were repeated in order to compare the effects of SCAN1 TDP1 and H493N TDP1 to that of complete Tdp1 deficiency.

As shown previously in Figures 4-5B and 4-9A, supplementing -/- extract with wild-type TDP1 reinstates the processing and repair of the pTyr nick substrate depicted in Figure 4-3F. However, supplementing -/- extract with SCAN1 TDP1 leads to an accumulation of radioactive signal in the wells of the gel, consistent with the presence of a protein-DNA adduct too large to migrate into the gel (Figure 4-15). Extract supplemented with SCAN1 TDP1 also shows more pTyr processing and more repair product than the unsupplemented -/- extract, and there is an additional novel band running beneath the wells and above the repair bands, which may represent a proteolyzed form of the protein(TDP1)-DNA adduct (denoted by the asterisk in Figure 4-15). A smaller increase in the repair product is seen when -/- extract is supplemented with H493N TDP1, and the well for the 20 min timepoint does display accumulation of radioactive signal. However, there is little or no increase in the band representing 18-hydroxyl, the product of the removal of the pTyr group. Thus, the H493N mutant retains
Figure 4-15. Complementation with either SCAN1 or H493N mutant TDP1 fails to restore the wild-type phenotype for processing pTyr end modifications. The pTyr substrate mimicking a SSB depicted in Figure 4-3F was treated with 30 µg of whole-cell extract from Tdp1+/+, Tdp1-/- MEFs for 5, 10, and 20 min. The substrate was also treated with 30 µg of Tdp1-/- MEF extract that had been supplemented with 50 ng of either wild type purified TDP1, the SCAN1 mutant form of TDP1, or the mutant H493N TDP1. Processed (OH), unprocessed (pTyr), and repaired forms of the substrate are indicated. The radioactivity remaining in the SCAN1 wells presumably represents an unresolved DNA-TDP1 adduct; bands running beneath the wells and above the repair bands may represent a proteolyzed form of the adduct (*).
a low level of pTyr-processing activity, and either may not form a persistent DNA adduct like SCAN1 TDP1, or may form an adduct but exhibits enzymatic kinetics that were not captured in the timepoints tested.

Supplementing -/- extract with wild type TDP1 also reinstates the processing and repair of the 3′ overhang PG DSB substrate, with the addition of more TDP1 resulting in greater conversion of the 14-PG to its processed intermediates, 14-hydroxyl, as well as repair products (Figure 4-16). Supplementing -/- extract with either SCAN1 TDP1 or H493N TDP1 does not lead to the appearance of either processed intermediates or repair bands; as in the -/- extract by itself, the unprocessed 3′-PG persists for at least 6 h of incubation in the extract. As identical aliquots of the SCAN1 TDP1 and H493N TDP1 proteins were also used to supplement cell extracts in Figure 4-15 and exhibited processing against the pTyr nick substrate, it is clear that lack of processed intermediates or repair bands with the 3′ overhang PG DSB substrate is a reflection of quantitative differences between PG and pTyr substrates in their susceptibility to the initial step in Tdp1 action, i.e. formation of the Tdp1-DNA adduct.

4.7 Characterization of MEF cell lines

Cells were initially passaged as a heterogeneous mix of cells derived directly from mouse embryos, as the cells could not be cloned out because they could not survive contact-independent growth. Early passages required a high inoculum at each transfer to promote continued growth in vitro, a phenomenon that has been observed by other researchers while developing a mouse fibroblast cell line (Reznikoff et al, 1973), as well as a sharp decline in growth in the initial ~10 passages of cells.
Figure 4-16. Complementation with either SCAN1 or H493N mutant TDP1 fails to restore the wild-type phenotype for processing 3’ PG end modifications. The substrate mimicking a DSB with partially complementary 3’ overhangs as depicted in Figure 4-12B was treated with 60 µg whole-cell extract from Tdp1+/+, Tdp1+-, and Tdp1-/- and Tdp1comp MEFs for 6 h. The substrate was also treated with Tdp1-/- MEF extract that had been supplemented with either 25 ng (1X) or 125 ng (5X) of either wild type purified TDP1, the SCAN1 mutant form of TDP1, or the mutant H493N TDP1. Labeled oligonucleotides on the right side of the gel include a 14-mer with a 3’PG, a 14-mer with a 3’-OH group, and a 42-mer. The experiment was performed 3 independent times, and a scan of a representative phosphor screen is shown. 34-base reaction products visible in Tdp1+/+, Tdp1+-, and Tdp1 -/- extracts complemented with WT TDP1 protein were seen in repeated experiments and are the result of DNA end resection prior to substrate repair.
After cells had been maintained in culture for approximately six months, different genotypes began to exhibited differences in growth rate, and in their requirements for local cell density (Figure 4-17). *Tdp1*<sup>−/−</sup> MEFs grew faster than either *Tdp1*<sup>+/+</sup> or *Tdp1*<sup>+/−</sup> MEFs, and earlier in As these cell lines became immortalized, they continued to have different requirements for local cell density or either contact-dependent proliferation, as demonstrated by the varying size of colonies (Figure 4-17B) and attachment efficiencies observed in the lines. For example, an initial attempt at a clonogenic survival assay after cells had been maintained in culture for 9 months showed that while *Tdp1*<sup>−/−</sup> and *Tdp1*<sup>+/−</sup> MEFs could form colonies and exhibited plating efficiencies of ~20-30%, *Tdp1*<sup>+/+</sup> MEFs did not survive plating, even at an inoculum of 10,000 cells in a 10cm dish. Because a limited number of clones of each genotype were maintained in culture over time, the possibility that the growth and attachment differences observed might have been the result of the *Tdp1*<sup>−/−</sup> MEFs immortalizing more rapidly than either *Tdp1*<sup>+/+</sup> or *Tdp1*<sup>+/−</sup> MEFs cannot be excluded. One could either speculate that cells without Tdp1 could experience a different rate of spontaneous immortalization as a result of the mutation, or alternately the observed differences might merely be the result of immortalization in culture being a stochastic process; growth rate being an output of the process that would follow certain probability distributions. Due to their requirements for local cell density, *Tdp1*<sup>+/+</sup> MEFs were passaged at a higher density (1:5) than *Tdp1*<sup>−/−</sup> MEFs (which could be passaged at 1:10 and still effectively recover). All MEF cell lines derived for these experiments are and remained sensitive to “postconfluence inhibition of cell division” a
term proposed by Martz and Steinberg in 1972 to describe mouse fibroblast cell lines of the 3T3 series (Martz & Steinberg, 1972).

“It is operationally defined as a pronounced depression of the mitotic rate in a postconfluent culture which displays a stationary density despite periodic nutrient renewal, the inhibition being locally reversible by removal of the adjacent cells.”

Figure 4-17. Partially-Immortalized MEFs proliferate at different rates. (A) To determine the growth rate of MEFs, 60-mm Petri dishes were seeded with $2 \times 10^4$ cells. At the time intervals indicated, 3 dishes were taken from each group, the cells were
trypsinized, and the number of cells was counted using a hemocytometer. This experiment was performed in March 2007, after the cells had been kept continuously in culture for ~150-180 days. Error bars indicate standard error and where not visible are contained within the symbol. (B) An initial attempt at a clonogenic survival assay after cells had been maintained in culture for 9 months indicated that Tdp1^/- and Tdp1^+/- MEFs could both form colonies and exhibited plating efficiencies of ~20-30%, though Tdp1^/- MEFs formed larger colonies. Images are of scanned, untreated 10cm plates.

4.8 Tdp1^- MEFs exhibited hypersensitivity to DNA damaging agents

All three genotypes of early-passage Tdp1 MEFs were characterized in their ability to respond to DNA damaging agents, including CPT, ionizing radiation (IR), and calicheamicin (Figure 4-18). MEFs plated in serial dilutions on 96-well plates were treated, then allowed to recover and proliferate for an additional 10 days post-treatment, after which viable cells were assessed with Alamar Blue. Experiments in triplicate or quadruplicate were repeated 2-4 times per each set of radiation or drug treatments, and typically Tdp1^- MEFs exhibited hypersensitization relative to Tdp1^+/- and Tdp1^+/- MEFs at all drug concentrations and radiation doses tested. However, the quantifiable extent of Tdp1^- hypersensitization was not consistently reproducible in repeated experiments, though typically Tdp1^- MEFs exhibited a 10-fold greater sensitivity than Tdp1^+/- MEFs at higher concentrations (0.1 and 1 µM) of CPT, 2-fold more sensitive to higher doses (5 and 10 Gy) of IR, and 2-fold more sensitive to higher doses (30 and 100 nM) of calicheamicin. That Tdp1^- MEFs exhibited the greatest degree of hypersensitivity to CPT reflects that specificity of CPT for TOP1cc, and fewer redundant pathways for repair of these DNA lesions, relative to the redundant pathways that exist for resolution of 3’-PG produced by IR and calicheamicin. Calicheamicin produces DSBs (at a lesion ratio of at least 20:1 DSBs:SSBs (Dedon et al, 1993)) with 2-base protruding PG ends.
Figure 4-18. *Tdp1−/−* MEFs typically displayed hypersensitivity to DNA-damaging agents. Alamar Blue assays were conducted on low-passage MEFs. Cells were exposed to either camptothecin for 1h, γ-radiation, or calicheamicin for 60h at doses indicated. Graphs represent two independent experiments performed for each drug or radiation treatment, each performed in triplicate (for drug studies) or quadruplicate (for radiation experiments) on 96-well plates.
(Chaudhry et al, 1999). Previously, it has been found that 0.2 mM of calicheamicin produced levels of PG comparable to those generated by 50 Gy IR. Extrapolated linearly, this would mean that 5 Gy IR would induce PG to a similar extent as 0.2 µM calicheamicin, and indeed, we found that the lethality in the Tdp1-/- MEFs is similar at those doses of calicheamicin and IR (Figure 4-18). The variability of hypersensitization seen from assay to assay reflects liable characteristics of each heterogeneous cell line as they continued to immortalize, in particular the doubling rate of the cells. However, the indicated differences in growth (Figure 4-17) and different requirements for cell density to promote growth (indicated by the initial poor plating efficiency of the Tdp1+/+ MEFs) somewhat compromises the validity of the Alamar Blue assay, as the measured endpoint relies on drug- or radiation-induced differences in proliferation. Despite that each cell line is measured relative to its own untreated control in the assay, it is known that IR has different effects on cells in different stages of the cell cycle. Mammalian cells in vitro are most sensitive to IR at or close to mitosis and in G₂, and are most resistant in S phase and early G₁ (Hall & Giaccia, 2006). Additionally, relative sensitivity to damage by radiation is correlated to the proliferation rate of that cell or tissue type. Though we have reported here that CPT can interact with transcription machinery with toxic effects, the predominant mode of CPT toxicity is S-phase specific (Holm et al, 1989). In general, radiation and radiomimetic drugs are more lethal in actively proliferating tissues than in non-proliferating tissues. Thus, the cells were allowed to further immortalize in culture for an additional several months in the hopes that this would further equalize growth and requirements for cell density. An alternative strategy was implemented to measure cell
survival using clonogenic survival assays. After the initial attempt at 9 months (discussed above), clonogenic survival assays were conducted again when the cells had been in culture for over 18 months using bleomycin, which has been reported to be “cell-cycle nonspecific” (Hall & Giaccia, 2006), but has also been noted to cause 2-3 times fewer DNA cleavages in S-phase cells than in G1- or G2/M-phase cells (Olive & Banath, 1993). This experiment was successfully repeated three independent times, and the survival curves obtained from graphing the surviving fractions of cells were as those previously described in the literature, without a “shoulder”, and slightly concave upward (Hall & Giaccia, 2006). $Tdp1^{-/-}$ MEFs exhibited approximately 5-fold greater sensitivity than $Tdp1^{+/+}$ MEFs to bleomycin, and overexpressing TDP1 in the $Tdp1^{comp}$ MEFs induced a 5-10 fold survival resistance to bleomycin toxicity when compared to $Tdp1^{+/+}$ MEFs (Figure 4-19).

All three genotypes of early-passage MEFs were also characterized immunohistochemically for their ability to respond to either 2 or 10 Gy of IR (Figure 4-20). After exposure to IR at times indicated, MEFs were probed with an antibody to phosphorylated H2AX ($\gamma$H2AX), a well-characterized marker of DSBs (Rogakou et al, 1998) that appears within minutes of DSB formation (Burma et al, 2001, Paull et al, 2000). Cells were imaged on a confocal microscope, and no gross differences in the formation or resolution of DSBs were noted between cell lines (Figure 4-20). However, an intriguing incidental finding was revealed as a consequence of the DAPI staining in the assay: after 6 months in culture, $Tdp1^{-/-}$ MEFs appeared to have nuclei that were twice the size of $Tdp1^{+/+}$ MEFs (Figure 4-20B), with $Tdp1^{+/+}$ MEFs possessing nuclei of intermediate size (data not shown). This observation was true in the untreated controls in
Figure 4-19. Tdp1/- MEFs trend towards hypersensitivity to bleomycin. Clonogenic survival assays were conducted on MEFs that had been in culture for 18 months. Cells were exposed to bleomycin for 4h at doses indicated, and colonies were counted by eye 10-12 days post-treatment. The graph represents data from three independent experiments. At 3 µg/mL bleomycin, no significant difference in survival was observed either between Tdp1+/+ MEFs (mean = 0.02433 ± 0.009025, n=3) and Tdp1/- MEFs (mean = 0.005333 ± 0.001333, N=3), two-tailed p=0.1057, or the Tdp1+/+ MEFs and the Tdp1COMP MEFs (mean = 0.1777 ± 0.07294, N=3). Error bars represent standard error and where not visible are contained within the symbol; ns, nonsignificant.
Figure 4-20. No gross differences in the formation or resolution of DSBs were noted between MEF genotypes. In (A) MEFs of all three genotypes that had been in culture for 90-120 days were allowed to grow on chamber slides, subjected to a large dose of radiation (10 Gy), and fixed with paraformaldehyde 30 min post-IR. Slides were stained with anti-γH2AX and DAPI, and imaged with confocal microscopy. Unirradiated cells were also fixed, stained, and imaged in parallel. Continued on the following page.
Figure 4-20. No gross differences in the formation or resolution of DSBs were noted between MEF genotypes. In (B), Tdp1+/+ and Tdp1−/− MEFs that had been in culture for 6 months were subjected to a lower, clinical dose of radiation (2 Gy) and fixed at 30 min, 4h, and 24h post-IR. All images were taken at 63X.
the assay, and so cannot be a product of IR treatment. It is tempting to speculate that this morphological difference could be related to the process of immortalization, and notably, a subsequent publication observed what might have been a similar effect in ∆tdp1 yeast *Schizosaccharomyces pombe*. Here it was noted that among non-dividing ∆tdp1 cells, about half were enlarged, indicating an unsuccessful attempt to re-enter the yeast vegetative cycle that was not observed in wild-type cells (Ben Hassine & Arcangioli, 2009).

4.9 TDP1 may be localized to the mitochondria as well as the nucleus

Given that mitochondria possess a specialized topoisomerase (TOPmt), similar to nuclear TOP1, that forms 3' covalent complexes to mitochondrial DNA, (Zhang et al, 2001), experiments were performed on preparations of purified mitochondria to detect 3’-pTyr processing activity indicative of the presence of TDP1, or an enzyme with similar function. To date, there have been no published studies indicating that TDP1 is localized to mitochondria. A mitochondrial extract from wild-type lymphoblasts (matched controls for the SCAN1 lymphoblasts), prepared by Tong Zhou using a method similar to that described by Stuart et al, 2004, but without a Percoll gradient, and an independently prepared mitochondrial extract graciously donated to the laboratory by Dr. Vilhelm A. Bohr, of the National Institute of Aging, were subjected to the same 3’-pTyr assay used for cell extracts. Both mitochondrial preparations displayed 3’-pTyr processing activity, less than that of a wild-type lymphoblast whole cell extract, but more than a commercially-prepared HeLa nuclear extract (Figure 4-21A). These observations prompted further work, including testing an additional preparation of isolated non-
Figure 4-21. Mitochondrial preparations possess 3′-pTyr processing characteristic of TDP1. (A) The nicked tyrosyl substrate depicted in 4-3F was treated 5-fold serial dilutions of purified TDP1, lymphoblast whole cell extract, purified mitochondria preparation, or commercially available HeLa nuclear extract (Promega) in buffer containing EDTA for 1h. Y-axis depicts the conversion from the starting product, the 3′-Tyr, to the 3′ phosphate group. Continued on the following page.
Figure 4-21. Mitochondrial preparations possess 3’-pTyr processing characteristic of TDP1. (B) The radiolabeled tyrosyl oligomer substrate depicted in 4-3A was treated with 5-fold serial dilutions of homogenate from mitochondria isolated from a 4-month old WT mouse (kindly donated by Michael Kieblish, Ph.D), and 5-fold serial dilutions of mitochondrial extract from JRL-2 cells (TDP1+/+ lymphoblasts). Substrate was also treated with Tdp1+/+ whole cell extract as a positive control. Reactions were treated for 1 h in buffer containing EDTA. (C) Graph depicts conversion from the starting product, the 3’-Tyr, to the phosphate or hydroxyl group products.
synaptic mouse brain mitochondria (from a 4-month old male C57BL/6J mouse) independently prepared and generously donated by Dr. Michael Kiebish (Kiebish et al, 2009) that also displayed 3′-pTyr processing activity (Figure 4-21B and C), and immunoblotting for TDP1 and COX4, a mitochondrial marker, in a similar set of mitochondrial and nuclear extracts (Figure 4-22). A band detected with an anti-TDP1 antibody migrating at approximately 60kDa was visible in a mitochondrial extract prepared from wild-type lymphoblasts (Figure 4-22), which could potentially represent a truncated or alternately-spliced form of TDP1 that localizes to mitochondria. However, preliminary confocal microscopy experiments have failed to demonstrate colocalization of TDP1 with mitochondrial markers (Figure 4-23). Experiments in this area are ongoing in an attempt to further demonstrate whether mitochondrial extracts possess 3′-pTyr processing activity, and that this activity is not due to contamination with nuclear proteins.
Figure 4-22. A band that reacts with anti-TDP1 antibody is present in a mitochondrial fraction that exhibits 3′-pTyr activity. Presence of TDP1 was confirmed in the HeLa nuclear extract (1), TDP1+/+ lymphoblast whole cell extract (2), and mitochondrial extract from TDP1+/+ lymphoblasts (2 dilutions, black triangle) used in the 3′-pTyr processing assay depicted in Figure 4-21A. The gel contains 10 µL each of HeLa nuclear extract and lymphoblast whole cell extract, and 76 and 38 µg of lymphoblast mitochondrial extract. Blotting was performed with the anti-TDP1 antibody also used in Figure 4-5A, and the same membrane was subsequently incubated with anti-COX4, a mitochondria-specific protein.
Figure 4-23. Immunohistochemistry fails to show TDP1 colocalizing with a mitochondrial marker. Presence of TDP1 (red) can be detected in the nuclei of both Tdp1COMP MEFs and HeLa cells using confocal microscopy. While COX4 (green) can be detected in HeLa cells, this protocol did not yield staining in MEFs. Staining was performed with the anti-TDP1 antibody also used in Figure 4-5A used at 1:1000, and anti-COX4 was used at 1:500. Cell fixation and membrane permeabilization were attempted with and without methanol treatment, and the best representative images from either treatment are shown (MEFs = no methanol; HeLa = with methanol). All confocal images were taken at 100X.
V. DISCUSSION FOR PART I

5.1 Tdp1 mouse phenotype

While there is little doubt that an H493R TDP1 mutation confers SCAN1 (Takashima et al, 2002), it is still unclear how the mutation elicits symptoms of the disease, and why symptoms are largely neurological. In an attempt to address these questions, we generated a Tdp1 knockout mouse. However, extensive behavioral analysis of Tdp1-/- mice up to 12 months of age did not reveal any detectable deficits in experimental correlates of human SCAN1, such as grip strength (muscle weakness), stride length or rotarod latency (ataxia), and indeed did not show any behavioral differences from wild-type mice (Figure 4-2). While our study was in preparation, two independent groups published their results on Tdp1 knockout mice (Hirano et al, 2007, Katyal et al, 2007), both derived from an identical embryonic stem cell clone generated from gene trapping available from BayGenomics (Ferrin, 2008, Stryke et al, 2003). A similar lack of overt behavioral symptoms was noted in both independently assessed Tdp1-/- mice, although in neither case were quantitative data reported (Hirano et al, 2007, Katyal et al, 2007). In one of these studies (Katyal et al, 2007), whole brain mass in 17-month old Tdp1-/- mice was reduced by 10% when compared to wild-type age-matched controls, and there was a progressive age-dependent reduction of the cerebellum-to-whole-brain ratio in Tdp1-/- mice when compared to their age-matched wildtype littermates (a reduction of 15% at 17 months, the latest time point reported). When comparing brain mass and gross brain histology, no differences were observed by eye between genotypes in our studies, corroborating the results of Hirano et al.. However, no attempt was made to quantify the cerebellar to whole brain ratio as was performed by
Katyal et al., who also noted that cerebellar morphology and foliation was indistinguishable between genotypes. Katyal et al. also observed in vivo hypersensitivity of the Tdp1<sup>−/−</sup> mice to the effects of the TOPI inhibitor topotecan, displaying significant weight loss due to intestinal progenitor cell hypersensitivity after six daily doses (Katyal et al, 2007). Hirano and colleagues found similar in vivo Tdp1<sup>−/−</sup> hypersensitivity to both camptothecin and the camptothecin derivative topotecan, and in both cases the sensitivity required repeated doses in a relatively short amount of time to manifest (Hirano et al, 2007). Hirano et al. also reported that Tdp1<sup>−/−</sup> mice are hypersensitive to bleomycin: after 10 days of intraperitoneal administration of 10 mg/kg/day, Tdp1<sup>−/−</sup> mice died 4 days after the final treatment. Tdp1<sup>−/−</sup> mice were not hypersensitive to etoposide, a chemotherapeutic inhibitor of TOPII (Hirano et al, 2007). In either study, even lethal treatments with TOPI inhibitors still failed to elicit any SCAN1-like symptoms; rather than showing sensitivity in post-mitotic neurons, histopathology showed apoptosis and cell loss in more rapidly proliferating lymphoid and hematopoietic tissues. Thus, overall, the Tdp1<sup>−/−</sup> mouse phenotype bears little resemblance to human SCAN1.

It is not unreasonable to anticipate that chemotherapeutic reagents could induce either acute or chronic neuropathology, as some patients treated with high-dose cytosine arabinoside (for leukemia) develop a cerebellar toxicity syndrome characterized by the death of nondividing Purkinje neurons (Vogel & Horoupian, 1993, Winkelman & Hines, 1983). Long-term survivors of acute lymphoblastic leukemia treated with chemotherapies (and not radiation) also display long-term deficits in motor timing (Mahone et al, 2007). Side effects of irinotecan, a CPT analogue, have included cholinergic syndrome (Abigerges et al, 1995, Gandia et al, 1993), though this may be
because of direct effects of irinotecan on acetylcholinesterase (Blandizzi et al, 2001).

Conversely, it has been shown that TOP1 activity is quickly modified in depolarized neurons in response to the excitatory neurotransmitter glutamate, which initiates Ca$_{2+}$-dependent activation of PARP, which in turn poly ADP-ribosylates TOP1, downregulating its activity (Homburg et al, 2000, Zehorai et al, 2008). It is, however, unknown what role this downregulation of TOP1 activity may have, though investigators have speculated that the interaction between neurotransmitters and TOP1 may be the basis for gene expression regulation in neurons (Zehorai et al, 2008). In agreement with the theory that TOP1 may be involved in neurotransmitter signaling, TOP1 inhibitors have been proposed for use as potential anti-seizure pharmaceuticals, especially for brain tumor patients who present with epilepsy (Song et al, 2008). Thus, TOP1 is likely to have further uncharacterized roles in the cerebellum, and one or multiple of these uncharacterized functions may be perturbed in the SCAN1 phenotype.

5.2 SCAN1 phenotype

While fundamental differences between human and mouse phenotypes of a given gene are not unusual, it is possible that rather than (or in addition to) the simple loss of TDP1 function, the symptoms of SCAN1 are dependent on the unique features of the H493R mutant enzyme. In particular, it has been proposed that the residual activity of this mutant enzyme may be sufficient to process many if not most TOP1cc that fail to religate, converting them to persistent TDP1-DNA adducts that may be more toxic and/or less amenable to alternative repair pathways than the initial TOPI-DNA adducts (Interthal et al, 2005b). On the other hand, there is evidence that the initial TOPI-DNA adducts are
also more persistent in SCAN1 than in normal cells, and that the difference is comparable to the overall deficit in SSB repair (Miao et al, 2006). In either scenario – an enzymatic deficit, a dominant negative effect of the SCAN1 mutation, or a combination of the two – given that TDP1 is expressed in a wide variety of tissue types (see Supplemental Data in (Hirano et al, 2007)), the neuronal-specific phenotype requires an explanation. Certainly, terminally-differentiated, non-replicating neuronal cells have relatively fewer alternative repair pathways than other cell types, have high levels of oxidative stress due to their energy demands (Caldecott, 2003), and elevated levels of transcription. Furthermore, it has been shown that in yeast the toxicity of SCAN1 TDP1 is dependent upon elevated levels of TOP1 (He et al, 2007), and TOP1 has been shown to be particularly highly expressed in Purkinje neurons of adolescent human brain (Gorodetsky et al, 2007, Holden et al, 1997). Purkinje neurons are very large cells with massive dendritic trees that allow each Purkinje neuron to integrate information from ∼ 200,000 granule neurons and are also the only type of neuron to send projections out of the cerebellar cortex (Brooks, 2002). TOP1 has also be shown to be highly expressed in the mouse cerebellum relative to other regions in the mouse brain (Plaschkes et al, 2005), and within the cerebellum was shown to be most highly expressed by Purkinje neurons (some of which are inhibitory neurons) (Zehorai et al, 2008). As discussed above, as the activity of TOP1 is regulated by neurotransmitters, it is also likely that there are further uncharacterized functions of TOP1, specific to the cerebellum. Curiously, Top1 has also been shown to be developmentally-regulated in mice, increasing in activity in the brain as mice age from birth, and is most highly expressed in 3 month old mice compared to 4 time points tested (1 day, and 1 month, 3 months, and 12 months) (Plaschkes et al, 2005). Although there
have been to date no autopsy studies on SCAN1 patients, other neurodegenerative disorders that feature ataxia among their symptoms have specific Purkinje neuron pathology (Sugawara et al, 2008, Yang et al, 2000). Thus, the ataxia seen in SCAN1 patients may be directly caused by Purkinje neuron pathology that results from the interaction of SCAN1 TDP1 in the presence of high levels of TOP1 (Hawkins et al, 2009).

5.3 Molecular actions of TDP1

In vitro complementation studies showed that extracts from \( Tdp1^{-/-} \) mouse fibroblasts largely could not process a 3′ tyrosyl-DNA substrate; these results correlated with previous observations made in extracts from SCAN1 lymphoblasts (El-Khamisy et al, 2005), astrocyte and cerebellar extracts (Katyal et al, 2007), and mouse neurospheres (Hirano et al, 2007). When supplemented with wild-type TDP1, processing of the tyrosyl-DNA substrate was reinstated, as also shown by Katyal et al. (Katyal et al, 2007). The generation of products of extremely low mobility in \( Tdp1^{+/+} \) extracts supplemented with TDP1-H493R (Figure 4-15), similar to the results seen by Interthal, et al. (Interthal et al, 2005b), under conditions where the wild-type enzyme promotes SSB repair, suggests that substantial amounts of persistent TDP1-DNA adducts would be formed during attempted repair of Top1 lesions in SCAN1 cells. Cosedimentation of TDP1 protein with DNA in CsCl-fractionated extracts of SCAN1 but not normal fibroblasts (Hirano et al, 2007) suggests that such complexes form in vivo.

Extracts of \( Tdp1^{+/+} \) cells are also completely deficient in processing of protruding 3′-PG DSB termini, and recombinant TDP1 can rescue that deficiency (Figure 4-16).
Previously, our research group has shown similar lack of processing of PG-terminated 3’ overhangs in extracts of TDP1-mutant human SCAN1 cells (Zhou et al., 2005), but due to low ligation efficiency in those extracts, generation of ligated repair products was not demonstrated. Much less 3’-PG processing occurred on DSB termini in nuclear extracts as compared to whole cell extracts, and it was hypothesized that this difference was due to the sequestration of DNA ends by DNA-PK (Zhou et al., 2005), which is present in higher concentrations in the nucleus than in the cytoplasm (Anderson & Lees-Miller, 1992). Likely too, the higher ligation efficiencies observed in MEF cell extracts are due to the different levels of DNA-PK in mouse cells as compared to human: human cells express 50X more DNA-PK than mouse cells (Finnie et al., 1995). Whereas the lymphoblast extracts required supplementation with XRCC4/ligase IV to exhibit alignment-based gap filling (Zhou et al., 2005), in MEF extracts these factors were present in sufficient amounts to exhibit products consistent with NHEJ, possibly again reflecting differences in expression of end-joining proteins in mouse vs. human cells, or perhaps a difference in cell-type expression, comparing lymphoblast vs. fibroblast expression. Since the initial studies with 3’-PG ends, further work by our research group has clarified some of the enzymatic properties of TDP1, showing that recombinant TDP1 expressed from an adenovirus processes a 3’-PG on a 6-base overhang 3 times more efficiently than 3’-PG on a blunt-end DSB (Zhou et al., 2009). This finding confirms results seen in Figure 4-14, in which a substantial fraction of 3’-PG on a blunt-end DSB substrate remained unprocessed even after 6h in Tdp1+/+ extracts, but no trace of 3’-PG remained in 3’-overhang DSB substrate processed by Tdp1+/+ extract (Figures 4-13 and 4-16). Additionally, adenoviral TDP1 exhibited activity against a 3’-Tyr substrate at
protein concentrations 200-fold less than those necessary to exhibit activity against a 3′-PG substrate (Zhou et al, 2009), similar to the 80-fold difference seen with TDP1 expressed in E. coli (Inamdar et al, 2002a). Furthermore, the hypothesis that DNA DSB ends are sequestered by DNA-PK and become refractory to TDP1 processing (Zhou et al, 2005) was shown to be accurate, in that after the initial 5 min of processing in extracts, DNA-PKcs and KU inhibited further processing (Zhou et al, 2009). Phosphorylation of DNA-PK partially abrogated the inhibition, indicating that conformational changes in DNA-PK might allow TDP1 to process 3′-PG after the end-bound repair complex is formed. Autophosphorylation of DNA-PK is known to facilitate end-joining (Reddy et al, 2004). Treating extracts with KU-55933, an ATM inhibitor, failed to inhibit end-joining (Zhou et al, 2009), indicating that the DNA-PK phosphorylation events necessary to make 3′-PG accessible for processing were not mediated by ATM, as some DNA-PK phosphorylation events have been proposed to be (Chen et al, 2007b). Thus, it is likely that in MEF extracts, 3′-PG DNA ends are processed more efficiently because they are not as readily sequestered into DNA-PK-containing repair complexes and remain accessible.

In contrast to the tyrosyl lesion, there is no evidence that the mutant enzyme can form covalent adducts at 3′-PG termini (Figure 4-16). Thus, presumably because the 3′-PG lesion is a much less favorable substrate than a 3′-pTyr (Inamdar et al, 2002a, Zhou et al, 2009), SCAN1 TDP1 displays a hypomorphic or perhaps even a null phenotype in PG processing. Tdp1<sup>−/−</sup> extracts also show partial deficiency in processing blunt 3′-PG DSBs, but no deficiency in processing 3′-PG SSBs. Although TDP1 has been shown to interact with SSB repair proteins (Plo et al, 2003), TDP1 is more active toward DSB ends
than SSB ends (Raymond et al, 2004) (though it has also been proposed that the double-stranded DNA becomes transiently single-stranded to properly bind to TDP1 (Davies et al, 2002a)). Inasmuch as bleomycin induces almost exclusively 3'-PG SSBs and (predominantly blunt-ended) 3'-PG DSBs (Povirk et al, 1989), it appears likely that unrepaired DSBs are responsible for the reported bleomycin sensitivity of Tdp1−/− mice and Tdp1−/− MEFs (Hirano et al, 2007). While neither Tdp1−/− astrocytes, Tdp1−/− cerebellar granule cells (Katyal et al, 2007), SCAN1 lymphoblasts (El-Khamisy et al, 2007) or Tdp1−/− MEFs (Figure 4-20) show any detectable deficit in repair of radiation-induced DSBs, a recent mass spectral analysis suggests that the fraction of radiation-induced breaks with 3'-PG termini may be as small as 10% (Chen et al, 2007a), much lower than the previous estimate of ~50% (Henner et al, 1982). Although SCAN1 cells have an apparent defect in repair of radiation-induced SSB (El-Khamisy et al, 2007) and exhibit small differences in radiosensitivity in survival assays (Zhou et al, 2005), as do Tdp1−/− MEFs (Figure 4-18), these deficits may reflect persistence of TOP1-mediated lesions formed at sites of base damage (Daroui et al, 2004, Pourquier et al, 1999), rather than failure to process direct free radical-mediated, PG-terminated SSBs. Additionally, though it was initially reported that TDP1 was phosphorylated on serine and threonine residues after ionizing radiation (Zhou et al, 2005), these experiments were performed with a FLAG-TDP1, and could not be reproduced in subsequent experiments with constructs without the FLAG-tag. Hence, it was concluded that the 32P incorporation seen after radiation may have been an artifact of the construct used. However, during the preparation of this manuscript, additional results reported by another research group have shown that TDP1 is phosphorylated on serine 81 after radiation, and that this
phosphorylation event stabilizes TDP1 and promotes its association with XRCC1 (Das et al., 2009).

Further supporting the requirement for TDP1 for 3′-PG processing, SCAN1 cells exhibit hypersensitivity to calicheamicin when measured by micronuclei formation, acentric chromosome fragments, or appearance of dicentric chromosomes in metaphase chromosome spreads (Zhou et al., 2009). Tdp1−/− MEFs exhibit hypersensitivity to calicheamicin in growth assays (Figure 4-18), and TDP1 knockdown HeLa cells exhibited a small enhancement in toxicity to calicheamicin (Zhou et al., 2009). Although Artemis is capable of processing 3′-PG on very long overhangs (Povirk et al., 2007b), taken together, these results indicate that TDP1 is relevant for processing 3′-PG on overhangs typically induced by chemotherapeutics and may prove a useful target for enhancing cytotoxicity of drugs in the clinic.

5.4 Cellular localization studies

Results on whether or not TDP1 is localized to the mitochondria, in addition to the nucleus, are inconclusive. While it is tempting to draw inferences regarding the additional 3′-PG processing activity seen in whole cell extracts compared to that of nuclear extracts (Zhou et al., 2005), as discussed above, these differences are likely to be due to DNA end sequestration by other repair factors, rather than additional TDP1 in the whole cell extracts. Three independently-prepared mitochondrial extracts or isolations exhibited 3′-Tyr processing (Figure 4-21), but it is difficult to discern if this activity originates from the isolated mitochondria or from contaminating nuclear TDP1. Additionally, if TDP1 is localized to the mitochondria, one must speculate on the
possible mechanism of its localization. The bands visible in the mitochondrial extracts in the western blot in Figure 4-22 (which may or may not be TDP1) suggest that a shorter isoform could direct intracellular trafficking of TDP1 to the mitochondria, as is the case for APE1 (Chattopadhyay et al, 2006). The APE1 isoform localized to the mitochondrial matrix is expressed from the same gene as nuclear APE1, but has a deletion of the first 33 amino acids, which removes the nuclear localization sequence, and the protein does not possess a canonical mitochondrial localization signal (MLS) (Chattopadhyay et al, 2006). The 33N cleavage is likely a post-translational modification executed by an uncharacterized serine protease associated with either the mitochondria or endoplasmic reticulum (Chattopadhyay et al, 2006). It is worth noting that approximately half of all proteins localized to the mitochondria do not contain a canonical MLS (A. Larner, in communication), an N-terminal stretch of positively charged amino acids that forms an amphipathic α helix. Other possible mechanisms of localizing TDP1 to the mitochondria include post-translational modifications, or a binding partner of TDP1 that may localize to the mitochondria accompanied by bound TDP1.

Why TDP1 should theoretically be localized to the mitochondria is clear; it is likely that its function would be similar to its function in the nucleus: resolving the mtTOP1cc that have been shown to occur in mitochondria (Zhang et al, 2001), and repairing 3′-PG on DNA overhangs that may occur as a consequence of elevated oxidative stress in mitochondria. While there are external sources of reactive oxygen species (ROS), the majority of ROS within eukaryotic cells are produced in mitochondria as by-products during the generation of ATP, through the process of oxidative phosphorylation (Schon & Manfredi, 2003). The nuclear DSB load due to mere oxygen
metabolism is quite substantial (Karanjawala et al, 2002). Due to proximity and a lack of complex chromatin organization with associated histone proteins, the nearby mitochondrial genome is much more susceptible to ROS-induced damage than the nuclear genome (Yakes & Van Houten, 1997). It is important to note that while BER has long been characterized as a repair process important for maintaining the mitochondrial genome (LeDoux et al, 1992, Pettepher et al, 1991), NHEJ has not yet been extensively characterized as a mitochondrial process in mammalian cells (Bacman et al, 2009, Lakshmipathy & Campbell, 1999, Larsen et al, 2005). However, multiple research groups have shown mitochondrial DNA repair after cells had been treated with bleomycin (Morel et al, 2008, Shen et al, 1995), indicating a probable role for TDP1.

5.5 Neuronal apoptosis

Integrity and fidelity of DNA is essential for the proper function and survival of neurons, and it is likely that compromised DNA repair is what leads to cell death in the cerebellum of SCAN1 patients, and perhaps Tdp1<sup>−/−</sup> mice. However, the current understanding of the signals, mechanisms, and developmental regulation that govern neuronal death after DNA damage is incomplete (McKinnon, 2009). Proper regulation of DNA repair and apoptosis is required for normal neuronal development during the expansion of neuronal stem and progenitor cells. If these cell types incur DNA damage without effectively repairing or fail to trigger apoptosis, injured neurons may be allowed to survive and expand only to degenerate during childhood, as McKinnon and colleagues have proposed is the case for AT patients (Herzog et al, 1998, Lee & McKinnon, 2000). As NHEJ plays an important role in later stages of neuronal development, likely after
cells have exited the cell cycle (Orii et al, 2006), the cell loss observed in the cerebellum of SCAN1 patients and \( Tdp1^{-/-} \) mice is probably a consequence of damage that begins to accumulate in later development.

A variety of apoptotic or cell death pathways exist for neurons, including the death receptor-mediated (extrinsic) signaling cascade, (ii) the mitochondrial pathway (intrinsic) involving the apoptosome, (iii) the endoplasmic reticulum (ER)-dependent stress response (Lindholm et al, 2006), which signals through the mitochondrial pathway, and (iv) autophagy (Danial & Korsmeyer, 2004, Yuan et al, 2003). DNA damage is thought to involve “inside-out” signaling (Valerie et al, 2007) the nuclear damage signaling to the interior of the cell. Specifically CPT, calicheamicin (Prokop et al, 2003), or oxidative stress-induced DNA damage can each induce mitochondrial-mediated apoptosis. This intrinsic apoptotic pathway proceeds via the outer mitochondrial membrane becoming permeable, allowing factors such as cytochrome \( c \) to be released into the cytoplasm that precipitate apoptosis (Danial & Korsmeyer, 2004). Typically the pro-apoptotic effector proteins of the (B-cell CLL/lymphoma 2) BCL-2 family – (BCL2-antagonist/killer) BAK and (BCL2-associated X protein) BAX – are activated and responsible for permeabilizing the mitochondrial membrane (Wei et al, 2001), but only a truncated BH3 domain-only form of BAK (N-BAK) is expressed in differentiated neurons (Uo et al, 2005). Thus, cerebellar neurons rely on BAX for CPT-induced apoptosis (Morris et al, 2001), and proceed to apotose by aberrantly re-entering the cell cycle via Cdc25A induction (Zhang et al, 2006). Other oxidative stressors induce neuronal death via a cell-cycle independent pathway, which can be abrogated by transiently inhibiting histone deacetylases (HDACs) via drug treatment (Langley et al,
This finding is somewhat at odds another recent finding that HDAC4 is neuroprotective: HDAC4-deficient mice have degeneration of Purkinje neurons, and those neurons that persist after P7 have duplicated cell bodies (Majdzadeh et al, 2008). This observation suggests that absence of HDAC4 allows aberrant cell-cycle progression and results in neuronal apoptosis; indeed, HDAC4 blocks proliferation in cell lines by inhibiting cyclin-dependent kinase (CDK) 1 (Majdzadeh et al, 2008). Inhibiting CDKs also has the effect of abrogating apoptosis in CPT-treated cells (Borgne et al, 2006).

Taken together, these data suggest that a likely source for the cell loss in the cerebellum of SCAN1 patients and Tdp1−/− mice is Bax-mediated apoptosis. Covalent TDP1-H493R-DNA complexes may be recognized by the cell in a manner similar to CPT lesions, and induce aberrant cell proliferation, and the 3′-PG oxidative lesions that result from the deficit in TDP1 activity against glycolate residues may induce cell cycle-independent apoptosis. Future lines of inquiry designed to examine modes of apoptosis in neuronal cells engineered to express SCAN1-TDP1 may prove informative; determining if SCAN1-TDP1 cells apoptose via a cell cycle-dependent or independent manner may provide clues as to the nature of the toxic lesions in SCAN1 TDP1-expressing cells.

5.6 Mitochondrial dysfunction-induced apoptosis

“…the possibility of a unifying principle based on mitochondrial dysfunction as a primary cause of neuronal cell death (Orth & Schapira, 2001) has become not only attractive, but even seductive.” (Schon & Manfredi, 2003).

If indeed TDP1 is localized to the mitochondria and plays a role in maintaining the integrity of the mitochondrial genome, it is possible that direct damage to the mitochondria may be mediating cerebellar degeneration in SCAN1. Products from the mitochondrial genome contribute to the electron transport complexes, and if the genome
is damaged, so too would be the electron transport chain (ETC). The consequences from this damage would amplify: defects in the ETC can increase reactive oxygen species production which can further damage the chain (Zhang et al, 1990) and lead to alterations in the transmembrane potential $\Delta\psi_m$, the electric gradient required for Ca$^{2+}$ internalization in the mitochondria (Brookes et al, 2004). If Ca$^{2+}$ sequestration by mitochondria is insufficient, excessive Ca$^{2+}$ influx into cells can be pathological, as is the case in glutamate excitotoxicity, in which excessive glutamate stimulation or excessive sensitivity to synaptic glutamate causes cellular toxicity. This toxicity can manifest as dark cell degeneration, a type of toxicity similar to both apoptosis and necrosis (Strahlendorf et al, 1998). A scenario such as this has been described in a mouse model of spinocerebellar ataxia type 28, in which a subunit of a complex in the inner mitochondrial membrane was engineered with a loss-of-function mutation, and the resulting phenotype is a progressive deficit in motor coordination and balance associated with mitochondrial dysfunction and Purkinje cell dark degeneration (Maltecca et al, 2009). This phenotype is due to a progressive, tissue-specific, reduction in ATP synthesis due to decrease assembly of ETC complexes I and III, specifically in mitochondria from cerebella tested at 6 and 12 months of age (brain and spinal cord mitochondria were no different from wild-type mouse mitochondria). Thus, the cerebellum possesses a hypersensitivity to mitochondrial dysfunction that can lead to SCAN1-like phenotypes.

5.7 Concluding hypotheses
At present, the most likely etiology of SCAN1 is that oxidative lesions in either nuclear or mitochondrial DNA promote transcription-mediated formation of trapped TOP1cc that are then converted to persistent SSBs or DSBs with a unique covalent 3′ linkage between DNA and the TDP1-H493R enzyme. A role for PG-terminated DSBs in either nuclear or mitochondrial DNA cannot be excluded from contributing to SCAN1 pathology. As demonstrated from other spinocerebellar ataxias, the cerebellum is uniquely vulnerable, likely due to the combination of a dearth of repair pathways, cellular longevity, and elevated levels of transcription and oxidative stress. Future work is necessary to determine if the SCAN1 phenotype may provide further evidence as to the normal function of human TDP1. The finding that a mouse Tdp1−/− phenotype is only seen under conditions of severe genotoxic stress, combined with the slow amount of 3′-Tyr processing seen in Tdp1−/− MEFs, suggests that there are redundant repair pathways for the major functions of Tdp1, as is likely the case for the human enzyme as well.
VI. INTRODUCTION FOR PART II

6.1 Ataxia Telangiectasia and Ataxia Telangiectasia Mutated

Ataxia Telangiectasia (AT) (OMIM #208900) is a complex, multisystem congenital disorder, the manifestations of which appear and develop gradually throughout childhood. The AT phenotype (reviewed in (Shiloh, 1997)) was first described in an initial report by Syllaba & Henner in 1926 and again by Louis-Bar in 1941; Boder & Sedgwick named the disorder “ataxia-telangiectasia” in 1957 (Boder & Sedgwick, 1958). AT is inherited in autosomal recessive manner with full penetrance, and estimated frequencies in various populations range between 1:40,000 and 1:300,000. It is of interest to note that heterozygotes for the AT gene are thought to comprise about 1% of the general population (Swift et al, 1976).

The major clinical sign of AT is neuromotor dysfunction. Ataxia (gross lack of coordination of muscle movements) typically appears when a child begins to walk, and gradually progresses until the patient is confined to a wheelchair around the age of 10 years (detailed early case reports can be found in (Centerwall & Miller, 1958). A second clinical indicator, for which the disorder is also named, is telangiectasia (dilated blood vessels) in the eyes and facial skin, which typically appear between the ages of 3-5. The major neuropathological finding is cortical cerebellar degeneration, particularly loss of Purkinje and granular cells. The disorder also includes major defects in the immune system (Peterson et al, 1966) later identified to be caused by deficits in both immunoglobulin class switching (Lumsden et al, 2004), and V-J recombination of T-cell receptor loci (Vacchio et al, 2007), and a predisposition to cancers, particularly leukemia and lymphoma (Peterson et al, 1964). Heterozygotes are also thought to possess an
elevated cancer risk, particularly for breast cancers (Swift et al, 1976, Swift et al, 1987). Attempts to treat malignancy in AT patients led to the identification of another characteristic of the disorder: profound acute clinical radiosensitivity (Gotoff et al, 1967). This radiosensitivity was later found to be consistent on the cellular level: fibroblasts from AT patients were killed at radiation doses 3-5 fold lower than those that kill normal cells (Taylor et al, 1975). Prior to identification of a causative gene, AT could be diagnosed based on the hypersensitivity of lymphocytes from AT patients to killing by γ-irradiation (Henderson et al, 1985). Death in AT patients is premature, in the second or third decade of life, and typically due to pulmonary complications, neurologic deterioration, or cancer.

Defects in cell cycle checkpoints after irradiation in cells from AT patients were observed (Painter & Young, 1980, Scott & Zampetti-Bosseler, 1982), and researchers identified several complementation groups in AT (Murnane & Painter, 1982). However, it was unclear if these complementation groups represented different genes, or different mutations within the same gene capable of intragenic complementation. Work on cell lines derived from AT patients showed that AT cells were not more susceptible to γ-irradiation-induced breaks than normal cells, nor do AT cells repair breaks more slowly than normal cells, but instead left 5-6x more unrepaired chromosome fragments after 6 Gy of x-rays compared to normal cells (27% vs. 5%) (Cornforth & Bedford, 1985). Further work identified that AT cells failed to induce p53 and G1 arrest after irradiation (Kastan et al, 1992), placing the AT defect proximal to the p53 response to DNA damage. After an initial linkage analysis narrowed the location of the gene defective in AT to chromosome 11q22-23 (Gatti et al, 1988), a group led by Yosef Shiloh used positional
cloning (Collins, 1992) to identify the causative gene in AT, and so named it and its protein product AT-mutated (ATM) (Savitsky et al, 1995). They also observed that the putative ATM protein exhibited similarity to other previously identified phosphoinositide 3 (PI-3) kinases. The ATM gene encodes a 350 kDa nuclear protein (Brown et al, 1997), and most mutations in AT patients inactivate the ATM protein via large deletions or truncations (rather than missense mutations) resulting in null alleles (Gilad et al, 1996). To further elucidate the role of the ATM gene in mammalian physiology, a mouse model of AT was created by disrupting the murine Atm locus by gene targeting. The Atm⁻/⁻ mouse phenotype has in common many of the features of the human disorder, including extreme radiosensitivity, immune abnormalities, and a predisposition to malignancy (Barlow et al, 1996), cementing the role of ATM as a tumor suppressor. However, it is notable that Atm⁻/⁻ mice homozygotes display no gross ataxia, but do exhibit significantly shorter stride lengths and latencies to fall off a rotor-rod when compared to wildtype mice. Additionally, Barlow et al. observed no histological evidence of neuronal degeneration in Atm-deficient mice (although dopaminergic neuron loss in the substantia nigra (Eilam et al, 1998) and altered Purkinje cell electrophysiology (Chiesa et al, 2000) were later found by other groups).

The biochemical function of ATM and ATR (Ataxia Telangiectasia and rad3-related) was clarified when Keegan et al. demonstrated the associated kinase function of both molecules, and the demonstrated the focal localization of ATM and ATR on meiotic chromosomes (though the proteins did not co-localize) (Keegan et al, 1996). Although complete knockout of the ATR gene is thought to be lethal and is associated with early embryonic lethality when knocked out in the mouse (Brown & Baltimore, 2000, de Klein
et al, 2000), a hypomorphic mutation in humans is associated with a rare disease known as Seckel syndrome (OMIM #210600). The homozygous mutation that causes the disease introduces a splicing defect that reduces the abundance of ATR to almost undetectable amounts, but the remaining protein is sufficient for viability (O'Driscoll et al, 2003). The phenotype of Seckel syndrome includes microcephaly and dwarfism. The lethality associated with lack of ATR and viability associated with lack of ATM indicates that ATM and ATR certainly must have some non-redundant functions.

ATM was then shown to directly phosphorylate p53 on serine 15 in response to DNA damage (Banin et al, 1998, Canman et al, 1998), and although protein levels of ATM do not increase after DNA damage (Brown et al, 1997), the kinase activity of immunoprecipitated ATM was activated after cellular exposure to ionizing radiation (IR) (Banin et al, 1998, Canman et al, 1998). Though the majority of early research on ATM was in response to radiation-induced damage, a wide variety of studies also showed ATM activation after other DNA damaging agents (elegantly summarized in Table 1 of (Kurz & Lees-Miller, 2004)). Of these studies, that of Bakkenist and Kastan (Bakkenist & Kastan, 2003) stands out for its original findings. The study demonstrated that in the absence of activating factors, ATM exists as a catalytically inactive dimer or multimer, and dissociating into catalytically inactive monomers is accompanied by self-phosphorylation (in cis or trans) on serine 1981 (later publications argued whether or not this phosphorylation event was required for the multimer-to-monomer transition (Lee & Paull, 2005)). Additionally, treatment of cells with chloroquine or trichostatin A (an HDAC) activated ATM in the absence of DNA damage, allowing the authors to posit that activation of ATM is not dependent on DNA damage, but rather through structural
changes in chromatin. (Bakkenist & Kastan, 2003). Subsequent papers demonstrated the interaction between the MRE11-RAD50-NBS1 (MRN) complex (Carney et al, 1998), which directly senses and binds to DSBs, and ATM, which the MRN complex and binds to and activates (Lee & Paull, 2004, Lee & Paull, 2005). Congenitally acquired hypomorphic NBS1 and MRE11 mutations also cause radiosensitivity and chromosome instability syndromes: Nijmegen breakage syndrome (Varon et al, 1998) and AT-like disorder: (Stewart et al, 1999). Our own research group demonstrated that ATM binds to DNA ends in endonuclease-produced DSBs and regulates HRR (Golding et al, 2004). Further work with a mouse model expressing a mutant form of Atm (a mutation of serine to alanine at position 1987, the mouse equivalent of serine 1981) as their sole Atm species demonstrated that Atm-dependent cellular and responses are functional without the autophosphorylation event (Pellegrini et al, 2006). The same research group created yet another mouse model in which all three conserved Atm serine autophosphorylation sites (serines 367,1899, and 1987) were replaced with alanine, and showed that these disruptions did not affect DNA damage-induced ATM kinase activity in vivo (Daniel et al, 2008). Together, these results have given way to the model that ATM autophosphorylation occurs simultaneously or as a consequence of ATM activation: once Atm is converted to a catalytically active conformation at a DSB, serine residues become accessible to phosphorylation, but are not the mechanism by which ATM is activated. Other research has identified an DNA-damage induced acetylation site on ATM (lysine 3016) regulated by Tip60 histone acetyltransferase that may be required for ATM’s kinase activity (Sun et al, 2007).
The basis of the radiosensitivity in AT cells and the AT phenotype continues to be researched (Loucas & Cornforth, 2004) and can be attributed to not only a deficit in DSB repair (Badie et al, 1995), but also to observed checkpoint deficiencies indicating that the defect in AT is also a failure to sense or adequately respond to DNA damage. Gradually it was discovered that ATM phosphorylates hundreds of cellular targets (Matsuoka et al, 2007) that regulate multiple cellular processes (reviewed in (Kurz & Lees-Miller, 2004)), and thus it is difficult to dissociate and analyze individual phosphorylation targets or checkpoint events and relate them to the radiosensitive phenotype of AT, though the literature is not lacking in examples of such (Chen et al, 1999, Khanna et al, 1995).

6.2 KU-55933 and KU-57788: small molecule inhibitors

“Some of the same qualities that make the defective AT gene so disturbing in relation to cancer causation have something of a positive spin when it comes to new possibilities for cancer therapy. The most obvious is that cancers could be made more sensitive to therapeutic x-rays by disabling the AT gene in the tumor cells. “It’s a natural target to think about,” Kastan says.” (Nowak, 1995)

Recently, highly specific small molecule inhibitors KU-55933 and KU-57788 have been developed against ATM (IC\textsubscript{50} = 13 nM) and DNA-PKcs (IC\textsubscript{50} = 14 nM), respectively, (Hickson et al, 2004, Leahy et al, 2004) for potential use as radiosensitizing agents. While these initial compounds are unlikely to find their way to the clinic due to issues related to solubility, KU-57788 exacerbated etoposide-induced tumor growth delay and displayed promising pharmacokinetics (Zhao et al, 2006). Targeting DNA repair kinases to enhance tumor killing has been in the literature for some time; earlier experiments found that caffeine, an inhibitor of ATM and ATR, sensitized p53-deficient cells to IR-induced killing (Powell et al, 1995). Indeed, even in 1980 it was observed that the radiation-induced response of AT cells was very similar to other mammalian cells.
irradiated in the presence of caffeine (Painter & Young, 1980). Later it was found that caffeine mediates its radiosensitizing effects by inhibiting ATM (IC$_{50}$ = 0.2 mM), ATR (IC$_{50}$ = 1.1 mM), and mTOR (IC$_{50}$ = 0.4 mM) in assays with immunoprecipitated proteins (Sarkaria et al, 1999). Our research group has shown that KU-55933 blocks HRR (Golding et al, 2007). It has also been observed that transient inhibition of ATM is sufficient to enhance sensitivity to IR (Rainey et al, 2008), and our research group recently published results on a new, highly specific inhibitor of ATM (Golding et al, 2009). This inhibitor, KU-60019, was 10-fold more effective than KU-55933 at blocking radiation-induced phosphorylation of key ATM targets in human glioma cells, blocked AKT phosphorylation on serine 473, and is a highly effective radiosensitizer of human glioma cells. Curiously, in addition to the strategy of radiosensitizing tumors via inhibiting ATM kinase, chloroquine (which activates ATM, among several other functions) has also been shown to have possible radiosensitizing and survival-enhancing effects in patients with glioblastoma multiforme (Sotelo et al, 2006). Taken together, these results show that is reasonable to pursue the study of DNA repair kinases as pharmacological targets to enhance radiation-induced killing of tumor cells.

6.3 ATM, DNA repair, and chromatin

In addition to ATM’s contribution to HRR (Golding et al, 2004), previous groups have also identified that ATM is required for a subset (approximately 10-25%) of end joining (Riballo et al, 2004). A distinct component of DSB rejoining (approximately 10%) occurs over a prolonged time and requires ATM, Artemis, and DNA-PKcs, representing an “ATM-dependent” component of end-joining. Both ATM and Artemis cells contained this same defect, suggesting that they were involved in the same required
pathway (Riballo et al, 2004). Originally, it was suggested that this proportion of DSBs might have represented especially complex breaks, typical of the lesion-clusters formed by ionizing radiation, and that because of this complexity, ATM would be required to phosphorylate Artemis (as it was shown to do, both in vitro and in vivo (Riballo et al, 2004)) such that Artemis could adequately mediate resection of long DNA overhangs. Since then, further work has shown that although ATM does phosphorylate Artemis on S645 (Goodarzi et al, 2006), it is the autophosphorylation of DNA-PK on what is known as the ABCDE cluster that mediates the endonuclease activity of Artemis. Work from this research group has gone on to support the hypothesis put forth by Bakkenist and Kastan (Bakkenist & Kastan, 2003): that ATM is required to facilitate access to DSBs within regions of more densely packed heterochromatin, rather than ‘complex’ breaks (Goodarzi et al, 2008, Kim et al, 2009). Electron microscopy has shown that generation of a DSB leads to a local, ATP-dependent decondensation of chromatin (Kruhlak et al, 2006), consistent with the idea that architectural changes in chromatin may be involved in the initiation of the DNA damage response.

6.4 KAP1

There is evidence that ATM mediates changes in local chromatin condensation via phosphorylation of KAP1 (Kruppel-associated box (KRAB) associated protein 1, also known as TIF1β or TRIM28) at DSBs, which promotes the relaxation of chromatin (Ziv et al, 2006). KAP1 is a molecular scaffold that coordinates activities to regulate chromatin structure, and interacts with the multi-subunit NuRD histone deacetylase complex, the histone H3 lysine 9-selective methyltransferase SETDB1 (reviewed in
(Urrutia, 2003)), and with heterochromatin protein 1 (HP1) (Ayyanathan et al, 2003, Sripathy et al, 2006). A model of KAP1 on chromatin is depicted in Figure 6-1. KAP1 is thought to be required for localized microenvironments of heterochromatin at gene-specific loci to repress gene transcription, and may require small ubiquitin-related modifier (SUMO) modifications on 3-6 of its lysine residues (554, 575, 676, 750, 779, and 804) (Mascle et al, 2007). In that same study, it was shown that the repressive activity of KAP1 was reduced 30-50% after treatment with sodium butyrate or Trichostatin A (both HDACs) in transfected cells. The SUMO-lations on KAP1 regulate histone 3 lysine 9 and lysine 14 (H3-K9 and H3-K14) acetylation and H3-K9 methylation on specific gene promoters in response to doxorubicin (Lee et al, 2007). KAP1 has been shown to be redundantly phosphorylated by DNA repair kinases after radiation on serine 824 (White et al, 2006), and neocarzinostatin-induced phosphorylation of KAP1 is ablated by treatment with either KU-55933 or shRNA against ATM in human neurons (Biton et al, 2007). Furthermore, ATM-deficient cells treated with siRNA against KAP1 restored the repair defect, as measured by γH2AX foci resolution and pulsed-field gel electrophoresis (Goodarzi et al, 2008). Curiously, KAP1 has been demonstrated to be important specifically for the functioning of the adult forebrain of mice: mice harboring a ‘‘floxable’’ KAP1 gene (Weber et al, 2002) were bred with mice expressing the Cre recombinase from a CamKIIa promoter, which induces recombination throughout the forebrain starting at approximately postnatal day 14. The resulting mice, with KAP1 expression abrogated in their forebrains, exhibit heightened levels of anxiety-like and exploratory activity and stress-induced alterations in spatial learning and memory (Jakobsson et al, 2008). If KAP1 is required for normal brain functioning in mice, it is
not unreasonable to suggest that normal KAP1-mediated functions may also be altered in
the brains of AT patients.

6.5 I-SceI-based repair assays

One of the continuing challenges in the field of DNA repair is identifying and
developing assays to measure DNA breakage and repair. The first and most basic assays
have already been discussed here – clonogenic survival curves – but clonogenic survival
as an endpoint integrates information not only about DNA repair, but also cell
attachment, growth, and apoptosis. Conventional techniques used to monitor DSB repair
in living cells, such as pulse-field gel electrophoresis (Schwartz & Cantor, 1984)
necessitate the use of high (~80 Gy) radiation doses that are arguably not clinically
relevant, particularly when delivered in a single dose. A commonly used, indirect
method of monitoring DSB that is effective at clinical doses of 2 Gy or less is monitoring
the appearance of γH2AX (Rogakou et al, 1998), a cellular response that occurs within 1-
3 minutes of introduction of DSBs (Paull et al, 2000). As is an indirect measure of
breaks, this method too has its disadvantages: H2AX phosphorylation is subject
regulation by the DNA repair kinases. Originally, ATM was identified as the kinase
responsible for phosphorylating H2AX (Burma et al, 2001), but since then, DNA-PK was
identified as having a redundant, overlapping role with ATM in phosphorylating H2AX
in both mouse and human cells (Stiff et al, 2004).

Other assays in this thesis have demonstrated processing or repair of radio-labeled
oligonucleotide or plasmid substrates in cell-free extracts, but these assays fail to reflect
information about repair in the context of the higher order structure of DNA, which ATM
Figure 6-1. A model mechanism for the involvement of ATM in NHEJ: KAP1-mediated repression. Histone (depicted in gray) acetylation (blue triangles) facilitates transcriptional activation and DNA repair by decondensing chromatin, whereas histone methylation (pink squares) silences transcription in the opposite manner. Histone H3 lysine 9 and 14 acetylation increases and Histone 3 lysine 9 methylation decreases on specific gene loci when KAP1 is de-SUMOylated. The opposite occurs when KAP1 is SUMOylated. KAP1 is phosphorylated in response DNA damage, and this results in a global decondensation of chromatin similar to the decrease in chromatin compaction evident in the absence of KAP1 (Ziv et al, 2006). Adapted from (Alter & Hen, 2008, Ayyanathan et al, 2003, Lee et al, 2007, Sripathy et al, 2006, Sun et al, 2005, Sun et al, 2007, Urrutia, 2003, White et al, 2006).
may play a role in regulating. Thus, to monitor the impact of ATM signaling on DSB repair, we employed an engineered DNA repair cassette to introduce DNA breaks in defined locations (Rouet et al, 1994a). These cassettes, pioneered by work by Maria Jasin, use a rare-cutting endonuclease, I-SceI to cleave recognition sequences that have been integrated into genomic DNA (Richardson et al, 1999, Rouet et al, 1994b). I-SceI has a long (18 bp) nonpalindromic recognition sequence, and generates staggered cuts with 4-base 3′-OH overhangs. The DSB ends formed after I-SceI cleavage have 5′-phosphate and 3′-OH groups suitable for ligation, whereas DSBs formed after exposure to IR, radiomimetic drugs, or oxidative damage have chemically heterogeneous ends that require processing by nucleases or other DNA modifying enzymes prior to repair by HRR or NHEJ. I-SceI repair cassettes have a number of advantages relative to other methods: first, the repair cassette is subject to chromatin structure; the DSB must be detected and then repaired under a more realistic cellular environment. Second, this allows for the direct monitoring of single DSBs, which are common occurrences in the cells, rather than overwhelming the cellular machinery with the repair response necessary at 80 Gy. These integrated repair cassettes with fluorescent reporter proteins (see Figure 6-2) have been used quite successfully by our group to monitor both HRR and NHEJ events (Golding et al, 2004, Golding et al, 2007, Golding et al, 2009).
Figure 6-2. Repair of NHEJ-red cassette activates DsRed expression. (A) NHEJ events can be monitored by DsRed expression (schematic). (B) U87/ NHEJ-red cells were infected with Ad-SceI (30 MOI) and imaged by fluorescent microscopy at 72 h post-infection.
6.6 Human glioma

The majority of the experiments described below were performed using the U-87 MG cell line (American Type Culture Collection Number HTB-14) (American Type Culture Collection), which was originally derived from a human malignant glioma (Ponten & Macintyre, 1968) and expresses wild-type p53 (Van Meir et al, 1994). Gliomas are tumors that arise from glial cells, and include astrocytoma, glioblastoma, oligodendrogliaoma, ependymoma, mixed glioma, malignant glioma NOS, and other neuroepithelial tumors. The annual incidence of malignant gliomas is approximately 5 cases per 100,000 people; each year, approximately 15,000 new cases are diagnosed in the United States (CBTRUS, 2009). The standard therapy for malignant glioma involves surgical resection when feasible, radiotherapy, and chemotherapy, but even with optimal treatment the median survival for patients with glioblastoma is only 12-15 months (Wen & Kesari, 2008). Relative to other tumor types, glioma is radioresistant and lethal. In placing DNA repair cassettes within the U87 cell line, we hope to examine a basic biological process in glioma that can be targeted to develop better cancer therapeutics.

6.7 Specific aims

Due to recently developed small molecule inhibitors (Hickson et al, 2004, Golding et al, 2009) it has become possible to pharmacologically target ATM for sensitization in radiotherapy. Although our research group has already characterized ATM’s role in HRR (Golding et al, 2004, Golding et al, 2007, Golding et al, 2009), the role of ATM in NHEJ has yet to be fully elucidated. In an attempt to further elucidate roles for ATM in DSB repair, we propose:
1. To determine whether and to what extent ATM is required for NHEJ. The DNA-PK and ATM inhibitors described above, as well as a novel assay based on I-SceI that allows for monitoring of NHEJ events, have provided a unique toolset to interrogate the signaling relationships between ATM, DNA-PKcs, and NHEJ. It was unclear what effect, if any, inhibiting ATM would have: previous research has suggested that since HRR and HHEJ are not independent, one may act as a compensatory mechanism for the other (Allen et al, 2002, Richardson & Jasin, 2000), and the perspective is further complicated by evidence for crosstalk that may occur directly between ATM and DNA-PKcs (Chen et al, 2007b, Peng et al, 2005), or that both kinases may phosphorylate identical end joining factors, but on different phosphorylation sites (Yu et al, 2008). To assess ATM’s effect on NHEJ, the incidence of repair will be measured via DsRed+ event monitoring by FACS, and a novel qPCR assay. The fidelity of repaired DNA will also be assessed by digesting PCR products with a restriction enzyme that exclusively cuts the DNA repaired by high-fidelity repair.

2. To determine if any effects of ATM inhibition on NHEJ are mediated through ATM’s effects on chromatin condensation. Other research groups have proposed that ATM signals to mediate repair of DSBs within heterochromatin (Ziv et al, 2006, Goodarzi et al, 2008). Thus, if inhibiting ATM with KU-55933 has an effect on NHEJ, histone deacetylase inhibitors such as Trichostatin A will be used in combination with KU-55933 to test if manipulating chromatin condensation either overcomes or exacerbates ATM’s effects on chromatin. Additionally, studies will be performed in
which expression of KAP1, a known chromatin modifier and phosphorylation target of ATM after DNA damage (White et al, 2006, Ziv et al, 2006), will be manipulated with shRNAs to determine its effect on NHEJ.
VII. METHODS FOR PART II

7.1 Generation of U87/NHEJ-red cells

The U87/NHEJ-red cells were generated by infection of U87 cells with a lentivirus (WPXld-2xISceI-DsRed) carrying a repair cassette (Figure 7-1) positioned upstream of the DsRed reporter gene that has been recently described (see Supplemental Methods in Golding, et al. (Golding et al, 2009)). Cells were grown in α-MEM medium with 10% fetal bovine serum (FBS) (unless otherwise noted) and 1% penicillin streptomycin solution. Cells were cloned by dilution and screened for the integration of WPXld-2xISceI-DsRed (hereafter referred to as the NHEJ-red cassette) by infection with adenovirus expressing the I-SceI rare-cutting homing endonuclease from Saccharomyces cerevisiae (Ad-SceI) (Rouet et al, 1994). The expression of I-SceI is driven by the cytomegalovirus immediate-early gene promoter, and vector also contains a nuclear localization signal, the HA epitope tag, and a deletion of the E1 adenoviral region (Anglana & Bacchetti, 1999). Ad-SceI infection was followed by subsequent analysis of DsRed expression by fluorescent microscopy and fluorescence-activated cell sorting (FACS) performed on live cells on a Beckman Coulter XL-MC flow cytometer with assistance from Dr. Sarah Golding at the Massey Cancer Center Flow Cytometry Facility.

To induce cleavage of the NHEJ-red cassette, Ad-SceI was added to the culture medium of cells at 70-90% confluency at a multiplicity of infection (MOI) of 30. For any infection, the number of virus particles that will enter any given cell on a dish is a statistical process, and some cells may absorb more than one virus particle while others may not absorb any. The viral titer was determined by Aaron Randolph or Barbara Szomju, Resource Managers of the VCU Virus Vector Shared Resource Facility.
Figure 7-1. Repair of the NHEJ-red cassette. The experimental outline is described in the Materials & Methods as indicated in (A).
In multiple experiments, 36-48 h prior to infection with Ad-SceI, cells were serum-starved (to induce growth arrest) by washing 4 x 10 min with serum-free media. Cells were incubated with virus while slowly rocking for 2 h at 37ºC. DNA repair kinase inhibitors KU-55933 and KU-57788 (Hickson et al, 2004, Leahy et al, 2004) or Trichostatin A (TSA) was added to the cell culture medium either at the time of Ad-SceI infection, or 2 h after introduction of Ad-SceI unless otherwise indicated, and in all cases left in the medium throughout the experiment. KU-55933 and KU-57788 were kindly donated by Graeme Smith (KuDOS Pharmaceuticals, a division of AstraZeneca, Cambridge, United Kingdom. TSA was purchased from Sigma-Aldrich. All drugs were dissolved in DMSO.

Two independently derived U87/NHEJ-red clones, presumably with different integration sites for the NHEJ-red cassette, were used in experiments: A2 and A3. The A2 clone also contains a separate I-SceI-inducible HRR cassette that has previously been characterized by our group (Golding et al, 2004, Golding et al, 2007, Golding et al, 2009) but this HRR cassette was not used in these experiments. In both cases the DNA repair cassettes are stably integrated into the genome of the U-87 MG cell line.

**7.2 Description of the NHEJ-red repair assay**

The NHEJ-red assay has been described previously (Golding et al, 2009). The assay relies on a genomically-integrated lentiviral vector containing two I-SceI recognition sequences flanking an ATG codon that when unperturbed, acts as a decoy initiation codon, preventing translation of the DsRed reporter gene (see Figure 7-1). Upon cleavage with I-SceI at both recognition sequences, the decoy codon is excised within a
25-base sequence. If DNA repair is then completed, the DNA is sealed, and DsRed expressed from a downstream, previously out of frame ATG codon. The two I-SceI recognition sites were placed in opposite orientations, such that when cleaved in both sites with I-SceI and the inner piece of the vector is excised, two partially complementary 3′ overhangs are generated: TTAT and TATT. If the generated partially complementary ends anneal without DNA-end resection, a two-base gap will result on either strand which must be filled in, likely by a gap-filling polymerase. This possible repair scenario would result in a repair joint sequence reading TTATAA, and is qualified as high-fidelity NHEJ. More extensive resection would still result in DsRed expression unless the deletion were to be so extensive that removes the downstream ATG codon or DsRed sequence, or removes the upstream promoter. NHEJ events can then be determined by FACS of live DsRed positive cells, or genomic isolation followed by PCR, cloning and DNA sequencing, or digestion with PsiI.

7.3 SDS-PAGE and western blotting.

To analyze I-SceI, hyperacetylated histone H4, phosphorylated and total KAP1, or XLF expression, the western blot procedure implemented was identical to that described in TDP1 Methods in Part I. Typically, cells were scraped directly into 1X Laemmli sample buffer prior to boiling and sonication. Where indicated, cells were delivered 5 Gy of radiation from a MDS Nordion Gammasell 40 (ON, Canada) research irradiator with a Cs-137 source delivering a dose of 1.05 Gy/min. Primary antibodies included anti-HA tag antibody (mouse monoclonal, Roche Applied Science, 1:1,000), anti-hyperacetylated Histone H4 (Penta) (rehydrated at unknown concentration, Millipore), anti-FLAG M
antibody (Sigma, mouse monoclonal, 1:1000), anti-KAP1 (Bethyl Laboratories, rabbit polyclonal, cat no. A300-275A; 1:10,000), anti-human XLF (Bethyl Laboratories, rabbit polyclonal, cat no. A300-729A; 1:2500), or anti-β-actin (Santa Cruz Biotechnologies, goat polyclonal, cat no. sc-1615; 1:1000). Additionally, anti-KAP1 and anti-phospho-KAP1 (both rabbit polyclonal antibodies; 1:1,000) were generously donated by David White, Ph.D., and his principle investigator Frank Rauscher III, Ph.D., of the Wistar Institute, and were shown to be functional in (White et al, 2006). In addition to the secondary antibodies specified in TDP1 methods, anti-rabbit IRDYE 800 (Rockland Immunochemicals; 1:10,000 - 1:5,000) was also used as a secondary antibody.

7.4 Real-time qPCR assay and assessment of DNA repair fidelity

After Ad-SceI infection at times indicated, cells were scraped directly into PBS, pelleted by 1 min centrifugation at 1500 rpm, and then frozen on dry ice or at -80°C. Cell pellets were resuspended in 200 µL PBS, then nucleic acids were extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics). Because the kit was designed for use on $10^4$ - $10^6$ cultured cells and the number of cells being used in these experiments varied slightly between experiments and cell types, the length of time that cells were digested in proteinase K and binding buffer was extended from the kit’s recommendation of 10 min to overnight. No other modifications to the kit protocol were implemented.

Real-time genomic amplification PCR was performed on an ABI 7900HT Real-time PCR instrument, using Sequence Detection Systems 2.2.2 software both as a user interface and as analysis software after the PCR was completed. The PCR primers used
were NHEJ-cassette
5′CACGAGACTAGCCTCGAGGTTT,
3′CTTGAAGCGCATGAACTCCTT, and β-actin
5′TCACCCACACTGTGCCCATCTACGA, and
3′CAGCGGAACCCTCATTGCCAATGG  synthesized by the VCU Massey Cancer Center Nucleic Acids Research Facility. Unless otherwise noted, reactions were amplified and detected using SYBR Green master mix (Applied Biosystems, Foster City, CA, cat. no. 4309155), in which SYBR Green allows for detection of the accumulation of double-stranded DNA (Zipper et al, 2004). As this method of detection is non-specific, β-actin reactions were loaded in parallel. The Relative Quantification Assay (ΔΔCt) was performed on the 7900HT Real-time PCR instrument, set on standard assay mode (as opposed to ‘Fast’ or ‘9600 Emulation’) and the cycling conditions were set to 50°C for 2 min, followed by 95°C for 10 min, then 40 cycles of 95°C for 15 sec followed by 60°C for 1 min. After the reaction, relative NHEJ levels were determined with Sequence Detection Systems software by the ΔΔCt method; Ct is defined as the cycle at which fluorescence becomes detectable above a user-defined level background fluorescence and is inversely proportional to the logarithm of the initial number of template molecules. For each target sample with a given reference sample, Sequence Detection Software then computes:

\[ \Delta Ct = Ct \text{ target (product of NHEJ-cassette primers)} - Ct \text{ reference (the product of } \beta \text{-actin primers)} \]

\[ \Delta \Delta Ct = \Delta Ct \text{ test sample (for example, U87/NHEJ-red cells treated with 10µM KU-55933)} - \Delta Ct \text{ calibrator sample (for example, untreated U87/NHEJ-red cells)} \]

Relative Quantity = \( 2^{-\Delta \Delta Ct} \)
As binding of SYBR Green to double-stranded DNA is non-specific, further testing such as DNA melting curve analysis, is generally recommended to assure that intended target amplified (Giglio et al, 2003). However, melting curve analysis is not likely to be informative in discriminating between the two NHEJ target amplicons (150 and 125 nucleotides), as that analysis relies on sufficiently different melting temperature ($T_m$) values, and the targets of the qPCR NHEJ assay have been predicted to vary by only 0.3°C (Figure 8-5B). The secondary structures, $T_m$, and Gibbs energies ($\Delta G$) of the 125- and 150-nucleotide amplicons were predicted with OligoAnalyzer 3.1 (Integrated DNA Technologies, available at http://www.idtdna.com/SCITOOLS/scitools.aspx) and calculated at 60°C, as this is the 1 min extension temperature dictated by the Sequence Detection Systems user interface and ABI 7900HT Real-time PCR system.

Thus, products of each real-time genomic qPCR are run out on non-denaturing acrylamide gels to distinguish amplified products. PCR bands were separated on a 9% non-denaturing polyacrylamide gel with a 1Kb DNA ladder (Invitrogen, Cat. No. 15615-016), detected by ethidium bromide staining, and imaged on a Typhoon 9410 variable mode scanner (General Electric Healthcare). The fidelity of the DNA repair of the 125-base product was assessed in one of two ways. In the first instance, genomic products were cloned into pCR2.1 vector by Dr. Konstantin Akopiants. Then using primers internal to the pCR2.1 vector, 40 clones were sequenced. The second method of assaying repair fidelity was to digest amplified genomic samples with $PsiI$, a restriction enzyme for which the restriction site is TTATAA, the exact sequence qualified as “high fidelity” repair. The percentage of the 125-nucleotide repair product that was digested with $PsiI$ can then be visualized via gel electrophoresis and quantified. When amplifying genomic
samples to be digested with PsiI, FastStart PCR Master mix (Roche Diagnostics) was used after SYBR Green 2X mix was excluded for experimental reasons (Figure 8-9). When amplifying genomic samples to be detected by $^{32}$P phosphorimaging, the 3’ DsRed primer was 5’ radiolabeled with $[\gamma^{32}P]$ ATP and T4-polynucleotide kinase for 1 h at 37°C, such that the label would be incorporated into the PCR amplification products. Where indicated, PCR products were eluted through QIAquick PCR purification kit columns (Qiagen), then digested overnight with the specified New England Biolabs digestion buffer, BamHI, or PsiI (New England Biolabs, Ipswich, MA) in 10 µL reactions. Digested bands were separated on a non-denaturing polyacrylamide gel, and stained and imaged as previous. Densitometric values were quantified using QuantityOne analysis software (Bio-Rad).

### 7.5 Ligation-mediated PCR to detect I-SceI incision

Adapting from methods published in (Pfeifer, 2006, Soutoglou et al, 2007, Villalobos et al, 2006), an asymmetric double-stranded DNA adaptor molecule was ligated to the I-SceI induced breaks, then subjected to quantitative real-time genomic PCR (Figure 7-2). Theoretically, the appearance and loss of the specific PCR product should allow for the detection of both the break and its repair. Genomic DNA purified from U87/NHEJ-red cells was ligated with T4 DNA ligase, 150 nmol of the double-stranded adaptor (approximately a 2000-fold excess of adaptor to genomic DNA), composed of 5’-CGGCATCACTACGATGTAGGATGATAA (top strand) and 5’-CATCCTACATCGTAGTGATGCCG (bottom strand) in 10X T4 Ligation buffer (New England Biolabs) for 16 h at 4°C. Ligated DNA and matched unligated controls
were amplified with a primer specific for the adaptor 5′-CGGCATCACTACGATGTAGGATG, and a second primer for sequence internal to the NHEJ-red cassette, 5′- GCGCATGAACTCCTTGATGA using the same quantitative real-time genomic PCR conditions as described above. The 84-nucleotide ligation-mediated PCR-amplified product was visualized on a 12% non-denaturing polyacrylamide gel with a 1kb DNA ladder (Invitrogen, Cat. No. 15615-016), detected by ethidium bromide staining, and imaged on a Typhoon 9410 variable mode scanner (General Electric Healthcare).

### 7.6 KAP1 manipulation in 293B and U87/NHEJ cells

The NHEJ-red lentiviral vector was modified to create a doxycycline (Dox)-inducible I-SceI-HA, stably transduced into 293B cell line and cloned. 293B/NHEJ-red cells were transfected using SuperFect (Qiagen, cat no. 301307) with pcDNA plasmids obtained from collaboration with Frank Rauscher III, Ph.D. and David White, Ph.D. Samples were harvested for qPCR analysis after 20 h of Dox induction and were otherwise treated and analyzed as described for U87/NHEJ-red cells. In other experiments, U87/NHEJ-red cells (A2 clone) were transfected with pSuper928 plasmid containing a short hairpin RNA (shRNA) against KAP1 (described in (Sripathy et al, 2006)), and two days later were selected with 10 µg/mL blasticidin. Selection pressure for U87/NHEJ-red cells pSuper928 KAP1 was maintained with 3 µg/mL blasticidin in their growth medium.
Figure 7-2. Ligation-Mediated PCR to detect I-SceI endonuclease cleavage. An asymmetric double-stranded DNA adaptor molecule was ligated to the I-SceI induced breaks. Using a primer designed to complement the adaptor (purple) and a primer designed to complement the top strand of the NHEJ-red cassette (dark green), a 84-base ligation-mediated product could be amplified with quantitative genomic PCR.
7.7 XLF<sup>-/-</sup> fibroblasts containing NHEJ-red repair cassette

XLF/Cernunnos-deficient fibroblasts were obtained from Jean-Pierre de Villartay, PhD. These cells were originally isolated from an RS-SCID (severe combined immune deficiency with radiosensitivity) patient, and were immortalized by h-TERT and SV40. Cells were grown in RPMI1640 medium plus 10% fetal bovine serum and contain a homozygous nonsense mutation at R178 of XLF/Cernunnos (Buck et al, 2006). XLF<sup>-/-</sup> fibroblasts were infected with the same lentivirus containing the NHEJ-red repair cassette described above, and a mix of cells were infected with Ad-SceI as described for U87/NHEJ-red cells, and analyzed as described above.

7.8 Statistics

As for all TDP1-related data from Part I, unpaired two-tailed t-tests were done on triplicate or quadruplicate data sets using GraphPad Prism 3.0. P values are indicated as follows: *, <0.05; **, <0.01; ***, <0.001. All error bars depict SE for triplicate or quadruplicate data sets.
VIII. RESULTS FOR PART II

8.1 DNA repair kinase inhibitors reduce NHEJ

After establishing that the NHEJ-red cassette functionally produces DsRed events, and thus proficient NHEJ, after Ad-SceI infection (Figure 6-2) (Golding et al, 2009), we assessed the effects of specific ATM (KU-55933) and DNA-PKcs (KU-57788) inhibitors on the frequency of the DsRed+ events. Inhibition of either ATM or DNA-PK resulted in reduced DsRed expression (Figure 8-1) at both 48 h and 72 h. At 72 h after Ad-SceI infection, KU-55933 (10 µM) reduced the number of cells expressing DsRed by ~95% compared to the percentage of DsRed expressing U87/NHEJ-red (A3 clone) cells infected with Ad-SceI alone. Similarly, KU-57788 (2.5 µM) reduced the DsRed-expressing population by 97% (Figure 8-1B). Both inhibitors induced dose-dependent decreases in NHEJ, which was reduced to 0%, 7.6%, and 23.7% of basal levels by 10, 5, and 2.5 µM KU-55933, and 1.6%, 8.4%, and 63% of basal levels by 2.5, 1.25, and 0.75 µM KU-57788 (Figure 8-2). To ensure that these reductions were not an artifact of an interaction between the repair kinase inhibitors modifying I-SceI endonuclease expression from the adenoviral vector, the kinetics of I-SceI expression were monitored via Western blotting against the hemagglutinin (HA) tag on I-SceI. Multiple experiments with the A2 clone of U87/NHEJ-red cells were used to show that I-SceI begins to express ~3-4 h after Ad-SceI infection and continues to increase in expression through 48 h. No I-SceI expression can be detected at 2 h and neither KU-55933 (10 µM) nor KU-57788 (1 µM) reduced I-SceI expression at any of the times (4, 8, 16 h; 1 – 8 h) tested (Figure 8-3).
Figure 8-1. Inhibition of ATM or DNA-PK suppresses NHEJ. (A) U87/ NHEJ-red (A3 clone) cells were infected with Ad-SceI and exposed to KU-55933 (10 μM) or KU-57788 (2.5 μM) 2 h post infection. DsRed-NHEJ+ events were analyzed by FACS 72 h after Ad-SceI infection. (B) DsRed-NHEJ+ events were analyzed by FACS at 12, 24, 48, and 72 h after Ad-SceI infection.
Figure 8-2. Inhibition of ATM or DNA-PK suppresses NHEJ in a dose-dependent manner. U87/ NHEJ-red (A3 clone) cells were infected with Ad-SceI and exposed to KU-55933 (10, 5, or 2.5 µM) or KU-57788 (2.5, 1.25, or 0.75 µM) 2 h post infection. DsRed-NHEJ+ events were analyzed by FACS 72 h after Ad-SceI infection. Fold (x) indicates the relative changes in repair levels compared to the Ad-SceI infected cells.
8.2 Repair of NHEJ-red cassette can be detected by novel qPCR assay

After validating the fluorescence-based NHEJ-red assay, the same integrated NHEJ-red cassette was used as the basis for a genomic qPCR assay that can more rapidly produce data. The qPCR assay relies on the unique combination of the NHEJ-red cassette, primer design, and qPCR conditions: after the vector is cleaved by I-SceI in both sites and resealed, it amplifies with much greater efficiency than the uncut vector starting material (Figure 8-4).

The difference in amplification efficiency between the uncut and cut amplicon is likely because the I-SceI palindromic sequences in the NHEJ-red cassette are complementary. After the initial DNA melting in the first step of PCR (90°C), during the subsequent annealing step at 60°C it becomes energetically favorable for the single-stranded NHEJ-red cassette to form a hairpin, as well as for primers to anneal to their designed target sequences. While the Gibbs Free Energies (calculated with software OligoAnalyzer 3.1) for both the 150- and 125-base amplicons to form hairpins are negative, indicating a spontaneously favorable reaction, the Gibbs Free Energy for the 150-base amplicon is more negative, indicating that it is more favorable for the uncut product to form a hairpin than the cleaved NHEJ-red cassette (Figure 8-5). The $T_M$ for both hairpin structures is also reported (68.9°C, 69.2 °C), a further indication that at the annealing temperature of 60°C it is likely these structures are present. Whereas with more traditional qPCR assays, this would be problematic, in this situation the difference in amplification efficiencies has proven to be quite fortuitous. As the 125 base product of I-SceI cleavage can be amplified and detected via SyberGreen fluorescence at levels 50-
Figure 8-3. Inhibition of ATM or DNA-PK does not inhibit I-SceI expression. (A) U87/ NHEJ-red (A2 clone) cells were infected with Ad-SceI, exposed to KU-55933 (10 µM) or KU-57788 (1 µM) 2 h post-infection, and harvested at 2, 4, 8, and 16 h after addition of adenovirus. Uninfected cells were harvested in parallel at 4 and 16 h. Membranes were blotted with anti-HA to visualize I-SceI expression, and with anti-β-actin as a control. In (B) U87/ NHEJ-red (A2 clone) cells were simultaneously treated with Ad-SceI, and KU-55933 (10 µM) and harvested at 1-8 h post-infection. Uninfected cells were harvested at 8 h. Membranes were blotted as in (A).
Figure 8-4. Repair of NHEJ-red cassette can be detected by qPCR. (A) U87/ NHEJ-red (A2 clone) cells were infected with Ad-SceI, and harvested at 4, 6, 8, 24, and 72 h after addition of adenovirus. Uninfected A2 cells and U87 cells were also harvested at 72 h. DNA extraction and qPCR were carried out as described in the Materials & Methods. PCR bands were separated on an 8% non-denaturing polyacrylamide gel and detected by ethidium bromide staining. (B) Samples visualized in (A) were amplified in qPCR reactions. Columns indicate the relative SyberGreen fluorescence levels detected when amplifying samples with NHEJ cassette primers normalized to β-actin signal in parallel reactions. Fold (x) indicates the relative changes in repair levels compared to the uninfected control. (C) Continued on the following page.
Figure 8-4. Repair of NHEJ-red cassette can be detected by qPCR. (C) U87/ NHEJ-red (A3 clone) cells were infected with Ad-SceI, and harvested at 1, 2, 3, 4, 5, and 6 h after addition of adenovirus. Uninfected A3 cells were also harvested at 6 h. DNA extraction and qPCR were carried out as described in the Materials & Methods. Fold (x) indicates the relative changes in repair levels compared to the uninfected control. (D) Samples visualized in (C) were amplified in qPCR reactions. Columns indicate the relative SyberGreen fluorescence levels detected when amplifying samples with NHEJ cassette primers normalized to β-actin signal in parallel reactions. Fold (x) indicates the relative changes in repair levels compared to the uninfected control.
100x higher than the uncleaved 150 base starting material (Figure 8-4), this allows for rapid, quantitative detection of resealing events of the NHEJ-red vector. As previously demonstrated in a recent publication (Golding et al, 2009), repair kinetics of the NHEJ-red vector were determined with U87 cells, into which the NHEJ-red vector had independently integrated, creating two separate clones of (A2, A3) of NHEJ-red U87 cells (Figure 8-4). Consistent with earlier findings, time course experiments showed that resealed DNA products can be detected between 4 – 8 h post Ad-SceI infection, but not at 3 h and earlier (see Figure 3A in (Golding et al, 2009)). The transition from the uncut DNA amplicon of 150 bases to the resealed DNA product of 125 bases is shown via gel electrophoresis (Figure 8-4A, C); these same genomic samples were amplified via the qPCR assay (Figure 8-4B, D). The relative change in fluorescence detected in these genomic sample illustrates the assay: using the same PCR primer set, the 125 base product is detected at levels 6, 75, and 120-fold higher compared to identical, but uninfected NHEJ-red U87 cells (clone A2) at 8, 24, and 72 h (Figure 8-4B). As SyberGreen is a non-specific dye that binds to double-stranded DNA, PAGE after qPCR allows for verification of the size of amplified qPCR products. Similar kinetics are observed in the A3 clone: fluorescent signal can be detected at levels 76-fold and 57-fold higher than uninfected cells at 5 and 6 h post-infection (Figure 8-4C), corresponding to an identical shift from the 150 base amplicon to the 125 base amplicon after Ad-SceI infection via gel electrophoresis (Figure 8-4D). No increase in signal over uninfected cells can be detected prior to 5 h post-infection. These kinetic results are typical, but not identical, to what can be observed each time the qPCR assay is repeated; the variations in
confluency and growth phase of NHEJ-red U87 cells may contribute to slight variations seen in the fold differences observed at times after infection.

The effects of inhibiting either DNA-PK or ATM were investigated with the qPCR assay and compared to results obtained when examining the DsRed+ events as different measures of NHEJ. After 20 h of Ad-SceI infection, KU-55933 (10 µM) caused a 76% reduction in NHEJ compared to cells infected with Ad-SceI alone (p = 0.0014) (Figure 8-6A). A similar result was observed after 22 h of Ad-SceI infection: an 81% reduction in NHEJ when cells were treated with KU-55933 (10 µM) (Figure 8-7). Inhibiting DNA-PK with KU-57788 (1 µM) reduced NHEJ by 31%, but this difference was not statistically significant (p =0.0750). This dramatic KU-55933-induced reduction in NHEJ was specific to the U87-A2 clone; whereas a reduction could also be observed in the U87-A3 clone, KU-55933 typically induced a 40-60% reduction in NHEJ in the U87-A3 clone (Figures 8-6B and 8-11B), under culturing conditions of either serum-starvation or serum-supplementation. Other modifications in performing the assay included adding DNA kinase inhibitors either 1 or 2 h after adding Ad-SceI to cell media, or simultaneously with Ad-SceI. Provided that the kinase inhibitors were added before the appearance of I-SceI protein, this variation did not appear to impact the reductions observed in NHEJ. The reductions measured by qPCR are comparable to the reduced amounts of DsRed+ events measured by FACS after exposure to lower concentrations of kinase inhibitors shown in Figure 8-2. Additionally, the reductions seen via the qPCR assay could be complemented by DsRed fluorescence imaging at 72 h, at which point a ~70% reduction in NHEJ can still be observed in KU-55933 treated U87/NHEJ-red A2 cells (Figure 8-7). Determining DNA break resealing via qPCR is a more direct approach
Figure 8-5. The uncut NHEJ-red cassette amplifies less efficiently than the cut NHEJ-red cassette. (A) Schematic of DNA melting and annealing stages of PCR with 150- and 125-base NHEJ-red amplicons. (B) Hairpin structures of 150- and 125-base NHEJ-red amplicons predicted with OligoAnalyzer 3.1 software with indicated Gibbs Free Energy values (in kcal/mole) calculated at 60°C, and $T_m$. 

**A**

pre-SceI digestion:
150 bp amplicon

post-SceI digestion and repair:
125 bp amplicon

melt at 95°C

re-anneal at 60°C

Forms hairpin rather than amplifying

**B**

150 bp amplicon
$\Delta G = -4.2$
$T_m = 68.9°C$

125 bp amplicon
$\Delta G = -3.0$
$T_m = 69.2°C$
that eliminates the possibility of transcriptional or translational effects that may compromise fluorescent protein based DNA repair assays (Golding et al, 2009).

8.3 Inhibiting ATM reduces the fidelity of NHEJ

In addition to assaying the incidence of NHEJ repair, it is also of great interest to assay another variable feature of NHEJ: the level of fidelity of the repair. U87/NHEJ-red (A2 clone) cells were infected with Ad-SceI and harvested for DNA extraction 24 h post-infection. If repair of the NHEJ-red cassette occurs in a manner determined to be “perfect” - consisting of annealing of the complimentary 3’ 4-base overhangs produced by I-SceI cleavage at both sites, followed by 2-base fill-in (AA) - the repair joint contained within the qPCR amplified sequence will read TTATAA, a sequence that corresponds exactly to the recognition sequence for endonuclease PsiI. A proportion of the PCR-amplified 24 h post-Ad-SceI infection sequence does exhibit this perfect repair sequence. The products of DNA extraction were cloned into pCR2.1, and 40 clones were sequenced. The five most common repair joint clones are shown in Table 8-1; perfect repair (TTATAA) represented 35.7%, or 10 out of 28 of the clones that contained the insert (28 clones out of 40). This indicates that perfect or “high-fidelity” NHEJ is not an uncommon event. Of these high fidelity repair clones, 4 were incubated with PsiI, an endonuclease specific for the sequence corresponding to the perfect repair joint: TTATAA. Clones that qualified as high fidelity were cleaved by PsiI endonuclease, whereas imperfect NHEJ repair clones remained intact. These differences in cleavage were visualized via gel electrophoresis (Figure 8-8).
Figure 8-6. ATM promotes repair of NHEJ-DsRed cassette. (A) U87-A2 clone cells were starved for 2 days prior to infection, infected with Ad-SceI, exposed to KU-55933 (10 µM) or KU-57788 (1 µM) 2 h post infection, then harvested at 20 h post-infection. DNA extraction and qPCR were carried out as described in the Materials & Methods. Samples (n=3) were loaded with β-actin controls in parallel. Fold (x) indicates the relative changes in repair levels compared to the mean of the Ad-SceI-infected samples. RQ values were calculated, set Ad-SceI 20 h infection = 1. (B) U87-A3 clone cells were either serum-starved for 2 days prior to infection or supplemented with serum, as indicated, infected with Ad-SceI and simultaneously treated with KU-55933 (10 µM). Cells were harvested at 20 h post-infection (n=3) and treated as in (A). Columns, Relative Quantities; Error bars, SE for data sets n=3. *, p<0.05; **, p< 0.01.
Figure 8-7. NHEJ repair accumulates and is promoted by ATM for up to 3 days. (A) U87/NHEJ-red A2 clone cells were serum-starved for 2 days prior to infection, infected with Ad-SceI and simultaneously exposed to KU-55933 (10 μM), then harvested at 22 h post-infection (n=4). An additional plate from each treatment group was allowed to continue and harvested 72 h post-infection. Immediately prior to harvesting, plates were imaged using the Typhoon with settings for DsRed fluorescence imaging. DNA extraction and qPCR were carried out as described in the Materials & Methods. Samples were loaded with β-actin controls in parallel. Fold (x) indicates the relative changes in repair levels compared to the mean of the Ad-SceI-infected samples. RQ values were calculated, set Ad-SceI 22 h infection = 1. Columns, Relative Quantities; Error bars, SE for data sets n=4. ***, p< 0.001. (B) Continued on following page.
Figure 8-7. NHEJ repair accumulates and is promoted by ATM for up to 3 days. (B) The 3-day timepoint was repeated in a qPCR assay and DsRed imaging in a subsequent experiment with U87/NHEJ-red A2 clone cells serum-starved for 2 days prior to infection, infected with Ad-SceI and simultaneously exposed to KU-55933 (10 µM). RQ values were calculated, set Ad-SceI 3 day infection = 1. Columns, Relative Quantities; Error bars, SE for data sets n=3. ***, p< 0.001.
Table 8-1. Fidelity of DNA Repair. U87/ NHEJ-red (A2 clone) cells infected with Ad-SceI were harvested for DNA extraction 24 h post infection. Recovered products were cloned into pCR2.1 vector. Using primers internal to the pCR2.1 vector, 40 clones were sequenced. The five most common sequences observed in clones that contained the insert are shown. Perfect repair of the cassette is qualified as reading TTATAA and is represented in (35.7%) of all clones that contained the insert.

<table>
<thead>
<tr>
<th>sequence</th>
<th>N</th>
<th>% from all inserts</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTATAA</td>
<td>10</td>
<td>35.7%</td>
</tr>
<tr>
<td>TTAT</td>
<td>4</td>
<td>14.2%</td>
</tr>
<tr>
<td>TATAAA</td>
<td>3</td>
<td>10.7%</td>
</tr>
<tr>
<td>TTATA</td>
<td>3</td>
<td>10.7%</td>
</tr>
<tr>
<td>ATAA</td>
<td>1</td>
<td>3.6%</td>
</tr>
</tbody>
</table>

21 74.9%
Figure 8-8: Fidelity of DNA repair can be assayed via $PsiI$-mediated cleavage. U87/NHEJ-red (A2 clone) cells infected with Ad-SceI were harvested for DNA extraction 24 h post infection. Recovered products were cloned into pCR2.1 vector. Using primers internal to the pCR2.1 vector, 40 clones were sequenced. Perfect repair of the cassette is qualified in the Materials & Methods as reading TTATAA and is represented above in clones 1 – 4. Repair joint sequence of clone 5 reads TTAT. DNA from each clone was treated with $PsiI$ endonuclease (+) or control buffer (-). Products from $PsiI$ cleavage were visualized on an agarose gel and detected by ethidium bromide staining.
This assay was extended to monitor high fidelity NHEJ after exposure to repair kinase inhibitors. U87/NHEJ-DsRed (A2 clone) cells were infected with Ad-SceI, exposed to KU-55933 (10 µM) or KU-57788 (1 µM), and harvested for DNA extraction 24 h post-infection (Figure 8-10). After PCR amplification, products were treated with PsiI endonuclease or control buffers and visualized via gel electrophoresis. This PsiI endonuclease treatment could not be used directly on DNA amplified in the SyberGreen qPCR assay, as samples amplified with reaction mix containing SyberGreen were found to be refractory to PsiI, but not BamHI, cleavage (Figure 8-9). Bands of interest including the 125-base resealing product, and a 77-base product known to be the product of PsiI cleavage of the 125-base product, were visualized by ethidium bromide staining, then quantified via densitometry (Figure 8-10A). As ethidium bromide binds DNA relative to the length of the DNA molecule, the densitometric measurements were adjusted accordingly. An alternate approach was also taken to visualize the bands of interest using 32P-labeled primer in the PCR reaction, resulting in products that could be visualized with phosphorimaging (Figure 8-10B and C), for which the signal would be linearly proportional to the number of DNA molecules present, rather than the number and length of DNA, as is the case with ethidium bromide staining. Relative to Ad-SceI infected cells, cells treated with 10 µM KU-55933 displayed a 40% reduction of the 77-base product, indicating a significant reduction in high fidelity repair at 16 and 24 h after Ad-SceI infection. Cells treated with 1 µM KU-57788 did not exhibit a consistent reduction of the 77-base product. Additionally, it is of note that the percentage of high-fidelity repair observed by sequencing (35.7%) was similar to the numbers obtained from
Figure 8-9: Inclusion of SyberGreen in PCR amplification prevents PsiI cleavage. (A) U87/ NHEJ-red (A2 clone) cells infected with Ad-SceI were harvested for DNA extraction 24 h post infection. Genomic DNA was amplified with SyberGreen 2X PCR mix and qPCR primers, one of which was 5’ radiolabeled with $^{32}$P. PCR products were eluted through QIAquick PCR purification kit columns (Qiagen) and divided into three aliquots. Samples were digested overnight with control buffer, BamHI, or PsiI. Products were subjected to denaturing gel electrophoresis with a radiolabeled 81-nucleotide marker (rightmost lane), and phosphorimaged. In (B), the same A2 clone 24h infection sample was treated identically, but amplified in 2X PCR mix without SyberGreen (Roche). The 77 base band that results from PsiI cleavage is indicated (red box).
Figure 8-10. ATM promotes high-fidelity NHEJ repair. (A) U87/ NHEJ-red (A2 clone) cells were infected with Ad-SceI, exposed to KU-55933 (10 µM) or KU-57788 (1 µM) 2 h post-infection, and harvested at 24 h after addition of adenovirus. Uninfected A2 cells were also harvested at 24 h. DNA extraction and qPCR were carried out as described. PCR products from each sample were treated with PsiI endonuclease (+) or control buffer (-). Products from PsiI-mediated cleavage were visualized on an 8% non-denaturing polyacrylamide gel and detected by ethidium bromide staining. Densitometry was performed on products of PsiI cleavage (+) as described in Materials & Methods. Densitometric information is reported below the 77 (red rectangles) nucleotide bands of interest as a percentage of the combined densitometries of the 125 (blue rectangles) nucleotide and 77 nucleotide bands. (B) Continued on following page.
Figure 8-10. ATM promotes high-fidelity NHEJ repair. (B) U87/ NHEJ-red (A2 clone) cells were infected with Ad-SceI, exposed to KU-55933 (10 µM) or KU-57788 (1 µM) 2 h post-infection, and harvested at 16 h and 24 h (depicted in (C)) after addition of adenovirus. DNA extraction was carried out as described, and qPCR was performed as described but with a radiolabeled 3’ primer, resulting in 32P-labeled PCR products. PCR products from each sample were treated with control buffer, BamHI, or PsiI endonuclease. Products from were visualized on an 8% non-denaturing polyacrylamide gel and detected by phosphorimaging. Densitometry was performed on products of PsiI cleavage (+) as described in Materials & Methods. Densitometric information is reported below the 77 (red rectangles) nucleotide bands of interest as a percentage of the combined densitometries of the 125 (blue rectangles) nucleotide and 77 nucleotide bands. (C) Continued on following page.
Figure 8-10. ATM promotes high-fidelity NHEJ repair. (C) U87/ NHEJ-red (A2 clone) cells were infected with Ad-SceI, exposed to repair kinase inhibitors, harvested, amplified, visualized and quantified as in (B). Depicted above are the 24 h post-infection samples.
PsiI digestion (22.7% - 48.5%), a further indication that high-fidelity NHEJ is not an uncommon event in cells.

8.4 Changes in histone modification can in turn modulate NHEJ

Histone modifications have long been associated with DNA repair, serving as instigators of architectural changes in chromatin, or acting as markers of DSBs (Rogakou et al, 1998). To test current hypotheses regarding how ATM signaling promotes access to DNA breaks located within regions of heterochromatin (Beucher et al, 2009, Goodarzi et al, 2008, Kim et al, 2009), we sought to determine if global changes in chromatin decondensation brought about by trichostatin A (TSA) treatment would reverse the inhibition of NHEJ seen after treatment with KU-55933. TSA is a histone deacetylase (HDAC) inhibitor which prevents the removal of acetyl groups from histone lysine residues, allowing chromatin to remain in a less condensed and transcriptionally active conformation. TSA has been characterized as radiosensitizer of glioma and colon carcinoma in vitro (Kim et al, 2004, Biade et al, 2001), but has proven to be too cytotoxic and unstable in vivo to be pursued clinically (Blagosklonny et al, 2002). However, valproic acid, another HDAC inhibitor, has shown to be more tolerable and is currently in a phase II clinical trial for high grade glioma in combination with temozolomide and radiation therapy (NCI-06-C-0112). U87/NHEJ-DsRed (A3 clone) cells were exposed to TSA for 24 h, then harvested and blotted against hyperacetylated histone H4 to show that TSA treatment does effectively induce acetylation in our cell model (Figure 8-11A). To test if this acetylation interfered with the inhibition of NHEJ seen after KU-55933 treatment, U87/NHEJ-DsRed (A3 clone) cells were treated with KU-55933 (10 µM) and
TSA (10 µM) 2 h after Ad-SceI infection, and the qPCR NHEJ assay was performed on samples harvested after 20 h infection. TSA treatment with Ad-SceI infection had no effect on NHEJ, but notably, the combination of both KU-55933 and TSA treatment reversed the observed KU-55933 inhibition seen in parallel samples (Figure 8-11B).

8.5 Inhibiting ATM had no effect on I-SceI incision of the NHEJ-red cassette

To confirm that any effects of ATM inhibition on chromatin modification did not prevent I-SceI cleavage of the NHEJ-red cassette, adaptor ligation-mediated PCR was performed to visualize NHEJ-red incision (Figure 7-2). Prior to PCR amplification, genomic samples were incubated with a double-stranded linker molecule designed to complement the 3’ 4-base overhangs produced by I-SceI. After ligation to the adaptor, qPCR reactions were performed with a 5’ primer specifically designed against the adaptor sequence. U87/ NHEJ-red (A3 clone) genomic samples previously shown to exhibit uncut and cut repair products (Figure 8-4C) were ligated and amplified with adaptor-specific primer, and exhibited incision beginning at 2-3 h post-infection. Samples that were not ligated to the adaptor prior to PCR did not amplify, nor did the 6 h sample that was not infected with Ad-SceI, demonstrating the specificity of the assay (Figure 8-12). The assay was then used to assess the effects of 10 µM KU-55933 on incision, where KU-55933 did not inhibit the generation of I-SceI-incision products (Figure 8-13) which again appeared beginning at 2-3 h post-infection. Considered together, these results indicate that inhibiting ATM does not inhibit either production of SceI or incision of the NHEJ-red cassette, leaving ATM-mediated repair of the NHEJ-red cassette as the likely target of KU-55933 inhibition, supporting the model depicted in Figure 8-14.
Figure 8-11. NHEJ can be modulated by changes in histone modifications. (A) U87/NHEJ-red (A3 clone) cells were exposed to 0, 1, 3, or 10 µM TSA for 24 h and harvested for Western blotting against hyperacetylated Histone H4. Membrane was also blotted against β-actin control. (B) U87/NHEJ-red (A3 clone) cells were infected with Ad-SceI, exposed to KU-55933 (10 µM) or KU-55933 (10 µM) and TSA (10 µM) 2 h post-infection, and harvested at 20 h after addition of adenovirus. Uninfected A3 cells were also harvested at 20 h. DNA extraction and qPCR was carried out as described. Fold (x) indicates the relative changes in repair levels compared to the uninfected control. Error bars, SE for data sets n = 4; ns, not significant; *, p< 0.05.
Figure 8-12. Ligation-mediated PCR can be used to detect I-SceI cleavage. U87/NHEJ-red (A3 clone) cells were infected with Ad-SceI, and harvested at times indicated. DNA extraction was carried out as described; genomic samples are identical to those depicted in Figure 8-4C. Ligation with an adaptor was performed as described in Materials & Methods, and PCR was carried out with both ligated and unligated controls with a primer specific to the adaptor (A). Relative Quantities were calculated with cells infected with Ad-SceI for 3h set=1.0. Products from each reaction were visualized on a 12% non-denaturing polyacrylamide gel and detected by ethidium bromide staining (B).
Figure 8-13. Inhibiting ATM kinase has no effect on I-SceI cleavage. U87/ NHEJ-red (A3 clone) cells were infected with Ad-SceI, and harvested at times indicated. Parallel samples were treated with 10 µM KU-55933. DNA extraction and ligation with an adaptor was carried out as described, and PCR was carried out with a primer specific to the adaptor (A). Relative Quantities were calculated with cells infected with Ad-SceI for 3h set=1.0. Products from each reaction were visualized on a 12% non-denaturing polyacrylamide gel and detected by ethidium bromide staining (B).
Figure 8-14. Potential targets of modification in NHEJ-red repair assay. U87-NHEJ cells are infected with the replication-incompetent adenovirus expressing I-SceI (Ad-SceI) (Anglana & Bacchetti, 1999) and then may be subject to regulation at several steps within our assay system. These measurable endpoints include: (1) expression of I-SceI, (2) cleavage of HNEJ-red cassette, and (3) NHEJ repair of the NHEJ-red cassette.
8.6 ATM may affect NHEJ repair via KAP1 phosphorylation

As the phosphorylation of KAP1 has been shown to be involved in the DSB response and regulated by ATM in a wide variety of cell types (White et al, 2006, Ziv et al, 2006), including neurons (Biton et al, 2007), U87/NHEJ-DsRed (A3 clone) cells were pre-treated with 10 µM KU-55933 and then tested for their response to 5 Gy IR. Cells were harvested 30 min, 1h, and 3h post-IR, and lysates were blotted for KAP1 and phosphorylated KAP1 (serine 824). Cells exhibited robust, but transient phosphorylation of KAP1 in response to IR; this response was completely abrogated in cells treated with 10 µM KU-55933 (Figure 8-15). Future experiments will be performed with U87/NHEJ-DsRed (A2 clone) cells that have been transfected with a vector that expresses blasticidin-resistance and a shRNA against KAP1, reducing KAP1 expression by ~60% compared to untransfected cells (Figure 8-16A). As human embryonic stem cells express ~60% less KAP1 than astrocytes within the same cell lineage (Figure 8-16B), but also do not respond to ATM inhibition affecting NHEJ (B. Adams et al, in preparation), U87 cells with reduced KAP1 expression will be tested for their capacity to engage in NHEJ via the assays described above.

8.7 Modulating KAP1 expression affects NHEJ in 293B cells

293B cells were also transduced with the NHEJ-red vector, and an expression vector containing doxycycline-inducible I-SceI-HA. While accumulation of a 125-base repair product could be observed by PAGE at 5-8 h after doxycycline (Dox) was added to cell media (Figure 8-17), the 2.5-fold relative change in SyberGreen fluorescence observed in Dox-treated cells (compared to untreated 293B-NHEJ-red cells) (Figure 8-17A) is substantially less compared to the differences observed in U87 clones, possibly
Figure 8-15. ATM kinase mediates phosphorylation of KAP1 after IR. (A) U87/NHEJ-red (A3 clone) was treated with 5 Gy IR and harvested at 30 min post-IR treatment. Black triangles indicate 2-fold serial dilutions of cell lysate. Membranes were first probed for phospho-KAP1, then both total KAP1 (C terminus) anti-β-actin antibody was subsequently applied to the phospho-KAP1 membrane. (B) U87/ NHEJ-red (A3 clone) with treated with 5 Gy IR and harvested at 30 min, 1h, and 3h post-IR treatment. Parallel samples were pre-treated with 10 μM KU-55933 for 1h prior to IR treatment as indicated. Cells were scraped directly into 1X Laemmli buffer and analyzed via western blot with anti-KAP1 (C terminus), anti-phospho KAP1 (serine 824), and anti-β-actin. Membranes were loaded in parallel to probe for both KAP1 and phospho-KAP1, anti-β-actin antibody was subsequently applied to the phospho-KAP1 membrane.
Figure 8-16. KAP1 manipulation in U87/NHEJ cells and unperturbed KAP1 expression varies with differentiation. (A) U87/NHEJ-red (A2 clone) cells with a vector containing a shRNA against KAP1 were analyzed for KAP1 expression with untransfected A2 cells. Black triangles indicate 2-fold serial dilutions of cell lysate. (B) Human embryonic stem cells and astrocytes derived from the same lineage as the human embryonic stem cells were probed for KAP1 expression. Densitometric values for KAP1 expression are displayed, Blast-KAP1 K928 value is relative to U87-NHEJ-red cells set=1.0; stem cell value set is relative to astrocytes set=1.0.
due to higher levels of non-specific amplification that can be observed in the untreated 293B-NHEJ-red cells, as compared to untreated U87-NHEJ-red cells. I-Sce-I expression was also observed to increase from 2 – 7 h after Dox was added to cell media (Figure 8-17C). After verifying the functionality of the NHEJ-red cassette in this cell type, 293B-NHEJ-red cells were transfected with plasmids expressing either the empty pcDNA vector, FLAG-tagged wild-type KAP1, an shRNA against KAP1 message, or FLAG-tagged S823A/S824A KAP1 (a mutant of KAP1 designed mimic constitutively dephosphorylated sites on a serine phosphorylated by ATM (White et al, 2006)) (Figure 8-18A). The shRNA against KAP1 reduced KAP1 expression by at least 30% (a western blot with reduced amounts of lysate might reveal a greater difference, due to the high abundance of this protein), though both the shRNA and the expression of the phosphorylation-mutant KAP1 had no statistically significant effect on NHEJ as measured by qPCR (Figure 8-18B). However, 293B-NHEJ cells transfected with wild-type KAP1 correlated with a reduction in NHEJ as measured by qPCR in samples harvested 20 h after Dox-induction. It should also be noted that 293B-NHEJ-red cells did not exhibit a statistically significant decrease after treatment with 10 µM KU-55933 (data not shown).

8.8 The NHEJ-red repair cassette is functional in XLF−/− fibroblasts

To extend the cell types in which the qPCR NHEJ-red cassette can be used to measure DNA repair deficits, XLF−/− fibroblasts were transduced with lentivirus containing the NHEJ-red cassette, and a mix of infected cells were used for the qPCR NHEJ assay. Cells were infected with Ad-SceI and assayed for NHEJ at 8, 24, and 72 h post-infection, and the SyberGreen fluorescence increased 7x, 38.5x, and 108.5x,
Figure 8-17. NHEJ cassette is functional and Dox-inducible in 293B cells. 293B cells were stably transduced with the NHEJ-red cassette and a doxycycline-inducible I-SceI expression vector. Cells treated with Dox were harvested 1-8 h after addition of Dox to the cell media. DNA extraction and qPCR were carried out as described in the Materials & Methods (A). PCR bands were separated on an 8% non-denaturing polyacrylamide gel and detected by ethidium bromide staining (B). Parallel samples were harvested for western analysis. Membranes were blotted with anti-HA to visualize I-SceI expression, and with anti-β-actin as a control (C).
Figure 8-18. Transflecting additional wild-type KAP1 into 293B cells correlates with reduced NHEJ. 293B-NHEJ-red cells were transfected with pcDNA plasmids expressing either the empty pcDNA vector, FLAG-tagged wild-type KAP1, a shRNA against KAP1 message, or FLAG-tagged S823A/S824A KAP1 and analyzed via western blotting with anti-FLAG, anti-KAP1, and with anti-β-actin as a control (A). Parallel samples were treated with Dox and harvested for qPCR after 20 h of Dox induction. Samples (n=3) were loaded with β-actin controls in parallel. Fold (x) indicates the relative changes in repair levels compared to the mean of the I-SceI-induced samples. RQ values were calculated, set Dox 20 h induction = 1x. Columns, Relative Quantities; Error bars, SE for data sets n=3; **, p< 0.01 (B).
respectively, at those times tested when compared to uninfected XLF$^{-/-}$-NHEJ-red cells (Figure 8-19). This indicates that the NHEJ-red cassette is also functional in cells without the gap-filling protein XLF. Further studies with these cells will involve transducing the XLF$^{-/-}$ fibroblasts with a lentiviral construct that expresses wild-type XLF to create an acceptable control, such that it will be possible to measure if the absence of XLF has an appreciable quantifiable effect on end-joining using the NHEJ-red repair cassette.
Figure 8-19. NHEJ-red repair cassette is functional in XLF⁻/⁻ fibroblasts. XLF⁻/⁻ fibroblasts were transduced with the NHEJ-red cassette, then infected with Ad-SceI and harvested for qPCR as described. Samples (n=3) were loaded with β-actin controls in parallel. Fold (x) indicates the relative changes in repair levels compared to the mean of the Ad-SceI-infected samples. RQ values were calculated, set Ad-SceI 8 h infection = 1. Columns, Relative Quantities; Error bars, SE for data sets n=3; **, p< 0.01 (A). XLF⁻/⁻ NHEJ-red cells were and analyzed via western blotting with anti-XLF and then anti-β-actin as a control (B).
IX. DISCUSSION FOR PART II

9.1 ATM promotes NHEJ repair

The major finding of this current study is that ATM inhibition suppresses NHEJ. This finding is at odds with previously published results from our research group and others (Golding et al, 2004, Morrison et al, 2000). Morrison et al. conducted an epistatic analysis by simultaneously disrupting ATM and a second protein involved in either HRR or NHEJ. Results demonstrated that KU70-deficient (and thus, NHEJ-deficient) $ATM^{-/}$ cells had a more severe radiosensitive phenotype than Rad54-defective (HRR-deficient) $ATM^{-/}$ cells, and thus researchers concluded that ATM and HRR were involved in the same pathway. (Morrison et al, 2000). In recent work from the Valerie laboratory, a genomically-integrated I-SceI repair cassette was used to assess DSB repair events in human glioma cells. Using caffeine and dominant-negative ATM expressed from an adenovirus, it was shown that ATM affected HRR and not NHEJ at 24 and 48 h after Ad-SceI infection, using genomic extraction and subsequent PCR or a fluorescent reporter, much in the manner of the studies described here (Golding et al, 2004). That ATM is required for HRR was later confirmed using KU-55933, in which it was also found that KU-55933 inhibits HRR, even under conditions of a single I-SceI-induced DSB (Golding et al, 2007). ERK kinase activity and ATM kinase activity appear to be in a regulatory feedback loop: PD184352 (which is a specific inhibitor of ERK1/2) compromises IR-induced ATM phosphorylation, but so too KU-55933 blocks ERK1/2 phosphorylation by 60% to 75% (Golding et al, 2007).

However, other research groups have also identified that ATM is required for a subset (approximately 10-20%) of slow DNA repair in both non-cycling G0 cells (Riballo
et al, 2004) and cycling cells in G₂ (Beucher et al, 2009, Deckbar et al, 2007). ATM was also found to be functionally epistatic with Artemis in regard to repair of DSBs in these studies, and Beucher et al. concluded that the DNA repair that occurs in G₂ that is dependent on ATM and Artemis is HRR, whereas the DNA repair dependent on ATM and Artemis in G₀/G₁ is NHEJ (Beucher et al, 2009). Originally, it was suggested that this 10-20% of DSBs might represent complex breaks that require end-processing by Artemis, and ATM would be required to phosphorylate Artemis (as it was shown to do, both in vitro and in vivo (Riballo et al, 2004)) such that Artemis could adequately mediate resection of long DNA overhangs. Since then, further work with in vitro assays with purified proteins has shown that although ATM does phosphorylate Artemis on S645, Artemis phosphorylation is dispensable for its own endonuclease function. Instead, it is the autophosphorylation of DNA-PK on what is known as the ABCDE cluster that mediates the endonuclease activity of Artemis by either remodeling the DNA or remodeling proteins around the DNA such that Artemis has access (Goodarzi et al, 2006). The authors suggested that ATM might be required for Artemis-dependent DSB repair in vivo (but notably is not required for Artemis-mediated V(D)J recombination) after IR due to its potential involvement in chromatin modifications that allow repair factors access to DSB sites. Further work by the same research group strengthened and clarified the hypothesis, which our results largely support: ATM is required to facilitate access to DSBs within regions of heterochromatin, rather than ‘complex’ breaks (Goodarzi et al, 2008).

Similarly, results from research groups headed by Jeggo and Löbrich and our results that KU-55933-mediated inhibition of ATM suppresses NHEJ also contradict
some of our previous findings in data obtained from cell-free extracts (Povirk et al, 2007a). Those findings showed that KU-55933 did not inhibit DNA end-joining or reduce repair fidelity, however, the disparities between these two sets of results highlight the advantages of the NHEJ-red repair cassette and further support our hypothesis. Results obtained from incubating short DNA oligomers or plasmid substrates in cell-free extracts would not have the capacity to evaluate the effects of signaling on chromatin structure. Given that we are proposing that ATM inhibition is being mediated via that particular cellular mechanism, not seeing an effect of KU-55933 in the instance in which there is no higher order chromatin to decondense supports our hypothesis. Similar to our current set of results, Povirk, et al. found that the fidelity of end joining was not affected by the mutant DNA-PKcs alleles or treatment with KU-57788 (Povirk et al, 2007a).

The fidelity with which NHEJ can repair ends is dictated in part by the type of ends present. The complementary cohesive ends generated by I-SceI have 5′-phosphate and 3′-OH groups that can be religated in an error-free manner, whereas more complicated breakage events, such as the lesion-clusters formed by ionizing radiation, may prove to be too damaged to sustain accurate repair. Other groups have previously shown with I-SceI-based repair cassettes that end joining events that are accurate or maintain 1 or more of the 4 3′-protruding nucleotides generated by I-SceI at the double-strand ends are Ku/XRCC4-dependent (canonical-NHEJ), whereas events associated with deletions that remove the 4 3′-protruding nucleotides at the resealed junction are hallmarks of alternative NHEJ (Guirouilh-Barbat et al, 2004, Guirouilh-Barbat et al, 2007) (see Supplementary Figure 2 in Rass et al, 2009). Our findings from sequencing
end-joining events in cells without DNA kinase inhibitors support that the NHEJ-red cassette is largely being repaired by canonical-NHEJ (Table 8-1).

9.2 Chromatin modulation after breaks

The hypothesis that ATM modifies chromatin structure is certainly not a new one. Relatively early in the literature, Painter and others suggested that the AT defect may be associated with differences in chromatin structure, proposing that the chromatin structure within AT cells was a suboptimal substrate for repair after damage compared to wildtype cells (Cornforth & Bedford, 1985, Painter & Young, 1980). This hypothesis was reinvigorated when it was shown that changes in chromatin structure (induced via treatment with chloroquine or TSA) activate ATM even in the absence of DSBs (Bakkenist & Kastan, 2003). Further results have demonstrated that ATM is also activated by heat shock, which occurs independently of DNA damage (Hunt et al, 2007). Bakkenist and Kastan proposed that even a single DSB would cause alterations in megabases of genomic DNA, and that these chromatin alterations would serve as a mechanism to amplify the repair response.

The best characterized DNA damage-induced histone modification is phosphorylation of the histone variant H2AX (γH2AX) (Rogakou et al, 1998), which extends to megabases of DNA flanking each DSB (Rogakou et al, 1999) and in foci colocalizes with other repair factors Rad50, Nbs1, and Brca1 (Paull et al, 2000). Although mice lacking H2AX are genomically unstable (Celeste et al, 2002), H2AX is dispensable for the initial recruitment of DSB response proteins to DSBs and the signaling of DNA damage (Celeste et al, 2003). However, after the initial recruitment to
DSBs, in the absence of γH2AX numerous factors, including Nbs1, 53BP1 and Brca1, subsequently fail to form radiation-induced foci. Thus, γH2AX is not the primary signal required for the redistribution of repair complexes to damaged chromatin, but may somehow provide a docking site within chromatin for DNA damage response proteins. It has been proposed that post-translational modification of histones may serve as a second order mechanism of information storage and processing beyond the linearly encoded information in the genome (Jenuwein & Allis, 2001). Additional findings on histone modifications in response to DNA damage have prompted a further model: DSBs trigger various histone modifications, including acetylation, deacetylation, methylation, and phosphorylation, and these modifications cooperate to control dynamic chromatin remodeling in the microenvironment surrounding a DSB, representing a DNA repair-specific histone code (Fernandez-Capetillo & Nussenzweig, 2004). Active chromatin reconfiguration could serve to increase the local concentration of end joining factors, or limit the diffusion of broken DNA ends until the break is repaired. In mammalian cells, it has been observed that immediately after DSB induction via UV laser or γ-irradiation, chromatin-containing DSBs undergo an energy-dependent local expansion (a 40% reduction in the density of chromatin fibers). The initial decondensation of chromatin at sites of DSBs occurs independently of ATM and γH2AX, but the histone modification may be essential for maintaining the decondensed and accessible state of chromatin (Kruhlak et al, 2006) or for maintaining other DNA repair proteins at sites of damage (Soutoglou & Misteli, 2008).

Using real-time fluorescence microscopy it was shown that these points of damage also exhibit limited diffusional mobility (Kruhlak et al, 2006). The limited
diffusion of the DNA ends is somewhat expected and has been found to be dependent on the DNA end-binding protein KU80 (Soutoglou et al, 2007), which forms an asymmetric ring around the two broken ends and functions to align broken chromosome termini at the site of repair (Downs & Jackson, 2004). The limited diffusion of DNA ends is highly advantageous for accurate DNA repair. While it is unknown how broken chromosome ends find their translocation partners within the cell nucleus, it has been observed that translocations preferentially occur among spatially proximal regions of the genome (Meaburn et al, 2007). The relative immobilization of broken chromosome ends supports the “contact-first” theory of how translocations form: end joining is likely to occur between DNA ends that are spatially proximal at the time of breakage, rather than DNA ends formed at distant areas scanning the nuclear space for potential partners (Soutoglou et al, 2007).

9.3 ATM signals to KAP1 to modulate chromatin

The mechanism proposed for ATM facilitating access to heterochromatin is via signaling to KAP1 (Goodarzi et al, 2008). Using a combination of ATM−/− (AT5BIVA) and DNA-PK−/− (MO59J) cells, and specific and non-specific inhibitors, KAP1 was shown to be redundantly phosphorylated by ATM, DNA-PK, and ATR on serine 824 (White et al, 2006), though it was suggested that the ATM/ATR pathway might be the dominant KAP1 signaling pathway, as MO59K and U2OS cells pre-treated with caffeine blocked phosphorylation of KAP1. White et al. also demonstrated that phosphorylated KAP1 rapidly localizes to sites of DNA damage. Other research has shown a dependence on ATM for the phosphorylation of KAP1 (Ziv et al, 2006) (Figure 8-15), and that the role
of KAP1 was downstream of ATM in the DNA damage response. When U2OS cells were treated with either siRNA against KAP1, or had endogenous KAP1 replaced with a non-phosphorylatable version (S824A) of KAP1, cells were hypersensitized to neocarzinostatin (sensitization of siKAP1 treated cells was equivalent to siATM treated cells, S824A cells were intermediate between siATM and untreated wild-type cells). Using microbeam laser-directed DNA damage, researchers demonstrated that within the first 5 minutes after irradiation, KAP1 phosphorylation was exclusive to damaged sites (denoted by $\gamma$H2AX foci), and within 15-45 minutes phosphorylated KAP1 became pan-nuclear. Researchers concluded that KAP1 maintains chromatin condensation, and that the phosphorylation of KAP1 inhibits its ability to condense chromatin, allowing DNA repair factors access to sites of damage (Ziv et al, 2006). These results were supported by a second study that demonstrated that KAP1 phosphorylation was eliminated by treatment with KU-55933 in human neurons (Biton et al, 2007), and our findings (Figure 8-15).

Goodarzi et al. reported that in immunofluorescent assays KAP1 stained more strongly within heterochromatin (versus euchromatin) regions, and also observed that the $\gamma$H2AX foci that persist in the presence of KU-55933 (or that are induced in ATM$^{-/-}$ fibroblasts) also juxtapose with heterochromatic regions (Goodarzi et al, 2008). Knockdown of KAP1 in cells treated with KU-55933 completed DSB repair with wild-type kinetics. This biochemical double-negative can be interpreted to mean that disruption of KAP1 removes the requirement for ATM for repair of the 20% of breaks within heterochromatin. Further evidence was shown that ATM signals to KAP1 to access breaks within compacted chromatin: the expression of the non-phosphorylatable
S824A mutant form of KAP1 induced a DSB repair defect with or without inhibition of ATM. Additionally the effect of knockdown of heterochromatin protein 1 (HP1) was similar to that of KAP1 knockdown, in that it induced normal repair kinetics in the presence of the ATM inhibitor. Although this work and other results described above supports the role of ATM signaling to heterochromatin, the possibility that DNA lesion complexity may play a role in some breaks being refractory to repair cannot be excluded: heterochromatin could interfere with the processing phase of NHEJ. The NHEJ-red repair cassette used in our studies would not have the capacity to evaluate this hypothesis, as it is unlikely that I-SceI generated breaks would require any Artemis-mediated processing prior to end joining.

Some of our past data were negative in regard to NHEJ repair of an I-SceI cassette being unaffected by caffeine-inhibited ATM (Golding et al, 2004). Here we also have reported inconsistent results in the two NHEJ-red U87 clones: ATM-mediated inhibition of end joining is greater in the A2 clone (76-81%) than the A3 clone (43-46%) at 24 h after Ad-SceI infection. These differences within our own data and data reported by others can be reconciled by hypothesizing that in the cases in which data was negative – that is, inhibiting ATM did not inhibit NHEJ - or less positive than the results found in the U87-A2 clone, the DNA repair cassettes in question had integrated into more transcriptionally active and accessible chromatin. Thus, NHEJ repair of these more accessible cassettes would not have required ATM-mediated chromatin remodeling.

Further results with our NHEJ-red cassette support the findings of Goodarzi et al., who demonstrated that siRNA (small interfering RNA) of key heterochromatic proteins, including KAP1, HP1, and HDAC 1/2, relieved the requirement for ATM for DSB repair
(Goodarzi et al, 2009). We have shown that treatment with TSA, an HDAC inhibitor, induces hyperacetylation of histone H4 and relieves the requirement for ATM signaling in repair of the NHEJ-red cassette. Furthermore, while we have shown that inhibiting ATM inhibits repair of the NHEJ-red cassette, we have also provided evidence that this inhibition is not an artifact of the NHEJ-red cassette. Data from western blotting has shown that inhibiting ATM with KU-55933 does not reduce the kinetics of expression of HA-tagged I-SceI (Figure 8-3), and data from ligation-mediated PCR supports that KU-55933 does not slow the expressed I-SceI from accessing and cleaving its recognition sequence within genomic DNA (Figure 8-13). It is also notable that the kinetics of the NHEJ-red repair cassette, in which repair products can be seen as early as 1 – 2 h post Ad-SceI expression (Figures 8-3, 8-4), are consistent with other research regarding the length of time required to accomplish NHEJ in mammalian cells (Mao et al, 2008). However, our data from 293B cells has failed to replicate the findings that the expression of the non-phosphorylatable S824A mutant form of KAP1 induced a DSB repair defect with or without inhibition of ATM (Figure 8-18B) (Ziv et al, 2006). This may be due to the high mitotic index of 293B cells, which fail to growth-arrest in the same manner as U87 cells in response to serum-starvation. It is also of note to consider the origin of 293B cells: embryonic kidney. KAP1 mediates developmentally-regulated functions, and while it is not required before embryonic day 5.5, KAP1−/− mouse embryos die soon after, indicating that KAP1 is required for early post-implantation development (Cammas et al, 2000). It has been demonstrated that over the course of differentiation in vitro, KAP1 localization changes from a diffuse nuclear distribution to localization to heterochromatin, likely mediated via its interaction with HP1 (Cammas et al, 2002).
have also shown that KAP1 expression increases over the course of *in vitro* differentiation (Figure 8-16B), which correlates with another finding: the extent which NHEJ is mediated by ATM also increases as cells differentiate *in vitro* (B. Adams and Valerie, manuscript in preparation). 293B cells may not be a suitable model cell line to interrogate repair in regions of heterochromatin, and have not been pursued further. Rather, future experiments are focused on modulating KAP1 expression in U87 clones to then examine the consequences on repair (Figure 8-16A).

Our finding that ATM inhibition reduces the fidelity of end-joining products (Figure 8-10) is novel. It is plausible that this reduction in fidelity is due to preserving the condensation of chromatin after DNA breakage, which may prevent canonical end joining factors from accessing DNA ends. However, one cannot exclude the possibility of another mechanism: that ATM may signal to specific end-joining factor(s) required for some particular aspect of end joining, such as limiting DNA end mobility, synapsis, or gap-filling. Surprisingly, initial experiments in XLF-/- fibroblasts containing the NHEJ-red cassette indicate that these cells are also able to form NHEJ repair products (Figure 8-19). Sequencing data from the 24 h Ad-SceI XLF-/- sample indicates that these repair products have substantially reduced fidelity at the repair joint compared to the sequenced repair products from the U87-A2 clone, but that some gap-filling did indeed take place (data not shown). This is in contrast to our group’s recent findings in cell extracts, that a DNA substrate requiring gap-filling (which would be performed by polymerase lambda or polymerase mu) prior to ligation of partially complementary 3’ overhangs required XLF/Cernunnos (Akopiants et al, 2009). However, considering the difference in cell types between glioma and fibroblasts, and the potential difference in integration site of
the NHEJ-red cassette (a difference that we propose is critical for determining the requirement for ATM signaling) in both cell lines, a more relevant comparison for the XLF<sup>−/−</sup> fibroblasts would be those same XLF<sup>−/−</sup> fibroblasts, complemented with wild-type XLF protein. This would control for both the cell-type and the integration site of the NHEJ-red cassette. Once the difference in repair efficiency and fidelity has been quantified between these cells, future experiments will include determining the affect of KU-55933 on these cells. Other research groups have shown that both ATM and DNA-PK phosphorylate XLF after radiation (ATM on serine 251, and DNA-PK on serine 245), though these phosphorylation events were not critical to complement the repair deficit in XLF<sup>−/−</sup> cells in regards to either recruitment of XLF to the DNA breaks or cell survival after irradiation (Yu et al, 2008). However, it would be of interest to determine if signaling from ATM to XLF impacts repair fidelity in vivo.

9.4 ATM signaling to AKT may regulate NHEJ via chromatin changes

AKT (also known as Protein Kinase B) promotes cell survival by phosphorylating and inhibiting components of the intrinsic cell death machinery. AKT translocates into the nucleus after exposure to insulin- and growth factor-induced signals, but the nuclear functions of AKT are still being elucidated. It has been shown that AKT’s response to radiation or insulin signaling is at least partially mediated through ATM (Viniegra et al, 2005). Recently in two separate publications our group has shown that repair of the NHEJ-red cassette is blocked by AKT inhibitors (Golding et al, 2009), and that KU-60019, a new highly specific inhibitor of ATM, reduces basal phosphorylation of AKT on serine 473, suggesting that ATM might regulate a phosphatase that acts on AKT
(Golding et al, 2009). Other research has shown that nuclear AKT regulates chromatin condensation and DNA fragmentation via at least one mechanism, by phosphorylation of acinus (Hu et al, 2005). Although this study focused on chromatin condensation associated with programmed cell death, during which time acinus undergoes apoptotic degradation, researchers proposed that nuclear AKT may have other downstream effectors that contribute to prevention of chromatin condensation. It is therefore not unreasonable to consider that ATM signaling to AKT contributes to DNA repair within heterochromatin.

9.5 Concluding hypotheses

The work presented here demonstrates that ATM promotes NHEJ repair. First, we demonstrated that the NHEJ-red cassette provides a working, *in vivo* model of DNA breakage and NHEJ repair at a physiologically relevant level of damage. This provided a system to assess the affect of ATM signaling on NHEJ. The result that inhibiting ATM inhibits NHEJ repair was not always consistent between clones that have repair cassettes integrated into presumably different genomic sites. We believe that the observed differences in inhibition can be accounted for by proposing that ATM signaling promotes NHEJ repair in sites of heterochromatin, and in agreement with others, propose that these chromatin-mediated effects are at least partially attributable to ATM signaling to KAP1. Showing that treatment with an HDAC overcompensates for the suppression of NHEJ seen after treatment with KU-55933 is as yet insufficient to fully support our hypothesis regarding the regulation of chromatin condensation by ATM. Certainly, it is possible that ATM mediates its effect through additional mechanisms: AKT is a likely candidate to
mediate chromatin-specific effects, and there are additional recently identified factors that participate in NHEJ that may be regulated by ATM phosphorylation, such as Aprataxin and PNK-like factor (APLF) (Macrae et al, 2008). To lend our hypothesis greater support, current and future directions of this work include modulating expression of KAP1 in already established glioma clones. With the NHEJ-red assay, we hope to show that KAP1 can directly modulate the efficacy of NHEJ, thus providing a mechanism of action for what may become a novel second generation chemotherapeutic drug.
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APPENDIX
In vitro complementation of Tdp1 deficiency indicates a stabilized enzyme-DNA adduct from tyrosyl but not glycolate lesions as a consequence of the SCAN1 mutation


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**ABSTRACT**

A homozygous H493R mutation in the active site of tyrosyl-DNA phosphodiesterase (TDP1) has been implicated in hereditary spino-cerebellar ataxia with axonal neuropathy (SCAN1). A homologous mutation in the active site of tyrosyl-DNA phosphodiesterase (TDP1) has been identified as the causative mutation in hereditary spinocerebellar ataxia with axonal neuropathy (SCAN1). SCAN1 is inherited as an autosomal recessive disorder.

1. Introduction

A homozygous mutation in the active site of tyrosyl-DNA phosphodiesterase (TDP1) has been identified as the causative mutation in hereditary spino-cerebellar ataxia with axonal neuropathy (SCAN1). SCAN1 is inherited as an autosomal recessive disorder that becomes apparent at adolescence; clinical features in these individuals include distal muscle weakness, absence of deep tendon reflexes, gait disturbances, and mild brain atrophy [1].

TDP1 has primarily been characterized as resolving trapped topoisomerase I cleavable complexes (TOP1cc) by hydrolyzing the normally transient tyrosyl linkage that forms between the active site of topoisomerase I (TOP1) and the 3' DNA terminus; this occurs during DNA relaxation that facilitates replication and transcription [2-4]. TDP1 can also process protruding 3'-phosphoglycolate (PG) termini on DNA double-strand breaks (DSBs) [5,6] that are formed in response to oxidative stress [7], ionizing radiation [8], and specific chemotherapeutic agents such as bleomycin [9].

Because the H493R TDP1 mutation associated with SCAN1 (hereafter referred to as SCAN1 TDP1) retains partial activity, SCAN1 cells do not provide a true null model of TDP1 deficiency. Moreover, it is uncertain how the mutation elicits the specific pathologies of SCAN1.
SCAN1, and in particular whether unresolved TDP1-linked breaks, unrepaired PC-terminated breaks, or covalent TDP1-DNA intermediates are the critical toxic lesions. As reported below, we generated a Tdp1 knockout mouse, carried out detailed behavioral analyses, and derived embryo fibroblast cell lines from these mice. These cells provided an in vitro system that permitted investigation of end-processing of these candidate DNA lesions.

2. Materials and methods

2.1. Generation of Tdp1 knockout mice

To generate both constitutive and conditional Tdp1 knockout (Tdp1<sup>−/−</sup>) mice, a Tdp1 targeting vector was constructed using pKO NT2K-1901 (Stratagene). This vector was modified by the insertion of loxp sites on either side of the PKR/neo/RGH cassette. A 4.8-kb region of the Tdp1 gene containing exons 5–7 (with 3.2 kb of intron 6 deleted) was generated by PCR from a 129/SvJ BAC clone, and a third loxp site (with an associated NheI site) was inserted into intron 5 (Fig. 1, panel ii). This arm was then inserted between the BgIII and XhoI sites of NT2K-1901. A 4.7-kb XmnI/Smal BAC clone fragment containing Tdp1 exons 8–12 was inserted into the NT2K-1901 Smal site. The Tdp1 targeting vector was linearized with Smal and electroporated into 129/SvJ embryonic stem (ES) cells. To identify the desired homologous recombinants, genomic DNA from ES cell clones resistant to both G418 and ganciclovir was screened by long-range PCR and Southern blotting. To delete the neo gene in the targeted Tdp1 allele and generate knockout and conditional knockout alleles (as shown in Fig. 1 panels iv and v), ES cells from a correctly targeted clone were electroporated with an MC1-cre expression vector and G418-sensitive clones selected. Knockout recombinants were identified by PCR and injected into blastocysts that were implanted into pseudopregnant females. Tdp1<sup>−/−</sup> mice were then generated from the resulting chimeras and were interbred to generate homozygous knockouts, as verified by PCR and Southern blotting (see Supplemental Methods and Supplemental Fig. 1 for additional details). These mice were subjected to a variety of behavioral tests, also described in detail in Supplemental Methods. In addition to a functional observational battery assessment [10], assays were employed to assess specific differences in motor performance and possible ataxia, including rotated performance and general locomotor activity measured in standard activity chambers.

All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University, and were conducted in accordance with the Animal Welfare Act, the PHS Policy on Humane Care and Use of Laboratory Animals, and the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

2.2. Generation and culture of embryonic fibroblasts

For generation of Tdp1<sup>+/−</sup>, Tdp1<sup>−/−</sup>, and Tdp1<sup>−/−</sup> mouse embryonic fibroblasts, males and females of the same or opposite genotype were mated, and 14-day embryos were dissociated, trypsinized, and plated on 0.1% gelatin. Cells were immortalized by continuous culture for 6 months in DMEM with 10% fetal bovine serum. A single Tdp1<sup>−/−</sup> clone was expanded and infected with

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(i) Wild-type Tdp1 locus (exons 5-12)

(ii) Tdp1 targeting vector

(iii) Targeted Tdp1 locus before Cre expression

(iv) Tdp1 knockout allele

(v) Tdp1 conditional knockout allele

Fig. 1. Generation of Tdp1<sup>−/−</sup> mice. Tdp1 targeting strategy. (i) Structure of the mouse Tdp1 gene, exons 5-12. (ii) Targeting vector for generation of both conditional and total knockout of the Tdp1 gene. (iii) Structure of the initial targeted Tdp1 allele prior to Cre expression. Structure of the knockout (iv) or conditional knockout (v) allele of Tdp1 following transient expression of Cre in the targeted ES cells.
a lentivirus expressing FLAG-tagged TDP1, and stable integration was verified by PCR. This infected clone was subsequently passaged as a separate cell line. Tdp1

2trimp and screened for expression of TDP1 by anti-TDP1 western blotting and an enzyme activity assay, described below. (See Supplemental Methods for further details.)

2.3. Preparation of Tyrosyl and 3' FG DNA Substrates

The 3'-FG-terminated 17-mer 5'-CGAGGAAACCGAAGACG-3' was prepared by bleomycin-mediated cleavage of 5'-CGAGGAAACCGAAGACG-3', and purified by polyacrylamide gel electrophoresis and HPLC [11,12]. The 3'-FG oligomers and a 3'-pTyr 18-mer (obtained from Midland Certified Reagents) and unmodified marker oligomers were 5',3'-P labeled with [γ-32P]ATP and T4 polynucleotide kinase, then annealed to complementary unlabeled oligos to create single strand-break (SSB) substrates [13]. A plasmid substrate containing a DNA double-strand break with FG-terminated AGC 3' overhang was constructed as described [14]. The substrate containing a DNA SSB with one 3'-FG-terminated blunt end and one recessed 3'-FG end [15] was constructed similarly.

2.4. Site-directed mutagenesis and enzyme purification

A PET plasmid expressing His-tagged human TDP1 was kindly provided by Howard Nash, NIMH. Mutants were generated using the QuickChange site-directed-mutagenesis kit from Stratagene and primers selected by the QuickChange Primer Design Program. Wild-type or mutant plasmids were freshly transformed into E. coli BL21(DE3) strain and cells were grown in 1 L LB medium supplemented with 100 μg/mL ampicillin. When the OD600 reached 0.4–0.5, 1 mM IPTG was added for induction. After 4 h, cells were harvested and dry pellets were stored at −80°C.

Cell pellets were resuspended in 12 mL lysis buffer (50 mM NaH2PO4, pH 8.0, 0.3 M NaCl, 10 mM imidazole, 1 mg/mL lysozyme, 1 mM phenylmethylsulfonyl fluoride (PMSF)). After 30 min on ice, cells were sonicated 6 × 15 s using a sonicator on 30% power from Ultrasonic Inc. Crude extracts were centrifuged at 14,000 × g at 4°C for 30 min. Soluble cell proteins were mixed with 1.5 mL Ni-NTA Superflow (Qiagen) and gently stirred 1 h at 4°C. The suspension was then added to a chromatography column and washed with 25 mL of wash buffer (50 mM NaH2PO4, pH 8.0, 0.3 M NaCl, 20 mM imidazole 1 mM phenylmethylsulfonyl fluoride (PMSF)). Bound proteins were then eluted with cell extracts containing 0.5 M imidazole. All four fractions were analyzed by SDS-PAGE electrophoresis and peak fractions were dialyzed against 21.20 mM Tris- HCl pH 8.0 overnight, passed through a 0.22 μm filter, loaded on a Pharmacia MonoQ FPLC column at 22°C and eluted with a 15-min gradient of 0–0.8 M NaCl in 20 mM Tris-HCl pH 8.0. Fractions were collected and analyzed by SDS-PAGE. Protein concentration was determined using a Pierce BCA assay (Thermo Fisher Scientific Inc.). Proteins were stored in 50% glycerol at −20°C.

2.5. Analysis of DNA end processing and repair

Organ homogenates were prepared by sacrificing mice of each genotype and dissecting the liver and brain, which were then flash frozen. Tissues were thawed and minced, then resuspended in lysis buffer (10 mM Hepes pH 7.8, 60 mM KCl, 1 mM EDTA, 0.5% NP-40, 0.5 M DTT, 100 mM PMSF, and Sigma protease and phosphatase inhibitor cocktails (catalogue numbers P8340 and P5726)) and homogenized with a Polytron tissue homogenizer (Kinematica). Extracts of MEs were prepared according to procedures described previously [16]. Protein concentrations of both tissue lysates and crude extracts were assessed with a BCA Assay (Pierce). Organ homogenates were diluted in buffer containing 50 mM Tris pH 8.0, 5 mM DTT, 100 mM NaCl, 5 mM EDTA, 10% glycerol, and 500 μg/mL BSA. A 0.5 μL aliquot of each dilution was allowed to react with 50 fmol of a radioactive 3'-pTyr oligomer substrate (see Fig. 2B), synthesized by Midland Certified Reagent Co. by a procedure described previously [17]. In 5 μL of reaction buffer containing 50 mM triethanolamine pH 7.5, 1 mM DTT, 5 mM EDTA, 100 μM dNTPs, and 1 mM ATP, and following incubation at 37°C for 1 h, samples were diluted in an equal volume of formamide containing 20 mM EDTA and denatured at 90°C for 5 min. For determining activity toward a pTyr nick substrate (see Fig. 3A), whole-cell extracts were serially diluted and incubated with 50 fmol of the substrate in the same reaction buffer described above, with the exception of 10 mM Mg2+/OAc2 substratizing for 5 mM EDTA. Typically, 3–5 μL of extract was added to a total reaction volume of 5–10 μL for 1 h unless otherwise noted. In some cases, serial dilutions of FLAG-tagged TDP1 purified from 293T cells was added to Tdp1−/− extracts [6]. To test the temporal requirements for pTyr processing, 50 ng of human TDP1 produced in E. coli was added to Tdp1−/− extracts in 5 μL reaction buffer and incubated with the radiolabeled substrate from 1 to 60 min, as indicated (Fig. 3D).

To assess activity toward PG nick and gap substrates, 20 μg of Tdp1−/− and Tdp1−/− whole cell extract was incubated with 50 fmol of either substrate in 5 μL of reaction buffer containing 50 mM triethanolamine pH 7.5, 1 mM DTT, 100 μM dNTPs, and 1 mM ATP, and either 5 mM EDTA, 2, or 10 mM Mg2+/OAc2. For the DSB substrates, extracts were incubated in 32 μL reaction buffer containing 50 mM triethanolamine pH 7.5, 1 mM DTT, 100 μM dNTPs, and 2 mM ATP, and 1.3 mM Mg2+/OAc2 with substrate for 6 h, unless otherwise noted. Some Tdp1−/− extracts were supplemented with 25 or 125 ng of wildtype, SCAN1, or H493N TDP1 protein purified from E. coli as noted. Samples were deproteinized with proteinase K, extracted with phenol and chloroform, and nucleic acids were precipitated, and then treated with BsaI and TaqI [18]. Samples were analyzed on a denaturing 20% polyacrylamide gel, wet gels were frozen and exposed on PhosphorImager screens for 1–2 days at −20°C. Phosphor images were developed with the Typhoon 9410 (General Electric) and analyzed with ImageQuant 3.3 software (Molecular Dynamics).

For fibroblast extract repair assays, unpaired two-tailed t tests were performed on triplicate data points using GraphPad Prism 3.0 (GraphPad Software Inc.). p values are indicated as: * < 0.05, ** < 0.01, *** < 0.001.

2.6. Western blotting

Extracts used in the DNA repair assays were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were exposed to anti-FLAG antibody from Sigma at a dilution of 1:1000, Abnova anti-human TDP1 (cat no. H00055775-A01) at a dilution of 1:2500, or Santa Cruz B-actin (cat no. sc-1615) antibody. Specific protein bands were detected using infrared emitting conjugated secondary antibodies; anti-mouse 680 Alexa (Invitrogen); or anti-goat IRDYE 800 (Rockland Immunochemicals), using the Odyssey infrared imaging system (LI-COR Biosciences).

3. Results

3.1. Generation of Tdp1−/− deficient mice

To assess the function of Tdp1 in vivo and to provide a source of Tdp1−/− deficient cell lines, we disrupted the endogenous mouse Tdp1 gene in ES cells by deleting exons 6 and 7, which also causes a frame-shift in translation of sequences encoded in the downstream exons (Fig. 1). The Tdp1 protein contains two HKD sequence motifs, encoded in exons 6–7 and 13–14, which together comprise the non-catalytic site of the enzyme [19]. Thus, the predicted truncated protein product of the targeted allele, which should contain only
Fig. 2. Behavior of Tdp1^{+/+} mice and deficiency in 3’ tyrosyl processing. (A) Motor activity and latency to fall from the rotarod was determined for male and female mice of each genotype and at each age: 3, 6, and 12 months in top, middle, and bottom panels, respectively. Asterisk indicates that at the 3-month assessment, Tdp1^{+/+} female mice were significantly less active than Tdp1^{+/+} males, p < 0.05. (B) A radiolabeled 5’3’-d-tyrosyl-tyrosyl substrate was treated with graded serial dilutions of brain tissue homogenates from Tdp1^{+/+}, Tdp1^{−/−}, or Tdp1^{−/−} mice for 1 h, subjected to denaturing gel electrophoresis, and phosphorimaged. The percent conversion from the tyrosyl substrate to its phosphate product was calculated by densitometry.

The first 253 amino acids of Tdp1 plus 10 out-of-frame amino acids, completely lacks the catalytic site. Our initial targeting vector also allowed for the generation of conditional Tdp1 knockout mice in which a flox site has been inserted into both introns 5 and 7 (Fig. 1, panel v), allowing for cell type-specific deletion of exons 6 and 7 upon interbreeding to lines of mice expressing Cre recombinase in specific tissues. However, only the conventional knockout mice (hereafter referred to as Tdp1^{−/−} mice) were used in the studies described below. Tdp1^{−/−} mice were born in the expected Mendelian ratio upon interbreeding of Tdp1^{+/+} mice, indicating that Tdp1 deficiency does not result in embryonic or neonatal lethality. In addition, Tdp1^{−/−} mice were fertile and had a normal lifespan. Reverse transcriptase-real-time PCR confirmed the presence of Tdp1 mRNA encoding exons 3–5 and exons 13–14, but not exons 6–7, in brain and liver tissue from Tdp1^{−/−} mice (Supplemental Fig. 1C).

3.2. Tdp1^{−/−} mice are phenotypically and behaviorally indistinguishable from Tdp1^{+/+} littermates

Visual inspection of the mice did not reveal any notable differences between genotypes. At each age, average weights were
similar across genotype for each sex, as were average body temperature and reflex sensitivity to a thermal stimulus (Supplemental Table 1). Similarly, phenotypic differences were not seen in average stride length or in any measure within the five domains of the functional observation battery, including CNS excitability, CNS activity, muscle tone/equilibrium, autonomic effects or sensorimotor effects (data not shown). Fig. 2A shows motor activity and latency to fall from the rotated male and female mice of each genotype and at each age. Statistical analyses of these data revealed that Tdp1−/− female mice (but not male mice) were significantly less active than Tdp1+/+ mice at the 3-month assessment (F(2,12)= 4.1, p<0.05). However, this difference was transient, as a difference in motor activity across genotype was not observed at later assessment times. In addition, although rotated performance declined markedly with age, rotated latencies were not significantly different across genotype at any age in either sex. Microscopic examination of brains sections did not reveal any overt differences between mice of any genotype. In particular, both granule cells and Purkinje cells in the cerebellum were present in equal numbers and appeared morphologically similar in brains of wild-type and Tdp1 knockout mice, both at 12 and 22 months of age (not shown). Altogether, under normal conditions, Tdp1−/− mice had no detectable behavioral phenotype.

3.3. Tdp1−/− cell extracts cannot process 3'-pTyr linkages

To confirm that Tdp1 activity was abolished by disruption of the Tdp1 allele, Tdp1 enzymatic activity was measured in tissue homogenates from Tdp1−/− mice (Fig. 2). Brain (Fig. 2B, Supplemental Fig. 2B) and liver (Supplemental Fig. 2C and D) tissue homogenates from a Tdp1+/+ and Tdp1−/− mouse efficiently converted a 3'-pTyr terminus on an 18 nucleotide oligomer (Fig. 2B) to a 3'-phosphate, while liver and brain homogenates from a Tdp1−/− mouse failed to process the oligomers at any detectable level. This 18-base 3'-labeled 3'-pTyr substrate mimics the cova-
dent DNA-protein linkage that would be formed between a tyrosine in TDp1 and DNA at sites of torsional stress; these linkages must be resolved and effectively rejoined by DNA ligase to prevent these sites from converting to DSBs. All the above reactions were performed in the presence of 5 mM EDTA, which prevents 3'-phosphate removal by polynucleotide kinase 3'-phosphatase (Pnkp), a magnesium-requiring enzyme.

To assess 3'-pTyr processing in the context of SSB repair, the nicked duplex shown in Fig. 3A was constructed and incubated in whole-cell extracts of MEFs derived from mice of each genotype. Accurate repair of this break would require cleaving the tyrosine-DNA linkage, removing a phosphate group, and ligating the reformed 3'-hydroxyl group to the 5' phosphate. Ligated repair products can be seen in samples treated with +/+ and −/− extracts, and these products display several distinct mobilities, possibly due to slight 3' resection at the end of the duplex (Fig. 3B; +/+, extract shown in Supplemental Fig. 3A). No such repair products were generated in −/− extracts; instead, these samples showed only a single band corresponding to the unprocessed 3'-pTyr 18-mer. Reactions with the SSB substrate were performed in buffer containing 10 mM MgCl₂, sufficient for apurinic/apyrimidinic endonuclease 1 (Apex1) and Pnkp activity. Thus, the band migrating slightly faster than the untreated substrate in the +/+ and −/− reactions is likely a 3'-hydroxyl 18-mer, generated by the combined action of TDp1 and Pnkp.

To verify that the lack of processing in −/− MEFs was due to TDp1 deficiency, the MEFs were stably transduced with a lentivirus expressing FLAG-tagged TDp1 (Supplemental Fig. 3B). As expected, only cells infected with the FLAG-TDp1 vector expressed FLAG at the expected molecular weight of TDp1, uninfected cells or cells infected with control viruses (LVTHM-DsRed, LV-IREs-DSRed) gave no signal. These same FLAG-TDp1 infected cells were subsequently shown to overexpress TDp1 in comparison to MEFs of other genotypes (lanes 3−/− comp' in Fig. 3C). Additionally, there was no evidence of any expression of a full-length or truncated TDp1 protein being expressed from the targeted knockout allele in −/− MEFs.

The ectopic TDp1 expression rescued both 3' processing and formation of repair products from the tyrosyl-modified SSB substrate (Fig. 3B). Lower concentrations of the complemented cell extract (5 and 10 μg of extract in a 10 μL reaction volume) produced bands that migrated just above the pTyr; these are likely the result of replacement synthesis after removal of the pTyr by the SSB substrate. Supplementation with +/− MEF extracts with purified TDp1 protein also effectively reinstated the ability to process and repair a 3'-pTyr SSB (Fig. 3B). Quantitative analysis of a reaction timecourse for the supplemented extract showed that the hydroxyl intermediate appeared almost immediately, substantial repair product was visible within 10 min, and repair appeared complete at 60 min (Fig. 3D). It is notable that under these conditions, a low level of slow processing and conversion to the repair product occurred in the unsupplemented +/− MEF extracts. This small amount of processing was seen in two independent experiments and may indicate an alternate, though less efficient, pathway of processing tyrosyl modifications in the context of SSBs. In summary, rescuing the TDp1 deficiency either by lentiviral infection or simply adding exogenous protein to cell lysate specifically reinstates TDp1-dependent 3'-pTyr processing.

Similar experiments were performed with duplexes that contained model free-radical-medicated SSBs, i.e., bearing 3'-pC and 5'-phosphate termini, either with or without a 1-base gap (Supplemental Fig. 4). There was no deficiency in repair of pG-terminated SSBs in TDp1−/− cells, presumably reflecting processing of these lesions by Apex1 [20]. However, the results also indicated that TDp1 could process 3' termini at these lesions when Apex1 activity was suppressed by MgCl₂ chelation (Supplemental Fig. 4B and C).

2.4. TDp1 is required for processing PG modifications on a subset of DNA DSBs

To assess whether TDp1 status affects processing of PG-modified ends on DSBs, model DSBs were constructed (Fig. 4A and B). These substrates were designed to mimic breaks that would commonly result from DNA sugar damage induced by either bleomycin [9], necrozinostatin, or radiation [8]. In which the deoxyribose frages, a base is lost, and a 3'-PG group remains. Accurate repair of a break within a 3' overhang (Fig. 4A) would require annealing the complementary CG sequences in each overhang, filling in the 1-base gap using the opposite overhang as a template, removal of the PG, and ligation. Substrates were constructed such that they were labeled with 32P either 14 (Fig. 4A) or 11 (Fig. 4B) bases from the 3' termini. These substrates were incubated in whole-cell extracts and then digested with restriction enzymes, which released short fragments that were analyzed on sequencing gels. The proposed accurate repair would therefore generate fragments of 42 nucleotides in the case of the 3' overhang DSB substrate, and 38 nucleotides in the case of the blunt-end DSB substrate.

When extracts from +/+ , −/−, +/−, and −/− comp MEFs were incubated for 6 h with the 3' overhang substrate, the +/+ , +/−, and −/− comp extract all produced shorter products that represented processing and resection of the 3'-PG 14-mer (Fig. 4C), and longer products of 16 and 34 nucleotides that represent repair of the DSB substrate (Figs. 4C and 5B), whereas no processing or repair could be observed in the −/− extract. In contrast, when extracts of all aforementioned genotypes were incubated with a radiolabeled plasmid substrate constructed to mimic a blunt-ended DSB, reactions of all genotypes formed processed intermediates and repair products (Fig. 4D). However, the −/− extract reactions uniquely exhibited a significant fraction of persistent unprocessed PG after 6 h of incubation (Fig. 4D–F), indicating a deficiency of DNA blunt-end processing in the −/− MEFs.

The longer repair product shown in Fig. 5B was seen in three independent experiments with the 3' overhang substrate and always migrated at approximately 34 bases, 8 bases shorter than the expected accurate repair product that dominates repair reactions in human cell extracts [14]. This result suggests that in MEF extracts a resection-based end joining pathway is utilized instead. Both the 3' overhang and blunt-ended substrate can be converted by 3' resection to an intermediate with self-complementary CCCG 5'-overhangs, the alignment of which would result in a 34-base repair product [14] containing an MluI cleavage site (ACCGGT). This scenario was confirmed by treating the DNA from these repair reactions with MluI, which completely eliminated the 34-base product, thus confirming its identity (data not shown).

Together, these results indicate that TDp1 is required for processing 3'-PG on overhangs, and also processes 3'-PG on blunt ends. In the absence of TDp1, other enzymes can process 3'-PG on blunt ends, but less efficiently than TDp1.

3.5. H493R (SCAN1) or H493N TDp1 cannot effectively rescue the defects in processing either 3' pTyr SSBs or 3'-PG DSBs

The mutant H493R TDp1 enzyme associated with SCAN1 has at least 25-fold lower activity than wild type, but it can under certain conditions cleave 3'-pTyr linkages and form a persistent (122−131 min) TDp1-DNA adduct intermediate [21]. In yeast, it has been shown that under conditions of elevated TDp1, H432N TDp1 (the yeast equivalent of H493N human TDp1 mutation) confers greater cytotoxicity than SCAN1 TDp1 [22]. To examine the action of mutant TDp1 in the presence of other DSB/SSB repair proteins, wild-type, SCAN1, and H493N forms of human TDp1 were generated in bacteria and purified. Since defects had been identified in both 3'-pTyr and 3' overhang PG processing in TDp1−/− extracts, these assays
were repeated in order to compare the effects of SCAN1 TDP1 and H493N TDP1 to that of complete Tdp1 deficiency.

As shown previously in Fig. 3B, supplementing −/− extract with wild-type TDP1 reinstates the processing and repair of the pTyr nick substrate depicted in Fig. 3A. However, supplementing −/− extract with SCAN1 TDP1 leads to an accumulation of radioactive signal in the wells of the gel, consistent with the presence of a protein-DNA adduct too large to migrate into the gel (Fig. 5A). Extract supplemented with SCAN1 TDP1 also shows more pTyr processing and more repair product than the unsupplemented −/− extract, and there is an additional novel band running beneath the wells and above the repair bands, which may represent a proteolyzed form of the protein(TDP1)−DNA adduct (denoted by the asterisk in Fig. 5A).

A smaller increase in the repair product is seen when −/− extract is supplemented with H493N TDP1, consistent with this less conservative amino acid change. However, there is little or no increase in the band representing 18-hydroxy, the product of the removal of the pTyr group. Thus, the H493N mutant retains a low level of pTyr-processing activity but does not form a persistent DNA adduct like SCAN1 TDP1.

Supplementing −/− extract with wild type TDP1 also reinstates the processing and repair of the 3′ overhang PG DSB substrate, with the addition of more TDP1 resulting in greater conversion of the 14-PG to its processed intermediates, 14-hydroxy, as well as repair

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**Fig. 4.** Tdp1−/− fibroblasts are deficient in processing PG on DSBS. Plasmid substrates mimicking DSBS with either partially complementary 3′ overhang (A) or blunt (B) DNA ends bearing PG modifications were incubated with whole-cell extract (or boiled extract) from Tdp1−/−, Tdp1−/−, and Tdp1−/− MEF cell lines for 6 h. Reactions were deproteinized, eluted acids were precipitated, digested with BsaXI and TaqI, and subjected to denaturing gel electrophoresis (C and D). In (C), the overhang substrate was incubated with 2.5 or 5 μg extract per μL reaction volume. In (D), the blunt ended substrate was incubated with 5 μg (or 10 μg) extract per μL reaction volume. In (D), rightmost lane contains unlabeled marker of 35 bases. The blunt-end DSB substrate was also incubated for 15 min, 1, 3, or 6 h with 7 μg of Tdp1−/− or Tdp1−/− cell extract per μL reaction volume, then deproteinized, precipitated, and digested (E). Rightmost lane contains radiolabeled marker of 11 bases. After 3 h incubation of the 3′-PG blunt end DSB substrate with extract, the percent of PG remaining in the lane was calculated by densitometry (F). Graph on left includes data from 4 independent experiments, performed with multiple preparations of cell extract per genotype; error bars indicate standard error and *** indicates p < 0.001. Rightmost graph shows quantification of (E), time course of PG processing with Tdp1−/− or Tdp1−/− cell extract. The 6-h time point, conducted in duplicate, includes error bars that are contained within the symbols.
Fig. 5. Complementation with either SCAN1 or H493N mutant TDPL fails to restore the wild-type phenotype for processing either pTyr or PG end modifications. The pTyr substrate mimicking a SSB depicted in Fig. 3A was treated with 30 μg of whole-cell extract from Tdp1\textsuperscript{WT}, Tdp1\textsuperscript{KO} MEFs for 5, 10, and 20 min (A). The substrate was also treated with 30 μg of Tdp1\textsuperscript{WT} MEF extract that had been supplemented with 50 ng of either wild type purified TDPL, the SCAN1 mutant form of TDPL, or the mutant H493N TDPL. Processed (OH), unprocessed (pTyr), and repaired forms of the substrate are indicated. The radioactivity remaining in the SCAN1 wells presumably represents an unresolved DNA-TDPL adduct; bands running beneath the wells and above the repair bands may represent a protected form of the adduct (*). The substrate mimicking a SSB with partially complementary 3' overhangs as depicted in Fig. 4A was treated with 60 μg whole-cell extract from Tdp1\textsuperscript{WT}, Tdp1\textsuperscript{KO} and Tdp1\textsuperscript{KO} MEFs for 6 h (B). The substrate was also treated with Tdp1\textsuperscript{WT} MEF extract that had been supplemented with either 25 ng (1X) or 125 ng (5X) of either wild type purified TDPL, the SCAN1 mutant form of TDPL, or the mutant H493N TDPL.

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products (Fig. 5B). Supplementing –/– extract with either SCAN1 TDP1 or H493N TDP1 does not lead to the appearance of either processed intermediates or repair bands; as in the –/– extract by itself, the unprocessed 3'-PG persists for at least 6 h of incubation in the extract.

4. Discussion

While there is little doubt that an H493R TDP1 mutation confers SCAN1 [11], it is still unclear how the mutation elicits symptoms of the disease, and why symptoms are largely neurological. In an attempt to address these questions, we generated a Tdp1 knockout mouse. However, extensive behavioral analysis of Tdp1−/− mice up to 12 months of age did not reveal any detectable deficits in experimental correlates of human SCAN1, such as grip strength (muscle weakness), stride length or rotated latency (ataxia), and indeed did not show any behavioral differences from wild-type mice (Fig. 2). A similar lack of overt behavioral symptoms was noted in two other independently derived Tdp1−/− mice, although in neither case were quantitative data reported [23,24]. In one of these studies [23], there was a progressive age-dependent reduction of cerebellar size in Tdp1−/− mice when compared to their wild-type littermates. When comparing brain mass and gross brain histology, no differences were observed between genotypes in our studies, corroborating the results of Hirano et al. However, no attempt was made to quantify the cerebellar to whole brain ratio as was performed by Kyatyal et al., who noted small differences in brain mass, but also that cerebellar morphology and foliation was indistinguishable between genotypes. Kyatyal et al. also observed in vivo hypersensitivity of the Tdp1−/− mice to the effects of the TOPI inhibitor topotecan, displaying significant weight loss due to intestinal progenitor cell hypersensitivity after six daily doses [23]. Hirano and colleagues found similar in vivo Tdp1−/− hypersensitivity to both camptothecin and the camptothecin derivative topotecan, and in both cases the sensitivity required repeated doses in a relatively short amount of time to manifest [24]. In either study, even lethal treatments with TOPI inhibitors still failed to elicit any SCAN1-like symptoms. Indeed, rapidly proliferating cells in the gut, liver, and spleen, rather than postmitotic neurons, appeared to be particularly sensitive. Thus, overall, the Tdp1−/− mouse phenotype bears little resemblance to human SCAN1.

While fundamental differences between human and mouse phenotypes of a given gene are not unusual, it is possible that rather than the simple loss of TDP1 function, the symptoms of SCAN1 are dependent on the unique features of the H493R mutant enzyme. In particular, it has been proposed that the residual activity of this mutant enzyme may be sufficient to process many if not most TOP1cc that fail to elimate, converting them to persistent TDP1-DNA adducts that may be more toxic and/or less amenable to alternative repair pathways than the initial TOP1-DNA adducts [21]. On the other hand, there is evidence that the initial TOP1-DNA adducts are also more persistent in SCAN1 than in normal cells, and that the difference is comparable to the overall deficit in SSB repair [4]. In either scenario, the neuronal-specific phenotype can be explained by the paucity of alternative repair pathways in terminally differentiated, non-replicating neuronal cells, the high levels of oxidative stress in neuronal tissue [25], and elevated levels of transcription in neuronal cells. Furthermore, it has been shown that in yeast the toxicity of SCAN1 TDP1 is dependent upon elevated levels of TOP1 [22] and that TOP1 is particularly highly expressed in Purkinje neurons of adolescence human brain [26,27]. Although there have been to date no autopsy studies on SCAN1 patients, other neurodegenerative disorders that feature ataxia among their symptoms have specific Purkinje neuron pathology [28,29]. Thus, the ataxia seen in SCAN1 patients may be directly caused by Purkinje neuron pathology that results from the interaction of SCAN1 TDP1 in the presence of high levels of TOP1.

In vitro complementation studies showed that extracts from Tdp1−/− mouse fibroblasts largely could not process a tyrosyl-DNA substrate; these results correlated with previous observations made in extracts from SCAN1 lymphoblasts [30], astrocyte and cerebellar extracts [23], and mouse neurospheres [24]. When supplemented with wild-type TDP1, processing of the tyrosyl-DNA substrate was reinstated, as also shown by Kyatyal et al. [23]. The generation of products of extremely low mobility in Tdp1−/− extracts supplemented with H493R TDP1 (Fig. 5A), similar to the results seen by Interal et al. [21], under conditions where the wild-type enzyme promotes SSB repair, suggests that substantial amounts of persistent TDP1-DNA adducts would be formed during attempted repair of Top1 lesions in SCAN1 cells. Coexpression of TDP1 protein with DNA in CsCl-fractionated extracts of SCAN1 but not normal fibroblasts [24] suggests that such complexes form in vivo.

Extracts of Tdp1−/− cells are also completely deficient in processing of protruding 3'-PG DSB termini, and reconstituted TDP1 can rescue that deficiency (Fig. 5). Previously, we showed similar lack of processing of PG-terminated 3' overhangs in extracts of TDP1-mutant human SCAN1 cells [6], but due to low ligation efficiency in those extracts, generation of ligated products was not demonstrated. However, in contrast to the tyrosyl lesion, there is no evidence that the mutant enzyme can form covalent adducts at 3'-PG termini (Fig. 5). Thus, presumably because the 3'-PG lesion is a much less favorable substrate than a 3'-pyr [5], SCAN1 TDP1 displays a hypomorphic or perhaps even a null phenotype in PG processing. Tdp1−/− extracts also show partial deficiency in processing blunt 3'-PG DSBs, but no deficiency in processing 3'-PG SSBs. Inasmuch as bleomycin induces almost exclusively 3'-PG SSBs and (predominantly blunt-ended) 3'-PG DSBs [31], it appears likely that unrepaird DSBs are responsible for the previously reported bleomycin sensitivity of Tdp1−/− mice and Tdp1−/− MEFs [24]. While neither Tdp1−/− astrocytes, Tdp1−/− cerebellar granule cells [23], nor SCAN1 lymphoblasts [32] show any detectable deficit in repair of radiation-induced DSBs, a recent mass spectral analysis suggests that the fraction of radiation-induced breaks with 3'-PG termini may be as small as 10% [33], much lower than the previous estimate of ~50% [34]. Although SCAN1 cells have an apparent defect in repair of radiation-induced SSB [32], this defect may reflect persistence of TOPI-mediated lesions formed at sites of base damage [35,36], rather than failure to process direct free radical-mediated, PG-terminated SSBs.

At present, the most likely etiology of SCAN1 is that oxidative lesions in DNA promote transcription-mediated formation of trapped TOPI cc that are then converted to persistent SSBs with a unique covalent 3' linkage between DNA and the H493R TDP1 enzyme. These lesions could lead to cytotoxicity either by promoting apoptosis or simply by inhibiting transcription. If this is the case, however, the SCAN1 phenotype may not be very informative with respect to the normal function of human TDP1. The finding that a mouse Tdp1−/− phenotype is only seen under conditions of severe genotoxic stress suggests that there are redundant repair pathways for the major functions of Tdp1, as is likely the case for the human enzyme as well.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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SUPPLEMENTAL MATERIALS AND METHODS

S.1 Generation and confirmation of Tdp1 knockout mice

To generate both constitutive and conditional Tdp1 knockout (Tdp1\(^{-/-}\)) mice, a Tdp1 targeting vector was constructed using pKO NTKV-1901, which contains both a PGK/neo/BGH cassette for positive selection of homologous recombinants with G418, and an MC1-tk cassette for negative selection of non-homologous recombinants with gancyclovir (Stratagene). This vector was modified by the insertion of loxP sites on either side of the PGK/neo/BGH cassette. A 4.8-kb region of the Tdp1 gene containing exons 5-7 (with 3.2 kb of intron 6 deleted) was generated by PCR from a 129/Sv BAC clone, and a third loxP site (with an associated NheI site) was inserted into intron 5 (Figure 1A, panel ii). This arm was then inserted between the BglII and XhoI sites of NTKV-1901. A 4.7-kb Xmal/SmaI BAC clone fragment containing Tdp1 exons 8-12 was inserted into the NTKV-1901 SmaI site. The Tdp1 targeting vector was linearized with Smal and electroporated into 129/SV embryonic stem (ES) cells. Genomic DNA from ES cell clones resistant to both G418 and gancyclovir was screened for homologous recombination by long-range PCR using intron 4 and BGH primers (for the 5′ arm), and PGK and intron 12 primers (for the 3′ arm). Retention of the intron 5 loxP site in PCR-positive clones was verified by NheI digestion of the 5′ arm PCR products. Southern blot analysis was performed on DNA from homologous recombinants using a PGK/neo probe to verify clone purity and integrity of both genomic arms.

To delete the neo gene in the targeted Tdp1 allele and generate the desired knockout and conditional knockout alleles (as shown in Figure 1A panels iv and v), ES
cells from a correctly targeted clone were subsequently electroporated with an MC1-cre expression vector. After initial growth in the absence of antibiotic selection, individual clones were picked, expanded, and then divided into two wells, one with and one without G418. The G418-sensitive clones were then screened by PCR to identify both type A recombinants (knockout allele) and type C recombinants (conditional knockout allele). Of 15 G418-sensitive clones screened, 10 were found to be knockout recombinants, and 5 were conditional knockout recombinants. One clone of each type was expanded and injected into C57BL/6 blastocysts, which were then implanted into pseudopregnant CD-1 recipients. The resulting male chimeras were bred to C57BL/6 females and tail genomic DNA from agouti offspring screened by PCR for the presence of the targeted \textit{Tdp1} allele. Germline transmission of both the conventional knockout and conditional knockout alleles was obtained.

\textit{Tdp1}^{+/-} mice were interbred to generate mice of the \textit{Tdp1} genotypes (+/+, +/- and -/-) used in this study. Offspring were screened by PCR using a common exon 8 anti-sense primer (GI:149292731/25190-25168: 5′-TTC TTG GGT ACA AAG GGC TCA AC-3′), an intron 5 sense primer for the knockout allele (16791-16813: 5′-CTC TAG TCA AAC AGC ACA AAT GC-3′), and an intron 7 sense primer for the wild-type allele (24700-24722: 5′-GTG GAA AGG AAT TGA TGA GAT GG-3′). Knockout and wild-type PCR products were 713 bp and 491 bp, respectively (Supplemental Figure 1A). Deletion of \textit{Tdp1} exons 6 and 7 in knockout mice was also verified by probing a Southern blot of XbaI digests of tail DNA from each of the three genotypes with 5′ (15945-17191) and 3′ (24986-25922) genomic fragments (Supplemental Figure 1B).
TDP1 transcript levels were quantified by real time PCR using cDNA reverse transcribed from RNA isolated from Tdp1+/+ and Tdp1−/− brain and liver. RT-PCR primers were designed to amplify part of the region deleted in the TDP1−/- mouse (exons 6 and 7), as well as regions 5′ and 3′ of the deleted exons, with one primer in each set derived from adjacent exons. The 5′ region primers were 5′-AGAAAGTTGTGGATAGAAGCC-3′ (exon 2 sense) and 5′-CAGAGGGATAAAATATCCTTG-3′ (exons 3/2 anti-sense), and yielded an RT-PCR product of 203 bp. The deleted region primers were 5′-GGAACACACCACACGAAAATG-3′ (exons 6/7 sense) and 5′-TTTGAAACGGGTGCTTGACTC-3′ (exon 8 anti-sense), and yielded an RT-PCR product of 183 bp. The 3′ region primers were 5′-CTCTCTTCCCTATAGCATCC-3′ (exon 12 sense) and 5′-CTGGACAGATTCGCACTTG-3′ (exons 14/13 anti-sense), and yielded an RT-PCR product of 184 bp. β-actin was used as an internal standard, using primers 5′-TCCTAGCACCATGAAGATCAAGATC-3′ and 5′-CTGCTTGCTGATCCACATCTG-3′, yielding a 118 bp product. Real-time PCR was performed in 15µl reactions on the 7900 HT Real-time PCR System (Applied Biosystems) using SYBR Green PCR Mastermix (Applied Biosystems) (Supplemental Figure 1C). PCR bands were separated on a 7% non-denaturing polyacrylamide gel, detected by ethidium bromide staining, and imaged on a Typhoon 9410 variable mode scanner (GE Healthcare) (Supplemental Figure 1C) to confirm the size of the amplified products.

S.2 Functional Observational Battery
The *in vivo* testing procedure involved a number of tests conducted sequentially on the same day. Following a general assessment of overall health and appearance, a functional observational battery (FOB) of behavioral tests [1] was undertaken. Adapted from a protocol for evaluation of neurotoxins [2], it contains tests similar or identical to those recommended for evaluation of behavioral phenotypes of transgenic and knockout mice [3-5]. The FOB consisted of observations of mice in an open field, their response to handling when removed from a locomotor activity chamber, and manipulative behavioral measures. Mice were placed in a 40 X 76-cm open field and scored on the following measures for the first 2 min of the test: posture, arousal, rearing, clonic movements, tonic movements, palpebral closure, gait, and gait abnormalities (see definitions in [2]). Piloerection, righting reflex, forelimb grip strength, the inverted screen task, landing foot splay, approach response, click response, touch response, tail pinch response and mobility were evaluated (in the order listed) over the next 4 min. Latency, in seconds, to climb to the top of the screen after inversion, was measured. Subsequently, when mice were tested in the locomotor activity chamber, they were evaluated for ease of removal and handling reactivity. Scoring of the FOB was done by a single trained technician who was blind with respect to the genotype of the mice.

Following the FOB assessment, three additional tests were employed to assess specific differences in motor performance and possible ataxia. First, general locomotor activity was measured in standard activity chambers interfaced with a PC-operated automated activity monitor. Mice were placed in individual activity chambers and spontaneous activity was measured for 10 min. Activity was expressed as total number of interruptions of 8 photocell beams per chamber for the 10-min session. Second, the
mouse was placed on the cylinder of a standard rotarod apparatus. The cylinder was programmed to rotate initially at 4 revolutions per min, gradually increasing to 40 revolutions per min over 5 min. Latency to fall off of the rotarod was recorded. Third, the hindfeet of each mouse were dipped in ink and it was then placed in a paper-lined 30 cm tunnel to assess stride length. Ataxia may be reflected as shorter stride length [6]. At the end of the FOB and motor performance testing, sensitivity to a thermal stimulus was assessed in a standard tail-flick apparatus. Latency to remove the tail from the stimulus was measured. A 10-s maximum latency was imposed in order to prevent damage to the tail. All in vivo tests describe above were conducted at 3, 6 and 12 months of age in the same mice, with the exception that additional mice were tested at 12 months in some groups due to low availability of these mice at earlier time points.

For behavioral assays, continuous and count measures were analyzed using separate ANOVAs for each time comparing the behavior of homozygous knockout, heterozygotes and wildtype mice. As appropriate, categorical data was analyzed with separate Chi square tests. Data for male and female mice were analyzed separately. For behavioral assays, separate ANOVAs for each sex were also used to assess motor activity, tail flick latency, rotarod latency, and stride length. For all significant ANOVAs, Tukey post hoc tests ($\alpha = 0.05$) were used to determine differences from wildtypes.

### S.3 Histologic analysis of brains

For histological analysis, 6 $Tdp1^{-/-}$, 2 $Tdp1^{+/+}$ and 3 $Tdp1^{+/+}$ mice were sacrificed at 12 months of age, and an additional 2 $Tdp1^{-/-}$ and 2 $Tdp1^{+/+}$ mice were sacrificed at 22
months of age. Animals were transcardially perfused with formalin, and the brains were sagitally blocked, embedded in paraffin and sliced at 10µm throughout the hemisphere. Twenty-four to twenty-eight uniformly spaced sections for each animal were deparaffinized and rehydrated, then stained with 0.05% cresyl violet, dehydrated and coverslipped. The microscopic examiner was blinded to the genotype and scanned through each section for evidence of gliotic scars or overt loss of cells in numerous brain structures including cerebellum (purkinje and granule cells), cortex, hippocampus, basal ganglia structures (subthalamic nucleus, striatum, globus pallidus).

S.4 Generation and culture of embryonic fibroblasts

For generation of Tdp1+/+, Tdp1+-, and Tdp1-/- mouse embryonic fibroblasts, males and females of the same genotype were mated, and the females were evaluated for the presence of a vaginal plug each morning. The day a plug was detected was defined as e0.5. A pregnant mouse of each genotype was sacrificed at e13.5 – e14.5 by cervical dislocation after anesthesia. Uterine horns were removed and dissected to remove the embryos, which were then washed and separated from any visceral tissue. Embryos were dissected free of brains, passed through a syringe, and the resulting cellular mixture incubated with trypsin to further digest the connective tissue. The mouse embryonic fibroblasts (MEFs) were then plated on plastic coated with 0.1% gelatin to facilitate attachment and were maintained in culture in DMEM (Mediatech) supplemented with 10% fetal bovine serum (Gibco). After being in culture continuously for six months, MEFs from Tdp1-/- mice were cloned from a single colony, then complemented with wild-type human TDP1 via infection with a lentiviral vector constructed from the pLV-
tTRKRAB-Red vector [7] and pFLAG-hTDP [8] to generate LV-FLAGhTDP1-Red, expressing FLAG-tagged human TDP1 protein. Tdp1-/- MEFs were also infected with lentiviral vectors that expressed only DsRed (LVTHM-DsRed and LV-IRES-DsRed) then examined under a fluorescent microscope to confirm infection. Infected cells were screened for expression of FLAG-tagged hTDP1 by western blotting, then cloned and screened by PCR using primers against the leader sequence of the lentiviral vector:

5'-GGAGCTAGAACGATTCGCAGTTA-3’

and

5'-GGTTGTAGCTGTCCCAGTATTTGTC-3’ which is part of LV-FLAGhTDP1-Red.

Cells infected with LV-FLAGhTDP1-Red were subsequently passaged as a separate cell clone, Tdp1-comp, and screened for expression of hTDP1 by anti-TDP1 western blotting and an enzyme activity assay, described in sections 2.5 and 2.6.

REFERENCES


Supplemental Figure 1. Tdp1-/- mice have one HKD site knocked out and produce aberrant transcript. (A) PCR analysis of genomic tail DNA from mice of each genotype. DNA was amplified with a common exon 8 anti-sense primer, an intron 5 sense primer for the knockout allele, and an intron 7 sense primer for the wild-type allele. Knockout and wild-type PCR products were 713 bp and 491 bp, respectively. (B) Verification of deletion of exons 6 and 7 in Tdp1+/- and Tdp1-/- mice. Southern blot analysis of genomic tail DNA from wild-type, heterozygous knockout and homozygous knockout mice. DNA was digested with XbaI (X) and probed with 5’ and 3’ genomic probes (indicated in Figure 1A panel iv). (C) TDP1 transcript levels were assessed by real time PCR using cDNA reverse transcribed from RNA isolated from Tdp1+/- and Tdp1-/- brain and liver. The transcript encoding the first HKD motif (exons 6-8) is absent in Tdp1-/- tissues. Transcripts encoding exons 2-3 and 12-14 were found to be reduced by approximately half in the Tdp1-/- tissues compared to their wildtype littermates based on real-time data (data not shown). The 7% acrylamide gel above depicts products from the real-time PCR reaction, confirming the size of the respective amplicons.
Supplemental Figure 2. Tdp1-/- mice are deficient in 3' tyrosyl processing. A radiolabeled (*) 3'-pTyr oligomeric substrate was treated with tissue homogenates from a Tdp1+/+ or Tdp1+/− or Tdp1−/− mouse for 1 h, subjected to denaturing gel electrophoresis, and phosphorimaged. (A) Diagram of 3'-pTyr 18-nucleotide oligomeric substrate. The substrate was treated with four-fold serial dilutions of brain (B), or five-fold serial dilutions of liver (C) homogenate from a Tdp1+/+, Tdp1+/−, or Tdp1−/− mouse. The percent conversion from the tyrosyl substrate to its phosphate product was calculated by densitometry and shown for liver (D) homogenates.
Supplemental Figure 3. Tdp1 +/- fibroblast cell line can also effectively process 3’ tyrosyl DNA ends and Tdp1 -/- fibroblasts were genetically complemented via lentivirus infection. The substrate indicated in Figure 3A was treated with (A) 40, 20, or 10 µg of whole-cell extracts from Tdp1 +/-, Tdp1 +/+, or Tdp1 -/- MEF cell lines for 1 h. 15 and 30 µL of each lentiviral-infected Tdp1 -/- MEFs lysate, harvested and identically prepared, were analyzed via Western blotting with (B) anti-FLAG antibody.
Supplemental Figure 4. Tdp1-/- fibroblasts are not deficient in processing PG on SSBs. TDP1 can also process 3’-PG termini, and under some conditions appears largely responsible for their removal [1]. MEF whole-cell extracts were incubated with radiolabeled substrates that mimic SSBs bearing phosphoglycolate end modifications to assess the removal of this 3’ blocking moiety in the -/- MEFs. (A) Diagram of 3’-PG SSB substrates; asterisk represents the 5′ radioactive tag on the 3’-PG 17-mer modification. Both the 42-base (B) and 43-base (C) substrates were treated with 20 µg of whole-cell extract from Tdp1 +/- and Tdp1 -/- cell lines for 5, 20, and 60 min in reaction buffer with 0, 2, and 10 mM Mg++. These conditions are either prohibitive (0 Mg++) or permissive (2, 10 mM Mg++) for the activity of Ape1 [2] or PNKP. In (B), rightmost lane contains radiolabeled marker of 35 bases. Generation of fully repaired 42- or 43-base products was evident for both substrates in both extract genotypes, and appeared to be greater in 10 mM Mg++, whereas the 3’-hydroxyl 17-mer and 1-base-elongated intermediates tended to accumulate in the presence of 2 mM Mg++. However, there was significant loss of label in 10 mM Mg++ at longer times, probably reflecting nucleolytic removal of the 5’-labeled nucleotide.

For both the nicked (B) and the gapped substrate (C), the efficiency and time course of PG removal from the labeled 17-mer in the presence of 2 mM or 10 mM Mg++ was similar in +/- and -/- extracts, suggesting that removal was catalyzed primarily by Ape1 rather than Tdp1. In the presence of 5 mM EDTA, there was significant processing of the glycolate end only in the +/- extracts, suggesting that Tdp1 can process these lesions when other enzymes do not. However, the results suggest that TDP1 is not the primary enzyme that processes 3’-PG linkages at SSBs.

References


**Supplemental Table 1**: Body weight, temperature and pain sensitivity in male and female mice of each genotype.

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<th>Genotype</th>
<th>Weight (g)</th>
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<td></td>
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</tr>
<tr>
<td>TDP1-/- (n=8)</td>
<td>24 (0.7)</td>
<td>38.3 (0.15)</td>
<td>7.4 (1.1)</td>
</tr>
<tr>
<td>TDP1+/- (n=9)</td>
<td>25 (1.1)</td>
<td>38.4 (0.17)</td>
<td>7.2 (0.7)</td>
</tr>
</tbody>
</table>

**Age: 3 months**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Weight (g)</th>
<th>Temperature (°C)</th>
<th>Tail flick latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype (n=5)</td>
<td>34 (1.8)</td>
<td>38.4 (0.32)</td>
<td>6.2 (1.0)</td>
</tr>
<tr>
<td>TDP1-/- (n=6)</td>
<td>36 (2.2)</td>
<td>38.6 (0.16)</td>
<td>6.6 (0.3)</td>
</tr>
<tr>
<td>TDP1+/- (n=6)</td>
<td>38 (2.3)</td>
<td>38.2 (0.36)</td>
<td>7.1 (1.0)</td>
</tr>
<tr>
<td><strong>Age: 6 months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype (n=3)</td>
<td>34 (5.2)</td>
<td>38.1 (0.37)</td>
<td>5.4 (0.7)</td>
</tr>
<tr>
<td>TDP1-/- (n=5)</td>
<td>30 (0.95)</td>
<td>38.2 (0.11)</td>
<td>6.4 (0.8)</td>
</tr>
<tr>
<td>TDP1+/- (n=5)</td>
<td>34 (2.7)</td>
<td>37.9 (0.14)</td>
<td>5.4 (1.2)</td>
</tr>
<tr>
<td><strong>Age: 12 months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype (n=7)</td>
<td>36 (2.1)</td>
<td>38.0 (0.23)</td>
<td>2.9 (0.4)</td>
</tr>
<tr>
<td>TDP1-/- (n=5)</td>
<td>42 (2.7)</td>
<td>38.3 (0.19)</td>
<td>2.8 (0.2)</td>
</tr>
<tr>
<td>TDP1+/- (n=5)</td>
<td>41 (3.8)</td>
<td>38.2 (0.33)</td>
<td>4.3 (0.9)</td>
</tr>
</tbody>
</table>

**Age: 3 months**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Weight (g)</th>
<th>Temperature (°C)</th>
<th>Tail flick latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype (n=4)</td>
<td>29 (3.1)</td>
<td>37.0 (0.28)</td>
<td>3.2 (0.2)</td>
</tr>
<tr>
<td>TDP1-/- (n=10)</td>
<td>32 (1.8)</td>
<td>37.7 (0.16)</td>
<td>3.4 (0.4)</td>
</tr>
<tr>
<td>TDP1+/- (n=7)</td>
<td>36 (3.8)</td>
<td>37.5 (0.21)</td>
<td>2.7 (0.3)</td>
</tr>
</tbody>
</table>
VITA

Amy Jane Hawkins was born on November 19, 1981 in Washington, D.C., and is an American citizen. She graduated from Thomas Jefferson High School for Science and Technology in Alexandria, Virginia, in 1999. She received her Bachelor of Science in Biology, with a minor in Bioethics from the University of Virginia, in Charlottesville, Virginia in 2003. While at the University of Virginia, Amy gained laboratory experience working in the laboratories of Dr. Emilie Rissman, Dr. Lisa Goehler, and Dr. James Garrison in the Departments of Biology, Psychology, and Pharmacology. Amy began her graduate studies in 2003 at Virginia Commonwealth University in Richmond, Virginia in the Department of Human Genetics. She joined the laboratories of Dr. Kristoffer Valerie and Dr. Lawrence F. Povirk laboratories as a PhD candidate in August, 2006. Amy is first author on a manuscript from her graduate work that was published in DNA Repair in 2009. Amy currently resides in Richmond, Virginia.