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**Project Title:** Investigation of the Disease Manifestations and Genetics of a novel TDP1 Mutation, the gene implicated in Spinocerebellar Ataxia with Axonal Neuropathy 1 (SCAN1)

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**Summary (250 words max single spaced):**

Tyrosyl-DNA phosphodiesterase 1 (TDP1) is a protein best-characterized in its DNA repair role via resolution of stalled Topoisomerase1-DNA (TOP1-DNA) complexes. These stalled complexes would otherwise collide with DNA replication machinery and cause DNA damage. Homozygous mutation of TDP1 (H493R) can result in spinocerebellar ataxia with axonal neuropathy (SCAN1, an autosomal recessive neurodegenerative syndrome characterized by early onset cerebellar ataxia that has been so far only characterized in one Saudi Arabian Family.

Recently, a novel TDP1 mutation has been found in an American patient, who has a SCAN1-like ataxic phenotype. Differences between this new phenotype and SCAN1 include later onset, potentially a predisposition to tumorigenesis, and an apparent autosomal dominant inheritance pattern. The patient has one wild-type TDP1 allele, and a point mutation in exon 4: 560-1G->A in the other TDP1 allele. The patient's mother had poor balance while walking, and the patient's son was afflicted by two tumours, the latter one claiming his life at age 33.

B lymphoblastoid cell lines were generated from the patient (PROP), the patient's daughter (DAU), and the patient's brother (BRO), for further investigation. Western blot analysis showed ~70% decreased baseline expression of TDP1. Comet assay with Topotecan treatment showed no increased DNA damage in PROP/DAU/BRO cells compared to control wild-type cells, unlike SCAN1 cells which showed increased DNA damage. Topotecan is an anticancer drug that stalls TOP1-DNA complexes; defective TDP1 in SCAN1 is unable to resolve the stall, and replication machinery causes DNA damage. This suggests that PROP/DAU/BRO cells have functioning TDP1.

Student Signature

Primary Supervisor Signature

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# Introduction and Background

**DNA damage and the nervous system.**

Defects in the response to DNA single-strand or double-strand breaks underpin many human diseases associated with disorders of the nervous system [1-4]. During nervous system development, endogenous DNA damage often results in apoptosis, although cell replacement can occur from germinal zones within this rapidly proliferating tissue. However, if this damage surveillance is faulty, cells with genomic damage may inappropriately become incorporated into the nervous system, and the subsequent demise of these cells may result in neurodegeneration.

**Cellular DNA strand-break repair pathways.**

DNA repair involves specific enzymes that address a multitude of different types of DNA lesions [5]. DNA single-strand breaks (SSBs) are one of the most common DNA lesions that occur within the cell. Cells use a specific single-strand break repair (SSBR) mechanism, distinct from the DNA DSBR pathway, to deal with this type of DNA lesion [6]. DNA SSBs can occur directly to DNA via reactive oxygen species (ROS) or indirectly as an intermediate during base excision repair (BER). DNA ends at a SSB usually have altered 3'- and 5'- ends, and a number of processing enzymes are used to deal with these DNA ends. ROS-mediated SSBs are detected by poly(ADP-ribose) polymerase (PARP), leading to recruitment of XRCC1/LIG3 and associated factors to resolve the damage. In the case of BER, excision occurs via DNA glycosylase, followed by apurinic site incision by the APE1 endonuclease, gap-filling by DNA polymerase b (Polb) and after short patch repair, re-ligation using XRCC1/LIG3.

**TDP1 processes Top1-DNA cleavage complexes.**

Tyrosyl-DNA phosphodiesterase 1 (TDP1) is involved in the repair of DNA strand breaks associated with a variety of DNA termini, the best- characterized of which are Topoisomerase-1 (Top1)-associated 3'-termini arising from abortive Top1–DNA complexes [7]. Top1 relieves torsional stress in DNA via formation of a transient intermediate known as the cleavage complex, whereby Top1 forms a covalent phosphodiester bond between an active site tyrosyl residue and the 3'-end of a DNA nick [8]. TOP1-DNA complexes are usually transient, but if TOP1 binds to DNA near pre-existing nicks and modified DNA bases, it will be unable to re-ligate the DNA, and will become stalled [9]. TDP1 displaces the stalled TOP1 fragment and forming a TDP1-DNA covalent bond, and then hydrolyzes the new bond, thereby removing Top1 peptide from the 3'-terminus and enabling repair of the DNA strand breaks [10]. If unresolved, TOP1-DNA complexes can be converted into Top1-linked DNA single-strand breaks (SSBs) or double-strand breaks (DSBs) by collision with the transcription or DNA replication machinery [11]. In this regard, TDP1 functions as a fail-safe to protect cells from the genotoxicity associated with this Top1-dependent lesion. TDP1 is also capable of hydrolyzing other DNA SSBs, such as 3'-phosphoglycolate, that result from genotoxic stress [12].

A search of the *PubMed.gov* database in August 2020 using search terms "TDP1 gene" yielded 106 citations. TDP1 is tyrosyl DNA phosphodiesterase 1, an important enzyme that participates in DNA repair. Breaks in DNA, if not repaired are deleterious, and can become spontaneous mutations or can interfere with gene transcription. The mutations can allow development of cancers and allow degeneration in the brain and peripheral nerves. Homozygous mutation of *TDP1* (H493R) can cause spinocerebellar ataxia with axonal neuropathy (SCAN1),

typically an autosomal recessive neurodegenerative syndrome [13].

**SCAN1 hypersensitivity to Camptothecin**

Camptothecin is an anticancer drug that causes TOP1 to stall on DNA, increasing the half-life of TOP1-DNA complexes, and therefore increases the likelihood of their conversion to DNA SSBs and DSBs [14]. This property of CPT and related compounds make them valuable as anticancer agents. This stall can be resolved by functional TDP1, but in SCAN1 patients, TDP1 is not functional, making SCAN1 patient cells unable to resolve the stall and hypersensitive to camptothecin. Other analogues to camptothecin, irinotecan and topotecan, have a similar effect in SCAN1 patient cells [15].

**SCAN1 disease characterization**

SCAN1 typically presents in late childhood, 13-15 years of age, initially as peripheral neuropathy, and progresses slowly over the patient's lifetime [14]. Cerebellar ataxic is the most notable symptom and first manifests as unsteady gait, which is then followed by areflexia, gaze nystagmus, cerebellar dysarthria, distal muscle weakness, and peripheral neuropathy [14, 16, 17]. Axonal neuropathy can be evidenced on nerve conduction studies, which show slower conduction speeds, and on nerve biopsy, which show axonal loss [14]. In general, there is also hypoalbuminemia and hypercholesterolemia [18]. Intellect and cognitive function is normal, lifespan and incidence of cancer is unaltered [13]. Despite the defective DNA repair, there is no predisposition to neoplasms, and no abnormalities in rapidly replicating tissues [19].

A Saudi Arabian family with history of autosomal recessive cerebellar ataxia was investigated in 2002; 3 individuals from the target family were selected for a more detailed work up and for the first time, SCAN1 was characterized [13]. Currently, only 1 mutation has been linked to the disorder; a homozygous A1478G mutation in the TDP1 allele leading to a H493R missense mutation was identified, which is thought to disrupt the active site of TDP1 [13]. The disorder has only been documented in one Saudi Arabian family, but the H493R mutation has recently been found in 2 apparently unrelated Omani families, who recessively inherit a phenotype similar to SCAN1 [18]. Differences between progenitor SCAN1 individuals and affected individuals in these 2 families are: later onset in adulthood (as opposed to late childhood onset), and impaired intellectual capacity and cognitive performance (as opposed to normal intellect) [18]. The reason for differences in phenotype despite the same mutation are not clear. As well, animal models such as TDP1 knock-down mice have similarities to, but do not reproduce, the SCAN1 phenotype [7, 16].

**A family with a novel *TDP1* mutation – the focus of this project**

Recently, an American-based neurologist presented to us a singular American patient displaying a slowly progressive cerebellar disorder that is manifest by poor balance while walking and abnormal eye movements. Examination did not show peripheral neuropathy. The subject's mother had similar problems walking, but lived to be 94. The patient's son was cured of a tumour at age 9, but died of a different tumor at age 33. Hence, three generations of the family may already be affected by a genetic abnormality that was passed from one generation to the next. Depending on chance alone, approximately half of the descendants of an affected family member may receive a copy of the gene; close relatives include the patient's daughter and the patient's two brothers. However, the gene identified in the patient who is the subject of this proposal, appears to be

dominant rather than recessive. That is, a single copy of the gene may be enough to cause the balance problems, and perhaps the cancer that has been identified in the family. Preliminary genetic analysis determined that the patient had one normal (wild-type) and one mutant *TDP1* allele, containing a point mutation within exon 4: 560-1G->A resulting in a change in the invariant AG acceptor splice site for exon 4 to an adenine, which is predicted to lead to aberrant splicing. An autosomal dominant form of SCAN1 elicited from a single mutant copy of the *TDP1* gene has not been previously described.

Subsequently, blood from the patient (**PROP**), the patient's daughter (**DAU**) and brother (**BRO**) were drawn and sent to UNC Lineberger Comprehensive Cancer Center (North Carolina, USA) wherein enriched B-cells underwent virus-mediated transduction and immortalization. The resulting B lymphoblastoid cell lines (LCLs) were sent to us for further analysis. Three additional LCLs containing no discernable mutations or abnormalities were also provided (used as normal wild-type controls). Western blot analysis of protein extracts derived from these cell lines revealed that the patient and the patient's daughter and brother all show an approximately 70% reduction in TDP1 protein relative to controls (Fig 1). These data indicate the possibility of all three family members being affected. With the emergence of more TDP1-involved syndromes, further investigation on pathogenic mechanisms is warranted.

# Materials and Methods

**To analyze TDP1 protein expression and activity via western analysis and DNA damage repair assays and methodology.**

Additional western analysis was performed to determine the nature of the mutant *TDP1* transcript and its affect on TDP1 protein expression. Preliminary data suggests that cells harboring the mutant *TDP1* allele show reduced TDP1 protein.

DNA damage repair analysis (COMET and gH2AX) was also performed, comparing patient cells to normal controls. COMET assays allow for real-time single-cell DNA damage analysis via electrophoresis of cellular DNA followed by microscopy to measure the "comet tail" which contains the trail of fragmented damaged DNA. Cells are first treated under various conditions, and given time to recover. Then, they are embedded in 1% low melting agarose on a 96-well plate, and immersed in a lysis buffer to remove membranes and histones from the DNA (Larson et al, 2016). The embedded DNA clumps are equilibrated in an alkaline electrophoresis buffer to unwind and denature the DNA, and then electrophoresed (Larson et al, 2016). Intact DNA clumps will not be spread apart by electrophoresis, but DNA clumps that have double-strand breaks will have different sized DNA fragments that move through the electric field at different rates. This creates a smear of DNA away from the initial DNA clump, much like a comet tail leading away from a comet head.

gH2AX assays utilize an antibody against phosphorylated histone H2AX, a protein normally associated with DNA in the cell nucleus, which becomes modified (phosphorylated) upon changes in DNA-protein structure that result from DNA damage. Cells are labeled with a fluorescent-tagged version of this antibody and visualized via epifluorescence microscopy. Increased gH2AX signal correlates with higher cellular DNA damage.

Unique to the Katyal lab is the development of automated high-throughput COMET and gH2AX analyses, techniques that has been well-validated, made to be highly user-friendly and easily acquired [20-22]. This system will be used to quantify and compare DNA damage repair rates amongst patient-derived controls and *TDP1* mutant LCLs using the Topoisomerase-1 inhibitor, Camptothecin (CPT), and hydrogen peroxide (for oxidative damage).

The well-characterized H493R SCAN1 patient LCL2 [7, 13] were used as a comparative control, to elucidate the extent that the novel *TDP1* splice site mutation impacts on overall cellular DNA damage repair. Furthermore, cell survival studies using CPT will be conducted to determine how dysregulated *TDP1* expression impacts DNA damage induced cell death. These studies will characterize the cellular impact of this novel TDP1 splice site mutation on overall TDP1 protein expression and cellular DNA damage.

**To target the mutant *TDP1* RNA transcript, using RNAi methodology, to rescue expression and activity of the remaining wild-type *TDP1* allele in patient cells.**

Short-hairpin RNA constructs will be generated, cloned into the TRC1.5-pLKO.1-puro vector, to specifically target the aberrant TDP1 allele. Transfected in both control and TDP1 mutant patient LCLs, allele specific qRT-PCR analysis will be performed to confirm knockdown specificity followed by western analysis to determine whether loss of the mutant TDP1 transcripts impacts overall TDP1 protein expression levels and can restore DNA repair activity. Recently, antisense morpholino oligonucleotides (AMO) have been used to suppress splice acceptor sites (through antisense oligonucleotide-mediated splicing modulation) and premature stop codon recognition in a variety genetic neurodegenerative syndromes including, Ataxia-telangiectasia (A-T), Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA) [23, 24]. In fact, small clinical trials using this methodology have yielded therapeutic success. Based on success of the above knockdown experiments, the AMO strategy could be used to derive a genetic treatment to restore TDP1 expression in the mutant *TDP1* patient cells. Collaboration with Dr. Richard Gatti (Pathology, UCLA, Los Angeles California), who has pioneered this treatment for A-T patients, will identify and generate mutant *TDP1*-specific AMOs. Following gene delivery into patient cells, western and DNA damage repair analysis on these modified *TDP1*-mutant patient cells will determine if nullifying the mutant allele can rescue the DNA repair defect in these patient cells.

# Results

**Lower levels of TDP1 in PROP and family cell lines**

Western analysis was repeated to confirm previous results (Fig 2); cell lines from the patient and patient's family members had lower amounts of TDP1. ImageJ quantification showed a baseline TDP1 reduction of 45% in PROP, 28% in DAU, and 39% reduction in BRO.

**Comet assay showed no hypersensitivity to Topotecan, but hypersensitivity to Methyl Methanesulfonate in the PROP and family cell lines**

Comet assays were performed on a wild-type (WT) LCL with no known TDP1 mutation, PROP LCL, DAU LCL, BRO LCL, and a well characterized H493R SCAN1 LCL, to evaluate DNA damage under different conditions. The first condition examined was exposure to Topotecan, a Camptothecin analog. Cells were exposed to concentrations of 0, 0.2, 1, and 5μM Topotecan for 5 mins at room temperature, and given a 1-hour recovery @37°C. Interestingly, WT and PROP,

DAU, and BRO cells showed similar minimal levels of DNA damage; untreated cells and cells treated with 5μM Topotecan had no appreciable comet tail (Fig 3). SCAN1 cells were highly sensitive to Topotecan, consistent with existing literature [14, 15]. SCAN1 cells treated at 0.2μM Topotecan had large comet tails, and cells treated at 5μM no longer had an appreciable comet head (Fig 3). Comet tail moments are not included in the results – explained further in the discussion.

The second comet assay condition examined was exposure to Methyl Methanesulfonate (MMS), an alkylating agent that predominantly causes N7-alkylguanine, and some N3-alkyladenine adducts (Beranek, 1990). MMS can cause DNA fragmentation through base mispairing and replication blocks, especially in homologous recombination deficient cells (Beranek, 1990). Cells were exposed to concentrations of 0, 0.014, 0.043, and 0.13mg/mL MMS for 10 mins @37°C and given no recovery. All cell lines showed a damage response to MMS, especially in the 0.13mg/mL group (Fig 4). However, WT cells showed considerably less DNA damage, with comet heads and tails still connected; PROP, DAU, BRO, and SCAN1 cells showed a high degree of DNA damage after 0.13mg/mL treatment, with comet heads occasionally non-existent (Fig 4).

# Discussion

**Western blot results**

Western blot analysis confirmed that cell lines from PROP and family produce less TDP1 under normal conditions. Next steps are to treat the cells under various conditions, such as protease inhibitors and camptothecin analogs, and do Western blot analysis to determine PROP TDP1 stability and proteolytic degradation, and PROP TDP1 response to cell stresses.

**Comet assay results**

Unlike TDP1 H493R mutant SCAN1 cells, which are hypersensitive to camptothecin analogs, PROP and family cells did not show significant DNA damage to camptothecin analog treatment. This suggests a different pathogenic mechanism of the PROP TDP1 mutation. Camptothecin analogs causes TOP1 to stall on DNA; if DNA replication occurs during the recovery period and the topotecan-induced TOP1-DNA complex is not resolved, it will replication machinery well collide and cause a double-strand break. This is the case in H493R SCAN1 cells, which do not have functional TDP1 due to a homozygous autosomal recessive mutation – hypersensitivity is seen (Fig 3). In PROP and family cells, topotecan did not cause more DNA damage compared to WT cells (Fig 3). This may mean that functional TDP1 is still present in PROP cells and is actively resolving topotecan-induced TOP1-DNA complexes. This is consistent with the proposed autosomal dominant inheritance of PROP's ataxia-like phenotype; the wild-type TDP1 allele is producing functional gene product, albeit at lower baseline quantities, that resolves TOP1-DNA complexes. Other interactions, such as masking, may be at play, and is causing TDP1 to be dysfunctional in a different capacity. Of note, the PROP TDP1 mutation does not result in the SCAN1 phenotype. Rather, the ataxic features manifest later in life, and there is an increased incidence in cancer. This delayed and tumorigenic phenotype may be a result of TDP1 failing to perform a crucial function other than TOP1-DNA complex resolution.

PROP and family cells are hypersensitive to MMS, more so than SCAN1 and WT cells. This is suggestive of homologous recombination impairment in both H493R SCAN1 and PROP

cells, but perhaps more so in PROP and family cells. More clinical tests on PROP and family, such as nerve conduction studies, may shed light on the effects of this new TDP1 mutation.

**Comet tail moment calculations**

Comet tail moments are not included because the test results did not allow for reliable and accurate moment calculation. Comet tail moment calculation is based on software identification of a comet head, and identification of an associated tail. Metrics involved in tail moment calculation include the ratio of DNA in the head vs tail, and the dimensions of the head vs tail. However, the software could not identify the head and tail of a comet in experiment conditions that caused extensive DNA damage. Either there was not enough DNA in the comet head for the software to recognize it, or the comet head and tail were separated too far to be recognized as DNA from the same cell (Fig 5a). Adjustment of software parameters could allow for the recognition of distanced comet heads and tails, and small comet heads; however, these parameter changes would disturb the recognition of intact DNA clumps that had virtually no comet tail. Since software parameters must stay consistent when evaluating all of the results in an experiment, it was not appropriate to give comet tail moment results that did not accurately represent the amount of DNA damage.

Another issue that affected comet tail moment calculation was density of cells. Some slides had <10 cells, which made it difficult to confidently assess the extent of DNA damage (Fig 5b). Some slides had comets overlapping each other, which made it difficult for the software to distinguish comets apart (Fig 5c).

Next steps are to titrate comet assay experiment conditions (treatment time and concentration of cells and treatment) that do not cause such extensive DNA damage that make comet tail moments uncalculatable, but still demonstrate the differences in DNA repair between cell samples, and allow for reliable and accurate comet tail moment calculation.

**Changes in project due to covid-19**

The remainder of planned experiments were not started, including gH2AX (although technique was learned), and use of RNAi to rescue the expression and activity of the remaining wild-type *TDP1* allele in patient cells. Anticipated results for gH2AX DNA damage repair assay would be used to compare with Comet assay results; PROP and family cell lines showing sensitivity to certain DNA damaging agents could further elucidate the effect of the novel TDP1 mutation. RNAi rescue of TDP1 expression could potentially represent a treatment strategy.

In order to mitigate the circumstances and to be productive, remaining time was spent on a literature review of DNA damage induced spinocerebellar ataxias (SCA), and investigation of related proteins' involvement in tumorigenesis. A draft will be submitted by Aug 10.

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# Figures

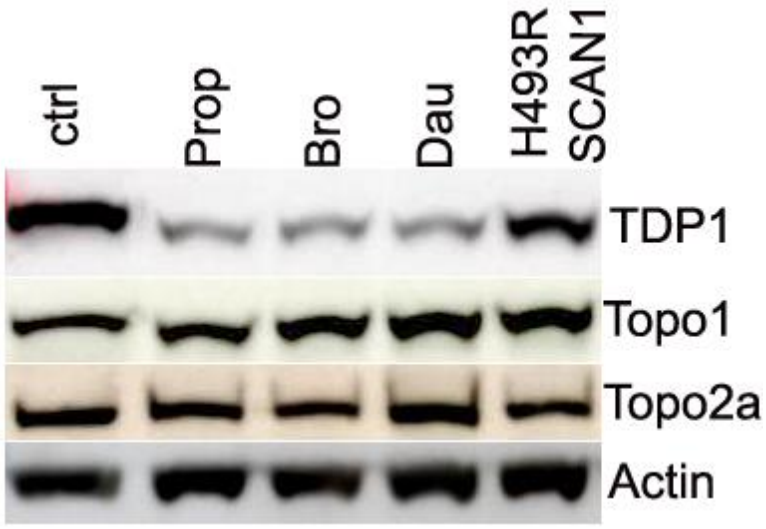


Figure 1. Western blot analysis of LCLs derived from family-members with a novel autosomal dominant inherited *TDP1* allele harboring an aberrant splice site mutation in exon 4. Protein extracts derived from control (ctrl), SCAN1 (H493R mutant), the patient (PROP), the patient's brother (BRO) and the patient's daughter (DAU) underwent BIS-TRIS (4-12%) SDS-PAGE and immunoblotting for the indicated protein antigens, done by Dr. Ali Saleh. TDP1 levels are noticeably reduced in all afflicted family members compared to the control and original autosomal recessive SCAN1 lines. Topoisomerase levels appear normal.

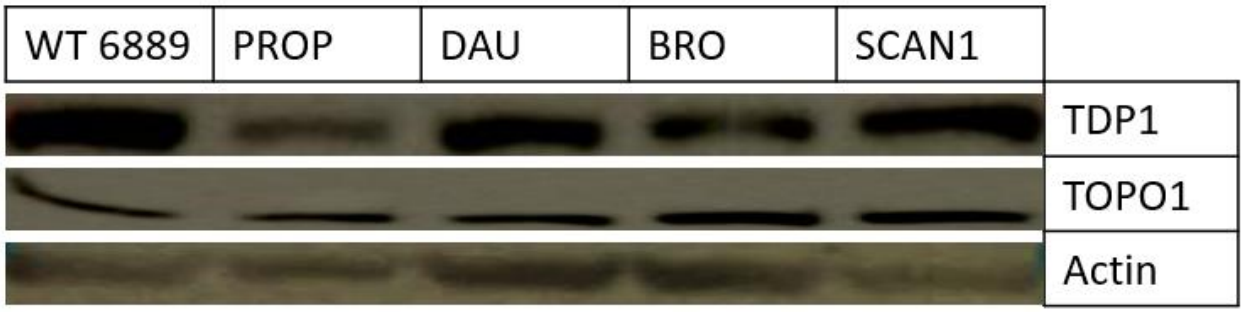


Fig 2. Western blot analysis of LCLs derived from a wild-type control, family members with a novel autosomal dominant inherited *TDP1* allele, and a H493R SCAN1 control, confirms the lower levels of TDP1 in patient family member cells, and normal Topoisomerase 1 levels. BIS-TRIS (4-12%) SDS-PAGE and immunoblotting was repeated for the indicated protein antigens by Alvin Janes.

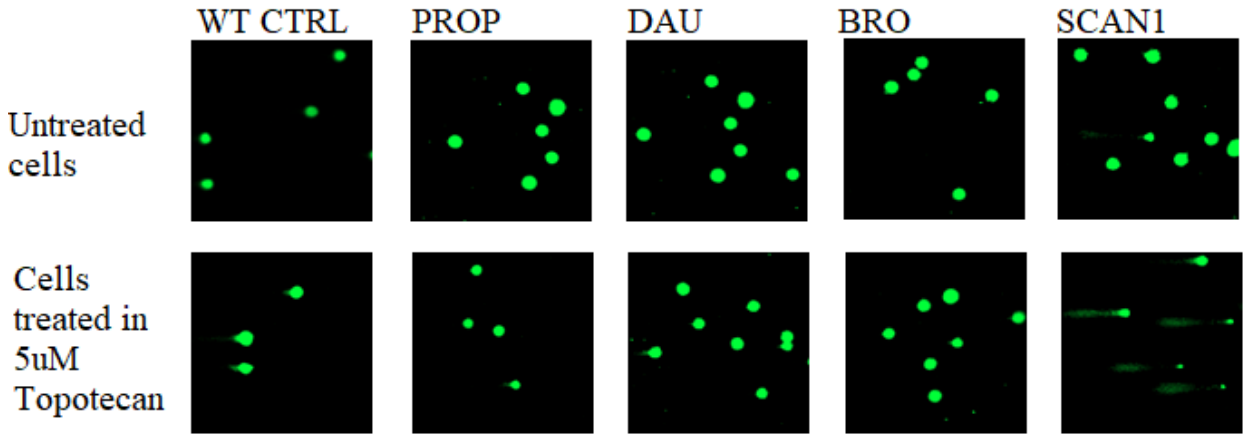


Fig 3. Sample images of Comet Assay with Topotecan treatment, and 1-hour recovery @37°C. Cells embedded in 1% low-melting agarose, electrophoresed @21V (1V/cm) for 40 min. A lack of comet tails in untreated cells indicate intact DNA and a healthy cell sample. Little DNA damage is seen in control, PROP, DAU, and BRO cells in both untreated and 5μM Topotecan treated conditions. SCAN1 is hypersensitive to Topotecan, consistent with the literature.

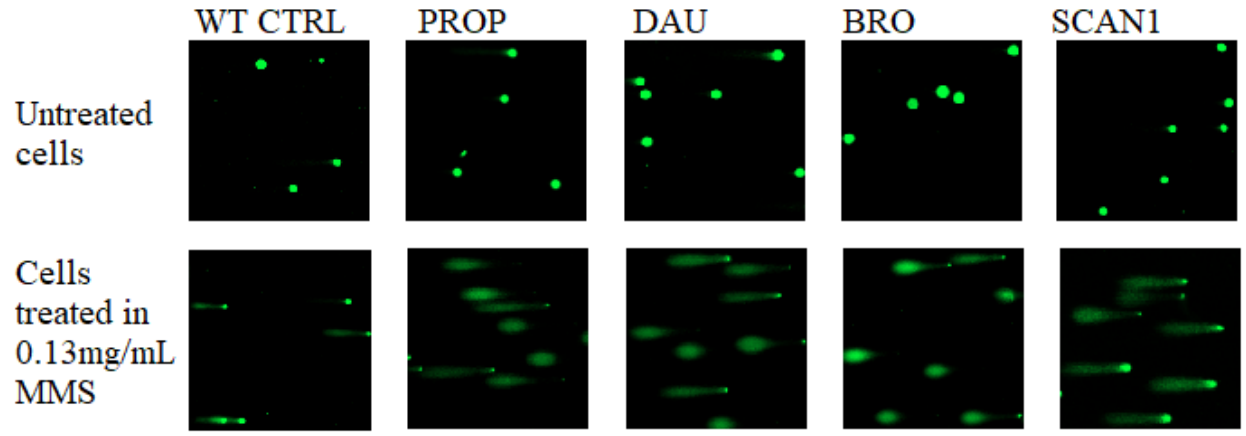


Fig 4. Sample images of Comet Assay with Methyl Methanesulfonate (MMS) treatment for 10 mins @37°C with no recovery. Experimental protocols were the same with the Comet Assay with Topotecan treatment. A lack of comet tails in untreated cells indicate intact DNA and a healthy cell sample. While all cell lines show DNA damage after MMS treatment, PROP, DAU, and BRO cell lines appear to show more DNA damage – comet heads and tails are visibly separated after 0.13mg/mL MMS treatment. On the other hand, control wild-type and SCAN1 comets tails are still linked to the comet head after 0.13mg/mL MMS treatment.

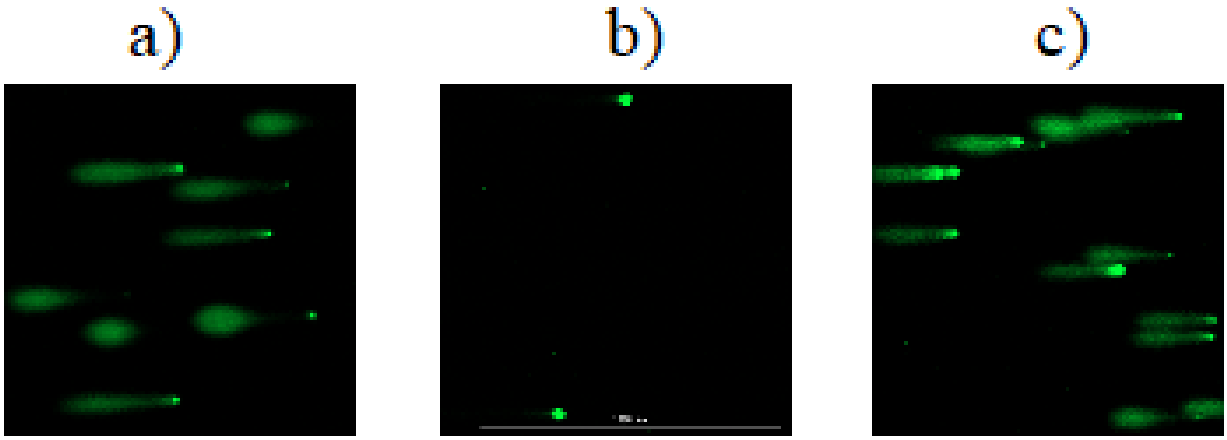


Fig 5. Issue with software identification of comets. 5a) shows comet tails that either do not have an appreciable comet head, or are too distanced from their comet head. As a result, the software is unable to correctly identify the comet head. 5b) shows a slide that had difficulty with cell adherence. As a result, not enough data was collected in these experiment conditions to confidently describe the extent of DNA damage. 5c) shows a slide that had a high density of cells, such that electrophoresis brought DNA fragments from one cell on top of DNA fragments from another cell. As a result, software is unable to distinguish the DNA from the two cells.