MYCN Affects DNA Repair Activity in Neuroblastoma

Jared Spitz

Senior Honors Thesis
in the laboratory of
Dr. Roland Kwok, Ph.D. and Dr. Valerie Castle, M.D.

ABSTRACT:

Despite being the most common pediatric solid tumor, neuroblastoma remains an enigma. Previous studies have indicated that MYCN gene amplification, an indicator of poor prognosis, may play a role in repair of double strand breaks and tumorigenicity. We hypothesized that MYCN increases the repair activity of double strand breaks, which results in genomic instability and increased tumorigenesis. To test this model, MYCN was overexpressed in one neuroblastic (N-type) and one stromal (S-type) neuroblastoma cell line. The levels of DNA repair factors and DNA repair activity were measured. While, MYCN overexpression increased the protein level of various DNA repair factors in both S and N-type cells, it did not increase repair activity in S-type cells. Interestingly, the increased level of MYCN did increase the level of an alternative, more error-prone non-homologous end joing pathway in S-type cells. Work to see the effect of MYCN levels on repair activity in N-type cells is in progress. These results suggest that MYCN increases the error rate during DNA repair and as a result increase genomic instability and tumorigenesis.

Key Words: Neuroblastoma; MYCN; classical non-homologous end joining; alternative non-homologous end joining; homologous recombination.

INTRODUCTION:

NEUROBLASTOMA:

Neuroblastoma is a pediatric solid tumor of the sympathetic nervous system and is derived from neural crest stem cells[1]. It is the most common extracranial solid tumor in children and the most commonly diagnosed malignancy in infancy[2]. It accounts for approximately 7.2% of malignancies in patients under the age of 15[2-4] with an incidence of 10.5 per million children[4]; in the United States alone there are about 650 new diagnoses each year[3]. While the incident of disease is higher amongst males and Caucasians, the overall survival rates do not vary amongst gender or race[5].

Histologically, neuroblastoma tumors are highly heterogeneous. They are composed of a variable amount of neuroblastic (neuronal lineage) and Schwannian-like cells (glial lineage)[6]. *In vivo* these cells arise from a common precursor in the neural crest stem cell lineage[7]; however, even if they are derived from the same origin, the chromosomal abnormalities found in neuroblastic cells are not shared by the Schwannian-like cells[8, 9].

It needs to be noted that *in vitro* three types of neuroblastoma cell line cultures exist: N (neuroblastic)-type, S (substrate-adherent)-type, and I (intermediate)-type[10]. N-type cells resemble neuroblastic cells and are tumorigenic; this is based on immunostaining, and thus N-type cells are similar to *in vivo* neuroblastic cells and thus serve as a surrogate model for them[6]. Further, many are MYCN amplified while many are MYCN non-amplified N-type cells[11]. These cells can be induced to differentiate into neuronal or neuroendocrine cells or to dedifferentiate into neural crest-like cells[12]. S-type cells are substrate adherent and non-tumorigenic and resemble Schwannian-like glial cells[13, 14]. As such, S-type cells serve as a surrogate model for Schwannian stromal cells. Additionally, the I-type cell has characteristics of

both neuronal and Schwannian, glial cells and *in vitro* has bipotentiality and can differentiate into a neuroblastic-like or glial-like cell[10].

Lastly, certain chromosomal aberrations are associated with neuroblastoma. The genetic abnormality that is most consistently associated with advanced stage disease, high risk categorization, and treatment failure is amplification of the oncogene MYCN[15-18]. It is found in approximately 20% of primary tumors[17, 18]. In approximately 25-30% of neuroblastomas, there is a loss of the short arm of chromosome 1 (1p)[19, 20]. Allelic loss of the long arm of chromosome 11 (11q) is present in 35-45% of primary tumors, and it is associated with non-amplified MYCN tumors[21]. A gain of a 1-3 copies of the long arm of chromosome 17 (17q) is also observed[22]. Finally, DNA ploidy serves as binary variable for assessment of risk. Most tumors are either diploid or hyperdiploid (normally triploid) with triploidy having a favorable prognosis[23, 24].

Treatment of disease is based on stratification of patients into three risk groups based on clinical variables, histological variables, and biological variables. Patients can be grouped into low, intermediate, and high risk groups. As seen in Figure 1[2], high risk patients have a far lower event-free survival rate than do those patients with low and intermediate risk.

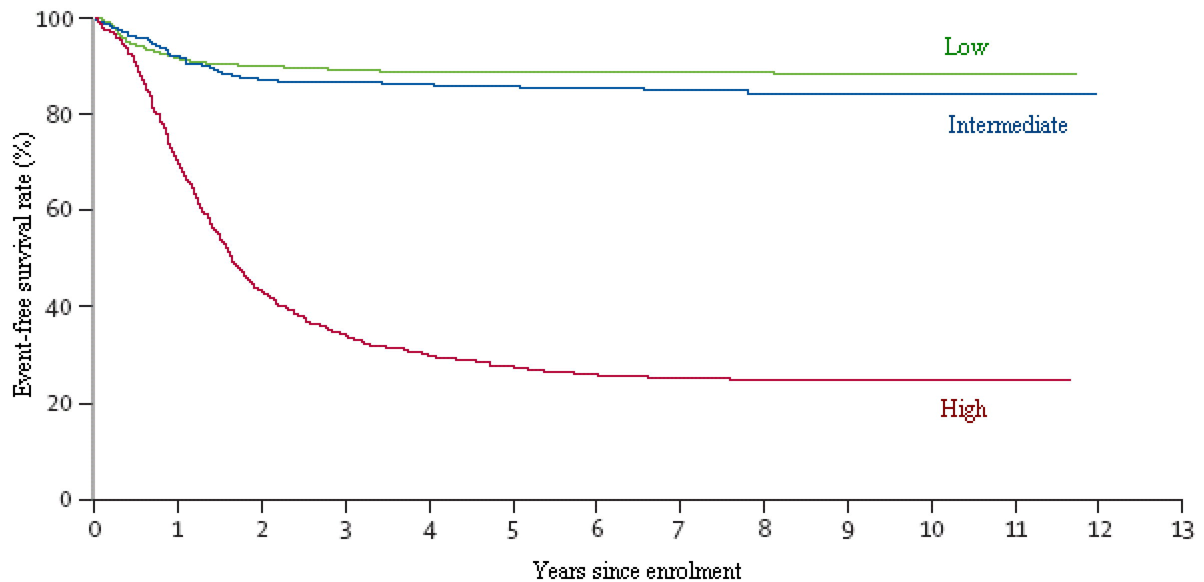

Figure 1: Patients treated between 1986 and 2001 in Children's Cancer Group, Pediatric Oncology Group, and Children's Oncology Group studies were classified as low-risk, intermediate-risk, and high-risk at diagnosis based on clinical and biological features. Kaplan-Meier survival analysis shows marked differences in event-free survival for these groups of patients. Data courtesy of W London, Children's Oncology Group statistical office.

MYCN:

As mentioned previously, amplification of the MYCN oncogene is the most frequent chromosomal anomaly associated with an aggressive phenotype in neuroblastoma. The MYCN gene is composed of two introns and three exons, two of which (exons 2 and 3) are coding[25, 26]. The gene is 6,435bp with the sequence predicting an mRNA transcript of 2914 nucleotides with a poly(A) tail[25, 27]. MYCN encodes a transcription factor with a short half life[28]. It is phosphorylated by casein kinase II (CKII)[29]. The protein is 456 amino acids with an unphosphorylated weight of 49 kDa[25] and a phosphorylated weight of 64 kDa. MYCN promotes transcription of numerous target genes[30] involved in a variety of cellular processes[31].

High levels of MYCN expression are seen in MYCN amplified tumors. These higher levels are thought to determine the aggressive and dedifferentiated phenotype that is often associated with MYCN amplification[27, 32-38]. Because MYCN amplified cells that have MYCN knocked down exhibit retardation of growth, they are not viable for a tumorigenic model[39]. Therefore, MYCN non-amplified cell lines with little or no endogenous MYCN expression are often used to evaluate the effect of high level MYCN and serve as excellent models for determining the biological effect of MYCN amplification[40, 41].

DNA REPAIR PATHWAYS:

Genomic stability is extremely important for normal development, growth, and the suppression of cancer[42, 43]. Therefore, breaks in DNA can be lethal to a cell with double strand breaks (DSBs) being perhaps the most lethal[43, 44]. These breaks can occur due to endogenous agents such as reactive oxygen species, exogenous sources such as chemotherapy and ionizing radiation, or when replication forks stall upon encountering a lesion in the DNA[45-47]. Double strand breaks are typically repaired through one of two pathways: homologous recombination (HR) and non-homologous end joining (NHEJ)[43, 48].

Homologous recombination uses a template strand to repair the DSB. It is most important during S and G_2 phases for error-free repair of DSBs as HR-mediate repair is extremely faithful[49, 50]. Homologous recombination is dependent on several proteins including Ligase I[51], Rad51[52], Rad52[53], Rad54[54], and RPA[55]. During HR, one strand invades another to align with its homologue. Rad54 is then thought to stabilize the resultant D-loop and extend the free ends of DNA[54] for relegation by Ligase I[51]. homologous recombination

The more important repair process in mammalian cells is non-homologous end joining, which is dominant in G_0, G_1, and the early part of S phase[56]. Unlike homologous recombination, NHEJ fuses free ends from broken DNA strands together. This process may end in deletion of terminal regions (those regions near to the break and religation sites) or chromosomal translocation[57]. As such, the absence or deregulation of NHEJ has been implicated in a number of diseases, including cancer[58-60]. Just like HR, NHEJ has two main pathways; however they differ in the proteins involved. These are the classical NHEJ and the alternative NHEJ pathways. The classical pathway depends on several proteins, including the Ku heterodimer (Ku70 and Ku80), DNA-dependent protein kinase (DNA-PK_{cs}), and Ligase IV[43, 61]. It is currently thought that the Ku heterodimer binds the free ends of the DNA and then recruits DNA-PK_{cs} [62, 63], which brings DNA Ligase IV[64, 65] to religate the free ends[64].

The alternative non-homologous end joining pathway is independent of the Ku heterodimer, DNA-PK_{cs}, and ligase IV[66, 67]. Rather, this pathway appears to be dependent on ligase III[68, 69] and Poly(ADP-ribose)polymerase 1 (PARP-1)[67, 69, 70]. This pathway is even more error-prone than classical NHEJ[67, 71, 72]. It appears that the decision between whether a cell uses classical NHEJ or HR depends in part on the phase the cell is in and competition for free-end DNA binding by Rad52 and the Ku heterocomplex[53]. Whether a cell will use classical or alternative NHEJ seems to be based predominantly on competition between PARP-1 and the Ku complex for binding to free DNA ends[70].

CURRENT RESEARCH:

Recent studies from our lab suggests that Ku70 acetylation status plays a role in release of Bax[73]. Bax is known to promote apoptosis[74]. Apoptosis is critical to normal

neurogenesis and nervous system development[75] and high levels of apoptosis is associated with favorable tumor biology in neuroblastoma[76] and neuroblastoma disease regression[76, 77]. Additionally, Ku70 is known to correspond with radiation and chemoresistance. Ku70 levels are inversely correlated with radiation sensitivity in cervical carcinoma[78] and overexpression of Ku70 has been shown to protect cells from radiotherapy[79] while lowering Ku70 levels has shown to enhance radiosensitivity via its role in double strand DNA repair and NHEJ[80, 81].

The fact that MYCN amplification is associated with disease progression and resistance to treatment and that Ku70 is involved in NHEJ, promotes radiation and chemoresistance, and can induce apoptosis in neuroblastoma made us ask if there was a relationship between MYCN expression and DNA repair. Specifically, prior data suggested that mRNA levels of various genes associated with NHEJ and HR were higher in pooled samples of MYCN amplified tumor than in MYCN non-amplified tumors[82] and that *myc* family genes could target DNA repair genes[83]. We took established neuroblastoma cell lines and overexpressed MYCN in them. Protein expression of various factors associated with NHEJ and HR were detected by Western blotting. Additionally, reporter plasmids were transiently transfected to allow us to measure the level of HR, classical NHEJ, and the error-prone alternative NHEJ. What was found was that the level of activity was not changed in the presence of MYCN expression however the error rate was increased.

MATERIALS AND METHODS:

Database:

Data was collected from Oncogenomics, a publically available, published database (http://pob.abcc.ncifcrf.gov/cgi-bin/JK). One part of the database shows the mRNA expression

of various genes from different patients. For neuroblastoma, these data can be grouped into MYCN amplified and MYCN non-amplified groups. Expression graphs for the DNA repair factors Ku70, Ku80, DNA-PK$_{cs}$, Artemis, XRCC4, DNA Ligase III, DNA Ligase IV, DNA polymerase lambda, and DNA polymerase mu were collected. For each factor, the database publishers determined the median expression level and the amount of mRNA in any given sample was reported relative to the median. For our preliminary analysis of the database, we calculated the percent of each patient that had overexpression of a given DNA repair factor in both the MYCN amplified and non-amplified groups.

Cell Lines:

Four cell lines were used throughout the experiments. The S-type cell that was selected was the MYCN non-amplified SH-EP1 cell line. It was chosen for two reasons. The first is that it is MYCN non-amplified and thus can be transfected with MYCN. The second reason was that a SH-EP1:MYCN cell line had previously been constructed by this lab through stable transfection of MYCN into SH-EP1 cells.

A transient transfection of MYCN into the N-type cell line, SH-SY5Y, was done.

Transfections:

Reporter plasmids:

Four DNA double strand break repair activity reporter plasmids were used to quantify DNA repair activity. These were gifts from Z. Mao of the University of Rochester, Department of Biology[84]. These are shown in Figure 2. The four plasmids are based on reconstitution of an intact GFP gene where positive GFP expression is a surrogate for successful repair. Three of the plasmids measure a form of non-homologous end joining. Figure 2A shows two plasmids, herein termed NHEJ-C and NHEJ-I. NHEJ-C measures repair of compatible DSB ends by

NHEJ and uses HindIII as the restriction endonuclease to create the DSB, while NHEJ-I, uses the restriction endonuclease I-SceI and shows NHEJ-mediated repair of incompatible DSB ends. Figure 2B shows a plasmid, herein named HR-I, which also uses I-SceI and measures repair by homologous recombination[84]. Lastly, Figure 2C shows a plasmid, herein named NHEJ-B, which measures "accurate" repair of DSB ends by NHEJ and uses the endonuclease BsrG1. NHEJ-B does not tolerate any end-processing before religation and therefore is a measure of accurate repair via a NHEJ-mediated process; it is used for quantifying the amount of error in NHEJ-mediated repair.

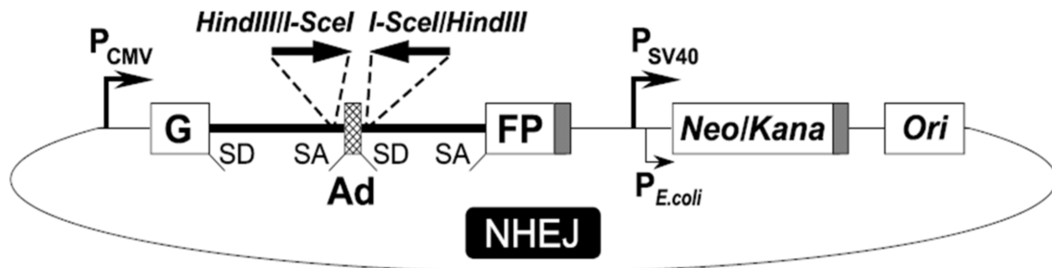

Figure 2A: A schematic of an NHEJ repair activity reporter plasmid showing the cut sites that would produce NHEJ-C (HindIII) and NHEJ-I (I-SceI).

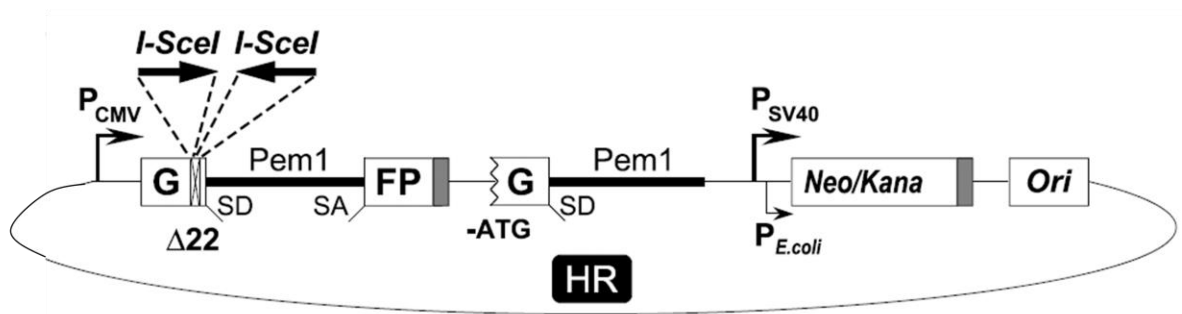

Figure 2B: A schematic of an HR repair activity reporter plasmid showing the cut sites that would produce HR-I (I-SceI).

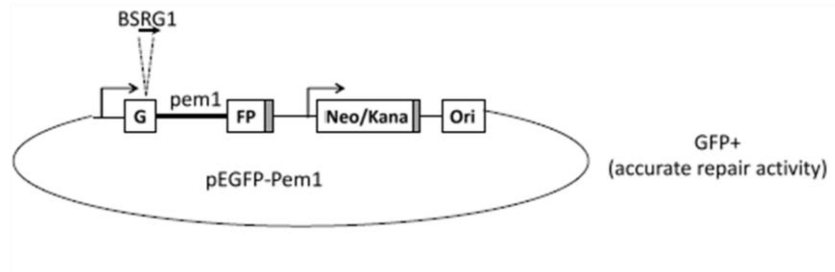

Figure 2C: A schematic of an NHEJ repair activity reporter plasmid showing the cut sites that would produce NHEJ-B (BsrG1).

Additional accessory pUC19 plasmids containing GFP and Cy5-labelling were used as positive and internal controls, respectively.

Reporter plasmids were transiently transfected into the two SH-EP1 cell lines. 200ng of the reporter assays were transfected into 75,000 cells using the *Trans*IT®-LT1 Transfection System (from Mirus®) per the Mirus® MIR 2300 transfection protocol. Cells were cultured in MEM media supplemented with 10% FBS. Transfections were carried out in Opti-MEMI Medium. A full protocol for transfection of the reporter plasmids can be found under Supplemental Protocol 1.

To ensure that sufficient enough data was collected to draw statistically meaningful conclusion, each cell line transfected with a given reporter plasmid was analyzed by flow cytometry in triplicate. This procedure was repeated three times.

Transient transfection of SH-SY5Y with MYCN:

A transient transfection of a MYCN construct into SH-SY5Y cells was done. Transfection was done per The General Protocol for Nucleofection® of Adherent Cell Lines (from Lonza). 0.5µg of a GFP plasmid and a puromycin resistance gene plasmid, along with 5µg of a pCMV or pCMV:MYCN plasmid (Figure 3) were transfected into 50,000 SH-SY5Y cells. These cells were cultured in MEM supplemented with 10% FBS. Transfection occurred in

a 15mM solution of the provided Nucleofection® transfection reagent in Opti-MEMI Medium. Cells containing the MYCN plasmid were selected using puromycin.

Western Blots:

Western blots were performed on all four cell lines. 40µg of protein was loaded and ran on a 7% or 10% denaturing SDS-polyacrylamide gel depending on the molecular weight of the target protein. Gels were run at 150V for 90 minutes. A wet transfer[82] was done for 60 minutes at 100V.

Blocking was done in a 5% w/v solution of dry milk in 1% TBST. Primary and secondary antibodies were applied in a 5% w/v solution of dry milk. Either ECL™ (Santa Cruz Biotechnology, Inc.) or ECL+™(GE Healthcare) was used as the chemiluminescent agent and was used per company instruction.

The following primary antibodies were used in a 1:500 dilution: anti-MYCN (mouse monoclonal from Santa Cruz Biotechnology, Inc.), anti-Ku70 (mouse monoclonal from Santa Cruz Biotechnology, Inc.), anti-Ku86 (rabbit polyclonal from Santa Cruz Biotechnology, Inc.), anti-DNA Ligase III (mouse monoclonal from Santa Cruz Biotechnology, Inc.), anti-DNA Ligase IV (rabbit polyclonal from Santa Cruz Biotechnology, Inc.), anti-DNA-PK_{cs} (mouse monoclonal from Santa Cruz Biotechnology, Inc.), and anti-Rad54 (mouse monoclonal from Santa Cruz Biotechnology, Inc.). Anti-α-tubulin (rabbit polyclonal from Abcam) and Anti-β-tubulin (rabbit polyclonal from Abcam) were used as loading controls.

Horseradish peroxidase-linked anti-rabbit or anti-mouse IgG secondary antibodies (Amersham™ from Thermo Scientific) were used at a 1:5000 dilution.

Statistical Analysis:

Statistics were done for all data measuring repair of DNA double strand breaks by either homologous recombination or non-homologous end joining. Comparisons involving the error rate of the NHEJ repair was also done. In each case, a t-tests was done.

RESULTS:

RNA Levels of Various DNA DSB Repair Factors are Elevated with MYCN Amplification in Pooled Database:

Initial evidence that MYCN affected DNA repair associated genes came from a database of neuroblastoma tumors that had been collected and analyzed for the mRNA levels of various genes. The database showed whether a particular tumor over or underexpressed a particular gene relative to the median of mRNA expression levels for that gene in all of the tumor samples. What our lab did was to calculate the percentage of samples that overexpressed a particular gene for both the MYCN amplified and non-amplified tumor samples. As seen in Figure 4, a higher percentage of patients with MYCN amplification had overexpression of various DNA repair genes than MYCN non-amplified patients. The factors that were increased in the presence of MYCN amplification had to do with homologous recombination and both classical and

alternative non-homologous end joining.

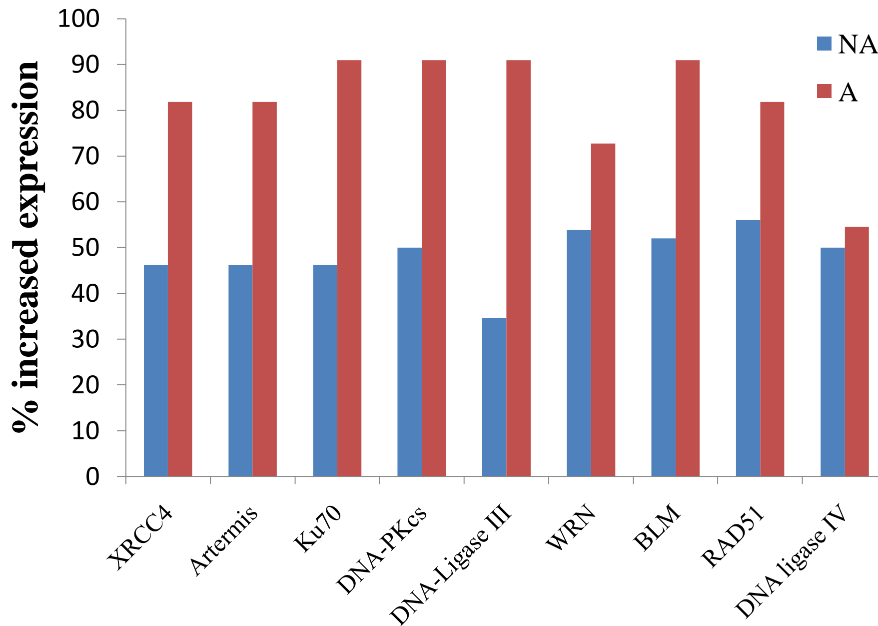

Figure 4: Expression of DNA repair genes in pooled MYCN amplified and non-amplified cells.

Due to the fact that the mediators of DNA repair are proteins, mRNA level is not sufficient for drawing conclusions about the effect of MYCN on DNA repair and therefore the protein levels of various repair factors need to be observed.

Protein Levels of DNA-PK$_{cs}$ and the Ku Heterodimer are Elevated with MYCN Overexpression in a S-Type Neuroblastoma Cell Line:

Western blots of proteins collected from SH-EP1 cells were done of various proteins known to be involved in HR and both classical and alternative NHEJ. The results are shown in Figure 5. As seen, this SH-EP1 construct over expresses MYCN. β-tubulin was used as a loading control. Neither the level of Rad54 nor ligase I showed any increase in protein level in SH-EP1 cells overexpressing MYCN. Several markers for classical NHEJ were observed at the protein level. The levels of Ku70 are increased along with a larger increase in DNA-PK$_{cs}$ in the

MYCN overexpressed cells; there is a very slight increase in Ku80 levels. However, there is a slight decrease in the protein level of ligase IV in the MYCN overexpressed SH-EP1 cells. The only marker of alternative NHEJ that was examined at the protein level was ligase III. As seen, there is no change in protein level in MYCN overexpressed SH-EP1 cells versus the non-overexpressed cells. Changes in protein levels were determined by visualization of the Western blot film. To determine the relative level proteins, densitometry studies need to be done.

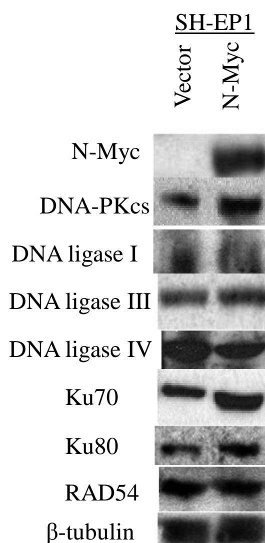

Figure 5: Western Blot Analysis of Various DNA DSB Repair Factors with MYCN Overexpression

However, as the main interest is in DNA repair activity, it still needs to be quantifiably measured whether MYCN overexpression actually affects DNA repair activity.

MYCN Overexpression Does Not Alter DNA DSB Repair Activity in SH-EP1 Cells:

In order to measure DNA repair activity, the stably transfected SH-EP1:MYCN cells were transiently transfected with the DNA repair activity reporter plasmids. The results are shown in Figure 6. Homologous recombination was measured with an I-Sce1-dependent HR reporter plasmid (HR-I). As seen, the level of homologous recombination increased slightly in

the SH-EP1 cells overexpressing MYCN. However, this increase was not statistically significant (p=0.14). Non-homologous end joining was measured using the two reporter plasmids NHEJ-C and NHEJ-I. As seen in Figure 6, the repair of compatible end DNA double strand breaks by NHEJ increased slightly in cells overexpressing MYCN. This increase was also not statistically significant (p=0.72). The repair of incompatible ends by NHEJ was actually decreased concomitantly with MYCN overexpression. As with HR and compatible end NHEJ repair, this decrease was also not statistically significant (p=0.16).

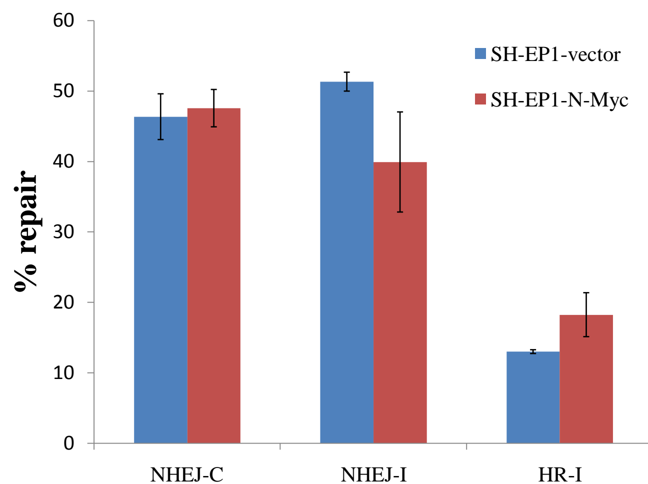

Figure 6: MYCN over-expression does not significantly affect DSB repair activity

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MYCN Overexpression Decreases Accurate NHEJ Repair Activity in SH-EP1 Cells:

Because there is both a classical and an alternative NHEJ pathway, it was important to look at the overall accuracy of NHEJ-mediated repair. As seen in Figure 7, MYCN overexpression decreased the accurate NHEJ repair activity as measured using BsrG1-dependent NHEJ reporter plasmid. This decrease was statistically significant (p<0.00001). This decrease in accurate repair led to a statistically significant increase of over 150% in repair error

($p<0.00001$). The overall repair activity, as measured by the HindIII-dependent NHEJ-C reporter plasmid, did not significantly change in the presence of MYCN overexpression ($p=0.72$); NHEJ-C was chosen as the measurement of total repair based on work done by the group that originally constructed these vectors[84].

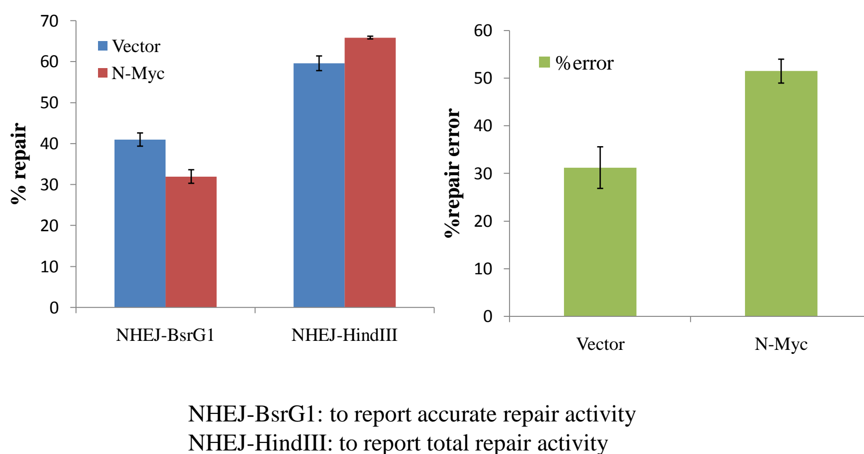

Figure 7: MYCN over-expression decreases repair accuracy

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Protein Levels of Ligase I, Ligase III, and Ku70 are Elevated with MYCN Overexpression in a N-Type Neuroblastoma Cell Line:

Based on the mRNA database, Western blots for DNA repair factors were done with proteins from the SH-SY5Y cells that were transiently transfected with MYCN. The results are shown in Figure 8. As seen, this SH-SY5Y cell line with the MYCN transfection over expresses MYCN. In this case, α-tubulin was used as a loading control to verify the high levels of various proteins. Ligase I, a marker of HR, was slightly increased in the presence of MYCN overexpression. No blot, as of this writing, has been done for Rad54. As of this writing, the only markers looked at for classical NHEJ were Ku70 and Ku80. While Ku70 showed a large

increase in protein level, Ku80 did not show an increase in protein level in the SH-SY5Y cells overexpression MYCN. The only marker of alternative NHEJ that was looked at was ligase III. As seen, there is an increase in ligase III protein level associated with MYCN overexpression. Again, the determination of the relative changes in protein levels was made by visualization; only densitometry studies can actually determine the relative changes in these protein levels due to MYCN overexpression.

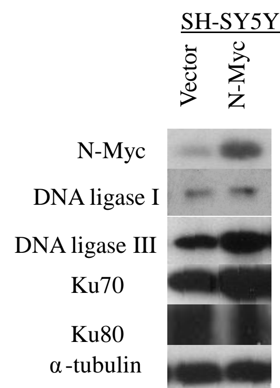

Figure 8: Western Blot Analysis of Various DNA DSB Repair Factors with MYCN Overexpression

Again, repair activity must still be quantified using the aforementioned reporter plasmids and the overall accuracy of this repair must also be quantified.

DISCUSSION:

Little is known about neuroblastoma biology, despite it being a common pediatric malignancy. The role of MYCN amplification and the mechanism of tumorigenicity are not well understood. Our hypothesis is that MYCN amplification increases tumorigenicity via a role in

DNA damage repair. Specifically, an increase in MYCN levels increases the protein level of certain factors involved in double strand DNA repair. The rise in protein level increases the activity level of DNA repair, which will increase the tumorigenicity of neuroblastoma tumors. The results of this current study showed that many proteins involved in DNA repair are upregulated by MYCN overexpression. To our surprise, while this did not change the level of repair activity, it did increase the amount of error in the non-homologous end joining that did occur. The increased error could potentially cause genomic instability, which would enhance the tumorigenicity of neuroblastoma.

Early evidence suggesting that this model could be accurate came from gene expression data found in a public database. The gene expression data supported the idea that MYCN played a role in modulating DNA repair factors because numerous repair genes showed an increase in expression in MYCN amplified tumors. Therefore, MYCN perhaps played a role in mediating double strand DNA break repair activity in neuroblastoma. However, there are several limitations to this data set. The first is that these are pooled sets and so contain Schwannian and Neuronal-type neuroblastoma cells. Therefore, no meaningful comparison can be done on the effect of MYCN amplification in DNA repair that takes into account the physiological and pathological differences between the two cell types. In addition to pooled data, there is no internal control for gene expression (such as expression of a housekeeping gene) making comparisons between genes impossible because over or underexpression was compared to the median mRNA level for each factor; without an internal control, the repair factors cannot be compared. Additionally, tumors are different than cell lines, which means that effects seen in tumors may not mirror what occurs in cell lines.

The second drawback is that, as it is data collected from a public database, it is hard to do any meaningful statistics. As such, no statistics are provided.

Lastly, because the mediators of DNA repair are proteins, mRNA level is not sufficient for drawing conclusions about the effect of MYCN on DNA repair. Thus, while this data set lent credence to the hypothesis that MYCN may affect DNA repair activity in neuroblastoma cells, the protein levels of various repair factors need to be determined.

RELATION OF MYCN TO DNA REPAIR PROTEIN LEVELS:

As MYCN is a transcription factor, its relevance in DNA repair activity would be in its ability to promote transcription of genes involved in DNA repair. Therefore, the protein levels of various DNA repair factors were studied using Western blot analyses. The importance of the protein expression data is that it strongly suggested that MYCN transcriptionally activates several genes important in double strand DNA repair. To the best of my knowledge, this has not been shown in MYCN. To definitively show this, however, MYCN needs to be shown to bind to the promoter regions of the genes whose protein levels were elevated in the presence of MYCN overexpression.

However, this data is similar to data about the transcription factor c-Myc (which is in the same family of transcription factors as MYCN) and its role in DNA repair. The study with c-Myc validated a role of the Myc family in DNA repair. Putative c-Myc binding sites have been shown to be present in the promoters of Rad54, DNA-PK_{cs}, and Ku70[85, 86]. Binding of c-Myc has been definitively shown to occur in the promoters of Rad51 (a protein related to Rad54 and needed for homologous recombination), DNA-PK_{cs}, Ku70, and DNA Ligase IV[83]. To verify that this binding can induce protein level increases, c-Myc binding to the promoters of Ku70 and Rad51was shown to increase the protein level of these two genes[83].

Given that MYCN and c-Myc have overlapping functions and MYCN can functionally replace c-Myc[87], it would seem logical to suggest that they would both upregulate the same proteins. Indeed, in both cell lines, Ku70 is upregulated and DNA-PK$_{cs}$ is upregulated in the SH-EP1 construct; of note, DNA Ligase IV was slightly downregulated in the same SH-EP1 construct. Based on these Western blots, it appears that while MYCN and c-Myc have similar functions and can be functional substitutes for the other, they do harbor distinct roles.

Importantly, MYCN was shown to upregulate protein level in the S-type cell line, SH-EP1, and the N-type cell line, SH-SY5Y. Interestingly, the same proteins are not upregulated in both S and N-type cells, suggesting that MYCN may have cell-specific roles in DNA repair.

However, while MYCN was shown to upregulate the protein levels of several genes associated with DNA repair, protein expression is not necessarily indicative of actual repair activity and therefore repair activity needed to be measured.

DNA REPAIR ACTIVITY LEVELS:

To evaluate the actual double strand DNA repair activity, special repair activity reporter plasmids based on reconstituted GFP expression were transiently transfected into the SH-EP1 cell line and flow cytometry was used to evaluate GFP expression. As shown, the activity levels of non-homologous end joining and homologous recombination were not affected by the level of MYCN overexpression to a statistically significant degree in the SH-EP1: MYCN cell line. Until similar data is generated for a SH-SY5Y:MYCN stable construct the following discussion will pertain only to S-type neuroblastoma cell lines.

Given the mechanism of homologous recombination and the protein expression results, it is not surprising that HR levels did not statistically change in the presence of MYCN overexpression. Homologous recombination is dependent on Rad54 stabilization of the D-loop

post strand-invasion and relegation of strand breaks by DNA Ligase I. Given that neither protein is upregulated by MYCN overexpression, it seems logical that the actual repair activity would not be upregulated either.

A similar argument to the one used for HR activity can be applied to non-homologous recombination. It must be noted that the decision between the use of classical NHEJ and the alternative NHEJ is based on a competition between the Ku heterodimer and the protein Poly(ADP-ribose)polymerase 1 (PARP-1)[70]. When Ku dominates then the classical NHEJ is favored and vice versa if PARP-1 dominates. The classical NHEJ pathway is dependent on Ku70 and Ku80 recognition of broken ends, recruitment of DNA-PK_{cs}, and religation by DNA Ligase IV. While in S-type cells the first three proteins listed are upregulated concurrent with MYCN overexpression, there is actually a decrease (albeit a slight one) in DNA Ligase IV. Since the entire mechanism is independently dependent on each protein, if just one protein were not upregulated, then the entire pathway should not be upregulated to a significant degree. Indeed, this is what was seen.

As for the alternative NHEJ, this pathway was statistically significantly upregulated in the presence of MYCN overexpression. This pathway is dependent upon PARP-1 recognition of the free ends of double strand breaks and subsequent ligation by DNA Ligase III. At this time, no argument can be put forth as to why there was such an increase. This is especially true in light of the fact that the protein level DNA Ligase III is not increased in the presence of MYCN overexpression.

However, one potential hypothesis can be offered for the increase in the alternative, more error-prone NHEJ pathway. While the Ku heterodimer protein level is upregulated in the SH-EP1 cells that overexpress MYCN, if the PARP-1 protein is increased to an even greater degree

by the increased MYCN levels then it is possible that the PARP-1 is out-competing the Ku heterodimer for binding to free DNA ends in the double strand break. In this case, the alternative NHEJ pathways would prevail, which was what was seen. However, this hypothesis would need to be tested to determine whether there is an increase in PARP-1 concurrent with MYCN overexpression and if this rise in PARP-1 is greater than that of the Ku heterodimer.

DNA REPAIR AND THE LINK TO TUMORIGENESIS:

As stated, our hypothesis proposes that the increased error-prone DNA repair causes an increase in genomic instability and tumorigenicity. The first is that PARP-1 and error-prone non-homologous end joining have been linked to tumorigenesis in several models. Error-prone NHEJ has been implicated in development of liver cancers[88], bladder cancer[89], and human leukemias[90]. These models all suggest that at some point, the error-prone nature of the alternative NHEJ pathway increases the mutation rate of cells and therefore increases tumorigenesis.

Other potential implications may stem from the new knowledge that there is a MYCN driven increase in error-prone DNA repair. To date, there is no known cause of MYCN amplification. Previous studies have suggested that NHEJ-mediated repair, in particular an error-prone version of it, has been linked to amplification of genes, including c-Myc in the context of certain lymphomas and leukemias[91]. While increased error-prone DNA repair has not been evaluated in ascertaining the origin of MYCN amplification in neuroblastoma, it is an attractive idea to investigate.

However, of perhaps more relevance clinically is the fact that with high-risk neuroblastoma, patients frequently relapse after chemo- or radiation therapy. The ionizing radiation used in conventional radiotherapy induces double strand DNA breaks. The same is true

of many conventional chemotherapeutics, including those used in neuroblastoma. An error-prone repair pathway may help drive tumor progression in the context of post-treatment relapse by haphazardly fixing the induced double strand breaks, which could then cause chemo- and radiation resistance. Therefore, there has been a thought that by inhibiting PARP-1 action in certain cancers, these tumors can be sensitized to chemotherapy or radiation therapy. Of great interest, a study of various cancer cell lines showed that PARP-1 inhibition significantly enhanced cell killing by gamma-radiation, X-ray radiation, and the chemotherapies temozolomide and topotecan, both of which induce DSBs[92]. While this current study did not evaluate the role of PARP-1 inhibitors in a neuroblastoma cell line, a later study showed that PARP-1 inhibition did increase the killing efficacy of temozolomide and topotecan in two neuroblastoma cell lines and xenograph models[93]. Given this evidence, the data presented above may hint at a mechanism for PARP-1-mediated delay of tumor relapse post-treatment and may warrant investigation of PARP-1 inhibitors in a clinical setting.

FUTURE DIRECTIONS:

Several further studies should be done to get a fuller understanding of double strand DNA break repair in neuroblastoma.

Densitometry:

As noted in the results above, to accurately determine the change in repair factor protein levels in response to MYCN overexpression, densitometry studies should be done. These studies should be done both for the SH-EP1:MYCN and SH-SY5Y:MYCN cell lines.

WORK IN SH-SY5Y:

As mentioned, protein levels are not a surrogate for activity levels in cellular models. As the results of this study indicated, increased protein expression of DNA repair factors does not

necessarily correlate to an increase in the repair pathway that utilizes those factors. Therefore, the repair activity must be quantified to fully understand how neuroblastoma cells repair double strand DNA breaks. As of this writing, as far as the other knows, no construct of SH-SY5Y N-type neuroblastoma cells that stably overexpress MYCN has been created. However, such a cell line is needed. Work should be done to create a MYCN overexpressing SH-SY5Y cell line. Once done, transient transfections of SH-SY5Y cells both overexpressing MYCN and not overexpressing MYCN should be done with the double strand DNA repair activity reporter plasmids and activity quantified per the above protocol. The flow cytometric data would give more accurate picture of DNA repair activity in N-type cells than the protein levels and would lead to a clearer overall picture of DNA repair in neuroblastoma overall.

Further, neuroblastic cells in neuroblastoma tumors are the actual tumorigenic cell line. S-type cells are non-tumorigenic and it is thought that Schwannian-like glial cells only continue to proliferate in neuroblastoma tumors in response to growing neuroblastic cell populations as Schwannian cells provide support and nutrients for neuronal cells. While it is true that the cross-talk between neuroblastic cells and Schwannian-like glial cells is crucial for tumorigenesis, staging of neuroblastoma tumors, and prognosis[94-96], the real culprit in tumor development is the neuroblastic cells. Therefore, only repair activity measured in N-type cells can give us a more accurate understanding of the role of MYCN in DNA repair in neuroblastoma.

Of note, MYCN is not normally expressed in S-type cells, while it is expressed in N-type cells. Therefore, forced MYCN overexpression in N-type cells represents a more physiologically relevant model than forced MYCN expression in S-type cells and therefore warrants measuring activity in an N-type cell line. Additionally, MYCN, as a transcription factor may have cell-

specific effects, which can only be understood by duplicating this work in the other main histologically distinct cell type.

Lastly, difficulties in obtaining high-specificity antibodies for DNA-PK_{cs}, DNA Ligase IV, and Rad54 prevented probing for these proteins in SH-SY5Y cells that are overexpressing MYCN. For a thorough understanding of the role of MYCN in double strand DNA repair and for a complete comparison of S and N-type cells, these last remaining Western blots should be completed as well.

PARP-1:

Any model of DNA repair in neuroblastoma must take into account the fact that in, at least, S-type cells, there is an increase in error-prone repair via the alternative non-homologous end joining pathway while there is no increase in the activity of the classical NHEJ pathway. As mentioned, the alternative NHEJ pathway is critically dependent upon the protein PARP-1. As discussed above, one hypothesis underlying the increase in error-prone NHEJ in the presence of MYCN overexpression is that it is driven by a substantial increase in PARP-1 that is greater than the increase seen in the Ku heterocomplex. Given the existence of both SH-EP1 and SH-SY5Y cell lines that express (at least transiently) MYCN, the protein level of PARP-1 should be evaluated to see if it is overexpressed in the presence of increased MYCN. Further, if possible, quantification of the amount of Ku heterodimer and PARP-1 proteins in the presence of forced MYCN expression should be carried out to assess which one would be present in excess and thus could out-compete the other. This way, the overactive error-prone pathway could be better understood in S-type cells.

Tumorigenicity:

The final link of our hypothesis states that the increase in error-prone DNA repair induced by MYCN overexpression leads to increased tumorigenicity. This putative increase in tumorigenicity needs to be measured. Assays to assess tumorigenicity in the presence and absence of MYCN overexpression should be done. These assays can include colony forming assays in soft agar and injecting mice with cells to measure tumor growth. Multiple assays need to be done to assess different characteristics of tumorigenicity as there is no specific tumorigenicity assay.

CONCLUSION:

Neuroblastoma is a devastating pediatric cancer. High risk neuroblastoma exhibits a high degree of mortality with treatment failure and disease progression being the norm. MYCN amplification is the most common genetic aberration associated with neuroblastoma and its presence is highly indicative (and diagnostic) of high risk disease. Additionally, evidence from our lab suggested a role for Ku70 in mediating response to radiation in neuroblastoma cell lines. Given this data, it was decided to investigate the role of MYCN in double strand DNA repair. To test our model that increased MYCN increases tumorigenicity via an increase in DNA repair, S and N-type cell lines that overexpress MYCN were probed for protein levels of DNA repair factors. The S-type cells were subsequently transfected with reporter plasmids in order to quantify the activity of various DSB repair pathways. MYCN, in its role as a transcription factor, was shown to increase the protein levels of several factors involved in DNA repair in both S- and N-type cells. However, the overall level of classical non-homologous end joining and homologous recombination were unaffected by MYCN overexpression in S-type cells. Rather, the alternative, error-prone non-homologous end joining pathway was increased concurrent with increased MYCN expression.

This pathway has been shown to increase genomic instability and has been linked to tumorigenesis and chemo- and radioresistance. Additionally, *in vitro* and *in vivo* studies have implicated PARP-1 inhibition as an attractive clinical avenue for decreasing tumorigenesis of neuroblastoma and for promoting sensitization of tumors chemotherapeutics and radiation. Our data helps to provide a mechanism for these observations.

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SUPPLEMENTAL PROTOCOL 1:

Double Strand DNA break repair reporter plasmids:

Transfection protocol per well of 12-well plate
A: Plate cells:
It has been found that the transfection efficiency of same-day plating cells is higher than that of cells plated overnight before transfection. Therefore, in this protocol, same-day plating is recommended.

1. The table below indicates the amount plated per well to achieve a confluence of 30-50%; this number of cells was resuspended in 1mL of the indicated complete medium.
2. Before transfection, allow around 4-6 h for the cells to adhere to the plate.

Cell Lines	Plated amount (cells/well)	Complete Medium	Plasmid transfected (reporter plasmid/Cy5-labelled pUC19)
SH-EP1 vector/MycN	$0.75x10^5$	MEM+10%FBS	200 ng/100 ng
SH-EP1	$0.75x10^5$	MEM+10%FBS	200 ng/100 ng
LAI-5s	$0.75x10^5$	MEM/F12K+10%FBS	200 ng/100 ng
SK-N-AS	$0.75x10^5$	DMEM+NEAA+10%FBS	200 ng/100 ng
SH-SY5Y	$1.5x10^5$	MEM+10%FBS	200 ng/100 ng
SK-N-Be(2)	$0.75x10^5$	MEM+10%FBS	100 ng/50 ng
IMR32	$0.75x10^5$	MEM+NEAA+Sodium Pyruvate+Sodium Bicarbonate	100 ng/50 ng
LAI-55n	$0.75x10^5$	MEM/F12K+10%FBS	100 ng/50 ng
Human Fibroblast I	$0.75x10^5$	RPMI+10%FBS	200 ng/100 ng
Human Fibroblast II	$0.75x10^5$	RPMI+10%FBS	200 ng/100 ng

B. Prepare TransIT-LT1 Reagent:DNA complex (immediately before transfection)
The exact amount of reporter plasmid/Cy5-labelled pUC19 is shown in the table above. Accordingly, the amount of pUC19 used will be the total amount of reporter plasmid and Cy5-labelled pUC19.

pUC19	
NHEJ-GFP /Cy5-pUC19	NHEJ-I-SceI/Cy5-pUC19-HindIII
NHEJ-GFP-BsrG1/Cy5-pUC19-HindIII	HR-GFP/Cy5-pUC19
NHEJ-HindIII/Cy5-pUC19-HindIII	HR-I-SceI/Cy5-pUC19-HindIII

Note: pUC19 is used as a negative control, NHEJ/pUC19 or HR/pUC19 can also used as a negative control.
NHEJ-GFP and HR-GFP are used as positive control for NHEJ/HR repair
Cy5-labelled pUC19 and Cy5-pUC19-HindIII are used to control the transfection efficiency.

1. Warm TransIT-LT1 Reagent to room temperature and vortex gentle before using.

2. Combined the indicated amount of plasmid in a sterile tube
3. Add 100 □L of Opti-MEMI Medium
4. Pipet gently to mix completely
5. Add 0.9 □L/0.45 □L TransIT-LT1 reagents to the diluted 300 ng/150 ng DNA mixture (The ratio of plasmid (□g) to TransIT-LT1 reagents (□L) is 1:3).
6. Pipet gently to mix completely
7. Incubate at room temperature for 30 min in shaker

C. Distribute the complexes to cells in complete growth medium
1. Add 100 □L TransIT-LT1 Reagent:DNA complexed (prepared in step B above) drop-wise to different areas of the wells
2. Gently rock the plate back and forth and from side to side to evenly distribute the transIT-LT1 Reagent:DNA complexes.
3. Incubate for 24 or 48 h. No need to change the complete growth medium.
4. Use trypsin to harvest cells, resuspend in ~250 □L medium, and incubate on ice.

Flow Cytometry to study the repair activity:

Using GFP-Cy5 program to study the repair activity
Cells transfected with pUC19 is used to set up the parameter of FL1 Channel (GFP) and that of FL4 channel (Cy5). For each cell line, the parameter should be set up once.
No compensation should be made between GFP and Cy5.
10,000 cell events are usually counted.

Flow cytometry analysis

Cy5 and GFP quarter analysis using WeAsel
%GFP positive and % Cy5 positive, then ratio A (%GFP+/%Cy5+) is calculated for each condition. The "% repair" is calculated by ratio of each condition to the positive control (either NHEJ-GFP or HR-GFP), that is $100*A_{pNHEJ-GFP-BsrG1}/A_{pNHEJ-GFP}$, $100*A_{pNHEJ-C}/A_{pNHEJ-GFP}$, $100*A_{pNHEJ-I}/A_{pNHEJ-GFP}$, $100*A_{pHR-I}/A_{pHR-GFP}$. The "% error" is calculated as $100*(A_{pNHEJ-C}-A_{pNHEJ-GFP-BsrG})/ A_{pNHEJ-C}$

ACKNOWLEDGEMENTS:

A great many thanks are owed to a number of people whose help, guidance, and support have been invaluable during the research for and writing of this thesis. To Dr. Li Wang, Ph.D. I owe an extremely large debt of gratitude. She was a tremendously hands on mentor who taught me much of the laboratory and analytical skills that I learned from this experience. Further, all of the work done with the SH-EP1 cell line was done under her guidance. To Dr. Roland Kwok, Ph.D., this thesis would not be possible without your support, encouragement, and guidance. Dr. Kwok patiently explained much of the thinking that went into my project and helped me throughout with understanding the nuances behind the assays I did and the analysis of the data. Further, he has read every draft of every word that I have written for this thesis and helped me understand the process by which science is written and how we must approach telling our story. To Dr. Valerie Castle, M.D., I would not be writing a thesis on neuroblastoma if it were not for you. You did not just open a door to the lab but also made time to mentor me, explain the importance of my work, and make me feel like an important member of our team. Your support throughout this whole process has been appreciated far more than I could ever express. To Dr. Chitra Subramanian, Ph.D. and Dr. Lijun Tan, Ph.D., I thank you for all of your help and patience. When I could not get the science to work, you helped and when I was under stress from the work, you listened and gave counsel. To Claudia Cao, Natalie Vandeven, Allyson Lieberman, and Manila Hadad, I thank you for putting up with my quirks and idiosyncrasies throughout this whole process. But more importantly, I thank you not just for being incredibly gifted and helpful scientists, but for being great friends. To Dr. Anthony Opipari, Dr. Rebecca Liu, and Dr. Erika Newman, M.D., thank you for always helping me when I needed help and for your support and camaraderie during my tenure here. Thank you also to my co-sponsor Dr.

Kenneth Cadigan, Ph.D. and my other reader, Dr. Laura Buttitta, Ph.D. for lending both your time and scientific acumen to help in the furtherance of my scientific career.

Lastly, and most importantly, thank you to my parents, Linda and Edward Spitz. Since the day I was born you have shown me nothing but unwavering love. From the day I decided I wanted to be a doctor, you have shown nothing but support, encouragement, and helped me to believe that I could achieve that goal even when the feat seemed insurmountable. Thank you both for all of your love, support, and patience not just during the writing of this thesis, but throughout my four years of undergraduate education and for my entire life.

This thesis is dedicated to my parents for all of the aforementioned reasons and for everything else that I could not possibly put down in writing. It is dedicated to Dr. Susan Cohn, M.D. for introducing me to the devastating disease known as neuroblastoma. It is a direct result of your mentorship, guidance, and support as well as your passion for your work and research that led me to pursue a thesis in neuroblastoma biology. This thesis is also dedicated in part to Dr. Mark Ratain, M.D. for first taking a chance on me and offering me a job. You introduced me to the world of cancer biology and I have not looked back since. Lastly, to all of the children suffering from this disease, this thesis is largely dedicated to you. May we all live to see the day that this disease is vanquished.