COCKAYNE SYNDROME B IS REQUIRED FOR NEURAL PRECURSOR SELF-RENEWAL AND NEURITEGENESIS AFTER DNA DAMAGE

BY

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A thesis submitted in conformity with the requirements for the degree of Master of Science, Graduate Department of Pharmacology and Toxicology, University of Toronto

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Master of Science, 2010
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ABSTRACT

Neural precursor cells self-renew and differentiate throughout development and in response to neural injury in the adult brain. The DNA damage response in NPCs has yet to be characterized. Patients with defective nucleotide excision repair (NER) demonstrate neurodegeneration dismyelination, and microcephaly, suggesting a potential link to defective NPC function with accumulating DNA damage. We observed reduced self-renewal in $Csb^{m/m}$ and $Xpa^{m/m}$ NPCs in response to UV damage. Serial passaging resulted in exhaustion of $Csb^{m/m}$ NPCs in the absence of exogenous DNA damage. In vitro neuronal differentiation resulted in abnormal neuritogenesis after UV DNA damage in $Csb^{m/m}$ NPCs, suggesting defects in the terminal differentiation process. Taken together, the results indicate that DNA damage can modulate the apoptotic, self-renewal and differentiation fates of NPCs.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Rebecca Laposa. You took an undergraduate student with limited research experience and helped craft me into the well-rounded scientist I endeavored to become. Your researched passions coincided with my own, and allowed me to merge my distinct interests in both DNA damage and regenerative medicine into a single research project. I am very grateful to have had the opportunity to have worked in your lab. Thank you for the patience, instruction and guidance you showed throughout the course of my degree.

I would like to thank Dr. John Peter McPherson and Dr. Denis Grant, who were willing to be part of my advisory committee and help guide the project from its inception. I would like to extend particular thanks to Dr. McPherson for always being helpful and allowing me to use his lab’s resources and equipment.

I would like to thank Dr. Vince Tropepe for his insights into neural stem cell biology, his invaluable demonstrations, and general guidance throughout the project.

I would like to give special thanks to Laura, our lab technician, whose technical knowledge, general wisdom, and limitless desire to help were instrumental to the completion of this project.

I have to thank all the other lab members from the Laposa lab, including Fernando, Kristel, Aileen and Sharanya. You were always willing to lend a helping hand when needed, and helped maintain a fun and cheerful lab environment.
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m/m

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m/m

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m/m

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m/m

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m/m

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m/m

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m/m

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m/m

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m/m

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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3 related</td>
</tr>
<tr>
<td>B[a]P</td>
<td>Benzo[a]pyrene</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>CS</td>
<td>Cockayne Syndrome</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EFG</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GE</td>
<td>Ganglionic eminence</td>
</tr>
<tr>
<td>GG-NER</td>
<td>Global genomic nucleotide excision repair</td>
</tr>
<tr>
<td>GO</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>MEF</td>
<td>Murine embryonic fibroblast</td>
</tr>
<tr>
<td>M1dG</td>
<td>3-(2-Deoxy-β-d-erythro-pentofuranosyl)pyrimido[1,2-α]purin-10(3H)-one</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural precursor cell</td>
</tr>
<tr>
<td>NSP</td>
<td>Neural stem cell</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3</td>
<td>phosphatidylinositol 3 kinases</td>
</tr>
<tr>
<td>Rock1/2</td>
<td>Rho-associated, coiled-coil containing protein kinase 1/2</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEL</td>
<td>Sub-ependymal layer</td>
</tr>
<tr>
<td>SVZ</td>
<td>Sub-ventricular zone</td>
</tr>
<tr>
<td>TC-NER</td>
<td>Transcription-coupled nucleotide excision repair</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Tuj1</td>
<td>tubulin, beta III isoform</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet radiation</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless and int-1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>XP</td>
<td>Xeroderma Pigmentosum</td>
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</table>
CHAPTER 1: INTRODUCTION

The human body is constantly exposed to numerous sources of potential DNA damage, including foods, drugs, environmental compounds and endogenous metabolic processes. The extent to which DNA damage disrupts normal cellular activity can depend on the type of damage, cell type, and DNA damage response. If left unrepaired, DNA damage has the potential to initiate carcinogenesis by forming mutations. Cell cycle arrest and apoptosis can prevent this outcome, but abnormalities in these processes may result in tissue degradation and disruption of physiological homeostasis (Friedberg 2006).

When DNA damage occurs in stem cells, which are responsible for both tissue development and maintenance, the effects can be significantly more devastating. In lineage-restricted stem cells, this damage can be transmitted both through clonal expansion, and during differentiation, with the possibility of generating tissue-wide pathology. Little is known, however, regarding the DNA damage response of lineage-restricted stem cells, particularly neural stem cells (NSCs). This thesis focuses on the consequences DNA damage on physiologically crucial NSCs. The introduction is divided into five sections. The first describes the formation of DNA adducts and potential consequences of these DNA lesions. Section two outlines the nucleotide excision repair (NER) pathway, which is responsible for repairing bulky DNA lesions. The third is a brief overview of stem cell biology, including the state of the field in understanding the DNA damage response in NSCs. Section four describes patient deficiencies in NER, their neuropathological manifestations and the potential correlations to aberrant NSC function. The final section details my hypothesis and the approaches I have taken to investigate the impact of DNA damage on NSC functional fates.
1.1 DNA Adducts: Types, Formation and Consequences

1.1.1 DNA adducts and bulky DNA lesions

DNA structural alterations occur in many forms, including abasic sites, pyrimidine dimers, oxidative lesions and adducts (Friedberg 2006). An important class of DNA lesions are bulky DNA adducts which can distort the DNA helix and interfere with replication and transcription to the point of causing mutagenesis or apoptosis (Dahlmann, Vaidyanathan et al. 2009). Sources of DNA adducts are the metabolism of xenobiotics including carcinogens and drugs, and the metabolism of endogenous compounds or macromolecules (Intro Fig. 1). Xenobiotic metabolism generates reactive intermediates capable of binding the DNA base at several locations (Friedberg 2006). The mutagenicity and genotoxicity of these DNA lesions can vary according to size, location within the chromosome, and rate of repair (Friedberg 2006).

1.1.2 DNA adducts can be generated by xenobiotic metabolism.

A classical model for describing adducting compounds is Benzo[a]pyrene (B[a]P), an aromatic hydrocarbon that results from the incomplete combustion of organic material. Through a series of enzymatic reactions via CYP1A1, epoxide hydroxylase, and CYP1A1 a second time, B[a]P is metabolized to a reactive metabolite called B[a]P-7,8-dihydrodiol-9,10-epoxide. Similar to many epoxides, this intermediate is reactive, and thus undergoes either \textit{cis} or \textit{trans} ring opening to bind to the N\textsuperscript{2} position of guanine and N\textsuperscript{6} position of adenosine respectively (Kinoshita, Lee et al. 1982). The base-adducts are very bulky, distorting the DNA helix.

Most DNA adducting agents follow a similar pattern to B[a]P, in which the compound is metabolized to an intermediate possessing some reactive functional group. The chemical reactivity of this functional group causes it to bind the DNA base, producing a sterically hindered alteration in base structure that distorts the helix. If the compound or its intermediate possesses
more than one reactive functional group, it may bind multiple macromolecules (Friedberg 2006). Humans have taken advantage of such compounds to produce chemotherapeutics designed to attack the DNA of cancer cells. For example, the chemotherapeutic Cyclophosphamide possesses two reactive chloride groups that can undergo substitution with the amino groups of DNA bases. If only a single DNA-cyclophosphamide link is generated, then the result is a DNA adduct. However, if cyclophosphamide binds to multiple bases, it can generate intra- or inter-strand DNA cross-links (Helge, Oberdisse et al. 1968; Ford and Warnick 1988).

1.1.3 DNA adducts can also be generated by endogenous cellular metabolism.

Not only xenobiotics bind DNA, but endogenous metabolites as well. Many metabolic processes generate reactive oxygen species (ROS) as by-products, which carry the possibility of modifying all macromolecules including DNA. Not only does this alter macromolecular structure but it also generates other metabolites that can cause indirect macromolecular damage. For example, the abstraction of the position 4 hydrogen atom by a hydroxyl radical can generate proenol. Proenol can then bind DNA bases at two points creating the DNA lesion 3-(2-Deoxy-β-d-erythro-pentofuranosyl)pyrimido[1,2-α]purin-10(3H)-one or M1dG (Breen and Murphy 1995). The peroxidation of lipids can generate malondialdehyde molecules that can similarly bind guanine to create M1dG (Dedon, Plastaras et al. 1998). M1dG is mutagenic in bacterial and mammalian cells as it induces frame shift mutations and base pair substitutions (M1dG→T and M1dG→A) (Otteneder, Knutson et al. 2006). Cellular oxidative stress can be introduced in culture via the glucose/glucose oxidase system (GO) according to published protocols (Limoli and Giedzinski 2003). Glucose oxidase (GO) catalyzes the oxidation of glucose to gluconolactone but generates intracellular hydrogen peroxide as a by-product.
1.1.4 **Short wavelength ultraviolet light generates bulky DNA lesions.**

Common to all these endogenous and xenobiotic agents is their propensity to generate DNA lesions that are bulky and distort the DNA helix. Ultraviolet light is another agent that can generate similar lesions, via both direct cross-linking and indirect ROS-induced base damage. Correlation studies between types of DNA lesions and UV wavelength demonstrate that short wavelength UV-light correlates well with DNA cross-link type lesions whereas high wavelength UV correlates with DNA modifications, strand-breaks and sites of base loss (Kielbassa, Roza et al. 1997). Short wavelength UV-A (~350nm) light indirectly generates DNA lesions, as it is only weakly absorbed by DNA (Jones, Huberman et al. 1987). Typically, radiation at this wavelength generates ROS, which then produce oxidative DNA lesions such as 8-oxoguanine or cause strand breaks. Low wavelength UV-C (254nm) and UV-B (~300nm) light directly excites functional groups within DNA structure, leading to the formation of intra- and inter-macromolecular photoproducts. The lesions that most commonly result are cyclopyrimidine dimers and (6-4) photoproducts (Ananthaswamy and Pierceall 1990). For experimental rigor, logistical simplicity and cleanliness, I used UVC as a model damaging agent within my self-renewal and differentiation assays, as it is a classical agent for interrogating NER DNA repair (see Sections 2.2.2 and 2.5.2).

1.1.5 **Molecular sensors and consequences of DNA damage.**

There are several reported means of detecting DNA damage within the cell, but their exact interactions, relationships to cellular outcomes, and relative importance are still under investigation and a matter of debate. The most well-characterized DNA damage sensors are the phosphatidylinositol 3 (PI3) kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related). Despite significant cross-talk, ATM typically recognizes and responds to strand-
breaking agents such as ionizing radiation, whereas ATR is involved in cellular recognition of lesions caused by UV radiation or inhibition of replication during S-phase (Abraham 2001; Durocher and Jackson 2001).

ATM normally exists as a dimer, and is recruited by the binding of the single stranded binding protein RPA to the staggered ends created by double-strand breaks. This induces ATM to phosphorylate and dissociate its dimerized partner, and then auto-phosphorylate, leading to its activation. It is then free to phosphorylate downstream targets involved in DNA repair, cell cycle arrest and apoptosis (Bartek and Lukas 2003). ATR, on the other hand, exists in a monomer form, and is recruited to sites of DNA damage by protein complex formation with other kinases and DNA binding proteins. Complex formation leads to phosphorylation of ATR, and downstream signaling cascades similar to, and sometimes involving, ATM (Kurz and Lees-Miller 2004).

DNA lesions can also be encountered by the replication and/or transcription machinery used in the normal steady-state activity of a cell. In the case of replication, upon encountering a bulky DNA lesion, the replication fork will stall due to helical distortion. Stalling of the replication fork effectively blocks DNA synthesis, causing the processive DNA polymerase to dissociate and recruit DNA damage sensors and effectors. The stalled replication fork is a strong recruitment signal for ATR, resulting in downstream activation of DNA repair, cell cycle arrest, and apoptosis (Bartek, Lukas et al. 2004; Yang, Xu et al. 2004). Other sensors of DNA damage and stalled replication are likely also involved, but have yet to be identified (Adams, Medhurst et al. 2006).

Another molecular consequence of bulky DNA lesions is the cessation of transcription through that lesion. Like the DNA polymerase, RNA polymerase will encounter the bulky
lesion, causing it to stall. At this point, depending on the cell type, the RNAP stalling can act as a strong signal for either apoptosis or transcription coupled repair, which will be discussed in subsequent sections.

1.1.6 Cellular outcomes of bulky DNA lesions

The molecular responses to DNA damage can result in several cellular outcomes that differ widely from cell type to cell type. These outcomes are not mutually exclusive, however, nor are they all available to every cell type. Different cell fates are preferentially activated depending on the type of damage and cell type. Below, I will briefly describe most of the potential cell fate outcomes that may result from DNA lesions (Intro Fig. 2).

1.1.6-A: DNA repair

DNA repair is the most relevant DNA damage-induced cellular outcome regarding my research. The recruitment of DNA repair pathways occurs both concomitantly or as a result of ATM and ATR activities, suggesting that some pathways possess their own damage sensors. I will briefly outline the different DNA repair pathways, the most important of which, NER, will be the focus of section 2 of the introduction. Mismatch repair primarily excises nucleotides that are incorrectly paired the correct nucleotides on the opposite DNA strand, its stimulus being small loops in the DNA strand created by the base mismatches. Base Excision Repair (BER) removes chemically altered bases via DNA glycosylases which are a family of DNA-repair-specific enzymes which recognize a single or a small subset or modified bases. Homologous recombination and Non-homologous end-joining are pathways recruited to repair DNA strand breaks (Friedberg 2006). The final and most important repair pathway related to my research is the Nucleotide Excision Repair (NER) pathway, which recognizes the bulky helix-distorting lesions produced by B[a]P, Cyclophosphamide, UV light, endogenous metabolism and numerous
other sources. A subset of NER is coupled to transcription, and thus, can sense DNA damage by the dissociation of the RNA polymerase from the template strand (Friedberg 2001). As mentioned, this pathway will be discussed in significant detail in the next section.

1.1.6-B: DNA lesion tolerance

An alternative to DNA repair is DNA lesion tolerance. As mentioned, cellular replication machinery may be the first DNA damage sensor to detect a DNA lesion. If the lesion is a small DNA base modification and substrate for BER, then the processive DNA polymerase will read through the lesion, with or without repair, and continue replication. However, if the processive DNA polymerase encounters bulky DNA-adducts, such as NER substrates, then it will stall and eventually dissociate. Two possible scenarios then arise. The replication blockage can lead to the recruitment and activation of ATR and signal other downstream cellular outcomes. A second possibility is “Trans-lesion synthesis” in which a low-fidelity trans-lesion polymerase is recruited to replace the processive polymerase and continue replication, effectively skipping over the lesion and inserting a nucleotide in the synthesized strand. The inserted nucleotide can be random or matched to particular types of lesions, but since it may not correspond to the correct nucleotide, trans-lesion synthesis can be either highly mutagenic or relatively error free. Thus, as an alternative to DNA repair, the cell can potentially tolerate the DNA lesion at risk of generating mutations at the site of the lesion.

1.1.6-C: Cell-cycle arrest

The prevention of cell division by cell-cycle arrest is intended to protect genomic stability by providing time for DNA repair mechanism to act. Replication in the midst of DNA lesions can lead to double-strand breaks, replication fork stalling, trans-lesion mutagenesis, and apoptosis. The most well characterized checkpoints in response to DNA damage are mediated
by the ATM and ATR kinases, and can occur in nearly every phase of the cell-cycle (Friedberg 2006).

Cell cycle arrest during G1-S-phase transition is initiated by the phosphorylation and activation of the Chk1/2 kinases. They increase p53 activity via phosphorylation and inhibition of the p53 negative regulator MDM2. This results in increased expression of p21, which accumulates and blocks Cdk2-cyclin E activity. As a consequence, RB1 is not hyperphosphorylated and inactivated, thereby preventing the E2F transcription factor controlling S-phase specific transcription from being released (Yuan, Huang et al. 1996; Bartek and Lukas 2001). Intra-S-phase arrest is again initiated by ATR phosphorylation of Chk2, which phosphorylates the phosphotase Cdc25A, resulting in its proteosomal degradation. The lack of Cdc25A allows Cdk2 to remain phosphorylated preventing S-phase initiation and progression. Alternative pathways initiated by the phosphorylation of the MRN complex, BRCA1 and FANCD2 can also culminate in S-phase checkpoint (Bartek and Lukas 2001; Bartek, Lukas et al. 2004). G2/M arrest can be both p53 dependent and independent. The activation of p53 can result in the transcription of both 14-3-3σ and p21, which result in increased inactivation and cytoplasmic sequestration of Cdk1, which is required for the G2-M-phase transition (Chan, Hermeking et al. 1999).

1.1.6-D: Apoptosis

DNA damage can also result in promotion of both intrinsic and extrinsic apoptotic pathways by way of p53. Recall that ATM/ATR activation can either directly or indirectly, via Chk1/2, phosphorylate p53 and its negative regulator MDM2, resulting in increased p53 levels (Friedberg 2006). The extrinsic pathway is mediated by death receptors such as FAS, which signal the downstream cleavage of the enzymatic caspases. DNA damage can enhance the
expression of both FAS and FAS-ligand via p53, suggesting a promotion of apoptosis via this death-receptor pathway (Kasibhatla, Brunner et al. 1998; Muller, Wilder et al. 1998). The intrinsic pathway relies on release of cytochrome c from mitochondria and binding to APAF-1, a process mediated by the BCL-2 family (Jiang and Wang 2004). Expression of these apoptotic mediators is regulated by p53, as it has been shown to activate pro-apoptotic BAX, NOXA and APAF-1 and repress anti-apoptotic Bcl-2 (Slee, O'Connor et al. 2004).

1.1.7 Molecular and cellular outcomes of DNA damage can be cell and tissue-specific.

We have discussed a variety of cell fates that may occur due to DNA damage, but not every cell type undergoes all of these processes. Depending on tissue and differentiation status, some cells are restricted to particular cell fate responses, while others strongly prefer one over others as a first response. The DNA damage response is highly tissue specific, depending on the cell’s physiological status, proliferative capacity, differentiation status and DNA damage consequence (Friedberg 2006). For example, proliferating myoblasts readily apoptose in response to γ-irradiation, whereas differentiated myotubes demonstrate radioresistance and reduced apoptosis (Latella, Lukas et al. 2004). When considered from a homeostatic perspective, this makes sense, as the more potent myoblasts can transmit the DNA damage during replication and differentiation, making their elimination more important. This concept will be discussed in detail in section 3, as it relates to the DNA damage response in stem cells.
1.2 Nucleotide Excision Repair: Subsets, Recruitment, and Molecular Players.

1.2.1 NER: a brief overview

My research investigates the impact that NER deficiency specifically has on NSC function. As mentioned, NER is specifically responsible for removing bulky DNA lesions that distort the DNA helix, such as those produced by the metabolism of xenobiotic and endogenous compounds, as well as UV light. The proteins involved in NER assemble in a step-wise fashion at the site of damage, creating a large multiprotein complex. Ultimately, the lesion is removed as part of an excised ~25-30nt long oligonucleotide, which is later re-synthesized correctly by DNA polymerase and sealed by DNA ligase (Friedberg 2001). However, there are actually two subsets of NER that possess slightly different stimuli for recruitment, involve different initiator proteins, but funnel into a common NER pathway beginning with TFIIH (Intro Fig 3).

1.2.2 Global genomic repair removes bulky lesions throughout the genome.

The first of two subsets, Global Genomic NER (GG-NER) acts throughout the genome to remove bulky DNA lesions and is considered the more general of the two. GG-NER is initiated by the XPC- hHR23B complex, which recognizes the lesions and recruits the other NER proteins. However, it is still poorly understood as to what exactly allows XPC to recognize such a wide range of NER substrates, some with little similarity in structure. The current model suggests that XPC recognizes the distortion created in the DNA helix by the bulky lesion, and then interacts with the lesion so as to further distort the helix. Thus, XPC would help promote single-strandedness around the lesion, facilitating the recruitment of TFIIH (de Laat, Jaspers et al. 1999). The XPC protein is specific to GG-NER, and its mutational inactivation results in the hereditary condition known as Xeroderma Pigmentosum, which will be discussed in detail in the next section.
1.2.3 Transcription-coupled repair removes bulky lesions from actively transcribed genes.

It was observed in the mid-1980s that the kinetics of NER was increased in transcriptionally active regions, particularly in the transcribed DNA strand as opposed to the non-transcribed strand. This led to the discovery of Transcription-coupled repair (TC-NER), wherein components of the transcription machinery doubled as the repair proteins responsible for recognizing DNA lesions (Hanawalt and Spivak 2008). The current model for TC-NER holds that recognition of the lesion is mediated by an elongating RNA polymerase complex. Upon encountering the lesion, the complex will stall, displacing the component proteins CSA and CSB. These proteins interact so as to further displace the TFIIH component of the RNAP complex and facilitate its recruitment to the lesion. Since the TC-NER initiator proteins are already part of the RNAP complex, the result is that more actively-transcribed regions receive more efficient recognition of potential DNA lesions, and thus faster repair kinetics (de Laat, Jaspers et al. 1999). The CSA and CSB proteins are specific to TC-NER, and mutational inactivation of the genes encoding these proteins leads to the hereditary disease called Cockayne syndrome, which will be discussed in detail in the next section.

1.2.4 The CSB Protein; Critical to TC-NER

As mentioned, the CSB protein, also known as ERCC6, is a critical component of both transcription and transcription-coupled repair. Located on chromosome 10q11-21, the 20 exon Csb gene encodes a 1493 amino acid protein containing both nucleotide binding and helicase motifs (Citterio, Rademakers et al. 1998). However, despite being capable of acting like a DNA-dependent ATPase, the helicase motifs do not translate into functional helicase activity in vitro (Selby and Sancar 1997). It is thought that CSB acts as a ATP-dependent chromatin-remodeling protein during both transcription and TC-NER (Lake, Geyko et al.). Cells with a CSB mutation
cannot remove/degrade transcription complexes that stall at sites of DNA damage, due to a failure in the necessary RNAPII ubiquitination (Bregman, Halaban et al. 1996). Mutations have been observed throughout the $Csb$ gene and are not localized to any particular mutational hotspots. Intro figure 4a the mutational spectrum of the $Csb$ gene.

As a caveat, it should be mentioned that several non-NER or transcription related functions of CSB are currently being investigated. It has been observed that some $Csb^{mm}$ cells are defective in the incision and removal of some BER substrate lesions, indicating a possible overlapping involvement of CSB in BER (Stevnsner, Muftuoglu et al. 2008). However, a very recent study demonstrated co-localization of CSB to mitochondrial membranes, as well as reduced incision of BER substrate lesions in mtDNA in $Csb^{mm}$ cells. This indicates that CSB may play a direct role in mitochondrial BER by helping recruit and stabilize the repair complexes associated with the inner mitochondrial membrane (Aamann, Sorensen et al.).

1.2.5 GG-NER and TC-NER channel into a common pathway resulting in the excision of the lesion.

In both GG-NER and TC-NER, the XPC and CSA/CSB proteins respectively facilitate the recruitment of TFIIH to the site of the lesion. TFIIH is a multiprotein of two helicases XPB and XPD, and is itself a component of the RNAP complex. It catalyzes open complex formation around the site of the lesion and facilitates the assembly of the repair complex. This recruits the $XPA$ protein, which binds the damaged DNA and stabilizes repair complex assembly, and the RPA protein, which binds the now single-stranded DNA and stabilizes the open complex. The TFIIH-$XPA$-RPA complex recruits XPG and ERCC-XPF, which catalyze the 3' and 5' incisions respectively. The result is a single stranded oligonucleotide containing the lesion that is released from the repair complex. This is followed by polymerization of a new strand using the
undamaged strand as a template, and ligation by DNA ligase. The oligonucleotide containing the lesion is then degraded, and the damage has been repaired (de Laat, Jaspers et al. 1999).
1.3 Neural Stem Cells and Neural Development

1.3.1 Stem cell hierarchy: From totipotent to differentiated cells

Stem cells possess two important qualities: the ability to clonally expand or self-renew and the ability to differentiate into other more committed cell types. Conception is essentially the generation of the first stem cell, the zygote, which is considered totipotent as it can be implanted into the uterus of an organism to generate another full organism. From the zygote, pluripotent embryonic stem (ES) cells are generated. These cells can spawn every cell type within the organism, with the exception of the trophoblast and placenta. ES cells cannot generate a full organism without the maternal context, explaining why these cells are not totipotent but are used in transgenic studies. Through a series of asymmetric cell divisions and differentiations, ES cells can give rise to multipotent lineage-restricted stem cells. These pools of multipotent stem cells persist throughout the life of the organism but can only differentiate into tissue-specific cell types (Gage 2000). For example, multipotent hematopoietic stem cells reside in the bone marrow, and can differentiate into all the blood cell types, including both the myeloid and lymphoid lineages. Thus, although the primary function of ES cells is “tissue building” in very early development, lineage-restricted stem cells become responsible for both “tissue building and maintenance” from development and throughout the life of the organism. The conclusion of this stem cell hierarchy resides in the final differentiated cells, such as the erythrocytes produced from HSCs (Rossi, Bryder et al. 2007).

1.3.2 From embryonic to neural stem cells

The exact steps driving neural development in the embryo are being continuously investigated and updated, but neural induction is known to be cued by a spatial and temporal gradient of positive and negative regulators. One model proposes that neural induction may be
the "default" pathway of embryonic differentiation in the absence of BMPs. Media devoid of any growth factors induces ES cells to express neuronal markers within hours. Additionally, mouse studies have demonstrated that ES cells can be induced to neural cell fate via inhibition of BMP/TGFβ signaling (Smukler, Runciman et al. 2006). In addition to BMP inhibition, FGF signaling promotes induction and survival of neural progenitors from ES cells. Finally, recent studies have identified Wnt as a negative regulator of neural induction (Aubert, Dunstan et al. 2002). In vivo, the spatial and temporal coordination of these factors and their respective inhibitors drives neural development. In vitro, the absence of FGF and inhibition of BMP and Wnt signaling can induce ES cells to differentiate into neural progenitors (Gaspard and Vanderhaeghen).

1.3.3 Neural stem cells: self-renewal and differentiation

Neural stem cells (NSCs) comprise a steady-state population of progenitor cells within the CNS that can clonally expand, self-renew, and differentiate into all the CNS cell types. NSCs are distributed throughout the developing embryo brain but are particularly localized to the subventricular zone (SVZ) of the lateral ventricle, the neocortex, and the hippocampus. In the adult mammalian brain, NSCs are localized to the dentate gyrus of the hippocampus, and the subependymal layer (SEL) overlaying the caudate nucleus, which is derived from the embryonic SVZ (Intro Fig 5b) (Curtis, Penney et al. 2005). These steady-state pools of precursors and stem cells are maintained by clonal expansion throughout the life of the organism, but can be induced to differentiate during development or in response to injury. NSCs can differentiate into the three main cell lineages of the CNS; neurons, astrocytes and oligodendrocytes by way of neuronal and glial precursor cells (Gage 2000). Intro figure 5a depicts hierarchical development of NSCs, and their self-renewal and differentiation capacities.
In vivo, the decision to self-renew or differentiate is directed by gradient concentrations of specific proteins, which act as molecular rheostats. Key molecular mediators of self-renewal, either via its direct stimulation or repression of differentiation, are the Notch, EGF, and FGF signaling pathways. The notch signaling pathway is an important repressor of both embryonic and adult neurogenesis (Hitoshi, Alexson et al. 2002). Notch activation results in downstream activation of transcriptional repressors of proneural genes, effectively inhibiting neuronal differentiation and promoting self-renewal (Bertrand, Castro et al. 2002). In vitro, the presence of FGF and EGF in culture promotes NSC self-renewal, while their removal and replacement with serum promotes differentiation (Temple 2001). Additionally, in the human adult brain, the receptors for EGF and FGF are up-regulated in response to injury (Yarden 2001; Zheng, Nowakowski et al. 2004, Alagappan, Lazzarino et al. 2009). Conversely, one crucial promoter of pro-neural gene expression and differentiation is the transcription factor Pax6. Overexpression of Pax6 can result in increased in vitro neurogenesis of mouse embryonic cortical NSCs, adult mouse SVZ NSCs, and human NSCs (Hack, Sugimori et al. 2004; Hack, Saghatelyan et al. 2005; Kallur, Gisler et al. 2008).

1.3.4 DNA damage responses in embryonic and lineage-restricted stem cells.

The current public and commercial interest in regenerative medicine has made elucidating the DNA damage response of stem cells a priority within the DNA repair field. In addition to the cell fates available to normal somatic cells, tolerance, DNA repair, cell cycle arrest and apoptosis, stem cells are capable of self-renewal and differentiation. The unique physiological roles of stem cells can exacerbate the deleterious impact of DNA damage, as the accumulation of DNA lesions can be transmitted horizontally, through clonal expansion, and vertically into downstream lineage cells. This makes determining the preferred DNA damage
response for such physiologically crucial for understanding development and regenerative biology.

Pluripotent embryonic stem cells are the primary source of all the different cell types comprising the organism. Their physiological importance makes tolerance of DNA damage a highly deleterious outcome, as clonal expansion and differentiation of these precursors could cause malformations throughout the organism. It seems logical, therefore, that a hyperapoptotic response to DNA damage has been observed in ES cells as compared to murine embryonic fibroblasts (MEFs) (Aladjem, Spike et al. 1998). The absence of NER significantly exacerbates this hyperapoptotic response in ES cells, indicating that this pathway is important in protecting these cells from DNA damage (Van Sloun, Jansen et al. 1999). Taken together, this indicates that the preferential response to DNA damage in ES cells is apoptosis.

ES cells differentiate into a variety of multipotent lineage-restricted stem cells and precursors, which are responsible for both tissue building during development and maintenance in adult tissues. Most research into lineage-restricted stem cells has occurred in hematopoietic stem cells (HSCs) as related to the aging phenotype. It has been observed in mice that with age, an accrual of DNA damage and a functional decline in HSC activity is observed, both of which are exacerbated by deficiencies in DNA repair pathways (Rossi, Bryder et al. 2007). The functional decline does not appear be caused by increased apoptosis, reduced cell cycle arrest, or cell cycle inhibition. One model proposes that accumulating DNA damage induces HSCs to remain in the quiescent G0 state outside of the cell cycle. This senescent DNA damage response results in a decrease in the steady-state pool of function HSCs, having a deleterious impact on tissue maintenance (Rossi, Seita et al. 2007).
1.3.5 DNA damage response in Neural Stem Cells

The DNA damage response of neural stem cells in the developing embryo or adult brain has only recently come under investigation, with two studies to date. The first study investigated the importance of ATM in apoptotic responses to $\gamma$-irradiation at different points of neurogenesis. Apoptosis was largely ATM-independent in irradiated precursor cells as opposed to post-mitotic neurons, where ATM was required indicating that the differentiation process within NSCs somehow modulated the upstream apoptotic signaling (Lee, Chong et al. 2001). A second study directly investigated DNA damage deficiency in relation to NSC self-renewal. Mutations in FANC proteins cause disruptions in double-strand break repair and are associated with Fanconi's anemia. An age-related decrease in ex vivo NSC clonal expansion was observed in fanca$^{-}$ mice, suggesting that accumulation of DNA damage with age results in disruption of NSC self-renewal (Sui-Felice, Etienne et al. 2008).

These studies demonstrate that apoptosis and abnormal self-renewal can occur as double-strand break responses in NSCs, but not how favored these responses are in relation to other cellular outcomes. Additionally, there have been no investigations of DNA damage affects on NSC differentiation. Thus, it seems prudent to investigate the impact that DNA damage has on all NSC fates, and try to determine how they are modulated in relation to one another.

The physiological impacts of DNA damage in NSCs could be severe, as a decline in the functional NSC number in the developing embryo could result in CNS malformations and potential neurodegenerative phenotypes. In the adult brain, depletion of the NSC pool would have a deleterious impact on the regenerative capacity in response to insult/injury. There are currently clinical manifestations of DNA repair disorders that already mimic these phenotypes, and they will be discussed in detail in the next section.
1.4 NER Deficiencies and the CNS

1.4.1 NER deficiencies: types and clinical manifestations

Of the 30 proteins involved in the NER pathway, 11 have been found to be associated with human pathology, several of which with overlapping manifestations. The two most common NER disorders are Xeroderma Pigmentosum (XP), which can be caused by mutations in the \textit{XPA}, \textit{XPC}, \textit{XPD}, \textit{XPE}, \textit{XPF} and \textit{XPG} proteins, and Cockayne Syndrome (CS), caused by mutations in \textit{CSA} and \textit{CSB}. Although these conditions can have diverse phenotypes with varying penetrance and severity particular importance will be placed on CS, as neurodegeneration is a primary feature of this disease.

1.4.2 Xeroderma Pigmentosum

XP is an autosomal recessive condition with an estimated incidence of approximately 1 in \(10^5\). The most severe form of XP, called XP with DeSanctis-Cacchione syndrome (XP-DSC) is caused by mutations in the \textit{XPA} gene, and is characterized by earlier onset and significantly higher incidence of neurological abnormalities. All XPs variants are associated with clinical and cellular sensitivity to UV-induced DNA lesions, with XP patients displaying a 1000-fold increased risk of developing sun-induced carcinomas. Additionally, ocular abnormalities including corneal clouding and neoplasms of the eye are common. Neurological abnormalities are reported in approximately 20\% of XP patients, characterized by mental retardation, microcephaly, growth retardation, spasticity, and ataxia, all of which are progressive. In XP, post-mortem analysis has revealed that it is primarily the neurons that are effected, as demyelination is not observed (Brooks 2002).
1.4.3 Cockayne Syndrome

CS is also an autosomal recessive disorder with a frequency less than that of XP but with a significantly more devastating phenotype, as nearly all cases demonstrate progressive postnatal growth failure, neurological dysfunction and an accelerating aging phenotype (Nance and Berry 1992). The average reported life span of CS patients is about 12.5 years, with a poorer prognosis associated with earlier onset of symptoms (Nance and Berry 1992). In utero development of CS patients is normal, but is followed by developmental abnormalities within the first postnatal year, particularly microcephaly. The earliest common neurological symptom of CS is delayed psychomotor development, followed by progressive spasticity, ataxia, tremors, and kyphosis of the vertebral column (hunchback) (Nance and Berry 1992). Although mental retardation ranges from mild to severe, the earlier the onset of neurological symptoms, the more pronounced the retardation becomes, suggesting a link between endogenous DNA damage, repair and neuronal development and maintenance.

A combination of CS symptom analysis and post-mortem patient analysis indicates that a progressive neuronal failure occurs throughout the CNS and PNS. Radiological analysis demonstrates intracranial calcifications, particularly of the basal ganglia, in combination with increased ventricular size and cerebellar atrophy. Post-mortem analysis has found the most severe neuronal loss to be localized to particular populations, including putamen/caudate, dentate nucleus and Purkinje cells (Itoh, Hayashi et al. 1999). The glial cells localized near these neuronal populations are also impacted in CS, as they are often pleomorphic, adopt irregular shapes or multinucleated (Rapin, Lindenbaum et al. 2000). Since some of these cells are responsible for myelinating neuronal axons to promote high velocity conduction, it is of little surprise that demyelination is common throughout the CS brain. This leads some to speculate
that the NER defect in CS affects the glial cells primarily, and neurons secondarily (Brooks 2002).

1.4.4 Modeling NER deficiencies

A general theme amongst mice modeling NER deficiencies is a marked reduction in neurodegenerative phenotypes as compared to human patients. XP can be modeled by at least 7 complementation groups, XPA through XPG. Mice lacking the XPA gene, denoted Xpa\textsuperscript{−/−}, completely lack NER activity and demonstrate increased susceptibility to UV light and carcinogens, as well as a 1000-fold increase in UV-induced skin cancer (Nakane, Takeuchi et al. 1995). Unlike human patients, however, Xpa\textsuperscript{−/−} mice do not show any neuropathological manifestations reminiscent of XP-DDC, a discrepancy that remains unresolved (de Vries, van Oostrom et al. 1995). One possible explanation is that the neurodegenerative pathology may require longer than the typical 2-3 year lifespan of these mice to develop. An additional explanation holds that the neurologic defects in these mice could also be very subtle, based on a report that they undergo delayed neuromotor recovery and cognitive deficits following experimental brain surgery (Wijnhoven, Hoogervorst et al. 2007).

Deletion of either the CSA or CSB genes result in CS, with the syndrome currently being modeled by Csb\textsuperscript{m/m} mice. The Csb\textsuperscript{m/m} mouse model utilized in my experiments was designed to closely mimic the K337Æ stop mutation observed in the CS-B patient CS1AN, shown to be nonlethal at the cellular level (Troelstra, van Gool et al. 1992). The pG7CSBko1 vector was constructed by the insertion of a dominant-selectable neo marker and a multiple reading frame insertion (MURFI) ochre stop codon linker in exon 5 of the Csb gene (Intro Fig. 4b). E14 ES cells were transfected with pG7CSBko1 to generate heterozygous ES cell lines carrying a mutated Csb allele. After verification of transfection, ES cells were injected into C57BL/6J blastocysts.
generating chimeric males. These were used to produce heterozygous offspring, that when crossed, yield homozygous mutant mice (van der Horst, van Steeg et al. 1997). RT-PCR of Csb mRNA levels verified that no wild-type Csb mRNA was present in cultured fibroblasts derived from the mutant embryos. Western blot analysis verified that the wild-type CSB protein could not be detected in extracts from the Csb<sup>m/m</sup> fibroblasts. Reduced repair of the transcribed strand of the p53 gene, compared to the non-transcribed strand, after irradiation verified that transcription-coupled repair was defective in the Csb<sup>m/m</sup> genotype (van der Horst, van Steeg et al. 1997).

The phenotype of Csb<sup>m/m</sup> is significantly milder than that of human patients, with progressive neurological abnormalities developing with age. Reported decreases in motor activity and impaired sensorimotor coordination indicate defects in the basal ganglia and cerebellum of the mice, similar to human patients. Additional symptoms including tremors, ataxia, cachexia and retinal degradation were observed, but demyelination of neurons was paradoxically absent (van der Horst, van Steeg et al. 1997).

The crossing of multiple mutant strains generates models that more adequately phenocopy the symptoms observed in human patients, including increases in neurological defects, cancer predispositions, and accelerated aging. Both Csb<sup>m/m</sup>/Xpa<sup>-/-</sup> and Csb<sup>m/m</sup>/Xpc<sup>-/-</sup> mice demonstrate a significant reduction in lifespan to a maximum of 3 weeks, coupled with progressive growth retardation and neuropathology, which includes tremors, dystonia, poor balance and ataxia (Murai, Enokido et al. 2001; Friedberg and Meira 2006). This indicates that a synergistic effect on neurological disease is the result of inactivating both GG-NER and TC-NER in mice, as opposed to humans where elimination of one pathway is sufficient to produce the same symptoms.
1.4.5 NER deficiencies, neurodegeneration and NSCs

The neurodegenerative manifestations of NER defects indicate that this pathway is critical in protecting the CNS from DNA damage. Indeed, differentiated neurons have been reported to be more UV sensitive than other cells, including fibroblasts and HeLa cells (James, Mansbridge et al. 1982). However, GG-NER activity is significantly attenuated during neuronal differentiation in vitro and in terminally differentiated neurons (Nouspikel and Hanawalt 2000). This suggests that when TC-NER activity is hampered in CS, GG-NER is unable to support DNA repair in neurons. The cellular implications of this are poorly understood however, as it fails to explain what molecular events correspond to functional neuronal decline, why multiple CNS lineages are affected, and why there are varying degrees of penetrance and severity.

One possible explanation of the neurodegenerative manifestations of NER syndromes is a progressive decline in the functional number of NSCs. The fact that multiple CNS lineages are affected implicates a possible precursor defect. Since the effects of DNA damage on NSCs have not been explored, potential abnormal cellular outcomes could explain the neuronal and glial abnormalities in CS patients. Ectopic or abnormal differentiation of these cells in response to DNA damage could result in irregular neuronal structure or lack of myelination. Imbalanced NSC self-renewal, hyperapoptosis or increased cell senescence would reduce the functional population of NSCs in a progressive fashion.

Although in utero development of CS patients appears normal, the perturbation of NSCs pools may not yet be significant enough to affect development. Additionally, the post-natal environment is more likely to introduce DNA damage to the patient than the in utero milieu, possibly explaining why neurodegeneration begins to manifest within the first post-natal year (Koren, Klein et al. 1992; Koren, Pastuszak et al. 1998; Kearns, Abdel-Rahman et al. 2003). The
progressive decline in functional NSCs would then impact both post-natal neurodevelopment (tissue building) and neuroregeneration (tissue maintenance), corresponding well with the progressive neuronal manifestations of CS.

A striking corroboration of this potential model is the co-localization of areas reportedly rich in NSCs and abnormal clinical manifestations. Recall that in both the developing embryo and adult brain, NSCs are localized to the SEL overlaying the caudate nucleus (SVZ in the embryo), the neocortex, and the dentate gyrus of the hippocampus (Curtis, Penney et al. 2005). Itoh et al. reported significantly enlarged ventricles within the brains of CS patients (Itoh, Hayashi et al. 1999), which could abnormally affect the niche of NSCs within SVZ/SEL.
1.5 Hypothesis

In somatic cells, DNA damage may elicit responses such as cell cycle checkpoints, DNA repair, apoptosis or lesion tolerance. All stem cells have two additional cell fates, self-renewal and differentiation, both of which define the roles of these cells within their tissue-niche. The DNA damage response has yet to be investigated in NSCs, but if it does impact these two functional NSC fates, then it may cause a decline in the effective NSC pool. NSCs would no longer persist throughout the life of the organism and be useful for both neurodevelopment and neuroregeneration. Thus it is important to identify the impact that DNA damage has on the functional fates of these physiologically crucial cells.

NER is clearly important in protecting the brain from bulky DNA lesions that distort the helix. Syndromes characterized by defects in the NER pathway carry neurodegenerative phenotypes of varying penetrance and severity. The neuropathology arises from abnormalities in neuronal and glial cell structure and function, with especially severe neuronal loss and demyelination in specific neuron populations and regions. These regions co-localize to the NSC-rich areas within the embryonic and adult brain, indicating that DNA damage-induced precursor defect may be present. This suggests that DNA damage may indeed impact the functional fates of NSCs, and that NER is critical in protecting these cells.

Thus, our hypothesis is that DNA damage adversely impacts or impairs neural stem cell fates, and that this is exacerbated in the absence of DNA repair. We have taken three approaches to evaluating this hypothesis, each relating to the impact that DNA damage has on one particular cell fate outcome within a particular DNA repair context. The first approach involves investigating the degree to which NSCs undergo cell death upon DNA damage induction in the presence/absence of NER. The second approach aims to evaluate self-renewal...
in the presence/absence of NER under different forms of DNA damage. This will involve both an *in vivo* comparison of NSC populations in developing embryos, and *in vitro* colony-forming assays under no, exogenous and endogenous damage conditions. The final approach evaluates the impact that DNA damage and NER activity have on NSC **multipotency** by inducing neuronal differentiation and both qualitatively and quantitatively comparing the neuronal lineage. The hypothesis and approaches are summarized in Intro Figure 6. These three cell fate outcomes, apoptosis, self-renewal and differentiation, were chosen as they most strongly affect the function of NSCs within the CNS, that is neurodevelopment and neuroregeneration. Abnormalities in these cellular outcomes could have broader neuropathological consequences, making elucidating the DNA damage response of NSCs of the utmost importance.
CHAPTER 2: MATERIALS AND METHODS

2.1 Ex vivo NPC isolation and Primary Neurosphere Generation

Csb<sup>+</sup> females and males were mated, with plug date used as E0.5. Ganglionic eminences (GEs) were dissected in cold PBS containing MgCl<sub>2</sub> and CaCl<sub>2</sub> from (E14) embryo brains. Embryonic tails were also removed to allow genotyping of each embryo. GEs were then dissociated using micropipetting to single cell suspensions in NeuroCult NSC Basal Medium (Stemcell Technologies). Cells were counted and seeded at clonal density (1x10<sup>5</sup> cells/mL) in T-25cm<sup>2</sup> flasks (Nunc). Cells cultured for four days in NSC proliferation media consisting of NeuroCult NSC Basal Medium (Stemcell Technologies), 10% NeuroCult NSC Proliferation Supplement (Stemcell Technologies), 20ng/mL rhEGF (Sigma), 10ng/mL rhFGF (Sigma) and 1% Penicillin-Streptomycin (Gibco). This allowed for selective expansion of NSCs/NPCs in the form of suspension cultures during which time NSCs/NPCs formed spheroid colonies ("neurospheres"; Results Fig. 1a).

2.2 Optimization of culture conditions for embryonic (E14) mouse neural precursor cell (NPC) self-renewal

For culture optimization, we utilized a commercial self-renewal culture protocol and reagents (Stem Cell Technologies, Vancouver) based on published formulations (Reynolds and Weiss 1992). Briefly this consisted of a serum-free basal media (NeuroCult NPC Basal, Stemcell Technologies) supplemented with proliferation supplements and EGF. Starting with freshly dissected E14 GEs, dissociated to a single cell suspension, we assessed densities of 2x10<sup>5</sup> cells/mL, 1x10<sup>5</sup> cells/mL and 5x10<sup>4</sup> cells/mL and concluded the optimal density for self-renewal to be 1x10<sup>5</sup> cells/mL, which is considered a clonal density. To determine the optimal interval for sub-passaging, we assessed 3, 4, 5, and 6 day intervals, with the best results obtained upon 4
days culture. Three day cultured neurospheres were too small, while five and six day cultures had dark centers rather than the bright centers indicating health. Several dissociation methods were assessed including enzymatic dissociation (Neurocult Enzymatic Dissociation Kit, Stemcell Technologies), dissociation through fire-polished glass Pasteur pipette, and dissociation through a p1000 micropipette. Of these, the micropipette provided the most consistent dissociation with the highest viability of single cells. To be as physiologically relevant as possible, oxygen concentrations within the incubator were held at 5% (in contrast to traditional 20% oxygen culture conditions). We observed a statistically significant increase in mean neurosphere number at 5% oxygen (mean +/- S.D.; p<0.005; Supplemental Fig. 1), consistent with published observations (Clarke and van der Kooy 2009). This lower oxygen concentration also produced healthier neurospheres as determined by visual inspection.

2.3 Ex vivo embryonic NPC self-renewal ("neurosphere") assay

2.3.1 Neurosphere assay in absence of DNA damage

At four days post isolation, primary neurospheres are dissociated to a single cell suspension and seeded at 1x10^5 cells/mL in 1mL of proliferation media in a 24 well plate (BD). Given 7 days to self-renew, the number of neuropheres in 200uL representative samples were counted, averaged and compared between genotypes.

2.3.2 Neurosphere assay upon exposure to UV light

At four days post isolation, primary neurospheres are dissociated to a single cell suspension and the volume corresponding to 1x10^5 cells was seeded into 24 well plates. Using the CL-1000 UVP Crosslinker, wells were then individually treated with varying doses of UVC light at a wavelength of 254nm. The doses chosen were untreated, 2, 5, 10, 25 and 50 J/m^2 and aluminum foil was used to cover and protect other wells as one was treated. NSC proliferation
media was then added to each well, producing a density of $1 \times 10^5$ cells/mL. At 7 days post treatment, the number of neurospheres in 200uL representative samples were counted, averaged and compared between treatments and genotypes.

2.3.3 Neurosphere assay with consecutive passaging

At four days post isolation, primary neurospheres are dissociated into a single cell suspension, which was then used to both continuously passage cells and produce neurosphere assays. From the single cell suspension, cells were seeded into a T-25cm$^2$ flask in NSC proliferation media and passaged at a density of $1 \times 10^5$ cells/mL. From that same single cell suspension, cells were also seeded at the same density into a 24 well plate. The cells within the 24 well plate were given 7 days to self-renew, at which point the number of neurospheres in 200uL representative samples were counted. The cells in the flask were given 4 days to generate the next passage, at which point the process would be repeated with some cells being used to passage and others to generate neurosphere assays.

2.4 Ex vivo adult NSC self-renewal assay

SVZs from the brains of 2 month old $Csb^{m/m}$ and $Csb^{+/+}$ mice were isolated and dissociated to a single-cell suspension of NPCs. Mechanical dissociation using a p1000 micro micropipettor was followed by enzymatic dissociation using the NeuroCult® Enzymatic Dissociation Kit for Adult Mouse CNS Tissue (Stemcell Technologies) according to manufacturer’s protocol. Adult NPCs were cultured for four days in the same NSC proliferation media used for embryonic NPCs. On the fourth day, primary neurospheres were dissociated and seeded at $1 \times 10^5$ cells/mL in 1mL of proliferation media in a 24 well plate (BD). Given 7 days to self-renew, the number of neurospheres in 200uL representative samples were counted, averaged and compared between genotypes.
2.5 Ex Vivo embryonic NPC survival assay

At four days post isolation, primary neurospheres are dissociated to a single cell suspension, from which the volume corresponding to 1x10^5 cells was seeded into two 24 well plates. Using the CL-1000 UVP Crosslinker, wells were then individually treated with varying doses of UV light at a wavelength of 254nm. The doses chosen were untreated, 2, 5, 10, 25 and 50 J/m^2 and aluminum foil was used to cover and protect other wells as one was treated. NSC proliferation media was then added to each well, producing a density of 1x10^5 cells/mL. At 24 hours post UV treatment, trypan (Invitrogen) was used to count the number of living and dead cells in one 24 well plate to generate a survival curve. This was repeated using the second 24 well plate at 48 hours post UV treatment.

2.6 Ex Vivo embryonic NPC differentiation

2.6.1 NPC Differentiation in the absence of DNA damage

NPCs were isolated from the GE of E14 embryos as outlined above, except they were dissociated into NSC differentiation media, which consists of ES grade KO DMEM (Gibco), 10% ES cell FBS (Gibco), 1% Glutamax (Invitrogen) and 1% penicillin-streptomycin (Gibco). Glass coverslips were sterilized, placed within 6 well plates (BD) and coated for 2 min with 500uL of 33% Growth Factor Reduced Matrigel™ Basement Membrane Matrix (BD) in KO DMEM. NPCs from the single cell suspension were seeded at a density of 3x10^5 cells/mL onto the coverslip at first within a 400uL droplet. This 400uL droplet was incubated at 37°C, 5% CO^2 and 5% O^2 for 1 hour to ensure that cells adhered to the coverslip rather than the 6-well plate. Another 1.1mL of media was then added to each well to achieve the 3x10^5 cells/mL density. Media was replaced with fresh differentiation media every 2 days to ensure continual differentiation for 4 days.
2.6.2 NPC Differentiation under exogenous DNA damage conditions

Primary NPCs are isolated and coverslips are coated in a similar method as outlined above. Upon obtaining a single cell suspension of NPCs and diluting to 3x10^5 cells/mL, 400uL is seeded in a droplet onto a coverslip. Samples on coverslips were arranged such that each plate corresponded to a single UV dose, to avoid error during exposure. Plates are then inserted into the CL-1000 UVP Crosslinker and treated with varying doses, 5, 10, 20, 30, 40 and 50 J/m², of UV light at a wavelength of 254nm. The 400uL droplet was incubated on the coverslip for 1hr to ensure that cells adhered to the coverslip rather than the 6-well plate. Another 1 mL of media was then added to each well to achieve the 3x10^5 cells/mL density. Media was replaced with fresh differentiation media every 2 days to ensure continual differentiation for 4 days.

2.7 Immunofluorescent labeling of neurons

After 4 days of differentiation, NPCs are fixed using 4% paraformaldehyde (Sigma). Cells are washed, undergo permeabilization with 0.04% Triton-X in PBS and blocked with goat serum. The cells are then incubated with a 1:1000 dilution of mouse anti-tubulin, beta III isoform (Tuj-1; StemCell Technologies), a monoclonal antibody that reacts with the C-terminus of the neuron specific beta III isoform of tubulin. Coverslips are then incubated with the goat anti-mouse Alexa555 antibody (Invitrogen) and mounted onto slides using Vectashield Mounting Media (Vector) containing DAPI. Slides are therefore labeled for total cell number with DAPI, and neurons with Tuj-1. When being imaged under the microscope, four fields are randomly chosen from each coverslip. The total number of cells and total number of neurons are then tabulated and compared between genotypes and treatments.
2.8 In Vivo Assessment of NPC Self-Renewal

*Csb+/-* females and males were mated, and at E14, the dam was injected with 10mg/mL of the nucleoside analogue 5-bromo-2-deoxyuridine (BrdU) in PBS (Invitrogen). One hour post injection, the dam was euthanized and all embryos were decapitated with heads being fixed in 4% paraformaldehyde (Sigma). The tails from these embryos were also removed to allow genotyping of the mixed genotype litter. Embryo heads were fixed for approximately one week and underwent coronal sectioning at the Pathology Research Program within Toronto General Hospital. From each embryo, three 5um coronal sections were selected to represent different relative depths of the forebrain. Endogenous Peroxidases were inactivated using 8mL 30% H₂O₂ (Sigma) in 200mL 100% Methanol (Caledon). Sections were incubated with 1:30 dilution of mouse anti-BrdU primary antibody (BD) overnight at 4°C, and with a horse anti-mouse biotinylated secondary antibody for 1h at room temperature. The avidin-biotin-horseradish peroxidase complex (ABC) (Vector) was then used to amplify the sensitivity of BrdU detection using the 3,3'-diaminobenzidine (DAB) substrate (Roche). Images of the neocortex were taken within each section of each embryo, as it is a region rich in stem cells that are easily quantifiable. The area, total number of cells, and number of proliferating cells (BrdU) were counted in each neocortex.

2.9 Embryonic Genotyping

As mentioned, each embryo from a mixed *Csb+/-* litter had their tails removed during dissections and labeled to correspond with the culture samples from that embryo. The tails were then digested, neutralized and amplified using the REDExtract-N-Amp™ Tissue PCR (XNAT) Kit (Sigma) according to the manufacturers instructions. *Xpa* and *Csb* primer sequences, cycling conditions and band sizes are summarized in Table 2.1. PCR products were run on a 2%
agarose gel at a Voltage of 110V for 1h and visualized using ethidium bromide and a UV transilluminator.

Table 2.1: Summary of genotyping primers, cycling conditions and band sizes.

<table>
<thead>
<tr>
<th>Xpa Primers</th>
<th>XPA Cycling Conditions</th>
<th>Band Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP26: 5’-GTGTCAGGCATAAGATCTATGACAA-3’</td>
<td>1) 95°C for 5min</td>
<td>WT – 300bp</td>
</tr>
<tr>
<td>XP47: 5’-AGGCAAGCACCTGCAGCTGT-3’</td>
<td>2) 94°C for 1min</td>
<td>KO – 200bp</td>
</tr>
<tr>
<td>PGK2: 5’-GGCCACTTGTGTAGCGCCAA-3’</td>
<td>3) 58°C for 1min - 30 cycles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4) 72°C for 2min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5) 72°C for 10min</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Csb Primers</th>
<th>CSB Cycling Conditions</th>
<th>Band Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSB4: 5’-GCTGTTATAATAATCCTGCATC-3’</td>
<td>1) 95°C for 5min</td>
<td>WT – 200bp</td>
</tr>
<tr>
<td>CSB5: 5’-ATCTGGGTGTTCAATTGCACATGC-3’</td>
<td>2) 94°C for 30sec</td>
<td>KO – 500bp</td>
</tr>
<tr>
<td>CSB6: 5’-GTCTTCTGATGACGTAGCTATGAG-3’</td>
<td>3) 58°C for 45sec - 35 cycles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4) 72°C for 1min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5) 72°C for 10min</td>
<td></td>
</tr>
</tbody>
</table>

### 2.10 Data Plots and Statistics

Data was both graphed and analyzed using Graphpad prism v5. Means, standard deviations, sample sizes and p-values are all provided with their respective results.
CHAPTER 3: RESULTS

3.1 Reduced Csb<sup>−/−</sup> embryonic NPC self-renewal ex vivo

Having optimized culture conditions for wild type embryonic NPC self-renewal in culture, the first objective was to characterize basal self-renewal capacity of Csb<sup>−/−</sup> embryonic NPCs. The number of secondary neurospheres resulting from 1x10<sup>5</sup> NPCs derived from primary neurospheres was compared between genotypes. Csb<sup>−/−</sup> embryos (n = 6; from 3 independent litters) generated 190 +/- 23 secondary spheres (mean +/- S.D.). This was significantly less than the number of secondary neurospheres (240 +/- 14, mean +/- S.D.) generated from wild type congenic littermate controls (n = 6; from 3 independent litters) (p < 0.05). When normalized to wild type controls, Csb<sup>−/−</sup> sphere forming capacity was 22% lower than wild type (Fig. 1c).

A related knockout mouse model was investigated as a positive control: Xpa<sup>−/−</sup> mice that are defective in the common pathway of NER. Xpa<sup>−/−</sup> embryonic NPCs/NPCs generated 20% fewer neurospheres than the wild type controls (n=6; from 4 independent litters, p<0.005) (Fig. 1d). Additionally, to verify that this was a self-renewal defect rather a defect in proliferation, we measured neurosphere size. There was no significant difference in the average neurosphere size generated by Csb<sup>−/−</sup> and WT NPCs, consistent with an equal rate of proliferation in vitro (Fig. 1b).

Taken together, the results of secondary sphere forming assays indicate that NER deficiency marginally impairs neural stem cell self-renewal in the absence of exogenous DNA damage. The observed reductions in NER<sup>−/−</sup> models were statistically different but likely not biologically significant at this early developmental timepoint. This slight defect prompted us to examine self-renewal capacity in Csb<sup>−/−</sup> mice under several conditions of stress: (a) a classical DNA damaging agent, UV light (b) chronic oxidative stress, (c) serial passaging, and (d) aging.
3.2 Reduced clonogenic survival of CSB<sup>m/m</sup> NPCs after UV-induced DNA damage

In order to assess the susceptibility of CSB<sup>m/m</sup> NPCs to exogenous DNA damage, UV light was chosen as a model DNA damaging agent, as it cleanly and directly introduces DNA lesions repaired by the NER pathway. We verified that increasing UV dose resulted in a linear increase in DNA lesions, specifically (6-4) photoproducts (Supplemental Figure 2). Over a dose range of 0 – 50 J/m<sup>2</sup> of UVB light, CSB<sup>m/m</sup> NPCs showed a progressive reduction in self-renewal (p<0.0001). At all doses of UV tested, NPCs from CSB<sup>m/m</sup> embryos generated fewer spheres than WT controls and this sensitivity of CSB<sup>m/m</sup> increased with increasing UV dose (p<0.0001) (Fig. 2). This indicates that upon UV-induced DNA damage, CSB<sup>m/m</sup> NPCs demonstrate reduced colony-forming ability. We inferred that the difference between CSB<sup>m/m</sup> NPCs and controls was due to an increased level of residual UV-induced DNA damage in transcriptionally active genes, although this was not tested directly. Since the observed reduction in colony formation has contributions from both UV-induced death and UV-induced reduction in self-renewal, these two contributing factors were examined independently, as indicated below.

3.3 Increased death of CSB<sup>m/m</sup> NPCs after exogenous DNA damage

To determine the relative contribution of cell death to the observed reduction in colony formation, dead and living cells were counted in dissociated cultures at 24 post-UV, using trypan blue, a dye that is excluded from viable cells. This time point was chosen because UV-induced apoptosis is maximal at 24 hours in a variety of cell types including mouse embryonic stem cells (Van Sloun, Jansen et al. 1999; Gentile, Latonen et al. 2003). CSB<sup>m/m</sup> NPCs demonstrated mildly reduced cell survival relative to littermate WT controls; the sensitivity of CSB<sup>m/m</sup> NPCs increased with increasing UV exposure (p<0.0005) (Supplemental Fig. 3a). These results were confirmed
with a related luciferase reporter assay (Cell-Titer-Glo, Promega) that rapidly but indirectly assessed viable cell number (Supplemental Fig. 3b).

3.4 Relative contributions of death and reduced self-renewal to reduced clonogenic survival after UV DNA damage

To compare cell death and reduced colony formation after UV, the two types of results were plotted together graphically, each on their own y-axis, both in logarithmic scale (Fig. 3). There are two crucial aspects of the data that require close inspection. First, there is a divergence between the percentage of cells surviving UV exposure and the percentage of cells undergoing self-renewal. At all doses, there is a higher percentage of cells surviving than generating spheres, indicating that there are cells present that are alive, but incapable of undergoing self-renewal. Thus, the UV-induced DNA damage is preventing the NPCs from self-renewing more than killing them. The second critical point to notice is that the divergence between survival and self-renewal is greater for the Csb\textsuperscript{m/m} NPCs than their WT counterparts. This indicates that in Csb\textsuperscript{m/m} embryos, NPCs have impaired self-renewal at sub-lethal doses of UV damage.

3.5 Reduced self-renewal of \textit{ex vivo} Csb\textsuperscript{m/m} NPCs with successive passages.

To evaluate if NER deficiency impacts NPC self-renewal in a physiologically relevant manner, it was necessary to approximate the endogenous damage presented to NPCs \textit{in vivo}. Our first attempt involved reproducing chronic cellular oxidative stress conditions through the glucose/glucose oxidase system according to published protocols, but this proved unamenable to NPC culture conditions (Limoli and Giedzinski 2003) (Supplemental Fig. 4). Thus, it was decided to evaluate self-renewal capacity after successive passages, with the assumption that each passage would allow DNA damage to accumulate in the NPCs lacking the capacity for...
repair. After a primary passage to generate a more homogenous culture, NPCs from each genotype were passaged six times with colony counts conducted seven days post-passage.

From the initial passage, \( Csb^{m/m} \) NPCs (n=4, from 2 independent litters) generated significantly fewer neurospheres than their littermate WT controls (n=4, from 2 independent litters; p<0.0001) (Fig. 4a). However, with each passage, the difference between \( Csb^{m/m} \) and WT neurosphere number diverged, culminating in a 44% difference in neurospheres formed (p<0.0001). WT neurospheres maintained a fairly consistent rate of neurosphere decline, generating about 16 fewer neurospheres per passage. \( Csb^{m/m} \) neurospheres, however, demonstrated varied rates of decline, with 27, 76, and 21 fewer neurospheres between passages 1-3, 3-4, and 4-6 respectively. Taken together, the data indicates that \( Csb^{m/m} \) NPCs demonstrate reduced self-renewal capacity with successive passaging as compared to WT controls, and that this is largely accounted for by dramatic changes in the rate of \( Csb^{m/m} \) neurosphere decline.

3.6 Reduced Self-renewal of \( Csb^{m/m} \) Adult NPCs ex vivo.

The reduction in \( Csb^{m/m} \) NPC neurosphere formation with successive passages indicated a potential stem cell exhaustion phenotype under simulated aging conditions. This led us to expand our self-renewal assays into NPCs isolated from adult mice. NPCs were harvested from 8 week old \( Csb^{m/m} \) and WT mice, passaged once to obtain neurospheres, and then seeded for the assessment of secondary sphere formation. \( Csb^{m/m} \) NPCs (n=3) generated a statistically significant 9% fewer neurospheres than WT controls (n=3, p<0.05) (Fig. 4b).

3.7 Equal numbers of BrdU+ cells in vivo in the neurogenic brain regions of \( Csb^{m/m} \) and WT E14 embryos.

In vitro studies revealed a self-renewal defect in repair deficient NPCs, making an in vivo analysis of self-renewal necessary. To accomplish this, we attempted to quantify the number
NSCs within the embryo brain at E14. However, a lack of agreed-upon NSC markers forced us to indirectly evaluate NSC number using the nucleoside analogue BrdU. BrdU incorporates into synthesizing DNA and is commonly used as a marker for proliferation. Thus, we investigated the number of BrdU+ cells within the NSC rich neocortex of Csb\textsuperscript{mm} and WT embryos (Fig. 5a). There is no significant difference in the number of BrdU+ cells within the neocortex of Csb\textsuperscript{mm} embryos (n=6; from 3 independent litters) as compared to WT littermate controls (n=6; from 3 independent litters) regardless of relative brain depth (Fig. 5b). The related Xpa\textsuperscript{-/-} model similarly revealed no difference in the number of BrdU+ cells (Supplemental Figure 5).

Additionally, \textit{in vivo} TUNEL assays demonstrated no significant differences in cell death between WT and Csb\textsuperscript{mm} E14 embryo brains (Supplementary Figure 6).

\textbf{3.8 Reduced multipotency of Csb\textsuperscript{mm} NPCs \textit{ex vivo}.}

Primary functions/fates of NPCs are cell death, self-renewal and multipotency. An established approach for evaluating multipotency is to induce NPCs to differentiate \textit{in vitro}, and then antibody-label the differentiated cells for lineage markers. The number of neurons and total cells were counted in three random fields and the percentage of neurons generated was compared between genotypes and doses (Fig 6b). The percentage of NPC-derived neurons was generally low, fluctuating between 3-12% of total cells. Two-way ANOVA analysis reveals that genotype does affect the number of neurons generated at all UV doses (p<0.05). Generally, neuronal percentage increased in the Csb\textsuperscript{**/+} genotype after UV, although a triphasic response was observed over increasing DNA damage. UV dose does not statistically correlate with the number of neurons generated, and so the interaction of genotype and UV dose is not considered significant. Differentiation requires that the NPCs adhere to the coated coverslips. We observed
no significant variation in the number of adherent NPCs with either genotype or UV dose (Supplemental Fig. 7).

With the goal of scoring neurons in a high-throughput and unbiased manner, we attempted to optimize a flow cytometry technique to evaluate neuronal differentiation. NPCs from either genotype were induced to differentiate in culture, incubated with an anti-Tuj-1 antibody and then subjected to cell sorting by flow cytometry. Preliminary experiments suggested no significant difference in the number of neurons generated by NPCs of either genotype (Supplemental Fig. 8). However, the lack of clear Tuj-1+/Fit-C fluorescent peaks and high background staining of Tuj1, indicated that this technique would require additional optimization. It was therefore discontinued as an alternative to manual counting of immunofluorescently-stained coverslips.

Immunocytochemical analyses have the advantage over flow cytometry in that the former allows for a visual assessment of cell morphology. We compared the morphology of neurons generated by Csb\textsuperscript{mm} and WT NPCs at 0-50 J/m\textsuperscript{2} (Fig 6a). We observed a reduction in the number of neurites with increasing UV dose, as well as decreased arborization. \textit{In vitro} differentiated Csb\textsuperscript{mm} NPCs were more sensitive, with reduced neuritogenesis observed by 20J/m\textsuperscript{2} and its complete abolishment at 50J/m\textsuperscript{2}. Impaired neuritogenesis only became obvious in the WT NPC-generated neurons at approximately 40 J/m\textsuperscript{2}.
CHAPTER 4: DISCUSSION

4.1 NSC self-renewal reduction under exogenous DNA damage.

Prior to an investigation of NPC self-renewal changes in response to DNA damage, it was important to identify any basal self-renewal differences within the repair deficient backgrounds. In vivo histological analysis revealed no significant differences in the number of BrdU+ cells within the embryonic neocortices of Csb\textsuperscript{m/m} mice as compared to littermate controls. The related Xpa\textsuperscript{-/-} model similarly revealed no difference in the number of BrdU+ cells (Supplemental Figure 5). We, therefore, concluded that there was no difference in the in vivo number of NSCs between repair backgrounds at early embryonic age.

Although incorporation of the thymidine analogue BrdU measures DNA synthesis, it is commonly used in the literature as a measure of the proliferative index of a particular tissue. For our purposes, we have maintained this assumption, using BrdU to label proliferating cells. Thus, equal incorporation of BrdU within the NPC-rich regions of repair proficient and deficient embryonic mice brains is consistent with the interpretation that there is an equal NPC abundance. Unfortunately, however, there are no definitive markers for NPCs within the field. The best candidate, nestin, has been used to label cortical NSCs (Lendahl, Zimmerman et al. 1990), but has also been reported to label other cell types, including radioglial cells (Doetsch 2003).

Self-renewal can also be evaluated in vitro via colony-forming assays. In these experiments, NPCs harvested from both Csb\textsuperscript{m/m} and Xpa\textsuperscript{-/-} embryos generated ~20% fewer neurospheres than their littermate controls, indicating that NPC self-renewal is somewhat reduced within these genotypes. The ~20% reduction in the number of neurospheres formed raises the issue of statistical significance versus biological relevance. Is a 20% difference in self-renewal capacity biologically relevant? It could be providing a subtle indication of a
compromised neural precursor phenotype in these repair deficient mice as early as E14. This may suggest possible developmental origins of repair deficiency syndromes, as these subtle differences may go unnoticed until they generate obvious clinical manifestations. Stronger phenotypes may become manifest only under stress conditions, prompting us to examine the impact additional DNA damage has on self-renewal.

UV light was chosen as a DNA damaging agent as it directly introduces DNA damage, removing the variability associated with enzymatic bioactivation and detoxification of drugs and chemicals. We verified that increasing UV dose resulted in a linear increase in DNA lesions, specifically (6-4) photoproducts (Supplemental Figure 2). Exposure of Csb<sup>m/m</sup> and Xpa<sup>-/-</sup> NPCs to UV light resulted in a dose-dependent reduction in the number of neurospheres generated from these cells, indicating a strong sensitivity to UV DNA damage. The sensitivity of many cell types derived from these repair deficient backgrounds has been well characterized in the literature. For example, both Csb<sup>m/m</sup> and Xpa<sup>-/-</sup> MEFs and ES cells have demonstrated a hypersensitivity to UV, but only in the MEFs does this correlate with increased apoptosis (de Waard, Sonneveld et al. 2008).

Similar to repair deficient ES cells, the hypersensitivity of Csb<sup>m/m</sup> and Xpa<sup>-/-</sup> NPCs is not entirely mediated by cell death (Fig. 3). Colony formation is both a function of self-renewal and cell death. When the results obtained from both the cell death and colony-forming assays are combined, it becomes obvious that only a small fraction of the NPCs are dying upon UV exposure. However, the dramatic reduction in neurospheres formed in Csb<sup>m/m</sup> NPCs indicates that many cells survived treatment but simply are not self-renewing.

Unlike other cell types, however, the hypersensitivity of Csb<sup>m/m</sup> and Xpa<sup>-/-</sup> NPCs to exogenous damage has more dramatic functional outcomes. In most other cell types, this...
sensitivity would result in apoptosis or quiescence, which would induce the replacement of these cells by their progenitors to sustain the tissue. However, when the progenitors themselves are hypersensitive, their populations begin to dwindle, impairing tissue maintenance. The observed reduction in self-renewal of NPCs upon exposure to exogenous DNA damage could result in a progressive decline in NPC abundance, impairing neural development and neuroregeneration.

4.2 Reduced self-renewal of Csb^{mm} NPCs after serial passaging.

It is important to evaluate NPC sensitivity under conditions of endogenous stress. Under endogenous stress conditions, in vivo TUNEL assays demonstrated no significant differences in cell death between WT and Csb^{mm} E14 embryo brains (Supplementary Figure 6). However, E14 may be too early to observe obvious differences in cell death in vivo.

Thus, we attempted to increase physiologic endogenous DNA damage in culture. First, we attempted to recreate chronic oxidative stress conditions within culture NPCs using a glucose/glucose oxidase system according to published protocols (Limoli and Giedzinski 2003). Glucose oxidase (GO) catalyzes the oxidation of glucose to gluconolactone but generates hydrogen peroxide as a by-product. Unfortunately, although some preliminary optimization experiments showed promise, the technique proved unamenable to culturing NPCs (Supplementary Fig. 4).

At this point, it was decided to increase endogenous DNA damage by serial passaging, as NPCs do not have an unlimited self-renewal capacity in the absence of transformation (Morshead, Craig et al. 1998). The assumption made regarding this technique was that DNA damage would accumulate with each successive passage in the repair deficient NPCs, and this would lead to a greater sensitivity in these cells. We did not directly measure DNA lesions with each successive passage, but an ELISA technique utilizing DNA lesion-specific antibodies could
be performed at the initial and final passages to verify differences in lesion quantity. This type of endogenous stress can generate a large number of NER substrate DNA lesions, thus it is unclear which lesion would be best to measure.

The serial passaging experiment demonstrated a progressive decline in the number of neurospheres formed with each consecutive passage, with the reduction exacerbated in the 

$Csb^{m/m}$ NPCs (Fig 4a). As in vivo TUNEL assays verified that cell death did not differ in $Csb^{m/m}$, this implicated a self-renewal defect, consistent with DNA damage experiments (Supplementary Fig. 6).

There are two possible interpretations of these results, one being that the $Csb^{m/m}$ NPCs simply exhaust more rapidly with endogenous DNA damage as compared to normal NPCs. A second explanation relies on the heterogenous nature of the neurosphere assay. In a hierarchical view of progenitors, NSCs and downstream precursor cells are distinct cell types. Although precursor cells are more restricted in their potency to differentiate relative to NSCs, NPCs are also limited in their self-renewal capacity, meaning they should exhaust upon fewer division cycles as compared to NSCs. Neurospheres derived from embryonic brain cultures contain both NSC and NPC cell-types (Morshead, Craig et al. 1998). Continuous passaging would result in a more rapid exhaustion of precursors than NSCs, and this could explain the divergence between $Csb^{+/+}$ and $Csb^{m/m}$ self-renewal curves. The slope of the $Csb^{m/m}$ line decreases dramatically between passages 3 and 4, and then returns to normal, whereas the $Csb^{+/+}$ remains consistent throughout. This change in slope could correspond to the exhaustion of downstream precursors within the culture, upon which only NSCs remain and continue to slowly exhaust with passage. If true, this suggests that the repair deficient cultures consisted of a higher percentage of precursors, relative to NSCs, as compared to the littermate control cultures.
4.3 NSC exhaustion and aging.

The serial-passaging experiment was intended to duplicate an *in vitro* aging scenario, and demonstrated a progressive reduction in colony formation, implicating an exhaustion phenotype. This prompted us to investigate self-renewal capacity as it related to *in vivo* aging, with a preliminary prediction that *Csb*\textsuperscript{m/m} adults would generate fewer neurospheres as compared to WT adults. The adult self-renewal assay demonstrated only a modest reduction in self-renewal in *Csb*\textsuperscript{m/m}.

However, prior to interpreting the results of the adult self-renewal assay, it is necessary to clarify the difference between normal *in vivo* and induced *in vitro* aging. The *in vivo* rate of self-renewal of sub-ependymal NSCs is approximately one division cycle every 30 days, indicating that these cells are normally relatively quiescent under normal homeostatic conditions (Morshead, Reynolds et al. 1994). However, these cells are induced to self-renew at a much faster, yet unmeasured, rate during neuroregeneration in response to injury. Therefore, NPCs self-renewed every four days *in vitro*, when they were dissociated and committed to generating neurospheres.

The 8 week old adult mice used to procure NPCs can then be considered to be at a relatively early division cycle number, as self-renewal occurs every 30 days in mice. The modest difference observed in the number of neurospheres generated by the NPCs of differing genotype is therefore consistent with the modest differences observed in the relatively equivalent second and third passages of the serial-passaging experiment.

The serial-passaging was designed to mimic endogenous DNA damage accumulation and NPC exhaustion as it relates to aging *in vitro*. The subtle reduction of NPC self-renewal in 8 week old *Csb*\textsuperscript{m/m} mice could represent the commencement of a progressive decline in self-
renewal with age, as was observed in vitro. Unfortunately, the procurement of NPCs from adult NSCs is difficult as they are less abundant and their subsequent culture is more challenging. Ultimately, however, self-renewal assays of NPCs from several different post-natal ages will be the most direct way to investigate NPC exhaustion and aging.

The accumulation of DNA damage as a possible mechanism behind stem cell exhaustion and physiologic aging has been reported within other tissues as well. The impacts of age on hematopoietic stem cell function has been well characterized, with reductions in self-renewal capacities, altered lineage potentials and decreased stress responses all being reported (Morrison, Wandycz et al. 1996; Kim, Moon et al. 2003; Pearce, Anjos-Afonso et al. 2007). Defects in the NER pathway, specifically Csb<sup>min</sup>, did not result in a depletion of HSC populations with age, but rather demonstrated a reduction in the number of cycling downstream precursors (de Boer, Andressoo et al. 2002; Rossi, Bryder et al. 2007). The proposed model describes increased DNA damage as a block to progenitor self-renewal, maintaining the hematopoietic stem and precursor cells in the quiescent G<sub>0</sub> state (Rossi, Seita et al. 2007). This is consistent with our serial-passaging results, where the data implicates a rapid exhaustion of neural precursors with a slow steady decline of NSCs in the repair-deficient genotype.

Neurogenesis has been reported to decline with age in the adult brain, but the reasons associated with this decline are unclear (Tropepe, Craig et al. 1997). A combination of both cell-intrinsic and niche-related signals (Enwere, Shingo et al. 2004), including DNA damage, may result in exhaustion of the NSC population. A putative correlation between stem cell exhaustion and DNA repair has undergone little investigation in NSCs. Mutations in the FANC proteins cause disruptions in double-strand break repair and are associated with Fanconi’s anemia. In vitro immunocytochemistry revealed a reduction in the number NSC-derived neurospheres, as
well as a dramatic 40% reduction in the number of precursor-derived neurospheres in aged mice (Sii-Felice, Etienne et al. 2008). Taken with the aforementioned Csb<sup>m/m</sup> HSC studies, and consistent with our results, this indicates that DNA repair deficiency may result in a rapid exhaustion of neural precursors concomitant with a slower progressive decline in NSCs.

Other non-repair related genetic models have reported a similar NSC exhaustion phenomenon. Loss of the cell cycle regulator p21 results in both <i>in vitro</i> and <i>in vivo</i> stem cell functional deficits. <i>In vitro</i> passaging of p21<sup>-/-</sup> neurospheres results in complete exhaustion by passage 5, with no subsequent NPCs generating neurospheres. Culturing of adult NPCs from multiple ages demonstrates a reduction in the number of primary neurospheres generated, as well as reduction in the rate of BrdU incorporation (Kippin, Martens et al. 2005). Taken together, these indicate a progressive reduction in <i>in vivo</i> longevity of p21<sup>-/-</sup> NSCs that is similar but more severe than what we report in Csb<sup>m/m</sup>. This is to be expected, as complete loss of cell cycle control would be more likely to result in NPC exhaustion then loss of DNA repair.

### 4.4 Decreased multipotency and reduced terminal differentiation of Csb<sup>m/m</sup> NPCs after UV DNA damage.

One of the most critical NSC functional capacities is multipotency as it allows NPCs to generate CNS tissue throughout development and in response to neuronal injury. Our differentiation assay evaluated differentiation capacity along the neural lineage. There was no statistically significant variation in the number of neurons generated with increasing UV dose for either genotype. However, the percentage of neurons generated by Csb<sup>+/+</sup> NPCs was significantly higher than those generated by Csb<sup>m/m</sup> NPCs (<strong>Fig 6b</strong>), particularly at the physiologically relevant UV doses below 20J/m<sup>2</sup>. This suggests that at low doses of UV damage, the typical NPC response is increased differentiation, whereas this is impaired in Csb<sup>m/m</sup> NPCs.
The most dramatic finding was that UV damage decreased the terminal differentiation of the NPCs into neurons, as indicated by the reduction of neuritigenesis and arborization with increasing UV dose (Fig 6a). Csb\textsuperscript{mn} NPCs were more sensitive to defective neuritigenesis. Defects in maturation were obvious at 20J/m\textsuperscript{2} and the mature phenotype was abolished at the highest UV dose (50J/m\textsuperscript{2}). DNA damage also decreased neuritigenesis in wild-types, but this could not be observed until 40-50J/m\textsuperscript{2}. This implies that DNA damage in NPCs can interfere with the terminal differentiation program.

Defective neuritigenesis during NPC neuronal differentiation has also been observed in at least one other genetic model, telomerase-deficient (Terc\textsuperscript{-/-}) mice. Telomerase activity is important in maintaining NSC self-renewal, as late generation adult Terc\textsuperscript{-/-} mice undergo NSC-exhaustion \textit{in vivo} (Ferron, Mira et al. 2004). However, telomere length also modulates multipotency, as telomerase expression is down-regulated during neuronal differentiation (Kruk, Balajee et al. 1996), and over-expression has been reported to inhibit the differentiation process (Richardson, Nguyen et al. 2007). Interestingly, the removal of telomerase activity in Terc\textsuperscript{-/-} NPCs results in defective neuritigenesis upon induction of \textit{in vitro} differentiation. Neuritic arborization can be rescued by the addition of p53 deficiency in Terc\textsuperscript{-/-}/p53\textsuperscript{-/-} mice, with γ-secretase inhibition of Notch signaling, and with Rock1/2 kinase inhibition, indicating them as mediators of neuritigenesis and terminal differentiation (Ferron, Marques-Torrejon et al. 2009). Since p53 is also involved in the DNA damage response at many points, it is a likely candidate mediator of the defective terminal differentiation of Csb\textsuperscript{mn} NPCs. DNA damage, particularly in the absence of DNA repair, could cause p53 to impair neuritigenesis via downstream Rock1/2 signalling, resulting in defective terminal differentiation. This could be investigated by introducing p53 and Rock1/2 inhibitors into our NPC-differentiation culture conditions, followed
by immunofluorescent staining and neurite evaluation. If p53 and Rock1/2 are inhibiting terminal differentiation in response to DNA damage, then abolishing their activity should return neuritogenesis to normal.

4.5 Overall NPC functional compromise in $Csb^{mm}$.

Taken together, the self-renewal and differentiation response to DNA damage of $Csb^{mm}$ NPCs differ from that of congenic wild-type controls. Upon exposure to DNA damage, $Csb^{++}$ NPCs demonstrate a modest reduction in self-renewal and a modest increase in differentiation. $Csb^{mm}$ NPCs, on the other hand, undergo a dramatic reduction in self-renewal with no change in multipotency, but rather a defect in terminal differentiation. In future studies, it will be important to identify the molecular factors and pathways that modulate these cellular outcomes.

Determination of stem cell fate can be modulated by the concentrations of specific factors, which act as molecular switches between self-renewal and differentiation (Gage 2000). I will discuss several here in the context of future studies. The Notch signaling pathway is an important repressor of both embryonic and adult neurogenesis (Hitoshi, Alexson et al. 2002). Notch activation results in downstream activation of transcriptional repressors of proneural genes, effectively inhibiting neuronal differentiation and promoting self-renewal (Bertrand, Castro et al. 2002). Conversely, the transcription factor Pax6 appears to be an important promoter of proneural gene expression and neuronal differentiation. Pax6 over-expression results in increased $in vitro$ neurogenesis of mouse embryonic cortical NSCs, adult mouse SVZ NSCs, and human NSCs (Hack, Sugimori et al. 2004; Hack, Saghatelyan et al. 2005; Kallur, Gisler et al. 2008). Finally, the growth factors EGF and FGF, and their respective receptors, are also required for maintenance of NSC self-renewal and are up-regulated in response to injury in the adult brain (Yarden 2001; Zheng, Nowakowski et al. 2004; Alagappan, Lazzarino et al.
The net effects of these three pathways modulate the NSC decision to self-renew or differentiate.

The effects we observed of DNA damage challenge on NPC function are likely mediated by combinations of Notch, Pax6, EGFR and FGFR. Table 4.1 summarizes our observed responses of NPCs to DNA damage, the predicted candidate proteins involved, their activities regarding stem cell fate, and their expected signaling or expression. The expected activity of these factors could be evaluated by introducing their chemical or genetic regulators into our self-renewal and differentiation assays. The regulators are predicted to interrupt the effects of these molecular players on cell fate, resulting in restoration of NPC function.

<table>
<thead>
<tr>
<th>Repair Background</th>
<th>Observed DNA damage response</th>
<th>Factor</th>
<th>Activity</th>
<th>Signaling/Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csb&lt;sup&gt;74+&lt;/sup&gt;</td>
<td>Slightly reduced self-renewal <em>(Fig. 2a)</em></td>
<td>Notch</td>
<td>Promotes Self-renewal</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>Slightly increased differentiation <em>(Fig. 6b)</em></td>
<td>Pax6</td>
<td>Promotes Differentiation</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGFR</td>
<td>Promotes Self-renewal</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGFR</td>
<td>Promotes Self-renewal</td>
<td>↓</td>
</tr>
<tr>
<td>Csb&lt;sup&gt;max&lt;/sup&gt;</td>
<td>Dramatically reduced self-renewal <em>(Fig. 2a)</em></td>
<td>Notch</td>
<td>Promotes Self-renewal</td>
<td>↓↓</td>
</tr>
<tr>
<td></td>
<td>Unaltered differentiation <em>(Fig. 6b)</em></td>
<td>Pax6</td>
<td>Promotes Differentiation</td>
<td>Unchanged</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGFR</td>
<td>Promotes Self-renewal</td>
<td>↓↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FGFR</td>
<td>Promotes Self-renewal</td>
<td>↓↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P53</td>
<td>Inhibits Neuritigenesis</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rock1/2</td>
<td>Inhibits Neuritigenesis</td>
<td>↑</td>
</tr>
</tbody>
</table>

### 4.6 Clinical implications for CSB Patients

Cockayne syndrome is characterized by seemingly normal *in utero* development, followed by post-natal onset of progressive growth failure, neurological dysfunction and an accelerating aging within the first year of life (Nance and Berry 1992). At the cellular level, CS patients show widespread neurodegeneration and glial cell abnormalities, as well as increased ventricular size (Itoh, Hayashi et al. 1999; Curtis, Penney et al. 2003). Since the embryonic SVZ and adult SEL are highly enriched for NSCs, enlargement of the ventricle could result in fewer,
more diffuse, NSCs. This, coupled with defects in multiple CNS lineages, suggests the potential involvement of a NSC defect in these patients.

The three most important functional outcomes for NSCs in response to DNA damage are apoptosis, self-renewal and differentiation. Hyperapoptosis or defective self-renewal would cause a gradual depletion in the NSC population with time. We observed a reduction in clonal expansion of NSCs in vitro, not as a function of apoptosis, but rather of dramatically reduced self-renewal. This stem cell exhaustion in response to DNA damage will likely deplete NSC pools over time, which is consistent with the progressive nature of CS. Gradual loss of NSCs could result in less tissue building and attenuated response to neural injury with time. Our serial-passage assay suggests that the most pronounced decline in self-renewal occurs as DNA damage accumulates, possibly explaining the post-natal onset of CS. In utero, DNA damage would begin to accumulate, but only begin to exert its exhaustive effect on NSCs within the first post-natal year. This would also account for the lack of significant differences in the number of BrdU-positive cells in embryonic Csb<sup>+/+</sup> and Csb<sup>−/−</sup> brains in vivo, as there may not have been sufficient time for the NSCs to exhaust at E14.

Aside from issues of NSC exhaustion, abnormal differentiation could also account for the malformations in different CNS lineage cells observed in CS patients. We report no change in neurogenesis with DNA damage in Csb<sup>−/−</sup> NPCs, indicating that commitment to neuronal fate was not affected. However, increasing DNA damage resulted in defective neuritigenesis and terminal differentiation in Csb<sup>−/−</sup> NPCs. Abnormal neuronal structure in vivo could result in incomplete synapses and interfere with action potential transmission, resulting in CNS functional deficits. Although we have yet to investigate glial cell differentiation, it is possible that similar defects in terminal differentiation could result in the abnormal glial cell populations already
reported in CS patients, and explain the demyelination observed in areas of neuronal loss (Rapin, Lindenbaum et al. 2000).

Thus, the observed cellular outcomes produced by DNA damage in Csb
m/m NPCs are consistent with the clinical picture of CS. Once DNA damage begins to impact NSC fate, a gradual exhaustion of NPCs coupled with irregular terminal differentiation would prevent continued CNS development and neuroregeneration in response to injury. This would contribute to the progressive neurodegeneration of Cockayne syndrome, resulting in microcephaly and growth failure.

4.7 Implications for Regenerative Medicine.

Advances in general stem cell biology have ignited interest in using stem cells as a means of regenerating damaged tissue throughout the body. The prospect of utilizing NSCs to restore CNS tissues after acute injury or chronic damage is especially enticing, as normal neuroregeneration can be slow (Biernaskie, Sparling et al. 2007). The advent of induced pluripotent stem cell (iPS) technology will allow somatic cells to be reprogrammed into ES-like cells, which could then differentiated into NSCs (Ellis, Bruneau et al. 2009). Once the technology has been optimized and approved, banks of iPS cells or NSCs will likely be generated in hopes of genetically matching donated NSCs to recipients. Genetic and functional screening of these banks will become a necessity to ensure their capacities to self-renew and differentiate are normal in vivo (Ibarra and Martinon 2009). This will likely involve chemical approaches and the use of small molecules that would allow precise manipulation of NSC fate in vitro (Xu, Shi et al. 2008). Our results suggest that screening for DNA repair capacity will also be necessary, as NSC functional fates are highly susceptible to DNA damage. If repair-deficient NSCs were transplanted into patients, they would likely rapidly exhaust and/or be unable to
regenerate the CNS tissue, essentially making them redundant. A corollary of this suggests that transplantation of normal NSCs may alleviate some of the neurologic symptoms observed in repair-deficiencies such as CS. However, with regenerative technologies in their infancy, current priority should be placed on the generation and screening of stem cell banks for functional abnormalities, including DNA repair capacity.

4.8 Conclusions

We have explored the cellular DNA damage responses in NPCs in order to determine DNA damage impact on NSC function. Despite an expected reduction in NPC survival after DNA damage, colony-forming assays reveal that cell death is not the prominent cellular DNA damage response (Fig. 3). Both exogenous DNA damage and cellular aging resulted in decreases in NPC self-renewal and gradual stem cell exhaustion (Fig. 2, Fig. 4). Finally, DNA damage also impaired the terminal neural differentiation of NPCs (Fig. 6). Thus, we can conclude that DNA damage impairs NSC function, specifically self-renewal and differentiation.

Repair-deficient NPCs demonstrate basal reductions in in vitro self-renewal compared to littermate controls (Fig. 1). These basal differences are likely incapable of producing the intense neurodegenerative phenotype observed in CS patients. However, these subtle differences are dramatically exacerbated after DNA damage accumulation, whereupon both self-renewal and differentiation are significantly compromised in the Csb<sup>mm</sup> genotype. Reductions in self-renewal could deplete the functional NSC population, and abnormal differentiation would impair neurodevelopment and neuroregeneration in the adult. Thus, the DNA damage-induced impairment of NPC function with age is consistent with the progressive neurodegeneration reported in CS patients.
4.9 Future Directions

There are other cellular responses to DNA damage that require exploration in NSCs, specifically cellular senescence. Cellular senescence describes a withdrawal from the cell cycle into a G₀ state, whereupon DNA content remains at G₁ levels and there is an upregulation of p53 and p21 (Friedberg 2006). We reported that Csb⁻/⁻ NPCs are not significantly vulnerable to cell death following DNA damage, but do demonstrate a reduction in self-renewal. It is possible that DNA damage either forces or retains NPCs in the senescent G₀ state. This could be evaluated by examining β-galactosidase activity, a common biomarker of senescence, in NPCs after DNA damage according to a published protocol (Debacq-Chainiaux, Erusalimsky et al. 2009).

Our investigation of NPC multipotency focused on neurogenesis as the most important function of NPC differentiation. However, the lack of myelin within the severely impaired neuronal populations in CS patients implicates a potential defect in gliogenesis. Therefore, it is important to evaluate the impact that DNA damage has on NPC differentiation into glial cells. This is easily amenable to our differentiation assay, by replacing Tuj1 immunofluorescent labeling with that of Glial Fibrillary Acidic Protein (GFAP) to detect astrocytes and O4 label to detect oligodendrocytes. This would allow us to quantify NPC commitment to glial differentiation as well as evaluate the terminal differentiation process.

The molecular mechanisms driving the DNA damage response in NSCs has undergone little investigation thus far. We demonstrated reductions in self-renewal and terminal differentiation in response to DNA damage, and provided some candidate proteins we predict to be involved in these responses (Table 4.1). The use of genetic and chemical regulators of these proteins could help confirm their involvement in the modulation of DNA damage responses. Overexpression of Notch, EGFR, and FGFR is predicted to rescue reduced the self-renewal
observed after DNA damage or aging. Additionally, inhibition of p53 and/or Rock1/2 could
restore neuritogenesis during neural differentiation. By investigating the molecular factors
driving NPC self-renewal and differentiation, one could elucidate the molecular mechanisms
linking DNA responses to cell fate decisions.
Figure 1: Exogenous and endogenous sources of bulky DNA adducts. DNA adducts are typically bulky lesions that distort the DNA helix and are repaired by the nucleotide excision repair pathway. Exogenous sources of DNA adducts include environmental carcinogens (B[a]P), drugs (cyclophosphamide) and radiation (UV light). DNA adducts can also be formed by endogenous metabolites, such as the addition of malondialdehyde, a by-product of lipid oxidation, to guanosine to form the M$_1$G adduct.
Figure 2: Molecular, cellular and physiological consequences of DNA damage. DNA damage typically results from the bioactivation of exogenous or endogenous compounds into reactive metabolites that bind DNA. Most normal somatic cells can undergo DNA repair, tolerance, cell cycle arrest, or apoptosis in response to DNA damage. In stem cells, defects in self-renewal and multipotency are also potential outcomes. The preferred cell fate outcome(s) after DNA damage depends on cell type, including tissue specificity and differentiation status. In stem cells, defects in cell cycle arrest, apoptosis and/or self-renewal can result in depletion of precursor populations, whereas defects in self-renewal and/or differentiation can result in abnormal downstream cell lineages.
Figure 3: The Nucleotide Excision Repair Pathway (NER). NER repairs bulky helix-distorting DNA lesions including DNA adducts. It is separated into two subsets that channel into a common pathway: transcription-coupled NER (TC-NER) and global genomic NER (GG-NER). TC-NER uses the CSA and CSB proteins, associated with the transcription machinery, to quickly recognize damage in actively transcribed regions of the genome. In GG-NER, the XPC protein recognizes DNA lesions and recruits the repair complexes. The NER complexes then assemble in a step-wise fashion, resulting in the excision of an oligonucleotide containing the lesion, and re-polymerization and ligation of a new oligonucleotide. Mutations particular NER proteins result in the repair deficiency syndromes Xeroderma Pigmentosum, Cockayne Syndrome, and Trichothiodystrophy, which are sometimes characterized by neurodegenerative phenotypes. (McKinnon, 2009).
Figure 4: Mutation of the Csb gene. The CSB protein is a critical component of TC-NER, as it remodels chromatin and recruits NER complexes to sites of DNA damage. A) The mutational spectrum of CSB demonstrates that there are no mutational “hotspots” within this protein. B) Generation of Csb mutant alleles via introduction of a stop codon into exon 5. Inactivation of the CSB protein results in defective TC-NER, and is associated with the neurodegerative Cockayne Syndrome (Modified from van der Horst, van Steeg et al. 1997; Citterio, Rademakers et al. 1998).
Neural Stem Cell (NSC)  

**Differentiation**

Neural Progenitor  

Glial Progenitor  

Neurons  

Oligodendrocytes  

Astrocytes

**Figure 5: Neural stem cells and Neurogenesis.**

A) Neural stem cells (NSCs) are a population of multipotent precursor cells that self-renew and differentiate throughout development and in response to neural injury in the adult brain. They can generate all three cell lineages of the CNS: neurons, astrocytes and oligodendrocytes.

B) Neurogenesis, occurs within distinct regions of the mammalian brain, including the subgranular zone (SGZ) lining the dentate gyrus of the hippocampus, and the subventricular zone (SVZ) surrounding the lateral ventricle.

(Modified from Teixeira et al., 2007).
Figure 6: Hypothesis and evaluative approaches. Our hypothesis is that DNA damage adversely impacts or impairs neural stem cell (NSC) fates, and that this is exacerbated in the absence of DNA repair. The hypothesis will be evaluated by investigating the impact that DNA damage has on apoptosis, self-renewal and differentiation.
Figure 1: Basal reduction in NPC self-renewal with defective NER. A) Neurosphere colony. B) Measurement of Csb<sup>m/m</sup> and wild type neurosphere size indicated no significant difference in proliferation (mean +/- S.D.). C) The number of secondary neurospheres resulting NPCs derived from primary neurospheres as a measure of basal self-renewal in the absence of DNA damage. When normalized to wild-type controls, Csb<sup>m/m</sup> NPCs generated ~20% fewer spheres (mean +/- S.D.; p < 0.05). D) Xpa<sup>-/-</sup> sphere-forming capacity after a single passage in the absence of DNA damage was ~20% lower than wild type controls (mean +/- S.D.; p < 0.005).
Figure 2: Reduced self-renewal capacity of NER-/- NPCs after UV-induced DNA damage. Csb<sup>m/m</sup> (A) and Xpa<sup>-/-</sup> (B) NPCs were exposed to increasing doses of UV light, and mean neurosphere number was measured 7-days post treatment. A) NPCs demonstrated a progressive reduction in neurosphere formation with increasing UV dose, and fewer Csb<sup>m/m</sup> neurospheres were generated at all doses (mean +/- S.D.; p<0.0001). B) Xpa<sup>-/-</sup> demonstrated reduced self-renewal with increasing UV dose (mean +/- S.D.; p<0.0001).
Figure 3: Acute death after UV-treatment is only a minor contributor to reduced clonogenic survival. Cell death and colony formation after UV are plotted together graphically, each on their own y-axis and in logarithmic scale, to compare the contributions of each to the observed reduction in self-renewal in Fig. 2.
Figure 4: Progressive reduction in NPC self-renewal is exacerbated in Csb\textsuperscript{m/m} with in vitro and in vivo aging. A) Csb\textsuperscript{m/m} NPCs generated progressively fewer neurospheres with passaging than their littermate controls, culminating in a 40% difference in neurospheres formed at the final passage (mean +/- S.D.; p<0.0001). B) Results of a neurosphere-forming assay using NPCs harvested from 8 week old Csb\textsuperscript{m/m} and wild-type mice. Csb\textsuperscript{m/m} NPCs generated a statistically significant 9% fewer neurospheres than WT controls mean +/- S.D.; p<0.05).
Figure 5: In vivo NPC abundance is unaffected by NER. The number of BrdU+ cells within the NSC-rich neocortex of Csb<sup>m/m</sup> and WT embryos were counted within coronal sections at three relative brain depths. A) Representative coronal section stained for BrdU. B) No significant difference in the number of BrdU+ cells within the neocortex of Csb<sup>m/m</sup> embryos as compared to wild type littermate controls was observed regardless of relative brain depth (mean +/- S.D.).
Figure 6: UV-induced DNA damage causes modest increases in Csb\(^{+/+}\) NPC neuronal differentiation, but reduced neuritogenesis and impaired terminal differentiation in Csb\(^{m/m}\) NPCs. A) Tuj1 labeling of NPC-derived neurons from Csb\(^{+/+}\) and Csb\(^{m/m}\) embryos demonstrating a progressive reduction in the number and arborization of neurites with increasing UV dose. Csb\(^{m/m}\) NPC-derived neurons were particularly sensitive, with reduced neuritogenesis observed by 20J/m\(^2\) and its complete abolishment at 50J/m\(^2\). B) The percentage of Tuj1+ cells was assessed in response to UV DNA damage. Csb\(^{m/m}\) neurogenesis remained consistent under all doses of UV. Two-way ANOVA analysis revealed that Csb\(^{+/+}\) NPCs generated more Tuj1+ neurons than Csb\(^{m/m}\) NPCs, particularly in the lower UV dose range (mean +/- S.D.; p<0.05).
Supplemental Figure 1: Increased neurosphere formation at 5% oxygen compared to 20% oxygen culture. Tissue culture incubator oxygen concentrations were held at 5%. Both Csb^{m/m} and Csb^{+/+} NPCs generated significantly more neurospheres under 5% oxygen than 20% oxygen, (mean +/- S.D.; p<0.005).
Supplemental Figure 2: Dose-dependent increase in 6-4 photoproducts after UV exposure. Hek293 cells were cultured, exposed to varying doses of UV light (0-20 J/m²), and harvested either immediately or 30min post-exposure. Genomic DNA was isolated using the QIAamp Blood Kit (QIAGEN) according to the manufacturer’s instructions. DNA solutions were then processed in 0.003% protamine sulfate-coated 96-well plates (Sigma) and incubated with anti-(6-4) photoproducts monoclonal antibody (CosmoBio) according to the manufacturers ELISA protocol. The absorbance at 650nm of each well in a spectrophotometer provided an indirect measure of (6-4) photoproduct abundance. As expected, with increasing UV dose, there was a corresponding increase in DNA lesions. Additionally, significantly fewer UV-lesions were present 30min post-treatment, as cells had time for some DNA repair to occur (mean +/- S.D.; p<0.05).
Supplemental Figure 3: Increased death of Csb\textsuperscript{mm} NPCs after exogenous DNA damage. A) Csb\textsuperscript{mm} and Csb\textsuperscript{+/+} NPCs were exposed to increasing doses of UV light and cultured for 24 hours. The vital stain Trypan Blue was then used to count living cells and generate a survival ratio. As expected, Csb\textsuperscript{mm} NPCs demonstrated reduced cell survival relative to littermate controls at all UV doses (mean +/- S.D.; p<0.0005). B) The CytoTox-Glo luciferase reporter assay (Promega) uses proteases released from dying cells to cleave and activate luciferase, providing an indirect measurement of cell viability. A dose dependent decrease in survival was observed with increasing UV dose, with the Csb\textsuperscript{mm} NPCs demonstrating increased sensitivity compared to littermate controls (mean +/- S.D.; p<0.0001).
**Supplemental Figure 4: WT NPCs undergo reduced self-renewal with chronic oxidative stress conditions.** In order to use chronic oxidative stress to introduce endogenous DNA damage, we attempted to utilize the glucose/glucose oxidase system according to a published protocol (Limoli and Giedzinski 2003). Glucose oxidase (GO) catalyzes the oxidation of glucose to gluconolactone but generates hydrogen peroxide as a by-product. Neurosphere formation after seven days of treatment was evaluated, and a significant dose-dependent reduction in self-renewal was observed (mean +/- S.D.; p<0.0001). However, this method proved unamenable to NSC culture conditions, and so was discontinued.
Supplemental Figure 5: No significant differences in the number of BrdU+ cells within the neocortices of Xpa^{+/+} and Xpa^{-/-} embryos. Pregnant dams were injected with the nucleoside analogue BrdU for 1 hour, sacrificed and their mixed litters were fixed in 4% PFA. Since, BrdU is commonly used as a marker for proliferating cells, coronal sections of Xpa^{+/+} and Xpa^{-/-} embryo brains underwent histology to label BrdU+ cells. The number of BrdU+ cells per area within the NSC-rich neocortices were counted and compared between genotypes at three relative brain depths, with no significant differences observed (mean +/- S.D.). These negative results were consistent with those for the related Csb^{-/-} model (Fig. 5).
Supplemental Figure 6: No significant difference in \( \text{Csb}^{+/+} \) and \( \text{Csb}^{m/m} \) \emph{in vivo} TUNEL labeling in neurogenic regions of embryonic brains. \emph{In vivo} TUNEL assay (Roche) was used to evaluate relative cell death in the brains of \( \text{Csb}^{+/+} \) and \( \text{Csb}^{m/m} \) NPCs. Coronal sections of E14 embryo brains were processed for TUNEL staining according to the manufacturer’s instructions. Immunofluorescent microscopy of the embryonic GEs reveals no significant difference in cell death within this NSC-rich region. Non-specific staining is due to blood vessels.
Supplemental Figure 7: NSC adherence to coverslips prior to neural differentiation induction was unaffected by genotype and UV dose. In order to evaluate the genotypic and dose-dependent impacts of DNA damage on differentiation, Csb^{m/m} and Csb^{+/+} NPCs were seeded onto coated glass coverslips, exposed to varying doses of UV light, and induced to differentiate. We compared the total number of adherent cells, which was quantified by nuclear DAPI staining. There was no significant variation in the total cell number with either genotype or dose (mean +/- S.D.).
Supplemental Figure 8: No significant difference in neuronal differentiation of Csb\(^{+/+}\) and Csb\(^{m/m}\) NPCs as measured by flow cytometry. We attempted to optimize a flow cytometry technique for evaluating neuronal differentiation. Csb\(^{+/+}\) and Csb\(^{m/m}\) NPCs were seeded onto thinly Matrigel-coated 10cm\(^2\) dishes (BD) at 1x10\(^5\) cells/mL in differentiation media (see 2.5.1 for composition). At 14 days post-seeding, cells were harvested using 0.05% trypsin (Gibco) and fixed in 4% PFA (Sigma) in PBS. They were then incubated with 1:1000 mouse-anti-Tuj1 antibody (StemCell Technologies) for 3 hours, followed by 30 min incubation with 1:500 goat anti-mouse Alexa488 antibody (Invitrogen). Gated Tuj1+ events were used to generate a percentage of total events, with preliminary experiments suggested no significant difference in the number of neurons generated by NPCs of either genotype (mean +/- S.D.). This technique was abandoned due to the lack of clear and consistent Tuj1+/Fit-C fluorescent peaks and the requirement for further optimization.
CHAPTER 6: REFERENCES


