An interplay between nonsense-mediated decay and DNA damage response pathways

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Summary

Nonsense-mediated decay (NMD) is an RNA processing pathway that helps maintain the quality of gene expression. It is best known as a surveillance system that detects aberrant mRNA molecules that result from mutations leading to premature stop codons. This pathway mainly operates in the cytoplasm and is linked to translation. Through cycles of phosphorylation and dephosphorylation of pathway proteins, mainly UPF1, a protein complex is assembled, which eventually degrades aberrant mRNAs. Thus, NMD prevents accumulation of truncated proteins.

In the Ciosk lab, a forward genetic screen was performed to discover novel components of the NMD pathway using *C. elegans* as a model organism. In addition to known NMD factors such as *smg* proteins, the screen uncovered *clk-2* as a novel NMD pathway component. *clk-2* is an orthologue of human TELO2 (telomere maintenance 2) with functions in DNA damage response. Interestingly, the Gartner lab reported *smg-1* (an NMD component) mutants to be hypersensitive to ionizing radiation (IR), which causes DNA double strand breaks. Therefore, there seems to be an interplay between the NMD and DNA damage response (DDR) pathways. DDR is a complex signaling network that mediates DNA repair while arresting the cell cycle. If the damage is extensive, DDR triggers apoptosis. Within this network, there are multiple overlapping pathways for repairing DNA double strand breaks. The two major pathways are homologous recombination (HR) and non-homologous end joining (NHEJ).

In this work, we aim to delineate the interplay between the components of the NMD pathway and the DDR network. We did this by first assessing whether NMD mutants other than *smg-1* and *clk-2* are hypersensitive to IR. We further investigated whether the components of any specific pathway in the DDR network interact genetically with the NMD pathway. Using specific reporters to quantify DNA repair, we checked which pathway is affected upon knockdown of NMD components. We discovered that *smg-2* mutants are also hypersensitive to IR, suggesting further involvement of NMD in DDR. The knockdown of NHEJ repair pathway proteins aggravated the phenotypes of *smg-1* and *smg-2* mutants, as opposed to HR pathway knockdowns which had no effect on phenotype. Additionally, we found that animals lacking *smg-1* or *smg-2* used homology dependent repair more, suggesting a decrease in NHEJ activity. In conclusion, considering the results of all the experiments, the NHEJ pathway is most likely linked with NMD.

Abbreviations

3’ UTR	3 prime untranslated region
5’ UTR	5 prime untranslated region
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
BER	base excision repair
BRC-1	breast cancer type 1
C. elegans	*caenorhabditis elegans*
cDNA	complementary DNA
CEP-1	*C. elegans* p53
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
CRISPR	clustered regularly interspaced short palindromic repeats
DDR	DNA damage response
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
DSB	double-strand break
dsDNA	double stranded DNA
EJC	exon junction complex
eRF	eukaryotic release factor

GFP	green fluorescent protein
GTP	guanosine-5'-triphosphate
HR	homologous recombination
HSP	heat-shock protein
IR	ionizing radiation
MMEJ	microhomology-mediated end joining
MMR	mismatch repair
MRN	MRE11-RAD50-NBS1
mRNA	messenger RNA
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NMD	nonsense-mediated decay
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinases
PTC	premature termination codon
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
SD	standard deviation
SDSA	synthesis-dependent strand annealing
SMG	suppressor with morphogenetic effect on genitalia

SSA	single strand annealing
SSB	single strand break
ssDNA	single stranded DNA
UPF	up-frameshift
UV	ultraviolet
WT	wild type
XRCC4	X-ray repair cross-complementing protein 4

1 Introduction

1.1 Nonsense-mediated mRNA decay (NMD)

1.1.1 Function of the NMD pathway

Each cell has an abundance of mRNA molecules at its disposal, not all of which are correctly transcribed, or even needed. Nonsense-mediated decay is one of the best-characterized RNA regulation pathways in the cell. If the translation of an mRNA molecule has been cut short by a premature stop codon, the NMD pathway will target and degrade it. In doing so, it prevents the overdue accumulation of unnecessary RNA molecules, or the synthesis of dysfunctional truncated proteins (Lykke-Andersen and Jensen, 2015). It also controls gene expression by degrading mRNA molecules with functional ORFs, through involvement with DNA rearrangements or pre-mRNA processing. NMD acts as a buffer that allows the cells better and tighter control over the expression levels of mRNAs (Hug et al., 2016).

1.1.2 Mechanism

When mRNAs are spliced, exons remain and exon-junction complexes (EJC) are deposited. As the name implies, EJC is a protein complex at the junction of two exons that have been fused together after the introns are excised. This complex surveils mRNAs and affects translation. During translation, the ribosome removes the EJCs as it moves forward. If the ribosome encounters a premature termination codon (PTC), it will stop at the PTC before it can remove any EJC downstream of the PTC. When it is time for translation to end, all EJCs should have been removed. If they are not, that means PTCs are present, and the translation complex and EJC will interact to start NMD. For a PTC to be registered as such by the NMD pathway, it needs to be a minimum of 50 nucleotides upstream of the last EJC, or the mRNA needs to have a long 3′ UTR (Lykke-Andersen and Jensen, 2015).

There are seven essential genes to this process that were identified in the nematode *Caenorhabditis elegans*, named *smg-1–7* (**S**uppressor with **M**orphological effect on **G**enitalia). They have orthologues in most mammalian species. It has been recently suggested that neither the presence of introns, nor the presence of EJC complexes are necessarily needed to set off the NMD pathway in nematodes (Longman et al., 2007).

UPF1 (SMG-2 in *C. elegans*) is the main effector and the central protein of the pathway. It is an ATP-dependent RNA helicase and all other SMG proteins are used to phosphorylate or de-phosphorylate UPF1/SMG-2 at some point in the pathway. These phosphorylation cycles are essential for NMD (Muir et al., 2018).

Translation normally ends when eukaryotic release factors 1 and 2 (eRF1, eRF3) along with the ribosome recognize a stop codon. eRF3 is a GTPase with a bound GTP that gets hydrolyzed. eRF3 and the newly-made polypeptide are released and the ribosome is dismantled (Dever and Green, 2012) (**Figure 1.1A**). If UPF1 interacts with eRF3, it will start the NMD process. UPF1 can bind to the mRNA anywhere along the sequence, except for the 3' end. Normally, since translation ends at the termination codon, which is generally located towards the 3' end of the mRNA, the spatial proximity to the poly(A) tail only allows UPF1 to bind to the mRNA transiently (Lykke-Andersen and Jensen, 2015). In this case, UPF1 does not interact with eRF3, and translation ends easily (Ivanov et al., 2008). In the event of a PTC, UPF1 can bind to the mRNA since there is a larger distance between the 3' end and the ribosome machinery. UPF1 then interacts with eRF3. The UPF1-eRF3 interaction thus starts the NMD process. UPF1-eRF3, with the help of the EJC at the untranslated 3′ end, recruit UPF2 and UPF3. UPF2 engages with the N- terminal domain of UPF1, thus freeing the central core domain for further reactions. Furthermore, UPF2 has distinct domains that link UPF1 and UPF3 (Gehring et al., 2005) (**Figure 1.1B**). At this stage, SMG1, and its regulators, SMG8, SMG9 are recruited to the complex (Yamashita et al., 2009). SMG1 phosphorylates UPF1, which allows for the recruitment of SMG6 (Kashima et al., 2006). The phosphorylation of UPF1 stops the translation process entirely (Isken et al., 2008). SMG6 has endonucleolytic activity that starts the mRNA degradation process. A SMG5-SMG7 dimer assists with deadenylation and also dephosphorylates UPF1, along with SMG6. A decapping complex decaps the mRNA. Finally, 5′-3′ and 3′-5′ exonucleolytic reactions degrade the entire mRNA and the process ends (Chang et al., 2007) (**Figure 1.1C**).

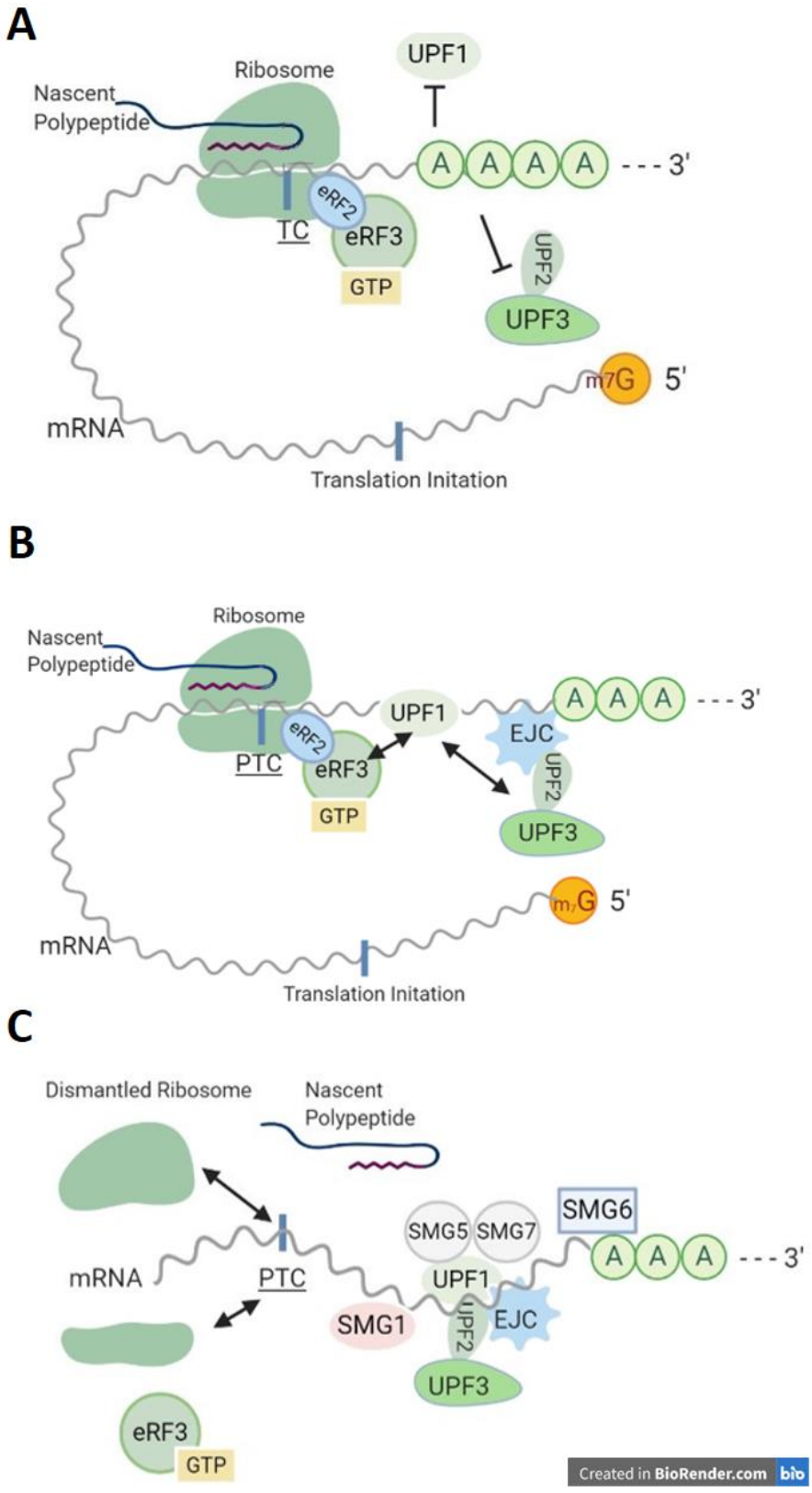

Figure 1.1. The components of the NMD pathway. A: If PTCs were not detected, translation would end normally. UPF1 cannot bind to the mRNA or interact with eRF3 while in physical proximity to the poly(A) tail. **B**: The activation of the NMD pathway after a PTC is detected. UPF1 binds to the mRNA and interacts with eRF3, and UPF2 and UPF3 are recruited by the EJC. **C**: All components of NMD gather and the translation

machinery is disbanded. (PTC: premature termination codon, TC: termination codon) [Figure adapted from: (Lykke-Andersen and Jensen, 2015)]

1.1.3 Project background

In the Ciosk lab, a forward genetic screen was performed to discover novel components of the NMD pathway using *C. elegans* as a model organism. In addition to the known NMD factors, the screen uncovered *clk-2*. *clk-2* is an ortholog of human TELO2 (telomere maintenance 2) with functions in DNA damage response (Ahmed et al., 2001). So far, there are no studies reporting *clk-2* as a component of the NMD pathway. Its function in NMD might be conserved. Interestingly, the Gartner lab reported *smg-1* (an NMD component) mutants to be hypersensitive to ionizing radiation (IR), which causes DNA double-strand breaks (González-Huici et al., 2017). Therefore, there seems to be an interplay between the NMD and DNA damage response (DDR) pathways. Since the two pathways are connected, we investigated how the NMD components interact with the DDR network.

1.2 DNA damage

The purpose of this thesis is to find possible connections between two cellular processes; the first one is nonsense-mediated decay and the second one is the DNA damage response network. The following chapter is an overview of different types of DNA damage and their response pathways.

1.2.1 Genetic integrity

Genetic information is stored in cells as chromatin. Double-stranded DNA is packed tightly around histone octamers and forms units called nucleosomes (Tremethick, 2007). Chromatin consists of multiple nucleosomes connected by DNA. Almost every cell in a living organism comes equipped with a copy of this code. Through the expression of genes, the cell manages to express mRNA, and subsequently, proteins (Alberts et al., 2002). Considering the ongoing urgent need for a cell to have DNA as a functional blueprint at all stages of its life, preserving the integrity of the genomic code is crucial to the function and well-being of any living cell.

DNA is known to be remarkably stable, despite its structural complexity. It is however still liable to spontaneous changes that could become mutations even under normal cell conditions, given the chance (Raff et al., 2002).

1.2.2 Sources of DNA damage

DNA damage is defined as the presence of an abnormal chemical structure in the DNA. It is distinct from mutation, which is only a change in the sequence of base pairs. Many kinds of DNA damages eventually become mutations, if left untreated (Köhler et al., 2016).

In cells, the source of DNA damage can be either endogenous or exogenous. Endogenous sources include endogenous cellular metabolites, or spontaneous cellular reactions. Furthermore, the genome is constantly being tested by imperfect replication, and mutagenic cellular processions, like transposition. Exogenous agents vary greatly and include anything from heat to different sources of radiation, and alkylating agents (Kaina and Fritz, 2006).

If left unrepaired, damaged DNA can accumulate in dividing cells, and lead to symptoms like aging. The most common causes of each type of damage are as follows:

1. **Endogenous damage.** This is mostly caused by reactive oxygen species (ROS), or incorrect replication (ROS are chemical byproducts of the normal metabolism of oxygen, for example, peroxides)

2. **Exogenous damage.** This can be caused by many things, chief among which are:

    - Ionizing radiation (UV, X-ray, Gamma)
    - Hydrolysis
    - Certain plant toxins
    - Viruses

1.2.2.1 Ionizing radiation (IR)

When atoms travel in the form of either electromagnetic waves or particles, they carry energy and can, therefore, knock off electrons from atoms they encounter, thus ionizing them (Humans et al., 2000). All living creatures are exposed to low doses of naturally produced IR. It is widely used in cancer radiation therapy and other procedures such as medical X-rays. (Hoeijmakers, 2001; Kastan and Bartek, 2004). IR can damage the DNA either by directly depositing energy, or indirectly, by producing hydroxyl radicals that attack the DNA (Mavragani et al., 2019). IR creates multiple types of damage, but mainly single strand breaks (SSB). If two SSBs occur on two complementing strands of DNA on the same spot, they will lead to a DSB (Milligan et al., 1995).

1.2.3 Types of DNA damage

DNA can be damaged in a myriad of different and sometimes naturally occurring ways, each with consequences of its own. To give an example, hydrolysis can lead to depurination and depyrimidination, or affect the bases themselves (**Table 1.1**). The common result of such errors is the alteration of genetic information. Nearly all of the DNA damage affected by bioactive molecules (drugs, toxins, etc.) can be categorized into two sections: 1. Alkylation or 2. Reaction of DNA with a radical (Gates, 2009).

Alkylating agents have the potential to react with DNA at multiple sites, considering that practically all the residues in the double helix can be alkylated (Mishina et al., 2006). In some

cases, it can lead to the DNA being bound by carcinogenic chemicals to form DNA adducts (La and Swenberg, 1996).

Table 1.1. The four main types of DNA lesions, and a list of the processes that can lead up to them. Many of the chemical processes that damage the DNA eventually result in the same types of mutations. These lesions either affect one or two bases, or break the DNA strands. They could also produce links between various DNA components. [Table adapted from: (Litwack, 2017)]

No.	Types of DNA Lesions	Examples
1)	Single Base Alteration	a) Depurination b) Deamination of adenine to hypoxanthine c) Alkylation of base d) Insertion or deletion of nucleotide e) Base-analog incorporation
2)	Two-Base Alteration	a) UV induced pyrimidine dimer b) Bi-functional Alkylating agent cross-linkage
3)	Single and Double-strand Breaks	a) Ionizing Radiation b) Radioactive disintegration of backbone c) Oxidative free radical formation
4)	Cross-Linkage	a) Between bases in same or opposite strands b) Between DNA and protein molecules

1.2.3.1 Double-strand breaks (DSB)

DNA double-strand breaks are perhaps the most dangerous type of lesion that can occur in a cell because they can cause genomic rearrangements such as deletions and fusions. A single strand break can be fixed easily, using the sister chromatid as a template, but not a DSB. Therefore, they must be repaired to ensure the continuity of normal cell functions, replication, and segregation of chromosomes. DSBs occur much less frequently than most other types of damage, and come to pass when the phosphate backbone of both strands of DNA are broken in the same spot (Mehta and Haber, 2014). They are specifically important, since even one DSB can be enough to lead to cell death (Mahaney et al., 2009). Well-known sources of DSBs include ionizing radiation, therapeutic anti-cancer drugs (used for chemotherapy), stalled replication forks, and topoisomerase poisons.

1.2.4 DNA damage response (DDR)

In the event of DNA damage, eukaryotic cells activate a network of complex biochemical signals collectively termed as DNA damage response. This network mediates several processes, of which the most prominent are: activation of repair mechanisms, cell cycle arrest, and in most multicellular organisms, if the damage is considerable, the induction of apoptosis (Kastan and Bartek, 2004). Together these pathways guarantee either the prompt repair of the created lesions or the eradication of the cell in more crucial cases (Stergiou and Hengartner, 2004). The proteins that are involved and upregulated in these cascades each have particular functions that are used to categorize them into several groups. Sensors are the initial responders that detect the damage, transducers transmit the signal, and effectors are those that enact the actual responses (Zhou and Elledge, 2000).

In mammalian cells, DDR is activated by the phosphorylation of 3 members of the phosphoinositide 3-kinase-like (PI3K-like) kinases family, ATM (ataxia telangiectasia mutated), ATR (ATM and Rad related), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit). ATR and ATM are the two major activators in DDR, with ATM being active in DSBs and ATR in SSBs. They recruit DNA repair proteins to the site of the damage. They also activate Chk1 and Chk2, which along with p53, are responsible for stopping the cell cycle temporarily (**Figure 1.2**) (Bartek and Lukas, 2003; Reinhardt and Yaffe, 2009; Yoshiyama et al., 2013).

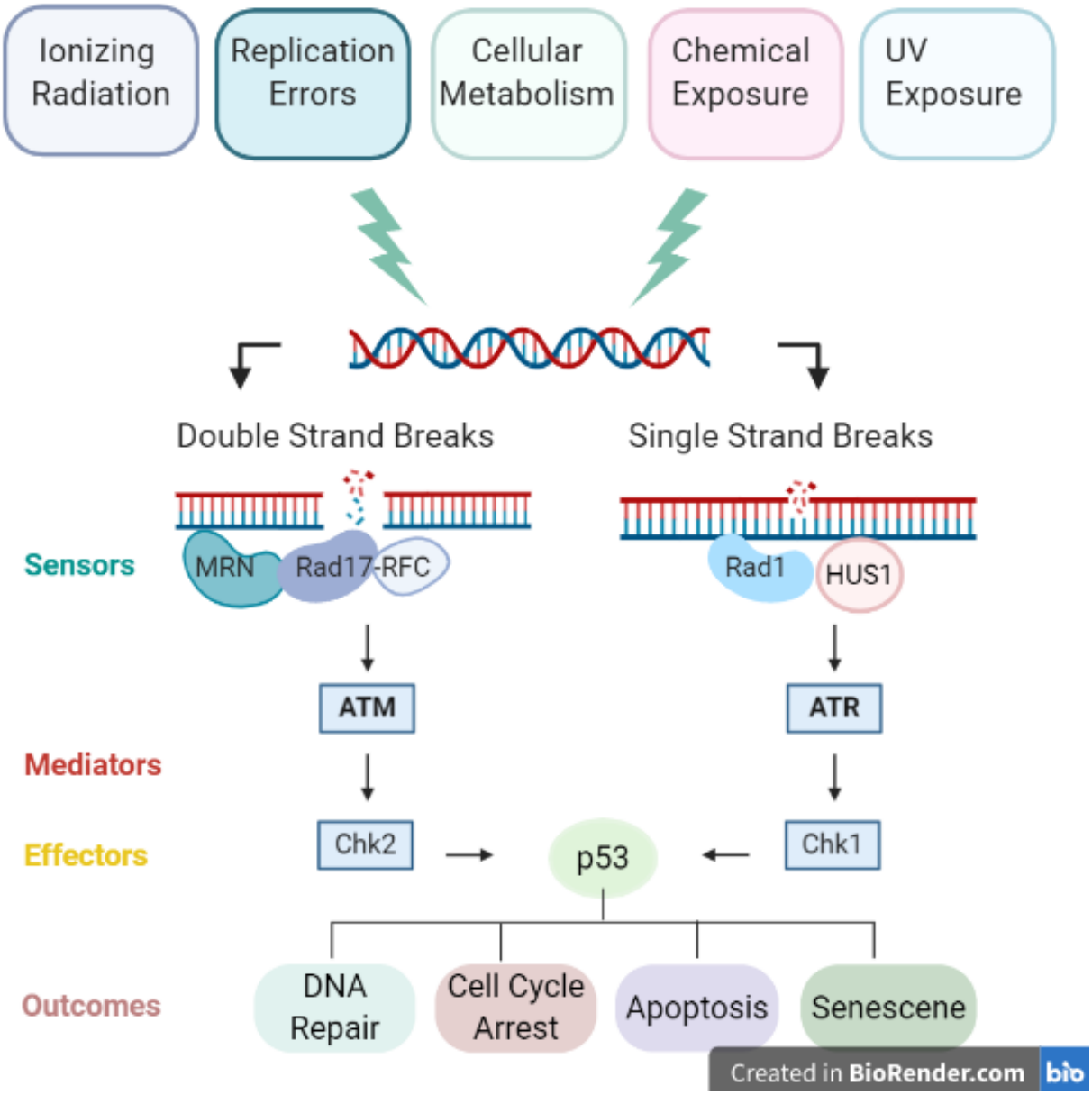

Figure 1.2. DNA damage response pathways, depending on the type of damage. Specialized sensors can each detect a different type of damage, and based on which one is activated, different cascades are put in motion. Eventually, the effector protein p53 is activated, which leads to different outcomes based on the pathway.

1.3 DNA repair

As many as one million DNA lesions can occur in each cell on a daily basis (Alberts et al., 2007). For a cell to endure such damage in one of its most crucial components, repair mechanisms need to be extremely rigorous. Indeed, the cells have many pathways and components with which to efficiently repair DNA damage.

Recent studies on human stem cells indicate that the accumulation rate of mutations is roughly 40 per year, in various tissues (Blokzijl et al., 2016). In *C. elegans*, it is possible to directly measure the amount of mutations in the germline. Since the worms have short life spans, a considerably large swath of DNA was observed in different generations, and it was determined that the mutation rate is around one mutation per $l0^9$ nucleotides every time the DNA replicates. This number is similar in humans, worms, and even *E. coli* (Alberts et al., 2007).

A variety of strategies are available to the cell, to recover the lost genetic information and restore the cell to normal function. Based on the type of lesion, different enzymes will be used. In broad terms, the cell has three options: 1. Direct reversal, 2. Elimination of the damaged bases, followed by synthesizing new DNA by either using the complementary strand or sister chromatid as a template, or doing so without a template, and 3. Trans-lesion synthesis.

1.3.1 Direct reversal

Direct reversal is a very simple yet efficient method of restoring the damaged DNA. As the name implies, the process involves the chemical reversal of the damage. It does not need a template, since each type of damage can only occur in one of the four nucleotides, and this reduces the errors that can occur greatly. Only three types of damage can be treated using this method: The formation of pyrimidine dimers after exposure to UV light, methylation of guanine bases, and certain types of adenine and cytosine methylation (Yi and He, 2013).

1.3.2 Repair of single strand damage

1.3.2.1 Base excision repair (BER) and nucleotide excision repair (NER)

Base excision repair is the pathway that corrects small lesions resulting from deamination, oxidation, or alkylation. A DNA glycosylase enzyme that recognizes and eliminates a faulty base initiates it. DNA polymerase then removes the damaged piece and rewrites the sequence. DNA ligase seals the nicks. Nucleotide excision repair works similarly, except the way the excision is made is different, and the damaged sites are bulkier. A complex of enzymes searches the DNA structure for irregularities, and once found, the two strands are separated by DNA glycosylase and the damaged section is cut away by AP endonucleases (de Laat et al., 1999; Krokan and Bjørås, 2013).

1.3.2.2 Mismatch repair (MMR)

If an error in the replication process somehow evades the proofreading that ensues afterward, it will be detected in this highly conserved pathway. At least two proteins are involved, one recognizes the mismatch, another recruits an endonuclease to cut away the error. DNA polymerase and ligase then correct it (Moriwaki et al., 2015).

1.3.3 Repair of double-strand breaks

There are multiple pathways for repairing DSBs, mainly: 1. Non-homologous end joining (NHEJ), 2. Homologous recombination (HR), and 3. Microhomology-mediated end joining (MMEJ).

1.3.3.1 Non-homologous end joining (NHEJ)

Out of all the pathways that treat DSBs, non-homologous end joining is the most extensively used, partly due to the fact that it can operate in all phases of the cell cycle. NHEJ does not need the DNA to be in a particular state because it just stitches the strands back together and religates them. This process is very efficient but naturally error-prone (Burma et al., 2006).

NHEJ is divided into four steps:

I. Recognition of the double-strand break and binding the NHEJ complex at the site of the break: The Ku heterodimer, consisting of Ku70 and Ku80 subunits, is recruited to the spot (**Figure 1.3**). The heterodimer can find the lesions exceedingly fast. In the case of laser-generated DSBs, the Ku proteins have been known to flock to the site within mere seconds, due to their affinity for DNA ends (Mari et al., 2006).

The Ku heterodimer then recruits other NHEJ factors, including DNA-PKcs and DNA Ligase IV (Costantini et al., 2007; Uematsu et al., 2007).

II. Bridging the broken DNA strands and stabilizing the ends: To protect the DNA ends from nucleases and non-specific processing, and preserve the position of the broken strands together, and hold them together. The XRCC4-XLF complex forms a filament-like structure over the lesion. The Ku complex, along with DNA-PKcs, and XRCC4-XLF gather and serve to protect the DNA (Hammel et al., 2010).

III. DNA end processing: This is done to create strand endings that can be ligated, which may not be necessary in every lesion. Depending on the kind of lesion that is formed, different enzymes, including Artemis and PNKP could be recruited by the Ku-XRCC4 complex to create suitable ends **(Figure 1.3)**. If the ends contain 5′ hydroxyl groups or 3′ phosphates, they are non-ligatable. PNKP can remove the 3′ phosphate groups and add phosphates to the 5′ OH ends (Bernstein et al., 2005). Artemis is another enzyme that has 5′ endonuclease ability. Different ends each require suitable enzymes (Ma et al., 2002).

IV. Ligation and disbandment of the NHEJ complex: As mentioned previously, DNA ligase IV is responsible for NHEJ end joining. XRCC4 and XLF both promote the ligases activities and make it more stable, so that it can even ligate ends that do not match (Grawunder et al., 1997; Gu et al., 2007; Lu et al., 2007).

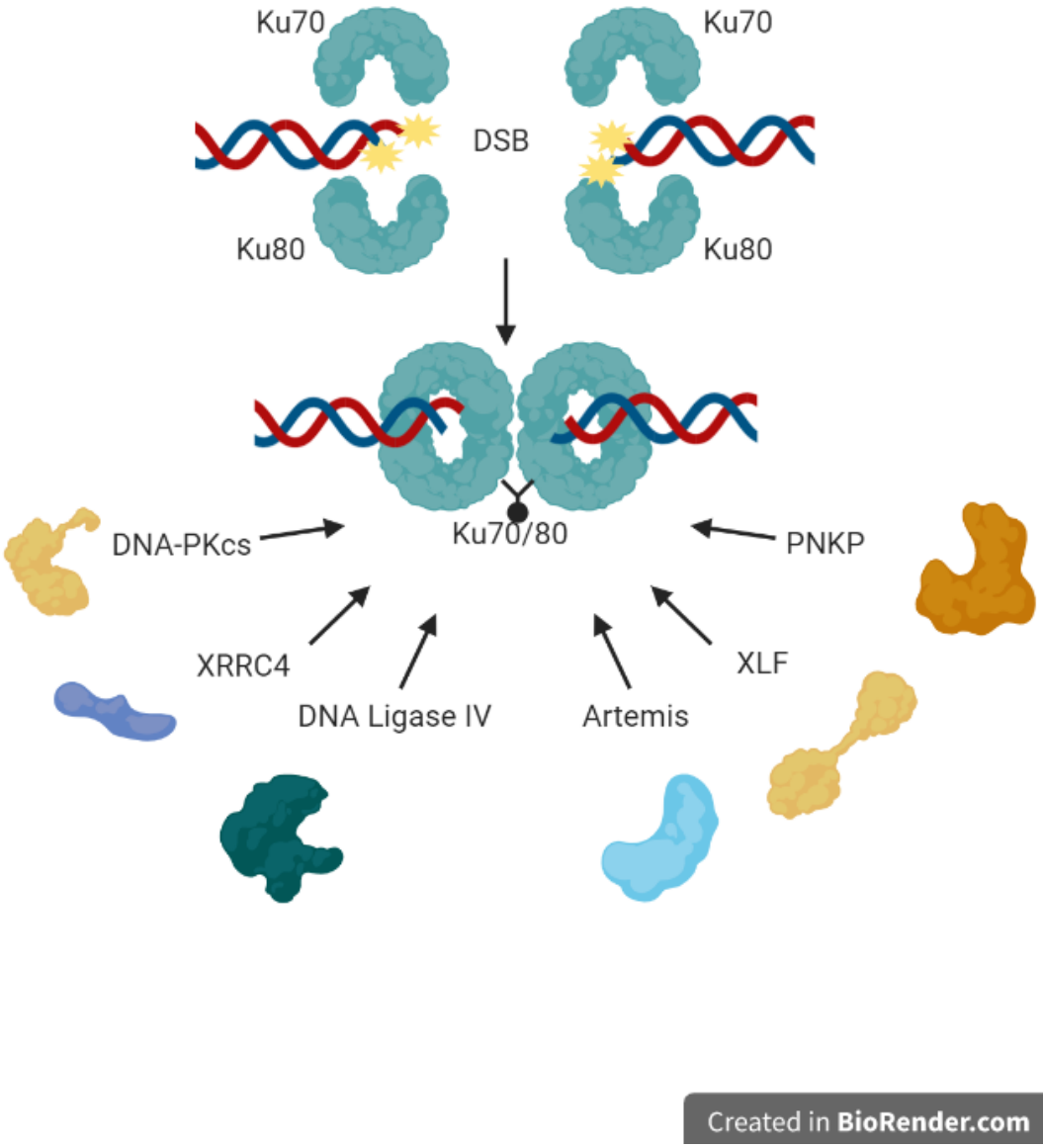

Figure 1.3. Ku70/80 heterodimer recognizes the ends of the broken strands due to high affinity, and then recruits the other factors to the DSB site for NHEJ. Enzymes, such as Artemis and PNKP, process the ends for ligation with DNA ligase IV, and XRRC4 and XLF stabilize the site and avoid non-specific processing by nucleases. [Figure adapted from: (Davis and Chen, 2013)]

1.3.3.2 Alternative end joining:

There are alternatives to what is considered the classical NHEJ (C-NHEJ), often called by the name alternate NHEJ (alt-NHEJ). These pathways are more error-prone than C-NHEJ.

1.3.3.2.1 Microhomology-mediated end joining (MMEJ)

Microhomology-mediated end joining is one of the alt-NHEJ pathways. It requires the strands at the site of the DSB to have homology and be aligned together. The size of the homologous region needs to be 1-16 nucleotides long. As can be expected of a pathway with only one strand to copy from, it is highly mutagenic (Sfeir and Symington, 2015). The general process, based on research on mammals and yeast, is as follows:

1. The first step is end resection, in which the 5' ends of the broken strands are processed so the microhomologue (MH) regions can align. In *S. cerevisiae* the Mre11–Rad50–Xrs2 (MRX) complex and Sae2 do this (Cannavo and Cejka, 2014). Then, the Dna2 nuclease and Sgs2 helicase, and the Exo2 exonuclease create long single-strand DNA (ssDNA) (Symington and Gautier, 2011).

2. Annealing of the corresponding MHs. In this step, insertions occur to increase the homology of the region, which increases the mutagenicity of the process.

3. DNA polymerases fill in the gaps.

4. Ligation. In mammals, MMEJ occurs in the absence of Lig4, which is a main ligase of C-NHEJ.

1.3.4.2.2 Single strand annealing (SSA)

If a DSB occurs and repeated sequences that have some homology are found flanking the break site in the ssDNA, a pathway named single strand annealing could be activated. SSA repairs DSBs in somatic cells, and takes advantage of many of the same intermediates and genetic networks as MMEJ, and HR (**Figure 1.4**). One of its important characteristics that marks a distinction from MMEJ is that the homology alignment of the strands covers a vaster area, between 30-400 nucleotides (Sugawara et al., 2000).

The process is simple, the flanking repeats around the DSB are aligned, whatever was in between them is degraded, and then ligation occurs. RAD-52 and XPF-1 are crucial to recognizing the repeats and recruiting the endonucleases and ligation enzymes. XPF-1 is used in HR as well. Although, in HR it is used only in the germline of the worm and not in somatic cells (Bhargava et al., 2016).

1.3.4.3 Homologous recombination (HR)

Homologous Recombination is a high-fidelity repair pathway found in all higher life forms. Apart from treating DNA damage, it is further essential to meiosis and for genetic exchange crossovers. However, the focus of this thesis is on the repair aspect of it. In somatic cells, it is the least error-prone repair pathway to treat DSBs and it has been extensively studied in many organisms (Li and Heyer, 2008). In this section, the *C. elegans* HR pathway is specifically described. Before HR starts, the presence of a template with high homology is needed. Usually, a sister chromatid serves as the predominant template, however, using the homologous chromosome is also feasible (Pâques and Haber, 1999; Rong and Golic, 2003). The process starts with the topoisomerase-II-like enzyme SPO-11, which introduces breaks around the double-strand lesion. Then, the MRN complex (consisting of MRE-11, RAD-50, COM-1) and EXO-1 resect the DNA ends to provide single-stranded regions. RAD-51 attaches to the ssDNA with the help of BRC-1 and BRC-2 and creates nucleoprotein filaments (Rieckher et al., 2016). The next step is strand invasion. Strand invasion is the initial pairing between two recombinant DNA molecules which is mediated by a number of proteins, including the helicase HELQ-1. This leads to the formation of D-loops (**Figure 1.4**). After that, one of two things can happen. In the classical double-strand break repair, the other DNA duplex which has no ssDNA yet, also performs strand invasion, and the DNA molecules are connected in what is called a Holiday junction, in which the two DNA molecules are inter-joined. In the second pathway, called synthesis-dependent strand annealing (SDSA), only the invasion of the first strand occurs, after which the invading strand is displaced, annealed back to its original spot, the gap in the other strand is filled, and ligated. During this process, there is no crossover (Clancy, 2008; Helleday et al., 2007). After the new DNA is synthesized based on the homologous strand, it is marked by MSH-4 and MSH-5, to be processed by nucleases. This process is called resolution. The Holiday junction is then believed to create either crossover (CO) or non-crossover (NCO) products, based on which points the cleavage occurs in.

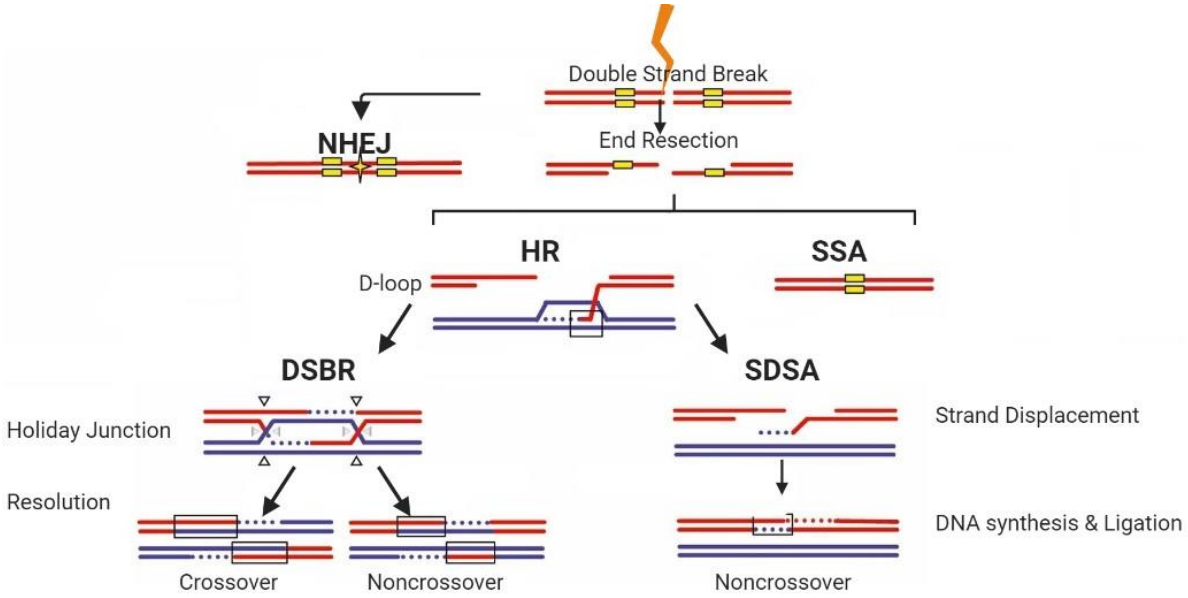

Figure 1.4. A depiction of some of the repair possibilities stemming from double-strand breaks. Based on the cell cycle, the sequence of the lesion site, and other factors, such as the presence of some proteins, the cell will activate one of multiple repair pathways. The repair mechanisms either occur through homologous templates or without it. Homologous repair has several branches, such as single-strand annealing, synthesis-dependent strand annealing, and double-strand break repair (the classically known HR pathway). [Figure adapted from: (Ertl et al., 2017)]

1.4 *Caenorhabditis elegans*

1.4.1 *C. elegans* as a model organism

Caenorhabditis elegans is a species of free-living nematode that is transparent, and can be found in soil (**Figure 1.5**). It was introduced by Sydney Brenner in 1963 as a model organism with a great potential for molecular biology research. The average size is about 1 mm, and it takes them 4 days to reach adulthood at 20 ºC. They can live up to 2-3 weeks in normal laboratory conditions. They are in many ways a great model organism for biological studies. They are easy to maintain and breed and produce many offspring in each generation. Their life-cycle consists of an initial embryonic stage, 4 larval stages dubbed L1-4, and adulthood (**Figure 1.6**). The population consists mostly of hermaphrodites and very few males. *C. elegans* are transparent and have an invariable cell line. All adults (hermaphrodites) have 959 cells, and each one can be tracked from the moment the egg hatches (WB, 1988). Genome-wise, the worms have five pairs of autosomal chromosomes, along with two sex chromosomes in hermaphrodites (XX), and only one sex chromosome in males (XO) (Hodgkin, 2005-2018). *Caenorhabditis elegans* was the first multi-cellular organism to have its genome sequenced and has served as a great tool for genetic studies. The nematode is considered a very convenient tool for forward genetic screens. RNA interference studies on worms have proven very accessible, and it is now widely used in genomics research (Kamath et al., 2003).

Figure 1.5. *C. elegans* adult hermaphrodite, imaged with DIC microscopy. [Image from Utrecht University]

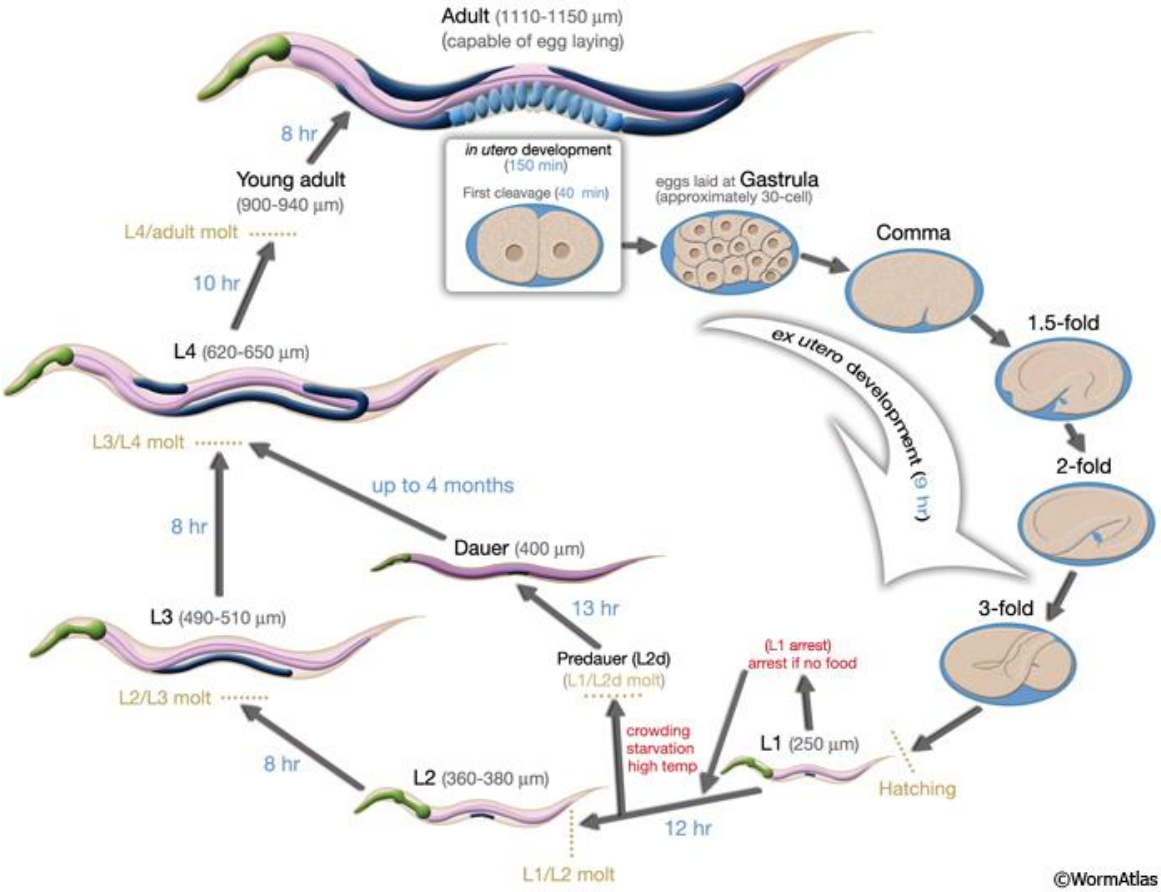

Figure 1.6. The life cycle of *C. elegans* at 22 ºC. It consists of an initial embryonic stage, 4 larval stages, and adulthood. This figure shows the life cycle of a hermaphrodite worm, and depicts the possibility of a dauer stage under stressful circumstances such as starvation as well. [Image taken from (http://www.wormatlas.org)]

1.4.2 *C. elegans* as a DNA repair model

Apoptosis and the genes that regulate it were discovered in *C. elegans*. DNA damage can activate both apoptosis and cell cycle arrest in the *C. elegans* germline. mRNA decay has been known to activate apoptosis. In worms, it is already known which cells will undergo programmed cell death during the development. This is because of their invariant cell line and is considered an advantage when studying DNA damage in an organism, since it will be easy to tell damage induced necrosis from apoptosis (Hengartner, 1999).

Most DNA repair processes are highly conserved between humans and *C. elegans* **(Table 1.2)**.

Table 1.2. DNA damage response proteins in *C. elegans* and their orthologues in mammals. [Table adapted from: (Stergiou and Hengartner, 2004)]

Protein function	***C. elegans***	**Mammals**
Sensors		
RFC1-like	HPR-17	RAD17
PCNA-like	HPR-9	RAD9
	HUS-1	HUS1
	MRT-2	RAD1
BRCT-containing	BRC-1	BRCA1
DSB recognition/repair		
	MRE-11	MRE11
	RAD-50	RAD50
		NBS1
Transducers		
PI3-kinases	ATM-1	ATM
	ATL-3	ATR
Rad3 regulatory subunit	1	1
Effector kinases	CHK-1	CHK1
	CHK-2	CHK2
Downstream effectors	CEP-1	p53

The pathways of DNA damage response are widely considered to be intertwined, with each component having roles in several responses. To investigate the steps and cellular signals involved in these events, *C. elegans* presents us with a multicellular organism that can be

studied to identify the mechanisms at work in more complex organisms (Stergiou and Hengartner, 2004). It is worth noting that DDR is highly conserved between *C. elegans* and humans (Boulton et al., 2002).

1.4.3 DNA damage repair in *C. elegans*

As a process highly conserved in many organisms, orthologues for almost all the DDR genes can be found in *C. elegans.* The process that they follow through is similar to those discovered in human cells, with some notable differences. During HR in *C. elegans,* the gene *rad-51,* encodes the protein RAD-51, which has the same name in human cells as well. After creating a filament with the ssDNA, RAD-51 is responsible for finding a homologous region for the ssDNA, and then helping the process of strand invasion, which makes it a crucial protein for the process (Alpi et al., 2003; Takanami et al., 2003). BRC-1 is another essential HR protein which has a conserved orthologue in mammals (Moynahan et al., 1999; Snouwaert et al., 1999). Non-homologous end joining (NHEJ) owes much of its function and accuracy to Ku80, however, recent studies show that MMEJ does not depend on Ku80 (Guirouilh-Barbat et al., 2004). In *C. elegans,* this protein is called CKU-80 encoded on gene *cku-80*. Exact MMEJ mechanics in *C. elegans* are still unknown (Sfeir and Symington, 2015). However, a number of polymerases are crucial to MMEJ specifically. *polq1* codes one such polymerase (Roerink et al., 2014).

1.5 Project objectives

The focus of this thesis is to confirm the connection between the DDR network and the NMD pathway and determine how they are connected. Although pathways that could repair SSBs may also be linked to NMD, they are not the focus of this work. In order to investigate the link between NMD and DSB induced DNA damage response we have the following objectives:

1. Confirming the hypersensitivity of *smg-1* mutants and checking the sensitivity of *smg-2* mutants to IR.

2. Investigating whether the components of the DDR network and NMD pathway interact genetically.

3. Quantifying the use of various repair pathways after *smg-1* and *smg-2* knockdown.

4. Quantifying the expression levels of several *smg* genes following IR-induced DNA damage.

2 Materials and Methods

The recipes for all the buffers and media mentioned in this section are available in the appendix. Some of the figures used throughout the thesis were made using the BioRender program.

2.1 Maintenance of *C. elegans*

The *C. elegans* were kept on agar plates with 2% Nematode Growth Media (NGM) which were seeded with the *E. coli* strain OP50, which has limited growth of NGM, using the standard procedure (Brenner, 1974). Unless specified otherwise, they were kept at 20 ºC.

2.1.1 Strains and constructs

N2 Bristol strain was used as the reference wild type. *smg-1(tm849)* and *smg-2(tm6028)* knockouts were generated by the National Bioresource Project, Tokyo, Japan, which is part of the International Gene Knockout Consortium. Both are deletions alleles. Reporter strains *XF0503* (HR/NHEJ reporter) and *XF0512* (SSA reporter) were kindly provided by the Marcel Tijsterman laboratory. The genotypes are mentioned in **Table 2.1**.

Table 2.1. The strain IDs of different worm strains in the Ciosk lab and their genotypes.

Strain ID in Ciosk lab	**Genotype**
N2	wild type (WT)
2145	*smg-1(tm849)*
2144	*smg-2(tm6028)*
2135	*elt-2::HR-reporter; hsp16-41::mCherry::I-SceI*
2136	*elt-2::SSA-reporter; hsp16-41::mCherry::I-SceI*

2.1.2 Synchronization

Several of the experiments in this thesis require the worms to be in the same life stage for accurate data collection. In order to do this, the worms are collected in tubes, and a "bleaching" solution containing sodium hypochlorite and potassium hydroxide is used to dissolve the adults, leaving only the eggs intact. After hatching, the worms remain arrested as larvae without food. Such synchronized larvae can be plated on seeded NGM 2% plates.

Procedure:

1. Use M9 buffer to "wash" adult worms, containing eggs, off, by tilting the plates and gathering the liquid. Transfer them to 15 ml falcon tubes.
2. Centrifuge at 1500g for 15 seconds to collect worms at the bottom of the tube.
3. Drain the liquid with a vacuum machine without draining any worms.
4. Add 5 ml bleaching solution, shake, leave on rocking platform machine for 7 minutes.
5. Centrifuge eggs at 1500g for one minute. Drain the bleaching solution.
6. Wash the eggs with M9 buffer three times.
7. Leave on rocking platform overnight, so hatched larvae get oxygen.
8. The next day, centrifuge tubes at 1500g for one minute and remove supernatant.
9. Add 300 µl M9 and plate worms.

2.2 *C. elegans* genetic methods

2.2.1 Worm lysis

To get access to the genomic DNA of *C. elegans,* the worms can be directly lysed, and used for further experiments as gDNA templates.

Procedure:

1. Put 2 adult worms inside an Eppendorf tube and add 10 µl of lysis buffer.

2. Run the lysis program on a PCR machine (**Table 2.2**).

Table 2.2. Format of the lysis program as input into the machine.

STEP	TEMP	TIME
1	60 °C	60 minutes
2	95 °C	15 minutes
3	4 °C	5 minutes

3. If the product is used for PCR afterward, 1 µl should be used as a template, since it will have less than 1 µg of DNA inside.

2.2.2 LacZ staining

A certain reporter construct was used to quantify single-strand annealing (SSA) in the event of DSB occurrence. This reporter would produce LacZ. In order to detect said LacZ, an X-Gal staining solution would be used (Fire, 1992; Zdinak et al., 1997).

Procedure:

1. X-Gal is added to an oxidation buffer (see buffers and media appendix) for a final concentration of 0.4 mg/ml.

2. L4 or young adult worms are placed inside Eppendorf tubes with BU buffer.

3. The tubes are dried in a SpeedyVac for 20 minutes, then -20 ºC acetone is added and removed after three minutes, and the worms are air-dried at room temperature.

4. Worms are placed on slides and staining solution is added to them. The slides are placed inside damp Petri dishes to avoid them drying out.

5. Slides are observed the next day under a bright-field microscope.

2.2.3 Generating males

To generate males among *C. elegans* populations, several methods exist. The first and most common one is through heat shock. Several plates of L4 worms were placed at 30 °C for an hour. In the next generation, the male occurrence rate increases greatly. Since some mutants do not react well to heat shock, another method was additionally attempted. *him-5* and *him-8* are genes that code proteins involved in sex determination and without them, almost 30% Of a hermaphrodite worm's eggs will be males (Hodgkin et al., 1979). Both were knocked down with RNAi and males were gathered from the next generation. After the first generation of males are generated, they must be maintained. 5 male worms along with two young adult hermaphrodites were placed on a small piece of seeded agar to maintain the male population.

2.2.4 Genetic crossover to create double mutants

After a strain has a steady population of males, they are crossed with another strain. Several young males were selected and placed on a small piece of seeded agar along with hermaphrodites of another strain. If the offspring are half males, that means the crossover was successful.

2.3 Characterization and phenotyping

2.3.1 Developmental assay

Worms were synchronized via bleaching solution and plated at L1 and observed to see how many hours it took them to reach different life stages.

2.4 Stress induction

2.4.1 IR assay

Ionizing radiation was used to treat worms at L1 and L4 stages. An Xstrahl X-ray tube (RS320 Research System Cabinet) was used in these settings: 300 kV, 10.0 mA, 1521 seconds. The final accumulated dose was 100 Gy. The worms were placed inside Eppendorf tubes filled with M9 solution and irradiated. They were then placed back on seeded plates until they grew to adults. When they were deemed to be at optimal egg-laying stage, three

worms were placed on a plate for three hours, and then removed. The number of the eggs they laid were counted and recorded.

In the reference study by González-Huici, 60 Gy was the maximum dose used. Both the N2 and *smg-1(tm849)* strains were not affected by 60 Gy, so a higher dose of IR (100 Gy) was used instead.

2.4.2 Reporter construct heat shock

In order to quantify the HR/NHEJ and SSA in *C. elegans*, two reporter constructs were used (Johnson et al., 2013).

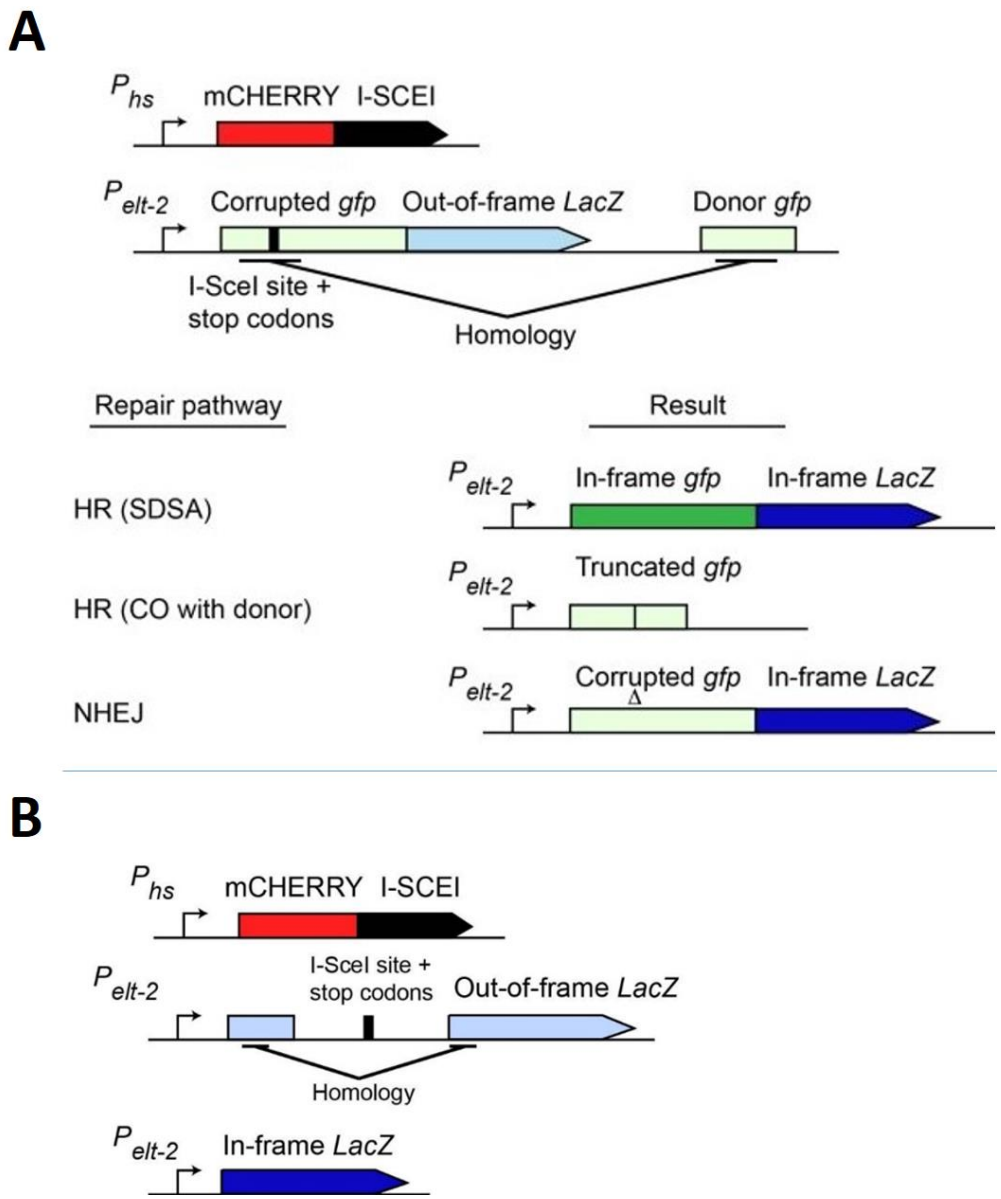

Figure 2.1. A: HR reporter with a corrupted *gfp* sequence and an out of frame *LacZ* sequence. It has an inducible nuclease with a cutting site in the *gfp* sequence. Once the restriction enzyme cut site is removed either through NHEJ or HR, *LacZ* will be back in frame. Only SDSA which relies on a homologous sequence will fix the *gfp* sequence. Crossover will result in incomplete *LacZ* and *gfp*. **B**: SSA reporter construct, with an inducible nuclease that has a cutting site in-between two homologous sequences. The first *LacZ* sequence is in frame, but incomplete. The second sequence contains the rest of the sequence but is out of frame. SSA will activate upon recognition of homology on both sides of the DSB and produce a complete and in-frame *LacZ*. [Figure adapted from: (Johnson et al., 2013)]

Both reporters contain heat-shock inducible nuclease sequences, which make the restriction enzyme mCherry tagged ISce-I.

In the HR/NHEJ reporter, this cutting site is located within a corrupted *gfp* sequence. A homologous repair template is available for repair. If the DSB in this lesion is fixed by synthesis-dependent strand annealing, the corrupted *gfp* sequence will be repaired based on the template and the cell will produce GFP. This will also put *LacZ* in frame, since *gfp* and *LacZ* share the same promoter and the cutting site was the only thing putting *LacZ* out of frame. If crossover occurs, an entire section containing LacZ and a part of the *gfp* sequence will be crossed out, and neither GFP nor LacZ will be produced. Non-homologous end joining will basically just stitch back the gap made by the restriction enzyme. This will not fix the *gfp* sequence but it will put *LacZ* back in frame, and LacZ will be produced (**Figure 2.1A**).

In the SSA reporter, the cutting site is placed between two homologous sequences which encode *LacZ*. This is the requirement for activating SSA. A double-strand break at this site has the potential to activate the single-strand annealing pathway, which will result in the elimination of the lesion and anneal the ends. This will create a complete *LacZ* sequence that is in frame (**Figure 2.1B**).

Procedure:

For the HR/NHEJ reporter: Activation by heat shock on L4 stage larvae at 34 °C for one hour, rest at 20 °C for half an hour, place back inside 34 °C for another hour. Image after 24 hours to check for mCherry expression. If mCherry has been produced, leave for another 24 hours before checking for GFP.

For the SSA reporter: L1 larvae were placed at 34 °C for 60 minutes, and then rested at 20 °C until adulthood.

2.5 RNA interference (RNAi)

RNAi is a method first developed in *C. elegans.* It is a method of genetic manipulation that works by altering the transcriptome and not the genome directly. RNAi will suppress expression and translation by targeting specific mRNA. The technique depends on the cell's pathway to defend itself from exogenous double-stranded RNA. dsRNA is processed by the Dicer complex into small interfering RNA (siRNA). This siRNA is then used by the RISC complex as a template to recognize complementary RNA strands and stop them from being translated by degrading them using a catalytic component of RISC named Argonaute 2 (Ago2).

Procedure:

1. Nematode Growth Media is made as per standard instructions, but before plating ampicillin and tetracycline are added for selection (see concentrations in buffers and media appendix). The plates are left to dry for several hours.

2. The desired bacterial strain is taken from a freezer and streaked on an LB plate containing ampicillin and tetracycline. After being grown at 37 ºC for 24 hours, colonies are selected to be inoculated in liquid LB media overnight. IPTG is added in the morning to induce expression for one hour. The resulting culture is then used to make a 50% glycerol stock for future use, and sent for sequencing just to make sure it is the desired strain.

3. Once the NGM agar plates are dry enough, they are seeded with 100-200 µl of the bacteria culture, depending on the amount the experiment needs.

4. Synchronized L1 worms are then placed on the RNAi plates. It is best to leave them for at least two days to observe results.

2.6 Molecular experiments

2.6.1 Polymerase chain reaction (PCR)

PCR is a technique used for DNA amplification. At first, a temperature of 95 ºC is used to denature the DNA and separate the two strands from each other. Then, an annealing temperature is used, so the primers anneal to the template strands. This depends on the primer melting temperature (Tm). In the afterward extension step, a DNA polymerase, usually *Taq* DNA polymerase, attaches to the primer-template part and synthesizes DNA by adding single nucleotides. This cycle is then repeated, so the new strands are denatured and new ones are made. Eventually, an extra extension step is added at the end, to fill in the protruding ends of newly synthesized DNA. A PCR buffer is used to create optimal conditions for the enzyme, including a catalyzer, $MgCl_2$ in this case. A list of used reagents is included in **Table 2.3**. Details of the PCR cycle as used in the machine are available in **Table 2.4**.

Procedure:

1. Mix the following reaction in PCR tubes:

Table 2.3. Ingredients of a typical PCR reaction. The amount of template DNA used is 500 ng, and the final concentration of dNTP is 200 µM. The final concentration of each primer is 0.2 µM.

Reagent	**Volume (µl)**
Template DNA	1 µl
PCR Buffer (5X)	4 µl
Taq Polymerase	0.15 µl
dNTP	0.5 µl
Primer mix (Forward and Reverse)	2 µl
Nuclease-free Water	12.35 µl
Total	20 µl

2. The following program is run on the PCR machine:

Table 2.4. Format of the PCR program used on the machine. The cycle includes the denaturation, annealing, and extension steps.

STEP	TEMP	TIME
Initial Denaturation	95 °C	30 seconds
30 Cycles	95 °C 58 °C 68 °C	30 seconds 60 seconds 1 minute/kb
Final Extension	68 °C	5 minutes
Hold	4-10 °C	-

2.6.2 Electrophoresis

Electrophoresis is a technique that separates macromolecules based on size and charge. In the case of DNA, which is what it has been used for in this thesis, since the charge per mass for DNA is the same, they are only separated based on size. An agarose gel is placed inside an electric field, and covered with a buffer with high conductivity, such as TAE buffer, and since the molecules are charged, they will move towards different electrodes. The pores inside the gel have different sizes, and so fragments move through them at different speeds.

Procedure:

1. Make 1% agarose mixture using TAE buffer and warm until all the agarose powder is dissolved.

2. Add GelRed DNA dye to the mixture, once it has cooled down to 50 ºC.

3. Put the mixture in a gel tray along with a comb, and wait for it to solidify before removing the comb.

4. Put the tray inside an electrophoresis unit and fill it with TAE buffer.

5. Add loading buffer to the samples and load them in the wells. This will help them settle in the wells because it contains glycerol and is dense, and additionally acts as a visible marker to track the progress on a gel.

6. Load a molecular weight ladder in a well next to the samples, which contains DNA fragments with known lengths and masses, as a way to estimate the length of the fragments in the sample.

7. Run the gel at 110 kV. The length of time depends on the fragment sizes. Typical run time is one hour. For pieces under 1 kB, half an hour is enough.

8. Turn off the machine and remove the gel. For imaging, take it to a device with a UV light. If pieces need to be cut out, put it on a UV screen and cut the pieces.

2.6.3 Cloning

In an attempt to make single-copy versions of the reporter constructs, a cloning experiment was designed. Since the exact sequence of the constructs is unknown, the pieces were amplified using PCR, separated by electrophoresis, and purified from the gel. Then the amplified inserts and vector both were digested with NotI restriction enzyme and the vector was dephosphorylated to avoid self-ligation. The ligation step was performed with an enzyme to connect the pieces. Once the vector and insert were assembled, transformation was performed using competent cells, and colonies were selected based on antibiotic resistance.

The first step was based on NucleoSpin® Gel and PCR Clean-up kit protocol from MN-net. The cloning protocol was adapted from NEBcloner v1.4.1 using NEBuffer™ 3.1, Quick CIP, and T4 DNA Ligase, all with kits from New England Biolabs.

2.7 RT-qPCR

2.7.1 Isolation of total RNA using TRIzol reagent

In order to gauge the expression level of different genes, the transcriptome must be examined. Total RNA is isolated using phase separation via centrifugation. *C. elegans* were collected with M9 solution in Eppendorf tubes. TRIzol™ was used to homogenize the worms. TRIzol contains phenol and acts as the organic phase in the separation. It inhibits RNase activity while disrupting cells and dissolving cell components. Then the samples were freeze-cracked to break cell membranes. Chloroform was used to separate the aqueous and organic phases, thus the RNA from DNA and proteins. Based on the pH of the mixture, DNA can either remain in the aqueous phase or organic phase (**Figure 2.2**).

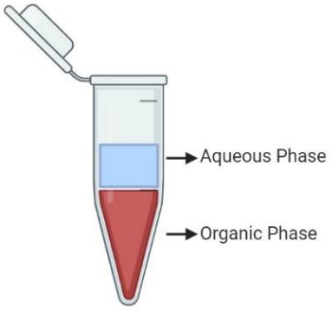

Figure 2.2. Separation of the aqueous phase and organic phase of TRIzol after chloroform is added. Nucleic acid is usually in the aqueous phase while proteins go to the organic phase.

Since we only need RNA, the pH is acidic (4-6) and DNA partitions into the organic phase. Total RNA is then precipitated from that aqueous layer using isopropanol. 80% ethanol is used to wash the sample, since it will dissolve any salts but not nucleic acids, and the RNA is solubilized in RNase-free water, and the concentration is measured.

The ΔΔCT method was used to analyze the expression levels. First, the expression of target genes was normalized against the housekeeping gene, *actin*, and then checked against the control worms that had not been irradiated. Then the following formula was used to calculate the fold:

$$Fold = 2\text{^}(-\Delta\Delta\text{CT})$$

Considering the formula, any upregulation of the target gene would result in a fold level above 1.

Procedure:

1. Add TRIzol to the Eppendorf tubes containing the worms.
2. Freeze crack for 10 cycles in liquid nitrogen and thaw at 37 ºC.
3. Add Chloroform. 1/5 of the amount of TRIzol in the tube. Mix well until it is dull pink.
4. Centrifuge lysate at 12000g for 15 minutes at 4 °C.
5. Remove the upper aqueous phase and add to isopropanol. Use isopropanol in 1:1 ratio.
6. Freeze at -20 °C overnight to precipitate RNA.
7. Centrifuge at 20000g for 20 minutes in the cold room and pour off isopropanol without losing pellet.
8. Add 80% ethanol. Centrifuge at 20000g for 5 minutes and pour off the supernatant, without losing pellet and let it dry.
9. Dissolve in 25 µl nuclease-free water.
10. Measure RNA concentration. It was calculated by measuring the light absorption at 260 nm using a spectrophotometer (Nanodrop ND-1000).

2.7.2 cDNA synthesis

RNA is not a stable template to use in further PCR steps, therefore it is first converted into cDNA, using the enzyme reverse transcriptase. This protocol is based on the Quick-Start protocol of the QuantiTect Reverse Transcription Kit. First, the samples were treated with a wipeout buffer to ensure the elimination of all genomic DNA. Then heat was applied to open secondary RNA structures and to allow the DNase to work more efficiently. A reverse transcription enzyme was used to make cDNA.

Procedure:

1. Mix the following reaction:

Component	Volume/reaction
gDNA Wipeout Buffer 7x	2 µl
Template RNA (1 µg)	X
RNase-free water	Up to 14 µl
Total volume	14 µl

2. Incubate for 10 min at 42 °C then place immediately on ice.

3. Reverse Transcription (RT) Master Mix:

Component	Volume/reaction
Quantiscript Reverse Transcriptase	4.5 µl
Quantiscript RT Buffer, 5x	18 µl
Oligo DT	4.5 µl

4. Make another similar reaction, except instead of the enzyme, add nuclease-free water. This will serve as the control reaction.

5. Mix the following Reverse Transcription Reaction:

Component	Volume/reaction
RT master mix	6 µl
Template RNA from step 1	14 µl

Total volume	20 µl

6. Incubate for 30 min at 42 °C.
7. Inactivate at 95 °C for 3 min.
8. Proceed directly with RT-qPCR or store at -20 °C.

2.7.3 Reverse transcription quantitative PCR (RT-qPCR)

This RT-qPCR assay was performed using a thermal cycler, LightCycler 96, using SYBR Green 1 (Roche). 96-well plates were used and sealed with transparent adhesive. SYBR green is a fluorescent dye with the ability to bind to double-stranded DNA, which emits fluorescence when it is bound. It is used for quantitative purposes because the fluorescence can be measured after every cycle, to determine how much original DNA was present in the sample and was amplified (Dragan et al., 2012).

The device recognizes a threshold for the detection of fluorescence, called the quantification cycle (Cq). After the exponential phase in which the quantification takes place, the process will stop. The more DNA there is in a sample, the faster this threshold is reached and fewer cycles are needed to reach it.

The ΔΔCT method was used to interpret the data accurately. A reference gene, which has stable expression in all the samples, is used, so that the resulting data from target genes can be normalized. In this experiment, *actin* was used as a reference gene. All samples were run in technical triplicates, and the average was used. Two out of three wells have to be positive for an expression level to be considered so. Each well contained 10 µl reactions containing the following:

Component	Volume/reaction
cDNA	1 µl
Primer	1 µl
5x reagent	2 µl
H2O	6 µl
Total	10 µl

A master mix was first made which contained everything except the primers, so the cDNA levels would be constant in all samples. After that, it was aliquoted and the primers were added.

The thermal cycle begins at 95 °C, in order to separate the chains of the double-stranded DNA. Then it goes down to 60 °C, to allow for the primers to bind to the newly available ssDNA, and lastly, the polymerization step at 72 °C. This cycle will repeat 23 times.

The following primers were designed based on the known sequences of the *smg* genes in *C. elegans* (**Table 2.5**).

Table 2.5. List of *smg* primers designed for qPCR.

Primer	Primer	Sequence (5’ to 3’)
smg-1	Forward	AAGGAACGGAAAGCGATTCT
smg-1	Reverse	CCAGCGTTTTCATGTCATTC
smg-2	Forward	TCTCAGTTCGCCTATGAGCA
smg-2	Reverse	GCGACACACAGAGGATCAGA
smg-3	Forward	ATGGCTGGAAGAGCTGAAAA

smg-3	Reverse	GACGCCATTTCCTCAACAAA
smg-4	Forward	GTTGCGTCGTCTTCCAAAAT
smg-4	Reverse	TGCAGCAGAATCATTTCCAC
smg-5	Forward	GGAAAAATACGGCCAAACTG
smg-5	Reverse	GGAAGCGAAAGCATTTCTTG
act-1	Forward	GTTGCCCAGAGGCTATGTTC
act-1	Reverse	CAAGAGCGGTGATTTCCTTC

2.8 Microscopy

2% agarose solution was made and heated up to 100 ºC, and then placed on microscope slides as a thin film. The worms were then mounted on this thin gel film to avoid damage during photography, and coverslips were placed on top to fix them in place.

Levamisole solutions (1 µg/ml) were used to render the worms motionless for the duration of the imaging. The Zeiss AxioImager Z1 research microscope was used for all images, used mostly for DIC (Differential Interference Contrast) and Fluorescent Imaging. The magnitude of the objective used for the photos is either 40 X or 100 X, with oil on the coverslip.

3 Results

SMG-1 and SMG-2 are the main components of the NMD pathway. Several *smg-1* gene mutant alleles are sensitive to ionizing radiation (IR) (González-Huici et al., 2017). Ionizing radiation (IR) is a known source of DSBs and activates the DDR pathway. In the Ciosk lab, we do not have any of the *smg-1* alleles that were used in the aforementioned study. Additionally, this study did not characterize any other NMD component. So to begin with, we checked if *smg-1(tm849)* and *smg-2(tm6028)* were hypersensitive to IR. Both are deletion alleles which result in loss of function.

3.1 *smg-1(tm849)* and *smg-2(tm6028)* are sensitive to ionizing radiation (IR)

L1 and L4 stage worms were exposed to 100 Gray (Gy) of X-ray and then allowed to develop to adulthood to check their fecundity. Eggs laid by three worms within a period of 3 hours were counted for analysis. The experiments were done in triplicates.

In contrast to N2, *smg-1/2* mutants laid nearly 50% fewer eggs after irradiation regardless of the developmental stage at which they were irradiated (**Figure 3.1A, B**). In addition, *smg-1/2* developed tumor-like protrusions in the vulva, and on some occasions, the worms burst out of the vulva (**Figure 3.1C**). Their developmental rate was affected and *smg-1* mutants took particularly longer (roughly 20 hours more) to reach adulthood.

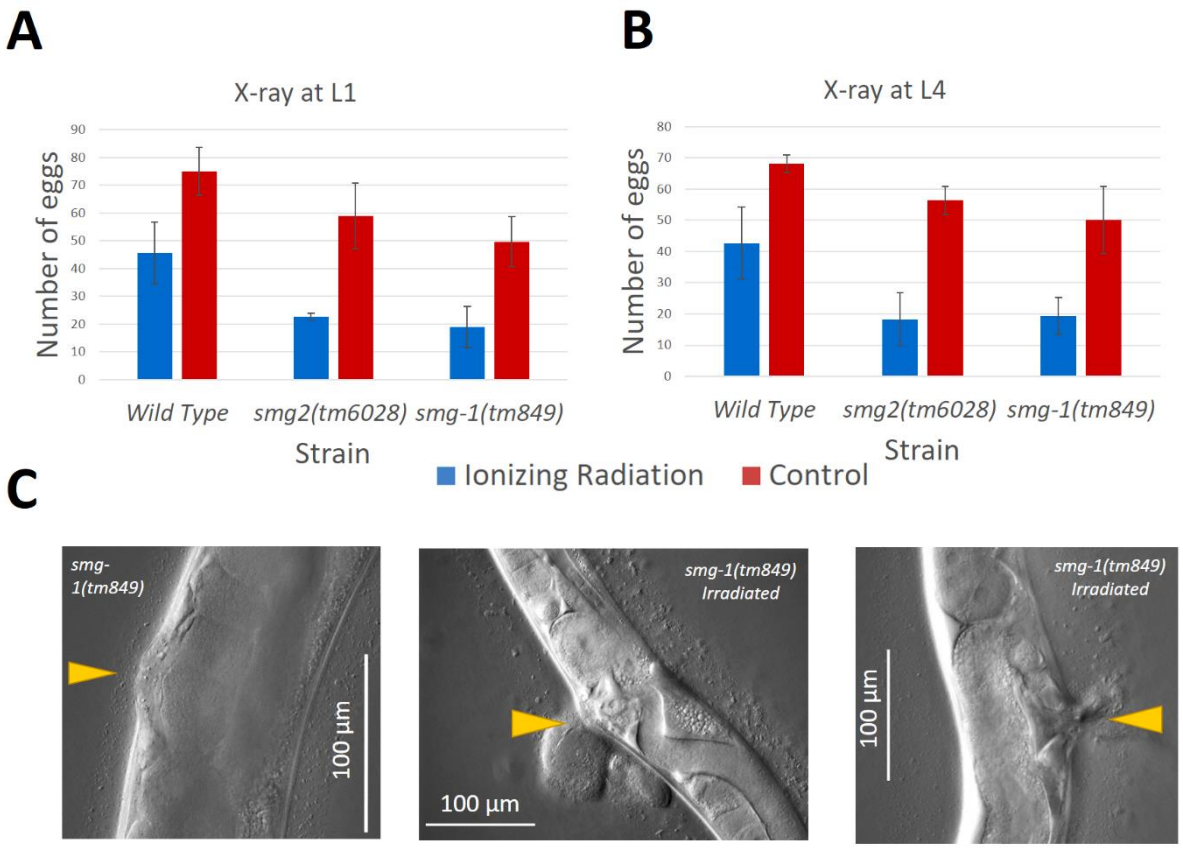

Figure 3.1. Effects of ionizing radiation of *smg-1(tm849)* and *smg-2(tm6028)*. A: Graph depicting the number of eggs that were laid by three worms that were irradiated at L1 stage with 100 Gy X-ray laid, over three hours, as compared to non-irradiated control. Each bar represents the mean of triplicate experiment data and error bars are standard deviation. **B**: This is the same experiment, except the worms were irradiated at L4, instead of L1. **C**: The morphological effect of IR was the aggravation of the protruding vulva phenotype, which in some cases burst. On the left, the control *smg-1(tm849)* mutant, which has a protruding vulva characteristic to this mutant, and on the right, worms with varying stages of swelling after being irradiated. The yellow arrows point to the vulva of each worm. The white bars represent the scale of 100 µm.

3.2 Characterization of *smg-1(tm849)* and *smg-2(tm6028)* knockout mutants

We investigated to see if the components of the DDR network and the NMD pathway interact genetically. This means checking if the effects of one gene were modified by other genes, which would show that the pathways interact. To do this, we knocked down specific components of various DDR pathways in the *smg-1* and *smg-2* mutants. If the effects of the DDR genes are modified by the *smg* genes, we expected changes in the phenotype of the worms.

We performed a developmental assay to first characterize the phenotypes of *smg-1(tm849)* and *smg-2(tm6028)* mutants. Henceforth, they will be referred to as *smg-1* and *smg-2* mutants (*smg-1/2* for short, when referring to both of them). For the assay, we obtained embryos by bleaching gravid adults and let them hatch without food. This resulted in synchronized stage 1 larvae (L1s).

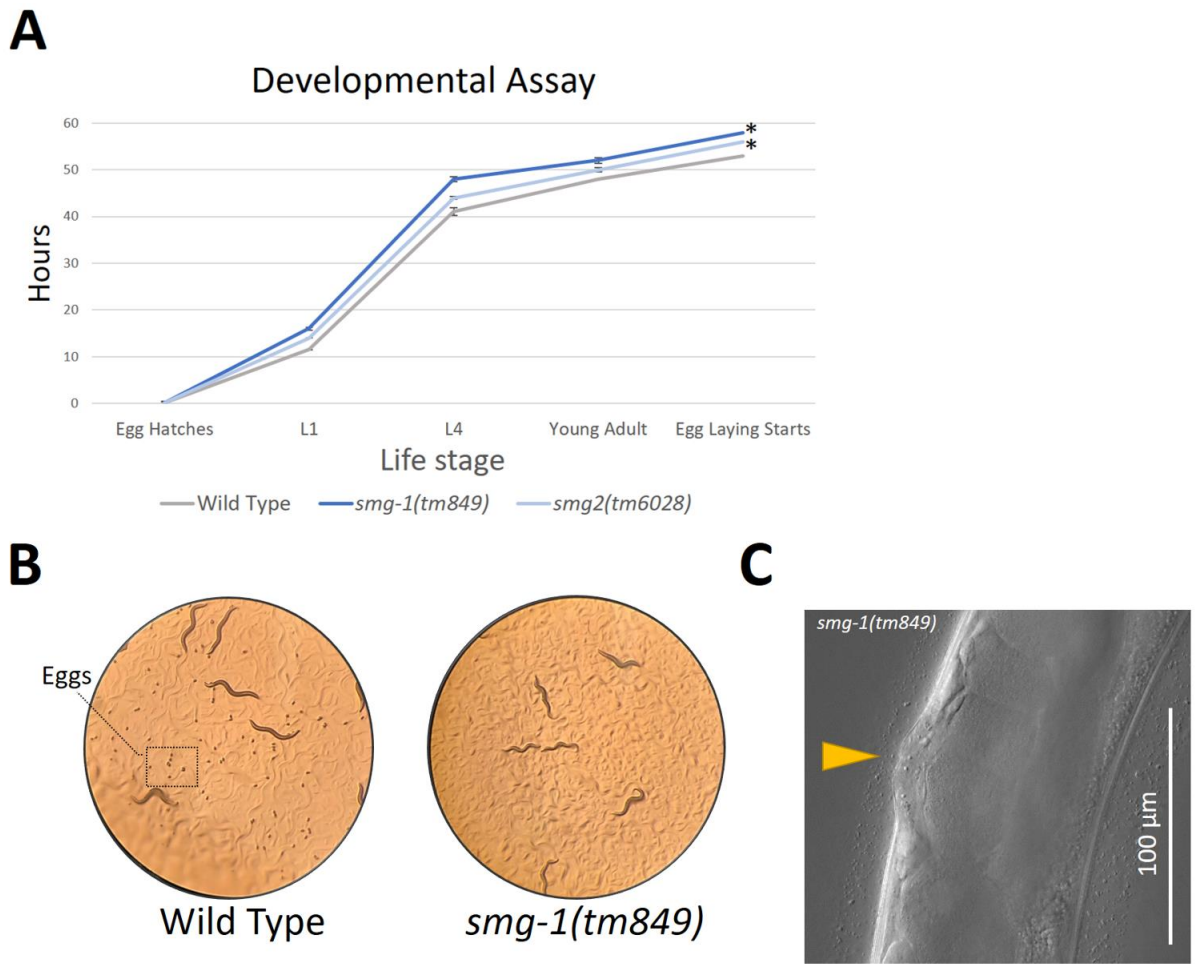

Figure 3.2. Characterization *of smg-1(tm849)* and *smg-2(tm6028)* mutants. **A**: A plot showing the number of hours taken by wild-type, *smg-1*, and *smg-2* worms to reach different larval stages. Both *smg-1(tm849)* and *smg-2(tm6028)* develop slower as compared to the wild type worms. The stars indicate that both experiments had p-values < 0.05 and the results were significant. Standard deviation was calculated for the error bars. **B**: Micrographs of culture plates, showing wild-type and *smg-1* worms at 54 hours. Wild-type worms have laid many eggs (boxed), while the *smg-1* worms have not. The mutant worms are also smaller than the wild type worms. **C**: Protruding vulva of a hermaphrodite *smg-1* mutant.

smg-1/2 mutants and N2 worms were observed to ascertain their developmental timeline (**Figure 3.2A**). Larval stages 2 and 3 do not have easily identifiable morphological features, therefore we used larval stage 4, adulthood, and the start of egg-laying as developmental

landmarks. The mutants took longer to develop to larval stage 4 and adulthood as compared to wild type worms. *smg-1* mutants took the longest, followed by *smg-2,* and WT worms were fastest to reach each stage. *smg-1* worms were slightly smaller in size than N2, while *smg-2* mutants were the same size as N2. When N2 worms had begun laying eggs, *smg-1* was yet to reach the adult stage, but *smg-2* worms were slightly faster and had laid eggs by that time (**Figure 3.2B**). As reported earlier, we observed protruding vulva in hermaphrodites and swollen bursa in males in the *smg-1/2* mutants (Hodgkin et al., 1989) (**Figure 3.2C**). Furthermore, *smg-1/2* had slower locomotion speed than the wild types, and the curvature of their movements was steeper. This was assessed qualitatively and no quantitative assays were performed to measure the speed or the angle of the curvatures. Slower locomotion has not been reported for *smg-2* mutants. *smg-2* and particularly *smg-1* mutants laid fewer eggs per worm than the wild type. This was further quantified in section 3.1.

3.3 Knockdown of DDR pathway genes in *smg-1(tm849)* and *smg-2(tm6028)*

DNA damage response (DDR) involves several overlapping pathways that are utilized to repair different kinds of DNA damages. To see if a specific pathway interacts with NMD components, we knocked down key proteins crucial to specific DDR pathways via RNA interference (RNAi) in *smg-1/2* mutants. We checked if the *smg-1/2* phenotypes were affected upon knockdown and if any synthetic phenotype appeared. The DDR components that we knocked down in *smg-1/2* mutants, their respective pathways, and the observed phenotypes are listed in **Table 3.1**.

Table 3.1. List of morphological and physiological phenotypes that occur in *smg-1/2* mutants after knocking down the essential proteins of several DDR pathways. All recorded phenotypes are noted in comparison to the mock RNAi control tests, for which bacteria with empty vectors were used. The aqua blue color is to highlight the pathways which need homology in order to activate.

smg-1(tm849)		
RNAi Type	**Pathway**	**Phenotypes after RNAi**
cku-80	NHEJ	Slower locomotion
lig-4	NHEJ	Slower locomotion, Developmental arrest in some worms
xpf-1	SSA	Enhanced protruded Vulva in only10% of worms
polq-1	MMEJ	No change in phenotype
brc-1	HR	No change in phenotype
rad-51	HR	No change in phenotype
cep-1	Cell cycle arrest	Slower locomotion

smg-2(tm6028)		
RNAi Type	**Pathway**	**phenotypes after RNAi**
cku-80	NHEJ	Slower locomotion
lig-4	NHEJ	Egg-laying variant (Lays fewer eggs)
xpf-1	SSA	Enhanced protruded Vulva in 10% of worms
polq-1	MMEJ	No change in phenotype
brc-1	HR	No change in phenotype
rad-51	HR	No change in phenotype
cep-1	Cell cycle arrest	No change in phenotype

The knockdown of proteins belonging to the NHEJ pathway yielded the most noticeable change in *smg-1/2* phenotypes. *cku-80* and *lig-4* knockdowns resulted in consistent and prominent aggravation in the phenotypes. The knockdown of pathways that required homology for repair displayed milder effects. The MMEJ knockdown resulted in an egg-laying variant with more eggs, but the increase was not significant, although it was consistent. Further statistical analysis is required to confirm this. When HR components were knocked down, no change in phenotypes was observed. The overall trend is that the knockdown of proteins from pathways that do not require homology for repair, namely NHEJ, aggravates *smg-1/2* phenotypes suggesting that these DNA repair pathways may interact with the NMD pathway.

3.4 Homologous recombination repair increases when *smg-1* and *smg-2* are knocked down

Hypersensitivity of *smg-1/2* mutants to IR could be due to inefficient repair of double-strand breaks. Our analysis of the genetic interaction between different DDR pathways and the NMD pathway suggests that NMD interacts with those repair pathways that do not require sequence homology. This can be further confirmed by quantifying activation of different DDR pathways after knocking down *smg-1/2*.

In the lab, we have two reporter strains to quantify HR/NHEJ, and SSA pathways (for details see methods section 2.4.2). In both reporter strains the restriction enzyme ISce-I is fused to mCherry and is driven by a heat-shock promoter (**Figure 3.3A**). If mCherry is expressed, the restriction enzyme is present and DSBs are being produced. Any worm that had even a single cell displaying GFP was considered positive for having used synthesis-dependent strand annealing (SDSA) HR to repair restriction enzyme induced DSBs (**Figure 3.3B**).

We knocked down *smg-1/smg-2* in the HR/NHEJ reporter and quantified GFP expression. Under normal circumstances, SDSA was used by less than 10% of the tested population to repair DSBs. After *smg-1* and *smg-2* are knocked down via RNAi, however, SDSA is used in over 40% of worms (**Figure 3.3C**). The SSA reporter requires a small amount of homology in the sequences surrounding the cutting site. If this is detected, the two sides are simply joined back together. Any cell that performed SSA would have a *LacZ* gene activated. Any worm with even one cell stained was considered positive for SSA occurrence (**Figure 3.3D**). In the SSA reporter, we quantified *LacZ* expression upon knockdown of *smg-1/2*. Initially, 20% of worms used SSA to repair the induced DSBs. After knocking down *smg-1/2* more than 40% of the worms used SSA to repair double-strand breaks (**Figure 3.3E**).

These results suggest that in the absence of *smg-1/2*, DNA repair happened mostly through the homology dependent pathways of HR (SDSA) and SSA. Due to lack of time, I could not check the expression of both GFP and LacZ in the HR/NHEJ reporter. However, the presence of GFP suggests that LacZ was also produced. This suggests that NHEJ was inactive after the knockdown, and the increase in HR repair could very well be due to the inactivation of NHEJ.

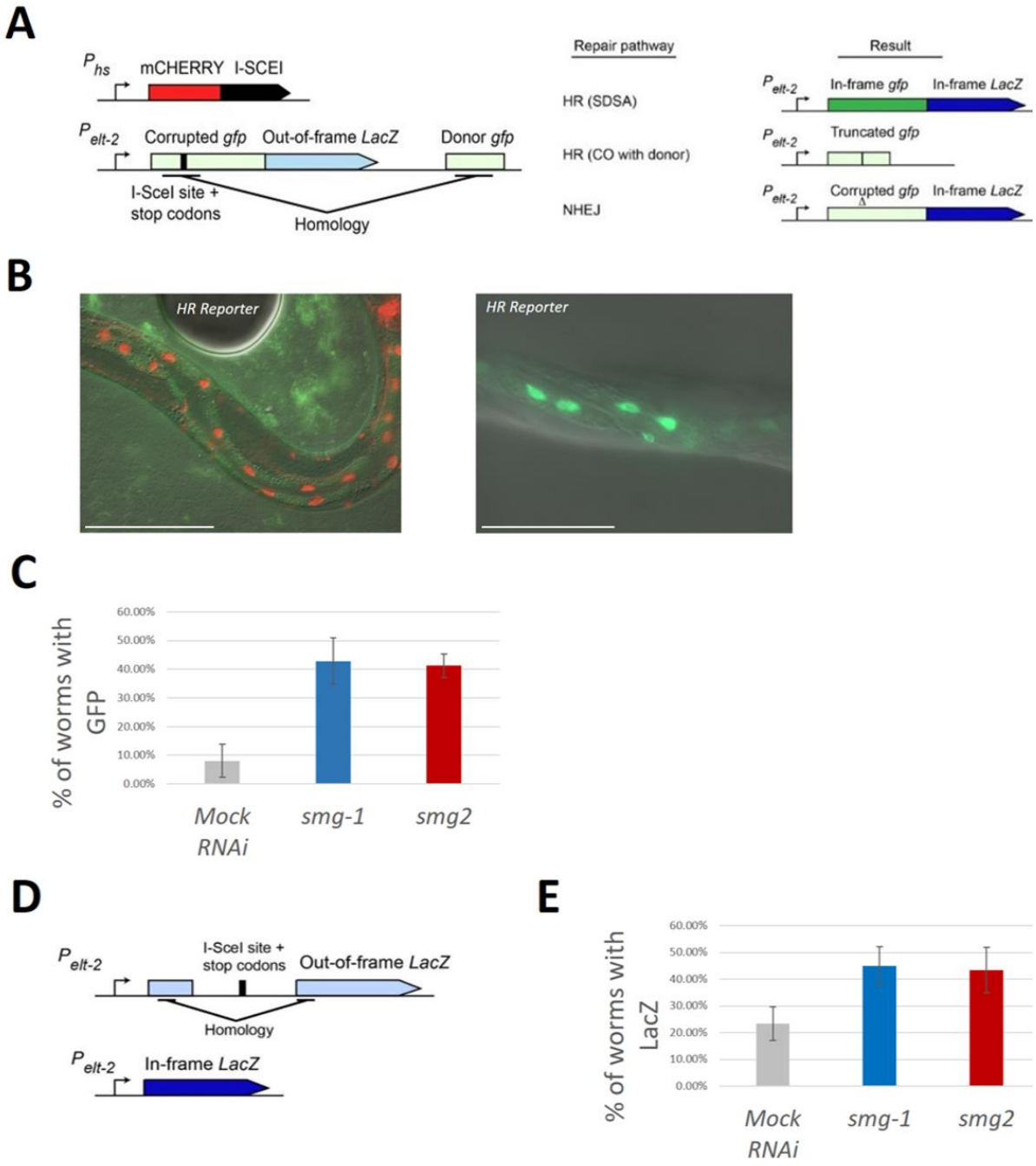

Figure 3.3. Quantifying HRR pathways SDSA and SSA using reporters. **A**: A heat shock promoter activates the expression of mCherry::ISce-I. The restriction enzyme ISce-I has a cutting site in a corrupted *gfp* sequenced. If it is fixed using a homologous sequence, GFP will be produced. Once the restriction enzyme cut site is removed either through NHEJ or HR, LacZ will be back in frame. Only SDSA which relies on a homologous sequence will fix the *gfp* sequence. Crossover will result in incomplete LacZ and gfp. **B**: mCherry is expressed after heat-shock is used to activate the reporter (left). Intestinal cells displaying GFP (right). The white bars represent a scale of 100 µm. **C**: The graph shows the percentage of worms that had at least one intestinal cell with GFP, implying SDSA has occurred. **D**: The SSA reporter requires a small amount of homology in the sequences surrounding the restriction site. If this is detected, the two sides are simply joined back together and LacZ will be in frame. **E**: Percentage of worms with at least one cell displaying LacZ after SSA.

3.5 *smg-1* and *smg-2* are upregulated after irradiation

To check if the expression of different NMD components is altered upon DNA damage, we quantified the mRNA expression of various NMD pathway genes by RT-qPCR. Wild type worms were irradiated with 100 Gy at two different developmental stages, L1 and L4. The worms were then raised until adult stage before being harvested for RNA isolation, and RT-qPCR. When worms were irradiated at L1 stage, expression of *smg-1* and *smg-2* was elevated, while expression of *smg-3*, *smg-4*, and *smg-5* did not change significantly. Upon irradiation at L4 stage, all *smg* genes were downregulated to different levels **(Figure 3.4)**. These results remain inconclusive and require further experimentation since protein levels and protein activity also need to be checked before any deductions are made based on this data.

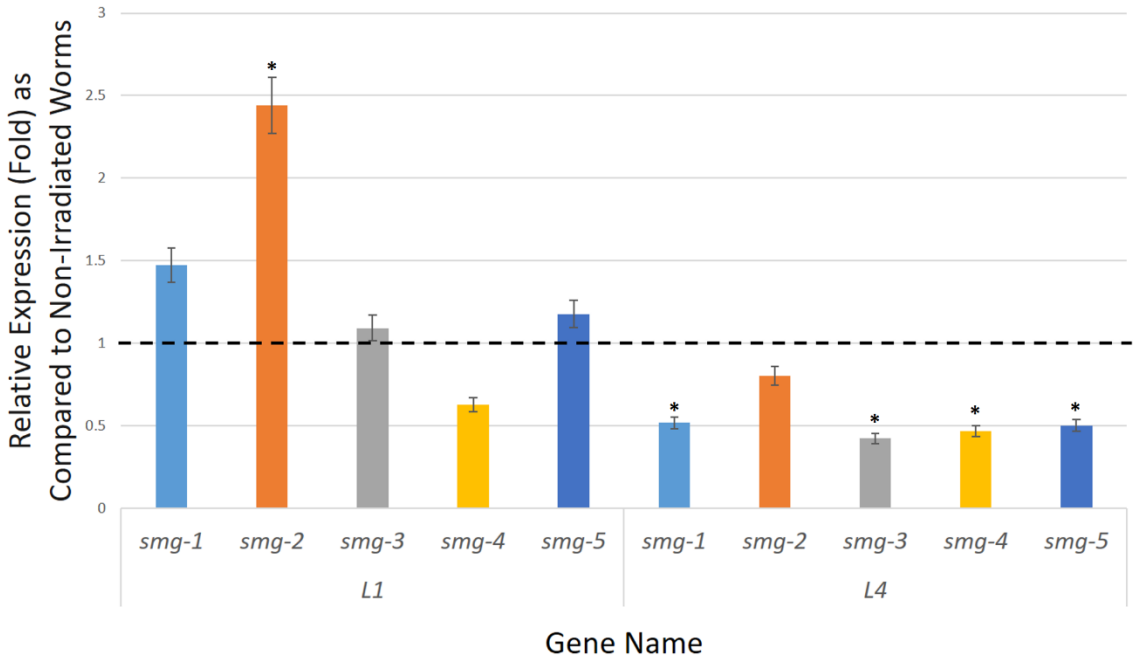

Figure 3.4. Relative Expression of *smg* Genes as Compared to Non-Irradiated Worms. Expression levels of N2 worms that had been irradiated with 100 Gy X-ray were obtained using RT-qPCR. Worms were irradiated at two life stages, L1 and L4. The dotted line represents the neutral fold. Any gene with a fold above 1 (one) has been upregulated. The stars (*) indicate that the sample had a p-value < 0.05, so the change was significant.

4 Discussion

4.1 Localization of NMD components

NMD is a cytoplasmic cellular process initiated during the translation of aberrant mRNAs (Karousis and Mühlemann, 2019). DNA repair takes place in the nucleus. Considering the disparity in their localizations, their interaction is somewhat unexpected and brings forth the question of how they can affect each other. However, some of the core NMD components, namely UPF1, UPF2, UPF3 (SMG-2, SMG-3, SMG-4 in *C. elegans*) travel back and forth between the nucleus and cytoplasm. UPF3 likely interacts with the EJC while in the nucleus (Le Hir et al., 2001; Lykke-Andersen et al., 2000). UPF1 harbors a nuclear localization signal and can even interact with the chromatin (Hong et al., 2019).

Many studies suggest that NMD has a nuclear aspect. If an mRNA containing a PTC was translated several times before being detected, truncated proteins would be produced in large quantities. Thus in some cases, they must be detected as early as possible, while they are still in the nucleus. There is a theory that a portion of pioneer translation occurs within the nucleus, accompanied by PTC recognition processes (Frischmeyer and Dietz, 1999). An early onset of NMD is initiated within the nucleus to recognize pre-mRNA that have PTCs, and then splice them. This is called “nonsense-associated altered splicing” (NAS). This is done so the cell can react to erroneous mRNA before any aberrant protein is produced (Chang et al., 2007; Hentze and Kulozik, 1999).

The phenomenon of transcriptional adaptation, which states that in the event of mutations the mRNA surveillance machinery upregulates the related genes, likewise implies that NMD must be able to somehow affect nuclear processes. The exact mechanism is yet to be discovered (El-Brolosy et al., 2019).

4.2 Hypersensitivity of NMD mutants to IR

Different *smg-1* mutant alleles are sensitive to ionizing radiation (González-Huici et al., 2017). Several phenotypes emerged after being irradiated. *Smg-1/2* developed tumor-like protrusions at their vulva. After irradiation, *smg-1* mutants took almost 20 hours more than usual to reach adulthood, while *smg-2* mutant development is not delayed (**Figure 3.1**). The

number of progeny in both mutants is affected. We hypothesize that *smg-1/2* knockouts are sensitive to IR because double-strand breaks are not being repaired as efficiently as in wild types. This could be due to these two proteins either affecting the repair process directly, or indirectly, by interacting with a component of the repair network.

The most prominent role of SMG-1 is an essential kinase of the NMD pathway. However, like many other kinases in the cell, SMG-1 is most likely involved in more than one pathway. Other members of the PI(3)K-related kinase family are active in stress signal pathways, in mammalian cells. In humans, SMG1 has a target sequence in p53, an important checkpoint protein, and has an overlapping function with ATM, a major DDR effector. (Brumbaugh et al., 2004). SMG-1, which is an NMD component, is then linked to DDR. An earlier screen in our lab had detected CLK-2 (known to be involved in DDR) to be a novel NMD component. To judge whether or not it was only SMG-1 that was affecting the stress response, or whether other members of the NMD pathway were involved, SMG-2 (UPF1 in mammalian cells) was tested as well. Our *smg-2* mutants displayed the same morphological phenotypes as *smg-1* worms, and the assays showed that *smg-2* mutants are sensitive to IR, although to a lesser extent compared to *smg-1* mutants. Thus, SMG-1, SMG-2, and CLK-2 are all NMD components with possible links to DDR.

4.3 Change in expression levels of *smg* genes upon irradiation

If the SMG proteins play a role in DNA repair, their expression or activity levels might be affected after irradiation. A change in mRNA expression does not always correlate to a change in the activity level of a protein. RNA level, protein level, and protein activity could each be affected by irradiation. In humans, SMG1 activation is potentiated by DSB induction. This leads to elevated phosphorylation of UPF1 (SMG-2 in *C. elegans*) proteins (Brumbaugh et al., 2004). Thus, we see an increase in the activity levels of both proteins. To further examine this, the expression levels of all the mRNA encoded by *smg-1* to *smg-5* were gauged by quantitative PCR. In specimens that were irradiated at L1, expression of *smg-1* and *smg-2* was elevated, while expression of *smg-3, smg-4,* and *smg-5* did not change significantly. Upon irradiation at L4, all *smg* genes were downregulated to different levels **(Figure 3.4)**.

We know that the DDR proteins in somatic cells and germline cells of *C. elegans* have different expression levels. Although DNA repair occurs in both cells, several signaling proteins including ATM (ATM-1 *C. elegans*) in are repressed in somatic cells (Vermezovic et al., 2012).

In times of stress, mammalian cells have been observed to downregulate the NMD components so they have more abundant copies of all the mRNAs that are suppressed in normal conditions. This is done to improve the cell's ability to fight off the source of stress. Hypoxia, nutrient deprivation, or infection all trigger stress responses that suppress NMD (Hug et al., 2016). However, the fact that we don't observe a consistent increase in expression levels of *smg* genes in both L1 and L4 implies that maybe their expression levels have not been increased or there is no correlation to the DNA damage. Before further deductions are made, the protein level and protein activity of both SMG-1 and SMG-2 need to be assessed after irradiation in *C. elegans* at L4.

4.4 Connection to DNA damage response network

In order to see if there is a link between the NMD pathway and a certain aspect of DDR and narrow down the direction of its effect, several knockdowns were performed with RNAi. It's known that if any of the *smg* components in the NMD pathway were to stop functioning, the entire pathway would lose its efficiency or stop working entirely (Gatfield et al., 2003; Rehwinkel et al., 2005). Both of the knockout mutant worms *smg-1(tm849)* and *smg-2(tm6028)* should then be considered to have no functional NMD pathway. It is worth noting that lacking a functional NMD pathway results in the aggregation of faulty mRNA and truncated proteins, which could have unforeseen effects on the cell.

One of the potential reasons that *smg-1* mutants are sensitive to IR is because SMG-1 phosphorylates CEP-1. CEP-1 is the *C. elegans* orthologue for p53; an important DDR effector protein that halts cell cycle progression when damage occurs. However, no significant phenotype changes were observed after CEP-1 was knocked down in the *smg* mutants. This implies that it is not SMG-1's ability to phosphorylate CEP-1 that makes the mutants sensitive to IR. The knockdowns that had consistently aggravated phenotypes in both

smg-1 and *smg-2* were *cku-80* and *lig-4*, both of which are essential specifically to NHEJ **(Table 3.1)**.

Two different reporter constructs with the capacity to detect certain repair pathways were used. The first one detected HR and NHEJ and the second, SSA. The reporter worms had *smg-1* and *smg-2* knocked down via RNAi, to see whether the reporters used these repair pathways more or less, in the absence of SMG-1/2. In both reporters, after *smg* genes were knocked down, repair via HR and SSA increased overwhelmingly (See **Figure 3.3**). One possible explanation is that there is a pathway that SMG proteins help to activate or maintain, and in their absence, the cell resorts to using other pathways. If the theory that SMG proteins are connected NHEJ in a way is correct, this would make sense. Another explanation could be that the NMD pathway regulates the transcriptome in a way that suppresses homology related pathways, and the absence of NMD leads to these pathways being used more. Since these constructs had multiple copies of the reporter sequences inserted inside them, quantifying the exact amount of repair was impossible. Ergo, we tried to extract these sequences and then make single gene copy versions of the reporter. However, the first attempt was unsuccessful, as the sequencing results did not match with the reporters. Due to the COVID-19 pandemic, we were unable to repeat this experiment.

5 Conclusion

NMD is an RNA processing and surveillance pathway. DDR is a network that responds to DNA damage using cell cycle arrest, DNA repair, and apoptosis. Using forward genetic screens with *C. elegans* as a model organism, *clk-2* and *smg-1* were uncovered as proteins involved in both NMD and DDR, suggesting the two are linked.

We investigate this by first assessing whether mutants of other NMD are hypersensitive to IR. We further investigated whether the components of any specific pathway in the DDR network genetically interact with NMD. We also checked which repair pathway is affected upon knockdown of NMD components. We discovered that *smg-2* mutants are also hypersensitive to IR, suggesting further involvement of NMD in DDR. Among all DDR pathways, only the knockdown of NHEJ repair pathway proteins aggravated the phenotypes of *smg-1* and *smg-2* mutants. Additionally, we found that animals lacking *smg-1* or *smg-2* used homology dependent repair more. This suggests a decrease in NHEJ activity in the absence of *smg-1* and *smg-2*. Considering the results of all the experiments, the most likely DDR pathway affected by NMD is NHEJ.

6 Future Prospects

To identify where and how the NMD and DDR pathways cross is a long term goal. The next step in confirming the theory that NHEJ is connected to NMD would be to properly quantify NHEJ activity after NMD components are knocked down to see if it is affected. This could be done using the reporters used for this thesis, although in that case LacZ cannot be visualized and may be quantified using methods such as antibody staining. Designing new reporters would increase the efficiency.

RNAi results should be confirmed by making double mutants. A strain with both NMD and NHEJ knocked out would likely yield interesting results to study. Although mating and maintaining male lines in NMD knockouts has proven arduous, other genetic manipulation methods, such as CRISPR can be used to create double mutants.

Another experiment that should be carried out is to visually check the localization of all SMG proteins in *C. elegans,* and see whether or not any of them enter the nucleus after irradiation. This is an experiment that has not been conducted before in *C. elegans* even under normal conditions. In addition, cell cycle checkpoints should be assessed on whether or not they still work properly in knockout worms.

We checked mRNA levels, but to make deductions from the data, information on the protein levels and protein activity, after an ionizing radiation event, is needed in worms of different life stages, especially L4.

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8 Appendix

8.1 Buffer and media

BU Buffer

70	mM	potassium phosphate
70	mM	NaCl, pH 7

Oxidation Buffer

5	mM	potassium ferricyanide
5	mM	potassium ferrocyanide
1	mM	$MgCl_2$

X-gal

20 mg/ml of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside in N,N'-dimethylformamide

Keep at -20 ºC.

10x M9 buffer (10 L)

300	g	KH_2PO_4
752	g	$Na_2HPO_4 \cdot 2H_2O$
500	g	NaCl
4.93	g	$MgSO_4 \cdot 7H_2O$

Dissolve substances in 7.5 L ddH_2O and then make up to 10 L with ddH_2O.

1x M9 buffer: dilute 10x M9 buffer in ddH_2O and autoclave.

S-basal buffer (1 L)

5.8	g	NaCl
50.0	ml	1 M Potassium Phosphate, pH 6.0 *
1.0	ml	Cholesterol (5mg/ml EtOH)

Dissolve substances in ca. 800 ml ddH_2O (~37 °C) and make it up to 1 L and autoclave.

*1M Potassium Phosphate is made by mixing 132 ml of 1M K_2HPO_4 and 868 ml of 1M KH_2PO_4 or 1M KH_2PO_4, pH 6.0 (adjusted by KOH).

NG 2% plates (5 L)

4.9	L	ddH_2O
100.0	g	Difco-Agar, Granulated (BD 214530)
12.5	g	Bacto-Peptone (BD 211677 / BD 211820)
15.0	g	NaCl

Autoclave and cool down to ~50 °C, then add:

5.0	ml	Cholesterol (5mg/ml EtOH) (sterile) (Sigma C3045)
5.0	ml	1.0 M $CaCl_2$ (sterile)
5.0	ml	1.0 M $MgSO_4$ (sterile)
125.0	ml	1 M Potassium Phosphate, pH 6.0 (sterile) *

Pour the plates.

*1M Potassium Phosphate is made by mixing 132 ml of 1M K_2HPO_4 and 868 ml of 1M KH_2PO_4 or 1M KH_2PO_4, pH 6.0 (adjusted by KOH).

NG 2% plates with 1 mM IPTG and 50 µg/ml Carbenicillin (5 L)

4.9	L	ddH_2O

100.0	g	Difco-Agar, Granulated (BD 214530)
12.5	g	Bacto-Peptone (BD 211677 / BD 211820)
15.0	g	NaCl

Autoclave and cool down to ~50 °C, then add:

5.0	ml	Cholesterol (5mg/ml EtOH) (sterile) (Sigma C3045)
5.0	ml	1.0 M $CaCl_2$ (sterile)
5.0	ml	1.0 M $MgSO_4$ (sterile)
125.0	ml	1 M Potassium Phosphate, pH 6.0 (sterile) *
5.0	ml	1 M IPTG (sterile)
2.5	ml	Carbenicillin (100mg/ml) (sterile)

Pour the plates.

*1M Potassium Phosphate is made by mixing 132 ml of 1M K_2HPO_4 and 868 ml of 1M KH_2PO_4 or 1M KH_2PO_4, pH 6.0 (adjusted by KOH)

Peptone rich plates (8 L)

7.8	L	ddH2O
200.0	g	Difco-Agar, Granulated (BD 214530)
160.0	g	Bacto-Peptone (BD 211677 / BD 211820)
9.6	g	NaCl

Autoclave and cool down to ~50 °C, then add:

8.0	ml	Cholesterol (5mg/ml EtOH) (sterile) (Sigma C3045)
8.0	ml	1.0 M $MgSO_4$ (sterile)

200.0	ml	1 M Potassium Phosphate, pH 6.0 (sterile) *

Pour the plates.

*1M Potassium Phosphate is made by mixing 132 ml of 1M K_2HPO_4 and 868 ml of 1M KH_2PO_4 or 1M KH_2PO_4, pH 6.0 (adjusted by KOH).

LB plates with Ampicillin and Tetracycline (1 L)

5	g	Yeast extract
10	g	NaCl
10	g	Tryptone peptone
1	L	ddH2O

Adjust pH to 7.2 with 5 M NaOH.

Add:

15	g	Agar

Autoclave and cool down to ~50 °C, then add:

1	ml	100 mg/ml Ampicillin
1	ml	12.5 mg/ml Tetracycline

Pour the plates.

Bleaching solution (1 L)

300	ml	Sodium hypochlorite, 5% Chlorine (ACROS organics 419550010)
150	ml	5M KOH (store in plastic bottle)
550	ml	ddH_2O

Store at 4C.

Worm lysis buffer for genotyping (50 ml)

625	µl	4M KCl (52 mM final)
500	µl	1M Tris pH 8.3 (10 mM final)
125	µl	1M $MgCl_2$ (2.5 mM final)
2.25	ml	10% NP40 (0.45% final)
225	µl	Tween 20 (0.45% final)
1.25	ml	2% Gelatin (0.05% final)
45.25	ml	ddH_2O

Prepare aliquots and store at 4 ºC.

8.2 Standard cloning protocol

Preparation of insert and vectors

Insert from a plasmid source

- Digest plasmid with the appropriate restriction enzymes to produce a DNA fragment that can be cloned directly into a vector. Unidirectional cloning is achieved with restriction enzymes that produce non-compatible ends.

Insert from a PCR product

- Design primers with appropriate restriction sites to clone unidirectionally into a vector
- Addition of 6 bases upstream of the restriction site is sufficient for digestion with most enzymes
- If fidelity is a concern, choose a proofreading polymerase such as Q5 High-Fidelity DNA Polymerase (NEB #M0491)
- Visit www.NEBPCRPolymerases.com for additional guidelines for PCR optimization
- Purify PCR product by running the DNA on an agarose gel and excising the band or by using a spin column (NEB #T1030, NEB #T1020)
- Digest with the appropriate restriction enzyme

Standard Restriction Enzyme Protocol

Restriction Enzyme	10 units is sufficient, generally 1µl is used
DNA	1 µg
10X NEBuffer	5 µl (1X)
Nuclease-free Water	To 50 µl

Incubation Time	1 hour*
Incubation Temperature	Enzyme dependent

* Can be decreased by using a Time-Saver Qualified enzyme.

Time-Saver Restriction Enzyme Protocol

Restriction Enzyme	1µl
DNA	1 µg
10X NEBuffer	5 µl (1X)
Nuclease-free Water	To 50 µl
Incubation Time	5-15 minutes*
Incubation Temperature	Enzyme dependent

* Time-Saver qualified enzymes can also be incubated overnight with no star activity.

Insert from annealed oligos

- Annealed oligos can be used to introduce a fragment (e.g., promoter, polylinker, etc.)
- Anneal two complementary oligos that leave protruding 5´ or 3´ overhangs for ligation into a vector cut with the appropriate enzymes
- Non-phosphorylated oligos can be phosphorylated using T4 Polynucleotide Kinase (NEB #M0201)

Typical Annealing Reaction

Primer	1 µg
10X T4 Ligase Buffer	5 µl
Nuclease-free Water	To 50 µl
Incubation	85°C for 10 minutes, cool slowly (30-60 min.)

Vector

- Digest vector with the appropriate restriction enzymes. Enzymes that leave non-compatible ends are ideal as they prevent vector self-ligation.

Dephosphorylation

- Dephosphorylation is sometimes necessary to prevent self-ligation. NEB offers four products for dephosphorylation of DNA:

    - Quick CIP (NEB #M0525), Shrimp Alkaline Phosphatase (rSAP) (NEB #M0371) and Antarctic Phosphatase (AP) (NEB #M0289) are heat-inactivated phosphatases.

Dephosphorylation of 5´ ends of DNA using Quick CIP

DNA	1 pmol of DNA ends
10X CutSmart Buffer	2 µl

Quick CIP	1 µl
Nuclease-free Water	To 20 µl
Incubation	37°C for 10 minutes
Heat Inactivation	80°C for 2 minutes

Dephosphorylation of 5' ends of DNA Using Shrimp Alkaline Phosphatase (rSAP)

rSAP Reaction Buffer (10X)	2 µl
DNA	≥ 1 pmol of DNA ends (about 1 µl of 3 kb plasmid)
rSAP (1 unit/ µl)	1 µl
Nuclease-free Water	To 20 µl
Incubation	37°C for 30 minutes
Heat Inactivation	65°C for 5 minutes

Note: Scale larger reaction volumes proportionally.

Blunting

- In some instances, the ends of the insert or vector require blunting
- PCR with a proofreading polymerase will leave a predominantly blunt end

- T4 DNA Polymerase (NEB #M0203) or Klenow (NEB #M0210) will fill in a 5´ overhang and chew back a 3´ overhang
- The Quick Blunting Kit (NEB #E1201) is optimized to blunt and phosphorylate DNA ends for cloning in less than 30 minutes
- Analyze agarose gels with longwave UV (360 nM) to minimize UV exposure that may cause DNA damage

Blunting with the Quick Blunting Kit

Blunting Buffer (10X)	2.5 µl
DNA	Up to 5 µg
dNTP Mix (1mM)	2.5 µl
Blunt Enzyme Mix	1 µl
Nuclease-free Water	To 25 µl
Incubation	15 minutes for RE-digested DNA/sheared or 30 minutes for nebulized DNA or PCR products
Heat Inactivation	70°C for 10 minutes

* PCR-generated DNA must be purified before blunting using a commercial purification kit (NEB #T1030), phenol extraction/ethanol precipitation or gel electrophoresis and subsequent extraction (NEB #T1020)

Phosphorylation

- For ligation to occur, at least one of the DNA ends (insert or vector) should contain a 5´ phosphate

- Primers are usually supplied non-phosphorylated; therefore, the PCR product will not contain a 5´ phosphate
- Digestion of DNA with a restriction enzyme will always produce a 5´ phosphate
- A DNA fragment can be phosphorylated by incubation with T4 Polynucleotide Kinase (NEB #M0201)

Phosphorylation With T4 PNK

T4 PNK	1 µl (10 units)
10X T4 PNK Buffer	5 µl
10 mM ATP	5 µl (1 mM final conc.)
DNA (20 mer)	1-2 µg
Nuclease-free Water	To 50 µl
Incubation	37°C for 30 minutes

Purification of Vector and Insert

- Purify the vector and insert before ligation by either running the DNA on an agarose gel and excising the appropriate bands or using a spin column (NEB #T1020, NEB #T1030)
- DNA can be purified using β-Agarase I (NEB #M0392) with low melt agarose or an appropriate spin column or resin
- Analyze agarose gels with longwave UV (360 nM) to minimize UV exposure that may cause DNA damage

Ligation of Vector and Insert

1. Use a molar ratio between 1:1 and 1:10 of vector to insert (1:3 is typical). Use NEBioCalculator to calculate molar ratios.

2. If using T4 DNA Ligase (NEB # M0202) or the Quick Ligation™ Kit (NEB #M2200), thaw and resuspend the Ligase Buffer at room temperature. If using Ligase Master Mixes, no thawing is necessary.

3. The Quick Ligation™ Kit (NEB #M2200) is optimized for ligation of both sticky and blunt ends

4. Instant sticky-end Ligase Master Mix (NEB #M0370) is optimized for instant ligation of sticky/cohesive ends

5. Blunt/TA Ligase Master Mix (NEB #M0367) is optimized for ligation of blunt or single base overhangs, which are the more challenging type of ends for T4 DNA Ligase

6. Following ligation, chill on ice and transform

7. DO NOT heat inactivate when using the Quick Ligation Buffer or Ligase Master Mixes as this will inhibit transformation

8. Electroligase (NEB #M0369) is optimized for ligation of both sticky and blunt ends and is compatible with electroporation (i.e., no cleanup step required)

9. Improved Golden Gate Assembly can be achieved by selecting high fidelity overhangs [Potapov, V., et al (2018) *ACS Synth. Biol.* 2018, 7, 11, 2665-2674, https://doi.org/10.1021/acssynbio.8b00333i]

The following three tables show ligation using a molar ratio of 1:3 vector to insert for the indicated DNA size. Use NEBioCalculator to calculate molar ratios.

Ligation with The Quick Ligation™ Kit

Quick T4 DNA Ligase	1 µl

2X Quick Ligation Buffer	10 µl
Vector DNA (3 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease-free Water	20 µl (mix well)
Incubation	Room temperature for 5 minutes

Ligation with Instant Sticky-end Ligase Master Mix

Master Mix	5 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	50 ng
Nuclease-free Water	To 10 µl
Incubation	None

Ligation with Blunt/TA Ligase Master Mix

Master Mix	5 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	50 ng
Nuclease-free Water	To 10 µl

Incubation	Room temperature for 15 minutes

Transformation

- To obtain transformants in 8 hrs., use NEB Turbo Competent *E. coli* (NEB #C2984)
- If recombination is a concern, then use the *recA*$^-$ strains NEB 5-alpha Competent *E. coli* (NEB #C2987), NEB-10 beta Competent *E. coli* (NEB #C3019) or NEB Stable Competent *E. coli* (NEB #C3040)
- NEB-10 beta Competent *E. coli* works well for constructs larger than 5 kb
- NEB Stable Competent *E. coli* (NEB #C3040) can be used for constructs with repetitive sequences such as lentiviral constructs
- If electroporation is required, use NEB 5-alpha Electrocompetent *E. coli* (NEB #C2989) or NEB 10-beta Electrocompetent E. coli (NEB #C3020)

Transformation with NEB 5-alpha Competent *E. coli*

DNA	1-5 µl containing 1 pg-100 ng of plasmid DNA
Competent *E. coli*	50 µl
Incubation	On ice for 30 minutes
Heat Shock	Exactly 42°C for exactly 30 seconds
Incubation	On ice for 5 minutes Add 950 µl room temperature SOC 37°C for 60 minutes, with shaking

8.3 Raw data

8.3.1 Developmental assay

	Wild Type	***smg-1(tm849)***	**P-value**	***smg2(tm6028)***	**P-value**
Egg hatches	0	0	-	0	-
L1	11.5	16	<0 .00001	14	0.000662
L4	41	48	< 0.00001	44	<0 .00001
Young adult	48	52	0.002664	50	0.011323
Egg-laying starts	53	58	0.000045	56	0.000662

8.3.2 Survival assay

Average number of eggs after three experiments:

X-ray at L1	**Ionizing Radiation**	**SD**	**Control**	**SD**
Wild Type	45.66667	11.08553	75	8.602325
smg2(tm6028)	22.66667	1.247219	59	11.77568
smg-1(tm849)	19	7.348469	49.66667	8.956686

X-ray at L4	Ionizing Radiation	SD	Control	SD
Wild Type	42.66667	11.55	68	2.828427125
smg2(tm6028)	18.33333	8.498366	56.33333	4.496912521
smg-1(tm849)	19.33333	5.906682	50	10.8012345

8.3.3 HR and SSA reporter assays

SSA:

	Mock RNAi	*smg-1*	*smg2*
1st	30.0%	50.0%	55.0%
2nd	15.0%	35.0%	35.0%
3rd	25.0%	50.0%	40.0%
Average	**23.33%**	**45.00%**	**43.33%**
SD	6.2%	7%	8.4%

HR/NHEJ:

	smg-1	*smg2*	Mock RNAi
1st	52.9 %	47.6 %	12.31%
2nd	39.3 %	50.0 %	4.00%
3rd	40.0 %	30.0 %	12.00%

4th	39.1 %	37.3 %	4.00%
Average	**42.8369%**	**41.2421%**	**8.07%**
SD	5.8%	8%	4%

8.3.4 RT-qPCR

L1	*smg-1*	*smg-2*	*smg-3*	*smg-4*	*smg-5*
	1.028114	2.219139	1.505247	0.959264	0.920188
	1.013959	1.853176	1.164734	0.926588	1.433955
	2.378414	3.24901	0.61132	0.002291	1
Average	***1.473496***	***2.440442***	***1.093767***	***0.629381***	***1.118048***
SD	0.6399	0.590942	0.368378	0.44362	0.225744
P-value	0.177209	0.013061	0.368534	0.151426	0.250317

L4	0.707107	0.939523	0.566442	0.429283	0.521233
	0.517632	0.888843	0.562529	0.5	0.598739
	0.332171	0.586417	0.145592	0.476319	0.392292
Average	***0.51897***	***0.804928***	***0.424854***	***0.468534***	***0.504088***
SD	0.15307	0.155889	0.197475	0.02939	0.085149
P-value	0.00564	0.075748	0.007313	0.00001	0.000592