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Potential of using CRISPR-CAS9 to treat BRCA1 and BRCA2 associated hereditary breast cancer

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POTENTIAL OF USING CRISPR-CAS9 TO TREAT BRCA1 AND BRCA2 ASSOCIATED HEREDITARY BREAST CANCER

by

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DEDICATION

I would like to dedicate this work to Swami, my wonderful parents Satish and Aruna, and Siddharth.

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I want to thank my readers, Dr. Atkinson and Dr. Engel, for their continuous support and encouragement through this process.

POTENTIAL OF USING CRISPR-CAS9 TO TREAT BRCA1 AND BRCA2 ASSOCIATED HEREDITARY BREAST CANCER

PREMSAI NAGABHYRAVA

ABSTRACT

Breast cancer is one of the major causes of death. About 22.9% of all cancer in women is breast cancer (Pohlreich et al., 2005). Although there are many known risk factors, genetics play a major role in breast cancer. Of which, germ-line mutations in BRCA1(BReast-CAncer susceptibility gene 1) and BRCA2 (BReast-CAncer susceptibility gene 2) genes are responsible for about 90% of all hereditary breast cancer cases, as well as most of hereditary ovarian cancer cases (Calderón-Garcidueñas et al., 2005). The BRCA1 and BRCA2 genes code for Tumor Suppressor Gene proteins which act as cell growth suppressors. BRCA1 and BRCA2 also help repair damaged DNA (Mehrgou & Akouchekian, 2016). Damage to one of these two genes could result in impaired repair of damaged DNA, which can lead to the accumulation of unrepaired DNA mutations and eventually lead to cancer. (Mehrgou & Akouchekian, 2016). The most common mutation forms for BRCA1 and BRCA2 are small insertions, deletions, nonsense mutations, missense mutations that cause premature transcription terminations and altered splicing (Pylkäs et al., 2008). Breast Information Core reports that the majority of cancerous mutations in BRCA1 and BRCA2 genes result in the generation of a truncated protein through nonsense, frameshift, and splicing mutations (Pohlreich et al., 2005). There is significant morbidity and mortality associated with BRCA1 and BRCA2-associated hereditary breast cancer.

Clustered regularly interspersed short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas), combined as CRISPR/Cas9 has garnered tremendous interest in the scientific community for its myriad applications from diagnostics, imaging, and most importantly in genome editing (Zhan et al., 2019). Two RNAs activate and guide Cas9, a DNA endonuclease, to bind specific DNA sequences. Cas9, then, subsequently cleaves the DNA at that site (Zhan et al., 2019). When a double-strand break is introduced in the target sequence by the Cas9 endonuclease, there are two different mechanisms of repair that are used by the host cell to respond. (1) The host cell may respond with a non-homologous end joining (NHEJ), a repair mechanism that often leads to insertions or deletions. This may be beneficial if the study, in question, aims to render a target gene functionless. (2) The second method is via homology-directed repair which recombines DNA donor templates to reconstruct damaged DNA (Zhan et al., 2019). This feature is helpful as it allows for site-specific editing, if the target gene in question, contains a mutation that leads to reduced expression, no expression, or results in cancer. In this thesis, I wish to explore the use of CRISPR-Cas9 as a possible tool for preventative therapy in BRCA1 and BRCA2-associated hereditary breast cancer.

The specific aims of the following thesis include:

1. A comprehensive review of literature on CRISPR-Cas9 and breast cancer, specifically due to mutations in BRCA1 and BRCA2 genes.
2. Investigation into current evidence for CRISPR-Cas9 use in diseases.
3. Conclusion on the challenges and limitations of CRISPR-Cas9 as a potential tool for preventative therapy for BRCA1 and BRCA2- associated hereditary breast cancer.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BRCA1	Breast-Cancer Susceptibility Gene 1
BRCA2	Breast-Cancer Susceptibility Gene 2
AAV	Adeno-Associated Virus
APLF	Aprataxin-And-PNK-Like Factor
BARD1	BRCA1-Associated RING Domain Protein
BER	Base Excision Repair
Cas	CRISPR-Associated Proteins
CRISPR	Clustered Regularly Interspersed Short Palindromic Repeats
CrRNA	CRISPR Targeting RNA
DALY	Disability-Adjusted Life Years
DCIS	Ductal Carcinoma *in Situ*
DNA PK	DNA-Dependent Protein Kinase
DNA PKcs	DNA-Dependent Protein Kinase Catalytic Subunit
DSB	Double-Stand Breaks
ENIGMA	Evidence-Based Network for The Interpretation of Germline Mutation Alleles
ER	Estrogen Receptor
HER2	Human Epidermal Growth Factor Receptor 2
HR	Homologous Recombination
IBS	Invasive Breast Cancer
IHC	Immunohistochemistry

LCIS	Lobular Carcinoma *in Situ*
Lig4	DNA Ligase 4
LNP	Lipid Nanoparticle
NHEJ	Non-Homologous End Joining
NUC	Nuclease
PALB2	Partner and Localizer of BRCA2
PAM	Protospacer Adjacent Motif
PD-1	Programmed Cell Death Protein 1
PR	Progesterone Receptor
REC	Recognition
RING	Really Interesting New Gene
ROS	Reactive Oxygen Species
RPA	Replication Protein A
SCD	Sickle Cell Disease
SCF	Skp-Cullin-Fbox
SgRNA	Single Guide RNA
Spcas9	Cas Protein from Streptococcus Pyogenes
TDLU	Terminal Duct Lobular Units
TDT	Beta-Thalassemia
TracrRNA	Trans-Activating RNA
TTR	Transthyretin
XLF	XRCC4-Like Factor

XRCC4	X-Ray Repair Cross Complementing 4

LITERATURE REVIEW

Epidemiology

The term cancer encompasses a large number of diseases characterized by uncontrolled cell proliferation. These abnormal cells have the ability to infiltrate and destroy normal body tissue, often resulting in death. Cancer is the second-leading cause of death in the world (Mayo Clinic, 2018). Cancers are classified based on the tissue of origin, e.g., breast cancer refers to a cancer that is initiated in breast tissue cells. In 2021, breast cancer became the most prevalent cancer in the world. It accounted for 12% of all new annual cancer cases (Breast Cancer Facts and Statistics, 2022) as illustrated by **Figure 1**.

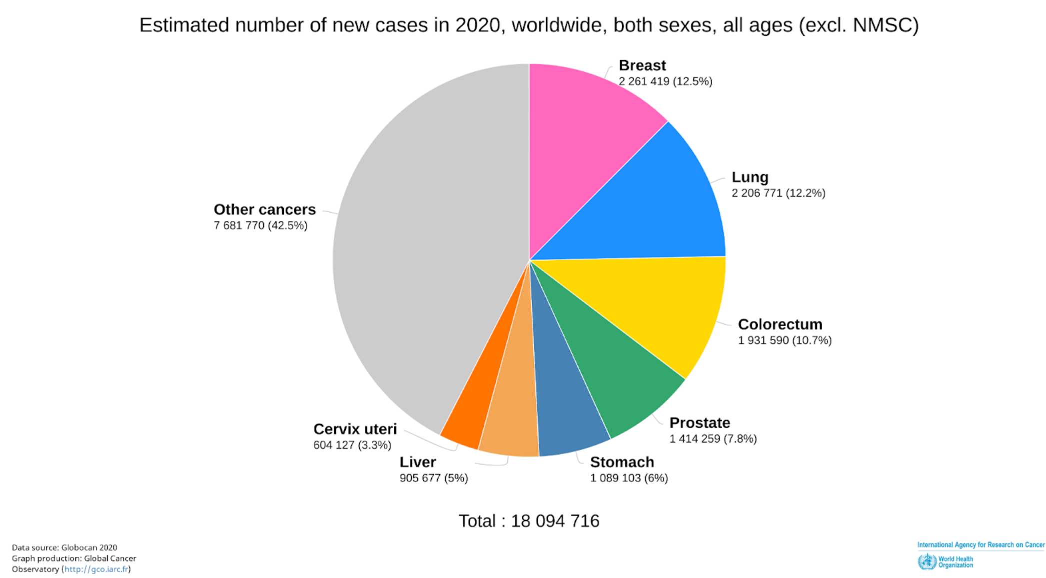

Figure 1. Estimated number of new cases in 2020, worldwide, for both sexes, all ages. This pie chart illustrates the breakdown of new cancer cases worldwide. As illustrated above, approximately 12% of all new cancer cases are breast cancers. Data from (Global Cancer Observatory: Cancer Today, 2020).

Globally about 2.3 million women were diagnosed with breast cancer and about 685,000 deaths occurred from breast cancer in 2020 (WHO, 2021). Although there is a higher prevalence of breast cancer in developed countries, developing countries seem to have higher mortality rates. Some examples are shown in **Table 1**.

Table 1. Age-standardized mortality rates per 100,000 of breast cancer. Data from (Momenimovahed & Salehiniya, 2019).

Mortality (age-standardized rate per 100,000) of breast cancer	
Regions	Mortality Rates (per 100,000)
Less Developed Regions	11.5
More Developed Regions	14.9
Australia/New Zealand	14.5
Northern America	14.8
Western Europe	16.2
Northern Europe	16.4
Eastern Asia	6.1
South-Central Asia	13.5

Multiple reasons account for the higher mortality rates in developing countries. Unlike developing countries, countries with high-income have better promotion of breast cancer management as well as advanced therapeutic and diagnostic methods. These interventions seem to dramatically reduce the mortality rate in such countries (Momenimovahed & Salehiniya, 2019).

In 2021, breast cancer is the most prevalent cancer among American women, except for skin cancer, according to the American Cancer Society (Breast Cancer Facts and Statistics, 2022). It is estimated that about 1 in 3 of all newly diagnosed female cancers in the USA will be breast cancer. Currently, breast cancer death rates are higher than those for any other cancer, besides lung cancer (Breast Cancer Facts and Statistics, 2022). Breast cancer causes more lost disability-adjusted life years (DALY) in women than any other type of cancer around the world (WHO, 2021). DALY is used to assess the overall burden of disease. It is a time-based measure that computes the years of life lost due to premature mortality and time lived in states of less than full health.

Male breast cancer is a rare malignancy. Yearly, 1,500 new cases are diagnosed in the United States (Giordano et al., 2002). Less than one percent of all breast cancers and less than one percent of all cancers in men constitute male breast cancer (Abdelwahab Yousef, 2017). Some of the risk factors for male breast cancer include testicular disease, advanced age, benign breast conditions, Jewish ancestry, BRCA2 mutation, family history, and Klinefelter syndrome (Abdelwahab Yousef, 2017).

Anatomy of the Breast

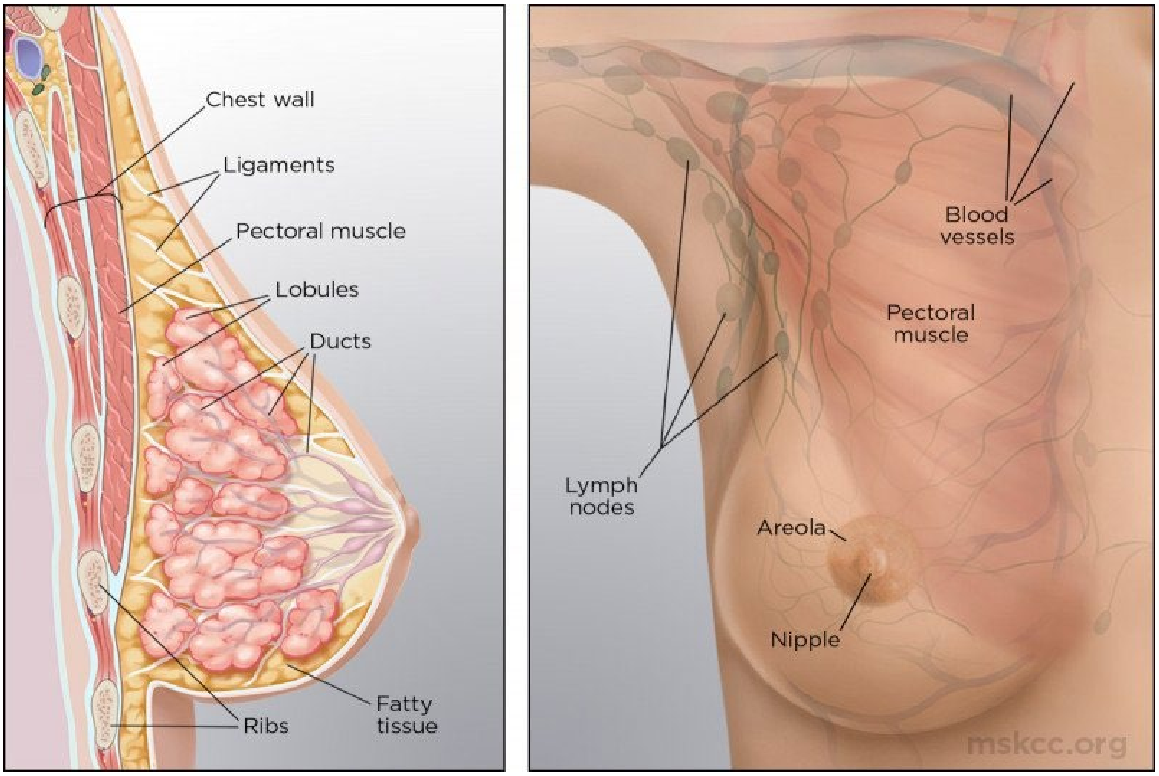

Figure 2. Image of the anatomy of the breast. The image on the left illustrates three main components of the breast- glandular tissue (lobules/ducts), adipose tissue (fatty tissue), and connective tissue (ligaments). Taken from (Anatomy of the Breast, 2020)

The breast is divided into three parts: glandular tissue, adipose tissue, and connective tissue illustrated in **Figure 2**. The glandular tissue contains a branching ductal network formed through a process of branching morphogenesis (Gudjonsson et al., 2005). It consists of stratified epithelium with two dissimilar cell populations: an outer layer of myoepithelial cells and an inner layer of polarized epithelial cells (Gudjonsson et al., 2005) as illustrated by **Figure 3**. The stratified epithelium is enclosed by a basement membrane and supported by a framework of connective tissue consisting of blood vessels, lymphatic, and stromal cells (Akram et al., 2017).

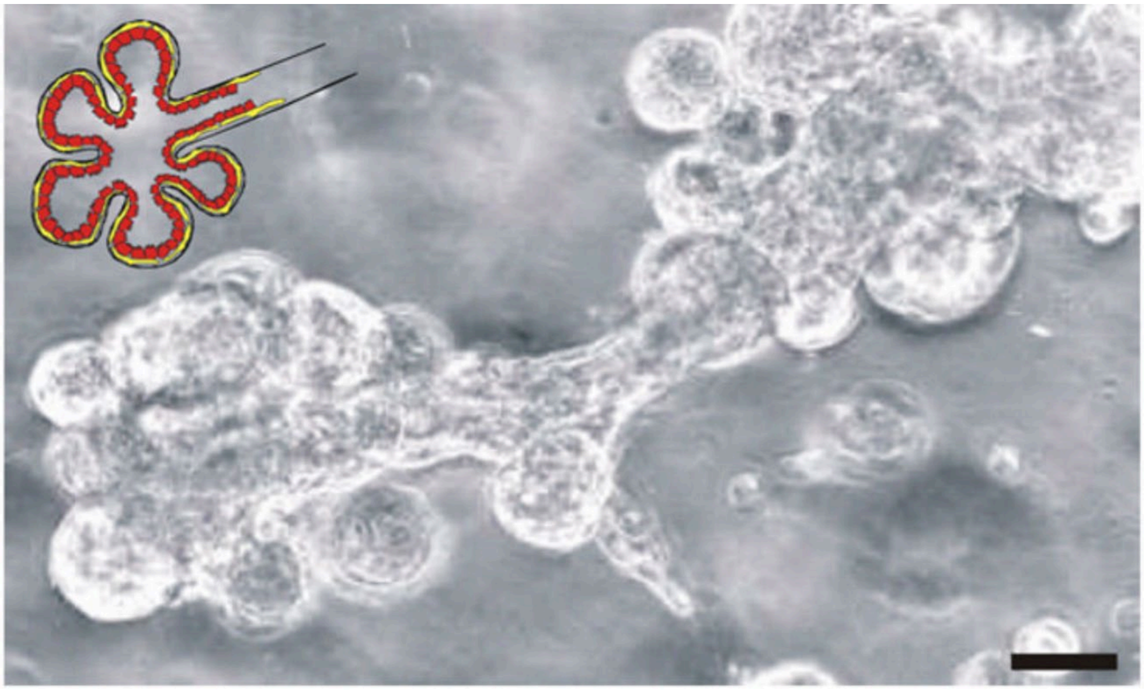

Figure 3. Terminal duct lobular units (TDLU) *in vivo*. A schematic drawing of TDLU is also present on the top left. The schematic drawing illustrates myoepithelial cells in yellow surrounding polarized luminal epithelial cells in red. The black line represents the basement membrane. Taken from (Gudjonsson et al., 2005).

The adipose tissue fills in the space between glandular and connective tissue. (Anatomy of the Breast, 2020). Female breasts contain about 12-20 lobes which further divide and terminate as terminal duct lobular units (Akram et al., 2017). Terminal duct lobular units contain secretory acini that function to secrete milk during lactation (Gudjonsson et al., 2005). The lobes and lobules are linked via milk ducts. About 15-20 milk ducts carry milk from glandular tissue to nipples (Akram et al., 2017). The branching ductal network is illustrated in **Figure 4**.

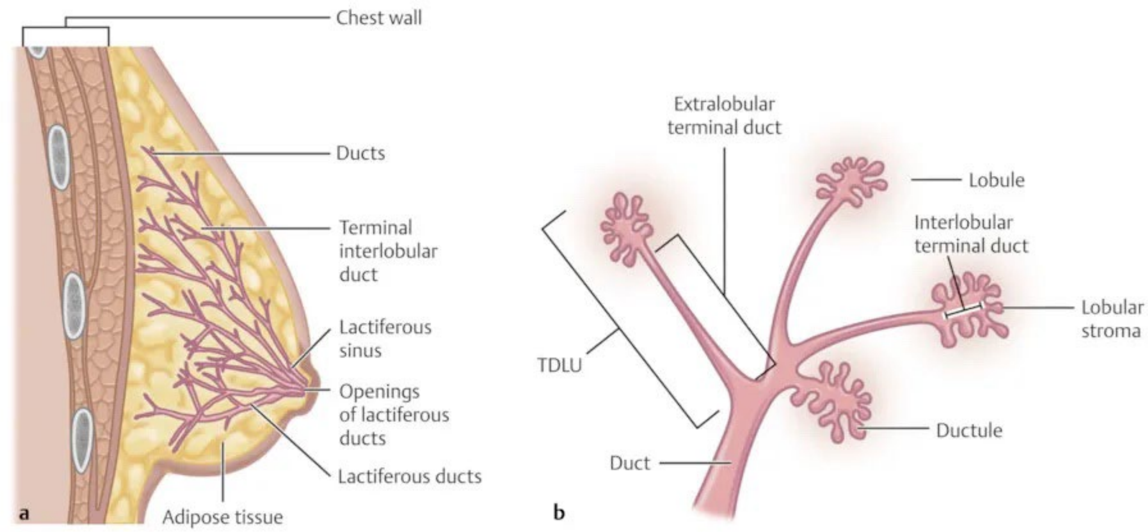

Figure 4. The branching ductal network of a female breast. The fundamental unit of the breast is a terminal duct lobular unit (TDLU). Any secretions from TDLUs flow into 12-20 lobes. The lobes and lobules are linked via milk ducts. About 15-20 milk ducts carry milk from glandular tissue to nipples. Taken from (Themes, 2021).

Until puberty, the male and female breasts are largely identical. However, during puberty, the increase in testosterone levels in males causes involution and atrophy of the ducts. This results in a poorly developed ductal system surrounded by adipose and connective tissue (Chau et al., 2016).

Histopathology

There are two types of breast cancer: invasive and non-invasive (Alkabban & Ferguson, 2018).

Non-invasive breast cancer refers to a cancer that does not extend outside of the lobule or duct in which it is situated (Akram et al., 2017). Non-invasive breast cancer can be classified into two categories: ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). The term "*in situ*" means "in place." Although LCIS and DCIS contain atypical cells that do not extend to tissues outside the lobules and ducts, respectively, these non-invasive breast cancers have the potential to develop into invasive breast cancers (Akram et al., 2017). LCIS and DCIS are illustrated in **Figures 5** and **6**.

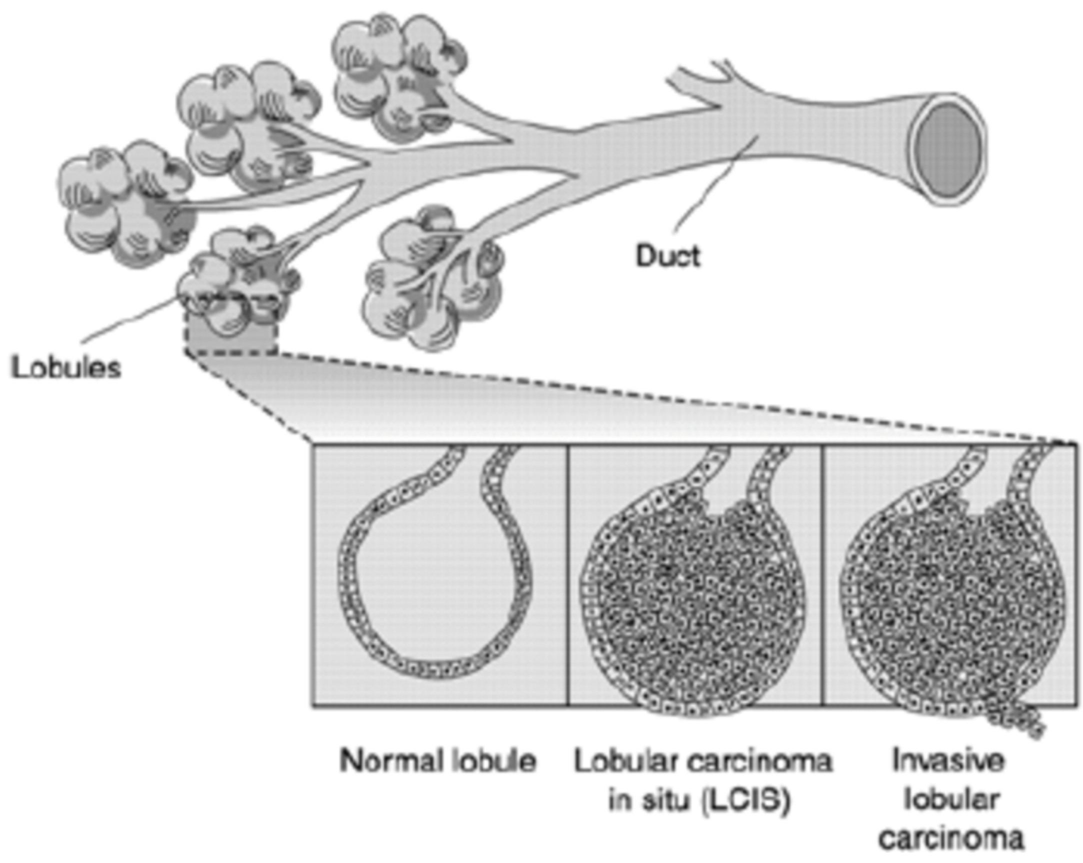

Figure 5. Lobular Carcinoma *in Situ* (LCIS). This type of carcinoma begins in the terminal duct lobular units as illustrated. Unlike LCIS, invasive lobular carcinoma extends to tissues outside the lobules. Taken from (Shah. et al., 2011).

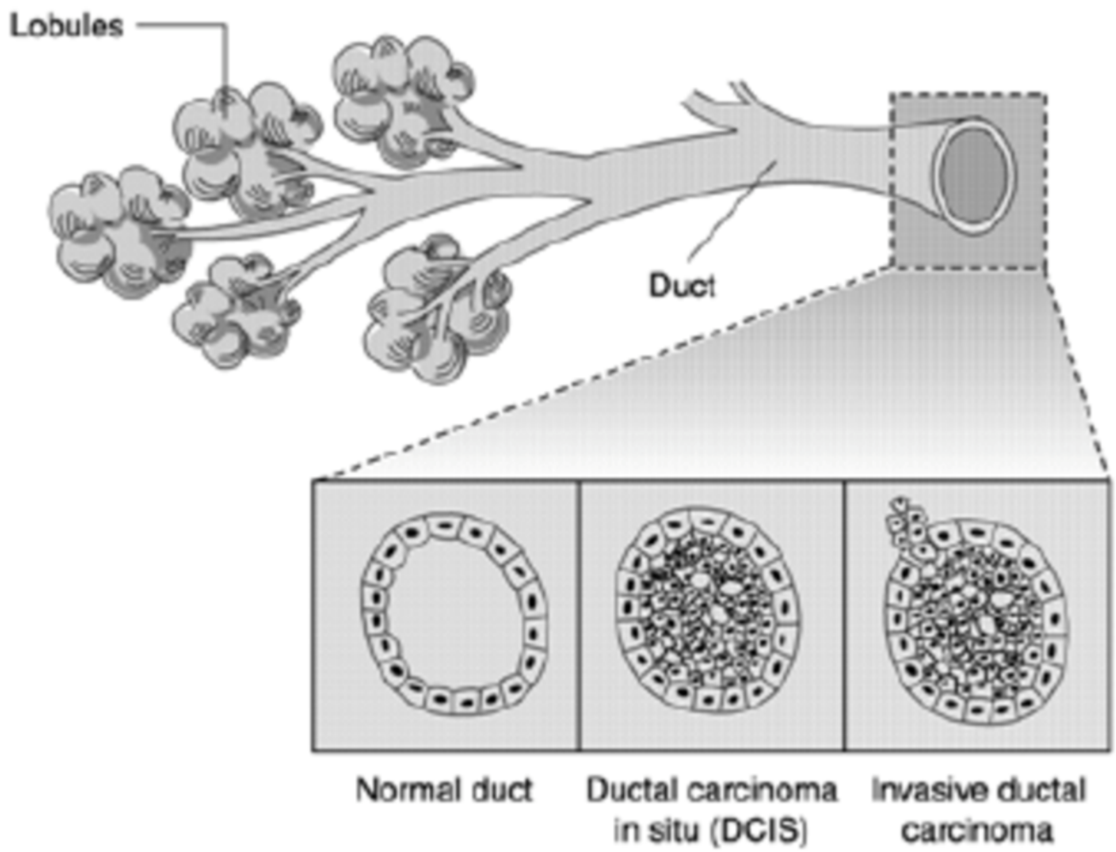

Figure 6. Ductal carcinoma *in Situ* (DCIS). This type of carcinoma is centered around the milk ducts. Unlike DCIS, invasive ductal carcinoma extends to the tissues outside the ducts. Taken from (Shah. et al., 2011).

LCIS is neoplastic cell proliferation centered around terminal duct lobular units, with enlarged and filled acini (Alkabban & Ferguson, 2018). DCIS, on the other hand, is a neoplastic cell proliferation within the milk ducts with normal myoepithelial cells at the innermost layer of the epithelium (Alkabban & Ferguson, 2018). Data obtained between 2012-2016 shows that DCIS constitutes 16% of all breast cancer diagnoses (American

Cancer Society, Breast cancer facts & figures). Unlike LCIS, DCIS is morphologically heterogeneous (Alkabban & Ferguson, 2018). Cribriform, comedo, papillary and solid are four broad types of DCIS. These classifications are based on the histological growth pattern, which are illustrated by **Figure 7**. Cribriform and papillary are the less aggressive forms of DCIS while the solid and comedo types have a greater malignant potential (Vaidya et al., 2015). If left untreated, DCIS has the potential to turn into invasive cancer. According to Cancer.org, only 20-53% of women with untreated DCIS were ultimately diagnosed with invasive breast cancer (American Cancer Society, Breast cancer facts & figures).

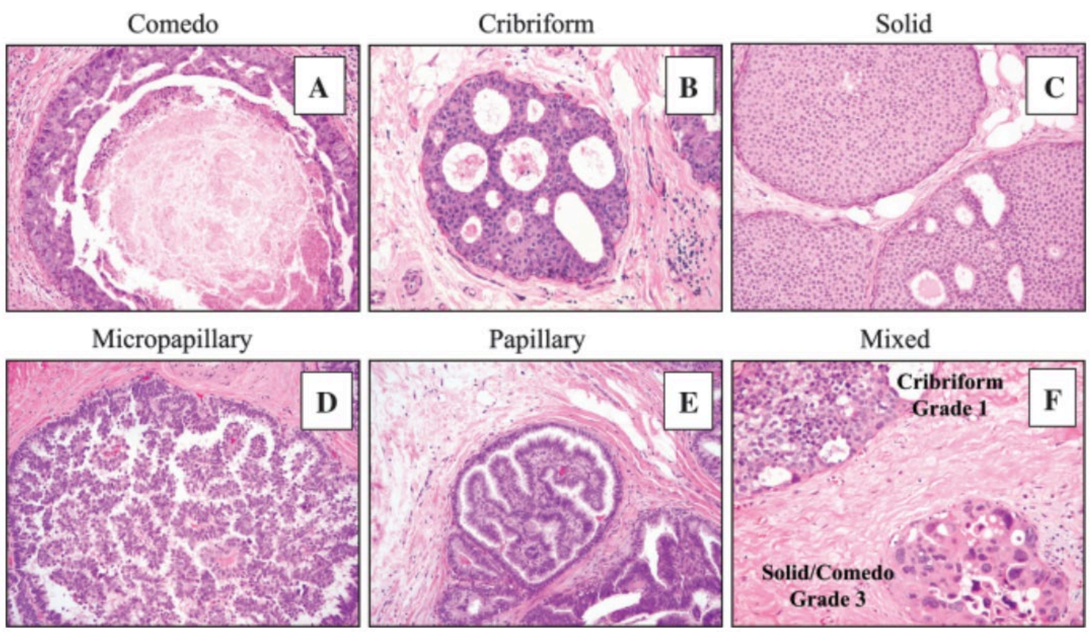

Figure 7. Different classifications of ductal carcinoma *in situ*. These classifications are determined by histologic growth patterns; this includes (a) comedo (b) cribriform (c) solid (e) papillary. Taken from (Allred, 2010).

Invasive cancer contains atypical cells that extend outside the basement membrane of the lobules or ducts where they originated and grow into the connective and adipose tissue (Sharma et al., 2010). Histologically, these types of cancers do not have an overall architecture. Invasive breast cancer is histologically illustrated by **Figure 8**. There are two types of invasive cancers: invasive ductal carcinoma and invasive lobular carcinoma (Sharma et al., 2010). Invasive ductal carcinoma originates in the milk ducts but penetrates the basement membrane and invades the adipose tissue (Akram et al., 2017). Similarly, invasive lobular carcinoma begins in the lobules but spreads beyond its origination point (Akram et al., 2017). Invasive ductal carcinoma often grows as a cohesive mass while invasive lobular cancer permeates the breast in a single-file nature. This difference results in easier detection of invasive ductal carcinoma while invasive lobular carcinoma remains clinically undetectable on mammography (Alkabban & Ferguson, 2018). Histologically, about 75% of all invasive breast cancers are categorized as invasive ductal carcinomas while only 15% of invasive breast cancers are categorized as invasive lobular carcinomas (American Cancer Society, Breast cancer facts & figures). Once invasive breast cancer spreads to the various organs in the body, it is referred to as metastatic breast cancer.

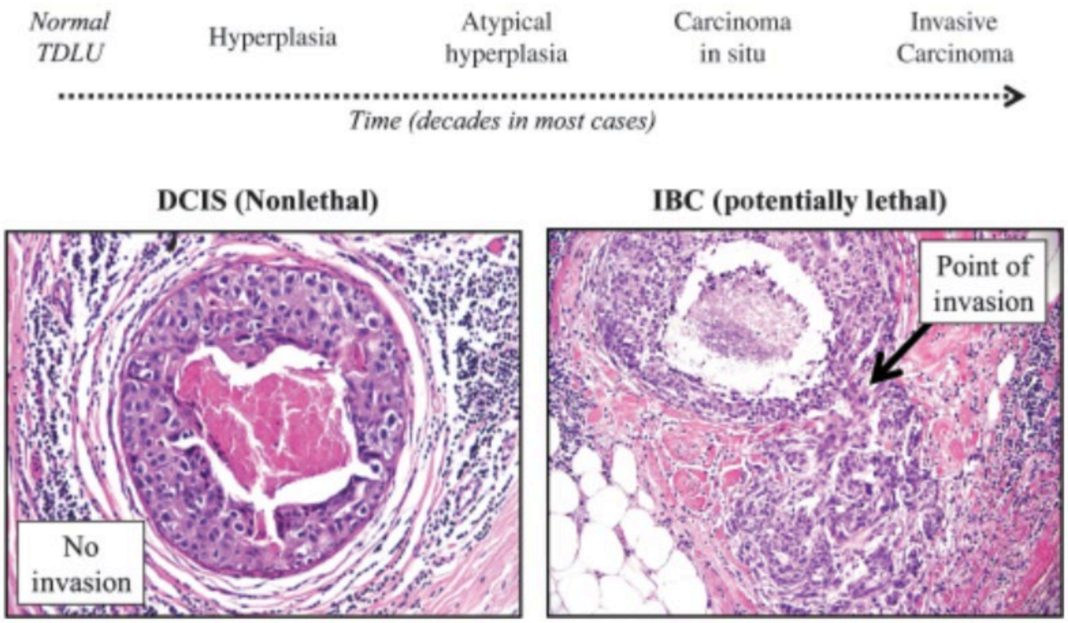

Figure 8. The progression from ductal carcinoma *in situ* to invasive breast cancer. Unlike DCIS, IBC (invasive breast cancer) extends outside the basement membrane of the lobule or duct into the connective or adipose tissue. The point of invasion demonstrates the exact location in which the carcinoma has broken past the basement membrane into the surrounding connective tissue which classifies this type of breast cancer as invasive. Taken from (Allred, 2010).

In addition to the histological subtypes mentioned above, breast cancer molecular subtypes are another important factor for treatment strategy decision-making. There are four subtypes; Luminal A, Luminal B, HER2 and Basal (see **Table 2**). These subtypes are based on the expression of biological markers in cancer cells. Breast cancers that are estrogen receptor positive (ER+) contain receptors that use the hormone estrogen to grow (Mayo Clinic, 2018). Similarly, cancers that are progesterone receptor positive (PR+) are receptive to progesterone. They contain receptors that use progesterone to grow. In addition to ER+ and PR+, human epidermal growth factor receptor 2 (HER2) provides prognostic and predictive information for breast cancer. HER2 is a membrane tyrosine kinase receptor that, when activated, provides cells with potent proliferative and anti-apoptotic signals. Cancer cells often contain too many copies of the HER2 gene, resulting in overexpression and gene amplification. These increases cause tumor development and progression (Gutierrez & Schiff, 2011). A new and controversial biomarker, Ki-67, is also used to describe molecular subtypes. Ki-67 is a nuclear protein expressed during all phases of the cell cycle, excluding the resting state of G0 phase (Zhu et al., 2020). Thus, Ki-67 expression is proposed to measure cell proliferation in breast cancer. High levels of Ki-67 (>14%) experimentally correlate to greater proliferation and poorer prognosis (Zhu et al., 2020).

Luminal A subtype cancers are the most common type of breast cancer with the most favorable prognosis (American Cancer Society, Breast cancer facts & figures). They are breast cancers that are hormone-receptor positive (HR+; estrogen-receptor and/or progesterone-receptor positive) and HER2 negative with low levels of Ki-67 index (Hu et al., 2021). Luminal B subtypes are higher grade than luminal A with a slightly worse prognosis (American Cancer Society, Breast cancer facts & figures). They are HR+ and HER2 positive or negative with high levels of Ki-67 index (Hu et al., 2021). The more aggressive subtype with a severe prognosis is basal-life or triple-negative. They are HR- and HER2 negative (Hu et al., 2021). This type of cancer is more common among African American women, premenopausal women, and those with BRCA1 gene mutation (American Cancer Society, Breast cancer facts & figures). Finally, HER2-enriched subtypes are HR- but HER2 positive (Hu et al., 2021). This information is depicted in **Table 2**.

Table 2. Molecular subtypes of breast cancer. There are four major subtypes: luminal A, luminal B, HER2- enriched, and basal-like. Each subtype varies based on immunohistochemistry (IHC). Taken from (Grimm et al., 2015).

Subtype	Receptor Status
Luminal A	ER positive and/or PR positive, HER2 negative
Luminal B	ER positive and/or PR positive, HER2 positive
HER2	ER negative and PR negative, HER2 positive
Basal	ER negative, PR negative, and HER2 negative

Pathogenesis of Breast Cancer

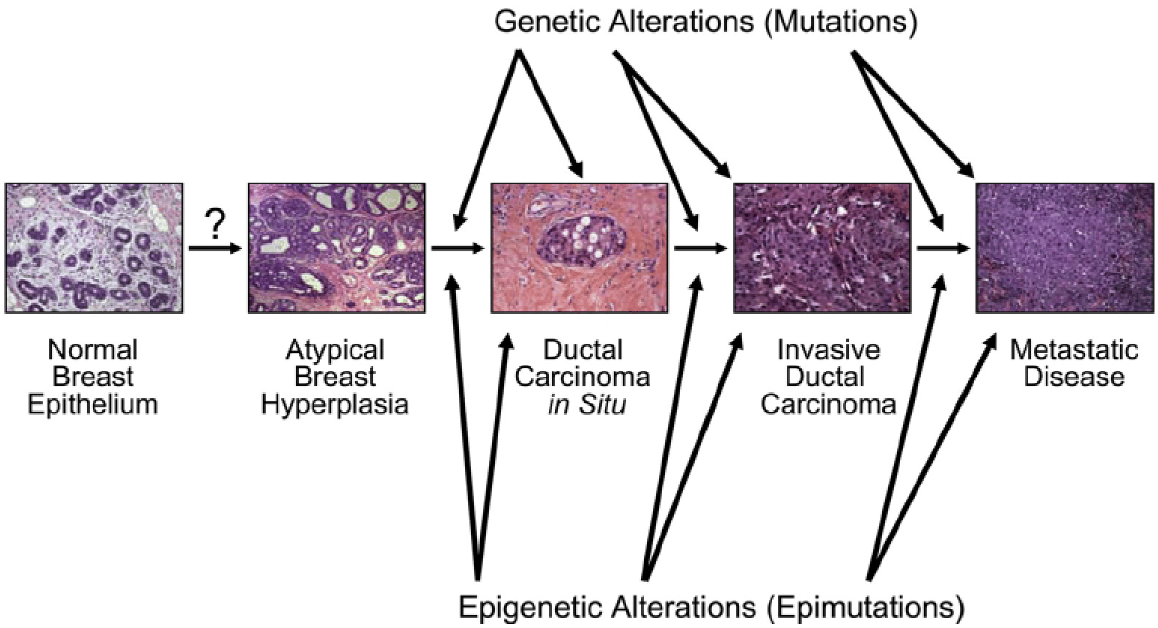

Figure 9. Natural history of breast cancer development. Genetic mutations of normal breast epithelial cells often result in atypical hyperplasia. If the immune system or other protective mechanisms fail to regulate, atypical breast hyperplasia evolves into ductal carcinoma *in situ* (DCIS). Additional genetic and epigenetic alterations, DCIS can result in invasive ductal carcinoma and finally to metastatic disease. Taken from (Rivenbark et al., 2013).

The pathogenesis of breast cancer has multiple origins. The natural history of breast cancer development is illustrated in **Figure 9**. In a normal individual, the body cells have limited growth promotion and regulation. Any cells with abnormal DNA or growth either undergo cell suicide or are attacked by the immune system and subsequently killed. However, this protective mechanism fails, it frequently leads to cancer. The evolution of breast cancer begins with a neoplastic change in epithelial cells, myoepithelial cells, or stem cells (Akram et al., 2017). Over time, these neoplastic cells incur numerous molecular mutations and altered phenotypic characteristics, resulting in a complex cellular landscape in clinical metastatic cancer (Rivenbark et al., 2013). This complexity reflects the molecular and cellular heterogeneity within breast cancer. Cancerous cells exhibit different characteristics from normal body cells. They have persistent or long-lasting proliferation without any external stimuli (Akram et al., 2017). This proliferation results in tumor growth and spread (Alkabban & Ferguson, 2018). Breast cancer forms from DNA damage and genetic mutations (Alkabban & Ferguson, 2018). Inheritance of mutations in tumor suppressor genes such as BRCA1 and BRCA2 increases the risk of breast cancer development (Alkabban & Ferguson, 2018). Furthermore, signaling pathways play a critical part in cellular function like regulation, proliferation, growth, and survival (Asati et al., 2016). Overactivation of signaling pathways due to mutations in the PI3K/AKT pathway and RAS/MEK/ERK pathway amplify the growth, survival, and metabolism of cancer cells (Asati et al., 2016). The RAS/MEK/ERK pathway and PI3K/AKT pathway is illustrated in **Figure 10**. In breast cancer, dysregulation of such pathways prohibits cells from committing suicide leading to the development of cancer. Mutations in the signaling pathways have been experimentally associated with estrogen exposure (Akram et al., 2017).

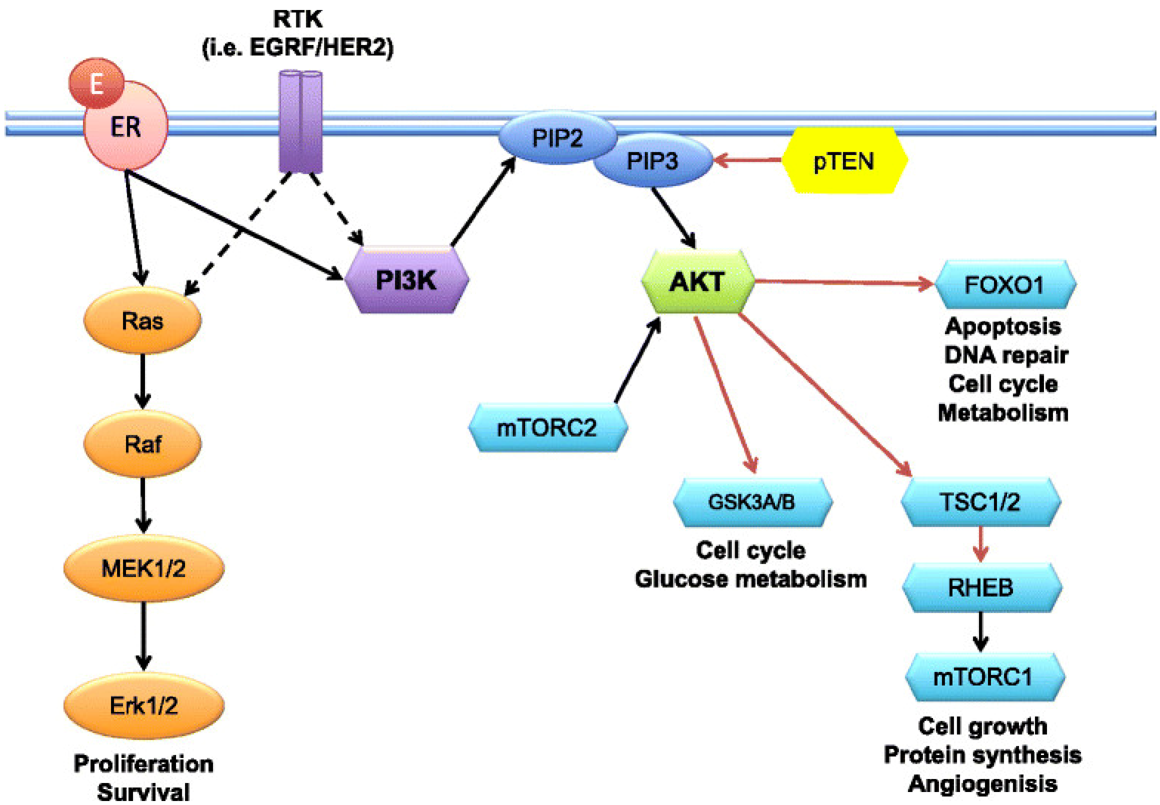

Figure 10. The PI3K/AKT pathway and RAS/MEK/ERK pathway. Dysregulation of these pathways leads to growth, survival, and metabolism of cancer cells. Taken from (Toss & Cristofanilli, 2015).

BRCA1 and BRCA2

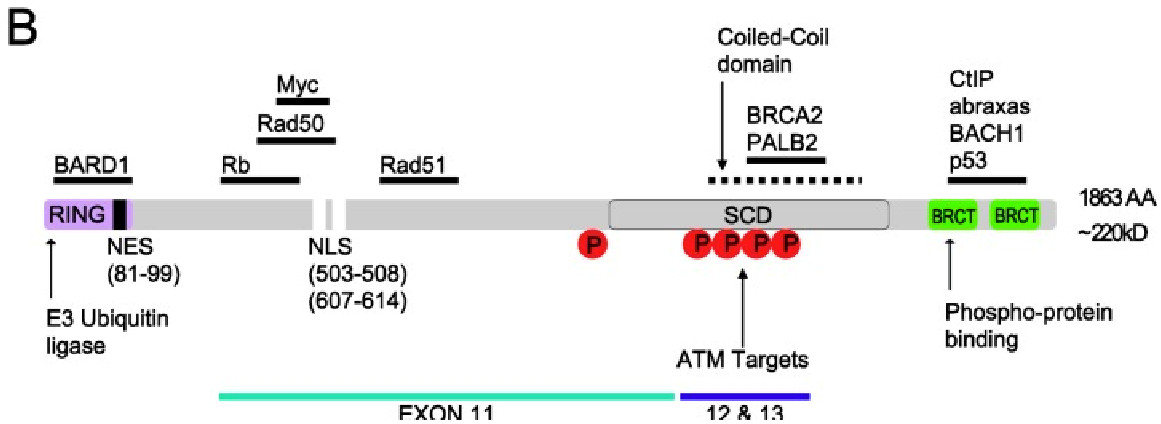

Figure 11. Schematic drawing of BRCA1. BRCA1 contains 3 regions - the RING domain, domain encoded by exons 11(teal), exons 12 and 13 (blue), and the BRCT domain. Taken from (Clark et al., 2012).

BRCA1 (BReast-CAncer susceptibility gene 1) and BRCA2 (BReast-CAncer susceptibility gene 2) are tumor suppressor genes associated with breast and ovarian cancer. The BRCA1 gene is located on chromosome 17q21 and has 24 exons while the BRCA2 gene is located on chromosome 13q12 with 27 exons (Alacacioglu et al., 2018). BRCA genes are implicated in transcriptional regulation of DNA synthesis, in addition to the recognition and correction of double stranded DNA breaks.

BRCA1 is a large multi-domain protein. It is made up of three domains: a zinc-binding RING domain at the amino terminus, BRCT domain, and domain encoded by exons 11-13 (see **Figure 11**). RING, or “Really Interesting New Gene,” domain of BRCA1 made up of RING finger and two flanking alpha helices. This domain serves as an E3 ubiquitin ligase and allows for interactions with various other proteins. The region of exons 11-13 also serves as a binding domain for a variety of proteins. Finally, phosphoproteins bind to the BRCT domain, especially those that are phosphorylated by ATM/ATR kinases. ATM/ATR kinases are activated by DNA damage.

The BRCT domain contains 2 BRCT repeats joined via a linker that is 22 amino acids long. Every BRCT repeat contains 3 alpha-helices packed around 4 strand beta-sheets. These two BRCT repeats work together in a tip-to-tail pattern via hydrophobic interactions between alpha-helix 2 of BRCT1 and alpha-helix 1 and 3 of BRCT2 (Clark et al., 2012). The complete molecular architecture of full-length BRCA1 is yet to be ascertained.

BRCA1 performs its duties of DNA repair in conjunction with BRCA1-associated RING domain protein (BARD1), a binding partner. BRCA-BARD1 heterodimer operates as an E3 ubiquitin ligase in the nucleus. This heterodimer transfers ubiquitin moieties to nuclear protein substrates. The ubiquitin-bound protein substrates move to diverging paths, one that corrects DNA damage (Liang et al., 2017). This is illustrated in **Figure 12**.

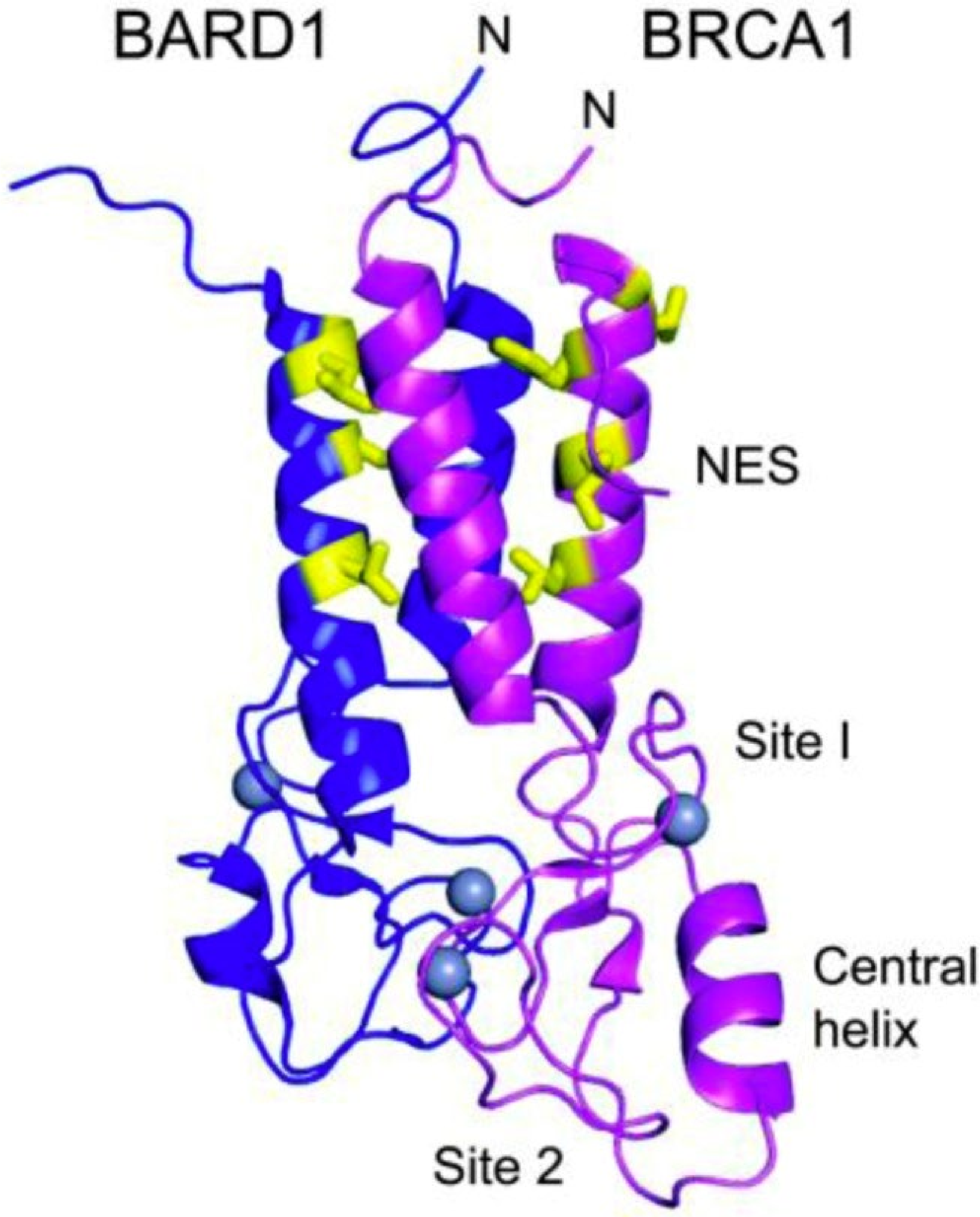

Figure 12. BRCA1 RING Domain (BARD1). Heterodimeric interactions between BRCA1 (purple) and the RING domain of the BARD1(blue) increases the E3-ubiquitin ligase activity of BRCA1. Taken from (Clark et al., 2012).

Using single-particle electron microscopy, Liang 2018 found subtle structural differences between mutated BRCA15382insC-BARD1 and wild-type BRCA1-BARD1 created from human breast cancer cells under normal growth conditions. The BRCT of mutated BRCA1 was slightly truncated compared to wild-type BRCA1-BARD1. However, during oxidative stress, the structure of the mutated BRCA15382insC-BARD1 deviated significantly from its wild-type component. Under such conditions, Liang 2018 found a region on BRCA15382insC, rich in lysine, that was easily ubiquitinated. This lysine-rich hotspot corresponded to the main type of modification on BRCA15382insC was K48-linked ubiquitin attachments (Gilmore et al., 2017). In cancer cells, lower functional levels of active BRCA15382insC correlated with increased levels of ubiquitination (Liang et al., 2017). The identified hotspot region allowed for increased levels of ubiquitination resulting in reduced amounts of active BRCA15382insC. Significantly, Liang, 2018, were able to prove that removing the ubiquitin moieties in the lysine-rich "hotspot" area of mutated BRCA1 returned their overall structure.

BRCA1 also plays an important role in base excision repair (BER). BER fixes small base lesions that do not significantly distort the helical structure of DNA. In oxidative environments, BRCA1 triggers BER pathways through ubiquitin signaling (Liang et al., 2017). Cells with BRCA1 gene mutations cannot appropriately handle the higher amounts of reactive oxygen species (ROS) from metabolism of estrogen. Over time, deficiencies in base excision repair can lead to an accumulation of DNA damage and genomic instability. DNA damage and genomic instability are often hallmarks of cancer (Hanahan & Weinberg, 2000).

The cell cycle largely determines the amount of phosphorylation and BRCA1 protein present in cells. In the late G1 and S phases, BRCA1 is hyperphosphorylated. However, after the M phase, BRCA1 is dephosphorylated (Alacacioglu et al., 2018). Phosphorylation of BRCA1 protein is critical for proper response of double-stranded DNA break repair (Cortez, 1999). S phase through G2/M phase has the highest amount of BRCA1 protein. However, it falls in early G1. Similarly, BRCA2 gene is expressed in the late G1/early-S phase of the cell cycle. (Alacacioglu et al., 2018).

BRCA2 (BReast CAncer susceptibility gene 2) is instrumental in homologous recombination pathway of DNA repair (Pendlebury et al., 2021). With BRCA2 mutations, the cell is forced to rely on a non-homologous end joining pathway to repair double-stranded breaks, resulting in greater number of errors. The various steps of NHEJ are illustrated in **Figure 13**. Defects in DNA integrity are hallmarks of cancer development (Pendlebury et al., 2021).

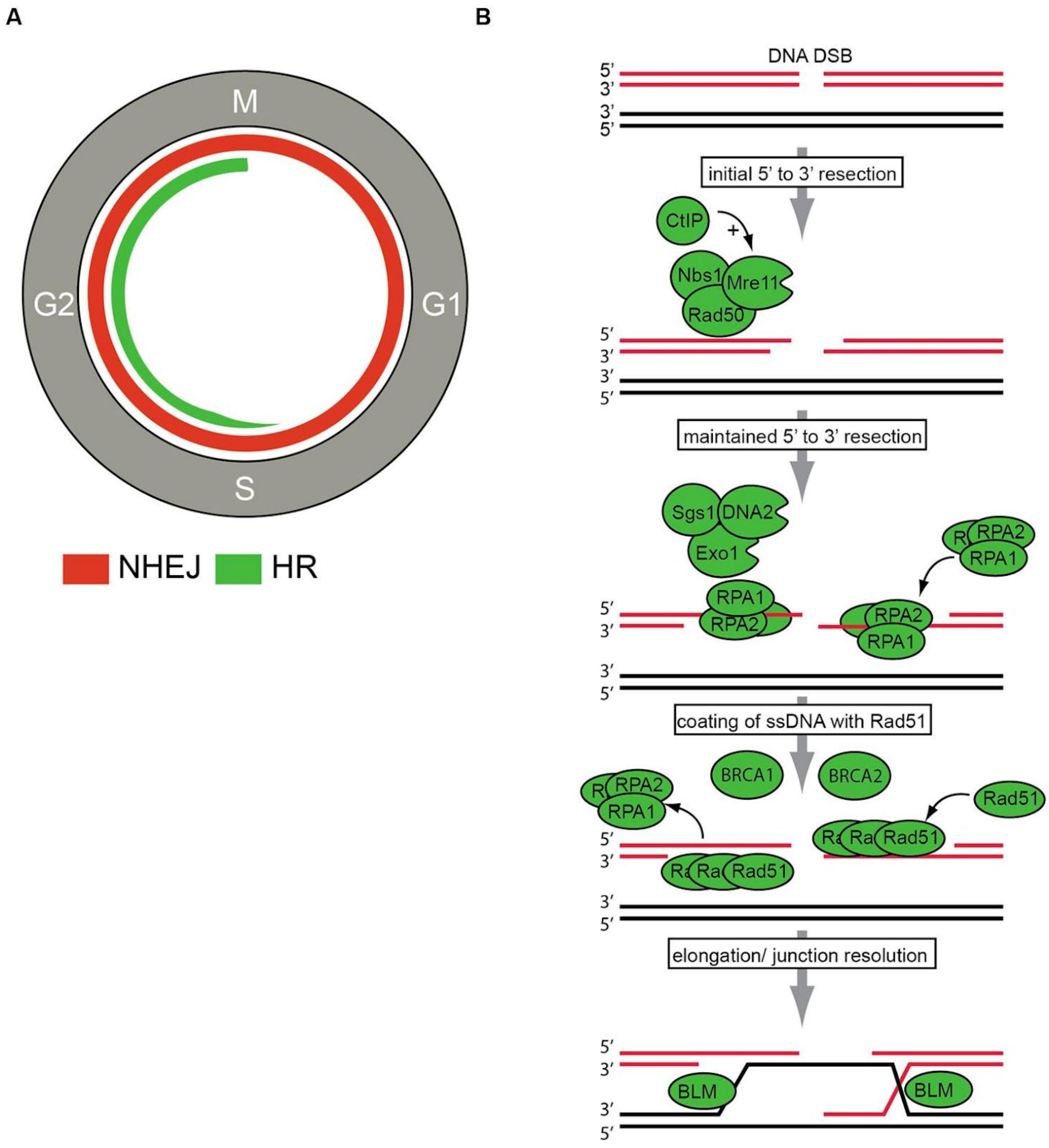

Figure 13. DNA double strand break (DSBs) repair. (A) Non-homologous end joining (NHEJ) often takes place during the entire cell cycle however homologous recombination (HR) occurs in S/G2 phase of the cell cycle. (B) The steps of homologous repair are illustrated. This repair begins with 5' to 3' resection followed by coating of the signal-stranded DNA ends by replication protein A (RPA) filaments. Finally, RPA is replaced by Rad51, a recombinase. This process is facilitated by BRCA2. Taken from (Krajewska et al., 2015).

Homologous recombination is essential for DNA integrity (Li & Heyer, 2008). There are three highly related steps of homologous repair. The first step involves generation of 3’ single-stranded DNA ends by nucleolytic degradation of the 5’ strands. This action is catalyzed by endonucleases, including MRN complex consisting of Mre11, Rad50 and Nbs1. The second step uses replication protein A (RPA) filaments to coat single-stranded DNA ends (Krajewska et al., 2015). Finally, RPA is replaced by Rad51 to assemble helical nucleoprotein filaments called presynaptic filaments (Li & Heyer, 2008). The RAD51 (also known as RecA) family of recombinases execute a key step in HR (Shahid et al., 2014). Recombinases are enzymes that work to pair and shuffle DNA sequences during HR repair. Unfortunately, nucleation of RAD51 onto ssDNA is a slow process so it allows for ssDNA binding protein RPA interference (San Filippo et al., 2008). BRCA2 works as a recombinase accessory factor to overcome the inhibitory effect of RPA on the assembly of the RAD51 presynaptic filament (San Filippo et al., 2008).

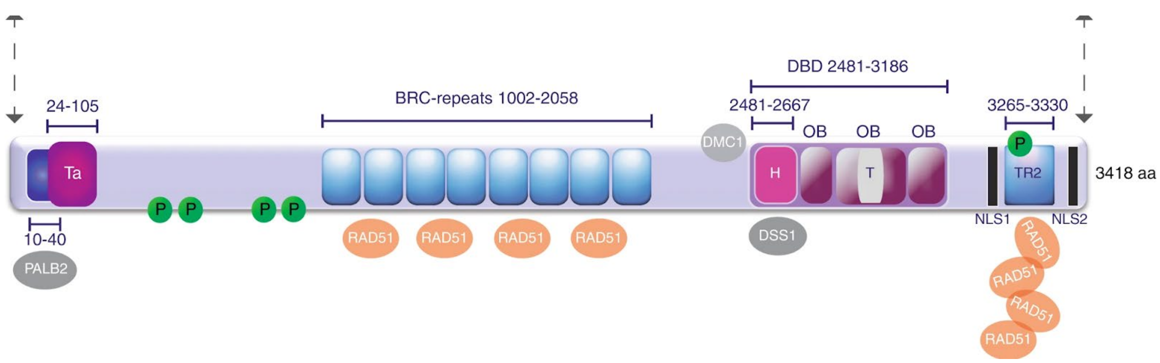

Figure 14. Schematic representation of BRCA2 gene. BRCA2’s N-terminal region communicates with the Partner and Localizer of BRCA2 (PALB2; in gray) while the central domain contains 8 copies of the BRC motifs (in blue). These motifs have a high affinity to RAD51 (in orange). The C-terminal region of the BRCA2 gene contains 3 oligonucleotide-oligosaccharide-binding folds (OB; in red). Taken from (Mesman et al., 2019).

Unfortunately, due to its large size, information on the structure of BRCA2 is limited. Current information indicates that PALB2 (Partner and Localizer of BRCA2) and the meiotic recombinase DMC1 interact with the N-terminal region and central domain of BRCA2, respectively. The C-terminal region contains an alpha helical domain followed by 3 oligonucleotide-oligosaccharide-binding folds that bind single-stranded DNA (Shahid et al., 2014). The schematic representation of the BRCA2 gene is illustrated in **Figure 14**. The BRCA2 protein has eight copies of the BRC motif, a 70 amino acid motif. The BRC motif repeats 1-4 appear to have a greater affinity for RAD51 than repeats 6-8. Approximately, four to six RAD51 molecules are bound by a BRCA2 monomer. BRCA2 exists *in vivo* as a dimer. Two RAD51 molecules oriented in opposite directions bind to the BRCA2 dimer (Shahid et al., 2014).

BRCA1 and BRCA2 Variants

In the general population, only 0.1-0.2% are carriers of BRCA1 and BRCA2 mutations (Alacacioglu et al., 2018). The prevalence of breast cancer for women who inherited a harmful BRCA1 variant at 70 years of age is 55%-72% while it is only 45%-69% in women who are carriers of BRCA2 gene mutation (National Cancer Institute, 2014). There is a 10-30 times higher prevalence of breast cancer in females with a BRCA1 and BRCA2 gene mutation than those that do not have the mutation (Akram et al., 2017). Despite a large increase in breast cancer prevalence associated with BRCA1 and BRCA2 genes, these germline mutations account for approximately 5% of all breast cancers (Talhouet et al., 2020). However, BRCA germline mutations account for approximately 10-15% of ovarian cancers. Of which, germ-line mutations in breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2) genes account for 90% of hereditary breast cancer cases as well as most of the hereditary ovarian cancer (Calderón-Garcidueñas et al., 2005). Moreover, many women who go on to develop ovarian cancers due to BRCA1 or BRCA2 have high-grade serous ovarian carcinomas (Talhouet et al., 2020).

The BRCA Exchange database catalogs distinct mutations, polymorphisms, and genetic variants of uncertain significance in the BRCA1 and BRCA2 genes identified around the world (BRCA Exchange, 2022). As of March 2022, there are approximately 34,001 variants of BRCA1 and 33,550 variants of BRCA2 genes. Of which, only 4,900 BRCA1 variants were determined to be pathogenic by an international consortium of investigators. This consortium is called Evidence-based Network for the Interpretation of

Germline Mutation Alleles (ENIGMA). 30,809 BRCA1 variants are yet to be reviewed by this consortium. Similarly, only 2,672 BRCA2 variants were determined to be pathogenic by ENIGMA, while 29,644 variants are yet to be reviewed. This information is depicted in **Figure 15**.

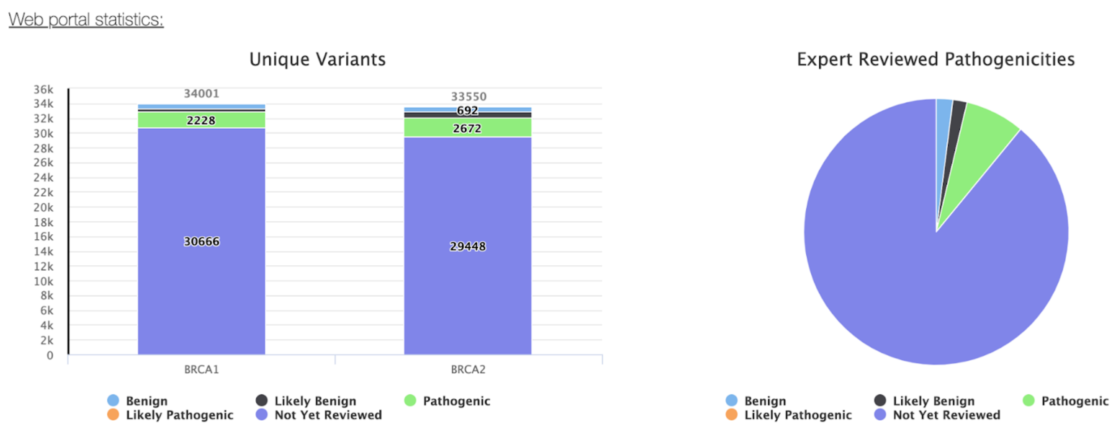

Figure 15. BRCA Exchange database. As of 2022, there are over 34,000 variants of the BRCA1 and 33000 variants of BRCA2 gene cataloged in the BRCA Exchange database. As seen by the pie chart on the right, about 89% of the variants are not yet reviewed (blue) while only 7.3% of the variants were determined to be pathogenic (green) by Evidence-based Network for the Interpretation of Germline Mutation Alleles (ENIGMA). Taken from (BRCA Exchange, 2022).

The most common BRCA1 mutations are 5382insC, 185delAG, 3819del5 and 4153delA. The commonly reported BRCA2 mutations are 4075delGT and 5802del4. However, the frequencies of specific mutations seem to differ according to different populations (Ramus & Gayther, 2009). The majority of the deleterious BRCA1 and BRCA2 mutations result from truncated protein from premature termination codons through small frameshift deletions or insertions, nonsense or splice junction alterations, or missense alterations at critical residues in functional domains.

For example, the most common mutations among the Ashkenazi Jewish population are BRCA1185delAG, BRCA15382insC, and BRCA26174delT. These mutations comprise about 98-99% of all identified mutations among the Ashkenazi Jewish population. In Iceland, BRCA1 mutations are rare. However, the BRCA2999del5 mutation accounts for the large bulk of ovarian and/or breast cancer families. BRCA15382insC is rather common in a few Eastern European countries including Poland, Russia, and Hungary (Ramus & Gayther, 2009). This information is illustrated in **Table 3**.

Table 3. Common founder BRCA 1 and BRCA2 mutations based on population. Taken from (Ramus & Gayther, 2009).

Population	Number common mutations	*BRCA1*	*BRCA2*	Proportion of BRCA1/2
Ashkenazi Jewish	3	185delAG 5382insC	6174delT	98–99% of BRCA1/2 mutations
Iceland	1		995delG	Vast majority of BRCA1/2 mutations, 7.9% of ovarian cancer
Russia	1	5382insC		94% of BRCA1 mutations, 11% of families
Poland	3	5382insC C61G, 4153delA		80% of BRCA1/2 mutations, 91% of BRCA1 mutations
Germany	3	5382insC 300T>G Del ex 17		38% of BRCA1 mutations
Germany	18			66% of BRCA1 mutations
Hungary	5	5382insC 300T>G 185delAG	9326insA, 6174delT	80 of BRCA1 mutations, 50 of BRCA2 mutations
Norway	4	1675delA 1135insA 816delGT 3347delAG		68 of BRCA1 mutations, 1675delA and 1135insA 3% of ovarian cancer

CRISPR-Cas9

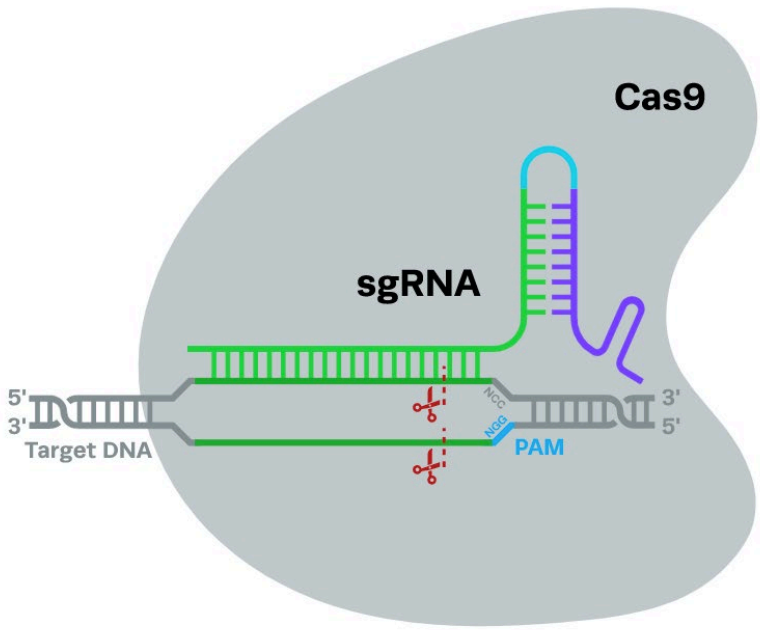

Figure 16. CRISPR-Cas9. The CRISPR-Cas9 complex consists of single guide RNA (sgRNA; green) and Cas9 endonuclease (gray). Cas9 endonuclease binds sgRNA and DNA and cleaves complementary and non-complementary strands of target DNA. Taken from (Synthego, 2019).

Clustered regularly interspersed short palindromic repeats (CRISPR) and the CRISPR-associated proteins (Cas), combined as CRISPR/Cas9 has garnered tremendous interest in the scientific community for its myriad applications from diagnostics, imaging, and most importantly in genome editing. Found first in E. coli in 1987, CRISPR, along with Cas9, utilize two distinct RNAs- the trans-activating RNA (tracrRNA) and the CRISPR targeting RNA (crRNA) (Zhan et al., 2019). Combining the crRNA and tracrRNA into a single guide RNA simplified this system. The single guide RNA (sgRNA) has RNA bases that base-pair with the target DNA sequence in the genome. DNA and guide Cas9, a DNA endonuclease, bind specific DNA sequences (Zhan et al., 2019). CRISPR-Cas9 is illustrated in **Figure 16**. Type II Cas Protein from Streptococcus pyogenes (SpCas9) was foundational for CRISPR/Cas9 use. Structure of Cas9 protein consists of two lobes: a recognition (REC) lobe and a nuclease (NUC) lobe. The recognition lobe contains three regions: (1) bridge helix (2) REC1 (3) REC2. The nuclease lobe also contains three regions: (1) RuvC (2) HNH (3) PAM-interacting domain. The recognition lobe of the Cas9 endonuclease binds sgRNA and DNA while the nuclease lobe with its RuvC and HNH nuclease domains cleaves complementary and non-complementary strands of target DNA, respectively. The PAM-interacting domain, like the name suggests, interacts with protospacer adjacent motifs or PAM. PAM is a 2-6-base pair DNA sequence that follows the DNA region of interest. This sequence is targeted by cleavage by Cas9. Structural and functional analysis of the Cas9 protein indicates that the 20 bp negatively charged heteroduplex of sgRNA: target DNA fits in a positively charged groove at the interface between REC and NUC lobes. Furthermore, the scissile phosphate group of the target DNA is appropriately placed for cleavage by the HNH domain (Nishimasu et al., 2014). The schematic representation of Cas9 protein is illustrated in **Figure 17**.

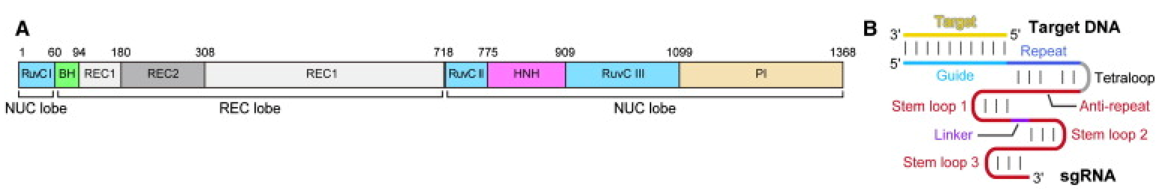

Figure 17. A. Schematic representation of Cas9 protein. The structure of Cas9 protein consists of two lobes: a recognition (REC) lobe and a nuclease (NUC) lobe. The recognition lobe contains three regions: (1) bridge helix (2) REC1(light gray) (3) REC2 (dark gray). The nuclease lobe also contains three regions: (1) RuvC(blue) (2) HNH (pink) (3) PAM-interacting domain (PI; yellow). Taken from (Nishimasu et al., 2014).

The Cas9 protein acts as a pair of 'molecular scissors' capable of cutting across both strands of the DNA. When a double-strand break is introduced in the target sequence by the Cas9 endonuclease, there are two different mechanisms of DNA repair that are used by the host cell to respond- non-homologous end joining (NHEJ) or homology-directed repair (Zhan et al., 2019. The NHEJ pathway is illustrated in **Figure 18**.

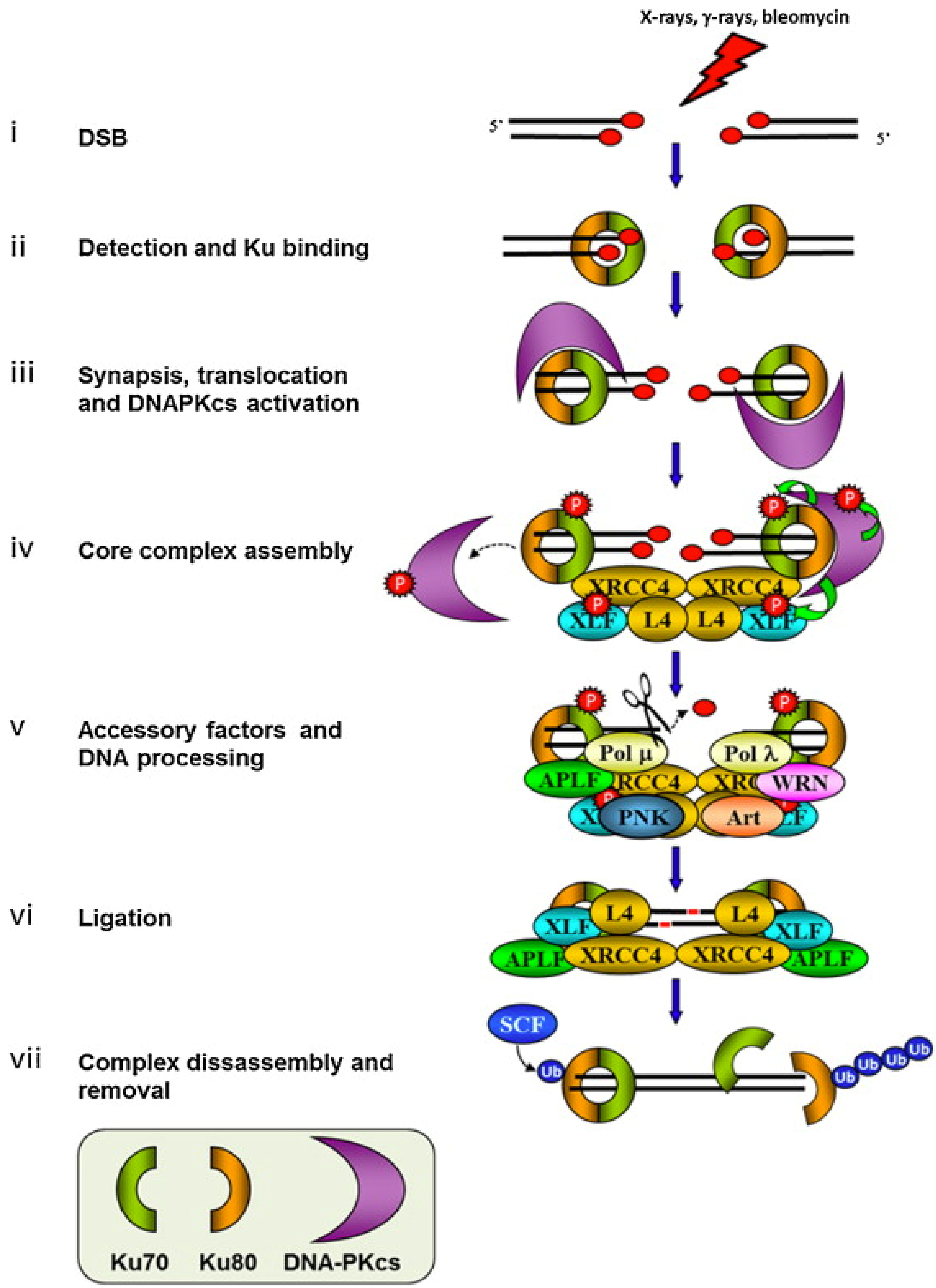
X-rays, γ-rays, bleomycin
5'
5'
i DSB
ii Detection and Ku binding
iii Synapsis, translocation and DNAPKcs activation
iv Core complex assembly
P
XRCC4
XRCC4
XLF
L4
L4
XLF
v Accessory factors and DNA processing
Pol μ
Pol λ
APLF
WRN
PNK
Art
vi Ligation
L4
L4
XLF
XLF
APLF
XRCC4
XRCC4
APLF
vii Complex dissassembly and removal
SCF
Ub
Ub
Ub
Ub
Ub
Ku70
Ku80
DNA-PKcs

Figure 18. Non-homologous end joining pathway (NHEJ).
In step 1, a double-strand break (DSB) occurs.
In step 2, the DSBs allow Ku heterodimers to establish a close interaction between DNA and the central core. Ku is a nuclear heterodimer with a hollow circular ring shape. This structure allows the protein to slide over the DNA ends. The function of this protein is to act as a recruiter for multiple interacting proteins so they all can act in a regulated manner to ensure proper DNA repair.
In step 3, the DNA-PKcs (DNA-dependent protein kinase catalytic subunit) binds to Ku and becomes activated via its proximity to the DNA end. DNA-PK (DNA-dependent protein kinase) holoenzyme is a protein kinase that is activated by double stranded breaks. DNA-PK consists of the catalytic subunit DNA-PKcs and Ku heterodimer. DNA-PKcs is necessary for efficient non-homologous end joining. DNA-PKcs can bind to DNA by itself. However, its association with Ku heterodimer enhances DNA affinity by a hundredfold.
In step 4, Ku, XRCC4 (X-ray repair cross complementing 4), Lig4 (DNA Ligase 4), and XLF (XRCC4-like factor) proteins comprising the "core" NHEJ are assembled and stabilized through phosphorylation by DNA-PKcs. DNA-PKcs also auto-phosphorylates and breaks away from the NHEJ complex. XRCC4 is a DNA repair protein. Lig4 is a DNA ligase. XLF encourages DNA ligation by XRCC4-Lig4 of both complementary and non-complementary double stranded break ends.
In step 5, core complex via Ku recruit DNA polymerases and DNA end processing factors. The DNA polymerases and end processing factors modify the DSB termini
In step 6, Lig4, facilitated by APLF and/or XLF, is used to carry out ligation. APLF is an Aprataxin-and-PNK-like factor.
In step 7, the Skp-Cullin-Fbox complex (SCF) ubiquitinates Ku heterodimer. Ku heterodimer is then destroyed by proteolysis. Taken from (Grundy et al., 2014).

NHEJ is a type of double-stranded break (DSB) repair mechanism. This type of repair mechanism is not restricted to a cell cycle phase since it does not require a homologous template. The general mechanism of NHEJ is as follows: (1) detection and Ku binding (2) synapsis, translocation, and DNAPKcs activation (3) core complex assembly (4) accessory factors and DNA processing (5) ligation and finally (6) complex disassembly and removal (Grundy et al., 2014).

Homologous recombination, another type of DSB repair, requires a homologous template for repair. This type of repair mechanism is restricted to S and G2 phases of the cell cycle when a homologous template via the sister chromatid is available. This is ideally suited for cells that are rapidly dividing. Homologous recombination is an important DNA repair mechanism that provides high-fidelity, template-dependent repair for DNA gaps, DNA double-stranded breaks, and DNA interstrand crosslinks. The host cell may respond with a non-homologous end joining (NHEJ), a mechanism that leads to insertions or deletions. This may be beneficial if the study, in question, aims to render a target gene functionless. (2) The second method is via homology-directed repair which recombines DNA donor templates to reconstruct damaged DNA (Zhan et al., 2019). This feature is helpful if the target gene, in question, contains a mutation that leads to reduced expression, no expression, or results in cancer. NHEJ and HR are illustrated in **Figure 19**.

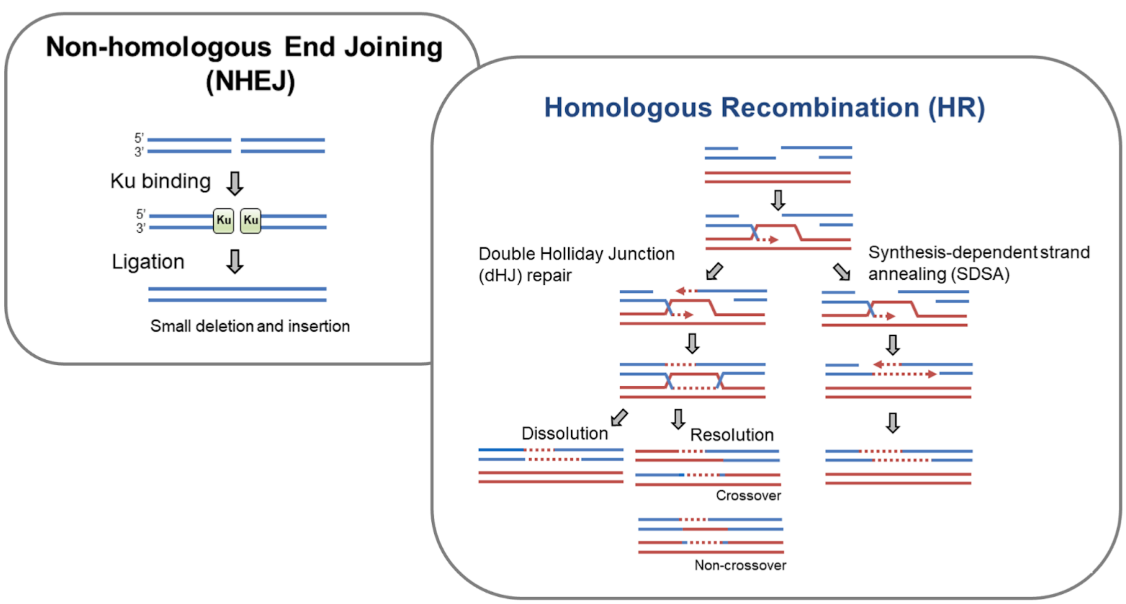

Figure 19. Non-homologous end joining (NHEJ) and Homologous recombination (HR). NHEJ and HR are two major repair mechanisms for double-stranded break. Taken from (Tedsall, 2022).

Current CRISPR-Cas9 Studies

There are many proposed applications for CRISPR-Cas9. Recently, a landmark CRISPR-Cas9 clinical trial shows promise against Transthyretin amyloidosis. Transthyretin amyloidosis is a deadly progressive condition caused by a buildup of transthyretin (TTR) protein misfolds in the form of amyloid fibrils in nervous and cardiac tissues. Current therapies focus on reducing the ongoing amyloid formation. Reduction of ongoing amyloid formation is done through TTR tetramer stabilizers or through inhibition of TTR protein synthesis by destruction of TTR messenger RNA. These treatments provide short-term relief. Moreover, exposure to these therapies, like Patisiran, a novel RNA interference therapeutic that specifically lowers the production of both wild-type and mutant TTR protein, has serious side effects. A long-term treatment is required for these patients. In the 2021 article from The New England Journal of Medicine, a new CRISPR-Cas9-based gene-editing therapy was administered to 6 patients with hereditary Transthyretin amyloidosis (Gillmore et al., 2021). NTLA-2001 is a novel *in vivo* gene-editing therapy that utilizes CRISPR-Cas9 technology. It contains a lipid nanoparticle (LNP) delivery system with liver tropism, sgRNA that targets human TTR and a human-codon-optimized mRNA sequence of Streptococcus pyogenes Cas9 protein. After intravenous administration, NTLA-2001 LNP is taken up by hepatocytes. Once inside the hepatocytes, the LNP is broken down and its contents are released into the cytoplasm. The native ribosomes in the hepatocytes use the human-codon-optimized mRNA sequence of Streptococcus pyogenes Cas9 protein to make Cas9 mRNA. The Cas9 mRNA is then used to make Cas9 endonuclease. Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 complex is formed with the use of TTR-specific sgRNA along with the Cas9 endonuclease. This complex targets and cleaves the DNA at the TTR gene. Endogenous DNA repair through NHEJ introduces indels in the TTR gene, resulting in frameshift mutations. This mutation results in the reduction of TTR protein production. This study is illustrated in **Figure 20**. This study showed that in the group that received a dose of 0.1 milligram/kilogram of the NTLA-2001, there was a 52% mean reduction from baseline serum TTR protein concentration by day 28. Moreover, in the group that received a 0.3 milligram/kilogram dose, there was an 87% reduction in serum TTR protein concentrations (Gillmore et al., 2021).

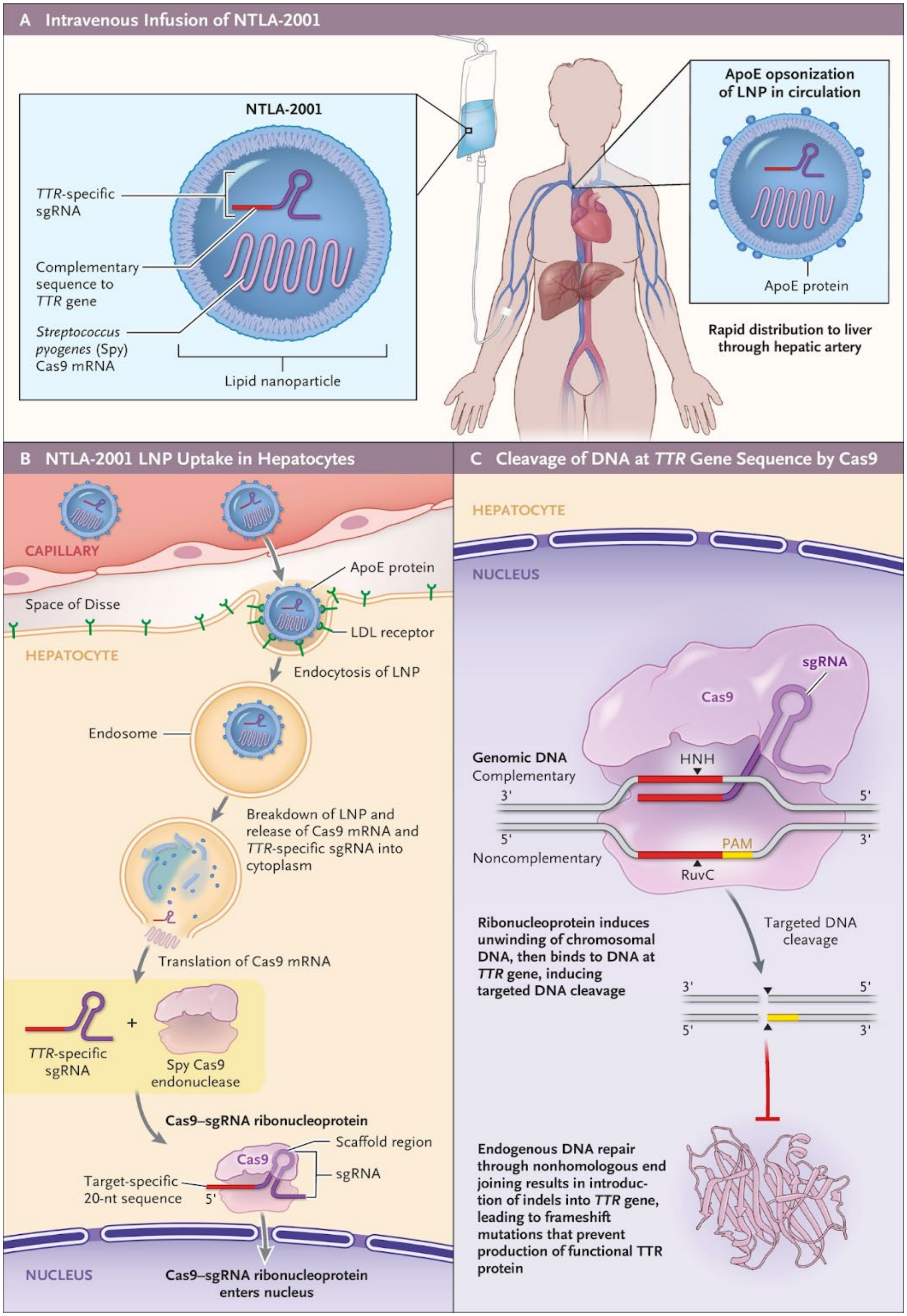
A Intravenous Infusion of NTLA-2001
NTLA-2001
TTR-specific sgRNA
Complementary sequence to TTR gene
Streptococcus pyogenes (Spy) Cas9 mRNA
Lipid nanoparticle
ApoE opsonization of LNP in circulation
ApoE protein
Rapid distribution to liver through hepatic artery
B NTLA-2001 LNP Uptake in Hepatocytes
CAPILLARY
ApoE protein
Space of Disse
LDL receptor
HEPATOCYTE
Endocytosis of LNP
Endosome
Breakdown of LNP and release of Cas9 mRNA and TTR-specific sgRNA into cytoplasm
Translation of Cas9 mRNA
TTR-specific sgRNA
+
Spy Cas9 endonuclease
Cas9–sgRNA ribonucleoprotein
Scaffold region
Cas9
sgRNA
Target-specific 20-nt sequence
5'
NUCLEUS
Cas9–sgRNA ribonucleoprotein enters nucleus
C Cleavage of DNA at TTR Gene Sequence by Cas9
HEPATOCYTE
NUCLEUS
sgRNA
Cas9
Genomic DNA
Complementary
HNH
3'
5'
5'
3'
PAM
Noncomplementary
RuvC
Ribonucleoprotein induces unwinding of chromosomal DNA, then binds to DNA at TTR gene, inducing targeted DNA cleavage
Targeted DNA cleavage
3'
5'
5'
3'
Endogenous DNA repair through nonhomologous end joining results in introduction of indels into TTR gene, leading to frameshift mutations that prevent production of functional TTR protein

Figure 20. CRISPR-Cas9 *In Vivo* Gene Editing for Transthyretin Amyloidosis. (A) A schematic drawing of NTLA-2001. NTLA-2001 is a novel *in vivo* gene-editing therapy that utilizes CRISPR-Cas9 technology. (B) NTLA-2001 LNP is taken up by hepatocytes in the liver. (C) Cas9 endonuclease binds and cleaves TTR gene which causes DSB repair through NHEJ. This process introduces insertions or deletions into the gene sequence ultimately resulting in frameshift mutations and reduced production of functional TTR protein. Taken from (Gillmore et al., 2021).

CRISPR-Cas9-based in-vivo gene editing therapy is also used to address sickle cell disease and beta-thalassemia. Sickle cell disease (SCD) and beta-thalassemia (TDT), like Transthyretin amyloidosis, are monogenic diseases (Frangoul et al., 2020). Mutation in the beta subunit gene causes decreased or nonexistent beta-globin synthesis in beta-thalassemia, which causes ineffective erythropoiesis. In sickle cell disease, an amino acid substitution takes place of valine for a surface exposed glutamic acid. Current treatment options include transfusions and iron chelation for those with beta-thalassemia. Individuals with sickle cell disease utilize pain management, transfusions, and hydroxyurea as temporary treatments. The only long-term cure for TDT and SCD is bone marrow transplant however only a few patients are eligible for this option. A study published in 2021 by the New England Journal of Medicine (Frangoul et al., 2020) discusses CRISPR-Cas9 gene-editing in progenitor cells and hematopoietic stem at BCL11A, an erythroid-specific enhancer region. The aim of this study was to decrease the expression of BCL11A in erythroid-lineage cells. BCL11A is a zinc-finger containing transcription factor that inhibits expression of gamma-globin and fetal hemoglobin in erythroid cells. By reducing the BCL11A expression, the study aimed to restore gamma-globin expression and bring back the production of fetal hemoglobin. CTX001 was manufactured from CD34+ cells that were edited using CRISPR-Cas9 and sgRNA. This study, as illustrated in **Figure 21**, found that after the administration of CTX001, both subjects, a subject with SCD and a subject with SCD, had sustained, substantial and early rise in fetal hemoglobin levels with more than 99% expression of HbF during a year (Frangoul et al., 2020).

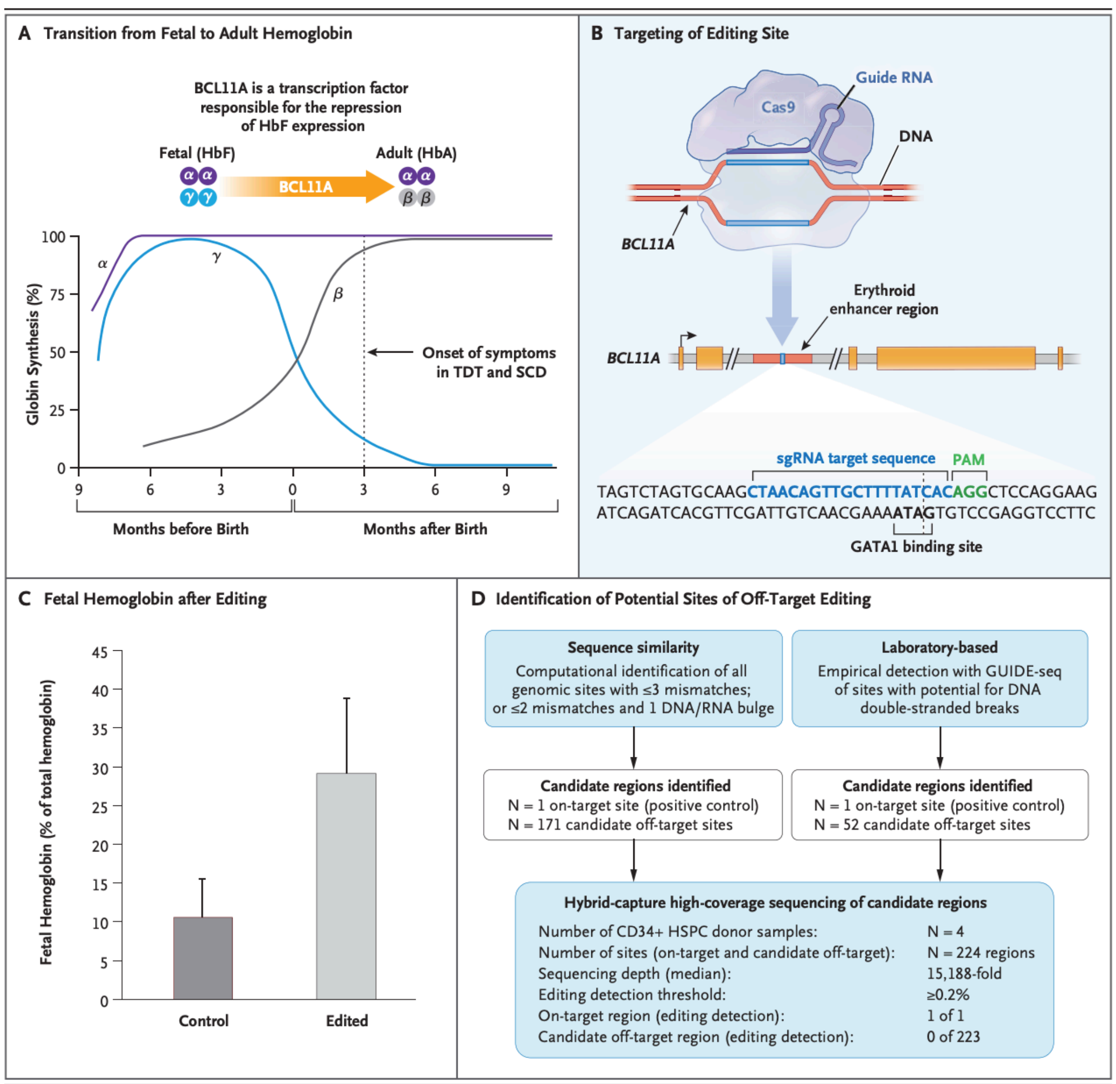

Figure 21. CRISPR-Cas9 Gene Editing for Sickle Cell Disease (SCD) and β-Thalassemia (TDT). (A) BCL11A is a zinc-finger containing transcription factor that inhibits expression of gamma-globin and fetal hemoglobin in erythroid cells. (B) CRISPR-Cas9 gene-editing in progenitor cells and hematopoietic stem at BCL11A, an

erythroid-specific enhancer region. CTX001 was manufactured from CD34+ cells that were edited using CRISPR-Cas9 and sgRNA. This study found that after the administration of CTX001, both subjects, a subject with SCD and a subject with SCD, had sustained, substantial and early rise in fetal hemoglobin levels with more than 99% expression of HbF during a year. Taken from (Frangoul et al., 2020).

The previous studies use CRISPR-Cas9 gene therapy in monogenic diseases. In 2019, the University of Pennsylvania conducted the first in-human phase 1 clinical trial in the United States that uses CRISPR-Cas9 technology. The goal of this study was to use patients' own immune cells to genetically modify them to better detect and kill cancerous cells. More specifically, T-cells from the patients were removed. These cells underwent four genetic modifications. The first modification was to insert the gene for the NY-ESO-1 receptor. NY-ESO-1 is a cancer-specific T-cell receptor transgene that is used to recognize tumor cells. Then the CRISPR-Cas9 technology was used. A mechanical transfection method is used to transfer CRISPR-Cas9 ribonucleoprotein as well as 3 sgRNAs into modified T-cells. This step removed endogenous T-cell receptors and programmed cell death protein 1 (PD-1). The rationale for these gene knockouts was to enhance the function and endurance of edited T-cells and reduce immunogenicity. The edited T-cells were grown in large numbers and infused to three patients (one with metastatic sarcoma and two with advanced refractory myeloma) at consistent levels for at least nine months. This approach is illustrated in **Figure 22**. Single-cell RNA sequencing of the infused CRISPR-engineered T-cells revealed that 30% of cells had no detectable mutations, while 40% had a single mutation and 20% had double mutated and 10% had triple mutated at the target sequences. There were some off-target edits, a major concern for any CRISPR-Cas9 technology. However, the article notes that none of the off-target edits seem to have the potential of becoming cancerous. Initial findings suggest that the treatment is safe. Although there were some side effects, there was no evidence of an immune reaction to the CRISPR-edited T-cells. Although biopsies indicate lingering tumors in patients with myeloma, a reduced LAGE-1 and/or NY-ESO-1 target antigens are present. This reflects on-target effects of edited T-cells. The author notes that although this clinical trial indicates that CRISPR-Cas9 use in gene therapy is safe, long-term effects will still need to be monitored (Stadtmauer et al., 2020).

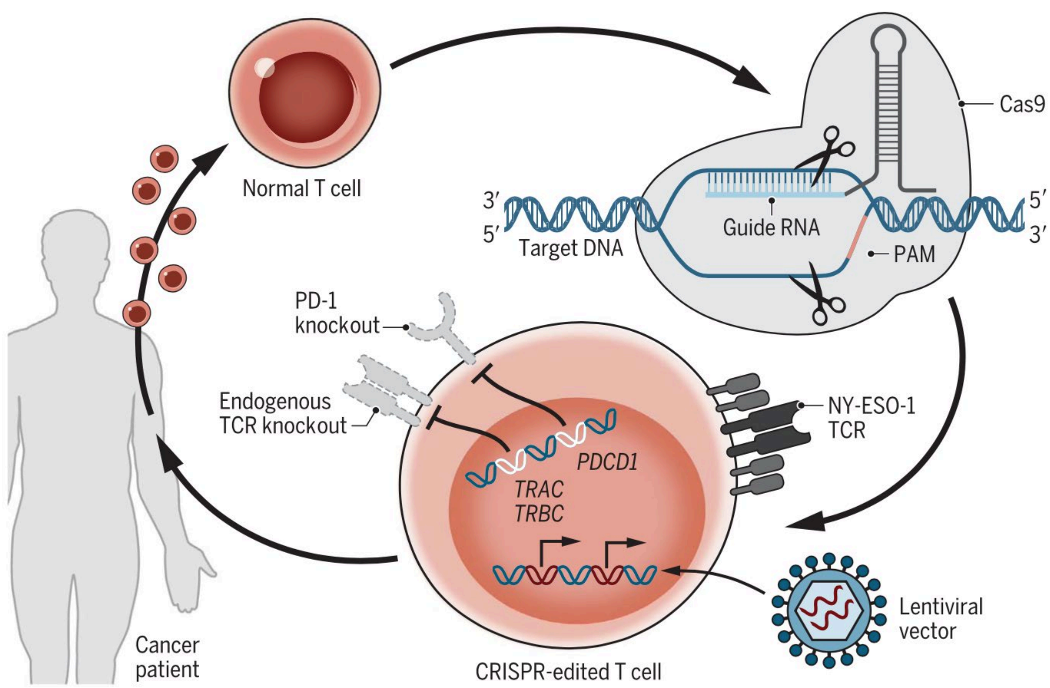

Figure 22. Phase 1, first in-human, clinical trial in the US that uses CRISPR-Cas9 technology. Normal T-cells are removed from a cancer patient. CRISPR-Cas9 technology is used to eliminate endogenous T-cell receptors as well as programmed cell death protein 1 (PD-1). The edited T-cells are grown in large numbers and infused back into the patient (Stadtmauer et al., 2020).

CRISPR-Cas9 Limitations

Although CRISPR-Cas9 is a powerful tool, there are many important limitations to its use. The first limitation is the difficulty in delivering CRISPR-Cas9 material to mature cells in large amounts. A successful delivery approach must consist of high editing capability, low probability to elicit an immune response and effective delivery of sgRNA/Cas9 to a specific location in the body. The first generation of CRISPR-Cas9 technology utilized plasmids to synthesize Cas9 and sgRNA. This approach, although efficient in *in vivo* application to mouse models, is limited and resulted in poor control over the Cas9 activity. One way to circumvent this limitation is the use of viral vectors or lipid nanoparticles. A common viral vector used in gene therapy is adeno-associated virus (AAV) vector. AAV is efficient for the following reasons: (1) its non-integrating nature (2) high transduction efficiency (3) serological compatibility with most of the human population. Although there are many pros to the use of AAV, it is also limited. AAV does not have the cargo size for Cas systems and sgRNA to be encoded on the same vector. In addition to AAV, lipid nanoparticles are also being considered as a viable delivery approach (Yang et al., 2021). Gillmore and his colleagues from Royal Free Hospital were able to use a proprietary lipid nanoparticle (LNP) delivery system (Gillmore et al., 2021). Although this study showed great promise, nanoparticle-based carriers do not have high editing efficiency (Yang et al., 2021).

The next limitation is CRISPR-Cas9's inefficiency. As seen from the 2019 study by University of Pennsylvania researchers (Stadtmauer et al., 2020), only 10% of all engineered T-cells contained triple mutations at the target sequence. Another limitation is its off-target edits. This is a big concern for many members of the scientific community. In most cases, the guide RNA is a 20-base sequence that is complementary to the target sequence of the gene in question. However, not all 20 bases need to be completely paired for Cas9 to cleave the DNA. If, for example, 19 of the 20 base sequences exist in another location of the DNA, there is a potential for the guide RNA to bind at this off-site. Researchers applied a variety of approaches to reduce off-targets (Yang et al., 2021). To pinpoint and anticipate off-target edits, online editing programs have been created and leveraged. Along with online editing programs, new technology like genome-wide next generation sequencing could play a major role in the future (Yang et al., 2021). Another approach to reducing off-target effects is to change the 20 nucleotide sgRNA. For example, extra two G nucleotides added to the 5' end of the sgRNA or short sgRNAs truncated at the 5' end have lowered the off-target impacts by 5000 times while keeping the high activity of on-target (Suleiman et al., 2021).

CONCLUSION

Breast cancer is one of the major causes of death. Approximately 2.3 million women were diagnosed with breast cancer and 685,000 deaths occurred throughout the world in 2020 (WHO, 2021). There is an obvious need for preventative therapies for breast cancer. I wished to investigate a potential preventative therapy for BRCA1 and BRCA2-associated hereditary breast cancer. CRISPR-Cas9 technology garnered tremendous interest in the scientific community for its myriad applications from diagnostics, imaging, and most importantly in genome editing. In this thesis, I wish to explore whether we can apply CRISPR-Cas9 as possible preventative therapy in BRCA1 and BRCA2-associated hereditary breast cancer. However, after much research, I conclude that current CRISPR-Cas9 technology might not be a possible preventative therapy. The reasons for this conclusion are as followed:

1. A large number of variants of BRCA1 and BRCA2 genes
2. Limited CRISPR-Cas9 technology

As of March 2022, there are approximately 34,001 variants of BRCA1 and 33,550 variants of BRCA2 genes. Of which, only 4,900 BRCA1 variants were determined to be pathogenic by Evidence-based Network for the Interpretation of Germline Mutation Alleles (ENIGMA) (BRCA Exchange, 2022). These variants also differ by population. Although, we can identify one specific mutation to target and correct, this approach would not be beneficial as a preventative therapy for all BRCA1 and BRCA2-associated hereditary breast cancers.

Although CRISPR-Cas9 technology is powerful, there are many limitations. These include difficulty in delivering CRISPR-Cas9 material to mature cells in large amounts, CRISPR-Cas9's inefficiency, and off-target edits. The first in-human phase 1 clinical trial in the United States by University of Pennsylvania in 2019 conducted that only 10% of all engineered T-cells contained triple mutations at the target sequence (Stadtmauer et al., 2020). This is not effective in targeting cancers. Even if researchers can correct 99% of all cells with BRCA1 or BRCA2 pathogenic mutations, the remaining genetically unstable cells can still become cancerous. So as of now, this technology can be used to manage the growth but not prevent cancer.

Although researchers are trying to find ways to circumvent these issues, a lot of advancements must be made to use CRISPR-Cas9 for BRCA1 and BRCA2-associated hereditary breast cancer. Most of the clinical trials that use CRISPR-Cas9 technology use its knock-out function. This was illustrated in the 2021 landmark CRISPR-Cas9 trial showing promise against Transthyretin amyloidosis. These researchers were able to use CRISPR-Cas9 to target and cleave the TTR gene. Endogenous DNA repair through NHEJ introduced insertions and deletions in the TTR gene, resulting in frameshift mutations. This mutation resulted in the reduction of TTR protein production (Gillmore et al., 2021). We cannot utilize CRISPR-Cas9's knock-out function on BRCA1 and BRCA2 genes. We need functioning BRCA1 and BRCA2 genes. So, we must rely on CRISPR-Cas9 knock-in functionality which is still in its nascent stage.

In the general population, only 0.1-0.2% are carriers of BRCA1 and BRCA2 mutations. BRCA1 and BRCA2 mutations are detected in 2-3% of all breast cancer cases

(Alacacioglu et al., 2018). The use of CRISPR-Cas9 for a disease that only a small cohort of patients might be impacted by might not accrue the same research funding. Many funding agencies might be hesitant to provide funds for such low-impact diseases.

So, what next? Instead of trying to fix BRCA1 and BRCA2 mutations, an alternative would be to use CRISPR-Cas9 technology to detect and kill cancerous cells.

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