Molecular and genetic studies of DLG2 in neuroblastoma and colorectal cancer

Simon Keane

Department of Laboratory Medicine
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Simon.keane@gu.se

Cover illustration: “Immunofluorescent staining of DLG2 isoform 7 (orange) and DLG2 isoform 2 (green) in neuroblastoma SKNBE cells”

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"The more that you read, the more things you will know.
The more that you learn, the more places you'll go."

– Dr Seuss,
I Can Read with My Eyes Shut!

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Gothenburg, Sweden

ABSTRACT

Neuroblastoma is one of the most common extra cranial solid tumors in children. It is one of the most common causes of death amongst the pediatric malignancies. There is however a large difference between the low risk neuroblastomas that are easier to treat compared to the high-risk neuroblastomas that are difficult to treat and account for a majority of the deaths. Two common groups of high-risk tumors have different genetic features, one with MYCN amplification and the other with loss of chromosome region 11q. Despite these two genetic alterations account for a high percentage of the total neuroblastoma cases they rarely occur together. Additionally, tumors with loss of 11q also tend to have decreased genome stability, resulting in increased DNA breakage. Currently, a number of candidate 11q tumor suppressor genes have been proposed, however, none of them have by themselves been able to explain the aggressive behavior of 11q-deleted neuroblastoma. For this reason, we continued the search and identified and characterized DLG2 a novel tumor suppressor gene residing in the 11q-deleted region.

DLG2 resides on the reverse strand at the proximal edge of the 11q deleted region. Within this thesis we have characterized some of the functions of DLG2 using large scale genetic data such as microarray and next generation sequencing in combination with various molecular techniques and tumor models. In addition to its importance in neuroblastoma behavior, we have also been able to show that DLG2 is altered early, during chronic inflammation, within the tumorigenesis of colon cancer, a vastly different tumor compared to neuroblastoma, indicating that DLG2 may be a common target of various cancers.

Through a combination of bioinformatic analysis and molecular methods we have shown that DLG2 affects the cell cycle and DNA repair pathways. We also discovered that the expression of DLG2 was equally affected either by 11q-deletion or by MYCN-amplification, making it a target in all high-risk neuroblastomas. We continued to show that there are a number of different isoforms of DLG2 with isoform 2 and isoform 7/8 the major isoforms expressed in neuroblastoma. The expression of isoform 2 remained stable so the decrease in DLG2 expression in neuroblastoma could be attributed to the loss of isoform 7/8, which alters the interactive ability of DLG2. In order to further elucidate the impact of DLG2-loss on DNA repair pathways, we investigated the relationship of DLG2 and genome stability by inducing dsDNA breaks by UVC irradiation or by etoposide, a topoisomerase II poison. We showed that loss of DLG2 was sufficient to result in dsDNA breaks without additional stimulus and that DNA breakage was prevented when DLG2 was present, by the removal of cells after the induction of breaks. We finally showed that DLG2 was silenced by inflammation early in the development of colon cancer. We showed that DLG2 activated the inflammasome and resulted in a decrease in STAT3 phosphorylation in adjacent cells.

To conclude, DLG2 with more research, may provide a treatment target to increase survival of high-risk neuroblastoma patients.

Keywords: Cancer, Neural crest, Neuroblastoma, colon cancer, DLG2, MYCN, LIN7A, inflammationISBN 978-91-8009-773-4 (PRINT)
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SAMMANFATTNING PÅ SVENSKA

Neuroblastom är en av de vanligaste solida extrakraniella tumörerna hos barn, och en vanlig barncancerrelaterad dödsorsak. Det finns dock en stor skillnad mellan olika typer av neuroblastom, lågriskneuroblastom är relativt lätt att behandla medan högriskneuroblastom är mycket svårbehandlade och står för en majoritet av dödsfallen. Högriskneuroblastomen kan delas upp i två grupper med olika genetiska egenskaper, en med MYCN-amplifiering och den andra med deletion av kromosomregion 11q. Trots att dessa två genetiska förändringar finns i en stor andel av de totala antalen neuroblastom, finns de sällan tillsammans i samma tumör. Tumörer med 11q-deletion uppvisar dessutom en minskad genomstabilitet, vilket resulterar i ökat antal DNA-brott. Hittills har ett antal gener i 11q-området föreslagits som kandidater till att vara viktiga tumörsuppressorgener, men ingen av dem har var för sig kunnat förklara det aggressiva förloppet hos 11q-deleterade neuroblastom. Mot denna bakgrund fortsatte vi sökandet och identifierade då DLG2, som en ny kandidattumörsuppressorgen som finns i den 11q-deleterade regionen.

DLG2 återfinns vid den proximala kanten av den 11q-deleterade regionen, den är alltså den första genen som alltid är påverkad av 11q-deletionen i neuroblastom. I denna avhandling har vi karaktäriserat några av funktionerna hos DLG2 med hjälp av storskaliga genetiska data, som microarray och NGS, i kombination med olika molekylära tekniker och tumörmodeller. Förutom att visa betydelsen av DLG2 för egenskaperna hos neuroblastom har vi också kunnat visa att uttrycket av DLG2 förändras tidigt i kolontumörer vid kronisk inflammation hos koloncancerpatienter. Kolontumörer är en mycket annorlunda tumör jämfört med neuroblastom, vilket indikerar att DLG2 kan vara viktig i många olika typer av cancer.

Genom en kombination av bioinformatisk analys och molekylära metoder har vi visat att DLG2 påverkar cellcykeln och DNA-reparation. Vi kunde också konstatera att uttrycket av DLG2 påverkades lika mycket av antingen 11q-deletion eller av MYCN-amplifiering, vilket gör DLG2 till ett intressant mål i samtliga högriskneuroblastom. Fortsättningsvis kunde vi visa att det finns ett antal olika isoformer av DLG2, där isoform 2 och isoform 7/8 var de som uttrycktes i neuroblastom. Uttrycket av isoform 2 hölls konstant, så minskningen i DLG2-uttryck i neuroblasom kunde tillskrivas isoform 7/8, denna isoform påverkar den interaktiva förmågan hos DLG2. För att få en fördjupad analys av effekten av DLG2 på DNA-reparation undersökte vi

förhållandet mellan DLG2-uttryck och genom-stabilitet genom att inducera dsDNA-brott genom UVC-bestrålning eller etoposidbehandling. Vi kunde visa att förlust av DLG2 var tillräckligt för att resultera i dsDNA-brott utan ytterligare stimulans och att DNA-brott förhindrades vid närvaro av DLG2, genom att celler med DNA-brott avlägsnades. Slutligen visade vi att DLG2 kunde tystas av inflammation tidigt i utvecklingen av koloncancer och att DLG2 kunde aktivera inflammasomen och resulterade i en minskning av STAT3-fosforylering i intilliggande celler.

Sammanfattningsvis skulle DLG2, efter mer forskning, kunna vara ett nytt mål för behandling för att öka överlevnaden för patienter med högrisk-neuroblastom.

Nyckelord: Cancer, Neural crest, Neuroblastom, koloncancer, DLG2, MYCN, LIN7A, inflammation

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Keane, S**., Améen, S., Lindlöf, A., & Ejeskär, K. (2020). Low DLG2 gene expression, a link between 11q-deleted and MYCN-amplified neuroblastoma, causes forced cell cycle progression, and predicts poor patient survival. Cell Communication and Signaling, 18(1), 1-14.

II. **Keane, S**., Martinsson, T., Kogner, P., & Ejeskär, K. (2021). The loss of DLG2 isoform 7/8, but not isoform 2, is critical in advanced staged neuroblastoma. Cancer Cell International, 21(1), 1-13.

III. **Keane, S**., de Weerd, H. A., & Ejeskär, K. (2022). DLG2 impairs dsDNA break repair and maintains genome integrity in neuroblastoma. DNA Repair, Apr;112:103302.

IV. **Keane, S**. Herring, M., Rolny, P., Wettergren, Y. & Ejeskär, K. (2022) Inflammation supresses DLG2, preventing inflammasome formation. Journal of Cancer Research and Clinical Oncology, *in press*

Publications not included in this thesis

V. Trindade, F., Saraiva, F., **Keane, S.,** Leite-Moreira, A., Vitorino, R., Tajsharghi, H., & Falcão-Pires, I. (2020). Preoperative myocardial expression of E3 ubiquitin ligases in aortic stenosis patients undergoing valve replacement and their association to postoperative hypertrophy. PloS one, 15(9), e0237000.

VI. Deland, L., **Keane, S**., Olsson Bontell, T., Sjögren, H., Fagman, H., Øra, I., ... & Abel, F. (2021). Discovery of a rare GKAP1-NTRK2 fusion in a pediatric low-grade glioma, leading to targeted treatment with TRK-inhibitor larotrectinib. Cancer biology & therapy, 22(3), 184-195.

CONTENT

ABBREVIATIONS

CAC	Colitis associated colon cancer
SCC	Spontaneous colon cancer
APC	Adenomatous polyposis coli
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
KRAS	Kirsten rat sarcoma virus
MSI	Microsatellite instability
CIN	Chromosomal instability
CIMP	CpG island methylation phenotype
HVA	Homovanillic acid
VMA	Vanillylmandelic acid
MIBG	meta-Iobenguane
INRGSS	International neuroblastoma risk group staging system
INSS	International Neuroblastoma Staging System
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CDKs	Cyclin dependent kinases
HR	Homologous Repair
c-NHEJ	Canonical non-homologous end joining
MMEJ	Microhomology-mediated end joining
PAMPs	Pathogen Associated Molecular Patterns

DAMPs	Damage Associated Molecular Patterns
GUK	Guanylate kinase
DLG2	Disks large homolog 2
ISO	Isoform
SRO	Smallest region of Overlap
ATRA	All Trans Retinoic Acid
CRC	Colorectal cancer
UC	Ulcerative colitis
IBD	Inflammatory bowel disease
DSS	Dextran sulfate sodium

1 INTRODUCTION

1.1 CANCER

In the western world, one in eight individuals between the age of 60 and 69 develop some form of cancer with this increasing to one in three individuals over 70 years of age [1]. As we live longer and longer the likelihood of genetic alterations accumulating and resulting in a cancer increases. In addition to the interplay of age and genetics, the final factor determining the development of cancers are the environmental factors [2].
Colon cancer is one of the most prevalent cancer types with almost two million cases and close to 1 million deaths worldwide each year [3]. As such it is the fifth most common malignancy resulting in mortality. The development of colon cancer is generally related to age, diet and environmental factors. Age is one of the most important factors with regard to disease development. With colorectal cancers generally affecting older adults, over 50 years of age, however, this is changing with more cases occurring and been detected under 50 years of age [4]. The environmental risk factors for developing colorectal cancer include; a sedentary lifestyle, obesity, heavy alcohol use, smoking and inflammatory bowel conditions [5, 6]. Moreover, it is thought that the increase in sedentary lifestyles and the changing dietary patterns are the driving factor in the younger cases.

1.1.1 ADULT CANCER: COLON CANCER

The colon, along with skin, is in close contact with a large population of microbes, and is therefore, one of the main sites in the human body that encounters high concentrations of pathogens. These pathogens can represent an infection risk if there is a disturbance in the commensal gut microbiota [7] or themselves can act to disrupt the commensal gut bacteria [8]. As a result, there is a large number of immune cells presence in the colon with one of the main immune defenses resulting in inflammation. Inflammation results in increased cell division to replace damaged cells [9]. If the inflammation is chronic the rate at which cells are replaced can exceed the required rate resulting in flattened cellular outgrowths. This type of development, which is the least common, is termed Colitis associated colon cancer (CAC) [10, 11] and referred to as inflammation-dysplasia-carcinoma-sequence [12]. The main colon cancer development pathway is spontaneous colon cancer (SCC) [13] also referred to as the adenoma-carcinoma-sequence [12]. This mutation and development sequence results in raised lesions that are detectable with sigmoidoscopy or colonoscopy. The limitations of these detection methods are cost and patient acceptance with the investigations tending to occur after additional symptoms such as blood in the stools are detected [14].

Some of the genetic factors driving disease development include mutation or silencing of the tumor suppressor genes; APC [15], p53 and MLH1 or MSH2 [16] and the activation of oncogenes such as KRAS [17]. The tumors can additionally be classified based on molecular characteristics, with three main groupings, microsatellite instable (MSI), Chromosomal instability (CIN) and epigenetic status determined by CpG island methylation phenotype (CIMP) [18]. The genes that are inactivated or activated are dependent on the development sequence that the tumor is following with CIMP targeting CDKN2A and MLH1, CIN targeting the amplification of AURKA and MSI correlated to increased fatty acid synthase (FASN) [19]. In addition, the cohort of patients with early onset colon cancers localized to the left-side frequently have losses of 11q14.1-14.3 [20], in combination with other chromosomal alterations resulting in a higher CIN. However, it is also worth noting that events can overlap between the different molecular types with the loss of CDKN1B appearing in both MSI and CIMP tumors [19] (Figure 1).

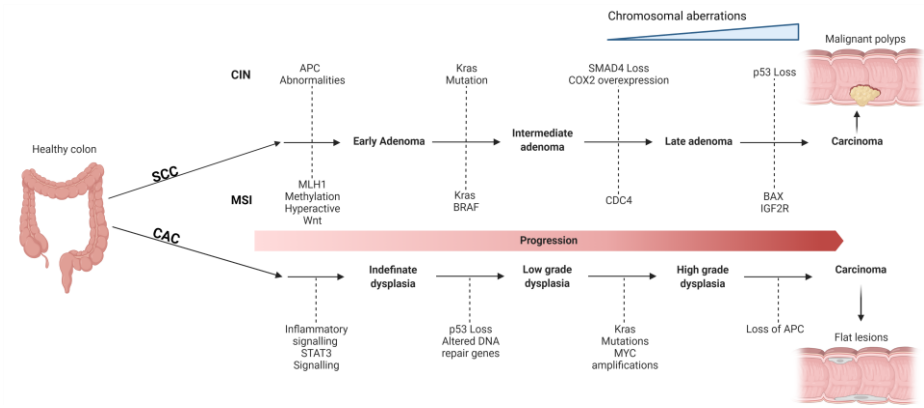

Figure 1. The progression of Colon cancer by either spontaneous colon cancer (SCC) or by Colitis-associated colon cancer (CAC). The progression of SCC has been further divided into Chromosomal instability (CIN) or Microsatellite instability (MSI). The progression of SCC results in polyp formation and invasive tumors whereas the CAC program results in dysplasia and flatter lesions. The common genes associated with both CAC and SCC include APC, KRAS, p53 and COX2. Created with BioRender.com

1.1.2 CHILDHOOD CANCER

In direct contrast to adult cancers there are children's cancers such as leukemias, gliomas and neuroblastomas (NB). For these types of tumors, it is difficult to identify the environmental factors leading to the disease. In part due to the relative rarity of the disease as well as the difficulty in identifying the exposure to potentially carcinogenic factors [21]. As a result of these difficulties the main focus for research has been on the genetics of the tumors. However, other factors that need to be considered when treating childhood cancers, that are of limited concern in adult tumors, is the lifelong effect that any treatment will have on the individual [22]. Treatment for childhood cancers can result in; weakness in the limbs, growth deficiency, scoliosis, seizures, hearing loss and blindness [23]. Hearing loss can be a result of radiation treatment as well as the cumulative dose of cisplatin [24] and cisplatin dose has also been shown to result in kidney damage in children treated for cancer [25]. It has previously been reported that the impact of these type of treatments on young developing children resulted in learning difficulties, impaired social skills, requirements for special education as well as fewer friends and low self-esteem [24]

1.2 NEUROBLASTOMA

Neuroblastoma is a tumor that forms within the sympathetic nerve tissue in young children. It is one of the most common forms of solid extra cranial malignant tumors found in children aged two years and under, with almost all cases presenting before five years of age [26]. Primary tumors most commonly form in the adrenal glands as well as in the parasympathetic ganglia of the thorax. Due to the age of the patient and the vague appearance of the symptoms the clinical diagnosis of neuroblastoma is difficult. The exact location of the tumor can alter the presenting symptoms, e.g. primary tumors that form in the chest can press against a lung and cause breathing difficulties, and tumors that are found in the paravertebral ganglion can press against the spinal cord causing difficulties related to movement [27]. Due to the vague symptoms it is common that the tumor metastasizes before diagnosis, with the bone marrow been the most common site for metastasis formation [28]. It is common that a combination of imaging, biopsy and biochemistry tests are required for neuroblastoma to be diagnosed. Most patients show high levels of blood or urinary catecholamine metabolites of neurotransmitters such as Homovanillic acid (HVA) or Vanillylmandelic acid (VMA). Imaging studies for neuroblastoma often make use of meta-Iobenguane (MIBG), a radiolabeled analogue of adrenergic neurotransmitters that the nerve cells will take up and allow the visualization the location of the tumor by scintigraphy [27]. Finally, the tumor biopsy will appear as small round and blue after Hematoxylin and Eosin (H & E) staining, due to the high number of undifferentiated cells and large nuclei [29].

There are two common staging systems for classifying neuroblastoma tumors; a presurgical and a post-surgical. The presurgical classification is performed according to the international neuroblastoma risk group staging system (INRGSS) [30]. INRGSS builds off the INRG stages L1, L2, MS and M where L tumors are localized, M are metastatic and MS is a special metastatic tumor that is limited to the skin, bone marrow or liver and the patient is under 18 months of age. Based off the; four stages, age, histology, differentiation, MYCN status and genetic profile, neuroblastoma patients are subsequently given a pretreatment risk group; very low, low, intermediate or high. The definition by risk group predicts the prospects for treatment and will often direct the therapy given, with very low, low and intermediate having good treatment prospects. The high-risk tumors include all tumors with MYCN amplification, all metastatic tumors except those that are hyperdiploid and all MS tumors except the patients under 12 months of age with a favorable genetic profile. These high-risk tumors have poor treatment prospects and are generally resistant to current therapies, this difficulty to treat results in a higher mortality and increased chronic health problems [31, 32]. Using the international neuroblastoma staging system (INSS) neuroblastomas can be post-surgically staged [33]. Stages 1 and 2 are complete or partially resected localized tumors that do not cross the midline, nor

have lymph node activity across the midline. Stage 3 denotes the larger localized tumors, crossing the midline or infiltrated proximal lymph nodes across the midline. Stage 4 tumors have advanced dissemination of the tumor to distant lymph nodes, bone marrow, liver, skin or other organs. In the cases where the patient is younger than one year old, with a one-sided tumor with metastasis to the liver or skin but less than 10% bone marrow involvement the patient is stages as special cases, Stage 4s [33]. As such if a MIBG scan has been performed there should be no visible infiltration into the bone marrow. The survival prognosis of the patients decreases as the stage increases with the exception of stage 4s patients whom have survival prognoses better than the stage 3 tumor group. Within the higher staged tumors there are also subtypes with highly specific but varying genetic alterations. Currently, the overall survival of the high-risk subgroup is between 40% and 50%, drastically lower than the 90-95% survival for the other risk groups [34].

1.2.1 GENETICS OF NEUROBLASTOMA

A limited number of NB tumors can be described as familial. The identification of mutations within PHOX2B in parents, associated with other diseases and conditions of the neural crest, increase the risk of the offspring developing neuroblastoma [27]. Initially, the PHOX2B mutation was identified as a driver of developmental disorders and congenital malformations due to the function in the developing nervous system. The whole picture of familial neuroblastoma is not completely explained by this one gene mutation with only 10% of all familial neuroblastomas containing this mutation indicating that there are other factors that influence the development in familial cases. Sporadic neuroblastomas account for the other proportion of neuroblastoma cases, with these tumors often containing one of a limited number of mutations, resulting in a relatively consistent gene signature. Common gene alterations include; ALK, ATRX, CD79B, and SOX9 [35], with CHK2 and APC germline alterations also noted within neuroblastoma tumors [26, 36]. Additionally, there are a number of recurrent chromosomal alterations that occur within neuroblastoma tumors. The loss of 3p25.3-p14.3 is present in approximately 15% of all neuroblastomas [37], and loss of this region is associated with deletion of chromosome 11q [38]. The 1p36-loss is present in a large number of tumors with some estimates showing that the deletion is present in around 35% of all neuroblastoma and 70% of the high-risk neuroblastoma tumors [39]. Deletion at this locus also shows preferential allelic deletion with the maternal 1p chromosome often deleted in non-MYCN amplified tumors. Tumors that have MYCN-amplification also have a larger SRO 1p36-ter as well as non-preferential deletion of the 1p36 segment [40]. The most common alteration in neuroblastoma is gain of 17q21-ter which is present in 66.3% of all cases [41]. The gain is often associated with advanced stage disease markers such as 1p-loss, MYCN-amplification and patient age older than 12 months. The high-risk neuroblastoma tumors often have either; MYCN-amplification or 11q-deletion (11q14.1-ter), which account for approximately 20% and 30%, respectively, of all cases [42].

1.2.2 MYCN

One of the earliest genetic alterations observed in neuroblastoma tumors was the amplification of MYCN. As previously discussed it also remains one of the key criteria for the classification of patients. The amplification of MYCN is thought to occur early in the tumorigenesis process, and if it is not present at diagnosis it will not appear later. Interestingly, the amplification of MYCN and the 2p24 chromosome cassette also results in increased copies of the adjacent gene ALK which as previously mentioned is also a driver of neuroblastoma [43]. Moreover, MYCN heterodimerizes with MAX to bind the consensus E-box DNA sequence to promote proliferation and cell cycle progression in the early developmental stages and should be essentially absent in adult tissues. MYCN has also been shown to suppress differentiation and immune surveillance [44].

1.2.3 11Q DELETION

Debate has raged over the smallest region of overlap (SRO) of the deletion of the 11q-deletion in neuroblastoma. Initially, the SRO was identified at 11q23 [45], with subsequent investigations showing that the deletion extended to 11q14 [46]. It has now been shown that there are three distinct SROs on 11q; the first, a small amplification from 11q13.2 to 11q13.4 which includes the cell cycle gene CCND1. The second, a deletion spanning from 11q14.1 to 11q22.2 where the patient does not have MYCN-amplification. The final region, for the rare tumors with both 11q deletion and MYCN-amplification is a deletion spanning 11q23.1 to 11q23.3 for the [47]. Patients with 11q loss are always heterozygous for the deletion and generally lack MYCN-amplification [46] unless they have the more distal breakpoint. As previously stated these two genetic alterations are common in neuroblastoma, however, they rarely occur together and as such they are considered to be mutually exclusive [28]. To date a number of 11q tumor suppressor genes have been proposed, including; CADM1 (11q23.3) [48], ATM (11q22.3) [49] and H2AFX (11q23.3) [28].

1.2.4 TREATMENT OF NEUROBLASTOMA

The current treatment protocol varies depending on the risk, stage and how aggressive the tumor is. For the tumors that are very low risk the patient is placed under observation as the tumor can spontaneously resolve [50]. Surgical intervention to remove the tumors can also be performed. However, if the tumor has spread across the midline or is unsafe to remove, more treatment alternatives need to be used. Radiation treatment is used after surgical intervention to destroy the remaining cells to minimize the risk of relapse to occur [51]. For aggressive tumors a phased combination approach is often used with surgery, radiation, chemotherapy and immunotherapy [28]. The chemotherapy drugs can include; platinum-based drugs such as cisplatin, drugs to induce DNA breaks (alkylating agents) as well as drugs that influence the cell cycle and cause DNA breaks, such as topoisomerase poisons like etoposide [28]. When treating the aggressive tumors with agents that break the DNA (alkylating agents and topoisomerase inhibitors) the treatment is often unsuccessful. Possibly, as the cells can repair themselves satisfactory, the treatment just acquire more mutations, and in some cases, make the tumor more aggressive. Currently, the only option available to physicians is to give higher cumulative doses of the chemotherapy agents.

1.3 GENES, GENETICS AND GENOME

When we are discussing the genome, we are referring to all genetic material that is within an organism. A human generally has 23 pairs of chromosomes with half of the pair coming from the mother and half from the father, resulting in 46 chromosomes in total. At the ends of each of the chromosomes there are protective caps, or telomeres, that help to maintain the chromosomes integrity by preventing the DNA from fraying, much in the same way that an aglet protects a shoelace. Chromosomes, during metaphase are condensed into ropey structures, and can be stained and visualized by producing a karyotype. Initially, to compare chromosomes, they were ordered and numbered based on size from largest, chromosome 1, to smallest, chromosome 22. Due to the limitations at the time of discovery, and their closeness in size chromosomes 21 is actually smaller then chromosome 22 but the naming convention has remained. Every chromosome has a number of genes with flanking regulatory sequences and coding sequences called exons, interrupted by introns. The classical approach, mendelian genetics [52], was to investigate how discrete inheritable units, genes, resulted in easily observable alterations to the phenotype. It wasn't until Watson and Crick and, Franklin and Wilkins that we discovered how the discrete inheritable units were structurally arranged [53] in the form of a DNA double helix. The mendelian genetics approach has led to the discovery of a number of monogenetic disorders, diseases that can be explained by a single gene alteration in either a dominant or recessive manner [54]. However, this approach is limited in its use of complex disorders and diseases where multiple genes may be affected [55]. Once the central dogma of biology was established, i.e. that DNA is transcribed into messenger RNA that is subsequently translated to protein, the study of genes and their effect was able to proceed. As the use of "omics", which is large scale biological data and associated computations used and applied to understand the data, has increased and the human genome has been sequenced, the total number of estimated genes has decreased but the understanding of the true complexity of the genome increased. The presence of different types of RNAs and the wide-reaching effects that they have on the activity of the genome has only begun [56].

1.3.1 HOMOLOGUES, PARALOGUES, ORTHOLOGUES AND ISOFORMS

Within our genome and the genomes of closely related mammals there are a number of genes closely related to each other through shared evolutionary lineages. These are termed as homologues, we can specify the type of homology further by determining how and when the duplicated gene came to be. Throughout the genome there are a number of genes that are highly similar in their genetic code and protein structure. These genes often arise from the duplication of a single ancestral gene and subsequently reside within different locations in the genome [57], these types of gene duplications are known as paralogues. Over time these paralogues may be able to diverge and slightly change their function. When a speciation event occurs the genes that are present in the ancestral species will be present in all of the progeny species. Over time these genes will slowly diverge and become sequentially dissimilar but remain functionally similar. For example, we can compare human genes sequences to the gene sequences of mice and fruit flies to establish common ancestral genes and determine functionality. The genes that arise from this type of gene duplication are termed as orthologs [58]. Lastly, a single gene that either has; multiple transcription start sites (TSS), variable exon structures or variable untranslated regions can result in different transcription products termed as gene isoforms [59]. Isoforms often have alterations within the exon structure can alter the protein domains that are present within the protein product and hence change the function.

1.4 THE CELL CYCLE

For a cell to divide a number of key steps must occur so that the daughter cells maintain the fidelity of the source genetic material. Due to the cellular logistics of copying all of the chromosomes, including epigenetic marks and subsequent sorting of the new material into new daughter cells, a number of checkpoints and signaling cascades must come together for the process to function [60]. There are two main protein families that are responsible for controlling the cell cycle, the cyclins which are active periodically and the cyclin dependent kinases (CDKs) [61]. A specific cyclin accumulates in each stage of the cell cycle which preferentially binds to the CDK which induces the signaling cascade and hence signals the cell to advance to the next stage in the cell cycle.

The cell cycle consists of four main phases, the G1 phase where the cell cytoplasm increases in size. Cyclin D accumulates throughout the first half of the cycle and decreases in the other half as such it is the dominant cyclin during the G1 phase. The accumulation of Cyclin E during the latter half of the G1 phase results in the start of the Synthesis phase (S-phase). The S-phase is where the whole genome is copied so that the cell now has two complete sets of chromosomes. This phase is completed as Cyclin A peeks and the cell enters the G2 phase. The G2 phase is where the cell continues to increase in size and gets ready to divide into two daughter cells by increasing the cellular machinery to ensure that there is sufficient material for two cells. The final step in cell division is the M-phase which is marked by the accumulation of Cyclin B. In this phase the chromosomes are segregated into the new daughter cells and the separation occurs [60]. Cells can also enter into the G0 phase or quiescence and generally not partake in further cell divisions. This is normally the state for fully differentiated cells. There are two key checkpoints that act to maintain control and prevent the cell from progressing through the cell cycle. The first is the G1 checkpoint, which is also known as the restriction checkpoint and is responsive to both internal and external signaling [62]. The external singling is often mitogenic signaling that targets Cyclin D to push the cell into the cell cycle. Internal signaling often targets the cell cycle proteins such as the RB protein. The other checkpoint is the G2/M checkpoint and ensures that DNA integrity is high by allowing the cell enough replication time to ensure that the S-phase is complete and any damaged DNA is repaired [63]. The accumulation of Cyclin B pushes the cells through this checkpoint and results in cell division. The accumulation of Cyclin B when the DNA is damaged or incompletely replicated can result in genome instability (Figure 2).

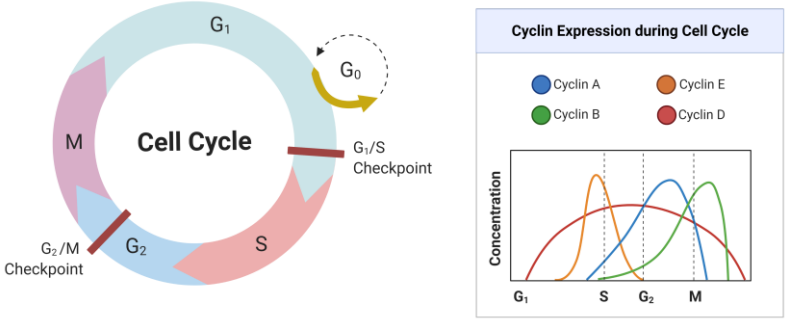

Figure 2. Cell Cycle: A schematic overview of the progression of a cell though the different phases of the cell cycle. Including quiescence or senescence (G0), Gap1 (G1), Synthesis (S), Gap2 (G2) and Mitosis (M). The relative expression of cyclins throughout the cell cycle, with cyclin E required to pass the G1/S checkpoint, cyclin A to pass the G2/M checkpoint and cyclin B for the M/G1 checkpoint. Throughout the cycle there are 3 checkpoints that ensure proper control and progression. The G1/S checkpoint is known as the Restriction checkpoint and the G2/M checkpoint is the DNA damage checkpoint. Created with BioRender.com

1.5 DNA REPLICATION AND REPAIR

DNA strand breaks occur in all types of cells and not only in cancer cells. A cell with a DNA break can either undergo apoptosis (cell death) or repair the damage [64]. Depending on the type of DNA break different mechanisms will be used. If only a single strand is broken the DNA repair will often be highly successful as template DNA still remains [65]. However, if both DNA strands are broken, known as a double strand break (dsDNA break), the repair can be mediated by one of three different mechanisms; Homologous repair (HR), canonical non-homologous mediated end joining (c-NHEJ) and microhomology mediated end joining (MMEJ). Generally, it is accepted that once dsDNA strand breaks occur H2AX is rapidly phosphorylated (γH2AX) which mark the broken ends of the dsDNA. Subsequently, the MRN complex consisting of MRE11a, Rad50 and NSB1 interacts with γH2AX. PCNA subsequently acts to clamp the DNA stands and the polymerase to facilitate repair. The MRN complex also interacts with ATM [49] and H2AX [28], both of which are established tumor suppressor candidates located in the 11q deletion of aggressive neuroblastoma. MRE11 and PARP1 expression are both upregulated in MYCN amplified neuroblastoma tumors [66, 67]. Treating these aggressive neuroblastoma tumors with agents that break the DNA (alkylating agents and topoisomerase inhibitors) is often unsuccessful, likely as these cells are highly adept at performing DNA repair, the effect of which can be genome instability and an aggressive tumor [28].

1.5.1 HOMOLOGOUS REPAIR

Homologous Repair (HR) uses the sister chromatid or homologous chromosome as a template and is highly accurate. The repair however, can only occur late in the cell cycle [68] after the S phase and generally during G2. The use of the redundant information on the sister chromatid to make the repair generally means that the repair is accurate. The break needs to be made into a single stranded sticky end section that can be used to find homology on the sister chromatid. For the homology to be found the intact sister chromatid DNA has to be prepared by topoisomerases [68]. Generally, this type of repair does not result in crossover of genetic material and maintains the heterozygosity of the regions. HR is determined by the activation and presence of RAD51 [69].

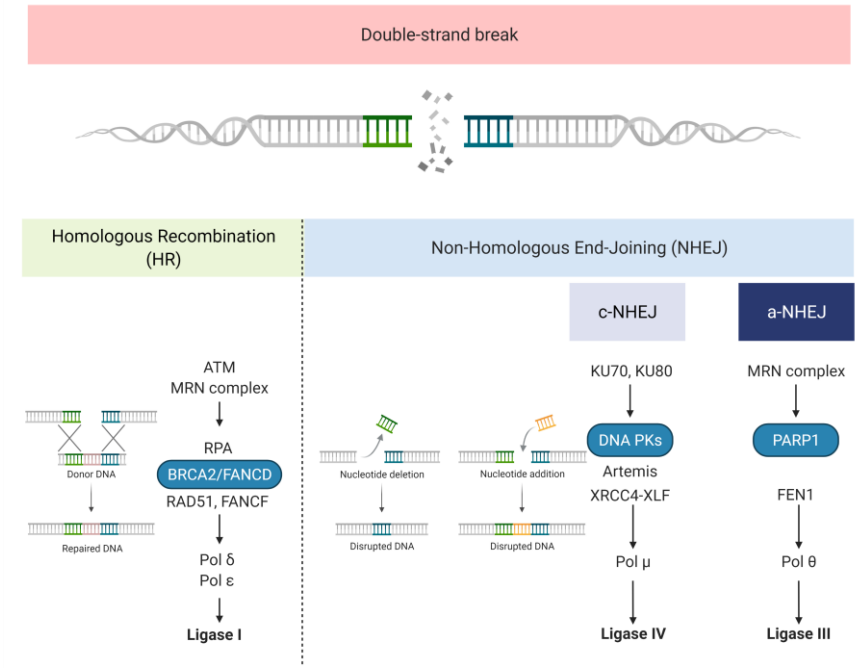

Figure 3. dsDNA damage repair: A schematic overview of the two major types of dsDNA repair process along with the two subtypes of NHEJ. The key proteins are highlighted in blue. The specific polymerase used in each type of repair is also listed along with the Ligase required. Important to note is that the HR requires donor DNA and the NHEJ mediated repair results in disrupted DNA sequences. Created with BioRender.com

1.5.2 NON-HOMOLOGOUS MEDIATED END-JOINING

Canonical non-homologous end joining (c-NHEJ) is generally active during the early phase of the cell cycle, before S phase and as such functions without the use of template DNA [70]. It can however be active throughout the cell cycle. As the repair does not use any DNA template, as with HR, but homology sequences, sequences that share similarity. This type of repair is considered to be mostly accurate but dependent on the overhang that is caused by the break [71]. If the repair is not precise and the overhangs are not compatible, translocations can occur [72]. The initial steps after DNA breakage are the same as HR with the MRN complex required to hold the broken ends in proximity. The presence of the Ku complex determines that c-NHEJ mediated repair will occur, with the Ku proteins forming a complex with DNA-PKcs [72], however the exact functions and mechanisms of this interaction are currently not known. The broken ends in this case need to be prepared, often by 5′ extension by DNA polymerase, resulting in blunt ends, followed by ligation of the blunt ends by LIG4 and XRCC4 [73].

1.5.3 MICROHOMOLOGY MEDIATED END JOINING

The least accurate of the DNA break repair mechanisms is microhomology-mediated end joining (MMEJ), also known as alternate-NHEJ (alt-NHEJ) and is active when HR is nonfunctional or will directly compete with HR by a mechanism that is currently not fully understood; however, it is known that MMEJ is active at the same time in the cell cycle as HR [74]. This type of repair will often be deleterious as there is no DNA template remaining for the repair [75, 76]. Repair by MMEJ will often result in microdeletions of a few base pairs due to the cleaving of overhangs adjacent to the homology sequences or microinsertions of a few base pairs where gaps have been filled [77]. The MMEJ pathway includes the genes; MRE11A, FEN1, LIG3 and PARP1 [76]. Colorectal cancers have been shown to rely on this type of dsDNA repair and with a number of MMEJ components shown to be upregulated [78]. FEN1 is also generally shown to be upregulated in all neuroblastomas whereas, in MYCN amplified neuroblastoma tumors LIG3 and PARP1 are often constantly shown to be upregulated [79]. Unique to this pathway is the POLQ gene (DNA-polθ) which is a unique polymerase that can fill sequence gaps [80].

1.6 INFLAMMATION

Inflammation is the collective name given to a complex response of tissues to harmful stimuli that can otherwise damage cells. In broad terms the goal of inflammation is to eliminate the initial stimuli, remove the damaged cells and activate tissue repair. The process by which this occurs depends on the initial stimuli, with the trigger mediated through pattern recognition receptors (PRRs), which include Toll like receptors (TLRs), RIG-I like receptors (RLRs) and the NOD like receptors (NLRs) [81]. These different receptor classes are subsequently activated by specific Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs) [82]. The function of PAMPS and DAMPS are to activate signaling cascades to initiate the formation of inflammasomes, which are large multimeric proteins that activate inflammatory responses [83]. One of the important steps in the activation of the inflammasome is paracrine signaling to the adjacent cells in the tissues to activate their inflammatory response in preparation for harmful stimuli. Generally, the initial signaling cell will undergo pyroptosis, a form of programmed cell death relying on inflammation, to promote the rapid signaling to the surrounding cells. These surrounding cells will also undergo pyroptosis, with the further adjacent cells been able to clear the stimuli [84]. Concise modulation of this process is required, as constitutive activation of the inflammasome can result in chronic inflammation. Dysregulation of the inflammasome has been shown in a number of cancers and other diseases, with proper function of the inflammasome been maintained within a narrow range. Inflammasomes are named after the NLRP that is bound into the complex, with the most common being NLRP3. Activation of the NLRP3 inflammasome has been shown to require NFKBIZ (IκBζ), a lesser known Iκβ. Chronic inflammation and inflammasome activation have been shown to result in an increase in IL-6 expression, which subsequently activates STAT3, a signaling pathway that when active has been shown to result in poor outcomes in colon cancer [85].

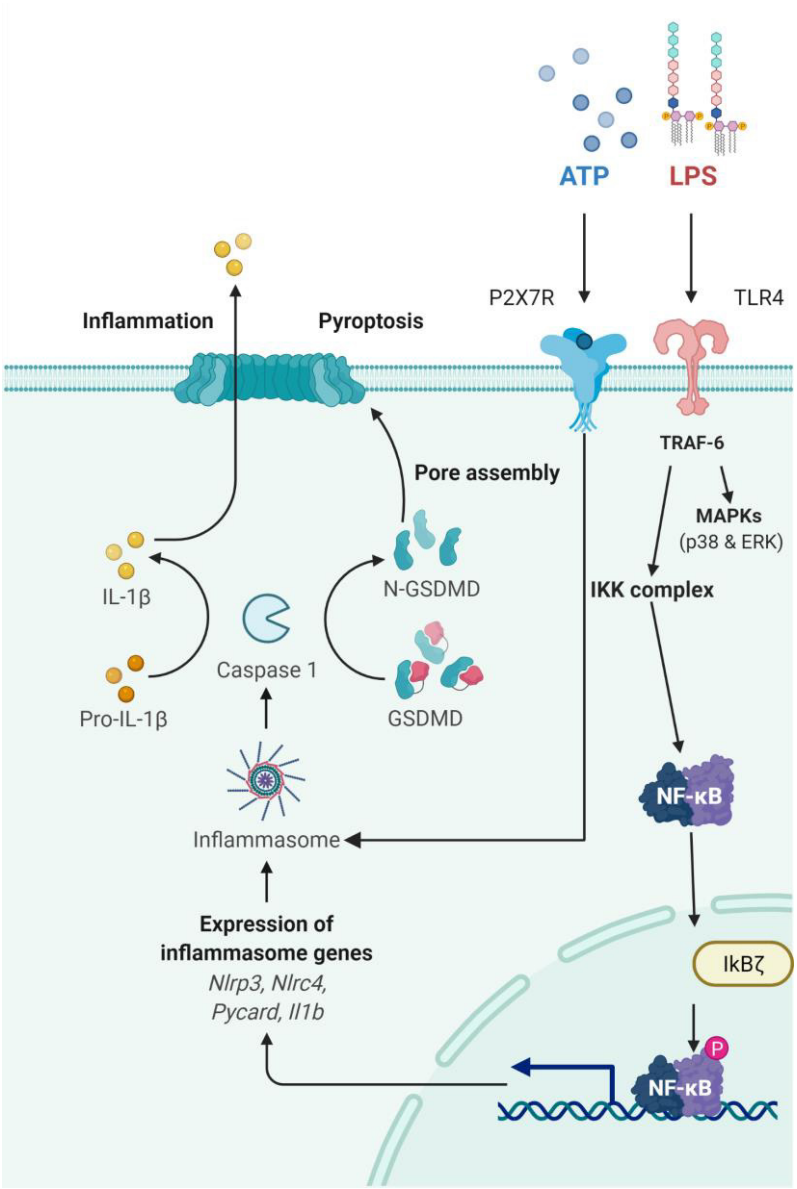

Figure 4. A schematic for the activation of the NLRP3 inflammasome. The priming of the complex occurs during the LPS stimulation, resulting in the transcription of inflammasome components by NFKB mediated DNA binding. The second signal to assemble the inflammasome occurs by the activation of the P2X7 receptor by the presence of extracellular ATP. The effect of inflammasome activation is the formation of pores by gasdermins and the cleavage of Pro-IL-1β into IL-1β which is subsequently released into the extracellular space. Created with BioRender.com

1.7 HALLMARKS OF CANCER

All multicellular organisms have developed distinct cell populations with specific functions, properties and spatial distributions, with maintenance of these tightly controlled. In essence the loss of control over a cell population is the underlying cause for all cancer forms. For a cell to begin the process of tumorigenesis a number of steps need to occur to allow for the transformation from a normal cell into a malignant version. Selection pressure akin to Darwinian evolution allows for the cell to adapt and develop strategies to survive. Whilst the exact mechanism that a tumor and even tumor subtypes use can differ, the general molecular steps and alterations are considered to be the same, and are referred to as the hallmarks of cancer [86]. The hallmarks have been added to and revised a number of times, with each of the updates been able to follow the trends in cancer research as well as providing a framework in which to view and study cancers. For a cell to undergo tumorigenesis it is generally accepted that it needs to acquire the following characteristics; resistance to cell death, sustained proliferative signaling, evasion of growth suppressors, tissue invasion and metastasis, replicative immortality and induce angiogenesis. Whilst the previously mentioned hallmarks have been accepted for more than 20 years, the next update identified two additional hallmarks; avoiding immune destruction and deregulation of cellular energetics, as well as two characteristics that allow for the hallmarks to develop; the creation of a tumor promoting microenvironment and increased genome instability and mutation rates [87]. Finally, the latest refinement of the hallmarks identified phenotypic plasticity and senescent cells as the latest hallmarks and non-mutational epigenetic reprogramming and polymorphic microbiomes as enabling characteristics [88] (Figure 5).

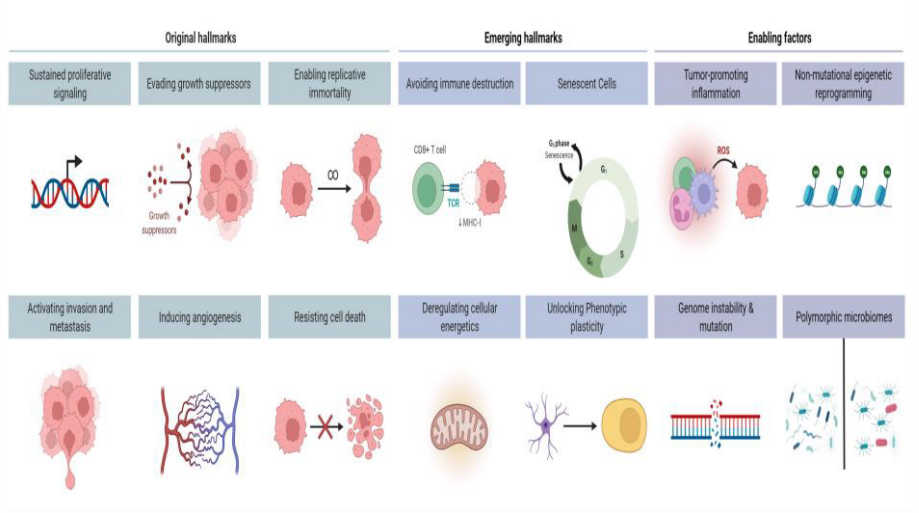

Figure 5. The hallmarks of cancer are a theoretical framework for simplifying the complex biology of tumors into six functional alterations that generally occur and are needed for a healthy cell to transform into a neoplastic cell. The hallmarks of cancer have been added upon twice over the previous 20 years. Each of the updates provided two new emerging hallmarks and two new enabling factors. Created with BioRender.com

1.7.1 RESISTING CELL DEATH

Once a cell reaches the end of its lifespan, specific signaling cascades are activated to destroy that particular cell. The most commonly studied mechanism is that of apoptosis, or programmed cell death. The programmed cell death mechanism uses a balance of BCL2 family members to either promote survival or initiate a caspase cascade to initiate intrinsic apoptosis [89], depending on the relative abundance [90]. Another type of programmed cell death is pyroptosis, a highly inflammatory lytic process that is controlled by the formation of inflammasomes [91]. For a cell to evade these types of cell death they can develop a number of strategies, the most common that appears across all cancers is dysregulation of p53 [92, 93]. p53 is often regarded as the guardian of the genome as this protein accumulates with genome damage and initiates apoptosis if the damage is too great to repair. Alternatively, suppression of the caspase proteins can avert the function of the apoptotic signaling cascade [94] (Figure 6).

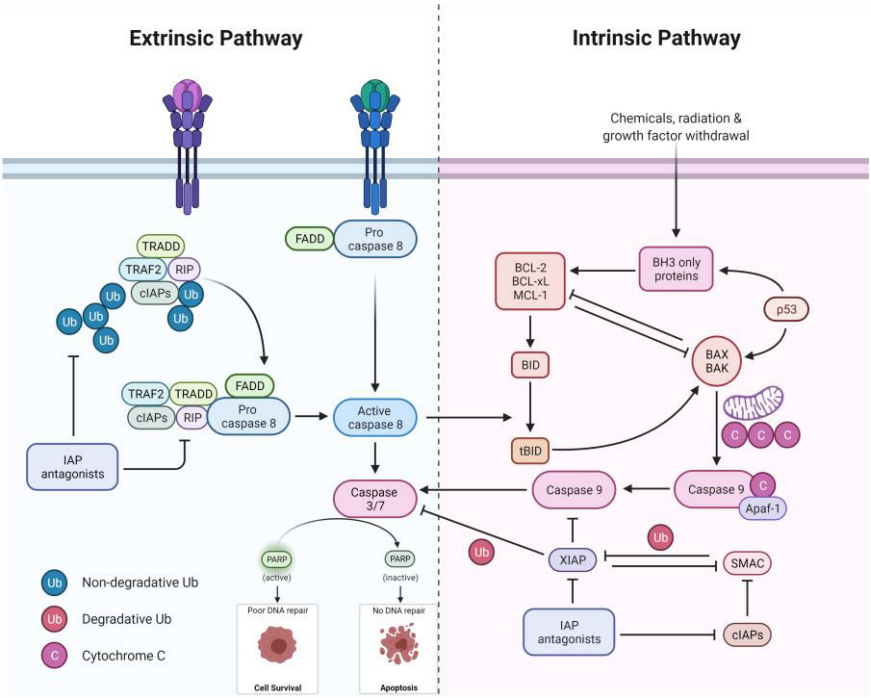

Figure 6. A comparison of the activation and the convergence of the extrinsic and intrinsic pathways of apoptosis. Activated Caspase 8 can activate intrinsic apoptosis as well as Caspase 3/7 to initiate early apoptosis. The intrinsic pathway feeds into the activation of Caspase 3/7 by the BAX/BAK mediated activation of Caspase 9. If PARP is active then the genome can be repaired and recover resulting in cell survival, if it is inactive then the cell will complete apoptosis. Created with BioRender.com

1.7.2 SUSTAINING PROLIFERATIVE SIGNALING

The maintenance of proliferative signaling is one of the more fundamental of the hallmarks. By forcing the cell to enter into the cell cycle and divide, resulting in increase in total cell number. The effect of which is to increase the probability of acquiring additional mutations that may be beneficial to the cancer, a common example of which are the RAS family [95] which drive sustain Proliferation signaling. Cell proliferation and signaling is modulated by growth factors interacting with membrane bound receptors [96]. Growth factors are typically released from a cell and can subsequently act on itself as well as adjacent cells, called paracrine signaling [97]. Once the growth factor interacts with the appropriate receptor, often a tyrosine kinase, the result is an internal signaling cascade and activation of proteins that once in the nucleus result in the upregulation of Cyclin D [98]. As this process can be impacted by adjacent cells, cancer cells can avoid reliance on external signaling by developing fusion proteins, the joining of two otherwise unrelated protein domains together, by joining unrelated gene segments. An example of this is the Philadelphia chromosome, commonly seen in leukemia, where a segment of chromosome 22 and chromosome 9 are fused and create a fusion of the genes BCR-ABL1 [99]. The net effect of this is a protein that is constitutively active allowing the cell to independently grow and divide [100].

1.7.3 EVADING GROWTH SUPPRESSORS

As the control and maintenance of cells and proliferation is critical to the success of multicellular organisms there are a number of mechanisms by which cells can prevent excessive proliferation and cell growth. The mechanisms are mediated by tumor suppressor genes and can act anywhere throughout the signaling cascade and serve to dampen and modulate the growth signaling. Tumor suppressors can be grouped into the three subcategories; gatekeepers, caretakers [101] and landscapers [102]. As previously discussed, p53 is an example of a gatekeeper as is can directly regulate cell growth by inhibiting the cell cycle and inducing apoptosis [93]. A caretaker gene maintains the stability of the genome by DNA repair and prevents the accumulation of mutations, an example of this are the BRCA genes which are responsible for DNA repair and often altered in breast cancer [103]. Finally, the most recently described subcategory of the tumor suppressor genes are the landscapers, which maintain the cellular microenvironment by maintaining cell to cell contact, the extra cellular matrix as well as the composition of the cell membrane. Within the colon, pTEN is suggested to have this role [104].

1.7.4 ACTIVATING INVASION AND METASTASIS

During normal embryogenesis cell populations need to be able to migrate to colonize new areas. An example of this is the migration of neurons from the trunk neural crest to the developing organs in organogenesis [105, 106]. This is an extremely controlled process which, as previously described, multicellular organisms need to maintain control over specific functions, properties and spatial distributions of cell populations. Generally, cells that are maintained within these conditions are also under the influence of the surrounding cells within the same population. If a cancer cell is to gain the ability to invade other locations and form metastases a number of key steps need to occur, collectively, referred to as epithelial-mesenchymal transition (EMT) [106, 107]. The first step is localized invasion and removing the influence of the adjacent cells. Once this has been achieved the cell then needs to be able to detach from the adjacent cells, remain viable and infiltrate into the vasculature. The next challenge is escaping from the vasculature and entering into a novel environment. The cell, once in this novel environment then need to be able to grow, reestablish cell to cell contact and cluster as a micrometastasis. The final step is colonization, this is reached once the micrometastasis has reached a size that it is large enough to be a macrometastasis, requiring a permissive tumor microenvironment [108].

1.7.5 ENABLING REPLICATIVE IMMORTALITY

It is widely accepted that most differentiated cells have a limited replicative potential that results in an upper limit to the number of cell divisions that can be performed [109]. As a cell replicates and divides the length of the chromosome shortens fractionally which results in the ends of the chromosome losing material. These areas of the chromosome, known as telomeres, are tandem hexametric DNA repeats that function to protect the ends of the chromosome during normal cell divisions. Once the telomeres have been shortened the cell will either enter into senescence and remain active but not proliferative, or undergo a crisis phase in with the cell generally is removed by apoptosis [110]. Naturally, the length of the telomeres corresponds to the number of cell divisions that can be performed. A cancer cell however, can avoid this by upregulating the telomerase enzyme, which is used in stem cells to lengthen the telomere [111]. Alternatively, a cancer cell can go through the crisis phase and then emerge without undergoing apoptosis and become immortalized [112], a process that has given rise to most of the cell lines currently used in biomedical research [113].

1.7.6 INDUCING ANGIOGENESIS

For cells to be viable they require nutrients and oxygen for respiration, the cell also needs to be able to remove the byproduct of respiration, carbon dioxide. The formation of new vasculature is highly important during morphogenesis but after this point vascular formation is generally dormant [114]. Angiogenesis can be transiently activated under certain conditions such as the vascularization of the placenta during pregnancy [115]. During tumorigenesis angiogenesis is almost always active to aid in the maintenance of neoplastic growth [116, 117].

1.7.7 AVOIDING IMMUNE DESTRUCTION

The conventional view of the immune system is that it maintains surveillance of cells and tissues preventing the development of neoplasms. Surface markers and antigens on cells often allow for the immune system, namely B and T cells, to recognize and destroy these aberrant cells. This process, on a large scale in a cancer context, is often referred to as spontaneous regression, something that neuroblastoma is well known to undergo [118]. Cancers can often alter the surface markers and antigens that they present to avoid B and T cell detection [119].

1.7.8 DEREGULATING CELLULAR ENERGETICS

Under aerobic respiration conditions cells will generally use and completely metabolize glucose as the favored fuel [120]. This process involves processing glucose into pyruvate by glycolysis and then subsequently fully metabolized carbon dioxide in the mitochondria. With abundant oxygen this process is highly efficient at producing ATP, the chemical energy source of most reactions within the cell. If there is a limited supply of oxygen then only glycolysis occurs, yielding a vastly lower amount of ATP. Cancer cells can reprogram their metabolism and despite conditions with an excess of oxygen these cells can limit energy production to solely glycolysis [121]. This phenomenon, called the Warburg effect, was first detected by Otto Warburg in the first half of the 20th century and has only recently reemerged within the research interest [120]. Whilst the exact mechanism and the function of the counterintuitive energy source switch is currently not agreed upon there are a number of hypotheses. One of the leading hypotheses is that the cell can subsequently use the byproduct in other bioprocesses and another that this process whilst yielding lower net energy is highly and rapidly adjustable allowing for the cell to adjust the energy production as required [120].

1.7.9 TUMOR PROMOTING INFLAMMATION

Inflammation is one of the critical processes for maintaining homeostasis by both promoting cell death and cell survival based on stimuli from the microenvironment. Acute inflammation can be caused by a diverse number of stimuli that can activate the chemokine receptor family or the Toll-like receptors (TLRs), these are the main routes to transfer signals into the cell. The response of this signaling can be; gene transcription, chemotaxis of neutrophils, macrophages infiltration of the inflamed area or degranulation of secretory vesicles [122]. Degranulation releases, along with other signaling molecules, cytokines into the tumor microenvironment. One of the common inflammatory markers in cancer is IL-6 which activates STAT3 [123]. When this process is uncontrolled and dysregulated, the signals can be maintained and propagated resulting in an environment permissive to tumor growth [124].

1.7.10 GENOME INSTABILITY AND MUTATION

As we have previously indicated throughout these hallmarks, several alterations need to occur in a cell for it to develop into a tumor. A helping factor can be gross alterations to the genome such as chromosome gains, losses and inversions or point mutations to activate proto-oncogenes or deactivate tumor suppressor genes [125]. Activations of proto-oncogenes by mutation can be caused by environmental factors, such as exposure to a carcinogen like cigarette smoke, which can potentially alter the DNA sequence resulting in an alteration to the gene product [126]. Another possibility is that a carcinogen deactivates a tumor suppressor gene, this often occurs at a lower frequency as the alteration needs to occur twice within the same gene within the same cell on both homologous chromosomes [127]. The basis for this is Knudsons two hit hypothesis [128], whereby humans, a diploid organism carries two copies of all genetic information with the exception of sex chromosomes. The exception to this is familial cancers where one of the inherited gene copies is already altered and therefore the likelihood of a single event occurring in the corresponding functional gene is higher [129]. Another common alteration that is observed in cancer is instability of the genome. Which, if affecting short repeating DNA sequences is called microsatellite instability [130] and larger alterations can be copy number variations (CNV) [131] or ploidy alterations [132] (Figure 7). This may be as a result of faulty DNA repair or issues during cell divisions resulting in the gain or loss of chromosome material. If an oncogene resides on one of the gained segments then there can be an increase in expression [44]. Alternatively, if a chromosome segment is lost there could be a tumor suppressor gene located on the segment [39]. As these events are rare and highly disruptive to the cell the probability is that there is no selective advantage to the cell and rather a disadvantage, thus only the alterations that are advantageous are maintained.

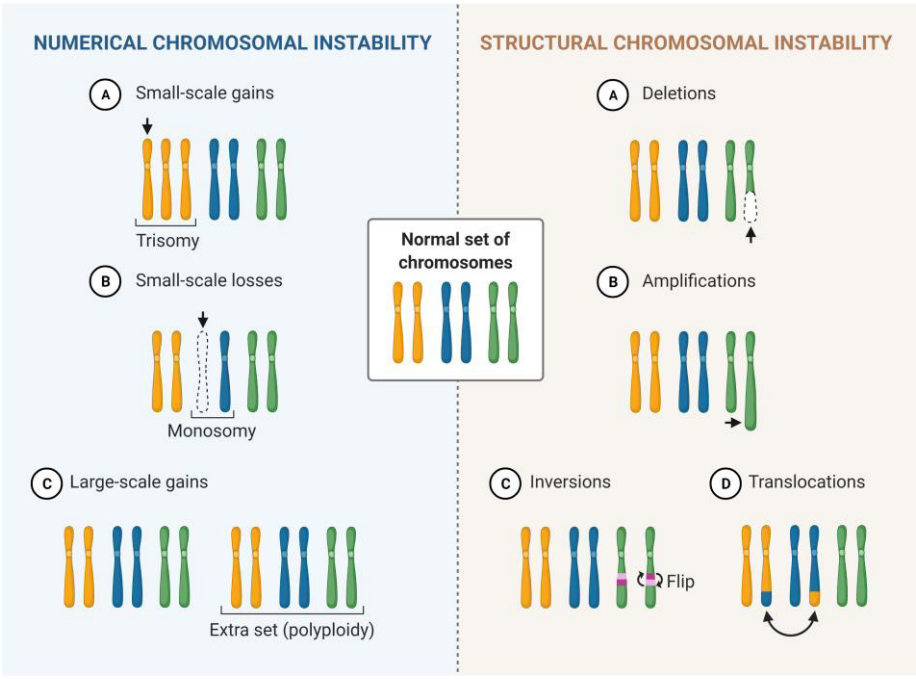

Figure 7. Types of Chromosomal instability: An overview of the different types of alterations that can occur to chromosomes divided into numerical and structural changes. A single whole chromosome gain as is called a trisomy whereas a single whole chromosome loss is a monosomy. A large-scale gain is where multiple chromosome sets are incorporated into the genome. These types of gains whilst damaging are not linked with the poor outcomes and higher risks that associated with the structural chromosomal instabilities. Deletions are when a segment of chromosome is lost and amplifications are when a segmental copy is inserted into the chromosome. Inversions are when a segment of the chromosome is reversed. Finally, a translocation is when material from one chromosome is swapped for material on another chromosome. Created with BioRender.com

1.8 EMERGING HALLMARKS AND ENABLING CHARACTERISTICS

1.8.1 PHENOTYPIC PLASTICITY

Stem cells are the name given to the undifferentiated cells of multicellular organisms. These cells can be partially differentiated and thus belong to a specific lineage, known as multipotency or undifferentiated also known as pluripotency which have the potential to be almost any type of cell in the body [133]. The differentiation of these cells into lineages generally limits the differentiation potential of that cell as the process is thought to be terminal [134]. During development such as embryogenesis or organogenesis the need to produce a large number of specialized cells results in an increase in proliferation and replenishment of these stem cells. The ability of a cell to regress and dedifferentiate or to gain new functions that are otherwise outside of the differentiated lineage can provide advantage to cancer cells [135]. These types of cells are known as cancer stem cells as they function in a similar mechanism to stem cells [136].

1.8.2 SENESCENT CELLS

As previously discussed the cell cycle is under tight control to maintain appropriate cell numbers. We have detailed the different stages of the cell cycle, however there is an alternative. Rather than continue to proliferate until the cell reaches the upper limit of cell divisions and undergo apoptosis, the cell can enter into senescence and avoid destruction [60]. Alternatively, cells can enter into senescence after microenvironmental pressures such as; DNA damage, nutrient deprivation or signaling imbalances [137]. The process of senescence is important in aging as we no longer produce the quantities of cells that are needed to replace all of the cells. The presence and accumulation of senescent cells leads to degeneration and aging associated diseases. Cancer cells are thought to have the ability to influence neighboring senescent cells and return them back to the dividing cell population [138].

1.8.3 NON-MUTATIONAL EPIGENETIC REPROGRAMMING

The structure and acquired changes in a cells genome is influenced by the surrounding microenvironment. Factors such as hypoxia [139] and paracrine signaling have been known to alter the tumor microenvironment [140] with alterations such as; methylations, histone modifications, alteration of chromatin accessibility, posttranslational modifications [141] and translation of RNAs [142]. These types of alterations can be reversed and altered depending on the prevailing conditions in the microenvironment and as such are not fixed [143]. The alteration by these factors may result in dysregulation in microRNA transcription and hence have larger transcriptomic consequences [144].

1.8.4 POLYMORPHIC MICROBIOMES

The microbiome has long been associated with development of some cancers. Colon cancer is the obvious place to start due to the close proximity of bacteria and colon cells [145]. The presence of certain bacterial microbiomes has been associated with an increased risk of colon cancer development [8]. The exact mechanism of action is not fully elucidated with the tumor effect thought to be as a result of bacterial toxins or other molecules that act to damage DNA or disrupt DNA repair [146]. It should also be no surprise that cancers such as melanomas are also influenced by the skin microbiome [147]. The influence of bacteria is not limited to organs with large bacterial contact. The presence of bacteria and the certain microbiome profiles has previously been established in solid tumors derived from; bone, brain, breast, lung, ovary and pancreas [148].

1.9 DLG FAMILY: DISCS LARGE HOMOLOGUES

The DLG family (DLG1-5) are a family of gene paralogues that have been shown to have significant functions governing; growth behavior, cellular structure and polarity [149-151]. These functions are thought to be achieved by stabilization of the supramolecular signaling and adhesion complexes through heterodimeric formation as well as protein trafficking to the cellular surface of epithelial cells [152, 153]. The human DLG family has a single orthologue in Drosophila melanogaster DLG gene (dmDLG). Previous research has shown that loss or mutation of dmDLG results in spontaneous neoplasms thought to be caused by sustained growth signaling, resisting cell death, evading apoptosis and maintenance of proliferative signaling [154]. Based off early studies in drosophila dmDLG was identified early as a tumor suppressor gene along with binding and complex forming partners scribble (Scrib) and lethal giant larvae (LGL), however, the mechanism of action was not established [155]. Knockdown of dmDLG was previously established to result in an early and abnormal exit from the cell cycle which results in binucleate cells [156]. Furthermore, dmDLG was shown modulate asymmetric neuroblast division by spindle alignment in Drosophila melanogaster [157].

1.9.1 DLG FAMILY ISOFORMS

The DLG family members were first found in the membrane of the post synaptic density of the nervous system, however, they have now been detected in the membrane of most epithelial cells [149]. To function in the membrane the protein requires multiple protein domains to allow for multiple binding partners. The DLG family members must contain multiple PDZ domains, a single SH3 domain and a C-terminal guanylate kinase (GUK) domain [158]. In addition to these standard domains DLG1, DLG2 and DLG4 all have isoforms that contain mutually exclusive “Lin2, Lin7” (L27) domain or N-terminal palmitoylated cysteines. DLG1 and DLG4 both have 2 exons encoding the L27 domain whereas DLG2 (encoding the protein PSD93) has 5 exons that encode the L27 region and the SH3 linker region. The DLG isoforms that contain palmitoylated cysteines are designated as the α-protein whereas the L27 domain are designated as the β-protein, derived from two codons that are mutually exclusive to the α-protein [159]. The currently accepted PSD93β protein, corresponding to DLG2 isoform 1 does not include exon 1 or the start of exon 2, but has been bioinformatically labelled as such to follow the standard exon structure of DLG1 and DLG4.

The L27 domain mediates binding into tripartite complexes and are thought to be localized to tight junctions with The L27 domain assembly important for signaling complexes and cell polarity complexes. These complexes are important for cell

architecture and growth signaling in all cells, including cancer cells [160]. The tripartite complex assembly is important for cell architecture and growth signaling in all cells, including cancer cells. Previously, it has been shown that the L27 domain can form L27 mediated tripartite complexes with the L27 domain either as Type A or Type B. Type A L27 domains show a preference for binding type B domains. The L27 mediated protein interaction required the interaction of three proteins forming a complex of four L27 domains. For the tripartite protein complex to form, a protein with two Type B L27 domains such as one of the members of the Membrane Palmitoylated Protein (MPP) or Calcium/Calmodulin Dependent Serine Protein Kinase (CASK) are first required. Subsequently, the Type A L27 domain is provided by the LIN7 family with a DLG family member providing the final type A L27 domain. The LIN7 family consists of three members, Lin7 Homolog A (LIN7A), LIN7B, and LIN7C; each containing an N-terminal L27 domain and a single PDZ domain. The L27 domain types have been shown to direct protein binding so that the resulting complex is diverse and does not contain homodimerization, which is otherwise common with the DLG α proteins. Parker et. al. in 2004 [161], first discussed and showed that isoforms 7 and 8 (encoding PSD-93ζ) are the full-length proteins and contain the first three exons resulting in the complete L27 domain with the next two exons corresponding to the linker domain. To better reflect the naming convention within the family it has been suggested that it should be renamed as PSD93β. The isoforms that have the L27 domain can bind with the MPP and LIN7 families to form the tripartite complexes.

The DLG isoforms encoding the α proteins have an N-terminal palmitoylated cysteine that result in homodimerization and target the resultant complexes to neural synapses to increase synaptic strength. The proteins corresponding to DLG3 and DLG5 proteins lack the L27 domain and N-terminal cysteines [151] in all isoforms. The DLG3 encoded protein SAP102 is thought to be regulated by the SH3 and GUK domains. Generally, DLG3 is often found in immature neurons, suggesting a specific role in neuron growth and development [162]. Overexpression of DLG3 results in decreased survival in breast cancer patients as well as a loss of adhesion properties in esophageal cells. [163]. DLG5, the most diverse member of the DLG family has been shown to be lost in breast cancer cells with restoration of DLG5 expression inhibiting cell migration and proliferation.

1.9.2 DLG2

Located within the 11q LOH region in neuroblastoma is the DLG2 gene (11q14.1) also known as the post synaptic density 93 protein (PSD93). At 2.169M bases from the start of the first exon to the end of the last exon, DLG2 is one of the longest coding sequences in the human genome. The gene contains 28 exons with an average length of 115.5 bases and 27 introns of average length 77.7K bases. The loss of all or part of DLG2 has previously been implicated in neurodevelopmental disorders [164], schizophrenia and other psychiatric diseases [165]. These studies generally focus on the PSD93-α protein and the tracking and maintenance of the post synaptic density. One of the mechanisms by which DLG2 is thought to maintain the post synaptic density is by forming heterodimers with DLG4 (PSD95), GRIN2B a subunit of the glutamate receptor (NMDA receptor) [161, 166, 167] and KCNJ12, an inwardly rectifying potassium pump protein [168]. A study by Sanders et. al. using a CRISPR knockout of DLG2 generated in nerve cells revealed impaired; migration, differentiation and synaptic signaling. This study also reported a pronounced general effect on the expression of protein coding genes with between 40-60% of genes altered after 30 days of knockout, indicating the scale that loss of DLG2 has on the transcriptome [169]. Recent studies have identified DLG2 as a tumor suppressor in; osteosarcoma [170], colorectal cancer and ovarian cancers [171]. Additionally, studies have shown that the breakpoint for 11q deleted neuroblastoma often occurs prior to or within DLG2 to disrupt function [172]. Treatment with All Trans Retinoic Acid (ATRA) has been shown to increase DLG2 expression in neuroblastoma [173] and experiments in PWR-1E prostate cells showing that DLG2 is under the control of RARRES1, the retinoid acid response element 1 [174]. DLG2 has been shown to be inhibited by miR-942-5p a microRNA upregulated in colorectal cancer [175], however this has not been investigated in neuroblastoma. Worth noting is that due to articles and manufacturers maintaining older naming conventions there is a risk that MPP2 (MAGUK p55 subfamily member 2) is also referred to as DLG2 [176] and thus resulting in a degree of confusion in regard to the function of DLG2.

2 AIM

With this thesis, the general objectives have been to establish and characterize the function of DLG2 as a tumor suppressor using molecular methods and genetic analysis in cancer models, namely neuroblastoma and colorectal cancer. The choice of DLG2 was derived heavily from the observed chromosome 11q deletion correlating to aggressive tumor behavior in neuroblastoma. The current therapeutic options available to this subgroup of patients is limited and therefore identification and characterization of genes of interest in this deletion is required to understand the biology driving the aggressive nature of the tumor. One of the additional aims to this project was to show that the impact of DLG2 expression is not only limited to a single cancer form.

PAPER I

- Identification of a candidate neuroblastoma 11q tumor suppressor gene based on clinical and pathological data.
- To determine the molecular and biological function of DLG2 based of bioinformatic results.
- To determine if DLG2 act as a tumor suppressor gene in neuroblastoma.

PAPER II

- Evaluation of the different isoforms of the DLG gene family.
- Determine differential expression of the DLG gene family and their isoforms in neuroblastoma.
- Characterize the function of the L27 protein domain in DLG2.

PAPER III

- Establish the basic state for dsDNA repair in neuroblastoma cells.
- Characterization of DLG2 expression in response to dsDNA damage.
- Identification of signaling pathways that are altered by DLG2 in response to DNA damage.

PAPER IV

- Confirm that DLG2 can act as a tumor suppressor gene in also in colorectal cancer.
- Identify the novel function of DLG2 in the NLRP3 inflammasome.
- To establish how DLG2 expression in macrophages influences the tumor microenvironment.
- Determine the effect of DLG2 expression on secondary inflammatory markers and signaling.

3 PATIENTS AND METHODS

3.1 PATIENT AND TUMOR MATERIAL

The investigation of heathy and tumor material derived from patients has been one of the most fundamental tools for research. The ability to look at a tumor and determine its origin, characteristics and molecular signature has provided insights into tumors and driven research forwards. When trying to obtain samples there are a number of steps that first need to occur. The most important step with regard to research on human material is obtaining ethical approvals as well as obtaining consent from the patient or in the case of children the legal guardian. This is done and enshrined into Swedish law (Swedish Ethical Review Act (SFS 2003:460)) to physically and mentally protect human beings and their integrity.

The next steps of obtaining the material is to ensure it is going to be viable and reflective of the condition that is to be studied. Often requiring the material to be verified, extracted and stored. Important to remember when dealing with any tumor sample, particularly when investigating dynamic events, is that a sample is a single snapshot in time and potentially not reflective of the larger picture. The same can also be said for the biopsies of large volume tumors, with a single biopsy difficult to capture the character of the whole tumor sample.

3.1.1 NEUROBLASTOMA

Twenty-two primary neuroblastoma samples were collected for which written or verbal consent was obtained according to the ethical permits approved by the Karolinska University Hospital Research Ethics Committee (Approval No. 2009/1369-31/1 and 03-763) (PAPER II)

3.1.2 COLORECTAL CANCER

Forty controls who underwent colonoscopy and twenty patients who were diagnosed with colorectal cancer were collected in accordance with the ethical permits approved by the Regional Ethical Review Board in Gothenburg under study number 233-10 and clinical trial (NCT03072641) [145] were included in the study (PAPER IV).

3.1.3 COLLECTION OF COLON TISSUE SAMPLES

Regarding the colorectal cancer study, at colonoscopy, the mucosa samples were obtained from the mid-portion of the ascending colon (right side samples) as well from the sigmoid (left side samples) using a regular biopsy forceps. If applicable, a

tissue sample was also collected from the tumor. The distance between the tumor and the matching cancer mucosa that was sampled on the same side was approximately 10 cm. The tissue samples were immediately frozen in liquid nitrogen, and stored at -80°C until required. The prerequisite for inclusion into the control group was ≥18 years of age, and a normal-appearing mucosa in the entire colon, e.g. patients with any significant pathology such as colonic polyps or adenomas, inflammatory bowel disease, malignancy, ischemic colitis etc. were excluded. The prerequisite for inclusion into the colon cancer group was the presence of at least one malignant tumor in the colon and ≥18 years of age. Tumors were classified according to the Tumor-Node-Metastasis (TNM) staging system.

3.2 CELL CULTURE

Cells can be cultured in a laboratory under favorable yet artificial conditions. A number of commercially available cell lines that originate from different tis-sues are available for purchase with each requiring somewhat unique growing conditions. The use of these cell lines and the highly controlled and reproducible results provides a backbone for a number of investigations and tests to allow for the determination of characteristics, functions and processes that occur after different treatments. It is also possible to insert specific DNA sequences, plasmids or small interfering RNA into the cells to alter the expression profile, which can then be quantified and measured.

3.2.1 GROWTH CONDITIONS

Human neuroblastoma cell lines; SKNAS, SKNBE(2), and NB69, Human Colorectal cell lines SW480 and COLO205, Human embryonic kidney cells HEK293 and Human monocyte derived cells THP 1-ASC-GFP were obtained from ATCC Cell Line Collection. The cells were cultured in a humidified incubator at 37 °C with 5% CO2. SKNAS, SKNBE(2), SW480, COLO205 and HEK293 cells were maintained in RPMI 1640 culture medium supplemented with 10% FBS, 1% L-Glutamine, 1% HEPES solution, and 1% sodium pyruvate. NB69 was cultured in RPMI 1640 medium supplemented with 15% FBS, 1% L-Glutamine, 1% HEPES solution, and 1% sodium pyruvate. THP 1-ASC-GFP cells were cultured in RPMI-1640 with L-glutamine, 10% heat inactivated premium grade FBS, 10mM HEPES, 1mM sodium pyruvate, 0,45% glucose and 100U/ml penicillin-streptomycin. Zeocin (200µg/ml) (Invivogen) was added to the culture medium as per the manufacturer's instructions.

3.2.2 TRANSFECTIONS

DLG2 isoform 2 (NM_001364) and DLG2 isoform 7 (NM_001351274.2) mammalian expression plasmids pcDNA3.1/C-(K)-DYK (OHu25658D and OHuq102626D respectively) were purchased from GenScript. LIN7A (NM_004664) mammalian expression plasmid pCMV6-AC-GFP (PS100010) was purchased from Origene (RG221902). siRNA targeting DLG2 (s4122), LIN7A (s16836) or Silencer™ Select Negative control No. 1 siRNA (4390843) was purchased from Ambion (ThermoFischer Scientific).
Cells were grown to 80% confluence and subsequently transfected with 100 ng of plasmid-DNA or 10 pmol of siRNA complexed with 0.3µl of Lipofectamine 2000 according to the Lipofectamine 2000 reagent forward transfection protocol.

3.2.3 INFLAMMASOME INDUCTION

Cell density was maintained between 5x105 and 1.5x106 cells/ml and cells were used up to passage number ten. Differentiation was conducted with 100ng/ml PMA (Merck) for 72 hours followed by 72 hours of rest in fresh media. Priming of differentiated cells was conducted with 500ng/ml ultrapure LPS (Invivogen) for 4 or 24 hours followed by activation of the inflammasome complex formation with 5 mM ATP (Merck) for 30 minutes.

3.3 CELLULAR ASSAYS

Once the cells have been altered or treated, a number of assays to determine the functions of the alterations can be performed. One of the most basic assays is to determine the number of cells in a given sample, with the assumption that the starting point is the same for all conditions and the difference in cell number is as a result of the given treatment. This can be assayed by proxy by determining the rate of conversion of a substance in a substrate. In the case of MTS, the reduction of the MTS tetrazolium compound to the measurable colored formazan product in the mitochondria of living cells can be used to estimate relative abundance of cells. The key assumption is that the rate metabolic conversion is maintained between samples. The same principle can be used to determine the relative abundances of the executioner caspases 3 and 7 to determine apoptosis, in this case by fluorescence. One of the classical assays that is performed is the comet assay. A comet assay relies on the principle that DNA is negatively charged and will migrate through a matrix at a variable speed depending on the fragment size and matrix density. This assay can be used to determine DNA breakages by measuring the migration of DNA through an agarose matrix under certain conditions. There are two components to the comet, the head and the tail. If the genome is intact then there will be minor migration and hence will be a large circular contiguous mass. The higher the number of breaks will result in a reduction in the volume that is seen in the head and an associated increase the directional spread of the tail.

3.3.1 MTS

The MTS assay was performed post transfection using the MTS/MPS Cell Titer 96® One solution Reagent (Promega, USA) and detected the variation in color (FLUOstar Omega, BMG Labtech, Germany) as per the manufacturer's recommendations. The absorbance values were normalized to the mock transfection and expressed as a percentage. In all MTS experiments 6 biological replicates in parallel were used and repeated three times.

3.3.2 CASPASE 3/7

Apoptosis levels were determined by the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega) in the transfected neuroblastoma cells using a FLUOstar Omega multiplate reader (BMG Labtech), as per the manufacturer's instructions. Apoptosis was calculated by subtracting the fluorescence for the average blank background and then normalizing against the mean of the control cells.

3.3.3 CELL CYCLE

Cell cycle analysis was performed using the cell-clock cell cycle assay (Bio-color) determined by colorimetric alterations for different cell cycle states. Images were analyzed subsequently using Image J image analysis as per the manufacturer's instructions. The data presented is the average of three biological replicates from one experiment. Each experiment series was repeated in triplicate.

3.3.4 COMET ASSAY

The comet assay determines the fragmentation of DNA, performed according to the manufacturer's protocol (Abcam, Cat. ab238544). In brief, aliquots of 10 000 cells were mixed with low melting point agarose and added to slides. The agarose was allowed to harden, and the cells lysed (pH 10; 4°C) in pre-chilled lysis buffer. The slide was subsequently washed 3 times in alkaline buffer and transferred into alkaline buffer for electrophoresis. The slides were run for 30 min at 1V/cm, 300 mA, washed 3 times with H_2O, incubated for 5 min with ice cold 70% ethanol, allowed to air dry, and stained with Vista green for visualization. Measurements and analysis were performed using Image J software version 1.52a. Data were normalized to the control transfection and expressed as a percentage of the control.

3.4 DROSOPHILA MELANOGASTER

One of the simplest whole organisms for the study of genetics is Drosophila melanogaster, the fruit fly. The fruit fly has been a staple of research over the past 100 years partly due to the high percent of homologous paralogues that can be found between it and the human. Additionally, 4 pairs of chromosomes result in a relatively simple genetic makeup. By associating genes to gene markers, we can use the phenotype, their physical appearance, to understand their genotype, something that can be particularly useful when making crosses between flies. Since the 1980s transgenes from foreign organisms have been inserted into flies to allow for control of gene expression. The UAS-GAL4- system is a method by which we can use inserted transgenes to study the expression of genes and importantly their effect. The GAL4-UAS system contains two parts, the GAL4 gene taken from yeast that is a transcription activator protein and the upstream activator sequence (UAS) an enhancer sequence that can be inserted upstream of the gene of interest to which GAL4 binds and drives gene transcription. Generally, the system is divided into two parts, the driver flies that have the GAL4 sequence and the flies that contain the UAS. By selecting specific cell lineages into which the GAL4 sequence is inserted we can control which cells will express the gene (Figure 8). Additionally, we can add a reporter tag such as green fluorescent protein (GFP) to enable visualization of the protein product of the gene.

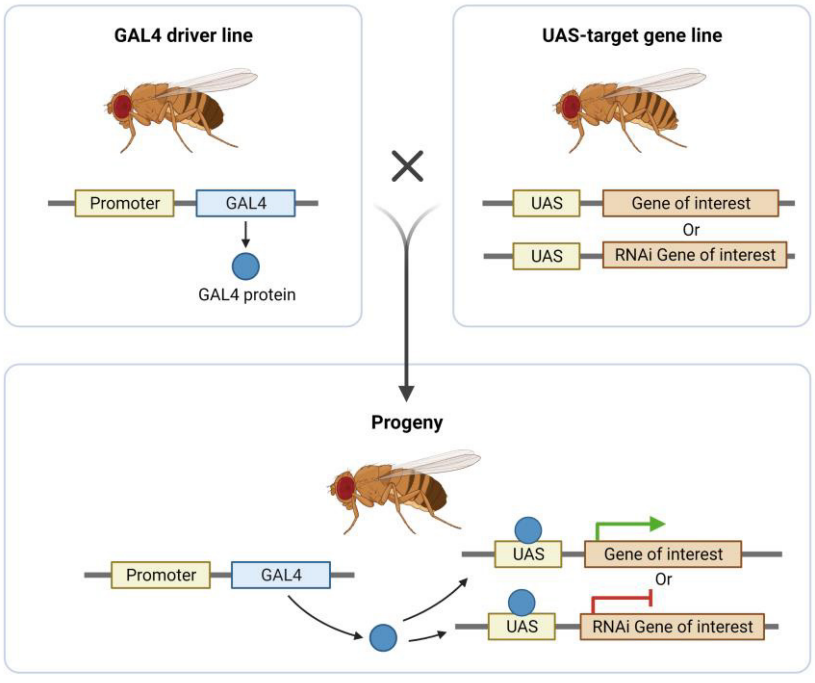

Figure 8. The mechanism for inducing gene expression or inhibiting gene expression in drosophila with the UAS-GAL4 driver system. A specific promoter is upstream of the GAL4 protein in the driver line that once crossed with the target line will result in the expression of a specific gene target for UAS or the repression of a specific gene in the case of the UAS-RNAi. Created with BioRender.com

3.4.1 STUDIES OF DLG IN DROSOPHILA MELANOGASTER

Commercially available UAS-RNAi-dlg1 flies or control white (w1118) flies were obtained from the Bloomington Drosophila Stock Center. The flies were crossed with the driver strain da-GAL4 to promote ubiquitous repression of dmDlg. Twenty female da-GAL4 flies were crossed with 10 male UAS-transgenic flies or control flies and the progeny was incubated at 25 °C on standard fly media. Five of the progenies were harvested after 72 h during the third instar larvae phase for gene expression or protein analysis (PAPER I). For another study ten progeny flies per cross were exposed 30 Seconds UVC and allowed 3 hours recovery, they were harvested for RNA and DNA preparation (PAPER III; Figure 9).

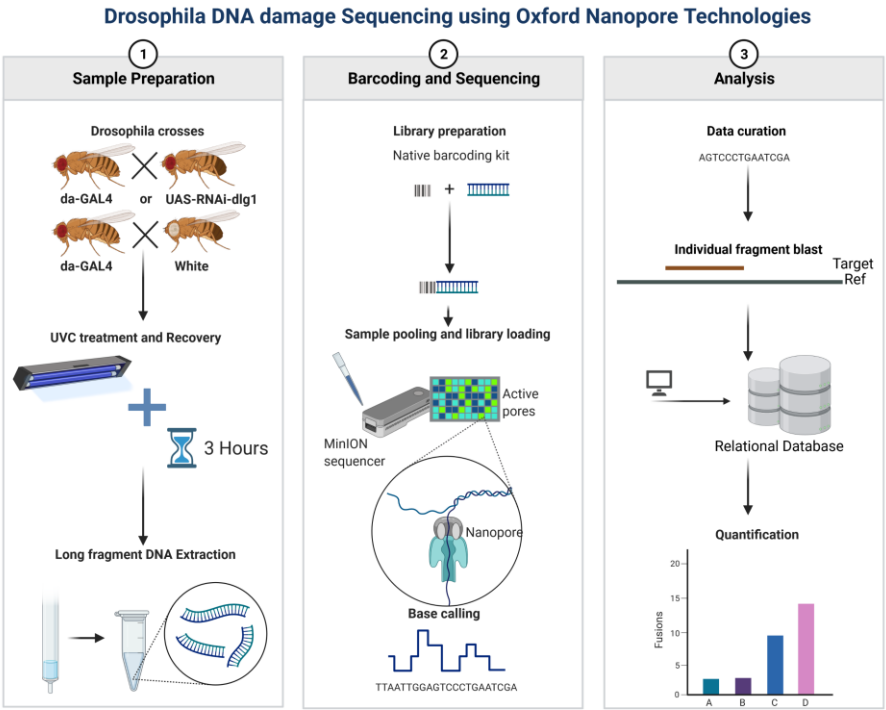

Figure 9. Schematic overview of the Drosophila crosses, DNA break induction, Extraction, nanopore sequencing and subsequent analysis from Paper III. Created with BioRender.com

3.5 GENE EXPRESSION AND METHYLATION ANALYSIS

To determine the differences in the expression of genes we need to be able to quantify the amount of specific mRNA and relate that quantity to the total amount of RNA. Using large scale data, we can determine the presence of multiple target mRNAs in a given sample. The use of hybridization probes to mark a known sequence to allow its detection has been an important way to determine the presence of specific mRNAs. To be able to compare samples we quantify the amount of a target sequence against the amount of a separate target sequence, in this context one or more reference genes. The purpose of these reference genes is to provide a stable reference point to which any given target can be relatively quantified. This is the basis for microarray, Taqman and SYBR green qPCR techniques. However, later technologies that sequence the whole transcriptome or exome such as RNA-sequencing (RNA-SEQ) provide both the relative amount of a sequence and the total amount of the RNA as well as providing additional information such as the exact sequence of the transcript. This allows for the quantification of the total of unknown sequences as there is no previous knowledge required. This method can be used for the detection of novel transcripts. There are a number of different methods for which quantification can be performed on this type of data and how it should be normalized; RPKM (reads per kilobase million), FPKM (fragments per kilobase million) or TPM (transcripts per kilobase million). Common to these is that the total number of reads or fragments have been normalized to a million reads as well as the length of genes measured as kilobases with the order of the mathematical operations altering. The use of TPM is beneficial when comparing between individuals whereas the other two methods can be beneficial when looking at isoforms or relative amounts within the same sample. Another method that can be used to determine information regarding the activity of a particular gene is the use of methylation arrays. If certain CpG islands are methylated, the binding of transcription factors and enhancers can be altered which results in decreased transcription, naturally this assay only measures the presence of the CpG methylation so it would need to be complimented with a separate gene expression analysis to confirm the expression level.

3.5.1 LARGE SCALE DATA

The datasets analyzed during the current study are available in the 'R2: Genomics Analysis and Visualization Platform repository, [http://r2.amc.nl]. The neuroblastoma datasets analyzed are 1): GSE49710 [177], 2): Neuroblastoma NCI TARGET data, GSE16476 [178], 3): GSE3960 [179] and 4): GSE73517 [180]. Neuroblastoma methylation datasets; 1): GSE73515 [180] and 2): GSE120650 [181]. The neuroblastoma cell datasets; 1): GSE89413 [182], 2): GSE28019, 3): GSE16478 [183], and a human embryogenesis dataset GSE15744 [184]. The study of colon cancer included five independent colon cohorts; 1): GSE4183[185], 2): GSE109142[186], 3): GSE75214[187], 4): GSE10950 [188] and 5): GSE8671 [189]. The inflammation time series mouse models, with DSS treatment GSE22307[190] and Colon T cell transfer GSE27302 [191] were also selected. The results generated from the NCI TARGET data was generated by the Therapeutically Applicable Research to Generate Effective Treatments (https://ocg.cancer.gov/programs/target) initiative, phs000218. The data used for this analysis are available at https://portal.gdc.cancer.gov/projects. The microarray data was downloaded as the centered log2 fold change. Methylation data was downloaded as raw values.

3.5.2 QPCR

The relative gene expression of select genes was quantified using TaqMan Assays labelled with FAM-MGB (ThermoFisher Scientific) or by SYBR green with specifically designed primers. Samples were run as duplicates in 96-well plates. Polymerase chain reactions were carried out in 5µl reactions scaled as per the manufacturer's instructions. The plates were run and analyzed using the Pikoreal qPCR System (ThermoFisher Scientific) according to the manufacturer's protocol. Thresholds and baselines were set manually and Ct values were extracted. The Ct values were normalized to the mean of the reference genes; ACTB, GAPDH, GUSB and PPIA (Delta Ct) for each sample. For fly studies RPL32 was used as control. GAPDH + GUSB (PAPER I), ACTB + GAPDH (PAPER II), GAPDH + GUSB (PAPER III) and ACTB, GAPDH, GUSB and PPIA (PAPER IV).

3.6 PROTEIN STUDIES

Following the central dogma of biology RNA is subsequently transcribed into protein. So logically, the next investigations that are generally performed are studies on proteins. Determining the presence or absence of a specific protein in a sample is generally a difficult procedure as we cannot amplify a specific protein like in gene expression studies. Rather the standard procedure is to detect the specific protein-based sequence epitopes that have been created to challenge and elicit an immune response in a host. This process hopefully creates antibodies that are highly specific to a particular protein and thus allows for its detection. This can even be extended to allow for the determination of post translational modifications to proteins and thus allow for the determination of activity status. Additionally, it can be interesting to determine the binding partners of specific proteins. Care must be taken with this procedure as to not disrupt the binding between the different proteins and preserve the overall protein structure. Generally, one protein is targeted and secured, in this case by conjugating the specific antibody to magnetic beads and preparing the sample on a high-power magnet. The non-bound proteins are subsequently depleted from the sample by washing, the proteins that remain are the ones that were bound to the protein target of interest. Finally, molecular tools allow for us to alter the composition of genes and hence the final protein product by adding fluorescent protein tags to the protein. These tags once excited and illuminated provide additional information on the location of the protein within the cell. The use of multiple fluorescent tags on multiple proteins can allow for the visualization of relative locations within cells or tissue.

3.6.1 PROTEIN EXTRACTION

In brief, protein was extracted from the transfected cells in 96 well plates (1 × 104 cells/well), 24 well plates (1×105 cells/well), or 6 well plates (1×106 cells/well), by aspirating the media and incubating on ice for 5 min then adding ice cold RIPA buffer (Thermo-fisher Scientific, 89900) (PAPER I, PAPER III and IV). For extractions from Drosophila five larvae were homogenized in 100ul RIPA buffer, followed by centrifugation (PAPER I). For co-immunoprecipitation studies protein was extracted from a 6 well plate, as described above, by aspirating the media and incubating on ice for 5 minutes then adding ice cold mPER buffer (ThermoFisher Scientific, 78505; PAPER II).

3.6.2 CO-IMMUNOPRECIPITATION (COIP)

The lysate was co-immunoprecipitated using µMACS magnetic labelling and isolation kits with antibodies for DYKDDDDK (FLAG®-tagged; Miltenyi Biotech, 130-101-591) and for GFP (Miltenyi Biotech, 130-091-288) as per the manufacturer's instructions.

3.6.3 IMMUNOBLOTTING

Western blot analysis was performed using Mini-PROTEAN® TGX™ 8-20% gradient gel (Bio-Rad), protein was blotted onto a LF-PVDF membrane (8 minutes, 25V and 2.5A) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad). Blots were subsequently blocked for 1 hour in 5% milk in TBST buffer (0.1% Tween-20 and 150 mM NaCl in 10 mM Tris–HCL, pH 7.4). Primary antibodies were diluted in PBST (0.1% Tween-20 in PBS) or TBST (0.1% Tween-20 in in tris-buffered saline) depending on the manufacturer's recommendations. After incubation with the primary antibodies, the membranes were washed 3x10 minutes in TBST 0.1% (0.1% Tween-20 in tris-buffered saline). Secondary antibodies; Starbright B520 goat anti-rabbit (12005870, 1:5000, BioRad) or Starbright B700 goat anti-mouse (12004159, 1:5000, BioRad) were incubated for 1 hr at RT. Western blot analyses were performed in triplicate as three independent experiments. Image detection was performed on ChemiDoc MP (BioRad) and band intensity was quantified using Image lab™ (v. 6.1, BioRad). Protein loading from the different experiments and gels were normalized against total loaded protein from stain free images. GAPDH was included as loading control.

4 RESULTS

4.1 PAPER I

4.1.1 Background

Located within the first breakpoint (11q14.1) is the Discs Large (DLG) homolog family member gene DLG2. The DLG family have functions governing polarity, cell structure and growth behavior. In Drosophila melanogaster DLG is known to be important in spindle alignment in asymmetric neuroblast division, and is known as a tumor suppressor gene along with binding partners SCRIB and LGL.

The potential tumor suppressor gene that is disrupted by 11q deletion should adhere to the observed characteristics of the 11q deleted neuroblastomas. The first characteristic is the inverse relation of 11q and MYCN amplification. Secondly, the tumor suppressor should maintain genome integrity and prevent the increased number of chromosomal breaks that are observed in the 11q deleted tumors. Finally, the age at diagnosis of the patients with 11q deleted neuroblastoma is often older than those with neuroblastoma with intact chromosome 11 [34].

4.1.2 Result and discussion

We began by using publicly available data to investigate DLG2 expression in primary neuroblastomas stratified by INSS stage and compared the low and high-risk tumors. We saw that the higher INSS staged tumors had a lower expression of DLG2, and we subsequently showed that patients with low tumor DLG2 expression had poorer overall and event free survival. Using the total gene expression for each patient, we used unsupervised k-means clustering to group the patients into four groups where we quantified the overall survival and subsequently saw that the groups with better survival had higher DLG2 expression.

Using publicly available datasets we created gene lists based on the correlation to DLG2 expression level for each of the separate neuroblastoma patient datasets, and an embryogenesis dataset as a non-cancer control. We identified that the following biological processes were all significantly enriched; Cell cycle, DNA replication and Mismatch repair. Furthermore, we presented a list of genes that were significantly correlated to DLG2 expression in all different neuroblastoma datasets. Two of the genes of particular interest were CCNA2 (cyclin A2) and CCNB1 (cyclin B1). These

bioinformatic results showed that DLG2 may be involved in regulation of the cell cycle and DNA repair as well as providing testable targets for further experiments.

To investigate the relationship between DLG2 and MYCN expression we subsequently investigated publicly available datasets, two datasets were from neuroblastoma primary tumors and three from cell lines. The expression of DLG2 was negatively correlated to the amplification of MYCN. When dmMYC, the fly homolog of MYCN, was induced in a fly model, the expression of dmDLG was decreased. There was also an increase in the cell cycling proteins Cyclin A and Cyclin B compared to the control. To evaluate if 11q deletion resulted in decreased expression of DLG2 we investigated two primary datasets for the expression of DLG2 in relation to 11q deletion. We also showed that the methylation of the DLG2 promoter region was not altered as a result of the deletion.

Finally, we investigated functional changes that occurred in neuroblastoma cells when DLG2 was over or under expressed. We could show that there was a difference in proliferation between the cells with 11q deletion and without after DLG2 transfection. Knockdown of DLG2 in the 11q deleted cells showed lower proliferation and fewer cells, this observation in combination with the unaltered cell cycle analysis indicated that DLG2 had a basal function in the 11q deleted cells. In both cell lines we could show that over expression of DLG2 resulted in lower proliferation, fewer total cells and an increase in the number of cells maintained in G2/M with an associated decrease in the number of cells in G1 phase. Using a fruit fly model, we could show that dmDLG silencing resulted in a similar expression of cyclin A and cyclin B to the over expression of dmMYC protein. These results reflect the results that were collected throughout the paper.

We propose that for a gene to satisfy the requirements to be the neuroblastoma 11q tumor suppressor gene it must fulfill the following criteria based on the known clinical and genetic alterations that are observed in neuroblastoma tumors. There should be an inverse relation between 11q deletion and MYCN amplification. Secondly, deletion of 11q results in an increase in the number of chromosomes with chromosomal breaks as well as increasing the total number of chromosomal breaks. Finally, there should also be a two-hit mechanism, considering the deletion always appears as heterozygous deletion [128, 192], something that previous attempts at tumor suppressor characterization on 11q have also considered. This was the first study in neuroblastoma that identified and begun to characterize the function of DLG2 and the association to MYCN. The bioinformatic results that were obtained in this article were subsequently used as a basis for investigations in Paper III and Paper IV

4.2 PAPER II

4.2.1 Background

In the first paper we established that loss of DLG2 results in poor outcomes in neuroblastoma patients. In this article we further evaluate the different isoforms of DLG2 as well as the different members of the DLG family and how they relate to neuroblastoma.

4.2.2 Result and discussion

We evaluated the expression levels of the various members of the DLG gene family in neuroblastoma using publicly available RNAseq data. We determined the difference in expression between the low- and high-risk neuroblastomas for each of the DLG family members. We could show that DLG1, DLG2 and DLG4 had lower expression in the high-risk tumors when compared to the low risk tumors. DLG3 showed higher expression in the high-risk tumors, whereas DLG5 was unaffected. The three DLG family members that showed low expression in the high-risk tumors are the three members that have isoforms with an L27 encoding domain. To determine if the expression level was associated with survival we also evaluated event free survival (EFS). We concluded that higher DLG2 expression resulted in better EFS whereas DLG1 and DLG4 did not.

To investigate whether the difference in expression was caused by different isoform expression we investigated the expression of isoform 1, isoform 2 and the total expression for DLG1, DLG2 and DLG4 divided by INSS stage. Combining the predominating isoforms should result in the total expression and any major differences between these two should be accounted for. For DLG1 we could see that the total expression was the sum of isoform 1 and 2, with neither of the isoforms showing a difference in expression between stage 3 and stage 4 tumors. We confirmed the result using an independent microarray dataset. We subsequently investigated the expression of DLG2 isoform 1 and 2 and compared them to the total expression. Here we saw that there was no difference in expression for either isoform but a significant difference between stage 4s and stage 4 in the total expression, indicating that we have not captured the major isoform. We subsequently investigated the expression of the remaining annotated isoforms using the GRCh37 chromosome build, which had not identified the isoforms 7/8. We could see that the expression of isoform 1, 3 and 4 were extremely low with sporadic expression in only some samples. The complete L27 domain, which was not annotated to be a complete isoform in this dataset, showed the next highest expression level following DLG2

isoform 2. After identifying isoform 7/8 as the only DLG2 isoform with the complete L27 domain we subsequently investigated the expression in relation to DLG2 isoform 2 and the total DLG2 expression using primary tumors. Here we were able to confirm that the expression of DLG2 isoform 2 was equal between different staged tumors. We could also show that DLG2 isoform 7/8 showed a large decrease in expression in the stage 4 tumors compared to stages 1+2 and stage 3. We analyzed the expression data for DLG4 and could show that there was no difference in expression across the stages in either dataset. The identification of the loss of DLG2 isoform 7/8 as an important player in neuroblastoma subsequently shifts the focus of the function away from neural stability as mediated by isoform 2 towards signaling mediated by the tripartite complexes as mediated by isoform 7/8. It also further complicated the matter due to the increase in the number of permutations of varying complex members. We therefore needed to identify the likely binding members of isoform 7/8 by working backwards through the formation of the complex, as experimental data for this was not available.

The relationship between DLG1 or DLG2 expression and each of the three members of the LIN7 family was investigated using 2D gene expression, as they are binding partners. We subsequently identified clusters of tumor subtypes based on the spatial coordinates. The DLG1 expression level didn't result in any clusters of subtypes forming for any of the LIN7 members. DLG2 however formed a number of clusters, particularly in tumors of neural origins, with higher expression of DLG2 and high expression of LIN7A. To test if the expression of one gene was dependent on the other we quantified the gene expression of LIN7A and DLG2 in primary neuroblastoma tumors, and subsequently over expressed and silenced DLG2 expression in neuroblastoma cells and quantified LIN7A gene and protein expression. We concluded that DLG2 isoform 7 over expression resulted in an increase in LIN7A expression and silencing of DLG2 resulted in a decrease in LIN7A. However, over expression or silencing of LIN7A had no effect on DLG2 expression. To confirm that DLG2 and Lin7a binding is modulated by the L27 domain we performed a co-immunoprecipitation, where we could show that DLG2 isoform 2 does not bind to Lin7a whereas DLG2 isoform 7 does. To further investigate the differences between DLG2 isoforms 2 and 7 we performed cell cycle analysis and showed that over expression of isoform 7 maintained the cells in S phase, in contrast to control and isoform 2 over expressed cells.

Finally, we investigated the expression of LIN7 paralogues in neuroblastoma in relation to survival. We could show that LIN7A had lower expression in the patients that were deceased, with no difference in LIN7B or LIN7C. We also determined that expression of LIN7A was lower in the higher stages compared to the lower INSS stage tumors. Over expression of LIN7A resulted in a substantial decrease in proliferation as well as increased number of non-viable cells, conversely silencing

LIN7A resulted in increased proliferation as well as an increase in the number of viable cells. We quantified the expression of BAX and BCL2 in response to LIN7A over expression and silencing. We could show that over expression of LIN7A resulted in an increase in BAX/BCL2, indicating inducing of apoptosis in the cells. Whereas silencing LIN7A resulted in a decrease in the BAX/BCL2 ratio, indicating cell survival.

These findings are consequent with the existing literature, with LIN7A depletion resulting in abnormal neuronal migration and loss of LIN7A resulting in cellular hyperplasia [193]. Previous reports have argued that LIN7A is oncogenic, seen in breast cancers [193], ovarian cancers [194] and in hepatocellular carcinomas [195]. The 2D results showed that these types of tumors are high in LIN7A and low in DLG2 and potentially no longer under the control of DLG2.

4.3 PAPER III

4.3.1 Background

Here, we continue on from the first paper, investigating if DLG2 influences the number of chromosomes with DNA breaks as well as the total number of chromosome breaks, something that is observed in 11q deleted neuroblastoma tumors [34].

Within the 11q deleted region of neuroblastoma there are a number of candidate tumor suppressor genes that are involved in DNA damage response including; ATM [49], H2AX [34], CHK1 [196] and MRE11A [197]. As we could show in the first article our candidate, DLG2, can regulate the cell cycle as well as potentially regulate DNA damage repair. As all of the previously mentioned candidates lack a second hit as per the Knudsons two hit hypothesis, a coalescence of haploinsufficiency within a single pathway may be sufficient for disease development and progression, however this has not currently been investigated.

4.3.2 Results and Discussion

We evaluated the gene expression of seven genes involved in the alternate non-homologous end joining (a-NHEJ), also called microhomology end joining (MMEJ), in four independent neuroblastoma datasets and stratified based on risk, survival and 11q status. We determined that PARP1 was over expressed in high-risk neuroblastoma tumors as well as in tumors of the deceased patients. There was no difference in the expression between the 11q deleted tumors and the non-11q deleted tumors. MRE11A showed no difference for risk group but showed decreased expression in tumors from the patients that survived as well as in one of the 11q deleted datasets. FEN1 was upregulated in high-risk neuroblastoma, the patients that died of disease as well as in the 11q deleted tumors. XRCC1 and NSB1 expressions both showed some alteration across the four datasets but were not consistently altered, LIG3 was stably expressed across all datasets. PCNA, a binding partner of XRCC1 and previously established to negatively correlate to DLG2 expression was also investigated, it showed increased expression in high-risk neuroblastomas, correlated to poor survival and higher expression in 11q deleted tumors. To determine if the DLG2 level influenced the expression of these MMEJ genes we over expressed or silenced DLG2 in neuroblastoma cells as well as silenced dmDLG expression in a fruit fly model. Here we could determine that silencing DLG2 and dmDLG resulted in increased PARP1 and FEN1 expression, as well as decreased MRE11A expression. The over expression of DLG2-iso7 in neuroblastoma cells resulted in a decrease in PARP1 and FEN1

expression as well as an increased MRE11A expression. The PARP1, FEN1 and PCNA results were also confirmed on protein level by immunoblot. We noted that FEN1 expression was identified in the first article as having a strong negative correlation to DLG2 expression.

In Paper I we identified that DLG2 is likely involved with DNA repair. To determine the effect of DLG2 in DNA reparation and maintenance of genome integrity a comet assay was performed. Neuroblastoma cells with either intact 11q or 11q deletion were transfected with DLG2 and then subjected to UVC irradiation followed by 4 hours recovery. Comet assay showed that silencing of DLG2 in the 11q normal cells resulted in a decrease in the average head diameter without UVC radiation compared to the control transfection, with an even smaller head diameter seen post UVC radiation. The experiments were subsequently repeated with an inducer of double strand breaks, etoposide, showing a similar result as UVC. This indicated that DLG2 expression in these cells, maintained genome integrity. Silencing of DLG2 in the 11q deleted cells showed no difference to the control. The over expression of DLG2 isoform 7 also resulted in a smaller head diameter with no difference after UVC-irradiation, which was seen in both 11q normal and deleted cells. Further analysis showed that these cells had markedly less γ-H2AX, a marker of dsDNA breaks, 2 hours after UVC treatment. Analysis of the comet tail length showed the same trend for the 11q deleted and 11q normal cells with the silencing of DLG2 resulting in a longer tail and over expression of DLG2 resulting in the shortest tail. Silencing of DLG2 resulted in prolonged γ-H2AX across all of our cell lines, indicating that there were dsDNA breaks still present. To determine if the repair was mediated by NHEJ we determined the activation of reporter cells post transfection. We could determine that silencing of DLG2 resulted in activation of NHEJ whereas DLG2 isoform 7 prevented the activation of NHEJ. Taken together, this showed that silencing DLG2 results in the activation of the NHEJ mechanism but repair was impaired as measured by duration of γ-H2AX as well as an associated decrease in S phase duration and increased rate of proliferation (Paper I). Potentially, providing a mechanism for the shotgun losses seen in primary neuroblastomas. Conversely, we showed that NHEJ was not active in DLG2 isoform 7 over expressed cells with increased S phase (Paper I) and G2/M phases (Paper II), conditions conducive for homologous repair if not apoptosis (Figure 10).

Apoptosis was investigated by determining the expression of BAX, BCL2 and caspase 3/7 activation after silencing or over expressing DLG2. We showed that there was no alteration in the cells with silenced DLG2 or in the control, but a large increase in the BAX/BCL2 ratio as well as caspase 3/7 activation, signals leading to apoptosis, in the cells with DLG2 isoform 7 over expression. We also determined that p53 expression was increased consistent with the correlation identified in paper I. In combination with increased p53 expression we could determine a strong increase

in S46 phosphorylation of p53, which strongly associates to severe genotoxic stress and results in apoptosis [198].

To investigate the effect of DLG loss on DNA repair we silenced the expression of dmDLG in Drosophila melanogaster and then exposed them to UVC irradiation followed by 3 hours recovery. We subsequently sequenced the DNA using long read sequencing to determine if there was any active DNA repair resulting in fusions or large-scale aberrations. The flies with dmDLG silenced but not exposed to UVC irradiation showed the same sequence distributions and mapping lengths as the untreated control, the effect on DNA repair was only apparent after a second trigger, in this case a UVC exposure. The combination of dmDLG silencing and UVC exposure resulted in more sequences that didn't map to the genome, longer sequences that mapped to multiple places in the genome as well as an increase in the number of fusion sequences with greater than 10000bp between the mapped fragments. With the increase in fusion sequences a direct result of low fidelity NHEJ type repair.

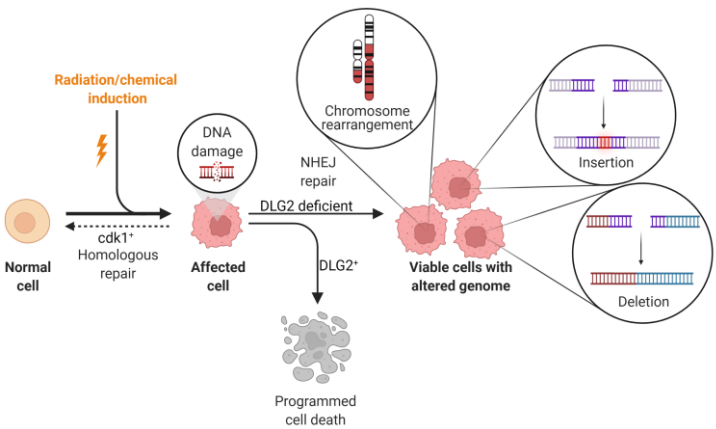

Figure 10. A schematic overview of the effect of DLG2 in the DNA repair process mediated by the NHEJ mechanism when activated by radiation or chemical induction. A normal cell if chemically induced can result in DNA damage. If DLG2 is present the cell will undergo cell death. If the cells are deficient in DLG2 then non-homologous end joining will occur, with the repair resulting in loss of DNA material, gain of DNA material or chromosome fusions. Created with BioRender.com

Finally, we investigated the phosphorylation of CHK1 at S317, the phosphorylation of CHK2 at T68 and the expression of ATM and DNA-PKcs in neuroblastoma cells

after UVC exposure followed by 2 or 24 hours of recovery. We could determine that there was no difference in CHK1 phosphorylation after 2 hours of recovery between the control and over expressed DLG2 cells. The silencing of DLG2 resulted in a lower phosphorylation of CHK1 at S317. We could determine that after 24 hours recovery there was a decrease in CHK1 S317 phosphorylation in the DLG2 over expressed and control cells and a maintained CHK1 S317 phosphorylation in the DLG2 silenced cells. The maintenance of CHK1 S317 phosphorylation can act to prevent cells from advancing through the G2/M checkpoint [63]. However, our previous results showed that this was not the case with DLG2 loss. We also investigated the phosphorylation of T68 CHK2. The control transfection showed that the phosphorylation of T68 CHK2 decreased over time. Silencing DLG2 resulted in lower T68 CHK2 at 0 hours, an increase after 2 hours and a decrease after 24 hours. The DLG2 isoform 7 transfected cells showed a lower degree of CHK2 phosphorylation after 0, 2 and 24 hours with no alteration over time. CHK2 has previously been shown to be phosphorylated by ATM [199], indicating that DLG2 and ATM have different mechanisms of action. Additionally, we showed that over expression of DLG2 results in low ATM expression consistent with the CHK2 phosphorylation results.

4.4 PAPER IV

4.4.1 Background

In this study we investigate the roll of DLG2 in colorectal cancer (CRC) and the effect that DLG2 has on inflammation and the inflammasome.

CRC is one of the leading causes of cancer related deaths in the developed world. Some of the risk factors for developing CRC are lifestyle factors such as; increasing incidences of obesity and the alteration and reduction of diversity of commensal bacteria in the gut. Other factors that increase the likelihood of developing CRC is advancing age as well as inflammatory diseases such as Ulcerative colitis and Crohns disease. Spontaneous and colitis associated CRC both result in the activation of COX-2 in response to inflammation along with altered NFκβ signaling which in this context results in survival, proliferation and progression through the cell cycle [200-202].

4.4.2 Results and Discussion

We evaluated the expression of DLG2, NFKBIZ and NLRP3 in the independent colon datasets to determine the effect of inflammation and cancer. We investigated the expression of these genes in different tissue types, ulcerative colitis (UC), active or inactive UC, and in tissues adjacent to tumor vs. tumor. DLG2 was consistently downregulated in inflammatory diseases such as inflammatory bowel disease (IBD) and UC, and a decrease was also seen during active UC compared to inactive UC. We could also show that DLG2 was downregulated in patients with IBD, adenomas and colon carcinomas when compared to healthy individuals. We additionally showed that patient paired mucosa and tumors had a large decrease in DLG2 expression compared to healthy controls. NLRP3 expression was equal between IBD, adenomas, colon carcinomas and healthy individuals. Patients with UC showed higher levels of NLRP3 compared to controls, with these levels been higher during active disease. Interestingly, in the colorectal cancer patients there was decreased NLRP3 expression in the tumor compared to in the adjacent healthy appearing tissue. A similar pattern was observed for NFKBIZ with an increase in expression observed for inflammatory diseases IBD, UC and active UC. With a decrease of NFKBIZ expression in tumors of patient paired mucosa and tumor samples.

To confirm these results we investigated mouse, cell and fly models. In mice that were treated with DSS (Dextran sulfate sodium) to induce a UC like phenotype [10, 191], a slight increase in DLG2 expression was observed after 4 days with expression

returning to base line levels after 6 days. NLRP3 and NFKBIZ showed an increase in expression 6 days after DSS treatment. When T-cells were transferred into mice colon a decrease in DLG2 expression was detected 4 weeks post transferal, that was maintained until 6 weeks. THP-1 cells were treated with LPS and showed an initial increase in DLG2 expression after 12 hours and a decrease after 24 hours exposure. The intestines of fly larvae were investigated after bacterial treatment (Bifidobacterium lactis and Lactobacillus acidophilus). We saw an initial increase in dmDLG expression after 1-day of treatment, trending to decrease over time to below the initial expression level after 4 days. The decrease in dmDLG and DLG2 in response to inflammatory stimuli is completely novel and requires further investigation. There is no orthologue in fly for NLRP3 or NFKBIZ, thus this could not be investigated in this model.

We subsequently investigated the expression of RELA, RELB, NFKB1 and NFKBIZ genes in response to inflammasome activation and DLG2 over expression. Here we could determine that there was no change in RELA expression when DLG2 was over expressed, however a consistent increase in RELB, NFKB1 and NFKBIZ expression in response to increased level of DLG2. We also confirmed that NFKBIZ was upregulated during the activation of the inflammasome. We also investigated the gene expression of IL1B, IL6 and the apoptosis genes BAX and BCL2. We showed that IL1B expression was increased in response to DLG2 over expression across all treatments and IL6 decreased in response to LPS and LPS+ATP, which was not seen in the control. Consistent with previous results and publications we detected an increase in BAX expression and a decrease in BCL2 expression [203]. DGL2 over expression also affected the phosphorylation of STAT and S6 at the sites S727 in STAT3 and S235/236 in S6. Finally, we determined ASC speck formation as a proxy for inflammasome formation in THP-1-ASC-GFP cells by microscopy. Silencing of DLG2 resulted in a large decrease in inflammasome formation, while DLG2 over expression resulted in an increase. Previous reports have highlighted the activation of the NLRP3 inflammasome as important in protecting against cancer progression [204] as well as increasing il18 expression [205].

To determine the effect of inflammasome formation on surrounding tissue we harvested media from THP1-ASC cells after over expression of DLG2 or DLG2 silencing, followed by IL-1b and IL-6 protein quantification. The media was subsequently used to treat COLO205 colon cancer cells, with the level of proliferation and G2/M status determined. We determined that the media from the THP-1 silenced DLG2 cells resulted in an increase in proliferation and percentage of COLO205 cells in G2/M phase and that over expression of DLG2 in THP-1 resulted in a decrease in proliferation and percentage of COLO205 cells in G2/M. We then investigated the protein expression in the COLO205 cells in response to the media. We determined that DLG2 over expression increased RELB, IκBζ, NFKB1 and BAX

protein levels whilst decreasing the phosphorylation of S727 STAT3, important to note that activation of STAT3 is common in colon cancer [123, 124].

Finally, the investigation of DLG2 expression of in primary colon cancer samples was quantified in paired healthy tissue from different locations within the colon and comparing it to the tumor tissue. Using a publicly available dataset we showed that lower DLG2 expression resulted in larger tumors and that silencing DLG2 in SW480 colon cancer cells resulted in an increased proliferation whereas an increase in DLG2 resulted in a decreased proliferation. We compared the gene expression level of NLRP3, NFKBIZ and FOXO3 in response to DLG2 over expression in the SW480 cell line, and saw that DLG2 over expression resulted in a decrease in NLRP3 expression whereas it resulted in an increase in NFKBIZ and FOXO3. We then quantified the protein level by immunoblot for AKT, FOXO3 and S6 which all showed decreases in phosphorylation in response to DLG2 over expression. The total amount of FOXO3 increased in these cells with the total level of S318/S312 FOXO3 remaining constant resulting in the relative decrease of FOXO3 phosphorylation [206]. Phosphorylation of FOXO3 has been shown to result in dysregulation of the DNA repair mechanism mediated by the ATM-Chk2-p53 axis, with phosphorylated FOXO3 required for apoptosis in response to DNA damage [206]. The decreased phosphorylation of FOXO3 observed here is consistent with the result that we obtained in Paper III showing that DLG2 over expression increased apoptosis and p53 expression. Additionally, the decrease in S236/236 S6 phosphorylation helps to reverse the stemness in dedifferentiated colon cancer cells by reducing protein synthesis and cell proliferation [207, 208].

5 CONCLUSION

The conclusions from the published articles and the manuscripts that have been included within this thesis are;

Paper I

- The expression of DLG2 is low in high stage neuroblastoma with low expression correlated to high mortality.
- DLG2 expression has an inverse relation to MYCN amplification; with MYCN amplification acting to disrupt DLG2 expression.
- DLG2 controls the cell cycle by regulating the protein expression of Cyclin A and Cyclin B.
- DLG2 is predicted to regulate DNA repair.
- We showed that DLG2 is expressed in all neuroblastomas, including 11q-deleted tumors and cell lines, without having altered promoter methylation.
- Loss of DLG2 increased cell proliferation and shortened the cell cycle S-phase length.

Paper II

- DLG2 has two predominating isoforms in neuroblastoma; isoform 7/8 encoding the β protein with a complete L27 domain, and isoform 2 encoding the α protein.
- DLG2 isoform 7/8 is downregulated in high INSS stage tumors whereas isoform 2 is unaltered.
- The downregulation of isoform 7/8 is the overall cause of the total DLG2 expression decrease.
- DLG2 isoform 7 binds to LIN7A by through the L27 domain, LIN7A is also lowly expressed in high stage neuroblastoma.
- High LIN7A expression decreases proliferation and cell number in neuroblastoma.

Paper III

- DLG2 over expression resulted in a decrease in PARP1, FEN1 and PCNA, key components in the NHEJ DNA repair pathway.
- Silencing dmDLG results in an increase in the number of fusion sequences in Drosophila in response to UVC treatment.
- DLG2 increases TP53 expression and increases pS46 p53 phosphorylation to induce apoptosis in both 11q deleted and MYCN amplified neuroblastoma cells.
- DLG2 over expression prevents the phosphorylation of T68-CHK2 in response to UVC treatment.

Paper IV

- DLG2 is downregulated in inflammatory precancerous conditions such as UC and adenocarcinoma indicating that DLG2 alteration could occur early in the tumorigenesis process.
- DLG2 is initially upregulated in response to inflammation and subsequently downregulated dependent on longer duration chronic inflammation.
- DLG2 overexpression results in higher expression of RELB, NFKB1, NFKBIZ.
- DLG2 over expression in macrophage like cells increases inflammasome formation and reduces the expression and release of il-6, resulting in decreased proliferation by inhibiting STAT3 phosphorylation in surrounding cells.

6 FUTURE PERSPECTIVES

Currently, the available treatments to patients with aggressive neuroblastoma are often not successful. The 5-year event free survival rates of patients with aggressive neuroblastoma are around 35% for both MYCN amplified and 11q deleted tumors. As these tumors can escape the current treatment strategies, the treatment ends up being both ineffective, invasive and often result in significant morbidity and long-term disability. The neuroblastoma patients that survive are subject to significant lifelong morbidity that impacts on the individuals' quality of life. To better treat the patients with the 11q deletion it is important to identify and characterize the genes affected by the deletion.

As previously discussed, the age of diagnosis for colorectal patients is decreasing. Combined with the increasing incidences due to older populations and lifestyle factors the total number of individuals at risk for colon cancer is increasing. The 5-year survival rates for colon cancer depend on the localization of the tumor with localized tumors having high survival rates and tumors with distant metastasis having less than 20% survival. The poor survival rate of these patients is of concern and highlights the ineffectual treatments currently available.

One of the fundamental issues that remains unanswered within high risk neuroblastoma is the inverse relationship between 11q deleted and MYCN amplified tumors. Whilst we used this inverse relationship to strengthen the claim that DLG2 is the 11q tumor suppressor we did not persist with the complete investigation to determine the manner and nature of this relationship. The identification of the interaction between the two may result in an alteration in how we approach the treatment of these individuals. In addition, we have presented the function of DLG2 within cell cycle regulation by the manipulation of cyclins, the mechanism of action for these alterations has fallen outside of the scope of the study. It may therefore, be of interest to revisit this to further elucidate how DLG2 interacts with cell cycle proteins and further investigate the other candidates that we identified with the bioinformatic screen that we performed.

During the investigation of DLG2 isoform 7 we began to look at the interactions between DLG1, DLG2 and the members of the LIN7 family. This top down approach did not account for the final member of the tripartite complex, the proteins containing two L27 domains. Most of which are members of the MPP family, of which there are seven, as well as CASK, all potential members for which the complex can be built upon. The variations in functions as well as the binding preferences for these permutations have not yet been investigated. The investigation of the construction of these complexes may be required to explain the phenomenon of various tumor

expression patterns observed in the 2D gene expression clustering or the haploinsufficient mechanism that was proposed in Paper I. Additionally, the investigation of the different DLG2 isoforms and the combined results presented in paper III present an opportunity to reevaluate the cellular location of the two main DLG2 isoforms.

Based on the results from Paper I we chose to further investigate the effect of DLG2 on DNA repair in Paper III. This identified a tentative negative correlation between DLG2 and ATM as well as the relationship between DLG2 and ATR identified in Paper I. We did not further evaluate the specific relationships that indicated that high levels of DLG2 expression suppressed the expression of ATM and ATR. This would further suggest that the presence of DLG2 prevents the repair of DNA breaks pushing the cells into apoptosis. The continuation of this study would be to determine the localization of DLG2 to ensure that it can localize to the nucleus and either interact with ATM/ATR or prevent the transcription of the genes. This research will feed into the broader context of cellular decision making within the DNA damage repair field.

The investigations in paper IV were focused on the effect of DLG2 and the formation of the inflammasome in colon cancer. The translation of this research between the colon cancer and neuroblastoma field may be of interest. As previously mentioned the loss of 11q14 in colon cancer is associated with higher CIN concordant with the neuroblastoma data discussed in Paper III. In neuroblastoma the loss of 11q results in a decrease in the immune infiltration with the mechanism of action remaining unknown. Recently, NLRP3 inflammasomes have been characterized in neural type cells including microglia, astrocytes and Schwann cells. Further studies into how DLG2 is modulated by inflammation in a biphasic manner, initially upregulating DLG2 and decreasing with chronic type inflammation remains currently unexplained. Further studies to investigate this may provide novel therapies for the modulation and or activation of the inflammasome in 11q deleted patients.

The future goal for this particular project is now to investigate pharmaceutical methods of inducing DLG2 expression, namely Isoform 7, first in models and subsequently in human patients. In addition, we can begin to determine the type of currently existing agents that may be used in combination with any DLG2 inducing treatment. As with much of the work already performed, publicly available databases may be used to identify drug candidates for initial testing in models. Based on the current knowledge available, combining an agent that induces DLG2 isoform 7 expression with etoposide to induce DNA strand breaks in addition to an agent that can aid in the activation of the inflammasome formation may yield interesting results that could be taken further.

Ultimately, the goal of cancer research is to participate in the improvement of treatment strategies and therapies to increase survival and reduce side-effects. The focus of these works has been to investigate a single gene and untangle the diverse networks and pathways in the hope that the work finds a clinical application.

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