Muthiah Bose

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF ABRAXAS AND PALB2 GENES IN HEREDITARY BREAST CANCER PREDISPOSITION
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MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF ABRAXAS AND PALB2 GENES IN HEREDITARY BREAST CANCER PREDISPOSITION

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Abstract

Hereditary mutations in DNA damage response (DDR) genes often lead to genomic instability and ultimately tumor development. However, the molecular mechanism of how these DDR deficiencies promote genomic instability and malignancy is not well understood. Thus, the specific aim of this thesis is to identify the functional and molecular framework behind the elevated breast cancer risk observed in heterozygous PALB2 and ABRAXAS mutation carriers.

The heterozygous germline alteration in PALB2 (c.1592delT) causes a haploinsufficiency phenotype in the mutation carrier cells. Due to PALB2 haploinsufficiency, elevated Cdk activity and consequently aberrant DNA replication/damage response was observed in the PALB2 mutation carrier cells. Excessive origin firing that is indicative of replication stress was also seen in the PALB2 mutation carrier cells. In addition to replication stress, PALB2 mutation carrier cells also experience G2/M checkpoint maintenance defects. The increased malignancy risk in females associated with heterozygosity for the Finnish PALB2 founder mutation is likely to be due to aberrant DNA replication, elevated genomic instability and multiple different cell cycle checkpoint defects.

The heterozygous germline alteration in ABRAXAS (c.1082G>A) causes a dominant-negative phenotype in the mutation carrier cells. Decreased BRCA1 protein levels as well as reduced nuclear localization and foci formation of BRCA1 and CtIP was observed in the ABRAXAS mutation carrier cells. This causes disturbances in basal BRCA1-A complex localization, which is reflected by a restraint in error-prone DNA double-strand break (DSB) repair pathway usage, attenuated DNA damage response, deregulated G2/M checkpoint control and apoptosis. Most importantly, mutation carrier cells display a change in their transcriptional profile, which we attribute to the reduced nuclear levels of BRCA1. Thus, the Finnish ABRAXAS founder mutation acts in a dominant-negative manner on BRCA1 to promote genome destabilization in the heterozygous carrier cells.

Keywords: ABRAXAS, BRCA1, breast cancer, cancer genetics, cancer predisposition, CtIP, DNA damage response, DNA double-strand break repair, G2-M checkpoint, heterozygous germline mutation, PALB2, replication stress, tumorigenesis
**Tiivistelmä**

Perinnölliset muutokset DNA-vauriovasteen geneessä johtavat usein genomin epävakautteeseen ja lopulta syövän kehittymiseen. Molekyyliytason mekanismeja, joilla vauriovasteen vajaatoiminta ajaa genomin epävakautta ja syöpää, ei kuitenkaan ymmäretä kunniolla. Tämän vääristäkin tavoitteena on tunnistaa solutoiminnan ja molekyyliytason vaikutajat heterotsygoottisten **PALB2**- ja **ABRAXAS**-geenimuutosten kantajien kohonneen rintasyöpäriskin taustalla.

Heterotsygoottinen ituradan suomalainen perustajamuutos **PALB2**-geenissä (c.1592delT) aiheuttaa haploinsuffiisiin kantajahenkilöiden soluissa. **PALB2**:n haploinsuiffiisiin seu-ruuksena kantajasoluissa havaittiin kohonnuttu Cdk-proteiinin aktiivisuutta ja siitä johtuvaan kiihtynyttään DNA:n kahdentumista. **PALB2**:mutaatiota kantavissa soluissa nähtiin myös liiallista replikaation aloituskohtien käyttöä, mikä viittaa replikaatiostressiin. Replikaatiostressin lisäksi **PALB2**:mutaation kantajasoluilla havaittiin vaikeuksia ylläpitää solusyklin G2/M-tarkastuspisteen toimintaa. Näiden solutoiminnan poikkeavuksien takia heterotsygoottisen **PALB2** c.1592delT-mutaation kantajilla todettiin genomin epävakautta ja kohonnut syöpärikkö.


**Asiakset:** **ABRAXAS, BRCA1, CtIP, DNA-korjaus, DNA-vauriovaste, G2/M-tarkastuspiste, heterotsygoottinen ituradan mutaatio, PALB2, replikaatiostressi, rintasyöpä, syöpäalitiittisuus, syöpäjen genetiikka, syövän kehitys**
Dedicated to my Family
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Copenhagen, March 2018

Muthiah Bose
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>53BP1</td>
<td>p53-binding protein 1</td>
</tr>
<tr>
<td>9-1-1</td>
<td>RAD9–RAD1–HUS1</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3-related protein</td>
</tr>
<tr>
<td>ATRIP</td>
<td>ATR interacting protein</td>
</tr>
<tr>
<td>BACH1</td>
<td>BTB domain and CNC homolog 1</td>
</tr>
<tr>
<td>BBA</td>
<td>Biochimica et Biophysica Acta</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer gene 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer gene 2</td>
</tr>
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<td>BRCA1-PALB2-BRCA2-RAD51</td>
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<td>BRCA1/BRCA2-containing complex subunit 36</td>
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<tr>
<td>BRCC45</td>
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<td>BRCT</td>
<td>BRCA1 C terminus</td>
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<tr>
<td>BRIP1</td>
<td>BRCA1 interacting protein C-terminal helicase 1</td>
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<td>C1QTNF5</td>
<td>C1q and tumor necrosis factor related protein 5</td>
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<td>Cas9</td>
<td>CRISPR associated protein 9</td>
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<td>CASP8</td>
<td>Caspase 8</td>
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<tr>
<td>CCDC98</td>
<td>Coiled-coil domain containing 98 (alias ABRAXAS, ABRA1, FAM175A)</td>
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<td>Cdc25</td>
<td>Cell division cycle 25</td>
</tr>
<tr>
<td>Cdc25C</td>
<td>Cell division cycle 25C</td>
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<td>CDH1</td>
<td>E-cadherin</td>
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<tr>
<td>Cdk</td>
<td>Cyclin-dependent kinase</td>
</tr>
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<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
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<td>CDKN1B</td>
<td>Cyclin-dependent kinase inhibitor 1B</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>ChAM</td>
<td>Chromatin-association motif</td>
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<tr>
<td>CHEK2</td>
<td>Checkpoint kinase 2</td>
</tr>
<tr>
<td>CHK1</td>
<td>Checkpoint kinase 1</td>
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<td>CHK2</td>
<td>Checkpoint kinase 2</td>
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<td><strong>Abbreviation</strong></td>
<td><strong>Definition</strong></td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
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<tr>
<td>COS</td>
<td>CV-1 (simian) in origin, and carrying the SV40 genetic material</td>
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<td>COX11</td>
<td>Cytochrome C oxidase assembly homolog 11</td>
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<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<tr>
<td>CtIP</td>
<td>C-terminal binding protein 1 (CtBP1) interacting protein</td>
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<tr>
<td>CYREN</td>
<td>Cell cycle regulator of NHEJ</td>
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<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DEGs</td>
<td>Differentially expressed genes</td>
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<td>D-loop</td>
<td>Displacement loop</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DSB</td>
<td>Double-strand break</td>
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<tr>
<td>DSBR</td>
<td>Double-strand break repair</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitinating</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>edgeR</td>
<td>Empirical analysis of digital gene expression in R</td>
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<td>EdU</td>
<td>Ethynyl-deoxyuridine</td>
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<td>eIF4F</td>
<td>Eukaryotic initiation factor 4F</td>
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<td>ESR1</td>
<td>Estrogen receptor 1</td>
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<tr>
<td>ETO</td>
<td>Etoposide</td>
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<tr>
<td>EXO1</td>
<td>Exonuclease 1</td>
</tr>
<tr>
<td>EZH2</td>
<td>Enhancer of zeste homolog 2</td>
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<tr>
<td>FA</td>
<td>Fanconi anemia</td>
</tr>
<tr>
<td>FAM175A</td>
<td>Family with sequence similarity 175, Member A</td>
</tr>
<tr>
<td>FAM84B</td>
<td>Family with sequence similarity 84 member B</td>
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<td>FANCD2</td>
<td>Fanconi anemia, complementation group D2</td>
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<td>FANCM</td>
<td>Fanconi anemia, complementation group M</td>
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<tr>
<td>FANCN</td>
<td>Fanconi anemia, complementation group N</td>
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<td>FGFR2</td>
<td>Fibroblast growth factor receptor 2</td>
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<td>G1-phase</td>
<td>Gap 1 phase</td>
</tr>
<tr>
<td>G2-phase</td>
<td>Gap 2 phase</td>
</tr>
<tr>
<td>GRM1</td>
<td>Glutamate receptor, metabotropic 1</td>
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<tr>
<td>HA</td>
<td>Human influenza hemagglutinin</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks cell line</td>
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<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human mammary epithelial cells</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>Indels</td>
<td>Insertions and deletions</td>
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</table>
IPA       Ingenuity pathway analysis
IR        Ionizing radiation
JAMM      JAB1/MPN/Mov34 metalloenzyme
K63       Lysine 63
KEAP1     Kelch-like ECH-associated protein 1
LCLs      Lymphoblastoid cell lines
IncRNA    Long non-coding RNA
LOH       Loss of heterozygosity
LSPL      Lymphocyte-specific protein 1
MAP3K1     Mitogen-activated protein kinase kinase kinase 1
MCF10A     Michigan Cancer Foundation-10A
MCF7       Michigan Cancer Foundation-7
MDC1      Mediator of DNA damage checkpoint 1
MEFs       Mouse embryonic fibroblasts
MERIT40    Mediator of RAP80 interactions and targeting subunit of 40 kDa
MMC       Mitomycin C
MMEJ      Microhomology-mediated end joining
MMR       Mismatch repair
MORF4L1    Mortality factor 4 like 1
M-phase    Mitosis phase
MPN       Mpr1 and PAD1 N-terminal
MRG15     MORF-related gene on chromosome 15
MRN       MRE11–RAD50–NBS1
mRNA      messenger RNA
MRPS30    Mitochondrial ribosomal protein S30
NA        Not applicable
NBS1      Nijmegen breakage syndrome 1 (Nibrin, NBN)
NEK10     NIMA (never in mitosis gene A)-related kinase 10
NER       Nucleotide excision repair
NES       Nuclear export signal
NHEJ      Non-homologous end joining
NLS       Nuclear localization signal or sequence
NPI       Nottingham prognostic index
NRAS      Neuroblastoma RAS viral (V-Ras) oncogene homolog
NRF2      Nuclear factor-erythroid 2-related factor 2
NST       No special type
PALB2     Partner and localizer of BRCA2
PARP  Poly (ADP-ribose) polymerase
PCR  Polymerase chain reaction
PI3K  Phosphatidylinositol 3-kinase
PLA  Proximity ligation assay
Pol η  Polymerase eta
POU4F3  POU class 4 homeobox 3
PP2A  Protein phosphatase 2A
PR  Progesterone
PRC2  Polycomb repressive complex 2
PTEN  Phosphatase and tensin homolog
PTM  Post-translational modification
qPCR  quantitative PCR
RAD51C  RAD51 paralog C
RAD51L1  RAD51-like 1
RAD54L  RAD54 like
RAP80  Receptor-associated protein 80
RB1  Retinoblastoma 1
RET  Rearranged during transfection
RIF1  Rap1-interacting factor 1
RIN  RNA integrity number
RNA  Ribonucleic acid
RNA-seq  RNA sequencing
RNF168  Ring finger protein 168
RNF8  Ring finger protein 8
ROS  Reactive oxygen substances
RPA  Replication protein A
Rpn10  Regulatory particle number 10
Rpn11  Regulatory particle number 11
Rpn8  Regulatory particle number 8
SCE  Sister chromatid exchange
SETD6  SET domain-containing protein 6
SFR  Stalled fork repair
siRNA  Small (or short) interfering RNA
SMAD4  Mothers against decapentaplegic homolog 4
SNP  Single nucleotide polymorphism
S-phase  Synthesis phase
SSA  Single-strand annealing

<table>
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<tr>
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<td>SSB</td>
<td>Single-strand break</td>
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<tr>
<td>STK11</td>
<td>Serine-threonine protein kinase 11</td>
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<td>SUZ12</td>
<td>Suppressor of zeste 12</td>
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<td>TAL1</td>
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<tr>
<td>T-ALL</td>
<td>T cell acute lymphoblastic leukemia</td>
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<td>TNBC</td>
<td>Triple negative breast cancer</td>
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<td>TOX3</td>
<td>Tox high-mobility group box family</td>
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<td></td>
<td>member 3</td>
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<td>TP53</td>
<td>Tumor protein 53</td>
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<td>TRPS1</td>
<td>Trichorhinophalangeal syndrome, type</td>
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<td>U2OS</td>
<td>Human osteosarcoma cell line</td>
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<td>Ub</td>
<td>Ubiquitin</td>
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<td>UIM</td>
<td>Ubiquitin-interacting motif</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<td>XRCC3</td>
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<td>ZP3</td>
<td>Zona pellucida glycoprotein 3</td>
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<tr>
<td>γH2AX</td>
<td>phospho-histone H2AX</td>
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Original publications

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1 Introduction

Cancer is not one disease, but a collection of related diseases, which originate from most of the cell types and organs in the human body (Stratton et al. 2009). Approximately 90% of human cancers arise from the epithelial cells (Cooper 2000). The risk of suffering any cancer before the age of 40 is ~2%; however, by the age of 80 this risk increases to 50% (Martincorena & Campbell 2015). Cancer is a disease of the genome which arises due to somatically acquired mutations (Fearon 1997, Michor et al. 2004, Stratton 2011, Yates & Campbell 2012). Most cancers carry around 1,000 to 20,000 point mutations along with a few to hundreds of insertions and deletions (indels) and rearrangements (Martincorena & Campbell 2015).

Breast cancer is the most commonly diagnosed cancer in women, and most importantly, it is the second leading cause of cancer mortality in women (after lung cancer) (Downs-Holmes & Silverman 2011, Makki 2015). It is estimated that in industrialized countries, approximately one in eight women are expected to develop breast cancer during her lifetime (Ellsworth et al. 2010). Worldwide, an estimated 1.7 million breast cancer cases and 521,900 breast cancer related deaths were observed in 2012 (Torre et al. 2015). In Finland, 5,161 new breast cancer cases and 841 breast cancer related deaths were reported in 2015 (Finnish Cancer Registry 2017).

Approximately 5–10% of all patients with breast cancer exhibit a strong genetic predisposition to the disease (Claus et al. 1996, Romero-Laorden & Castro 2017, Thull & Vogel 2004). The main indicators for a hereditary disease predisposition are: 1) early disease onset, 2) familial clustering of breast cancer, 3) occurrence of multiple primary tumors in the same individual, 4) occurrence of bilateral breast cancer and 5) male breast cancer incidences in the family (Thull & Vogel 2004). Mutations in high-risk breast cancer susceptibility genes such as BRCA1 and BRCA2 together account for 15% of hereditary breast cancer cases. Mutations in the rest of the known breast cancer susceptibility genes account for an additional 21% of hereditary breast cancer cases (Couch et al. 2014).

Genes that, when mutated, promote cancer development can be classified into dominant-acting proto-oncogenes and recessive tumor suppressor genes, the latter including also the caretaker gene sub-class. However, tumor suppressor gene mutations are not always completely recessive, as they can alternatively be either haploinsufficient or dominant-negative (Payne & Kemp 2005). Haploinsufficiency represents the special circumstance in which one functional
allele is insufficient to maintain the normal cellular process (Berger & Pandolfi 2011, Payne & Kemp 2005). On the other hand, any mutant protein which disrupts the activity of the wild-type protein is classified as dominant-negative mutant (Herskowitz 1987).

It is well known that hereditary mutations in DDR genes associated with important cellular caretaker functions often lead to genomic instability and ultimately tumor development (Negrini et al. 2010). However, the molecular mechanisms of how these DDR deficiencies promote genomic instability and malignancy are not well understood. Thus the specific aim of this thesis is to better understand how genomic instability and tumorigenesis arise in the heterozygous PALB2 and ABRAXAS germline mutation carriers.
2 Review of the literature

2.1 Cancer

Cancer encompasses more than 100 distinct diseases which originate from most of the cell types and organs in the human body (Stratton et al. 2009). Approximately 90% of human cancers arise from the epithelial cells (Cooper 2000). It is estimated that the risk of suffering any cancer before the age of 40 is ~2%; however, by the age of 80 this risk increases to 50% (Martincorena & Campbell 2015). Cancers are characterized by abnormal proliferation of cells that can invade beyond normal tissue boundaries and metastasize to distant organs (Stratton et al. 2009).

Most importantly, cancer is a disease of the genome which arises due to somatically acquired mutations (Fearon 1997, Michor et al. 2004, Stratton 2011, Yates & Campbell 2012). Mutation in genes can have both in cis and in trans effect on cancer development. If a mutant gene directly impacts on cancer development then the mutation is acting in cis fashion. However, if a mutation promotes cancer development through some other genes encoded elsewhere then the mutation is acting in trans fashion. In addition to genetic changes, cancer can also arise due to epigenetic alterations. Mutation in genes that regulate epigenome as well as epigenetic alteration results in abnormal gene expression, thereby promoting tumorigenesis (You & Jones 2012).

In addition to genetic and epigenetic changes, several studies have recently shown that the tumor microenvironment also plays a critical role during tumor initiation and progression (Chen et al. 2015). For tumorigenesis, genes that control critical cellular processes such as cell proliferation and differentiation, DNA replication, cell cycle and DNA repair are frequently targeted. Currently, there are several models available to describe how tumorigenesis arises (reviewed in Meng et al. 2011), but in simple terms, biological changes in the epithelium alone or along with changes in stroma/extracellular matrix are necessary for malignancy (Vineis et al. 2010).

The human genome is a very dynamic structure with somatic mutations occurring in all cells during fetal development and tissue regeneration (Ju et al. 2017, Luning Prak & Kazazian Jr 2000). It is estimated that each normal cell in our body undergoes more than 20,000 DNA-damaging events and over 10,000 replication errors per day (Loeb 2011). Fortunately, most of these mutations are
efficiently repaired by the cellular DNA repair machinery, and it is estimated that only 2–10 mutations per each cell division remain unrepaired (Loeb 2016, Martincorena & Campbell 2015, Rayner et al. 2016). Thus, the gradual accumulation of mutations can lead to the transformation of a normal cell into a cancerous one (Loeb 2016).

As shown in Figure 1, for malignant transformation, a normal cell has to acquire the following six essential physiological changes known as hallmarks of cancer: 1) sustaining proliferative signaling, 2) evading growth suppressors, 3) resisting cell death, 4) enabling replicative immortality, 5) inducing angiogenesis and 6) activating invasion and metastasis. Since cancer development is a multistep process, acquisition of one cancer hallmark accelerates the attainment of the remaining hallmarks in succession. Additionally, two emerging hallmarks of cancer, reprogramming energy metabolism and evading immune destruction, are also important for malignant transformation. Furthermore, genome instability and tumor-promoting inflammation are considered as enabling characteristics for malignant transformation (Hanahan & Weinberg 2000, Hanahan & Weinberg 2011).

Fig. 1. The hallmarks of cancer. Modified with permission from Elsevier (Cell), Hanahan & Weinberg (2011) (Copyright 2017).
When compared to the mutagenic processes such as chromosomal rearrangements, copy number changes and indels, point mutations are the most frequent lesion observed in cancer cells (Martincorena & Campbell 2015, Nik-Zainal & Morganella 2017, Vogelstein et al. 2013). Although the numbers vary widely, most cancers carry around 1,000 to 20,000 point mutations along with a few to hundreds of indels and rearrangements (Martincorena & Campbell 2015). Among others, leukemias and pediatric brain tumors typically have the lowest numbers of mutations. On the other hand, tumors induced due to exposure to mutagens such as lung cancers (tobacco) or skin cancers (UV rays) have the highest numbers of mutations. Nevertheless, some cancers acquire dramatically increased mutation rates due to the loss of genome maintenance pathways such as DNA repair and cell cycle checkpoints (Martincorena & Campbell 2015, Vogelstein et al. 2013).

Using various statistical methods (as reviewed in Vogelstein et al. 2013), mutations accumulating during tumorigenesis can be broadly classified into two different groups known as driver mutations and passenger mutations. Mutations that confer a selective growth advantage to the tumor cells are known as driver mutations. Out of all the 20,000 protein-coding genes studied to date, driver mutations have been estimated to be present in 138 genes. Of these, 64 are proto-oncogenes and 74 are tumor suppressor genes. In common adult tumors such as pancreatic, colorectal, breast and brain cancers, mutations are often observed in three to six driver genes. However, in pediatric tumors such as medulloblastomas, the number of driver gene mutations seems to be low (zero to two).

On the other hand, mutations that do not confer any selective growth advantage to the tumor cells are known as passenger mutations. Over 99.9% of the somatic alterations observed in the tumors are due to passenger mutations (Vogelstein et al. 2013). In addition to the above-mentioned “driver” and “passenger” genes, a new class of genes known as “epi-driver” has been proposed by Vogelstein et al. (2013). Epi-driver genes are not frequently mutated in tumors but are rather expressed aberrantly, thereby providing selective growth advantage to the tumor cells (Vogelstein et al. 2013).

### 2.1.1 Genes involved in cancer development

Genes that promote cancer development when dysregulated can be classified into two major classes known as proto-oncogenes and tumor suppressor genes (anti-oncogenes) (Michor et al. 2004). Based on their biological functions, tumor suppressor genes can be further classified into caretakers, gatekeepers and

Proto-oncogenes

A proto-oncogene is a normal cellular gene that encodes a protein which is usually involved in the regulation of cell growth or proliferation. A key genetic alteration in the proto-oncogene results in the activation of the oncogene. At least four distinct genetic mechanisms can produce oncogenes from the corresponding proto-oncogenes: 1) point mutation, 2) gene amplification, 3) chromosomal translocation and 4) hypomethylation. Point mutations in a proto-oncogene may lead to encoding of an oncoprotein, which is constitutively active and differs slightly from that of the normal protein. In contrast, the oncoprotein resulting due to gene amplification, chromosomal translocation and hypomethylation is identical to that of normal protein but expressed at much higher concentrations, thereby conferring the oncogenic potential (Botezatu et al. 2016, Croce 2008, Lee & Muller 2010).

Chromosomal translocation can activate oncogenes by the following two mechanisms: 1) by creating an oncogenic fusion protein and 2) by translocating the proto-oncogene in close proximity with an active promoter region. Hypomethylation that usually results in overexpression of oncoprotein has been frequently observed in the promoter and enhancer regions of the proto-oncogene (Botezatu et al. 2016). Mutations in proto-oncogenes are a gain-of-function mutation which acts in a dominant fashion, that is, mutation in only one of the two alleles is sufficient for cancer development (Croce 2008, Lee & Muller 2010).

Although a majority of inherited cancer syndromes arise from mutations in tumor suppressor genes, a small number of them also occur due to mutations in proto-oncogenes (Fearon 1997, Frank 2001). So far, inherited mutations in proto-oncogenes such as RET (mutated in medullary thyroid carcinoma), MET (hereditary papillary renal cell carcinoma and familial colorectal cancer), NRAS (Noonan syndrome) and CDK4 (familial malignant melanoma) have been observed (Ekvall et al. 2015, Marsh et al. 1996, Neklason et al. 2011, Schmidt et al. 1997, Zuo et al. 1996). Inherited mutations in proto-oncogenes are relatively
rare because such mutations are often lethal during embryogenesis and thus lead to spontaneous termination of pregnancy (Frank 2001).

**Tumor suppressor genes**

Tumor suppressor genes encode proteins that normally help to prevent unrestrained cellular growth and promote apoptosis, DNA repair and cell cycle checkpoint activation (Lee & Muller 2010). In contrast to proto-oncogenes, defective tumor suppressor genes act recessively, that is, both alleles need to be mutated for cancer development. In addition to being recessive, tumor suppressor genes can also be either haploinsufficient or dominant-negative for cancer development (discussed in more detail in chapter 2.4) (Payne & Kemp 2005). Based on their biological functions, tumor suppressor genes are further classified into caretakers, gatekeepers and landscapers (Ashworth et al. 2011, Kinzler & Vogelstein 1997, Naik et al. 2015). Caretaker genes (e.g. **BRCA1**, **BRCA2** and **PALB2**) encode proteins that are responsible for maintaining genome stability. The caretaker genes confer efficient repair of DNA damage that arises due to endogenous and exogenous agents (Ashworth et al. 2011). If the damaged DNA is not efficiently repaired, the gatekeeper genes (e.g. **PP2A**, **RB1**, **PTEN** and **TP53**) that regulate cell growth and apoptosis either stop the cell from proliferating further or eliminate the cell via apoptosis (Kaur & Westermarck 2016, Kotnis et al. 2005). Defects in landscaper genes (e.g. **SMAD4**) do not directly affect cellular growth, but generate an abnormal stromal environment that contributes to the neoplastic transformation of cells (Ashworth et al. 2011, Soussi & Wiman 2015).

**2.1.2 Cancer initiation due to non-genomic mechanisms**

In addition to genetic changes, cancer can also be initiated due to non-genomic mechanisms such as aberrant transcription and translation along with aberrant post-transcriptional and post-translational regulation. Overexpression of oncogenic transcription factors can cause changes in the transcriptional regulatory switches, thereby promoting cancer initiation and metastasis. The oncogenic transcription factor known as TAL1 is frequently overexpressed in approximately 50% of T cell acute lymphoblastic leukemia (T-ALL) cases (Lee & Young 2013). TAL1 forms transcriptional regulatory networks with other key transcription factors that control hematopoiesis. Thus, overexpression of TAL1 results in
sustained overactivation of TAL1-mediated transcription, which drives the oncogenic program in T-ALL (Sanda & Leong 2017).

In addition to overexpression of oncogenic transcription factors, suppression of transcription factors that have a tumor suppressor role is also frequently observed during cancer initiation and development (as reviewed in Van Vlierberghe & Ferrando 2012). Besides transcription factors, aberrant expression of long non-coding RNAs (lncRNAs) that regulate transcription can also alter the transcriptome, thereby promoting cancer initiation (Lee & Young 2013). Finally, epigenetic alterations and chromatin modifications also play a crucial role in transcriptional regulation and are frequently targeted during cancer initiation and development (Ellis et al. 2009).

Differential regulation in the post-transcriptional mechanisms such as mRNA stability control also plays a pivotal role in cancer initiation and development. Regulation of mRNA half-life plays a central role during normal development as well as during disease progression. Significant changes in the half-life of mRNA encoding growth factors, cell cycle regulators, cytokines and oncogenes are frequently observed in breast cancers. Increased mRNA half-life (or stability) will result in the mRNA being available for longer time for translation, resulting in higher levels of the protein products. IncRNA, microRNA, alternative polyadenylation and mRNA binding proteins have all been shown to regulate the half-life of mRNA (Griseri & Pagès 2014).

Translation is a highly conserved process during which proteins are synthesized using the information present in the mRNA. Translation is one of the most energy-consuming processes in the cell which is frequently targeted during cancer initiation and development. In the vast majority of malignancies, increased protein synthesis is a common feature due to translational dysregulation (Topisirovic & Sonenberg 2015). Several oncogenic pathways such as Myc, Ras and PI3K have been shown to promote cancer development by deregulating the translational machinery (Ruggero 2013). Overexpression of eIF4E (a translation initiation factor) that leads to oncogenic transformation has been frequently found in the majority of human cancers. Moreover, overexpression of eIF4E in cancer patients correlates with a poor prognosis. In addition to eIF4E, another translation initiation factor known as eIF4G was also found to be overexpressed in squamous cell lung carcinoma (Grzmil & Hemmings 2012). These studies collectively emphasize the importance of translational dysregulation for cancer initiation and development.
Post-translational modification (PTM) refers to the enzymatic modification of proteins after their synthesis (Cooper et al. 2015). To date, more than 300 different types of PTMs have been identified. Among them, the most common and well-studied ones are ubiquitination, sumoylation, methylation, glycosylation, acetylation and phosphorylation (Lu et al. 2016). PTMs are crucial for normal cellular maintenance and they are frequently exploited during cancer initiation and development.

Among the above-mentioned PTMs, protein phosphorylation has been well studied as many enzymes and receptors are activated/deactivated by either phosphorylation (kinases) or dephosphorylation (phosphatases) (Ardito et al. 2017). An aberrant phosphorylation-dephosphorylation cascade is frequently observed in various types of malignancies. As reviewed in Kauko & Westermarck (2018), cancer cells inhibit the phosphatase PP2A (tumor suppressor) by non-genomic mechanisms such as PTMs and overexpression of PP2A inhibitor proteins. In human cancers, PP2A is predominantly targeted by non-genomic inhibition and not genetic mutation (Kauko & Westermarck 2018). Thus, taken together, these studies further indicate the prevalence and importance of non-genomic mechanisms for cancer initiation and development.

2.2 Breast cancer

2.2.1 Epidemiology and risk factors of breast cancer

Breast cancer is a common term used to address all malignancies that arise from the breast tissue. Breast cancer is the most common form of cancer in women in the developed world, and most importantly, it is the second leading cause of cancer related deaths (after lung cancer) in women (Downs-Holmes & Silverman 2011, Makki 2015). It is estimated that approximately one in eight women will develop breast cancer during her lifetime (Ellsworth et al. 2010). Worldwide, an estimated 1.7 million breast cancer cases and 521,900 breast cancer related deaths were observed in 2012. Among women, 25% of all cancer cases and 15% of all cancer deaths are due to breast cancer (Torre et al. 2015).

Developed countries alone account for nearly 50% of all breast cancer cases and 38% of breast cancer related deaths (Torre et al. 2015). Breast cancer incidence and deaths are generally high in Western and Northern Europe, Northern America, Australia and New Zealand; intermediate in Caribbean, Latin
America, Central and Eastern Europe; and low in most of Africa and Asia (Ferlay et al. 2013, Torre et al. 2015). International variation in breast cancer incidence is due to the differences in the availability of early malignancy detection programs as well as disease risk factors (Torre et al. 2015).

In Finland, 5,161 new breast cancer cases were diagnosed during 2015, which is approximately one-third of all female cancers (16,028) diagnosed that year. Since 1953, breast cancer incidence rates have been rising annually in Finland. Among women, 841 breast cancer related deaths were observed in Finland during 2015, compared to all 5,777 cancer related deaths in that year. Similarly, in Finland during 2015, 30 new breast cancer cases and 7 breast cancer related deaths were observed among men (Finnish Cancer Registry 2017). Although the incidence of breast cancer is increasing, the associated mortality is fortunately declining (Althuis et al. 2005, Hery et al. 2008, Peto et al. 2000). The relative decline in breast cancer mortality is most likely due to improved early diagnosis and efficient use of adjuvant and neoadjuvant therapies (Althuis et al. 2005, Hery et al. 2008, Karim-Kos et al. 2008, Peto et al. 2000).

Breast cancer has a complex etiology where susceptibility is influenced by both genetic and environmental factors. Epidemiological evidence suggests that lifetime exposure to endogenous hormones such as estrogens (ER) and androgens promotes breast carcinogenesis (Ellsworth et al. 2010). Increased ER exposure throughout a woman’s lifetime due to use of oral contraceptives, hormone replacement therapy, early menarche and late menopause has been associated with ~2-fold increase in breast cancer risk among premenopausal women (Ellsworth et al. 2010, Kaaks et al. 2005a, Kaaks et al. 2005b, Key et al. 2002).

Late age (>30 years) at first pregnancy or never being pregnant, high breast density and family history are also important in defining the risk for breast cancer (Ellsworth et al. 2010). Lifestyle choices such as tobacco use, alcohol consumption, nutrition and physical inactivity also contribute significantly towards breast cancer risk (Ellsworth et al. 2010, Torre et al. 2015). Most importantly, age is the main risk factor as most breast cancer cases (81%) occur in women aged 50 years or older (Barrett 2010).

Breast cancer is generally treated via surgery, radiotherapy, chemotherapy, hormonal therapy and targeted therapy. The choice of treatment depends on a multitude of factors such as age, hormone receptor status, menopause status, human epidermal growth factor receptor 2 (HER2) status, overall health and various other parameters including personal needs and preferences. Surgery (lumpectomy) is offered to patients with early breast cancer to remove the
primary lesion and the associated regional lymph nodes. However, in patients with advanced disease, total removal of the breast (mastectomy) is often recommended. After lumpectomy and mastectomy, breast reconstruction surgery is offered to the patients to restore the normal shape of the breast (Barrett 2010).

Radiotherapy is usually recommended for patients who have undergone lumpectomy and mastectomy to reduce the risk of disease recurrence. Chemotherapy is usually offered to patients either after surgery (adjuvant chemotherapy) or before surgery (neo-adjuvant chemotherapy). Adjuvant chemotherapy is given to reduce the risk of disease recurrence whereas neo-adjuvant chemotherapy is given to facilitate surgery. Similarly, hormonal therapy is given to breast cancer patients who are hormone receptor positive either after surgery (adjuvant) or before surgery (neo-adjuvant). Finally, using HER2 targeted therapy, breast cancer patients who are HER2 positive can be efficiently treated with high specificity (Barrett 2010).

### 2.2.2 Classifications of breast cancer

Breast cancers with different biological and histopathological features display distinct behaviors, which results in contrasting therapeutic outcomes. Thus, accurate grouping of breast cancers into clinically relevant subtypes is of paramount importance for efficient therapeutic decision-making (Dai et al. 2015). Breast cancers are classified into different categories based on their histopathological type, tumor grade, tumor stage and molecular subtype (expression profile of specific proteins) (Rakha et al. 2010).

Breast carcinoma is primarily classified based on the histological appearance of the tumor tissue. Breast cancer originates most commonly in the epithelial cells lining the inside of lobules and ducts. Milk-producing lobules supply the milk via ducts, and cancers originating from the ducts are known as ductal carcinomas (~80%), while those originating from the lobules are known as lobular carcinomas (~10%) (Makki 2015). Ductal and lobular carcinomas are further subdivided into in situ or invasive disease depending on the disease progression (Vuong et al. 2014). There are more than 21 subtypes of invasive breast carcinoma defined in the latest edition of the WHO classification of tumors of the breast (Lakhani et al. 2012). The most frequent among them is the invasive carcinoma of no special type (NST), also known as invasive ductal carcinoma NST, which accounts for 40–75% of the invasive cases (Vuong et al. 2014). The remaining invasive cases are morphologically distinct and well defined, including
special subtypes such as invasive lobular, tubular, mucinous, metaplastic carcinoma and carcinoma with medullary, neuroendocrine or apocrine features. When a tumor does not fulfill the criteria for a histological special subtype, it is classified as invasive carcinoma of no special type (Sinn & Kreipe 2013, Vuong et al. 2014). For a complete list of breast carcinoma types based on their histological appearance, please see the review by Sinn & Kreipe (2013).

All breast carcinomas irrespective of their histological type should be histologically graded (Vuong et al. 2014). The most widely used grading system is based on that of Bloom and Richardson (Bloom & Richardson 1957), which was modified by Elston and Ellis (Elston & Ellis 1991). Scores are assigned for the proportion of tubule formation (score of 1–3, with 3 being poor), the degree of nuclear pleomorphism (1–3, with 3 showing a high degree of pleomorphism) and the mitotic count (1–3, with 3 indicating a high mitotic count). The scores are then combined to give a grade of 1 (total score of 3 to 5), 2 (scores 6 or 7) or 3 (score 8 or 9) (Vuong et al. 2014). Tumor grade along with lymph node status is used to predict the 5-year survival of patients using the Nottingham prognostic index (NPI) (Blamey et al. 2007). Patients with high-grade breast cancers (which tend to recur and metastasize early) have a poor clinical outcome when compared to patients with low-grade tumors who generally have a very good clinical outcome (Vuong et al. 2014).

Determination of tumor stage is most important for effective therapeutic decision-making. Breast cancer is staged using the TNM system, which includes information such as tumor size (T), the status of regional lymph nodes (N), and spread to distant metastatic sites (M). The presence of biomarkers such as ER, progesterone (PR) and HER2 are also routinely evaluated during clinical management of breast cancer patients (Vuong et al. 2014).

Using microarray-based gene expression profiling, breast cancers can be divided into five molecular subtypes: luminal A-like, luminal B-like (HER2-negative), luminal B-like (HER2-positive), HER2-type and basal-like (Table 2) (Cho 2016, Goldhirsch et al. 2013). The most common molecular subtype of breast cancer is luminal A (71%), with luminal B (8%), HER2-type (6%) and basal-like (15%) subtype accounting for the remaining cases (Wiechmann et al. 2009). Genomic complexity, key genetic alterations and prognosis differ between the different subtypes. Patients with luminal A tumors have better survival rates when compared to the other subtypes. On the other hand, the basal-like group cancers are mostly grade 3 and show aggressive clinical behavior (Vuong et al. 2014). Basal-like subtype is often referred to as triple-negative breast cancer
(TNBC) due to the absence of ER, PR and HER2. Most importantly, basal-like subtype is more common in individuals carrying mutations in DDR genes such as BRCA1 and BRCA2 (Couch et al. 2015).

Table 2. Intrinsic subtypes of breast cancer

<table>
<thead>
<tr>
<th>Intrinsic subtype</th>
<th>ER</th>
<th>PR</th>
<th>HER2</th>
<th>Ki-67</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A-like</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Low</td>
</tr>
<tr>
<td>Luminal B-like (HER2-negative)</td>
<td>+</td>
<td>- or low</td>
<td>-</td>
<td>High</td>
</tr>
<tr>
<td>Luminal B-like (HER2-positive)</td>
<td>+</td>
<td>Any</td>
<td>Over-expressed</td>
<td>Any</td>
</tr>
<tr>
<td>HER2-positive</td>
<td>-</td>
<td>-</td>
<td>Over-expressed</td>
<td>NA</td>
</tr>
<tr>
<td>Basal-like</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: not applicable

2.3 Inherited predisposition to breast cancer

Already in the nineteenth century, it was recognized that women from certain families were prone to developing breast cancer (Nielsen et al. 2016). In 1866, the French physician Pierre Paul Broca first published a report on a family with increased predisposition to breast cancer (Broca 1866). His wife acquired breast cancer at an early age, and pedigree analysis showed that four generations had breast cancer (Broca 1866). However, it was only in 1948 that two independent studies showed that the transmission of a specific genetic factor is a major cause of breast cancer in certain families (Penrose et al. 1948, Smithers 1948). Several twin studies also further confirmed that clustering of breast cancer in certain families is due to strongly penetrant genetic factors (Lichtenstein et al. 2000). In general, a monozygotic twin is at an increased risk to develop breast cancer when compared to a dizygotic twin (Mucci et al. 2016, Peto & Mack 2000).

Approximately 5–10% of all patients with breast cancer exhibit a strong genetic predisposition to the disease (Claus et al. 1996, Romero-Laorden & Castro 2017, Thull & Vogel 2004). Hereditary breast cancer patients are characterized by 1) early onset of the disease, 2) familial clustering of breast cancer, 3) occurrence of several primary tumors in the same patient, 4) occurrence of bilateral breast cancer and 5) incidence of male breast cancer in the family (Thull & Vogel 2004).

As shown in Table 3, breast cancer predisposing factors are classified into three different categories, namely high-penetration genes, moderate-penetrance genes and low-penetrance variants. The level of penetrance describes the
probability of an individual to develop breast cancer. Usually, an inverse correlation exists between the risk of breast cancer conferred by a particular genetic variant and its prevalence in the population. In other terms, enrichment of mutations in high- and moderate-penetrance susceptibility genes is often rare in the population whereas the low-penetrance variants can be very common.

Table 3. Recognized high-, moderate- and low-risk breast cancer susceptibility genes.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene/Locus</th>
<th>Disease risk (fold)</th>
<th>Population frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-penetrance</td>
<td><em>BRCA1</em>, <em>BRCA2</em>, <em>PALB2</em>, <em>TP53</em>, <em>PTEN</em>, <em>STK11</em>, <em>CDH1</em></td>
<td>&gt;6</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Moderate-penetrance</td>
<td><em>CHEK2</em>, <em>ATM</em>, <em>BRIP1</em>, <em>NBS1</em>, <em>RAD50</em></td>
<td>2 – 4</td>
<td>0.1 – 0.5</td>
</tr>
<tr>
<td>Low-penetrance</td>
<td>Multiple common SNPs including:<em>CASP8</em>, <em>FGFR2</em>, <em>MAP3K1</em>, <em>TOX3</em>, <em>LSP1</em>, <em>MRPS30</em>, <em>FAM84B</em>, <em>NEK10</em>, <em>COX11</em>, <em>NOTCH2</em>, <em>RAD51L1</em>, <em>ESR1</em>, <em>CDKN2A</em></td>
<td>&lt;1.5</td>
<td>&gt;1.0</td>
</tr>
</tbody>
</table>


As shown in Figure 2, mutations in high-risk breast cancer susceptibility genes such as *BRCA1* and *BRCA2* are observed in 15% of hereditary breast cancer cases. Additionally, mutations in the rest of the breast cancer susceptibility genes account for 21% of hereditary breast cancer cases (Couch et al. 2014). Nevertheless, despite decades of medical research and appreciable technological advancements, currently only 36% of hereditary breast cancer cases have an identified causative gene mutation (Figure 2) (Couch et al. 2014, Rousset-Jablonski & Gompel 2017). Thus, further studies are warranted to find new breast cancer susceptibility genes and other factors/mechanisms responsible for missing disease heritability. Regardless of the shortcomings, these genetic studies have already given us valuable information to design a breast cancer specific gene panel for routine use in clinical diagnostics. Panel-based testing enables more women and their family members to be tested efficiently and swiftly for heritable germline mutations. Currently, in Finland, a breast cancer specific gene panel that includes high-risk genes such as *BRCA1*, *BRCA2*, *CDH1*, *PALB2*, *PTEN*, *STK11* and *TP53* is available for commercial use.
2.4 Tumorigenesis due to inherited mutations in tumor suppressor genes

As discussed in section 2.1.1, cancer genes can be categorized into recessive tumor suppressor genes and dominant-acting proto-oncogenes (Payne & Kemp 2005). Interestingly, most inherited cancer syndromes arise due to genetic alterations in tumor suppressor genes (Fearon 1997). In 1969, using somatic cell hybridization experiments, Harris et al. (1969) demonstrated that tumor suppressor genes must be completely inactivated (being recessive) for malignancy to occur. In 1971, by comparing retinoblastoma (unilateral vs. bilateral) patients, Knudson proposed his two-hit hypothesis indicating that both the hereditary and the nonhereditary form of retinoblastoma are mechanistically linked. In the hereditary form of retinoblastoma, one mutant allele is inherited from either of the parents while the second allele is somatically mutated, which significantly accelerates the onset of retinoblastoma. However, in the nonhereditary form, somatic alterations must occur simultaneously in both alleles prior to retinoblastoma initiation (Knudson 1971). Further epidemiological studies on cancer solidified the belief that tumor suppressor genes act recessively, i.e. both alleles need to be inactivated (Payne & Kemp 2005).
However, tumor suppressor gene mutations are not always completely recessive, as they may alternatively be either haploinsufficient or dominant-negative. In diploid (2n) organisms, barring X and Y chromosome genes, two functional copies (or alleles) of all other genes are present. For a majority of genes, one functional copy is sufficient to maintain normal cellular functions. However, certain genes are sensitive to gene dosage levels (i.e. being haploinsufficient) and require both functional alleles to maintain normal cellular functions (Payne & Kemp 2005). Haploinsufficiency describes a special circumstance in which one functional allele is insufficient to maintain the normal cellular process (Berger & Pandolfi 2011, Payne & Kemp 2005). In 1996, three independent studies identified \( p27^{kip1} \) (CDKN1B) as a haploinsufficient tumor suppressor gene, with the \( p27^{+/−} \) heterozygote mice showing an intermediate size between the small \( p27^{+/+} \) and large \( p27^{−/−} \) homozygote mice (Fero et al. 1996, Kiyokawa et al. 1996, Nakayama et al. 1996). \( p27^{+/−} \) mice also showed an intermediate phenotype in both tumor-free survival and tumor multiplicity when compared to \( p27^{+/+} \) and \( p27^{−/−} \) littermates (Fero et al. 1998, Philipp-Staheli et al. 2002).

Under certain circumstances, \( TP53 \) is inactivated as expected by Knudson’s two-hit hypothesis; however, in other cases it shows clear evidence for haploinsufficiency (Venkatachalam et al. 1998). \( p53^{+/-} \) mice also showed an intermediate phenotype in both spontaneous chromosomal aberrations and apoptosis activation when compared to \( p53^{+/+} \) and \( p53^{−/−} \) littermates (Bouffler et al. 1995, Clarke et al. 1994). Studies on tumorigenesis due to haploinsufficiency have traditionally been based on murine models or cellular knockdown approaches. However, using patient-derived materials, three recent independent studies have successfully shown genomic instability and tumorigenesis due to \( BRCA1 \) and \( PALB2 \) haploinsufficiency (Nikkilä et al. 2013, Pathania et al. 2014, Vaclová et al. 2015). An extensive list of known haploinsufficient tumor suppressor genes is reviewed in Berger & Pandolfi (2011) and Payne & Kemp (2005).

\( PTEN \) is a critical tumor suppressor gene whose function is frequently disrupted in cancer. Like \( CDKN1B \) and \( TP53 \), \( PTEN \) is also a haploinsufficient tumor suppressor gene. Mice heterozygous for \( PTEN \) develop autoimmunity disorder and are susceptible to multiple tumor types. Interestingly, although complete loss of \( PTEN \) promotes tumorigenesis it has been shown to be less tumorigenic than heterozygous loss of \( PTEN \). This is because complete loss of \( PTEN \) activates cellular senescence mediated by p53 that limits the tumorigenic
potential of the cells. In contrast, cells heterozygous for *PTEN* have a high proliferation rate without the activation of cellular senescence. Thus, complete loss of *PTEN* is less tumorigenic than heterozygous loss of *PTEN* due to p53 mediated cellular senescence; this paradigm is known as obligate haploinsufficiency (Berger & Pandolfi 2011).

In addition to haploinsufficiency, mutations in tumor suppressor genes can also lead to dominant-negative effects as described below. By definition, any mutant protein that disrupts the activity of the wild-type protein is classified as a dominant-negative mutant (Herskowitz 1987). By nature, it is difficult to differentiate a dominant-negative mutation from a haploinsufficient mutation. In both these cases, the wild-type allele is retained, with the reason for the retention being different in each case. In the case of a haploinsufficient mutation, the wild-type allele is not lost since the reduced dosage of the wild-type protein is sufficient for tumorigenesis (Payne & Kemp 2005). In the case of a dominant-negative mutation, the wild-type allele is not lost since the mutant protein often disrupts the activity of the wild-type protein (Payne & Kemp 2005, Veitia 2007).

A dominant-negative mutation in *RAP80* was recently shown to promote genomic instability and tumorigenesis in U2OS cells (Nikkilä et al. 2009). Similarly, dominant-negative mutations in *SETD6* and *WT1* have been shown to predispose the mutation carriers to familial colorectal cancer type X and Wilms tumor, respectively (Martin-Morales et al. 2017, Pelletier et al. 1991). Besides cancer, the dominant-negative disease mechanism is also observed in late-onset retinal degeneration (due to mutation in *C1QTNF5*), spinocerebellar ataxia type 44 (*GRM1*) and empty follicle syndrome (*ZP3*) (Chen et al. 2017, Stanton et al. 2017, Watson et al. 2017).

A simple classification of tumor suppressor gene mutations as either recessive, haploinsufficient or dominant-negative is currently being challenged. In addition to being either recessive, haploinsufficient or dominant-negative, mutations in tumor suppressor gene can also have a gain-of-function phenotype (Payne & Kemp 2005). For example, mutations in *TP53* can under certain circumstances also have a gain-of-function phenotype. These gain-of-function *TP53* mutants acquire new oncogenic properties that are independent of wild-type p53 (Zhang et al. 2016). Depending upon the mutation, p53 can behave as a classical (Knudson two-hit hypothesis), haploinsufficient, dominant-negative or gain-of-function tumor suppressor gene.
2.5 DNA damage response

Maintenance of genome stability is a fundamental process for cell homeostasis. As mentioned in section 2.1, the genome of a cell is constantly challenged by both exogenous and endogenous agents. In the presence of DNA damage, to maintain genome integrity, cells activate the DNA damage response (DDR) pathway to sense and repair the DNA damage. Upon activation, the DDR pathway coordinates a response that includes activation of DNA repair pathways, cell cycle control, apoptosis and transcription (Figure 3) (Ghosal & Chen 2013).

DNA damaging agents can be categorized into two main classes based on their origin: endogenous and exogenous (Chatterjee & Walker 2017). A majority of the endogenous DNA damage arises due to hydrolysis, exposure to reactive oxygen substances (ROS) and other reactive metabolites (Ali et al. 2017, De Bont & van Larebeke 2004). In addition, inherent DNA replication processes are also a frequent source of endogenous DNA damage (Georgoulis et al. 2017). On the other hand, exogenous DNA damage occurs when environmental, physical and chemical agents damage the DNA (Chatterjee & Walker 2017). Among the environmental DNA damaging agents, ultraviolet (UV) light causes significant DNA damage in humans. Although the ozone layer absorbs the most hazardous UV-C radiations, residual UV-A and UV-B in strong sunlight can induce ~100,000 lesions per exposed cell per hour. Ionizing radiation (IR) generates the most toxic and lethal DNA damage of all, known as double-strand break (DSB) (Jackson & Bartek 2009).

As shown in Figure 3, conceptually, DDR facilitates a key four-step process. (1) Recognition of the DNA damage; a group of specialized proteins known as sensors sense the DNA damage and activate the appropriate DNA repair system. Next, (2) amplification and transduction of the DNA damage signal; once the DNA damage is sensed, the cell must transduce this signal down to its appropriate effector. A group of proteins known as transducers performs this signal amplification and transduction. (3) Activation of genome maintenance pathways; upon activation, a group of proteins known as effectors activate the DNA repair pathways, transcription, cell cycle control and apoptosis. Finally, (4) reversal of the previous steps; once the DNA is faithfully repaired, the genome maintenance pathways will be turned off for normal cell cycle progression (Georgoulis et al. 2017, Harper & Elledge 2007).
Fig. 3. Outline of the DNA damage response signal transduction pathway

The DNA repair pathways can be broadly classified into two groups based on the type of damage they repair: repair of DNA damage affecting only one strand (single-strand break (SSB) repair) and repair of DNA damage affecting both DNA strands (DSB repair). Nucleotide excision repair (NER), base excision repair (BER) and DNA mismatch repair (MMR) pathways are prominent to repair different types of single-strand DNA damage. Whereas, the DNA DSBs are repaired by non-homologous end joining (NHEJ), homologous recombination (HR), microhomology-mediated end joining (MMEJ) and single-strand annealing (SSA) pathways (Ghosal & Chen 2013, Jackson & Bartek 2009, Tubbs & Nussenzweig 2017). In addition to DNA repair pathways, DDR also activates cell cycle checkpoints in order to delay the cell cycle progression, thus preventing the cells from entering the next cell cycle phase with unrepaired DNA damage (Ghosal & Chen 2013, Houtgraaf et al. 2006). If the DNA damage is irreparable, then apoptosis is activated to eliminate the cells carrying DNA lesions and to preserve genomic integrity (Al-Ejeh et al. 2010, Houtgraaf et al. 2006).
2.5.1 DNA double-strand break repair pathway

DNA DSBs are cytotoxic lesions that threaten genome stability, and failure to repair such a lesion will induce genomic instability and cell death. To repair DSBs, cells employ four main mechanisms known as NHEJ, HR, MMEJ and SSA. NHEJ repairs the DSBs without a need of the homologous sequence by directly ligating the broken ends (Figure 4). NHEJ is active throughout the cell cycle but predominantly in G0/G1 and G2. Alternatively, the DSB end can be resected resulting in 3′ single-stranded DNA overhangs, which can be repaired by three possible mechanisms: HR, MMEJ and SSA (Figure 4). HR predominates during the mid-S and mid-G2 cell cycle phases when the sister chromatids become available. Since HR uses sister chromatid DNA sequence information to repair the DSBs, it requires strand invasion mediated by RAD51 (Figure 4). When compared to NHEJ, HR is an error-free repair pathway with slow repair kinetics (Ceccaldi et al. 2016).

![Fig. 4. Simplified representation of the DNA DSB repair pathways. LOH; loss of heterozygosity. Modified with permission from Elsevier (Trends in Cell Biology), Ceccaldi et al. (2016) (Copyright 2017).](image)

Besides HR, the resected DSB can also be repaired by mutagenic repair pathways such as SSA and MMEJ. SSA mediates end joining between the interspersed nucleotide repeats in the genome with the help of RAD52 protein (Figure 4). Thus, SSA is a highly mutagenic process resulting in the loss of intervening sequence between the nucleotide repeats (Ceccaldi et al. 2016). Finally, MMEJ uses...
microhomologous sequences on either side of the DSB to ligate the broken DNA ends (Figure 4). Similar to SSA, MMEJ is also a highly mutagenic process resulting in the deletion of DSB flanking regions (Wang & Xu 2017). Moreover, MMEJ can also join DSBs located on different chromosomes, thereby generating chromosomal translocations and other mutagenic rearrangements (Ceccaldi et al. 2016).

**Homologous recombination**

As mentioned above, HR repair is limited to S and G2 phases of the cell cycle, since it requires sister chromatids as templates to repair the DSB. A simplified model of HR repair mechanism is presented in Figure 5. As shown in Figure 5, there are four main key steps in the HR repair pathway. In the first step, 3′ single-stranded DNA overhangs are generated by nucleolytic degradation of the 5′ strands. This first step is catalyzed by endonucleases such as MRE11–RAD50–NBS1 (MRN) complex, CtIP and EXO1. In the second step, the single-stranded DNA ends are coated with replication protein A (RPA). In the third step, RPA-coated ssDNA filaments are replaced by RAD51 in a BRCA1-PALB2-BRCA2 dependent manner, leading to strand invasion into an undamaged homologous DNA template (Krajewska et al. 2015). In the final step, after strand invasion, the concerted action of DNA polymerase, ligase, helicase and resolvase results in resolution of HR intermediates to yield an intact and repaired DNA strand.
Fig. 5. Simplified representation of HR repair pathway. Modified with permission from Nature Publishing Group (Nature Structural and Molecular Biology), Buisson et al. (2010) (Copyright 2017).

The role of PALB2 and ABRAXAS in HR will be discussed in more detail in chapters 2.6.1 and 2.6.2, respectively.

**DSB repair pathway choice**

For the precise repair of DSBs, the repair pathway that is optimal for the particular condition needs to be selected (Shibata 2017). The specific DSB repair pathway is activated based on the cell cycle stage and resection status. End resection plays an important role in differentiating NHEJ from the other DSB repair pathways. As shown in Figure 4, since NHEJ directly ligates the broken DNA ends, it does not require end resection. Once end resection is activated, the DSB is committed to be repaired by either HR or MMEJ or SSA. The initial phase of end resection known as ‘end clipping’ is carried out by MRN complex together with CtIP. During this phase, a relatively small number of base pairs (20 bp in mammalian cells) are removed, thereby making the resected DNA ends
available for MMEJ. In the next phase of end resection (known as ‘extensive
resection’), the concerted actions of helicases and exonucleases generate long
stretches of single-stranded DNA, thereby committing the DSBs to be repaired by
either HR or SSA (Ceccaldi et al. 2016).

The cell cycle also plays a major role in the DSB repair pathway choice. CtIP
itself is activated in a cell cycle dependent manner; CDK-dependent
phosphorylation of CtIP favors the BRCA1–CtIP interaction (BRCA1-C complex)
during the S/G2 phases (Ceccaldi et al. 2016). CDK-dependent phosphorylation
of EXO1 also promotes end resection during S/G2 phases of the cell cycle.
Consistently, inhibition of EXO1 phosphorylation attenuates end resection, HR
and cell survival but augments NHEJ activity (Tomimatsu et al. 2014). In
noncycling cells, DSB end resection is greatly reduced, thereby favoring NHEJ
over the major resection-dependent repair pathways (such as HR, MMEJ, and
SSA) (Ceccaldi et al. 2016).

The balance between 53BP1 and BRCA1 also plays a major role in the DSB
repair pathway choice by either preventing or promoting end resection. 53BP1 is
a pro-NHEJ factor as it blocks end resection by preventing access of CtIP to the
broken DNA ends (Ceccaldi et al. 2016). Recently, REV7 and RIF1 were also
shown to promote NHEJ by inhibiting DNA end resection (Boersma et al. 2015,
Escribano-Díaz et al. 2013, Xu et al. 2015). Arnoult et al. (2017) has recently
identified an S/G2 cell cycle specific inhibitor of NHEJ known as CYREN. The
cell cycle also controls the interaction of BRCA1 with PALB2-BRCA2 to restrict
HR activity to the S/G2 phases only (Orthwein et al. 2015).

2.5.2 Cell cycle checkpoints

The induction of DNA damage in dividing cells results in the activation of cell
cycle checkpoints. In contrast, in terminally differentiated cells (such as muscle
cells) cell cycle checkpoints are not activated upon DNA damage. Checkpoints
generally halt the cell cycle progression in order to give ample time for the DNA
repair machinery to efficiently repair the damaged DNA. Thus, cell cycle
checkpoints avoid transmission of incorrect genetic information from one cell
generation to the next (Houtgraaf et al. 2006).

As shown in Figure 6, the eukaryotic cell cycle consists of four phases known
as gap (G)1, synthesis (S), G2, and mitosis (M). In G1 phase, the cell increases in
size and starts synthesizing RNA and proteins that are required for DNA synthesis.
During S phase, DNA is replicated carefully to produce an almost identical copy
of the genome for the subsequent daughter cells. In G2 phase, the cell will grow further and synthesize more proteins to ensure that two viable daughter cells can be formed. Finally, the cell will enter into M phase to accurately distribute (segregate) its chromosomes into two daughter cells. If required, cells can also stop dividing and remain in G0 phase of the cell cycle (Figure 6) (Houtgraaf et al. 2006).

![Fig. 6. Schematic representation of the cell cycle and its DNA damage checkpoints](image)

As shown in Figure 6, there are three DNA damage checkpoints in the cell cycle known as G1/S, intra-S, and G2/M (Houtgraaf et al. 2006). The G1/S checkpoint ensures that DNA damaged in the G1 phase is not replicated in the subsequent S phase (Dasika et al. 1999). The intra-S checkpoint ensures accurate replication of the genome (Gaillard et al. 2015). Finally, the G2/M checkpoint ensures that cells do not initiate mitosis with persistent DNA damage (Houtgraaf et al. 2006).

Although distinct, all these checkpoints respond to DNA damage in a similar way and share many common proteins. A more simplified image of the DNA damage induced checkpoint response is shown in Figure 7 (Houtgraaf et al. 2006). Depending upon the cell cycle stage and DNA lesions, the sensor proteins such as MRN complex and RAD9–RAD1–HUS1 (9-1-1) complex will sense the DNA damage and activate the checkpoint response. ATM and ATR are further activated by the MRN complex and 9-1-1 complex, respectively (Cimprich & Cortez 2008). ATM is primarily activated in response to IR, whereas ATR is primarily activated
in response to UV and hydroxyurea (HU). ATR and ATM carry out the signal transduction to the respective effector kinases such as CHK1 and CHK2. Depending upon the cell cycle phase, CHK1 and CHK2 either activate p53 or inactivate Cdc25A-C, which leads to cell cycle arrest. Finally, cell cycle checkpoints have predominantly evolved to prevent transmission of DNA damage to the daughter cells, and defects in checkpoint responses can thus result in increased genomic instability (Houtgraaf et al. 2006).

![Simplified representation of the DNA damage induced checkpoint response.](image)

### 2.5.3 Replication stress response

The DNA replication machinery successfully carries out accurate genome duplication even in the presence of numerous obstacles. Under chronic stress or after loss of key cellular signaling pathways (which help to deal with replication stress), a range of deleterious events can occur (Zeman & Cimprich 2013). In eukaryotes, DNA replication originates at thousands of individual replication origins in a bi-directional way (Técher et al. 2017, Zeman & Cimprich 2013).
Already prior to S-phase, each replication origin is licensed by a combination of replication initiation proteins to prepare the chromatin for replication (Gaillard et al. 2015, Zeman & Cimprich 2013). Once they enter the S phase, the replication origins will be fired after activation by S phase-specific kinases and DNA replication commences. To complete the replication in an efficient manner, cells need to balance accuracy and the speed of replication. Most importantly, cells need to monitor the consumption and distribution of relevant resources such as nucleotides, energy and replication factors for efficient replication (Zeman & Cimprich 2013).

Interestingly, in unperturbed S phase, most of the licensed replication origins do not fire at all. Instead, these dormant origins are only activated after disturbances of progressing replication forks, and thus represent a back-up resource to ensure the completion of DNA replication (McIntosh & Blow 2012, Woodward et al. 2006, Zeman & Cimprich 2013). Thus, presence of surplus replication origins is direct evidence of the presence of slowing or stalling of the replication fork progression. Limitations of replication resources such as nucleotides or damage of the DNA during S phase augments fork slowing and stalling and thus induces replication stress (Mazouzi et al. 2014, Zeman & Cimprich 2013). The manifestation of stalled replication forks thus indicates the presence of replication stress.

Upon sensing replication stress, cells will activate the intra-S phase checkpoint (as shown in Figure 7) in order to alleviate the replication stress. ATR activation is initiated in the presence of single-stranded DNA that is generated at stalled replication forks (Gaillard et al. 2015). Single-stranded DNA generated at stalled replication forks is generally coated with RPA protein, which independently brings together ATR and its co-factor ATRIP to activate ATR (Mazouzi et al. 2014). Upon persistent replication stress, ATM may also be activated by DSBs that are generated by collapse of stalled replication forks (Gaillard et al. 2015). As shown in Figure 7, ATM and ATR further activate the effector kinases CHK2 and CHK1, respectively, to activate the intra-S phase checkpoint as described above.

Even in the absence of exogenous challenges, cells that are deficient in various checkpoints or DNA repair proteins display reduced replication fork speed and increased replication origins (Técher et al. 2017). Cells deficient in ATR, CHK1, RAD51, BRCA1 and BRCA2 display an increased number of replication origins and stalled replication forks (i.e. replication stress) (Eykelenboom et al. 2013, Maya-Mendoza et al. 2007, Pathania et al. 2014,
Petermann et al. 2010, Técher et al. 2016, Wilhelm et al. 2014, Wilhelm et al. 2016). Increasing evidence suggests that several DNA repair and damage response proteins are involved in the replication of the genome. Thereby, defects in the corresponding genes accelerate genomic instability and tumorigenesis. This led to the proposal that DNA replication stress should be considered as a hallmark of cancer in its own right (Macheret & Halazonetis 2015).

2.6 PALB2 and ABRAXAS

2.6.1 PALB2

In response to DNA damage, both BRCA1 and BRCA2 are rapidly recruited to the DNA damage sites to facilitate DNA repair (Chen et al. 1996, Scully et al. 1996, Yuan et al. 1999). BRCA1- and BRCA2-deficient cells exhibit similar phenotypes with defects in HR, hypersensitivity to DNA damaging agents and chromosomal instability, indicating that both proteins have similar biological functions despite their lack of structural homology (Moynahan et al. 2001a, Moynahan et al. 2001b, Nepomuceno et al. 2017). In line with this, both BRCA1 and BRCA2 have been shown to facilitate DNA repair through their interaction with RAD51 (Scully et al. 1997, Sharan et al. 1997). Interestingly, it was later shown that both BRCA1 and BRCA2 coexist in the same RAD51-containing protein complexes (Chen et al. 1998). However, it was not clear whether the interaction between BRCA1 and BRCA2 is due to a direct or indirect association (Chen et al. 1998, Nepomuceno et al. 2017, Park et al. 2014a, Tischkowitz & Xia 2010). In 2009, three independent studies reported that BRCA1 and BRCA2 interact indirectly with PALB2 acting as a linker (Sy et al. 2009b, Zhang et al. 2009a, Zhang et al. 2009b).

Shortly after its functional characterization, PALB2 was identified as a major breast cancer susceptibility gene (Antoniou et al. 2014, Erkko et al. 2007, Erkko et al. 2008, Foulkes et al. 2007, Rahman et al. 2007, Southey et al. 2016, Tischkowitz et al. 2007). In addition to inactivating genetic changes, epigenetic modifications such as promoter hypermethylation have also been observed in the PALB2 gene in both inherited and sporadic breast and ovarian cancer (Potapova et al. 2008).

Xia et al. (2006) showed that the identified FLJ21816/LOC79728 protein was a major BRCA2-interacting factor and subsequently named it PALB2, for ‘partner
and localizer of BRCA2’. The *PALB2* gene, which encodes a protein of 1,186 amino acids (aa, 131 kDa) is located on chromosome 16p12.2 and consists of 13 exons (Pauty *et al*. 2014). As shown in Figure 8, PALB2 contains a coiled-coil and WD40 domain in the N- and C-terminus, respectively. In addition, the central portion of PALB2 contains the chromatin-association motif (ChAM) (Park *et al*. 2014a).

Fig. 8. Schematic protein structure of PALB2. PALB2 contains three structural domains, one each in the N-terminal, central, and C-terminal of the protein. Interacting proteins and the region of PALB2 required for their associations are also depicted. Modified with permission from Elsevier (Biochimica et Biophysica Acta (BBA) – Reviews on Cancer), Park *et al*. (2014a) (Copyright 2017).

The amino-terminal (approximately, aa 1–394) of PALB2 contains one prominent structural element, a coiled-coil domain (aa 9–44), which is known to mediate protein-protein interactions (Park *et al*. 2014a). The coiled-coil motif in PALB2 mediates interactions with BRCA1, disruption of which results in impaired localization of BRCA2, PALB2 and RAD51 to the DNA damage sites (Sy *et al*. 2009b, Zhang *et al*. 2009a, Zhang *et al*. 2009b). Interaction between BRCA1 and PALB2 is crucial for efficient HR-mediated DNA repair and resistance against MMC treatments (Foo *et al*. 2017, Simhadri *et al*. 2014, Sy *et al*. 2009b, Zhang *et al*. 2009a, Zhang *et al*. 2009b). Several studies have shown that BRCA1 acts upstream of PALB2 and facilitates its localization to the DNA damage sites,
which in turn recruits BRCA2 and RAD51 to sites of DNA damage (Xia et al. 2006, Zhang et al. 2009a, Zhang et al. 2009b).

In addition to interacting with BRCA1, the N-terminal coiled-coil domain of PALB2 also facilitates its oligomerization (Buisson & Masson 2012, Sy et al. 2009c). Monomeric PALB2 showed higher efficiency to bind DNA and promote RAD51 filament formation when compared to dimeric PALB2 (Buisson & Masson 2012). Moreover, Buisson & Masson (2012) have shown that there is competition between PALB2 self-interaction and PALB2–BRCA1 interaction after DNA damage. The switch from PALB2–PALB2 to PALB2–BRCA1 interaction allows activation of HR in the presence of DNA damage. Thus, regulation of PALB2 self-interactions could be a critical step in controlling HR to prevent aberrant recombination events (Buisson & Masson 2012).

Ma et al. (2012) have recently shown that PALB2 directly interacts with KEAP1, an oxidative stress sensor that binds and targets NRF2 for cytoplasmic degradation. NRF2 is a master transcription factor that induces the expression of several antioxidant genes upon oxidative stress to mitigate oxidative damage. Intriguingly, both PALB2 and NRF2 contain an identical LDEETGE sequence, which enables direct binding to KEAP1. Thus, PALB2 can effectively compete with NRF2 for KEAP1 interaction, thereby promoting NRF2 accumulation and subsequent anti-oxidant response (Ma et al. 2012). In addition, Orthwein et al. (2015) have shown that the interaction between PALB2 and KEAP1 is critical for cell cycle dependent regulation of HR.

The ChAM domain present in PALB2 promotes its chromatin association in both unperturbed and damaged cells. Deletion of ChAM domain resulted in cellular hypersensitivity to MMC treatments and abrogated PALB2 and RAD51 accumulation at DNA damage sites. Thus, the ChAM domain has a clear role in the function of PALB2 in response to DNA damage (Bleuyard et al. 2011).

Additionally, four independent studies have identified interaction between PALB2 and MRG15 both in protein complexes isolated from cells and through yeast-two hybrid screens (Bleuyard et al. 2017, Hayakawa et al. 2010, Martrat et al. 2011, Sy et al. 2009a). MRG15, also known as mortality factor 4 like 1 (MORF4L1), has been described as a component of the NuA4 histone acetyltransferase complex acetylating histones H4 and H2A. NuA4 has been implicated in transcriptional regulation and may have a direct role in the regulation of HR (Dhar et al. 2017, Lu et al. 2009). Sy et al. (2009a) suggested that abrogation of PALB2 and MRG15 interaction results in hyperrecombination and elevated sister chromatid exchange (SCE) with no change in sensitivity to
MMC. In contrast, Hayakawa et al. (2010) have shown that depletion of PALB2 and MRG15 interaction results in a decrease of HR and SCE formation along with hypersensitivity to MMC treatment. Thus, taken together, both of these studies support the role of PALB2-MRG15 interaction in regulating HR, but disagree about whether MRG15 is a positive or negative regulator (Park et al. 2014a). Further studies are therefore needed to clarify whether MRG15 is a positive or negative regulator of HR.

The carboxy-terminal of PALB2 (approximately, aa 791–1186) contains a seven-bladed WD40-type domain, which is known to mediate protein interactions (Park et al. 2014a). The WD40 domain of PALB2 facilitates direct interactions with many of the key proteins involved in HR, such as BRCA2, RAD51, RAD51C, XRCC3 and RNF168 (Luijsterburg et al. 2017, Park et al. 2014b). Additionally, the WD40 domain also facilitates direct interaction between PALB2 and Pol η to mediate recombination-associated DNA synthesis (Buisson et al. 2014). Recently, a nuclear export signal (NES) was identified to be located in the WD40 domain between amino acids 928 – 945 (Pauty et al. 2017). This motif is usually masked and allows export of PALB2 under specific conditions. The WD40 domain is critical for the tumor suppressor function of PALB2 as truncation of even the last four amino acids of the WD40 domain destabilizes the entire PALB2 protein (Oliver et al. 2009).

PALB2 in conjunction with BRCA2 plays a crucial role in HR and in resistance to MMC (Xia et al. 2006). Additionally, PALB2 together with BRCA2 also regulates both the intra-S phase checkpoint and G2 checkpoint in response to IR (Menzel et al. 2011, Xia et al. 2006). PALB2 has two different regions that can directly bind to RAD51 and stimulate D-loop formation: 1) at the N-terminus (aa 101–184) and 2) in the WD40 domain (Buisson et al. 2010, Dray et al. 2010). Of these two RAD51 binding sites in PALB2, the WD40 domain appears to be the primary binding site for RAD51. Further studies are warranted to delineate the roles of these RAD51-binding sites in mediating HR (Park et al. 2014a).

It has been shown that PALB2 promotes D-loop formation by inhibiting the association of RPA with the invading DNA, thereby promoting the binding and stabilization of RAD51 to DNA (Buisson et al. 2010). The interaction between the full-length PALB2 and APRIN appears to be essential for the PALB2-mediated RPA displacement and RAD51 filament formation during HR (Couturier et al. 2016). Moreover, three independent studies have recently shown that ATM/ATR-mediated phosphorylation of PALB2 at S59, S157, and S376 is

Finally, the phenotype of PALB2 knockout mice resembles those of BRCA1 and BRCA2 knockout mice, suggesting that a key function of PALB2 is to facilitate communication between BRCA1 and BRCA2 (Rantakari et al. 2010). In addition, it was recently shown that tumors originating due to PALB2 mutation displayed a similar mutational signature (signature 3) to that observed in BRCA1 and BRCA2 mutant tumors (Alexandrov et al. 2013, Nik-Zainal et al. 2016, Polak et al. 2017).

**PALB2 is a Fanconi anemia gene**

Shortly after its identification and initial characterization, PALB2 (FANCN) was found to be biallelically mutated in nine Fanconi anemia (FA) families (Reid et al. 2007, Serra et al. 2012, Xia et al. 2007). FA patients are characterized by growth retardation, congenital abnormalities, infertility and progressive bone marrow failure (Auerbach 2009, Kutler et al. 2003). All nine FANCN patients developed cancer in their early childhood, including five medulloblastomas, four Wilms tumors, two cases of acute myeloid leukemia (AML), one kaposiform hemangioendothelioma and two neuroblastomas. Cancer treatment was unsuccessful in eight FANCN patients, all of whom died before four years of age (Reid et al. 2007, Serra et al. 2012, Xia et al. 2007).

The occurrence of multiple pediatric malignancies along with early mortality in the FANCN patients is very similar to that of FANCD1 (BRCA2) patients (Reid et al. 2007, Serra et al. 2012, Skol et al. 2016). The phenotypes of FANCN and FANCD1 patients differ greatly from other FA subtypes, in whom progressive bone marrow failure predominates, often in advance of blood malignancies (Howlett 2007, Jacquemont & Taniguchi 2007, Patel 2007). The reason for the occurrence of multiple pediatric malignancies only in FANC-N/D1 patients, but not in other FA subtypes is unclear and has to be explored further (Ghazwani et al. 2016, Hirsch et al. 2004, Miele et al. 2015, Reid et al. 2007, Wagner et al. 2004).

Observations of similar clinical phenotypes in FANCN and FANCD1 patients indicate that both PALB2 and BRCA2 define a distinct genetic, clinical and functional entity in the Fanconi anemia pathway (Patel 2007). Of note, although all FANCN patients displayed a severe form of FA, two FANCN patients expressing a hypomorphic PALB2 allele were recently diagnosed with a mild form of FA (Byrd et al. 2016). Finally, the recent discovery of BRCA1 (FANCS) as
a new FA gene further exemplifies the importance of BRCA1-PALB2-BRCA2 interaction during DNA DSB repair (Sawyer et al. 2015).

### 2.6.2 ABRAXAS

**ABRAXAS** (*ABRA1, CCDC98*, or *FAM175A*) is a tumor suppressor gene in the BRCA pathway, mutations or copy number loss of which occur in several human cancers (Castillo et al. 2014). ABRAXAS was identified by three independent groups while searching for novel BRCA1 interacting proteins using the BRCA1 C-terminal (BRCT) domain as a bait (Her et al. 2016, Kim et al. 2007b, Liu et al. 2007, Wang et al. 2007). As shown in Figure 9, the gene encodes a protein of 409 amino acids (47 kDa) containing four recognized key elements such as a Mpr1 and PAD1 N-terminal (MPN) like domain (aa 11 – 121), a coiled-coil region (aa 206 – 260), a NLS (aa 358 – 361) and a pSPxF motif (aa 406 – 409) (Wang 2012, Wang et al. 2007).

![Fig. 9. Schematic protein structure of ABRAXAS. Interacting proteins and the region of ABRAXAS required for their associations are depicted. The NLS domain is important for translocation of BRCA1-A complexes into the cell nucleus and subsequently to DNA damage sites.](image)


As one of the imminent responses upon DNA DSBs, histone H2AX will be phosphorylated at serine 139 (γH2AX) by the DDR sensor kinases ATM/ATR (Burma et al. 2001, Rogakou et al. 1998). Subsequently, MDC1 is recruited to the DNA damage sites through binding to γH2AX and ATM (Stucki et al. 2005). Subsequent phosphorylation of MDC1 by ATM recruits ubiquitin (Ub) ligases, such as RNF8 and RNF168, which generates K63-linked poly-Ub chains on histones right at the damage site (Doil et al. 2009, Goldberg et al. 2003, Huen et al. 2007, Kolas et al. 2007, Mailand et al. 2007, Stewart et al. 2009). These act as a docking site for the BRCA1-A complex, mediated by RAP80 through its ubiquitin-interacting motif (UIM) (Harper & Elledge 2007, Hu et al. 2011b, Sobhian et al. 2007).

The exact role of the BRCA1-A complex in DNA DSB signaling is still not entirely clear (Wang 2012). It is suggested that the BRCA1-A complex regulates the DNA damage response process by deubiquitinating (DUB) the K63-linked poly-Ub chains (Shao et al. 2008, Wang 2012). Interestingly, the BRCA1-A complex shows striking similarity with the lid of 19S proteasome regulatory complex (Wang et al. 2009). The 19S proteasome lid cleaves ubiquitin from its substrates and subsequently facilitates the entry of substrates into the proteasome core for degradation (Wang 2012). Like Rpn11 in the 19S proteasome regulatory complex, BRCC36 also contains an active MPN+/JAMM domain that possesses DUB activity (Dong et al. 2003, Wang et al. 2009). BRCC36 displays DUB specificity towards the K-63 linked poly-Ub chains (Cooper et al. 2009, Sobhian et al. 2007). Similarly, like Rpn8 in the 19S proteasome regulatory complex, ABRAXAS also contains a MPN domain (Wang et al. 2009). Rpn10/S5a in the 19S proteasome regulatory complex contains both a UIM and VWA domain, as here RAP80 possesses a UIM domain and MERIT40 contains a VWA domain (Wang et al. 2009, Yan et al. 2006).

In addition to ABRAXAS, the BRCA1 BRCT domain also directly interacts with BACH1 and CtIP by binding a pSPxF motif in a CDK-phosphorylation dependent manner, forming BRCA1-B and BRCA1-C complexes, respectively (Cantor et al. 2001, Manke et al. 2003, Rodriguez et al. 2003, Wong et al. 1998, Yu et al. 1998, Yu et al. 2003). In addition to BRCA1-A, B & C complexes, as discussed in section 2.6.1, by direct interaction with PALB2, BRCA1 can also
form the BRCA1-PALB2-BRCA2-RAD51 (BRCC) complex (Savage & Harkin 2015, Sy et al. 2009b, Zhang et al. 2009a, Zhang et al. 2009b). All these four BRCA1-containing complexes compete for the availability of endogenous BRCA1 protein. Disruption of BRCA1-A complex formation by knockdown of RAP80 or ABRAXAS propagates BRCA1-B, BRCA1-C and BRCC complex formation and subsequent assembly at the DNA damage sites (Coleman & Greenberg 2011, Hu et al. 2011b, Typas et al. 2015).


**BRCA1-A complex and Cancer**

Our team recently reported the presence of a recurrent heterozygous germline alteration in the ABRAXAS gene (c.1082G>A) in Northern Finnish breast cancer families (Solyom et al. 2012). This specific c.1082G>A (Arg361Gln) alteration resides in the NLS domain of ABRAXAS and impairs the nuclear localization of the BRCA1-A complex and thus disrupts the BRCA1-mediated DNA damage response (Solyom et al. 2012). Although the appearance of the ABRAXAS germline alteration in other populations seems to be relatively rare, c.1082G>A is a founder mutation in the Finnish population (Solyom et al. 2012). Interestingly, according to the exome aggregation consortium, the same c.1082G>A alteration
is also observed in non-Finnish Europeans and Latinos (Lek et al. 2016). Intriguingly, the NLS domain of *ABRAXAS* is identified as a mutational “hot spot” which is frequently targeted by somatic missense mutations in several types of cancer (Castillo et al. 2014).

The average age of disease onset for this particular *ABRAXAS* c.1082G>A mutation carriers is 46 years (variation, 35 to 53 years), which is similar to that of Finnish *BRCA1* (46 years; variation, 32 to 57 years) and *BRCA2* (48 years; variation, 45 to 67 years) mutation carriers (Sarantaus et al. 2000, Solyom et al. 2012). This indicates that carriers of the *ABRAXAS* c.1082G>A alteration possess a similarly increased cancer risk as that observed for *BRCA1* and *BRCA2* mutation carriers. For comparison, the mean age of disease onset for the *PALB2* c.1592delT mutation carriers is 52.9 years (variation, 39 to 73 years) (Erkko et al. 2007).

In addition to breast cancer, families with *ABRAXAS* c.1082G>A mutation also display certain rare types of cancers such as lip and lung cancer along with throat lymphoma (Solyom et al. 2012). Similarly, in addition to breast cancer, germline mutations in *ABRAXAS* have also been associated with upper aerodigestive tract and ovarian cancers (Hamdi et al. 2016, McKay et al. 2011, Pennington et al. 2014, Solyom et al. 2012).

Members of the BRCA1-A complex are frequently targeted by many clinically important germline mutations. Germline mutations in the BRCA1 BRCT domain disrupt either the binding surface to pSPxF-containing phosphopeptides or the ability of the BRCT domain to dimerize and form BRCA1 complexes (Clapperton et al. 2004, Wu et al. 2016). Apart from *BRCA1* and *ABRAXAS*, germline mutations in *RAP80* and *MERIT40* are also associated with inherited predisposition to breast and ovarian cancers (Antoniou et al. 2010, Bolton et al. 2010, Couch et al. 2016, Nikkilä et al. 2009). These studies collectively emphasize that, in addition to BRCA1, other BRCA1-A members are also frequently targeted for tumorigenesis.
3 Aim of the study

It is well known that hereditary mutations in DDR genes often lead to genomic instability and ultimately tumor development. However, the molecular mechanism of how these DDR deficiencies promote genomic instability and malignancy is not well understood.

Thus, the specific aims of the study were:

1. To identify the functional and molecular framework behind the elevated breast cancer risk observed in heterozygous \textit{PALB2} mutation carriers.
2. To elucidate the molecular mechanisms by which the \textit{ABRAXAS} Finnish founder mutation causes an elevated breast cancer risk for the heterozygous carriers.
4 Materials and methods

4.1 Subjects

4.1.1 Study I

Altogether eight previously identified female individuals carrying the Finnish PALB2 c.1592delT founder mutation (Erkko et al. 2007) were analyzed in the current study. Among them, six individuals had been diagnosed with breast cancer while the other two persons were disease-free, representing two different breast cancer families (Erkko et al. 2007). Samples from the breast cancer patients were collected at least 3 years after the initial cancer diagnosis. Six healthy non-carrier female individuals served as controls; three of them were healthy members of two different PALB2 mutation carrier families and the other three were population controls. All control individuals were ethnically age-matched and originated from the same geographical region as the mutation carriers. Similarly, all control individuals were cancer-free at the time of donation and no follow-up was performed on the health status of the controls. The studied cell lines are presented in Supplementary Table S1 of study I.

4.1.2 Study II

Altogether eight individuals carrying the Finnish ABRAXAS c.1082G>A founder mutation were analyzed in this study. Among them, five individuals had been diagnosed with breast cancer and one with lymphoma, and the other two persons were disease-free siblings of one breast cancer patient. Samples from the breast cancer patients were collected at least 3 years after the initial cancer diagnosis. Six healthy non-carrier female individuals used in study I (Nikkilä et al. 2013) along with one individual who is a healthy family member of a patient served as controls. All control individuals were cancer-free at the time of donation and originated from the same geographical region as the mutation carriers. No follow-up was performed on the health status of the controls. The studied cell lines are presented in Supplementary Table S1 of study II.
4.2 Methods

4.2.1 Cell culture

In order to generate lymphoblastoid cell lines (LCLs), Epstein-Barr virus (EBV) was used to immortalize B-lymphocytes obtained from fresh peripheral blood samples of the participating individuals. LCLs were cultured in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 20% fetal bovine serum (Gibco, Invitrogen), 1% L-glutamine (Gibco, Invitrogen) and 10 μg/mL gentamycin (B. Braun). The MCF7 breast cancer cell line (ATCC HTB-22™, Manassas, Virginia, U.S.A.) was cultured in Dulbecco's modified Eagle Medium (DMEM), high glucose, with sodium pyruvate (Gibco, Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma Aldrich).

4.2.2 Plasmids and transfection

Wild-type and c.1082G>A mutant ABRAXAS carrying an N-terminal HA/FLAG-tag were cloned from pOZ-N-ABRAXAS vectors into pcDNA3.1/Hygro(-) backbone. MCF7 cells were transiently transfected with wild-type ABRAXAS, ABRAXAS mutant or empty pcDNA3.1/Hygro(-) vector using Cell Line Nucleofector kit V (Lonza) according to the manufacturer’s instructions.

4.2.3 Etoposide and hydroxyurea treatments

Cell lines treated with either 0.5 or 5 μM etoposide (ETO) (Etopofos, Bristol–Myer Squibb and/or Etoposide, Pfizer) and 2 mM hydroxyurea (HU) (Sigma-Aldrich) were cultured for up to 24 h in the presence of the agent. Samples were then collected at different time points (0 h, 0.5 h, 1 h, 2 h, 3 h, 5 h, 7 h and 24 h).

4.2.4 Western blot analysis

A standard western blot (WB) protocol was followed as mentioned in Studies I & II.
4.2.5 RNA extraction, cDNA synthesis and Quantitative PCR

Samples were collected from untreated cells and total RNA was extracted using RNeasy Mini Kit (Qiagen), and reverse transcription was done as described in the iScript™ cDNA synthesis kit (Bio-Rad). Quantitative PCR (qPCR) was performed using iQ™ SYBR® Green Supermix (Bio-Rad) as per the manufacturer’s instruction in a CFX96 real-time PCR system (Bio-Rad). *CLK2* and *GUSB* were used as calibrators.

4.2.6 DNA damage signaling PCR array

Samples were collected from untreated cells and total RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA quality was determined using RNA 6000 nanochips (Agilent Technologies) with an Agilent 2100 bioanalyzer, and only samples with an RNA integrity number (RIN score) of 10 were used further. Genomic DNA elimination and reverse transcription was done as described in the RT² First Strand Kit (Qiagen). Gene expression was studied using the DNA damage signaling pathway RT² profiler PCR array (Qiagen).

4.2.7 RNA sequencing

Samples were collected from untreated cells and total RNA was extracted using the RNeasy Mini Kit (Qiagen). The average RIN score of the samples was 8.7, ranging from 5.1 to 10. Around 1 ug total RNA was used for library preparation using Illumina’s TruSeq Stranded mRNA Library Prep Kit (Illumina, CA, USA). The libraries were sequenced (multiplexing five per lane) using an Illumina HiSeq2500 in high-output, single-ended, 51 cycle mode, followed by read extraction (FastQ format) using bc12fastq v1.8.4 (supported by Illumina). This resulted in an average of 43 million reads per sample (32.8–57 million).

4.2.8 DESeq2 and Ingenuity pathway analysis

DESeq2 (v1.14.1) in Bioconductor/R package was used to identify differentially expressed genes (DEGs) between the two cohorts and by using a negative binomial test. The parameters used in our DESeq2 analysis were: mode=“Union”, singleEnd=TRUE, ignore.strand=FALSE. The false discovery rate-adjusted P-value (FDR) was estimated based on Benjamini Hochberg correction. Changes in
gene expression with a FDR (Padj value) of <0.05 were considered statistically significant. To evaluate the significance of the identified DEGs, Ingenuity Pathway Analysis (IPA®, QIAGEN Bioinformatics) was conducted to find enriched biological, molecular and cellular functions as well as associated diseases and network functions.

### 4.2.9 edgeR and ENRICHR analysis

In order to find a wider range of DEGs, we performed R package edgeR (v3.3.1) analysis. In edgeR analysis, default parameters were used (DGEList: remove.zeros=FALSE, lib.size = NULL, norm.factors = NULL, genes = NULL; estimateCommonDisp: tol=1e-06, rowsum.filter=5; exactTest: pair=NULL, dispersion=NULL, common.disp=TRUE). P-values were corrected for multiple testing using the Benjamini Hochberg method, and genes with FDR (Padj value) <0.01 were considered to be differentially expressed and statistically significant.

To evaluate the importance of the DEGs identified by edgeR, the official gene names of these DEGs were fed into the web-based gene set enrichment tool service ENRICHR (http://amp.pharm.mssm.edu/Enrichr).

### 4.2.10 Flow cytometric analysis

Unexposed and 0.5 µM ETO treated cell lines were cultured for 24 h and samples were collected at different time points (0.25 h, 1 h, 7 h and 24 h). Cells were stained with thymidine analogue 5-ethynyl-2’-deoxyuridine (EdU) to measure DNA synthesis and analyze cell cycle distribution as described by the manufacturer of Click-iT® EdU Alexa Fluor® (AF) 647 Flow Cytometry Assay Kit (ThermoFisher Scientific). All measurements were performed with LSRFortessa (BD Biosciences) flow cytometer and FlowJo software was used for quantification.

### 4.2.11 Immunofluorescence

A standard immunofluorescence protocol was followed as mentioned in Study II to perform high content protein localization analysis and high content foci analysis.

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4.2.12 **DNA fiber spreads**

Exponential growing cells were pulse-labeled with 25 μM CldU (Sigma) followed by 250 μM IdU (Sigma) for the indicated times. For fiber analysis, after damage induction CldU and IdU media was given for the indicated time periods and 10 μM ETO was performed during the last 30 min of the CldU pulse. Labeled cells were harvested and DNA fiber spreads were prepared on microscope glass slides by addition of 200 mM Tris-HCl pH 7.4, 50 mM EDTA and 0.5% SDS. At least 200 replication forks were analyzed.

4.2.13 **Caspase activation assay**

Untreated cell lines and cell lines treated with 0.5 μM ETO were cultured for 24 h and samples were collected. Furthermore, caspase activation assay was performed using the 660 Polycaspase Assay kit (ImmunoChemistry) according to the manufacturer’s instruction. All measurements were done in LSRFortessa (BD Biosciences) flow cytometer and FlowJo software was used for quantification.

4.2.14 **Repair pathway analysis**

Different DNA mixtures containing expression plasmid for the endonuclease I-SceI (pCMV-I-SceI) together with one of the DSB repair substrates (EJ5SceGFP, EJ-EGFP, 5’EGFP/HR-EGFP, HR-EGFP/3’EGFP, HR-EGFP/5’EGFP) and pBS filler plasmid (pBlueScriptII) or wild-type EGFP expression plasmid (for determination of transfection efficiencies) were introduced into the cells by use of a Gene Pulser with Pulse Controller from Bio-Rad (Munich, Germany). Recombination frequencies were measured by quantification of green fluorescent cells containing reconstituted EGFP within the live cell-population (SSC/FSC gate).

4.2.15 **Proximity ligation assay**

A standard proximity ligation assay (PLA) protocol was followed as mentioned in Study II.
4.2.16 **Cell survival analysis**

A standard cytotoxicity assay protocol was followed as mentioned in Study II.

4.2.17 **Cytogenetic analysis**

The blood samples from the studied breast cancer patients were collected at least 3 years after the initial cancer diagnosis. A minimum of 50 Giemsa-banded metaphases for each sample were evaluated by light microscopy and photographed with an automatic chromosome analyzer (CytoVision version 7.2, Applied Imaging). Chromosomal aberrations were divided into five classes: 1) telomeric associations, 2) chromatid/chromosome breaks and deletions, 3) simple chromosomal rearrangements, 4) complex chromosomal rearrangements, and 5) total rearrangements.

4.2.18 **Statistical analysis**

Statistical differences for normally and non-normally distributed samples were determined using independent samples Student’s t-test and Mann-Whitney U-test, respectively. Mean values are reported for normally distributed samples; in the case of non-normally distributed samples, median values are reported. Correlation analysis was done using Spearman's rank correlation coefficient and rho scores were reported. All P-values were two-sided and statistical evaluations were done with IBM SPSS Statistics (version 20.0).

4.3 **Ethical issues (Study I & II)**

An informed consent to participate in the study was obtained from each individual. Samples obtained from the participants were used for research purposes only and no information on the outcome of studies was given to the participants or their family members. The studies have been approved by the Ethical Board of the Northern Ostrobothnia Health Care District (Studies I and II) and Ethics Committee of the Helsinki University Hospital (Study II).
5 Results

Key results from both Study I and II are presented below in this section. The results presented here show the mechanistic insight behind the elevated breast cancer risk observed in heterozygous *PALB2* (Study I) and *ABRAXAS* (Study II) mutation carriers.

5.1 *PALB2* c.1592delT mutation carriers display DNA replication and cell cycle checkpoint defects (Study I)

LCLs were established from both controls and heterozygous *PALB2* c.1592delT mutation carriers (Neitzel 1986). A list of the LCLs used is presented in the Supplementary Table S1 of Study I.

5.1.1 Heterozygous mutation in *PALB2* c.1592delT results in *PALB2* haploinsufficiency

Using WB analysis, endogenous full-length PALB2 protein levels were quantified and found to be decreased by 48% in the mutation carriers (carriers: 52.1% vs. controls: 100%, Figure 10, Figure 1a in Study I) indicating *PALB2* haploinsufficiency. Since the *PALB2* c.1592delT allele results in a truncated protein, after long exposure, we were able to detect the truncated PALB2 in all mutation carriers but not in the controls (Figure 1b in Study I). Consistent with the WB results, mRNA analysis also indicated that the transcript of the *PALB2* c.1592delT allele was able to escape nonsense-mediated decay. Thus, taken together, the *PALB2* c.1592delT heterozygous mutation results in *PALB2* haploinsufficiency, which could be responsible for the increased breast cancer risk observed in the mutation carriers.
5.1.2 PALB2 haploinsufficiency results in elevated replication stress

Cell cycle distribution analysis in a subset of LCLs showed that the carrier cells incorporated larger amounts of EdU, and concurrently, had a smaller fraction of S-phase cells (Supplementary Figure S1 in Study I), indicating that the mutation carrier cells are replicating their DNA faster than the control cells. Due to this, DNA replication phenotypes such as replication origin firing and elongation were studied in the LCLs by using the DNA fiber assay technique. No significant change was observed in the replication elongation rates (Figure 2a in Study I) between the PALB2 cohorts. However, we noticed closely spaced replication origins in the PALB2 mutation carrier cells (carriers: 43 kb vs. controls: 62 kb, Figure 2b in Study I). Simultaneously, there was a significant increase in the frequency of 1st pulse replication origins in the mutation carrier cells (carriers: 21.7% vs. controls: 11.8%, Figure 2c in Study I). The observation of closely spaced replication origins and excessive origin firing indicates the presence of replication stress in the mutation carriers due to PALB2 haploinsufficiency.
5.1.3 Increased replication origin firing is due to elevated Cdk activity

To understand more about the observation of increased replication origin firing in the PALB2 mutation carrier cells, we studied the regulation of cyclin-dependent kinase (Cdk), as S-phase Cdk activity constitutes a critical regulator of replication firing (Araki 2010, Beck et al. 2012). From WB analysis, we found that the inhibitory phosphorylation of Cdk at threonine 14 is significantly reduced in the PALB2 mutation carrier cells (carriers: 73% vs. controls: 100%, Figure 11, Figure 3a,b in Study I).

Fig. 11. Heterozygous PALB2 mutation carriers display increased Cdk activity

In the presence of roscovitine (a Cdk inhibitor), the frequency of 1st pulse origins in the PALB2 mutation carrier cells dropped dramatically to that of the control levels (carriers: from 26% to 10.5% vs. controls: from 14.2% to 8.8%, Figure 12, Figure 3c in Study I). Taken together, these results indicate that the increased replication origin firing observed in the PALB2 mutation carrier cells is dependent on increased Cdk activity in the S phase.
5.1.4 PALB2 haploinsufficiency results in overexpression of ATR

Since the ATR/CHK1 pathway is essential for the regulation of origin firing (Maya-Mendoza et al. 2007, Petermann & Caldecott 2006, Petermann et al. 2010, Syljuåsen et al. 2005) changes in its expression were monitored by WB analysis. Though no change was observed in the CHK1 levels (Figure 4a in Study I), ATR levels were significantly increased by almost threefold in the PALB2 mutation carrier cells (carriers: 275% vs. controls: 100%, Figure 4a,b in Study I). In addition, we also observed a strong inverse correlation between the ATR levels and PALB2 levels (rho = -0.846, Figure 4c in Study I) and inter-origin distance (rho = -0.698, Figure 4d in Study I). These results further validate the presence of aberrant origin firing in the mutation carrier cells, and the overexpression of ATR could be partly related to it.

5.1.5 PALB2 haploinsufficiency results in G2/M checkpoint leakiness

Since PALB2 is a key regulator of G2 checkpoint maintenance (Menzel et al. 2011), leakiness in the G2/M checkpoint was assessed in the LCLs in the presence of 0.5 μM ETO (Theunissen & Petrini 2006). After 24 h of ETO addition, nearly all control cells indicated an efficient G2/M checkpoint; however, 57% (4/7) of the mutation carriers had more mitotic cells indicative of G2/M
checkpoint leakiness (Supplementary Table S2 in Study I). As CHK2 is essential for efficient G2/M checkpoint maintenance (Reinhardt & Yaffe 2009, Zannini et al. 2014), phosphorylation of CHK2 at threonine 68 was monitored using WB analysis. CHK2 phosphorylation was significantly reduced in the PALB2 mutation carrier cells (carriers: 55.0% vs. controls: 108.3%, Figure 6b in study I) after 5 h in the presence of 5 μM ETO. Thus, the observation of reduced CHK2 phosphorylation further supports the G2/M checkpoint leakiness identified in the PALB2 mutation carrier cells.

5.1.6 Genomic instability due to elevated replication stress and G2/M checkpoint leakiness in the PALB2 mutation carrier cells

Aberration in the maintenance and regulation of DNA replication and cell cycle checkpoint pathways often leads to genomic instability. Therefore, occurrence of genomic instability was assessed in the PALB2 mutation carriers using untransformed primary cells (fresh peripheral blood lymphocytes). Cytogenetic analysis of the primary cells indicated a significant increase of chromosomal rearrangements in the PALB2 mutation carriers (carriers: 8.83 vs. controls: 0.67, Table 1 in Study I). The observation of increased genomic instability in the mutation carrier cells is due to defective DNA replication and/or cell cycle checkpoint (Hanahan & Weinberg 2011).

5.2 ABRAXAS c.1082G>A mutation carriers display transcriptional misregulation and aberrant DNA damage response (Study II)

LCLs were established from both controls and heterozygous ABRAXAS c.1082G>A mutation carriers (Neitzel 1986). A list of the LCLs used is presented in the Supplementary Table S1 of Study II.

5.2.1 Presence of dominant-negative mutant ABRAXAS in the ABRAXAS c.1082G>A mutation carrier cells

To characterize the tumorigenesis pattern, it is important to first establish whether both the mutant ABRAXAS mRNA and protein are stably expressed in ABRAXAS c.1082G>A mutation carrier cells by not undergoing nonsense-mediated mRNA decay or proteolysis. From qPCR analysis, we did not observe any significant change in the endogenous ABRAXAS mRNA levels between our case-control
ABRAXAS cohorts (Figure S3A in Study II). RNA sequencing (RNA-Seq) data showed that the ABRAXAS c.1082G>A transcripts were stably expressed in the ABRAXAS mutation carrier cells and present at levels comparable to those of the wild-type allele (46.5% ± 6.7% of total ABRAXAS reads including the c.1082 site; Table S5 in Study II). From WB analysis, we did not observe any significant change in ABRAXAS protein expression between our cohorts (Figure S3B,C in Study II), indicating that the mutant ABRAXAS and wild-type protein have comparable stability and expression levels, as already suggested by previous studies (Solyom et al. 2012, Vikrant et al. 2015). This indicates that the dominant-negative mutant ABRAXAS could be responsible for the increased breast cancer risk observed in the mutation carriers.

5.2.2 BRCA1 mislocalization due to mutant ABRAXAS in the mutation carrier cells

Since the observed ABRAXAS single nucleotide variant resides in the NLS domain responsible for nuclear localization, we checked for potential changes in the sub-cellular localization of ABRAXAS and BRCA1 between our cohorts. As expected, we observed significantly decreased nuclear localization of ABRAXAS (z-scores: carriers: -1.97 vs. controls: 0.00, Figure 13A, Figure 1A in Study II) and BRCA1 (z-scores: carriers: -2.12 vs. controls: 0.00, Figure 13B, Figure 1B in Study II) in the ABRAXAS mutation carrier cells. Cytoplasmic mislocalization of ABRAXAS and BRCA1 limits the nuclear concentrations of the respective proteins for normal functioning of the DDR pathway.

Fig. 13. Heterozygous ABRAXAS mutation carriers display cytoplasmic mislocalization of ABRAXAS and BRCA1
5.2.3 Reduced ABRAXAS and BRCA1 foci formation in the ABRAXAS mutation carrier cells

During DDR, both ABRAXAS and BRCA1 are known to form foci around the DNA damage sites (Solyom et al. 2012, Wang et al. 2007). Thus, we compared the foci formation pattern of both ABRAXAS and BRCA1 in our study cohorts. As expected, we observed a reduced number of spontaneous ABRAXAS (z-scores: carriers: -0.57 vs. controls: 0.00, Figure 1C in Study II) and BRCA1 foci (z-scores: carriers: -0.96 vs. controls: 0.00, Figure 1D in Study II) in the ABRAXAS mutation carrier cells. In addition, ABRAXAS and BRCA1 foci co-localization was also significantly diminished in the ABRAXAS mutation carrier cells (co-localizing foci per nuclei: carriers: 0.83 vs. controls: 2.13, Figure 1E in Study II). PLA also indicated weakened interaction between ABRAXAS and BRCA1, 4 h post IR in the ABRAXAS mutation carrier cells (Figure 1F,G in Study II). Observation of fewer ABRAXAS and BRCA1 foci together with diminished co-localization between them is due to the cytoplasmic mislocalization of respective proteins.

5.2.4 Impairment of BRCA1-A complex affects DNA repair pathway choice

Since the BRCA1-A complex is crucial for DSB signaling, we set out to assess differences in the DSB repair pathway usage between our cohorts. No significant changes were noticed in either NHEJ or HR repair pathway usage between our cohorts (Figure 2A,B in Study II). However, we observed a significant decrease in the usage of MMEJ (carriers: 61% vs. controls: 100%, Figure 2C in Study II), SSA (carriers: 86% vs. controls: 100%, Figure 2D in Study II) and SSA+HR (carriers: 76% vs. controls: 100%, Figure 2E in Study II) in the ABRAXAS mutation carrier cells.

When analyzing γH2AX (marker for DSBs), whose phosphorylation precedes the choice of DSB repair pathway, we found no significant changes in foci numbers between our cohorts (Figure S5A in Study II). Instead, we observed a significant decrease in γH2AX foci intensity (z-scores: carriers: -2.44 vs. controls: 0.00, Figure 3A in Study II) and global γH2AX levels (carriers: 90.4% vs. controls: 100%, Figure 3B in Study II) in the ABRAXAS mutation carrier cells. Cell cycle specific analysis indicated a significant decrease in γH2AX intensity.
only in the G1 (carriers: 89.8% vs. controls: 100%, Figure 3C in Study II) and S populations (carriers: 90.1% vs. controls: 100%, Figure 3C in Study II).

5.2.5 Reduced CtIP recruitment in cells from ABRAXAS mutation carriers

Since CtIP is essential for the activation of the MMEJ, SSA and HR pathways (Bennardo et al. 2008), we checked for potential changes in its sub-cellular localization and foci formation pattern between our cohorts. Interestingly, CtIP also showed significantly increased cytoplasmic mislocalization (z-scores: carriers: -1.12 vs. controls: 0.00, Figure 3D in Study II) and reduced number of spontaneous foci (z-scores: carriers: -1.17 vs. controls: 0.00, Figure 3E in Study II) in the ABRAXAS mutation carrier cells.

5.2.6 Attenuated G2-M checkpoint maintenance in ABRAXAS mutation carrier cells

Since the BRCA1-A complex is a key regulator of G2/M checkpoint maintenance (Solyom et al. 2012, Wang et al. 2007), leakiness in the G2/M checkpoint was assessed between our cohorts. After 24 h of ETO addition, ABRAXAS mutation carriers had significantly more mitotic cells, which is indicative of G2/M checkpoint leakiness (relative M/G2 ratio; carriers: 151% vs. controls: 100%, Figure 4A in Study II). Since ATM and CHK2 play a key role in the regulation of G2/M checkpoint, when analyzing the total protein levels, we found significantly reduced ATM (carriers: 79% vs. controls: 100%, Figure 4B,C in Study II) and CHK2 protein levels (carriers: 72% vs. controls: 100%, Figure 4D,E in Study II) in the ABRAXAS mutation carrier cells 24 h post ETO.

5.2.7 DNA damage persistence in ABRAXAS mutation carrier cells

We next looked at the efficiency of DSB repair between our cohorts. At 24 h post ETO treatment in the mutation carrier cells, we found significantly increased numbers of 53BP1 (z-scores: carriers: 3.48 vs. controls: 0.00, Figure 5A in Study II), γH2AX (z-scores: carriers: 1.66 vs. controls: 0.00, Figure 5B in Study II) and RAD51 foci (z-scores: carriers: 2.14 vs. controls: 0.00, Figure 5C in Study II) indicating more residual DNA damage. Apart from a slight increase in the frequency of 1st pulse replication origins fired (carriers: 17.7% vs. controls:
11.9%, Figure 6D in Study II), no other DNA replication aberrations were observed in the ABRAXAS mutation carrier cells. Similarly, apart from a possible slight increase in simple chromosomal rearrangements (carriers: 2.9 vs. controls: 0.0, Table S6 in Study II), no other chromosomal aberrations were observed in the ABRAXAS mutation carrier cells.

5.2.8 Cell lines from ABRAXAS mutation carriers display an aberrant transcriptional profile

In unchallenged cells, the BRCA1 (carriers: 80% vs. controls: 100%, Figure 7A,C in Study II), ATM (carriers: 84% vs. controls: 100%, Figure 7B,D in Study II) and BRCC36 (carriers: 81% vs. controls: 100%, Figure 7B,E in Study II) protein levels appeared to be significantly reduced in the mutation carriers. Concurrently, qPCR analysis also indicated downregulation of ATM (carriers: 82% vs. controls: 100%, Figure S14B in Study II), BRCC36 (carriers: 63% vs. controls: 100%, Figure S14C in Study II) and PALB2 (carriers: 79% vs. controls: 100%, Figure S14D in Study II) mRNA in the mutation carriers. RNA expression analysis using a RT² profiler PCR array also further indicated transcriptional misregulation of several genes functioning in the DNA damage signaling pathway (Table S7 in Study II).

Therefore, we performed RNA-Seq analysis to evaluate differences in the global transcriptome profile between our cohorts. DESeq2 analysis identified 60 statistically significant DEGs in ABRAXAS mutation carriers (Table S8 in Study II). In this set of DEGs, Ingenuity Pathway Analysis (IPA) identified “Cancer” to be the top-ranked disease associated with the mutation carriers (Figure 14, Figure 8A and Table S8 in Study II).
Next, we performed \textit{edgeR} analysis to find a wider range of DEGs to look for enrichment of particular transcription factor binding/chromatin binding factors and epigenetic modifications in the mutation carriers. \textit{EdgeR} analysis identified 586 genes to be differentially regulated between our cohorts (Table S9 in Study II). \textsc{ENRICHR} analysis in this set of DEGs primarily implicated enriched binding of PRC2 subunits such as SUZ12 and EZH2 to our gene set (Table S9 in Study II). Simultaneously, \textsc{ENRICHR} analysis against the ENCODE histone modification database indicated enrichment of the H3K27me3 epigenetic mark to our dataset (Table S9 in Study II).
6 Discussion

It is well known that hereditary mutations in DDR genes often lead to genomic instability and ultimately tumor development (Negrini et al. 2010). However, the molecular mechanism of how these DDR deficiencies promote genomic instability and malignancy is not well understood. It is of paramount importance to learn how genomic instability arises at the cellular level in the carriers of various specific DDR gene mutations. Understanding how genomic instability arises will provide us a greater understanding about cancer development. More importantly, these studies could also provide new targets for therapeutic interventions during cancer treatments.

As discussed in section 2.4, genomic instability and tumorigenesis can, in addition to the well-documented two-hit hypothesis (Knudson 1971), also arise due to haploinsufficiency and dominant-negative phenotype (Payne & Kemp 2005). To better understand how a specific hereditary susceptibility gene mutation results in genomic instability due to haploinsufficiency and/or dominant-negative phenotype, studies have to be performed in patient-derived material or models. However, functional characterization of breast cancer promoting mutations has traditionally been based on analysis of tumor-derived cell lines, cellular knockdown or knockout approaches, mouse models and permanently or transiently induced mutant allele overexpression.

So far, only a small number of studies have attempted to analyze the molecular and biological effects of germline mutations at the heterozygous state using patient-derived material or models. This may be due to genetic heterogeneity of study material or limited availability of suitable human samples along with observation of less pronounced or recessive phenotypes in the heterozygous state. Nevertheless, even in the few studies performed using patient-derived materials, increasing evidence points to genomic instability and tumorigenesis driven by haploinsufficiency (or possibly mild dominant-negative) phenotypes (Nikkilä et al. 2013, Pathania et al. 2014, Vaclová et al. 2015).

6.1 Genomic instability and tumorigenesis driven by haploinsufficiency in PALB2 c.1592delT mutation carriers (Study I)

Endogenous full-length PALB2 protein levels were found to be decreased by 48% in the mutation carriers when compared to controls. This indicates that the defects
observed in DNA replication and cell cycle checkpoint are indeed due to \textit{PALB2} haploinsufficiency in the mutation carriers. The observation of \textit{PALB2} c.1592delT mRNA and truncated PALB2 protein in the mutation carriers indicates that a dominant-negative phenotype could also be partly responsible for the defects observed in DNA replication and cell cycle checkpoint activities.

Larger amount of EdU incorporation along with smaller fraction of S-phase cells indicates a more rapid overall DNA replication in the \textit{PALB2} mutation carrier cells. Simultaneously, the observation of closely spaced replication origins and excessive origin firing further supports the rapid DNA replication seen in the \textit{PALB2} mutation carrier cells. In an independent study, it has been shown that homozygous inactivation of \textit{FANCM} also results in excessive origin firing without affecting the elongation rates as observed here (Schwab \textit{et al.} 2010). Similarly, cells deficient for \textit{XRCC2} or \textit{RAD51} also show closely spaced replication origins and excessive origin firing as observed in this study (Daboussi \textit{et al.} 2008).

Previously, it has been shown that elevated levels of Cdk activity cause an increase in global DNA replication due to excessive origin firing and reduced inter-origin distances (Beck \textit{et al.} 2012). Similarly, in the current study, we have also observed an increased Cdk activity in the \textit{PALB2} mutation carrier cells. Thus, the increase in Cdk activity might be responsible for the observation of excessive origin firing in the \textit{PALB2} mutation carrier cells. Cdk inhibition using roscovitine in the \textit{PALB2} mutation carrier cells dramatically reduced the number of replication origins fired, further indicating the dependence of origin firing upon Cdk activity.

Thus, taken together, our results indicate that the elevated Cdk activity is responsible for the excessive origin firing, which in turn is responsible for the rapid DNA replication observed in the \textit{PALB2} mutation carrier cells. These results further confirm the presence of replication stress due to \textit{PALB2} haploinsufficiency in the mutation carrier cells. Interestingly, in a later independent study, replication stress was also observed due to \textit{BRCA1} haploinsufficiency in heterozygous \textit{BRCA1} mutation carriers (Pathania \textit{et al.} 2014). Defective stalled fork repair (SFR) and consequently, higher frequency of collapsed forks, indicative of replication stress, was observed in the \textit{BRCA1} mutation carrier cells (Pathania \textit{et al.} 2014).

Notably, these studies (Study I of this thesis and Pathania \textit{et al.} (2014)) used slightly different approaches to uncover the presence of replication stress in the assessed mutation carrier cells. In the current study (Study I), we have analyzed \textit{PALB2} mutation carrier cells for differences in origin activation, whereas Pathania
et al. (2014) analyzed BRCA1 mutation carrier cells for differences in replication fork protection. Of note, Pathania et al. (2014) found replication stress in primary human mammary epithelial cells (HMECs) and fibroblasts, further indicating that the replication stress observed in our current study is not LCLs-specific.

Activation of dormant origins has been observed during replication fork arrest (Karnani & Dutta 2011), which is tightly regulated by ATR and its downstream kinase CHK1 (Liu et al. 2000, Zhao & Piwnica-Worms 2001). Both ATR and CHK1 play a key role in the restriction of distant origin firing and simultaneous activation of dormant origins during low levels of replication stress (Ge & Blow 2010). In addition, ATR and CHK1 are also responsible for normal S phase progression and replication timing (Maya-Mendoza et al. 2007, Petermann & Caldecott 2006, Petermann et al. 2010, Syljuåsen et al. 2005). Though we did not observe any significant change in the CHK1 protein levels between our PA LB2 cohorts, ATR levels were significantly increased (by almost threefold) in the PA LB2 mutation carrier cells.

A genetic screen performed by Menzel et al. (2011) identified both BRCA2 and PALB2 as key regulators of G2 checkpoint maintenance. siRNA-mediated downregulation of PALB2 resulted in the abrogation of G2 checkpoint (Menzel et al. 2011). Similarly, in the current study, we have observed G2/M checkpoint leakiness already in the heterozygous PA LB2 mutation carrier cells. Most importantly, ATM and its downstream effector kinase CHK2 are responsible for the efficient activation and maintenance of G2/M checkpoint (reviewed in (Guleria & Chandna 2016, Zannini et al. 2014)). In response to DNA damage, CHK2 is rapidly phosphorylated and thereby activated by ATM; active CHK2 further phosphorylates Cdc25C on serine-216 to prevent entry into mitosis (Matsuoka et al. 1998). In the PA LB2 mutation carrier cells, we have observed significantly reduced levels of active CHK2 at late time points after DNA damage, further supporting the finding of G2/M checkpoint leakiness.

Abnormalities or maintenance defects in key cellular pathways such as DNA replication and cell cycle checkpoints often lead to increased genomic instability (Hanahan & Weinberg 2011). Since the LCLs used in the functional analysis were transformed using EBV, we used primary untransformed cells to test for the presence of increased genomic instability in the PA LB2 mutation carriers. As expected, indeed both simple and complex chromosomal rearrangements were found to be significantly higher in the PA LB2 mutation carriers. The observation of genomic instability in primary untransformed cells indicates that the described mechanisms of genome destabilization operate at the organism level as well.
In a recent report from our group, we found that the *PALB2* mutation carrier cells use both MMEJ and SSA DSB repair pathways to a significantly higher extent compared to control cells (Obermeier *et al.* 2016). Both MMEJ and SSA are highly mutagenic DSB repair pathways and their augmented use results in increased chromosomal aberrations (reviewed in Bhargava *et al.* 2016, Seol *et al.* 2017). Thus, results from both of these studies indicate that the overuse of the highly mutagenic MMEJ and SSA pathways could be responsible for the observation of increased chromosomal aberration in the *PALB2* mutation carrier cells.

In summary, as shown in Figure 15, *PALB2* c.1592delT causes a haploinsufficiency phenotype in the mutation carrier cells resulting in elevated replication stress and G2/M checkpoint maintenance defects. Due to these aberrations, elevated genomic instability and eventually tumorigenesis are observed. Thus, genomic instability and tumorigenesis are driven by haploinsufficiency in *PALB2* c.1592delT mutation carriers. Therefore, it appears that loss of tumor suppressor/caretaker gene heterozygosity is not always mandatory for tumorigenesis.

![Fig. 15. Proposed model on how the heterozygous Finnish *PALB2* c.1592delT mutation may drive early-stage tumorigenesis in the carrier individuals.](image)

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In summary, as shown in Figure 15, *PALB2* c.1592delT causes a haploinsufficiency phenotype in the mutation carrier cells resulting in elevated replication stress and G2/M checkpoint maintenance defects. Due to these aberrations, elevated genomic instability and eventually tumorigenesis are observed. Thus, genomic instability and tumorigenesis are driven by haploinsufficiency in *PALB2* c.1592delT mutation carriers. Therefore, it appears that loss of tumor suppressor/caretaker gene heterozygosity is not always mandatory for tumorigenesis.
The presence of ‘conditional haploinsufficiency’ as proposed by Bartek et al. (2007) was observed in the heterozygous BRCA1 mutation carriers (Pathania et al. 2014). No significant change in the sensitivity to olaparib (PARP inhibitor) was observed between BRCA1\textsuperscript{mut/+} and BRCA1\textsuperscript{+/+} cells when they are not pre-exposed to DNA damaging agents like UV (Pathania et al. 2014). However, after pre-exposure to UV, BRCA1\textsuperscript{mut/+} cells became olaparib-sensitive, indicating ‘conditional haploinsufficiency’ for HR-DSBR in BRCA1\textsuperscript{mut/+} cells facing sufficient replication stress (Pathania et al. 2014). Thus, Pathania et al. (2014) reported the existence of innate haploinsufficiency for SFR and ‘conditional haploinsufficiency’ for HR-DSBR in their BRCA1\textsuperscript{mut/+} cells.

Similar to Pathania et al. (2014), ‘conditional haploinsufficiency’ has also been observed in the PALB2 mutation carrier cells, where G2/M checkpoint leakiness was only observed after 24 h of ETO treatment, but not in the undisturbed state or at early time points after ETO addition (Supplementary Table S2 of Study I). On the other hand, the observation of replication stress in the PALB2 mutation carrier cells is due to innate haploinsufficiency as increased origin firing was already observed in the undisturbed state. Thus, taken together, the results of the current study support the existence of innate haploinsufficiency for replication stress and ‘conditional haploinsufficiency’ for G2/M checkpoint maintenance in the PALB2 mutation carrier cells.

Genomic instability and tumorigenesis due to haploinsufficiency has previously been shown by several studies using isogenic cell lines, tumor-derived cell lines or mouse models (Barrett et al. 2015, Cochran et al. 2015, Coschi et al. 2014, Gonzalez-Vasconcellos et al. 2013, Konishi et al. 2011, Savage et al. 2014, Tan et al. 2017). However, to our best knowledge, this is the first study to show how haploinsufficiency promotes genomic instability and tumorigenesis in a patient-derived cohort. Since PALB2 facilitates the interaction between BRCA1 and BRCA2, we expect to see similar phenotypic changes also in cells heterozygous for mutations in BRCA1 and BRCA2.

6.2 Early-stage tumorigenesis in ABRAXAS c.1082G>A mutation carriers due to a dominant-negative effect mutation (Study II)

From RNA sequencing, we found stable expression of mutant ABRAXAS mRNA in all the carrier cell lines, indicating that the mutant mRNA is not undergoing nonsense-mediated mRNA decay. WB analysis also showed comparable ABRAXAS protein expression among our cohorts, indicating that the mutant
protein is also not undergoing proteolysis. This suggests that the defects observed in transcription, cell cycle checkpoint and DSB repair pathway choice could be due to the dominant-negative effect of mutant ABRAXAS in the carrier individuals.

In a previous report from our group, it was shown that the epitope-tagged mutant ABRAXAS protein expressed at near endogenous levels in HeLa, U2OS and MCF10A cells was stable and did not undergo proteolysis (Solyom et al. 2012). In a different study, it was shown that both the ABRAXAS mutant and wild-type protein forms have similar secondary structural patterns with the same number of α-helices and β-sheets, and also similar tertiary structure with comparable Tm (protein folding pattern) (Vikrant et al. 2015).

The ABRAXAS and BRCA1 cytoplasmic mislocalization observed in the ABRAXAS mutation carrier cells is due to the missense single nucleotide mutation that resides in the NLS domain of ABRAXAS. Similar to this observation, we reported earlier that the HA-tagged mutant ABRAXAS protein predominantly localized in the cytoplasm of HeLa, U2OS and MCF10A cells (Solyom et al. 2012). The NLS domain is a short stretch of amino acid responsible for the nuclear import of proteins (Kosugi et al. 2009). Recently, a missense mutation in the NLS sequence of POU4F3 (c.982A>G, p.Lys328Glu) was found to be associated with deafness in a Taiwanese family (Lin et al. 2017).

In the ABRAXAS mutation carrier cells, the observation of fewer ABRAXAS and BRCA1 foci together with diminished co-localization between them is due to the cytoplasmic mislocalization of the respective proteins. Similar to this, in our previous report, we also observed significantly less ABRAXAS and BRCA1 foci in Hela, U2OS and MCF10A cells overexpressing mutant ABRAXAS protein (Solyom et al. 2012). Most importantly, in our earlier report, co-immunoprecipitation experiments revealed that the epitope-tagged mutant ABRAXAS protein formed BRCA1-A complexes as efficiently as the wild-type protein, but the BRCA1-A complex containing the mutant ABRAXAS was localized predominately in the cytoplasm (Solyom et al. 2012). This indicates that only the localization, but not the formation of the BRCA1-A complex is altered in the ABRAXAS mutation carrier cells.

Several studies have shown that the BRCA1-A complex is responsible for DNA DSB signaling. In addition, this complex plays a major role in the choice of DNA DSB repair pathway (Coleman & Greenberg 2011, Hu et al. 2011b, Typas et al. 2015). Although in our current study no change was observed in NHEJ and HR repair pathway usage, activation of SSA and MMEJ is suppressed in ABRAXAS mutation carrier cells. These results indicate that activation of DSB
repair pathways, which utilize excessive nucleolytic end trimming such as SSA and MMEJ, is suppressed in ABRAXAS mutation carrier cells. As reported earlier, this result is exactly the opposite to the repair pathway usage observed in the Finnish heterozygous PALB2 c.1592delT carriers where a significantly higher usage of the MMEJ, SSA and SSA+HR repair pathways was seen (Obermeier et al. 2016).

γH2AX is a well-known marker for DNA DSBs and its phosphorylation precedes the choice of DSB repair pathway. The presence of less γH2AX in the ABRAXAS mutation carrier cells was unforeseen to us and the exact cause of it cannot be explained. However, similar to this result, another study reported significantly reduced γH2AX foci in ABRAXAS knockdown MCF7 cells transfected with ABRAXAS mutants, such as p.Gly39Val and p.Thr141Ile (Renault et al. 2016). Results from these two studies indicate that ABRAXAS, by a mechanism yet to be discovered, is responsible for the efficient phosphorylation of H2AX.

Several studies have shown that activation of CtIP and its recruitment to DNA damage sites is essential for the activation of both MMEJ and SSA pathways (Bennardo et al. 2008, Clerici et al. 2005, Lee-Theilen et al. 2011, Rass et al. 2009, Zhang & Jasin 2011). Surprisingly, similar to ABRAXAS and BRCA1, we have also observed cytoplasmic mislocalization of CtIP and consequently fewer CtIP foci in the ABRAXAS mutation carrier cells. Thus, the presence of reduced CtIP in the nucleus and its compromised recruitment to DNA damage sites could be the reason why both MMEJ and SSA are suppressed in our carrier cells. The compromised CtIP recruitment observed in the ABRAXAS mutation carrier cells is due to the compromised recruitment of BRCA1, similarly to that recently shown by Livingston and colleagues in their studies of BRCA1 mut/+ cells (Pathania et al. 2014).

Several studies have recognized that the BRCA1-A complex is essential for the regulation of the cell cycle, particularly G2/M checkpoint control (Solyom et al. 2012, Wang et al. 2007). Similar to the PALB2 mutation carrier cells in study I, we have observed G2/M checkpoint leakiness also in the ABRAXAS mutation carrier cells. In line with this, G2/M checkpoint leakiness was also observed after irradiation in 293T cells transfected with epitope-tagged mutant ABRAXAS protein (Solyom et al. 2012). Most importantly, in the ABRAXAS mutation carrier cells, we have also observed significant downregulation of ATM and its downstream effector kinase CHK2. As discussed in section 2.5.2, both ATM and
CHK2 play a key role in the efficient activation and maintenance of the G2/M checkpoint.

The presence of significantly higher 53BP1, γH2AX and RAD51 foci indicates persistence of DNA damage in the ABRAXAS mutation carrier cells. These results demonstrate that the ABRAXAS mutation carrier cells are unable to efficiently repair the DNA DSBs and are therefore left with more residual DNA damage. In an independent study, it was shown that ABRAXAS-deleted mouse embryonic fibroblasts (MEFs) had much higher percentage of unrepaired DNA damage after IR (Castillo et al. 2014).

In the ABRAXAS mutation carrier cells, there is only a marginal increase in the frequency of 1st pulse replication origins fired. It was previously shown that ABRAXAS is not required during replication as no changes were observed in stalled replication forks in ABRAXAS-deleted MEFs (Castillo et al. 2014). We believe that the observation of marginally increased origin firing in ABRAXAS mutation carrier cells is possibly due to the limited availability of BRCA1 as it is critically needed during replication (Schlacher et al. 2012).

Similarly, there was a potential increase in simple chromosomal rearrangements in the ABRAXAS mutation carrier cells, but limitations in the availability of further study material precluded a conclusive evaluation. Increased numbers of simple chromosomal aberrations have been reported after knockout or knockdown of multiple BRCA1-A complex members (Castillo et al. 2014, Coleman & Greenberg 2011, Hu et al. 2011b). Taken together, ABRAXAS mutation carrier cells display only a moderate increase in origin firing and chromosomal instability. This suggests that the phenotypes observed in the mutation carriers are not primarily driven by DNA replication stress.

As discussed in section 2.5.1, increased DNA repair by MMEJ and SSA as well as extensive replication stress such as that observed in the PALB2 mutation carriers has been shown to promote gross chromosomal aberration (Burrell et al. 2013, Iliakis et al. 2015, Nikkilä et al. 2013, Obermeier et al. 2016). Unlike the situation for PALB2, in ABRAXAS mutation carrier cells we did not observe any gross chromosomal aberrations which could be due to the reduced MMEJ and SSA pathway usage along with absence of extensive replication stress.

Previously, the BRCA1-A complex has been implicated in transcriptional regulation through its association with RAP80 (Wang et al. 2007). WB, qPCR and RT² profiler PCR array analyses indicated transcriptional misregulation of several genes in the ABRAXAS mutation carrier cells. Namely, a large number of genes implicated in DNA repair and damage response were found to be moderately, but
significantly downregulated. IPA analysis of the RNA-Seq data identified “Cancer” as the top-ranked disease associated with the ABRAXAS mutation carriers, which fits well with the observation of multiple tumors in these families (Solyom et al. 2012). The observation of hematological and immunological diseases as high-ranking IPA hits was not surprising because of the hemapoietic origin of the LCLs (Vaclová et al. 2015).

On the other hand, EdgeR and ENRICHR analysis primarily implicated enriched binding of PRC2 subunits, such as SUZ12 and EZH2 to our gene set in the ABRAXAS mutation carrier cells. The EZH2 subunit in the PRC2 complex trimethylates histone H3 at K27 to form H3K27me3, which is a major reversible epigenetic mark for transcriptional silencing (Hansen et al. 2008). Interestingly, ENRICHR analysis against the ENCODE histone modification database also simultaneously indicated enrichment of H3K27me3 in the same set of DEGs in ABRAXAS mutation carrier cells. Thus, taken together, transcriptional downregulation of multiple genes in the ABRAXAS mutation carrier cells could be due to globally increased PRC2 binding and H3K27me3 trimethylation.

Several studies have shown that BRCA1 plays an important role in transcriptional regulation (reviewed in Mullan et al. 2006, Savage & Harkin 2015). Most importantly, it was shown that BRCA1 inhibits the methyltransferase activity of PRC2 complex by targeting EZH2. BRCA1 inhibition increased EZH2 targeting and subsequently elevated H3K27me3 levels at PRC2 target loci (Wang et al. 2013). In our ABRAXAS mutation carrier cells, we have also noticed a moderate but significant downregulation of BRCA1 and this, in connection with the partial cytoplasmic mislocalization of BRCA1, could be the reason for the observation of PRC2 complex enrichment and elevated H3K27me3-mediated epigenetic silencing of several genes.

In summary, as shown in Figure 16, ABRAXAS cytoplasmic mislocalization and its compromised recruitment to the DNA damage sites is due to the mutation in the NLS sequence of the ABRAXAS gene. Furthermore, cytoplasmic mislocalization of BRCA1 and its compromised recruitment to the DNA damage sites is due to the dominant-negative effect of mutant ABRAXAS. Reduced BRCA1 localization and foci formation in the ABRAXAS mutation carrier cells results in attenuated BRCA1 signaling, altered DSB repair pathway choice, deregulated G2/M checkpoint maintenance, and transcriptional misregulation.
Complex combinations of rather small changes in the indicated pathways may be collectively required to promote tumorigenesis in the mutation carriers. As observed in the current Study (II), we expect to see similar phenotypic changes in cells heterozygous for mutations in other BRCA1-A members. Finally, the NLS sequence of ABRAXAS is frequently targeted in sporadic cancers (Castillo et al. 2014), suggesting that also somatic mutations disrupting the NLS are biologically relevant and may thus represent a driver of sporadic cancers.
7 Concluding remarks

Currently, only ~30% of hereditary breast cancer cases have been explained by mutations in the known breast cancer susceptibility genes (Rousset-Jablonski & Gompel 2017). A majority of the known breast cancer susceptibility genes encode proteins with integral roles in the DDR pathways (Mantere et al. 2017). It is well established that hereditary mutations in the known DDR genes often lead to genomic instability and eventually to tumorigenesis (Negrini et al. 2010). Nevertheless, the molecular mechanism of how these DDR deficiencies promote genomic instability and tumorigenesis is not well studied. Thus, the purpose of this thesis was to acquire mechanistically a better understanding of how genomic instability and tumorigenesis arise in the heterozygous PALB2 and ABRAXAS mutation carriers. Listed below are the major observations and conclusions from studies I and II:

1. **PALB2** c.1592delT causes a haploinsufficiency phenotype in the mutation carrier cells, resulting in elevated replication stress and G2/M checkpoint maintenance defects.
2. Elevated replication stress together with G2/M checkpoint leakiness is responsible for the observation of genomic instability in the **PALB2** mutation carrier cells. Therefore, loss of tumor suppressor/caretaker gene heterozygosity is not always mandatory for tumorigenesis.
3. **ABRAXAS** cytoplasmic mislocalization and its compromised recruitment to the DNA damage sites is due to the mutation in the NLS sequence of the **ABRAXAS** gene. Furthermore, BRCA1 cytoplasmic mislocalization and its compromised recruitment to the DNA damage sites is due to the dominant-negative effect of mutant **ABRAXAS**.
4. Reduced BRCA1 localization and foci formation in the **ABRAXAS** mutation carrier cells results in attenuated BRCA1 signaling, altered DSB repair pathway choice, deregulated G2/M checkpoint maintenance and transcriptional misregulation.
5. Finally, the tumorigenesis pattern observed in the **PALB2** mutation carriers is completely different from what was observed in the **ABRAXAS** mutation carriers.
8 Future prospects

1. The observance of genomic instability in the PALB2 mutation carrier cells due to elevated replication stress offers a new window for therapeutic targeting. Thus, future studies have to be performed to find out whether replication stress can be targeted to delay the onset of tumorigenesis or target the tumor clones in the latter stages.

2. Similarly, since G2/M checkpoint leakiness was observed in both ABRAXAS and PALB2 mutation carrier cells, future studies have to be conducted to find out whether it can be targeted to delay the onset of tumorigenesis, or alternatively, target the tumor clones in the latter stages.

3. Mechanistically, suppression of MMEJ and SSA activation in the ABRAXAS mutation carrier cells is not fully explained. Thus, future studies have to be performed to assess why specifically MMEJ and SSA are suppressed in the ABRAXAS mutation carrier cells.

4. Both PALB2 and BRCA2 define a distinct genetic, clinical and functional entity in the Fanconi anemia pathway. Thus, similar studies have to be performed to check whether the phenotype observed in the heterozygous BRCA2 mutation carrier cells is similar to that of PALB2 mutation carrier cells.

5. In addition to BRCA1 and ABRAXAS, germline mutations in other BRCA1-A complex members, such as RAP80 and MERIT40, are also associated with inherited predisposition to breast and ovarian cancers. Thus, similar studies have to be performed to check whether the phenotype observed in the heterozygous RAP80 and MERIT40 mutation carrier cells is similar to that of ABRAXAS mutation carrier cells.

6. Finally, the tumorigenesis pattern observed in the PALB2 mutation carriers is completely different from what was observed in the ABRAXAS mutation carriers. This indicates that though both ABRAXAS and PALB2 function in the same biological pathway (but in different niches), the pattern of tumorigenesis between them remains starkly different from what was previously envisioned. This observation warrants further investigations to analyze the tumorigenesis pattern both for different mutations in the same gene as well as in different tumor suppressor genes.


Original publications


Original publications are not included in the electronic version of the dissertation.

Contribution by the doctoral candidate:

For Study I – I was responsible for performing the lab works for Figure 3 and preparing that figure for publication. Additionally, I significantly contributed in making parts of the manuscript revision needed for its publication.

For Study II – I conducted the following experiments: WB analyses, quantitative PCR, flow cytometry analyses, protein localization analysis, DNA damage signalling PCR array, caspase activation assay, statistical analyses and the foci analysis. In addition, I was also actively involved in analysing the RNA-sequencing data. In terms of Figures included in the publication, I have generated the following main figures; Figure 1 A – E, Figure 3, 4, 5, Figure 6 A & B, Figure 7, 8 and 9. And also the following supplementary figures: S2, S3, S4, S5, S6 A – F, S7 A – D, S8, S9, S10, S11, S12, S13 C, S14 and S15. Additionally, I drafted the initial manuscript and upon Prof. Robert Winqvist and Adj. Prof. Helmut Pospiech suggestions, I prepared the final manuscript.
1449. Kajula, Outi (2018) Periyvyvän rintasyöpäalttiusmutaation (BRCA1/2) kantajamisten hypoteettinen perinnölöysysneuvontamalli
1452. Capra, Janne (2018) Differentiation and malignant transformation of epithelial cells: 3D cell culture models
1453. Panjan, Peter (2018) Innovative microbioreactors and microfluidic integrated biosensors for biopharmaceutical process control
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MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF ABRAXAS AND PALB2 GENES IN HEREDITARY BREAST CANCER PREDISPOSITION