

**Molecular and cellular basis for transformation associated
with BRCA1-haploinsufficiency**

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Abstract

Individuals with inherited mutations in *BRCA1* have a ~ 80% chance of developing breast and ovarian cancer within their lifetimes. Although *BRCA1* function appears to be essential for maintaining genomic integrity in all cell types, it is unclear why increased risk of cancer development in individuals with mutations in *BRCA1* is restricted to only a select few tissues. Moreover since loss of the remaining wild-type allele has been presumed to be a necessary and rate limiting event driving increased genomic instability and cancer formation, the tissue- and cell-type specific consequences of *BRCA1* haploinsufficiency responsible for tumorigenesis remain poorly understood. Here we show that human mammary epithelial cells (HMECs), but not other cell types from individuals harboring deleterious mutations in *BRCA1* (*BRCA1*^{mut/+}), exhibit increased genomic instability and rapid telomere erosion in the absence of tumor suppressor loss. Furthermore, we uncover a novel form of haploinsufficiency-induced senescence (HIS) specific to epithelial cells, which is triggered by pRb pathway activation rather than p53 induction. HIS and rapid telomere erosion in HMECs is mediated by misregulation of the NAD⁺-dependent deacetylase SIRT1 that leads to increased levels of acetylated pRb as well as acetylated H4K16 both globally and at telomeric regions. These results identify a novel form of cellular senescence and provide a molecular basis for the cell and tissue specific predisposition of breast cancer development associated with *BRCA1* haploinsufficiency.

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List of Abbreviations

ABC ATP-binding cassette

Akt protein kinase B

APC adenomatous polyposis coli

APC/C anaphase promoting complex/cyclosome

AROS active regulator of SIRT1

ATM ataxia telangiectasia mutated

ATR ataxia telangiectasia and Rad3-related protein

BACH1 transcription regulator protein

BARD1 BRCA1 Associated RING Domain protein 1

bFGF basic fibroblast growth factor

BLG B-lactoglobulin

BRCA1 breast cancer 1, early gene

BRCA1^{mut/+} BRCA1 germline mutation carrier

BRCA2 breast cancer 2, early gene

BRCC36/45 ubiquitin hydrolase

BRCT BRCA1 C-terminal

BRD7 bromodomain-containing protein 7

BRG1 ATP-dependent helicase SMARCA4

BRIP1 BRCA1-interacting protein 1

BubR1 mitotic checkpoint serin/threonine-protein kinase BUB1 beta

cAMP/PKA cyclic AMP/protein kinase A

CDH1 cadherin 1

CDK2/4/6 cyclin dependent kinases

CDKN1A cyclin-dependent kinase inhibitor 1A, p21

CHEK2 checkpoint homolog 2, serine/threonine-protein kinase

ChIP chromatin immunoprecipitation

CHK1 checkpoint homolog 1, serine/threonine-protein kinase

CK cytokeratin

CK2 Casein kinase 2

CKI cyclin-dependent kinase inhibitors

Cre tyrosine recombinase enzyme

CS cowden syndrome

CtIP CtBP-interacting protein

Cyclin D1 G1/S specific cyclin-D1

DBC1 deleted in breast cancer 1

DDR DNA damage repair

DNA deoxyribonucleic acid

DSB double strand breaks

DYRK1A/DYRK3 dual specificity tyrosine phosphorylation-regulated kinases

EGF endothelial growth factor

ER estrogen receptor

ERBB2/Her2/neu human epidermal growth factor receptor 2

FANCD1 Fanconi anemia associated gene BRCA2

FasL fas ligand

FOXO forkhead box

G1 gap1 phase of cell cycle

G2 gap 2 phase of the cell cycle

GADD45 growth arrest and DNA damage 45

GFP green fluorescent protein

GI gastrointestinal

GRO growth-related oncogene

GSEA gene set enrichment analysis

H1K26 lysine 26 on histone 1

H2A one of five histones in nucleosome

H2B one of five histones in nucleosome

H3 histone 3, one of five histones in nucleosome

H3K27 lysine 27 on histone 3

H3K56 lysine 56 on histone 3

H3K9 lysine 9 on histone 3

H4 histone 4, one of five histones in nucleosome

H4K16 lysine 16 on histone 4

H4K20 lysine 20 on histone 4

HDAC1/2 histone deacetylase 1/2

HDEs human dermal epithelium, keratinocytes

HDFs human dermal fibroblasts

HGF hepatocyte growth factor

HIS haploinsufficiency induced senescence

HMECs human mammary epithelial cells

HMFs human mammary fibroblasts

HR homologous recombination

hTERT human catalytic subunit of telomerase

ICAM intercellular adhesion molecule

IF immunofluorescence

IFN γ interferon gamma

IGF insulin-like growth factor

IGFBP insulin-like growth factor-binding protein

IHC immunohistochemistry

IL-6/7/1a and **b/8** interleukins

INK4a family of proteins containing structural motifs called ankryn-like repeats that inhibit the activity of cyclin-dependent kinases

IP immunoprecipitation

IR ionizing radiation

IRF7 interferon regulatory factor 7

JNK1 C-Jun N-terminal kinase 1

LFS Li-Fraumeni syndrome

LOH loss of heterozygosity

M mitosis

Mad2 mitotic arrest deficient 2

M0 stasis

M1 senescence

M2 agonescence

MCP membrane cofactor protein

MDC1 mediator of DNA damage checkpoint 1

MIP macrophage inflammatory protein

MMP matrix metalloproteinase

MMTV mouse mammary tumor virus

MRI magnetic resonance imaging

MRN DSB detection complex consisting of MRE11, RAD50, and NBS1

mRNA messenger ribonucleic acid

mTOR mammalian target of rapamycin

MYC v-myc myelocytomatosis viral oncogene homolog (avian)

NAD nicotinamide adenine dinucleotide

NBA1 new component of the BRCA1A complex

NES nuclear export sequence

NF1 neurofibromatosis type 1

NLS nuclear localization sequence

NOD/SCID immunocompromised strain of mice

NPM1 nucleophosmin

NUFIP nuclear fragile X mental retardation-interacting protein

OIS oncogene-induced senescence

p53/TP53 tumor protein 53

p53BP1 p53 binding protein 1

PAI plasminogen activator inhibitor

PALB2 partner and localizer of BRCA2

PARP poly(ADP-ribose) polymerase 1

PDs population doublings

PGE2 prostaglandin E2

PR progesterone receptor

pRb retinoblastoma protein

PTEN phosphatase and tensin homolog

q-FISH quantitative fluorescence *in situ* hybridization

qPCR quantitative PCR

Rad50 DNA repair protein

Rad51 DNA repair protein

RAP80 ubiquitin-interacting motif-containing receptor-associated protein 80

Ras small GTPase

RbAp46/48 retinoblastoma associated proteins 46/48

RING finger really interesting new gene domain

RNAPII rna polymerase II

RNF168 E3 ubiquitin ligase

RNF8 RING finger protein 8

ROS reactive oxygen species

S synthesis phase of cell cycle

S100A7 S100 calcium-binding protein A7

SAHFs senescence associated heterochromatic foci

SASFs senescence associated secretory factors

SA- β -galactosidase senescence associated-beta-galactosidase enzyme

SCD serine containing domain

SDF-1 stromal cell-derived factor

Sir2 silent information regulator 2

SIRT1 sirtuin (silent mating type information regulation 2 homolog) 1

SSB single stranded breaks

ssDNA single stranded DNA

STAT1 signal transducers and activators of transcription

STK11/LKB1 serine/threonine kinase 11/liver kinase B1

SUV39H1 histone-lysine N-methyltransferase

SV40 simian vacuolating virus 40

SWI2/SNF2 switch/sucrose non-fermentable 2

TER telomere erosion rate

Terc telomerase RNA component

TIFs telomere dysfunction induced foci

TIMP tissue inhibitor of metalloproteinase

TOPBP1 DNA topoisomerase 2-binding protein 1

TopoII α topoisomerase II α

TRF1/2 telomeric repeat-binding factor 1

UBC13 ubiquitin conjugating enzyme 13

uPAR urokinase-type plasminogen activator receptor

UV ultraviolet light

VEGF vascular endothelial growth factor

VHL Von Hippel-Lindau tumor suppressor

WAP whey acidic protein

α SMA alpha smooth muscle actin

γ H2AX phosphorylated histone H2AX on serine 139

CHAPTER 1.

INTRODUCTION

1.1 Brief overview of anatomy and physiology of the human breast

The breast is a structurally dynamic organ that partially develops during embryogenesis, and undergoes further tissue remodeling during puberty as well as pregnancy. After the first round of pregnancy, the breast gland is considered fully mature (Gusterson and Stein, 2012). It is organized into 15-20 lobes (glands) arranged radially around the nipple and embedded in a mix of connective and adipose tissues. Each lobe contains a number of lobules, which are branched tubulo-alveolar structures with secretory acini (Figure 1.1A, B). These drain into intralobular ducts that are connected to a single large duct, the lactiferous duct, which drains each lobe via a separate opening on the surface of the nipple (Hassiotou and Geddes, 2013).

The ducts and lobules are composed of stratified epithelium containing two distinct types of mammary epithelial cells. Luminal epithelial cells line the inner lumen, express cytokeratins (CK) 8, 18, and 19, are responsive to hormones, and produce milk (Figure 1.1A, B). Basal/myoepithelial cells underlie the luminal cells and are in direct contact with basement membrane - a barrier between mammary parenchyma and the stroma. These cells express alpha smooth muscle actin (α SMA), CK14, and CK5, and provide the contractile force to expel milk during lactation (Figure 1.1A, B) (Hassiotou and Geddes, 2013). Increasing evidence suggests that there is a differentiation hierarchy within breast epithelial cells. In simplified terms, it is thought that there is a common mammary stem cell that gives rise to early bipotent progenitors. These cells can differentiate into either luminal or basal progenitor cells. While luminal progenitors can give rise to ductal and alveolar luminal lineage, the basal progenitors give rise to the basal/myoepithelial cells (Visvader, 2009). This notion has recently been revised with lineage tracing experiments showing that bipotent progenitors become committed to a particular lineage at birth. Bipotent

progenitor activity in post-natal development and tissue maintenance seems to be induced primarily in the context of transplantation or tissue injury (Van Keymeulen *et al.*, 2011; Zeng and Nusse, 2010).

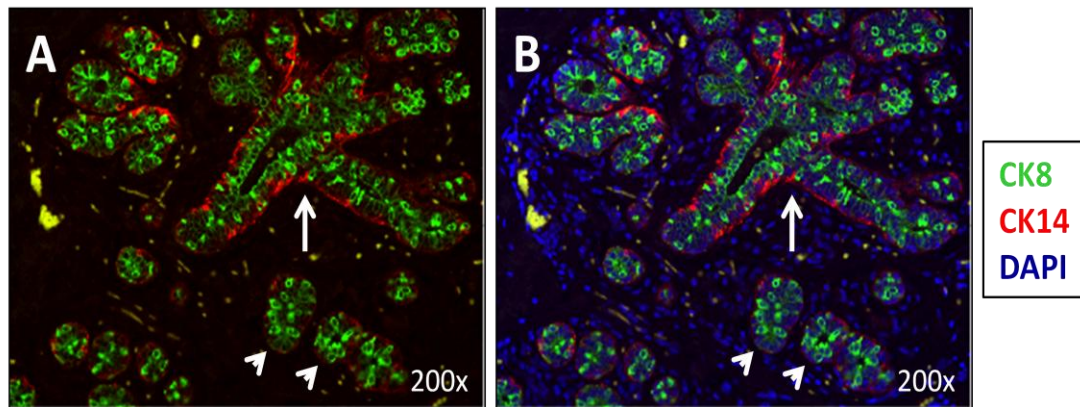


Figure 1.1. Human breast tissue. Cross sections of human breast lobe stained for basal and luminal cytokeratins using immunofluorescence. The extended arrow marks the intralobular duct, while the short arrows mark lobules. A) Luminal epithelial cells are CK 8 positive (green) whereas basal cells are CK14 positive (red). B) CK8, CK14 and DAPI (DNA, blue) stains merged. Image courtesy of Lisa Arendt.

In addition to different cell types present in breast parenchyma, local cell-cell interactions as well as systemic hormones play a major role in breast development and function. The cell-cell communication is mediated by a variety of growth factors, including epidermal growth factor, transforming growth factor, and fibroblast growth factor. Furthermore, growth and proliferation of breast epithelium is stimulated by estrogen and progesterone hormones, while epithelial differentiation, milk production and secretion are induced by prolactin and oxytocin. Other hormones necessary for the production of breast milk include: insulin, cortisol, thyroid hormone, parathyroid hormone, parathyroid hormone-related protein, and human growth hormone (Neville *et al.*, 2002).

1.2 Breast cancer

Breast cancer forms in tissues of the breast, specifically in the epithelial cells of the ducts and the lobules. In the United States, it is the most frequently occurring cancer (31% of all cancer cases) and the leading cause of death in women. Although over the last decade (1999-2009) breast cancer incidence and mortality rates have declined, it is estimated that in 2013 alone there will be 232,340 new breast cancer cases and 39,620 breast cancer related deaths. The median age at breast cancer diagnosis is 61, while the median age at death is 68 (Howlader *et al.*, 2013). Even though age represents a major risk factor, other important aspects of risk include hormones (both endogenous and exogenous), genetics (family history), reproductive history (age of menarche, childbearing, and menopause), obesity, and radiation exposure (Key *et al.*, 2001). Because of complexity and largely unknown molecular etiology of breast cancer, the majority of breast cancer cases are considered to be sporadic (70-80%). This means that a large number of individuals that develop breast cancer do not have a family history of this disease. The rest of the cases (20-30%) are categorized as familial types, since they are often seen in families with high incidence of breast cancer (Ellsworth *et al.*, 2010).

1.2.1 *Genetics of sporadic breast cancers*

Sporadic breast cancers are thought to arise from a serial stepwise accumulation of somatic mutations without evidence of any germline mutation that could be involved. Therefore, major factors that could fuel the propagation of genetic errors and development of sporadic breast cancers are considered to be age and exposure to sex hormones. It is presumed that the events during the progression of

sporadic breast cancers involve mutational activation or amplification of oncogenes, often coupled with loss of tumor suppressor genes. Many different genes have been implicated in breast cancer tumorigenesis, implying that in breast cancer there are no clear cancer driver genes as there are in other types of cancer such as melanoma or colon cancer. Here, we will focus on several genes that are found to be mutated in large number of sporadic breast cancer cases: *MYC*, *CCND1* (Cyclin D1), *ERBB2* (Her2/neu), *TP53*, *CDH1*, and *ER α* (Ingvarsson, 1999; Kenemans *et al.*, 2004). *MYC* oncoprotein is a transcription factor that regulates expression of genes mainly involved in cell proliferation, metabolism, differentiation and apoptosis. In breast cancers *MYC* deregulation involves gene amplification, overexpression, as well as mRNA and protein stabilization (Xu *et al.*, 2010). *Cyclin D1* is an important regulator of cell-cycle progression, and in breast cancers it is mainly found to be overexpressed or amplified (Roy and Thompson, 2006). *ERBB2/Her2* is a member of epidermal growth factor receptor family, which contains an intrinsic tyrosine kinase activity. Similarly to Cyclin D1, it has been shown that amplification or overexpression of Her2 plays an important role in pathogenesis of breast cancer (Tan and Yu, 2007). *TP53* is an important tumor suppressor gene essential for controlling cell-cycle progression and apoptosis. In sporadic breast cancer it is often inactivated by point mutations resulting in proteins that are defective for sequence-specific DNA binding and activation of p53-responsive genes (Ko and Prives, 1996). *CDH1* gene located on chromosome 16 encodes E-cadherin protein – a calcium-dependent cell adhesion molecule, involved in homophilic cell-cell interactions. It is found mutated in sporadic lobular breast tumors (Berx *et al.*, 1995). *ER α* encodes one of the most important growth factor receptors implicated in development of hormone-dependent sporadic breast cancers. Estrogen receptor α is involved in activation of transcription

of genes such as cell-cycle control proteins that induce cell proliferation. ER α is frequently found to be overexpressed in early stages of breast cancer (Hayashi *et al.*, 2003).

Even though breast cancer is referred to as one disease, large scale gene expression profiling to stratify breast cancers into similar clusters has revealed a considerable heterogeneity within this disease. Several histological and molecular subtypes have been identified, each of which displays a unique set of markers, responds differently to various treatment regimens and has a different outcome (Figure 1.2). This analysis is closely associated with a previously described classification distinguishing tumors according to the hormone receptor status (Sørli *et al.*, 2001).

Hormone receptor positive breast cancers include luminal-A and luminal-B molecular subtypes. Luminal tumors express genes similar to the luminal mammary epithelial cells as well as estrogen and progesterone receptors. They are considered to be more differentiated with a relatively low proliferation index. Luminal tumors encompass the majority of breast cancers and generally have the best prognosis across all subtypes (Figure 1.2). Hormone receptor negative breast cancers include Her2-positive and basal-like subtypes. Amplification or overexpression of Her2 gene as well as the genes on the same amplicon as Her2 are the main characteristic of Her-2 positive tumors. Features commonly observed in the basal-like breast cancers include expression of genes typical of basal mammary epithelial cells and absence of ER, PR and Her2 expression. Claudin-low tumors, a subtype of basal-like breast cancers, have gene expression comparable to the mammary stem cell signature, decreased expression of genes implicated in cell-cell contact and display features of mesenchymal rather than epithelial cells. Because basal-like breast cancers are

typically of high histological grade (low differentiation and high proliferation indexes) they overall have a poor prognosis (Figure 1.2) (Sørli *et al.*, 2001; Hennessy *et al.*, 2009).




Hormone status	+	-
Subtypes	Luminal A Luminal B	Her2 Basal Claudin low
Characteristics	 differentiation	
	 proliferation	
	 prognosis	

Figure 1.2. Heterogeneity of breast cancer. Luminal A and B breast cancers are hormone receptor positive, with high differentiation and low proliferation indexes and overall good prognosis. Her2, basal, and claudin low are hormone receptor negative breast cancers, with low differentiation and high proliferation indexes and overall poor prognosis.

1.2.2 Genetics of hereditary breast cancers

Women who develop breast cancer and have two or more family members diagnosed with breast cancer before 60 years of age are considered to have a familial type of this disease (Figure 1.3). Hereditary breast cancers account for approximately half of all familial breast cancer cases and are associated with germline mutations in high penetrance susceptibility genes such as BRCA1, BRCA2, TP53, PTEN and STK11 as well as moderate penetrance susceptibility genes such as ATM, CHEK2, BRIP1, and PALB2 (Figure 1.3). According to Knudson’s “two-hit” hypothesis, an early event in development of the hereditary type of breast cancer requires

inactivation of the second allele of these tumor suppressor genes. Also as predicted by Knudson, most hereditary types of breast cancers are diagnosed early in life, well before mean age at diagnosis of sporadic cancers (Kenemans *et al.*, 2004, Lalloo and Evans, 2012).

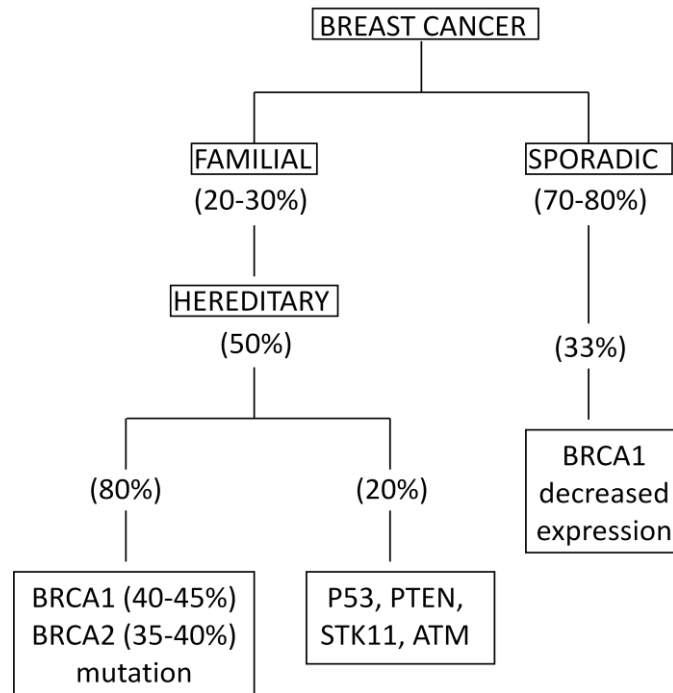


Figure 1.3. Genetics of breast cancer. 70-80% of breast cancers are sporadic, while 20-30% of cases are familial. Half of all familial breast cancers are hereditary. Germline mutation in BRCA1/2 genes is associated with 80% of hereditary breast cancers, while germline mutation in other high and moderate penetrance susceptibility genes is associated with 20% of cases. In addition, BRCA1 protein expression is decreased in 33% of sporadic invasive breast cancers. Adapted from Kennedy *et al.*, 2004.

BReast Cancer Associated gene 2 (BRCA2) was cloned in 1995, immediately after BReast Cancer Associated gene 1 (BRCA1). BRCA2 germline mutations account for 35-40% of cases in families with breast and ovarian cancer. Female BRCA2 mutation carriers have 30- 60% risk of developing breast cancer and 30%

risk of developing ovarian cancer. In addition, 14-20% of male BRCA2 mutation carriers are predisposed to prostate and breast cancers. The relative risk of cholangiocarcinoma, melanoma, pancreatic and gastric cancers are also increased. BRCA2 gene is located on chromosome 13 and encodes a protein crucial for DNA double strand break repair via homologous recombination pathway. Frameshifts, missense mutations and large gene rearrangements occur throughout the gene in BRCA2-mutation carriers. Biallelic mutations in BRCA2 are found in Fanconi anemia (FANCD1) patients, a syndrome causing short stature, microcephaly, childhood solid tumors and haematological malignancies (Nathanson *et al.*, 2001; Hemel and Domchek, 2010; Lalloo and Evans, 2012).

	Gene	Syndrome
High penetrance	BRCA1	HBOCS
	BRCA2	HBOCS
	TP53	Li-Fraumeni
	PTEN	Cowden
	STK11	Peutz-Jeghers
Moderate	ATM	Louis-Bar
	CHEK2	Li-Fraumeni
	BRIP1	Fanconi Anemia J
	PALB2	Fanconi Anemia N

Figure 1.4. Genetics of hereditary breast cancer. BRCA1/2 are high penetrance susceptibility genes associated with hereditary breast and ovarian cancer syndrome (HBOCS). Listed are also other genes associated with specific syndromes and high breast cancer risk.

Germline mutations in the TP53 gene, located on chromosome 17, are associated with Li-Fraumeni syndrome (LFS) (Figure 1.4). LFS is inherited in an

autosomal dominant manner and is a rare (1 in 20,000 people) cancer predisposing syndrome characterized by early-onset breast cancers, sarcoma, acute leukemias, brain tumors and adrenal cortical tumors. A woman with LFS has a breast cancer risk of ~ 90% (Nathanson *et al.*, 2001; Hemel and Domchek, 2010; Lalloo and Evans, 2012).

Cowden syndrome (CS) is associated with germline mutations in PTEN gene located on chromosome 10. Like LFS, CS is also a rare cancer predisposition (1 in 200,000 people) and is characterized by facial/buccal lesions, GI hamartomas, thyroid and endometrial lesions and macrocephaly. CS is inherited in an autosomal dominant fashion, as well. Lifetime risk of breast cancer for women with CS is estimated to be between 25% and 50%. PTEN encodes a lipid phosphatase enzyme that functions in tumor suppression by also regulating the cell-cycle progression and apoptosis (Nathanson *et al.*, 2001; Hemel and Domchek, 2010).

Germline mutations in STK11/LKB1, inherited in autosomal dominant manner, cause Peutz-Jeghers syndrome characterized by childhood GI hamartomas and cancers, pigmentation of lips, buccal mucosa, hands and feet. The relative risk of breast cancer associated with this syndrome is 55% compared to non-carriers. STK11/LKB1 is a tumor suppressor gene encoding serine-threonine kinase (Nathanson *et al.*, 2001).

Homozygous mutations in ATM, a protein kinase involved in the response to double-stranded DNA breaks, are linked to ataxia telangiectasia. This is an autosomal recessive condition characterized by hypersensitivity to ionizing radiation, increased incidence of malignancies (primarily hematologic), progressive cerebellar ataxia, facial and conjunctival telangiectasias, combined immunodeficiency, and growth retardation. It has been estimated that heterozygotes have 15.7-fold increase in breast

cancer risk compared to non-carriers (Nathanson *et al.*, 2001; Lalloo and Evans, 2012).

CHEK2 encodes a checkpoint kinase that is a signaling component in the cellular response to DNA damage. Recent meta-analysis suggests that in carriers of a particular germline mutation, 1100delC, breast cancer risk is as high as 37% by age 70 (Lalloo and Evans, 2012).

BRIP1 encodes a helicase which is a binding partner of BRCA1 and therefore functions in double strand break repair. Truncating mutations in this gene were identified in samples from breast cancer families. Segregation analysis assessed a relative risk (RR) of breast cancer of 2.0 (95%, CI 1.2-3.2), although it has been reported that the risk maybe higher in some families. Biallelic mutations of BRIP1 cause Fanconi anemia complementation group J (FANC-J), a condition that increases the likelihood of bearing childhood solid tumors (Lalloo and Evans, 2012).

PALB2 encodes a protein that interacts with BRCA2 during double strand break repair. PALB2 mutations are associated with relative risk of breast cancer of 2.3 (95%, CI 1.4-3.9). Biallelic mutations in PALB2 have been found in patients with FANC-N, a condition associated with increased risk of pancreatic cancer (Lalloo and Evans, 2012).

In summary, considering that breast cancer is one of the most common and deadliest types of cancer, it is surprising how little we know about genetics and the early events that are involved in the progression of this disease. This is true for sporadic as well as for hereditary cases. The advances in understanding of breast cancer have been largely stalled by the fact that it is not one and the same disease in all patients. Only recently we have come to realize the extent of heterogeneity of breast cancer, and that perhaps many cell intrinsic (genetics, cell-types, etc.), as well

as, extrinsic factors (exposure to hormones and carcinogens) play an important role in the formation of different types of this disease. Furthermore, a common theme in hereditary types of breast cancer is that the disease is associated with mutations in genes involved in DNA damage repair, implying that processes that function to maintain genome integrity are particularly important for tumor suppression in breast epithelium. Thus, studies of hereditary types of breast cancer may help delineate genetic events that are also important in sporadic breast tumorigenesis.

1.3 Early events in malignant transformation

1.3.1 Bypass of proliferative barriers is required for transformation

Normal cells must acquire certain biological alterations in order to transform into cancer cells. These biological changes seem to occur over a long period of time and in a multistep process involving several genetic and epigenetic events. The process is best exemplified by the progression of colon carcinomas (Figure 1.5). Extraction and analysis of genomic DNA from various stages of colon cancer revealed that the path of progression from benign to malignant lesions correlated with an increase in the number of altered genetic loci (Figure 1.5). Further analysis of cancer genomes led to the precise identification of oncogenes and tumor suppressor genes that are found to be commonly mutated in cancer cells. These genes are key regulators of cellular processes related to cell growth and division and disruption of their normal function is thought to confer growth advantage to cancer cells (Vogelstein and Kinzler, 1993; Weinberg, 2007). This idea was further supported by *in vitro* studies where transformation of human and rodent cells in culture was achieved by defined genetic elements involving overexpression of oncogenes and

inactivation of tumor suppressor genes. Interestingly, transformation of rodent cells required two cooperating oncogenes to be expressed, while human cells required disruption of additional intracellular pathways involved in cell-cycle regulation and survival, revealing that there are fundamental differences in biology of human and rodent cells (Land *et al.*, 1983; Hahn *et al.*, 1999). Furthermore, these and subsequent studies helped delineate cellular events that are important in tumorigenesis. There are at least six essential alterations in cell physiology that enable cells to become tumorigenic and ultimately malignant. These features/hallmarks of cancer cells include sustained activation of growth signaling, evasion of growth suppressors, resistance to apoptosis, ability to replicate indefinitely, induction of angiogenesis and activation of invasion and metastasis (Hanahan and Weinberg, 2000). These concepts have been recently updated to add two new emerging hallmarks capabilities which include reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg 2011).

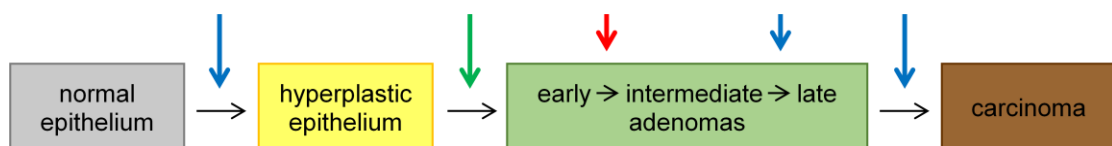


Figure 1.5. Progression of colon carcinoma. Differently colored arrows represent genetic and epigenetic events in progression of colon carcinoma from benign to malignant lesions. Blue arrows represent loss of tumor suppressor genes, red arrow represents activating mutation of an oncogene, and green arrow represents an epigenetic event involving DNA hypomethylation. Adapted from Weinberg, 2007.

Evasion of growth suppression is an important early event in tumor progression. Evidence for this is provided by the fact that the genes involved in control of cellular proliferation, pRb and p53 (also referred to as the gatekeepers), are the most prominent tumor suppressors disrupted in cancer. pRb is a master regulator

of G1-S transition and cell-cycle progression. p53 acts as a critical sensor of cell intrinsic pathways involved in genomic maintenance and metabolism. In a scenario where a cell encounters aberrant growth stimulus, multiple rounds of cell division can greatly increase the probability of propagating mutated or damaged genetic material. Activation of p53 and pRb can counteract this by inducing a specific type of permanent cell cycle arrest, termed senescence. Loss of any one or both of these checkpoints leads to continued proliferation, telomere attrition, telomere-end fusions and chromosome breakage-fusion-bridge cycles that finally trigger crisis or cell death. Therefore, two distinct states – senescence and apoptosis – emerge as important barriers standing in the way of uncontrollable proliferation and cellular transformation. Apoptosis causes death and elimination of damaged cells from the tissue. In contrast, senescence induces permanent growth arrest without eliminating those cells from the tissue. It is thought that these two proliferative barriers are important anti-tumorigenic mechanisms occurring *in vivo*, limiting replicative division of mutant, pre-neoplastic cells (Newbold, 2002; Hahn, 2002; Shay and Wright, 2005).

1.3.2 Cellular senescence is an important tumor-suppressive barrier

Cellular senescence is a state in which cells have permanently lost their ability to proliferate, but are still viable and metabolically active. The cell cycle arrest associated with senescence is mediated by active tumor suppressor network of genes such as p53 and pRb, as well as cell cycle inhibitors such as cyclin-dependent kinase inhibitors (CKIs: p21^{Cip1/Waf1} and p27^{Kip1}) and INK4a family members (p15^{INK4b} and p16^{INK4a}) (Lowe *et al.* 2004; Ben-Porath and Weinberg, 2005; Campisi, 2005). Senescent cells display considerable morphological changes; they become enlarged,

flat and multinucleated. They also exhibit increased SA- β -galactosidase activity, which becomes detectable at pH 6 (Dimri *et al.*, 1995). In some cells senescence is accompanied by regional chromatin condensation making nuclei appear dotted after DNA staining (Narita *et al.*, 2003). It is thought that these changes in chromatin structure substantially alter gene expression profile of senescent cells, resulting in increased expression and secretion of cytokines and chemokines (Shay and Wright, 2005; Kuilman *et al.*, 2010). Over the recent years a number of studies have found senescent cells in mouse models of tumor progression as well as in human lesions suggesting that senescence stands as an important barrier in tumor progression *in vivo*. However, it is of increasing concern that the secretome of senescent cells can create a pro-inflammatory environment that can have pro-oncogenic effects on neighboring cells (Coppe *et al.*, 2010). Given these conflicting features of senescent cells it unclear whether senescence is more beneficial or more harmful to the tissue. Several mechanisms have been proposed to induce cellular senescence - most of them converge on pathways involving stress, telomere biology, and DNA damage response (Shay and Wright, 2005; Kuilman *et al.*, 2010).

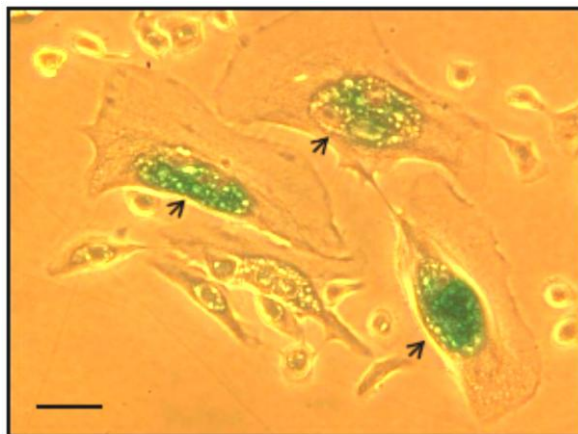


Figure 1.6. Senescent cells in culture. Arrows point to cells in culture that display typical features of senescence: enlarged, flat and SA- β -galactosidase positive.

1.3.3 Replicative senescence (M1)

Permanent growth arrest that ensues after multiple rounds of cellular replication and persistent telomere erosion is termed replicative senescence or Hayflick limit (Hayflick and Moorhead, 1961). Telomeres are specialized DNA structures that protect the ends of chromosomes from the double strand break repair machinery. With each round of cell division telomeres shorten because DNA polymerase fails to complete the replication of the lagging strand (Olovnikov, 1971; Watson, 1972). Since most human cells do not express telomerase (a specialized enzyme capable of extending telomeres), the telomere sequence is never fixed. Therefore, when telomeres become critically short, their protective structure is disrupted resulting in activation of DDR and p53 signaling pathway, as well as upregulation of p16^{INK4a} levels. Both pathways lead to activation of pRb, cell-cycle arrest and induction of senescence. Inhibition or loss of p53, p16^{INK4a}, pRb, as well as ectopic expression of catalytic subunit of telomerase (hTERT) can help overcome this proliferative barrier (D'Adda di Fagagna *et al.*, 2003; Shay and Wright, 2005; Kuilman *et al.*, 2010).

1.3.4 Stress-induced senescence in vitro (M0)

Expplanting cells from tissues and placing them in culture often triggers stress induced senescence. This is usually due to inadequate culturing conditions (such as abnormal concentration of nutrients, growth factors and presence of ambient O₂) and failure of cells to adapt to an artificial environment. Mouse embryonic fibroblasts (MEFs), for example, undergo premature senescence after several passages in culture even though they retain long telomeres (Loo *et al.*, 1987). This type of senescence is not associated with telomere attrition and is characterized by cell-cycle arrest mediated by active pRb signaling pathway. Stress-induced senescence can be

alleviated and cellular lifespan increased by changing culturing conditions, such as using serum-free media and/or a feeder layer (Shay and Wright, 2005; Kuilman *et al.*, 2010).

1.3.5 Oncogene-induced senescence

Overexpression of proto-oncogenes such as H-ras^{V12}, BRAF^{E600}, Myc and E1A alone in primary cells induces cell-cycle arrest and changes in morphology resembling that of cells undergoing replicative senescence (Serrano *et al.*, 1997). This proliferative barrier is known as oncogene-induced senescence (OIS). Characteristics of OIS include p38-MAPK activation as well as increased DNA damage response and activation of p53 and pRb signaling pathways (Wang *et al.*, 2002, Di Micco *et al.*, 2006). However, in some human cells, such as freshly isolated fibroblasts, OIS depends solely upon increased p16^{INK4a} levels. This suggests that OIS can vary according to the cell type, and with regards to senescence in some cells upregulation of p16^{INK4a} can play a more prominent role than p53. Consequently, if the p16^{INK4a} – pRb pathway is abrogated in these cells OIS is bypassed. In addition, OIS does not seem to be associated with telomere shortening since ectopic expression of hTERT in cells undergoing OIS does not lead to OIS bypass (Ben-Porath and Weinberg, 2005; Kuilman *et al.*, 2010).

1.3.6 Tumor suppressor loss-induced senescence (TSLIS)

Loss of tumor suppressor genes such as pRb results in increased rates of cellular proliferation and confers proliferative advantage during neoplastic transformation. However, not all tumor suppressor genes function the same. Studies of various tumor suppressors revealed that these genes can function in different ways to restrain tumor growth. For example, inactivation of some of tumor suppressors

leads to the induction of premature senescence, further suggesting that not all tumor suppressor genes are alike. Given these properties, tumor suppressors were further divided into groups of gatekeeper genes and caretaker genes. Generally, gatekeepers directly regulate tumor growth by inhibiting proliferation and promoting cell death. On the other hand, caretakers inhibit tumor initiation indirectly by maintaining genome integrity and lowering the rate of mutation in cells (Kinzler and Vogelstein, 1997).

It has been shown that loss of NF1, VHL, PTEN or BRCA1 can lead to premature senescence in human and mouse cells. Loss of each tumor suppressor gene engaged different mechanism in induction of premature senescence. NF1 loss results in activation of Ras and its downstream effectors ERK and Akt. Strong auto-inhibitory loops shut off downstream responses of Ras soon after initial activation and induce cell-cycle arrest (Courtois-Cox *et al.*, 2006). Premature senescence in VHL-deficient cells seems to be dependent on pRb pathway and SWI2/SNF2 p400 chromatin remodeling activity (Young *et al.*, 2008). On the contrary, PTEN loss induces senescence through mTOR and potent p53 stabilization completely independent of DNA damage repair (DDR) (Alimonti *et al.*, 2010). Furthermore, BRCA1 deficiency in mouse embryonic fibroblasts also induces premature senescence in p53-DDR-dependent manner (Cao *et al.*, 2002). Recently, it has been shown that BRCA1-knockdown in human fibroblast resulted in premature senescence due to increased association of BRG1 with chromatin and pRb, leading to increased sites of chromatin condensation (Tu *et al.*, 2013). Collectively, these studies suggest that tumor-suppressors are more complex than previously thought and that loss of their function can often induce potent cell-cycle arrest and premature senescence due to aberrant activation of cell signaling circuits as well as chromatin remodelers.

1.3.7 Cellular immortalization and requirements needed to bypass proliferative barriers

Another important event in tumor progression is acquisition of immortalized growth. *In vitro* experiments helped establish important events that result in senescence bypass and cellular immortalization in mammalian cells (reviewed by Rangarajan and Weinberg, 2003). Normal murine fibroblasts frequently undergo spontaneous immortalization in culture. However, normal human fibroblasts rarely immortalize in culture, suggesting that human cells are more resistant to spontaneous immortalization when compared to mouse cells (Newbold *et al.*, 1982). These observations led to an important discovery regarding telomere length and telomere maintenance in mice and humans: telomeres in mouse cells are 40-60 kilobases long, versus 10 kilobases in human cells. In addition, mouse cells have endogenous telomerase activity, while most human cells do not (Prowse and Greider, 1995; Kim *et al.*, 1994). Therefore, it has been shown that one of the important requirements needed for immortalization of human fibroblasts was telomerase (Vaziri and Benchimol, 1998). Furthermore, requirements for immortalization of normal human epithelial cells such as mammary epithelial cells (HMECs) and keratinocytes (HDEs) have also been examined. Unlike fibroblasts, ectopic expression of hTERT alone was not sufficient to immortalize human epithelial cells. Instead, immortalization of HMECs required additional events such as loss of p16^{INK4a} together with hTERT overexpression, suggesting that epithelial cells were even more resistant to spontaneous immortalization when compared to fibroblasts (Kiyono *et al.*, 1998; Newbold, 2002; Shay and Wright, 2005). Therefore, cellular requirements for

immortalization *in vitro* may reflect the steps needed for the bypass of proliferative barriers *in vivo*.

1.4 BRCA1 and breast cancer

A number of studies suggest that germline mutations in *BRCA1* predispose women to breast and ovarian cancers. *BRCA1* locus was first identified in genetic linkage analysis of families with multiple cases of early onset of breast cancer. In 1990 *BRCA1* was mapped to chromosome 17q12-q21 and in 1994 the gene was cloned. Approximately 15%-20% of women with a family history of breast cancer, and 60%-80% of women with a family history of both breast and ovarian cancers are identified as *BRCA1*-mutation carriers. Their lifetime risk of breast cancer is estimated to be as high as 60%-80%, while their lifetime risk of ovarian cancer is estimated to be 20%-40%. These numbers are extremely high compared to the lifetime risk of breast and ovarian cancers for the general population of women (~8% and 3%, respectively). In addition, risk of contralateral breast cancer (64% by age 70) is also high in *BRCA1*-mutation carriers. Furthermore, it has been reported that male *BRCA1*-mutation carriers have increased risk of prostate and colon cancer (Miki *et al.*, 1994; Nathanson *et al.*, 2001; Hemel and Domchek, 2010; Lalloo and Evans, 2012).

BRCA1 is a large gene containing a total of 24 exons. Germline mutations in this gene are inherited in autosomal dominant manner. To date over 1700 unique mutations have been reported to the Breast Cancer Information Core Database. Approximately 858 have been designated as “clinically significant,” implying that these mutations are associated with an increased risk of cancer. Mutations are found throughout *BRCA1* coding sequence of the gene. They include frameshift mutations that result in protein truncation, as well as large rearrangements (including large

deletions, insertions or duplications) and missense mutations that result in a protein with reduced function. Most of these mutations are localized within exons 16-24, followed by exons 2-7, and exons 11-13 (Figure 1.7) (Miki *et al.*, 1994; Grade *et al.*, 1996; Clark *et al.*, 2012).

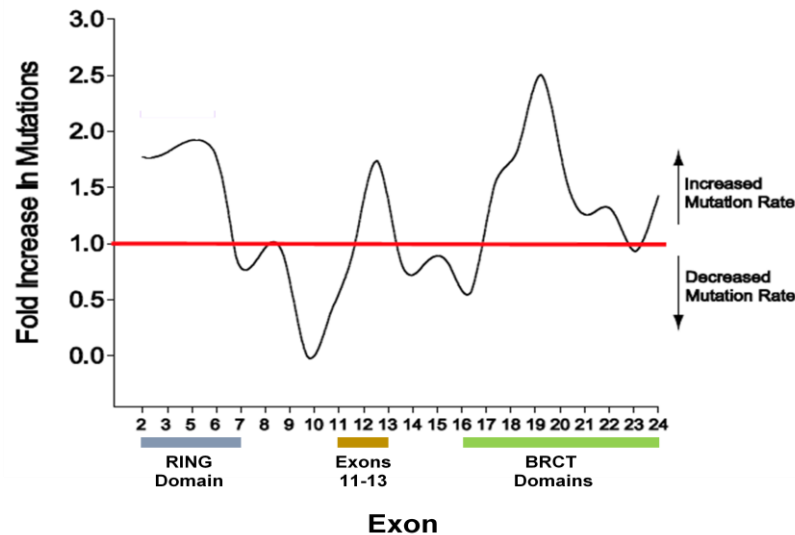


Figure 1.7. Frequency of mutations found across different regions of BRCA1 gene. BRCA1 mutations occur at the highest rate in the BRCT domain, followed by the RING domain, and exons 11-13. BRCA1 clinically relevant mutations were obtained from the Breast Cancer Information Core (BIC). Fold increase in mutations were calculated as the number of mutations per codon length of each exon/total mutations for all BRCA1 codons. 1.0 on the y-axis indicates the total average mutations per codon for BRCA1. Adapted from Clark *et al.*, 2012.

Certain mutations (also called founder mutations) appear to be more common within particular patient population, such as 185delAG and 5382insC which are found in Ashkenazi Jewish population and eastern European populations. Whether a mutation is deleterious or not is mainly determined by the family history of cancer. However, for a number of mutations family history is unavailable and the penetrance is not determined. These mutations are also known as variants of uncertain significance (VUS) and their characterization, currently underway, is based on a set of

functional tests for mutated BRCA1 protein (Hemel and Domchek, 2010; Lalloo and Evans, 2012).

The most common histological types of *BRCA1*-associated breast cancers are invasive ductal carcinoma and medullary carcinoma. These tumors are frequently found to have a prominent lymphocytic infiltrate, foci of necrosis, and pushing margins (Figure 1.8). Other common characteristic of *BRCA1*-tumors include high histological grade showing less tubule formation and poor differentiation as well as high degree of nuclear pleomorphism and mitotic frequency (Figure 1.8). At diagnosis *BRCA1*-associated breast cancers are frequently found to be of higher grade than their age-matched sporadic breast cancer controls. In addition, 70%-80% of *BRCA1*-associated breast cancers are of triple negative or basal-like subtype, meaning that they do not express hormone receptors or Her2, but they express CK5/6 and CK14 (Rahman and Stratton, 1998; Honrado *et al.*, 2005; Campeau *et al.*, 2008). The rest of the *BRCA1*-breast cancers (20-30%) are ER-positive and therefore of luminal subtype. *BRCA1*-associated breast tumors display frequent somatic mutations such as loss of PTEN, mutations or loss of TP53, as well as loss of heterozygosity (LOH) of *BRCA1* WT allele. It is thought that the loss of *BRCA1* WT allele is the rate limiting step in progression of *BRCA1*-associated breast cancers (Campeau *et al.*, 2008; Martins *et al.*, 2012). In addition, it was recently reported that genomic disruption of pRb gene is a major target in *BRCA1*-deficient hereditary breast cancer (Jonsson *et al.*, 2012). It is of note that somatic mutations in BRCA1 gene are not commonly found in sporadic breast and ovarian cancers. However, it has been observed that BRCA1 expression is frequently compromised by methylation of the *BRCA1* promoter in triple negative sporadic breast cancers (Matros *et al.*, 2005).

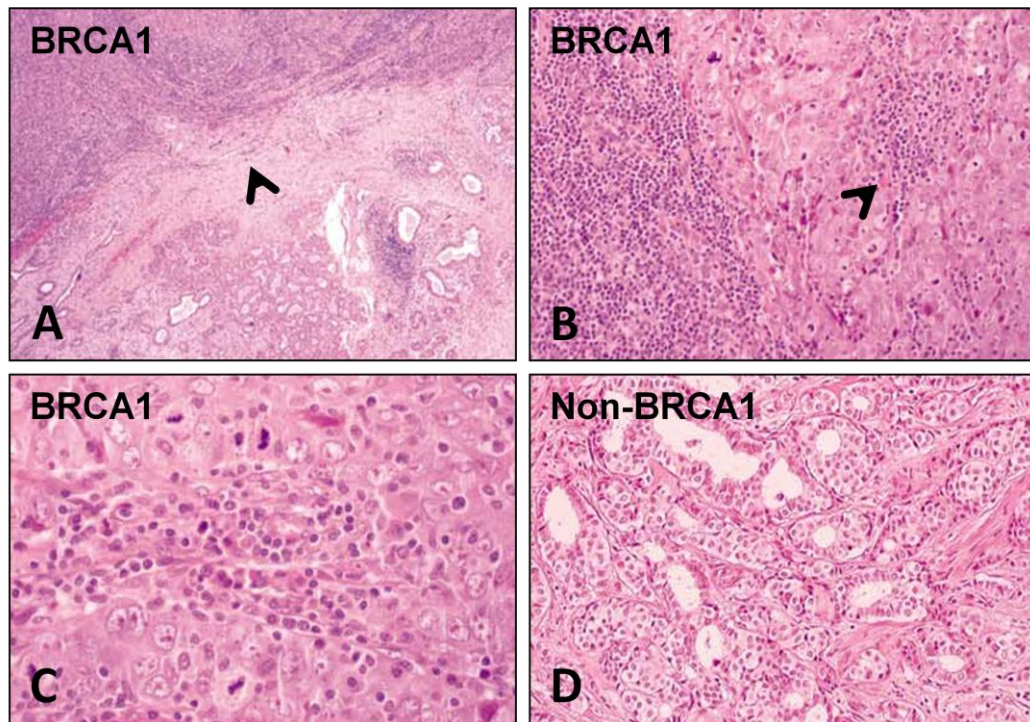


Figure 1.8. Histology of *BRCA1*-associated breast cancers. Typical histological patterns include: A) pushing margins, B) lymphocytic infiltrate, C) pleomorphism and numerous mitosis. D) Non-*BRCA1* tumor with well-formed tubules, low pleomorphism and low mitosis numbers. Adapted from Honrado *et al.*, 2005.

Family history, clinical data and histopathological features of tumors are all important indicators in determining a good candidate for *BRCA1* testing and genetic counseling. Currently the only source of commercial testing in the United States for *BRCA1* mutations is a company called Myriad Genetics (Salt Lake City, UT). This company, in fact, holds a patent on *BRCA1* gene (Hemel and Domchek, 2010). Types of analysis available through Myriad Genetics include: a) comprehensive, where a full sequence and/or large-rearrangements of the gene are analyzed; b) single site, where a specific mutation site is chosen for analysis based on previously identified mutation(s) in another family member; c) multisite, which is a test for three most common mutations found in people of Ashkenazi ancestry (Myriad Genetics, 2013). The testing costs over \$3000 and is covered by most healthcare insurers. However, over the last three years the patent validity as well as the cost and quality of Myriad's

genetic testing have come in question. Whether a company is entitled to own the rights to a human gene has become a public ethical debate involving the federal appeals court (since July 2011 ruled in favor of the company). Furthermore, the current cost of the testing is considered unjustified because of the newer DNA-sequencing techniques, which are now faster and less expensive. In addition, the analysis that is offered by Myriad Genetics is far from comprehensive since it does not include testing for any other gene predisposing to familial breast cancers. In fact, analysis of additional genes is available through supplemental tests each costing \$700, which are not covered by healthcare insurers (Pollack, 2011). In summary, it is surprising to see that a company with technologies considered to be obsolete in this day and age is still able to maintain a monopoly over the genetic testing of *BRCA1*.

Identification of a germline mutation in *BRCA1* provides useful information for women at high risk of hereditary breast cancer because they may begin cancer screening as early as 25 years of age. It is recommended that they have yearly mammograms and MRIs, twice-yearly clinical breast examinations, and monthly breast self-examinations. At present, no effective screening for ovarian cancer exists (Hemel and Domchek, 2010). All in all, there are relatively few clinical options available to prevent and treat cancer in *BRCA1*-carriers. Currently the most effective and only means of prevention is the extreme prophylactic surgical removal of breast and/or ovarian tissues. This surgery reduces risk of breast and ovarian cancers by 85% and 50%, respectively. However, risk-reducing surgery (specifically, oophorectomy) may have serious long-term consequences of estrogen deprivation such as osteoporosis, cardiovascular disease, and stroke (Drost and Jonkers, 2009; Hemel and Domchek, 2010; Domchek *et al.*, 2010).

Breast cancers in *BRCA1*-mutation carriers are treated similarly to sporadic breast cancers, where treatment decisions depend on tumor size, lymph node status and hormone receptor expression. Current therapeutics aimed for the treatment of hormone receptor-positive or Her2- expressing tumor cells are not applicable for patients with triple negative *BRCA1*-associated breast cancers. However, *BRCA1*-deficient cells show sensitivity to DNA damage inducing agents such as topoisomerase inhibitors, bleomycin, radiation and interstrand cross-linking agents such as platinum-based drugs. Efficacy *in vitro* is also seen for poly(ADP-ribose) polymerase 1 (PARP) inhibitors such as iniparib, which cause DNA double-strand breaks. These agents are currently in different phases of clinical trials. Furthermore, similarly to sporadic basal breast cancers, *BRCA1*-associated breast cancers have a poor prognosis (Kennedy *et al.*, 2004; Campeau *et al.*, 2008; Drost and Jonkers, 2009; Hemel and Domchek, 2010).

In summary, it is established that the carriers of germline mutation in *BRCA1* are at a high risk of developing breast and ovarian cancer. Mutations occur throughout the gene and frequently result in protein truncations. Since breast cancers associated with germline mutation in *BRCA1* commonly exhibit LOH of WT *BRCA1* allele, it is thought that this is the rate limiting step in tumor progression. In addition, most *BRCA1*-associated breast cancers are triple negative and aggressive, which greatly limits the treatment options for this particular group of patients. Thus, in order to develop better therapeutics, it is of great importance to understand the tumor suppressive function of *BRCA1* in breast (as well as ovarian) epithelium.

1.5 The function of BRCA1

1.5.1 BRCA1 structure

BRCA1 encodes a 220kDa phospho-protein consisting of three main regions: 1) an amino-terminal RING (Really Interesting New Gene) finger domain, 2) exons 11-13, and 3) two BRCT (BRCA1 C-terminal) domains (Figure 1.9). The RING domain of BRCA1 encompasses amino acids 1-109 (exons 2-7). It consists of a RING finger and two flanking alpha helices coordinating together two Zn²⁺ atoms to stabilize the structure. The RING finger domain is responsible for the interaction of BRCA1 with BARD1 (BRCA1 Associated RING Domain protein 1) (Figure 1.9). The heterodimer of BRCA1 and BARD1 functions as an E3-ubiquitin ligase. This interaction sequesters nuclear export sequence (NES) on both BRCA1 and BARD1, which ensures that both proteins are retained in the nucleus. BRCA1 E3 ubiquitin ligase acts solely as a scaffold to facilitate the transfer of ubiquitin from E2 to the substrate (Meza *et al.*, 1999; Brzovic *et al.*, 2003). Known *in vivo* DNA damage-related targets of BRCA1 E3 ligase are: CtIP and histone protein H2A involved in DNA repair and de/condensation. In addition, BRCA1 can also undergo mono or poly-ubiquitination *in vitro*. This autoubiquitination leads to BRCA1/BARD1 having an increased affinity for binding to DNA repair intermediates. Thus, one of the outcomes of BRCA1 autoubiquitination could be to modify its own activity in the DNA damage response (Yun and Hiom, 2009; Clark *et al.*, 2012).

Exons 11-13 account for over 65% of the sequence of BRCA1 (Figure 1.9). Out of three major domains of BRCA1, this is the least known region (Deng and Brodie, 2000). Three large exons encode: 1. two nuclear localization sequences (NLS1=501-507aa and NLS2=607-614aa), important for interaction with importin-alpha and proper transportation of BRCA1 from cytosol to nucleus; 2. coiled-coil domain (1364-1437aa) which mediates interactions with PALB1 and is important for DNA repair via homologous recombination (HR); 3. serine containing domain (SCD, 1280-1524aa), which acts as a substrate for ATM/ATR kinases and is important for BRCA1 recruitment to the sites of double strand breaks (DSB). In addition, binding domains for several proteins such as pRb, Rad50, Rad51 and c-Myc are also found in this region (Figure 1.9) (Clark *et al.*, 2012).

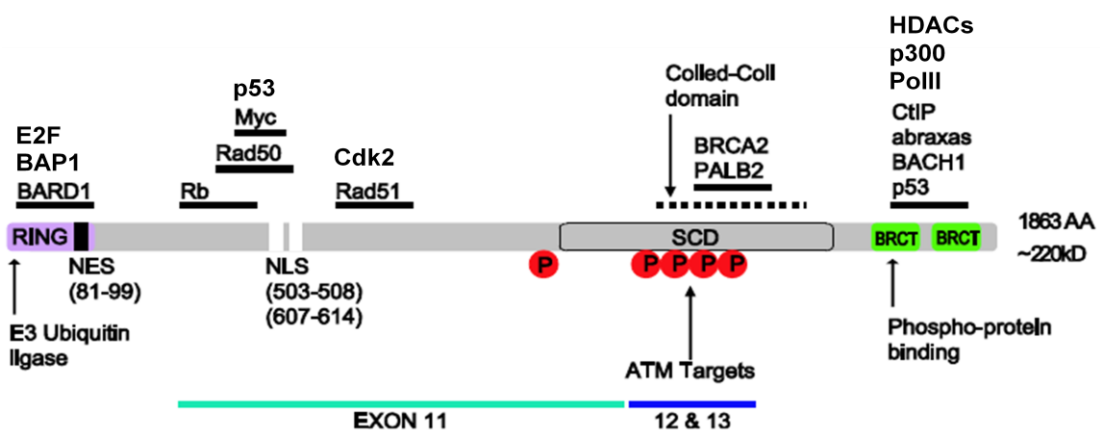


Figure 1.9. Functional domains and binding partners of BRCA1. RING domain, serine containing domain (SCD), BRCT domains, NES and NLS sequences are indicated on BRCA1 protein. Note the size of BRCA1 protein encoded by exons 11-13. Red circles represent sites of phosphorylation by DNA damage response kinases. Horizontal solid black lines indicate protein binding domains for the listed binding partners. Adapted from Clark *et al.*, 2012.

BRCA1 C-terminal (BRCT) domain spans 1650-1863aa and consists of two tandem BRCT repeats connected by a 22 amino acid linker (Figure 1.9). BRCT domain mediates phosphoprotein interactions between BRCA1 and proteins

phosphorylated by ATM and ATR upon DNA damage. It recognizes the sequence pSer-X-X-Phe in its binding partners and is therefore identified as Class I BRCT domain (Williams *et al.*, 2001; Williams *et al.*, 2004; Clapperton *et al.*, 2004). Proteins that bind BRCA1 BRCT domain include BACH1, CtIP, and Abraxas (Figure 1.9). In addition, BRCT domains seem to be important for DNA binding as well as non-phosphoprotein interactions of BRCA1 such as transcriptional regulation (Yun and Hiom, 2009; Clark *et al.*, 2012).

1.5.2 BRCA1's role in DNA damage repair

Initial observations that BRCA1 can be found at the sites of DNA damage indicated that BRCA1 functions in DNA damage repair pathway (Figure 1.10) (Scully *et al.*, 1997; Scully *et al.*, 1997). A model of how BRCA1-BARD1 complex is recruited to the site of DNA damage has been proposed. Double-strand DNA breaks are detected by MRN complex (consisting of MRE11, RAD50, and NBS1). This complex serves to tether the broken ends of DNA molecule and to recruit the checkpoint kinase ATM. Interaction of MRN with ATM promotes the autophosphorylation (at Ser1981, Ser367, Ser1893) and monomerization of ATM. Active ATM phosphorylates several downstream kinases as well as histone H2AX (γ H2AX). γ H2AX serves as an initial recruiting factor for various DNA repair proteins such as mediator of DNA damage checkpoint 1 (MDC1) and E3 ubiquitin ligase complex RNF8 - UBC13 (RING finger protein 8 - ubiquitin conjugating enzyme 13) (Stucki *et al.*, 2005; Bekker-Jensen *et al.*, 2006). These complexes mediate the initial round of ubiquitination of histones and other substrates on or near the site of DNA break to propagate the damage signal (which can span up to 1Mb). Next, E3 ubiquitin ligase RNF168 recognizes ubiquitinated histones and together with

UBC13 amplifies further the local ubiquitination signal. Ubiquitin-interacting motif-containing receptor-associated protein 80 (RAP80) recruits BRCA1 in complex with other proteins to initiate the repair (Yan *et al.*, 2007; Yun and Hiom, 2009; Huen *et al.*, 2010).

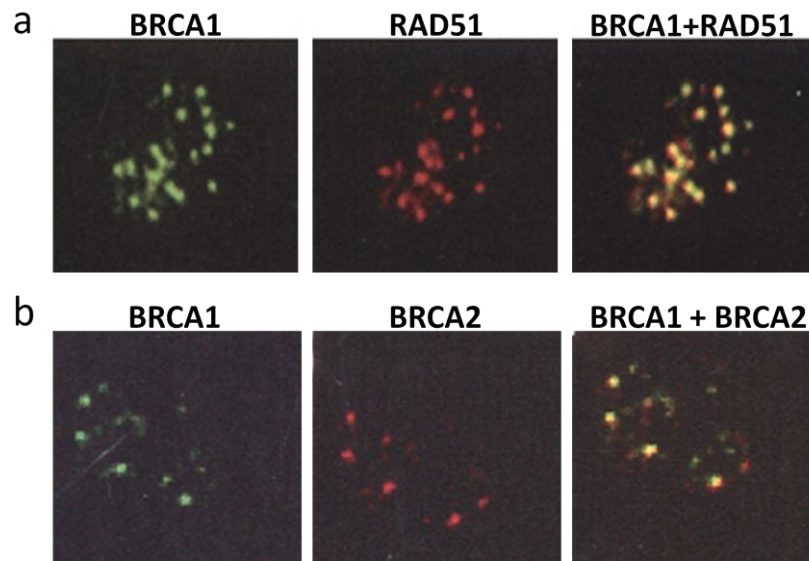


Figure 1.10. BRCA1 colocalizes with RAD51 and BRCA2 at DNA damage sites to initiate HR repair. a) BRCA1 colocalizes with RAD51, the eukaryotic RecA homologue involved in homologous recombination repair of DNA double strand breaks. The interaction was captured in S-phase-G2-phase nuclear foci of MCF7 cells using immunofluorescence. b) BRCA1 also colocalizes with BRCA2 in discrete nuclear foci. The interaction was captured in S-phase-G2-phase nuclear foci of DU145 cells using immunofluorescence. Adapted from Chen *et al.*, 1999

There are several different BRCA1 complexes that are found in cells. BRCA1A consists of Abraxas, BRCC36, BRCC45 and NBA1 proteins (Figure 1.11). Abraxas interacts with RAP80 and helps recruit BRCA1A to DNA damage sites (Wang *et al.*, 2007). BRCC36 is an ubiquitin hydrolase that seems to be involved in regulation of local histone modifications. BRCA1A participates in G2-M checkpoint control by promoting CHK1 phosphorylation (Yarden *et al.*, 2002). Interestingly, the structure of

BRCA1A complex resembles the 19S complex of the proteasome, further suggesting that many of the components bind ubiquitin and therefore may control the dynamics of local ubiquitination (Huen *et al.*, 2010; Roy *et al.*, 2012).

BRCA1 also forms a BRCA1B complex with BRCA1-interacting protein C-terminal helicase 1 (BACH1)-DNA topoisomerase 2-binding protein 1 (TOPBP1) (Figure 1.11) (Greenberg *et al.*, 2006). This complex is important for the G1-S and intra S-phase checkpoint in response to stalled or collapsed replication forks and DNA repair during replication (Kumaraswamy *et al.*, 2007). It may also help mediate ATR-CHK1 signaling, but other significance is not well known (Huen *et al.*, 2010; Roy *et al.*, 2012).

Finally, another way BRCA1 could be recruited to DSBs is through BRCA1C complex. In this complex, BRCA1 associates with CtBP-interacting protein (CtIP) and MRN complex (consisting of MRE11, RAD50, and NBS1) to sense and process DSBs in preparation for HR-mediated repair (Figure 1.11). Specifically, the complex seems to couple DNA end-resection and single stranded DNA generation with G2-M checkpoint activation (Chen *et al.*, 2008; Huertas *et al.*, 2009; Yun *et al.*, 2009; Huen *et al.*, 2010; Roy *et al.*, 2012).

HR repair of DSBs occurs in proliferating cells during S and G2 phases of the cell-cycle. It works by finding a homologous sequence (preferably intact sister chromatid) to carry out high-fidelity repair of predominantly replication-associated DNA double strand breaks. The homology directed pathway is initiated by DNA end-resection and single stranded DNA generation. Following resection, a number of proteins are recruited to form a nucleoprotein filament. The resulting filament invades the sister chromatid resulting in the formation of an intermediate structure called the displacement loop (D-loop). On the other side of the loop are Holliday junctions,

which allow the structure to slide in both directions. DNA is synthesized beyond the original break site to restore the missing sequence. The newly synthesized strand is released resulting in resolution of the recombination intermediate. Final processing includes removing flaps, filling in gaps and ligating the remaining nicks (Helleday *et al.*, 2007, Roy *et al.*, 2012).

To facilitate HR mediated repair, BRCA1 seems to be involved in several different steps. Depending on when in cell cycle DSB was detected, BRCA1A and BRCA1B can halt the cell-cycle by intra-S and G2-M checkpoint activation. In BRCA1A and BRCA1C complex BRCA1 can be recruited to DSBs. BRCA1C complex participates in efficient generation of ssDNA regions at DSBs by promoting CtIP-mediated 5'-end resection. Phosphorylation of BRCA1 by CHK2 is required for BRCA1-PALB2-BRCA2 complex formation and recruitment of DNA recombination repair protein RAD51 to the sites of DSB responsible for subsequent nucleoprotein formation, homology searching and strand invasion during HR (Scully *et al.*, 1997, Scully *et al.*, 1997, Chen *et al.*, 1999; Helleday *et al.*, 2007, Roy *et al.*, 2012).

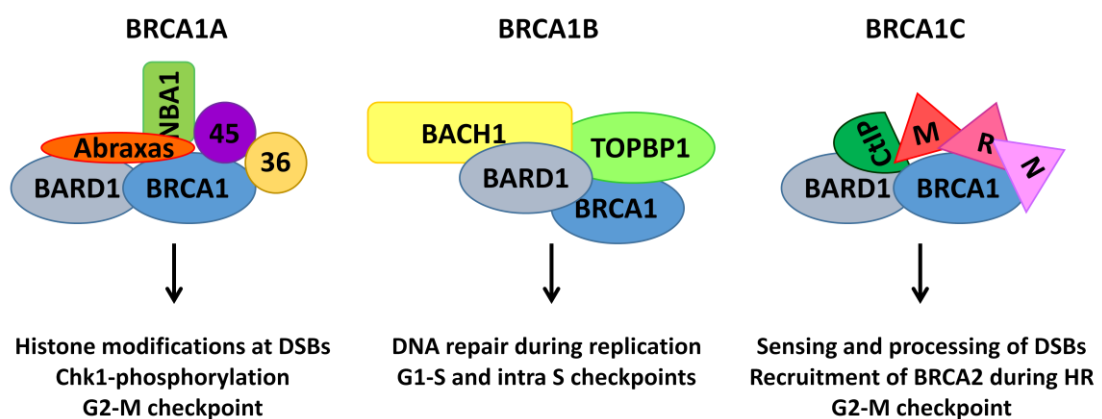


Figure 1.11. BRCA1 is found in several different complexes involved in DNA damage repair. Proteins assembling hypothetical BRCA1A, BRCA1B and BRCA1C complexes are depicted. An arrow points to a list of putative roles that each complex has in DNA damage repair pathway.

In sum, dissecting the role of BRCA1 in DNA damage repair has not been an easy task. BRCA1 is a multifaceted protein containing (at least) three functionally different domains that work in concert to repair damaged DNA. But, how exactly each region functions in DNA damage repair is not well known. In addition, BRCA1 is a component of many complexes that regulate separate events during DNA damage response and homologous repair. However, it is still not clear what dictates the formation of different BRCA1 complexes and what each one of them does once it is recruited to the sites of DNA damage. Besides facilitating recruitment of other DNA damage repair proteins at the sites of DSBs, BRCA1 also seems to communicate with the cell-cycle machinery to elicit cell cycle arrest and allow adequate time for DNA repair to occur. Since loss of control over these processes results in genomic instability, it is hypothesized that this is the main tumor suppressive function of BRCA1.

1.5 Other putative functions of BRCA1

Besides acting as a scaffold and mediator in DNA damage repair, BRCA1 has been implicated in other important processes in the cell such as ubiquitination, transcriptional regulation, cell-cycle control, and chromatin remodeling. Interestingly, some of these functions are related to DNA damage repair pathways, while some appear to regulate separate cellular processes.

1.6.1 *Ubiquitination*

To date, a number of substrates for BRCA1 E3-ubiquitin ligase have been identified: RNAPII, TopoII α , p53, CtIP, histones H2A and H2B, ER α , PR, γ -tubulin and NPM1. Since BRCA1 can form mono- and poly-ubiquitin chains, ubiquitination

by BRCA1 in particular cases may function in signal propagation, while in other cases it may result in protein degradation.

RNAPII: Once DNA damage is detected, RNAPII is phosphorylated, ubiquitinated, and sent for degradation. BRCA1 can directly ubiquitinate and thus inhibit RNAPII activity in presence of DNA damage. However, it is also possible that BRCA1 ubiquitinates RNAPII at a DNA lesion to initiate DDR (Scully *et al.*, 1997; Starita and Parvin, 2006).

TopoII α : TopoII α interacts with BRCT domain of BRCA1 in a phosphorylation-dependent manner. BRCA1 ubiquitinates and thereby positively regulates the activity of topoisomerase II α to properly decatenate DNA, which would otherwise form bridges between dividing cells (Starita and Parvin, 2006).

CtIP: CtIP transiently interacts with phosphorylated BRCT domain of BRCA1. BRCA1 ubiquitinates CtIP upon DNA-damage to promote its recruitment to DNA damage foci. ub-CtIP also activates Chk1, which results in activation of G2/M-checkpoint (Yu *et al.*, 2006).

H2A and H2B: In vitro studies showed that BRCA1 can ubiquitinate both free H2A and H2B histones in the context of nucleosomal particles (Thakar *et al.*, 2010). Recent study showed that BRCA1 binds to satellite DNA regions and ubiquitinates H2A in vivo. This interaction seems to be important in repression of satellite DNA expression. Ectopic expression of satellite DNA can phenocopy BRCA1 loss resulting in centrosome amplification, cell-cycle checkpoint defects, DNA damage and genomic instability. These results implicated BRCA1 in maintenance of constitutive heterochromatin (Zhu *et al.*, 2011).

ER α : BRCA1 ubiquitination of ER α is involved in cyclical stimulation of ER α transcription. In the proposed model the presence of estrogen activates ER α and the

recruitment of BRCA1 and RNAPII to ER α -regulated promoter regions to activate a pulse of transcription. ER α and RNAPII are then ubiquitinated by BRCA1, leading to dissociation of all factors from the promoter. This necessitates reassembly of all of these factors at the promoter for subsequent rounds of transcription resulting in cyclical activation of estrogen-responsive promoters (Eakin *et al.*, 2007; Heine and Parvin, 2007).

PR: BRCA1 regulates the transcriptional activity of PR by ubiquitinating PR and stimulating its degradation. In addition, BRCA1 is present at the hormone responsive regions of PR target genes where it increases the levels of monoubiquitinated histone H2A resulting in chromatin silencing at PR regulated promoters (Heine and Parvin, 2007; Calvo and Beato, 2011).

γ -tubulin and NPM1: BRCA1 functions in regulating the centrosome number by ubiquitinating several centrosome proteins including γ -tubulin and NPM1. The data suggest that loss of BRCA1 can cause both fragmentation of the centrosomes and reduplication in late S and G2 phases of the cell-cycle. The idea is that by ubiquitinating centrosome proteins, BRCA1 blocks a second inappropriate round of duplication (Sato *et al.*, 2004; Sankaran *et al.*, 2005; Starita and Parvin, 2006).

1.6.2 Regulation of gene transcription

BRCA1 co-purifies with RNAPII and associates with zinc-finger containing nuclear protein NUFIP, which stimulates activator-independent transcription by RNAPII. It is thought that BRCA1 and RNAPII complexes stimulate transcription and also monitor for DNA fidelity (Scully *et al.*, 1997; Cabart *et al.*, 2004; Starita and Parvin, 2006). In addition, BRCA1 can act as a co-activator and co-repressor of transcription in complex with known transcription factors. By interacting with the C-terminus of STAT1 following stimulation of IFN γ pathway, it stimulates interferon

inducible genes such as p21 and IRF7. BRCA1 stabilizes p53 levels when overexpressed or upon DNA damage. By interacting with C-terminus of p53 it induces cell-cycle arrest and expression of DNA repair genes such as p21, 14-3-3 δ and GADD45 (Somasundaram *et al.*, 1997; Wang *et al.*, 2000; Mullan *et al.*, 2006). On the other hand, it acts as co-repressor of growth promoting genes such as hTERT and S100A7 when bound to c-Myc. Furthermore, it represses ER α inducible genes such as VEGF (Mullan *et al.*, 2006).

1.6.3 Chromatin remodeling

In addition to pRb, BRCA1 was found to interact with RbAp46, RbAp48, HDAC1 and HDAC2. These complexes regulate histone modification, but the targets have not yet been identified. Nevertheless, this suggests that BRCA1 may act in concert with pRb to repress transcription through histone deacetylation. However, BRCA1 may also function to sequester the complex from DNA promoters, which can then activate transcription of certain genes (Yarden and Brody, 1999; Wang *et al.*, 2000; Mullan *et al.*, 2006). Moreover, BRCA1 binds members of SWI/SNF complex such as BRG1 and BRD7. These are thought to function in chromatin remodeling and gene transcription activation, since loss of this interaction failed to activate expression of genes such as ER α (Bochar *et al.*, 2000, Starita and Parvin 2003; Harte *et al.*, 2010). As mentioned above, BRCA1 ubiquitination of histones H2A and H2B plays an important role in maintenance of constitutive heterochromatin (Zhu *et al.*, 2011). Consistent with this is the observation that BRCA1-associated breast cancers show decreased promoter methylation pattern compared with sporadic breast cancers (Suijkerbuijk *et al.*, 2008).

1.6.4 Cell-cycle checkpoint regulation (G1-S, S, G2-M)

BRCA1 is involved in transcriptional activation of p21 and p27 which induce G1-S phase cell-cycle arrest (Somasundaram *et al.*, 1997). In addition, phosphorylation of C-terminal of BRCA1 is involved in activation of important cell-cycle checkpoints upon DNA damage. Specifically, phosphorylation of Ser1387 by ATM is required for the activation of intra-S-phase checkpoint, while phosphorylation of Ser1423 is important for G2-M cell cycle arrest. BRCA1 is also involved in transcriptional activation of GADD45, which functions to induce G2-M cell-cycle arrest (Mullan *et al.*, 2006).

Collectively, these studies reveal that BRCA1 might be involved in regulation of many cellular processes that seem to be independent of DNA damage repair pathway. However, understanding the physiological relevance of some of these functions is only in its infancy. Also, further investigation of their role in tumor suppression is necessary. Nevertheless, these studies point out that, in addition to DNA damage repair, there might be other ways that BRCA1 is suppressing tumor formation. Thus, by focusing our attention on DNA damage repair pathway only, we might be missing other important aspects of the BRCA1 function.

1.6 Lessons learned from BRCA1 mouse models

The full-length mouse BRCA1 protein contains 1812 amino acids, which is 51aa shorter than the human BRCA1 protein. Even though RING finger domain of mouse BRCA1 is 100% identical to the human protein, the overall mouse BRCA1 shares only 58% aa identity with the predicted human protein. However, homology increases to 73% if conservative substitutions are included. Furthermore, mouse and human BRCA1 share similar charge and hydrophilic character (Lane *et al.*, 1995).

Whereas amino terminus has several clusters of basic residues, middle and carboxyl terminus of the protein are generally acidic. Coding sequence of 5' end and Exon 11 is conserved in mouse *BRCA1* indicating extensive similarity in the organization of the *BRCA1* locus. The map location on mouse chromosome 11 is in synteny with chromosome 17q21-22 where human *BRCA1* is found. It has been observed that mouse *BRCA1* is expressed in a variety of cell lineages. However a notable increase in *BRCA1* expression was seen during specific stages in the development of a variety of ectodermally derived tissues. These tissues include: brain, skin, whisker pads, and mammary epithelial cells. Also, *BRCA1* transcripts were abundant in mesodermally derived tissues such as kidney epithelial cells, lymphoid organs, reproductive organs, and cultured fibroblasts. Interestingly, *BRCA1* expression in mammary epithelium was particularly elevated in mid-pregnancy, paralleling the expression of milk protein mRNA (Figure 1.12). Because little proliferation is seen by late pregnancy, this suggested that *BRCA1* plays a role in cell differentiation perhaps by regulating gene transcription and inhibiting cell-cycle progression (Lane *et al.*, 1995).

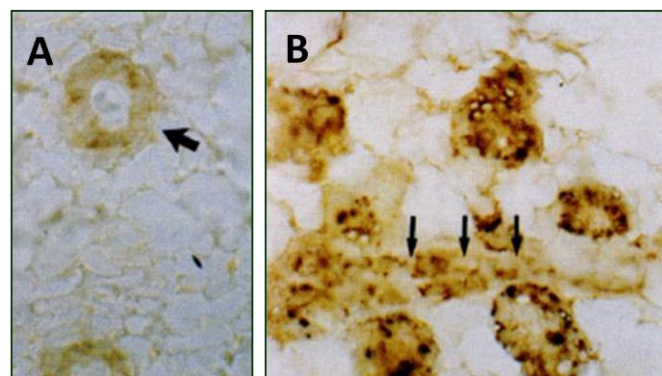


Figure 1.12. *BRCA1* expression in mouse mammary epithelium during development. Cells expressing *BRCA1* mRNA were identified by in situ hybridization on sectioned mammary glands at different stages of development. A) mRNA levels of *BRCA1* in virgin mammary glands was low but detectable. B) mRNA levels of *BRCA1* in mammary gland after day 17 of pregnancy were dramatically increased. Adapted from Lane *et al.*, 1995.

In order to model the human condition associated with germline mutation in BRCA1 gene, *BRCA1*^{+/-} mice were developed. However, BRCA1 heterozygous mice do not exhibit any apparent phenotypes. In addition, these mice do not develop spontaneous mammary tumors, which is in stark contrast to women with heterozygous *BRCA1* germline mutations. The reason for this discrepancy is not clear. Thus, *BRCA1*^{+/-} mice are rarely used for any types of studies of BRCA1 function in tumor suppression. Several ideas have been proposed to explain this inconsistency between human and mouse condition associated with *BRCA1* mutation. One idea argues that the lifespan in mice might be too short to develop *BRCA1*-associated mammary tumors. Another hypothesis is that the rate of LOH might be too low in mice to acquire additional mutations necessary for tumor development. Nevertheless, the field has moved on to study *BRCA1*-associated tumorigenesis in mice with biallelic inactivation of *BRCA1* (Bouwman and Jonkers, 2008; Drost and Jonkers, 2009).

BRCA1 knockout in mice results in embryonic lethality at midgestational stage (between embryonic day 7.5 and 13.5) due to reduced cellular proliferation, apoptosis, aneuploidy and chromosome damage (Evers and Jonkers, 2006). Deletion of p53 concomitant with *BRCA1*-null mutation delays embryonic lethality by several days suggesting that p53 activation is in part responsible for the embryonic lethality (Cressman *et al.*, 1999; Xu *et al.*, 2001). Therefore, observed embryonic lethality is not compatible with the anticipated tumor suppressor functions of BRCA1, but it emphasizes an essential role of BRCA1 in maintaining genome integrity in mice. This is also in agreement with the fact that no homozygous *BRCA1* mutation carriers have been reported to date (Bouwman and Jonkers, 2008; Drost and Jonkers, 2009).

It has been observed that humans and mice express different splice variants of BRCA1 (Miki *et al.*, 1994; Drost and Jonkers, 2009; Dine and Deng, 2012). Thus, the

function of various splice variants was examined in more detail by using mouse models. Three BRCA1 splice variants have been found and functionally analyzed in mice: BRCA1- Δ 11, BRCA1-IRIS and BRCA1 Δ 22 (Xu *et al.*, 1999; Kim *et al.*, 2006; Pettigrew *et al.*, 2008). Mouse embryos with one intact BRCA1 splice variant survive significantly longer than BRCA1-null embryos. Splice variant BRCA1- Δ 11 is similar to full-length BRCA1 except it is missing exon 11. It appears to be regulated in a cell-cycle dependent manner and localized in nuclear foci. This splice variant is not phosphorylated and does not promote formation of Rad51 foci upon DNA damage. Mice that carry homozygous BRCA1- Δ 11 alleles are viable, but develop various tumors including mammary carcinomas after long latency. In addition, mice expressing mammary-gland specific homozygous BRCA1- Δ 11 alleles also develop mammary adenocarcinomas characterized by genetic instability. Cells with BRCA1- Δ 11 isoform display G2-M cell-cycle checkpoint abnormalities, centrosome duplication, chromosome damage, and massive apoptosis. These data suggest that BRCA1- Δ 11 can compensate for some of the functions of full-length BRCA1 during embryogenesis but is unable to properly maintain genomic stability and prevent tumor formation. In contrast, mice that lack BRCA1- Δ 11 isoform, but retain the full-length BRCA1 form, display mammary gland abnormalities and uterine hyperplasia after 1 years of age with spontaneous tumor formation. In this mouse model mammary epithelial cells exhibit abnormalities in G1-S cell-cycle transition and centrosome duplication (Xu *et al.*, 1999; Kim *et al.*, 2006; Pettigrew *et al.*, 2008; Drost and Jonkers, 2009; Dine and Deng, 2012).

BRCA1-IRIS transcript spans exons 1-11, encoding a protein that has a unique C-terminus compared to full length BRCA1. This variant seems to be exclusively chromatin associated with positive influence on DNA replication. Also, this variant

cannot compensate for the loss of full-length BRCA1 and BRCA1- Δ 11 expression, since the embryos expressing only BRCA1-IRIS die at E10.5. BRCA1- Δ 22 leads to a loss of second BRCT repeat and thereby loss of transcriptional activity of this BRCA1 variant. However, embryos expressing BRCA1- Δ 22 survived longer in comparison to BRCA1-null mutants (Xu *et al.*, 1999; Kim *et al.*, 2006; Drost and Jonkers, 2009; Dine and Deng, 2012).

In order to circumvent the embryonic lethality of BRCA1 homozygous mice, mice with hypomorphic *BRCA1* alleles on a p53-null or p53-heterozygous background have been developed. These mice survive to adulthood, but they develop lymphoma before 3 months of age (Dine and Deng, 2012). Intriguingly, *BRCA1* ^{Δ 11/ Δ 11} *p53*^{+/-} mice display features of premature ageing and senescence such as reduced life-span, body weight, dermal thickness, adipose tissue, hair regeneration and pronounced osteoporosis and kephosis. Senescence in this model seems to be triggered in response to excessive DNA damage in a p53/p21^{Cip1/Waf1} – dependent manner (Cao *et al.*, 2002).

Furthermore, to avoid the whole body degeneration due to BRCA1-deficiency, conditional *BRCA1* knockouts have been created. Cre mediated deletion of exons *BRCA1*^{F5-6}, *BRCA1*^{F5-13}, *BRCA1*^{F11}, and *BRCA1*^{F22-24} abrogate BRCA1 function (Mak *et al.*, 2000, Liu *et al.*, 2007, Xu *et al.*, 1999, McCarthy *et al.*, 2007). In these models mammary tissue-specific Cre expression is driven by either MMTV or WAP promoters (Drost and Jonkers, 2009; Dine and Deng, 2012). Conditional deletion of exon 11 of *BRCA1* in mouse mammary epithelial cells results in increased levels of apoptosis and impaired ductal growth but not in the acceleration of mammary tumor development (Figure 1.13). Rather, these mice develop mammary tumors at a low frequency, late in life (23% by 15 months of age). Latency of tumorigenesis is

accelerated only in the presence of additional genetic mutations such as heterozygosity for p53 (Figure 1.13) (Xu *et al.*, 1999). Mammary tumors in these models show genomic instability and ER-negativity. However, a large proportion of tumors also overexpress ERBB2, which is not commonly observed in human *BRCA1*-associated breast cancers (Drost and Jonkers, 2009). Because recapitulating human tumors associated with *BRCA1* mutation has been one of the challenges in mouse models, various other mammary epithelium specific promoters have been tested to drive the Cre expression. For example, K14 Cre-mediated deletion of both *BRCA1* and *p53* in mice lead to high incidence of mammary carcinomas that displayed important hallmarks of human *BRCA1*-associated breast tumors: basal type, ER-negative, poor differentiation, high proliferation and increased genomic instability (Liu *et al.*, 2007). Also, B-lactoglobulin (BLG)-Cre deletion of *BRCA1* and *p53* resulted in mammary tumors that recapitulate many of the features of *BRCA1*-associated breast cancers (McCarthy *et al.*, 2007). These models are useful in preclinical therapeutic intervention studies (reviewed in Drost and Jonkers, 2009).

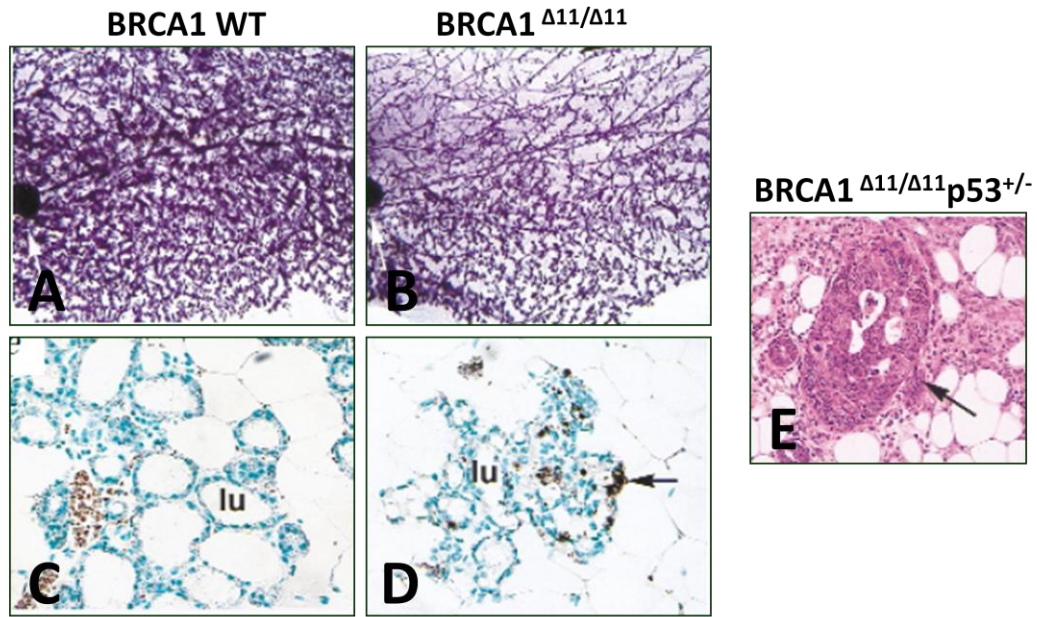


Figure 1.13. Abnormal mammary gland development in $BRCA1^{\Delta 11/\Delta 11}$ and mammary tumors in $BRCA1^{\Delta 11/\Delta 11}p53^{+/-}$ mice. A) Branching of mammary epithelium in BRCA1 WT mice (P16.5). B) Branching of mammary epithelium in $BRCA1^{\Delta 11/\Delta 11}$ mice is decreased (P16.5). TUNEL assay for apoptosis revealed that C) none of the epithelial cells in BRCA1 WT mice undergo apoptosis, while D) 20% of epithelial cells in $BRCA1^{\Delta 11/\Delta 11}$ were apoptotic (arrow). E) A ductal carcinoma *in situ* developed in $BRCA1^{\Delta 11/\Delta 11}p53^{+/-}$ mice. lu, lumen. Adapted from Xu *et al.*, 1999.

Several groups used mouse models to study the influence of estrogen in *BRCA1*-associated tumorigenesis. Treating postpubertal $BRCA1^{FL/FL}$ MMTV-Cre or $BRCA1\Delta 11p53$ -mutant mice with estrogen resulted in higher cellular proliferation than in the wild type mice and accelerated mammary tumor growth (Jones *et al.*, 2008). These studies support the notion that *BRCA1*-loss leads to upregulation of estrogen/ $ER\alpha$ activity and increased mammary cell proliferation resulting in tumorigenesis. These models also revealed that $ER\alpha$ is highly expressed in the premalignant mammary gland and the initiating low grade tumors. However, $ER\alpha$ expression progressively decreased as the stage of tumors increased (reviewed in Dine and Deng, 2012). These findings are also in agreement with a study that examined

correlation of ER α expression and tumor stage in *BRCA1*-associated cancers. Interestingly, low grade *BRCA1*-mutant breast cancers were mostly ER α positive (71.4%), while grade 3 breast cancers were mostly ER α negative (84.3%) (Foulkes *et al.*, 2004). These studies suggest that ER α signaling might be significant in early stages of formation of *BRCA1*-associated mammary tumors, but for later stages of tumorigenic progression it is not as important. Furthermore, cancer prevention studies have been performed examining the effect of oophorectomy on tumor initiation and progression in *BRCA1*^{Flox11/11}; MMTV-Cre; *p53*^{+/-} mice (Bachelier *et al.*, 2005). Surprisingly, while tumor initiation was not significantly different between oophorectomized and control mice (during the first 135 days post-surgery), a 50% reduction in tumor growth was seen after 180 days post-surgery (Dine and Deng, 2012). Nevertheless, these studies support the notion that hormones play an important role in *BRCA1*-associated tumorigenesis.

Conditional models that develop mammary tumors with strong resemblance to human *BRCA1*-mutated breast tumors have been used to predict the response to conventional and targeted therapeutics. Responses to various conventional chemotherapeutics such as doxorubicin, docetaxel and cisplatin were studied as well as mechanisms of acquired resistance (Rottenberg *et al.*, 2007). Heterogeneity in the responses of individual mouse mammary tumors was seen. Eventually, most of the tumors become resistant to doxorubicin and docetaxel. The main mechanism of resistance is via upregulation of ATP-binding cassette (ABC) drug transporters (Rottenberg *et al.*, 2007). Interestingly, acquired resistance to platinum compounds was never observed in mouse models, but tumors are never completely eradicated (Drost and Jonkers, 2009). Platinum resistance, however, is a major issue in clinic. In *BRCA1*-mutated human tumors resistance to platinum compounds is associated with

secondary mutations in *BRCA1* that restore the open reading frame. Thus, the reason platinum resistance is never seen in mice is because large mutations of *BRCA1* reading frame can never be restored as it occurs in human tumors (Drost and Jonkers, 2009; Dine and Deng, 2012). In support of this idea is Shaffe *et al.* (2008) study where platinum resistance was reported in one mouse model. Tumors recurred after a second round of treatment with platinum drugs and had a faster growth rate. An important difference between this and other studies was that the hypomorphic allele *BRCA1-Δ11* isoform was intact in these tumors leading to partial *BRCA1* activity. This study indicates that restoration or partial activity of *BRCA1* can lead to resistance to platinum agents (Shaffe *et al.*, 2008).

Mouse models have been used to assess the response of *BRCA1*-deficient tumors to PARP inhibitors. *BRCA1*-deficient cells were found to be highly sensitive to chemical inhibitors of Poly(ADP-ribose) polymerase-1 (PARP1) (Farmer *et al.*, 2005). PARP1 is a key molecule in the repair of DNA single-strand breaks. By inactivation of SSB repair, DSBs are induced by replication fork collapse at SSB during S phase. The effect is synthetic lethality in cells with *BRCA1* loss, serving as a specific therapy for *BRCA1*-mutated tumors (Drost and Jonkers, 2009; Dine and Deng, 2012). As in case of platinum resistance, *BRCA1*-deficient tumors in mice showed prolonged response to the clinical PARP inhibitor, olaparib. However, tumors became resistant as a consequence of upregulation of the P-glycoprotein drug efflux pump (Rottenberg *et al.*, 2008). A combination therapy with platinum and olaparib showed increased relapse-free survival suggesting that PARP inhibition may enhance the effects of DNA-damaging agents (Drost and Jonkers, 2009; Dine and Deng, 2012).

Although modeling BRCA1-associated tumorigenesis in mice has had its pitfalls, it still provided a great deal of information about the function of BRCA1 *in vivo*. It served to validate a number of observations reported by *in vitro* studies. The analysis of *BRCA1*-mutant mice supports the notion that BRCA1 plays an essential role in maintaining genome integrity through DNA damage repair, centrosome duplication, and cell-cycle checkpoints regulation. Furthermore, models that recapitulate the phenotype of human tumors can be used to study the response as well as resistance of *BRCA1*-tumors to various drugs. In the future, it would be beneficial to develop better models of *BRCA1*-associated ovarian tumors as well as mouse models with specific mutations that are found to be deleterious in humans.

1.7 Unresolved questions of BRCA1

There are several unusual observations involving the phenotype as well as the progression of *BRCA1*-associated cancers that the current knowledge about BRCA1 function cannot explain. *BRCA1*-associated cancers have an early onset compared to the sporadic breast and ovarian cancers, which has been poorly investigated. Since BRCA1 functions in DNA damage repair it is not known why *BRCA1*-associated cancers develop predominantly in breast and ovarian tissues. In addition, it is unclear why the majority of *BRCA1*-associated breast cancers are mainly of the basal subtype. Also, as we continue to investigate *BRCA1*-associated cancers with more sophisticated technologies, doubts have been raised whether LOH is indeed the rate limiting step in *BRCA1*-tumor progression.

Large population-based studies have reported that on average *BRCA1*-mutation carriers are younger at the time of breast cancer diagnosis compared to noncarriers. *BRCA1*-mutation carriers may develop breast cancer as early as 18 years of age.

Specifically, by age 40 the estimated cumulative risk of breast cancer in carriers is 10%, whereas in noncarriers it is 0.2%. Moreover, at the highest cumulative risk for noncarriers (6.8% at age 80) the risk for carriers is 73.5%, implying that the lifetime risk of developing breast cancer in *BRCA1*-mutation carriers is overwhelmingly high compared to the general population (Whittemore *et al.*, 1997). Early onset of breast cancers, as mentioned previously, is observed in germline mutation carriers of other high and moderate susceptibility genes. Because most of these genes function in genome surveillance and repair, the idea is that these individuals have a highly unstable genome leading to rapid mutator phenotype which results in faster cell proliferation and tumor progression (Musolino *et al.*, 2007).

Risk of developing breast and ovarian cancers specifically in *BRCA1*-mutation carriers is unusually higher than the risk of developing any other type of epithelial cancer. Since *BRCA1* is important in DNA damage repair, the increased risk of developing cancer has generally been attributed to compromised DNA damage repair activity in cells. However, this proposed function of *BRCA1* is not specific to breast epithelial cells and therefore, it remains unknown why *BRCA1*-mutations are preferentially associated with increased incidence of cancer in only a small subset of tissues rather than a generalized increase in all cancer types, as is observed with other tumor suppressor proteins involved in DNA damage repair (e.g. p53, ATM). Several ideas have been proposed. In unaffected tissues, other proteins might serve to compensate for improper *BRCA1* function in the context of DNA damage repair (for example). If this is the case, then such “compensation” for *BRCA1* loss is absent in ovarian and breast epithelial cells. However, severe embryonic lethality in *BRCA1*^{-/-} mice is inconsistent with this hypothesis (Scully and Livingston, 2000). Another possibility is that *BRCA1* performs a unique function in breast and ovarian epithelial

cells and this function is only relevant for tumor suppression. BRCA1's role in downregulation of estrogen receptor would fit the requirements for the tissue specificity. However, majority of *BRCA1*-tumors are ER-negative and this presents a challenge for this hypothesis (Zheng *et al.*, 2001; Monteiro, 2003). Also, it has been proposed that breast and ovarian cells might exhibit a delay in apoptosis compared to other rapidly dividing cells. Therefore, loss of WT *BRCA1* allele is longer tolerated in these tissues compared to unaffected tissues (Elledge and Amon, 2002). In addition, it has been suggested that the rate of LOH might differ between tissues. In support of this idea are studies in mice where it has been shown that rates of mitotic recombination vary in a tissue-specific way and seem to be higher in females as compared to males (Holt *et al.*, 1999; Monteiro, 2003). It is most likely that a combination of these factors is contributing to tissue specificity of *BRCA1*-associated cancers. However, the research efforts on this front are still in infancy because it requires comparative studies of BRCA1 function in different tissues and cell-types. Thus, it has been challenging to address this question since BRCA1-heterozygosity in mice does not result in tissue-specific tumor development.

Another perplexing observation is that *BRCA1*-associated breast cancers often develop rapidly between screens, and at the time of diagnosis are more likely to exhibit aggressive features compared to cancers developed in noncarriers. Several studies have reported that nearly 50% of *BRCA1*-mutation carriers who undergo close surveillance develop malignant disease in less than a year after having normal findings on screening mammography (Komenaka *et al.*, 2004). Also, a study of clinical characteristics and outcomes of breast cancers that arise in *BRCA*-mutation carriers and noncarriers suggested that *BRCA*-associated breast cancers were significantly more likely to be histologic grade III (100% vs 59%, $p=.04$) including

having other adverse clinical and histopathological features when compared with cases not associated with *BRCA* mutations (Robson *et al.*, 1998). At present, it is not known what is the underlying cause of this rapid mutator phenotype.

In addition, *BRCA1*-mutation carriers preferentially develop poorly differentiated, basal subtype of breast cancers. Unlike other unresolved questions related to *BRCA1*, this one has received more attention in recent years. A role of *BRCA1* in differentiation of mammary epithelium has been proposed in early studies of *BRCA1* function in mouse and human cells (Lane *et al.*, 1995; Kubista *et al.*, 2002). Because of the considerable similarities between basal-like and *BRCA1*-mutant breast cancers, one of the initial hypothesis was that *BRCA1*-mutation carriers have increased number of basal/stem progenitor cells that are also more likely to transform because of *BRCA1*-dysfunction (Foulkes, 2004). Several subsequent studies have observed changes in differentiation in human mammary epithelial cells (HMECs) following *BRCA1* knockdown, proposing that dysfunction in *BRCA1* leads to an increase in progenitor or stem-like populations of mammary epithelial cells (Furuta *et al.*, 2005, Liu *et al.*, 2008). In addition, recent studies using mammary epithelial cells from *BRCA1*-mutation carriers reported defects in the luminal progenitors, which are also likely to contain the cell of origin of *BRCA1*-associated breast cancers (Lim *et al.*, 2009, Proia *et al.*, 2011). These studies suggest that germline mutation in *BRCA1* perturbs differentiation of breast epithelium and that this aberrant differentiation program dictates the subtype of breast cancer in *BRCA1*-mutation carriers.

According to the Knudson two-hit hypothesis, loss of heterozygosity in tumor-suppressor genes is the limiting step in tumor development. LOH is frequently observed in *BRCA1*-associated cancers and the regions around the cancers as well.

However, there have been conflicting reports regarding LOH in healthy and preneoplastic breast tissues. Some reports found it to be rare, while others found that the number of samples with evidence of LOH was as high as 50%. The difference in observations might be due to tissue sections examined, since in some studies the tissue was obtained from prophylactic mastectomies, while in others the tissue was adjacent to cancer. More recent observations, using laser microdissection to isolate cells from various pathologic lesions and corresponding normal tissues, suggest that there is considerable heterogeneity in loss of heterozygosity (affecting wt as well as mutant allele) within and between preinvasive lesions and invasive cancers from *BRCA1*-mutation carriers (Cavalli *et al.*, 2004, Clarke *et al.*, 2006, King *et al.*, 2007, Martins *et al.*, 2012). Also, it has been reported on several occasions that hallmarks of *BRCA1*-associated cancers (increased genomic instability, inefficient DNA damage repair, PTEN and p53 loss) can be already observed in *BRCA1*-haploinsufficient cells, and therefore prior to *BRCA1* loss (Baldeyron *et al.*, 2002; Rennstam *et al.*, 2010; Konishi *et al.*, 2011; Martins *et al.*, 2012). Taken together, these data suggest that LOH is a stochastic (albeit still potentially selected) event and not an obligatory first step for initiation of *BRCA1*-linked breast tumors.

1.8 Speculations and evidence for *BRCA1*-haploinsufficiency phenotype in human cells

No obvious developmental phenotype is apparent in human or murine carriers of heterozygous mutations in *BRCA1* since both species develop normally. However, studies looking at genomic stability in healthy breast tissue from *BRCA1*-mutation carriers as well as genetically engineered human *BRCA1*-heterozygous cells revealed that certain phenotypes associated with DNA damage repair exist. In a study by

Rennstam *et al.* (2010) the types of genomic aberrations frequently found included low copy number gains and losses. Some of these gene copy number changes were similar across samples from different patients and linked to biological functions such as transcriptional regulation and DNA binding. In addition, studies addressing the capacity for DNA damage repair have been conducted in cells isolated from *BRCA1*-mutation carriers as well as genetically engineered *BRCA1*-heterozygous cells. They observed increase in genomic instability and substantial deficiency in error free double strand end joining as well as homologous repair. Also, *BRCA1*-heterozygous cells frequently exhibited spontaneous hyper-recombination and sensitivity to genotoxic stress (Baldeyron *et al.*, 2002; Cousineau and Belmaaza, 2007; Konishi, 2011). Altogether, these data indicate that *BRCA1*-haploinsufficient cells exhibit defects in maintenance of genomic stability. But, it is difficult to discern from these studies whether this is a general or tissue/cell-type specific phenotype of *BRCA1*-haploinsufficiency.

Other phenotypes associated with *BRCA1*-haploinsufficiency have also recently been described. Lim *et al.* as well as Proia *et al.* examined properties of breast epithelium derived from *BRCA1*-mutation carriers and showed altered differentiation in their cells. These studies showed that proportions of mammary progenitor cells in the breast tissue from *BRCA1*-mutation carriers were significantly altered compared to wildtype. While Lim *et al.* reported expansion of luminal progenitors, Proia *et al.* observed an increase in basal progenitors. The differences in observations could be explained by the fact that luminal progenitor cells in *BRCA1*-mutation carriers appear to co-express luminal as well as basal markers, as described by Proia *et al.* (Figure 1.14). Consistent with this, several differentiation markers in breast epithelium from *BRCA1*-mutation carriers were aberrantly expressed and

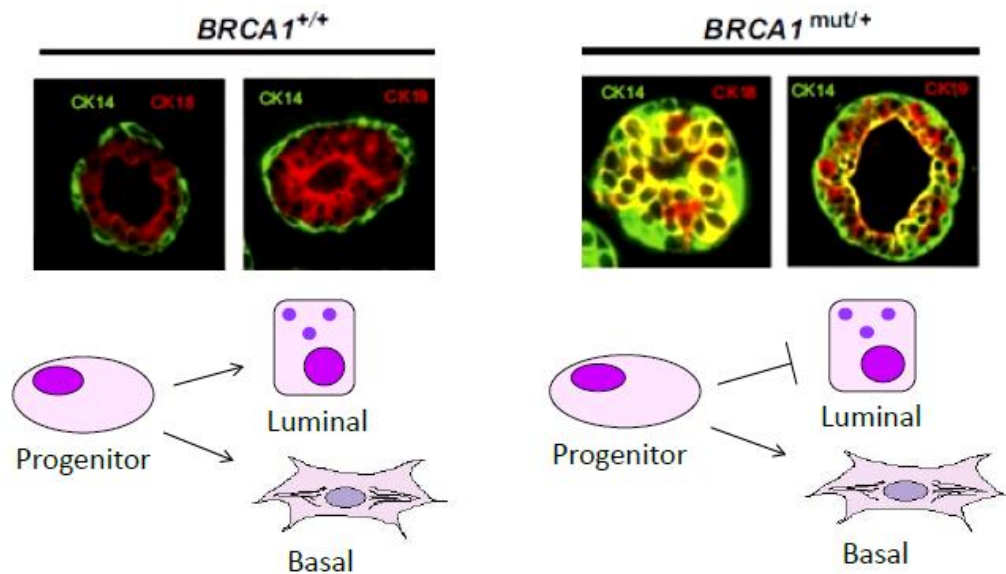


Figure 1.14. BRCA1-haploinsufficient epithelium exhibits defects in full commitment to luminal lineage. Cross-sections of epithelial outgrowths formed *in vivo* from breast epithelial cells isolated from *BRCA1* WT and *BRCA1*^{+/^{mut} patients. Sections were stained for markers of mature breast epithelial lineages using immunofluorescence. *BRCA1* WT outgrowths have clearly distinct basal (CK14-positive) and luminal (CK18- and CK19-positive) cells forming the epithelial bilayer. *BRCA1*^{+/^{mut} outgrowths have distinct basal cells (CK14-positive), however the “luminal” cells display immature phenotype by expressing both basal and luminal markers. Adapted from Proia *et al.* 2011.}}

notable changes in genes associated with DNA transcription and chromatin remodeling were found suggesting defects in lineage commitment. Taken together the accumulating evidence is suggesting that mutation in one *BRCA1* allele, in addition to other phenotypes, results in haploinsufficiency for mammary epithelial fate commitment.

Mechanistically, it is unclear how *BRCA1*-haploinsufficiency leads to these various phenotypes. One possibility is that mutant *BRCA1* may interfere with the function of wildtype *BRCA1* in *BRCA1*-heterozygous cells. While this issue has not been extensively examined to date, a few studies propose that certain full-length mutant *BRCA1* proteins may function incorrectly in the cell. In particular, Fan and colleagues (2001) found that carboxy-terminal truncated *BRCA1* proteins can abrogate certain functions of WT *BRCA1* such as chemosensitivity, susceptibility to

apoptosis, and inhibition of estrogen receptor transcriptional activity. In addition, Ye *et al.* (2001) reported that mutations in 3' region of BRCA1 gene enhance its recruitment to chromatin and chromatin unfolding compared to the wildtype. Therefore, it possible that some mutant BRCA1 proteins may act in dominant negative fashion and thereby actively promote tumorigenesis, but further research into this topic is needed.

1.9 Significance, purpose and innovation of the project

A number of mouse models have been unable to reveal why mutations in *BRCA1* lead to breast and ovarian cancer in humans. The differences are apparent in at least two ways: *BRCA1*-heterozygosity in mice does not increase the risk of cancer, while *BRCA1*-dysfunction leads to profound effects on proper development of the whole organism rather than the specific tissue. Moreover, *BRCA1*-heterozygous mice do not exhibit developmental, cellular or molecular defects, nor do they develop spontaneous mammary or ovarian tumors. Thus, while these studies have been extremely insightful, they suggest that *BRCA1* function is considerably different in mice compared to humans, which challenges the idea of using mice to model this particular human condition.

Our lab was among the first to report how mutations in *BRCA1*, in part, predispose for the formation of aggressive breast cancers in humans. Proia *et al.* found that breast epithelial cells from *BRCA1*-mutation carriers exhibited perturbations in differentiation, and that these defects acted prior to evidence of breast cancer incidence to disrupt the lineage commitment programs thereby influencing tumor phenotype. Our findings along with others provide strong evidence that additional molecular and cellular features associated with *BRCA1*-haploinsufficiency may be

unique to breast epithelial cells and may help explain the increased propensity for neoplastic transformation. Thus, we hypothesize that, in addition to DNA damage repair, BRCA1 has other functions that are specific to breast epithelium. We decided to test this hypothesis by comparing the behavior of epithelial cells and fibroblasts ex-vivo from breast and skin tissues obtained from prophylactic mastectomies (from *BRCA1*-mutation carriers) and reduction mammoplasties (*BRCA1*-non carriers) (Figure 1.15). Identifying BRCA1 function and the molecular events associated with it that are unique to the breast epithelial cells from *BRCA1*-mutation carriers is a highly innovative approach. Further understanding of the behavior of different cell types from *BRCA1*-mutation carriers will provide insight(s) into tissue-specificity of BRCA1's tumor-suppression and may ultimately lead to development of more efficient and less invasive treatments for *BRCA1*-mutation carriers.

Model: Isolate cells from

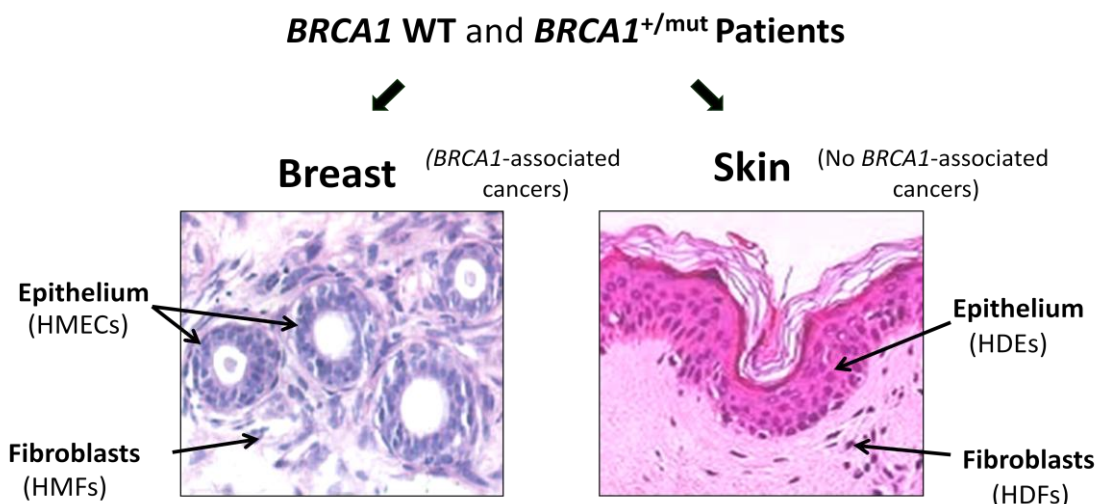


Figure 1.15. Model to test the hypothesis that BRCA1 has distinct functions in breast tissue. Breast and skin tissues are obtained from *BRCA1* WT and *BRCA1*^{+/-mut} patients that underwent reduction mammoplasty and prophylactic mastectomy, respectively. Tissues are further broken down into fractions of cells enriched for epithelium as well as fibroblasts. Different cell-types (HMECs, HMFs, HDEs, and HDFs) were plated and their behavior in culture was compared.

1.10 Specific Aims

1. To identify a phenotype in breast epithelial cells associated with BRCA1-haploinsufficiency and higher probability of transformation.
2. To determine whether the phenotype is tissue/cell-type specific.
3. To characterize the underlying mechanism of the phenotype associated with BRCA1-haploinsufficiency in order to understand BRCA1's function in tissue/cell-type specific manner.

CHAPTER 2.

MATERIALS AND METHODS

2.1 Cell Lines and Tissue Culture

All human breast tissue procurement for these experiments was obtained in compliance with the laws and institutional guidelines, as approved by the institutional IRB committee from Brigham and Women's Hospital and Tufts Medical Center. Disease-free prophylactic mastectomy (4 fresh, 10 formalin-fixed paraffin embedded) and skin tissue derived from women carrying a known deleterious *BRCA1* heterozygous mutation were obtained with patient consent from the Surgical Pathology files or immediately following prophylactic mastectomy surgery. Tissues in which *BRCA1* mutation was confirmed but not known were submitted for sequence/genotyping at Myriad Genetic Laboratories to confirm *BRCA1* mutation. Non-*BRCA1* tumor tissues were obtained from discarded material at Tufts Medical Center undergoing elective reduction mammoplasty at Tufts Medical Center. *BRCA1* mutation status is listed in Table 3.1. The range of patient ages for fresh *BRCA1*^{+/+} tissue used in this study was 30-54 with a median age of 40; the range of patient ages for fresh *BRCA1*^{mut/+} tissue used in this study was 35-53 with a median age of 44. All disease-free breast tissues were verified by surgical pathologists prior to use in these studies.

HMECs were isolated as previously described (Proia 2011) and cultured in MEGM (Lonza) supplemented with bovine pituitary extract (BPE), insulin (5 µg/mL), EGF (10 ng/mL) and hydrocortisone (1 µg/mL). These cells were immortalized with the catalytic subunit of human telomerase (hTERT) as previously described (Elenbaas 2000). Human mammary fibroblasts (HMFs) were isolated as previously described (Proia 2011) and cultured in DMEM (Invitrogen) supplemented with 10% Calf Serum. Keratinocytes (HDEs) and dermal fibroblasts HDFs were isolated as

previously described (Normand and Karasek 1995). Briefly, skin tissue was chopped up into 0.5cm cubes using a razor blade, and incubated overnight for digestion in a Dispase-containing solution. The following day, epidermis and dermis layers were separated and incubated in Collagenase-containing solution for 20min at 37°C. Tissue/cell suspensions were pelleted, resuspended in trypsin, and frequently agitated to promote dissociation of cells. Dissociated epidermis layer was pelleted, plated and cultured in KGM-2 (Lonza) supplemented with bovine pituitary extract (BPE), insulin (5 µg/mL), hEGF (10 ng/mL), hydrocortisone (1 µg/mL), GA-1000 (gentamicin, amphotericin-B), Epinephrine, and Transferrin. Dissociated dermis layer was pelleted, plated and cultured in DMEM (Invitrogen) supplemented with 10% Calf Serum.

2.2 Lentiviral Constructs and Virus Production

The VSV-G-pseudotyped lentiviral vectors were generated by transient cotransfection of the vector construct with the VSV-G-expressing construct pCMV-VSVG (Miyoshi 1998) and the packaging construct pCMV DR8.2Dvpr (Miyoshi 1998), generously provided by Inder Verma, into 293T cells together with FuGENE 6 transfection reagent (Roche). Lentiviral shRNA constructs targeting BRCA1, SIRT1 and pRb (Sigma-Aldrich MISSION shRNA SHCLNG-NM_007294, SHCLNG-NM_012238 and SHCLNG-NM_000321, respectively) were prepared as previously described (Gupta 2005).

2.3 Western Blot Analysis

Cultured cells were harvested by trypsinization, pelleted and incubated in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche) to obtain whole cell lysates. Cellular debris was removed by centrifugation at 13,000rpm for 10min. 30µg of the whole cell lysate was used per sample. Western blotting was performed according to the manufacturer's protocol (BioRad). Briefly, 12% and/or 4-12% pre-cast gels (depending on the kDa size of the proteins) and XT-MOPS running buffer were used for SDS-PAGE electrophoresis. 0.2 or 0.45µm nitrocellulose membrane was used for protein transfer. Membranes were incubated overnight at 4°C with primary antibodies diluted in 1% bovine serum albumin in TBS-T. Secondary antibodies were applied for 1hr at room temperature. The antibodies used included p16 (Santa Cruz), p53-Ser15 (Cells Signaling), p53-total (Santa Cruz), p21 (Santa Cruz), γH2AX (Cell Signaling), p27 (Santa Cruz), pRb-Ser795 (Cell Signaling), pRb-total (Santa Cruz), Cyclin E (Santa Cruz), Cyclin A (Santa Cruz), SIRT1 (Millipore), and β-actin (AbCam).

2.4 Immunoprecipitation (IP)

shRNA-expressing WT HMECs (shScr, shBRCA1 and shSIRT1) were lysed in IP buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Roche). For immunoprecipitation assays, protein lysates (200-600 µg) were combined with 2µg of antibody and 25 µl of Protein A/G-Plus agarose beads (Santa Cruz, sc-2003). Following an overnight incubation at 4°C, agarose beads were extensively washed in IP buffer, resuspended in SDS sample buffer (125 Mm Tris pH 6.8, 2.5% SDS, 10%

glycerol, 2.5% 2-mercaptoethanol, 0.01% bromo-phenol-blue) and loaded into a protein gel. Antibodies used in these experiments included anti-pRB (BD Pharmingen, #554136) and anti-Acetylated lysine (Cell Signaling, #9441).

2.5 Histone Acid Extractions/Blots

Cells were harvested by trypsinization and acid extraction of histone proteins was carried out as described previously (Shechter 2007). Briefly, cells were lysed in PBS with 0.5% Triton X-100, 2mM PMSF and 0.02% NaN₃, nuclei were pelleted by centrifugation at 1000x g and the nuclear pellet was incubated at 4°C overnight in 0.2N HCl. Western blotting was carried out as described above using 5 ug of acid-soluble lysate per sample. Antibodies used: anti-histone H3 (Cell Signaling #9715, 1:1000), anti-acetyl-histone H3K9 (Cell Signaling #9649P, 1:1000), anti-histone H4 (Millipore 07-108, 1:250), anti-acetylated histone H4K16 (Millipore 07-108, 1:500).

2.6 Senescence Associated β -galactosidase Assay

Senescence associated β -galactosidase staining was performed according to the chromogenic assay as previously described (Debacq-Chainiaux 2009). Briefly, cells were cultured in 6-well plates and fixed with formaldehyde/glutaraldehyde solution. After fixation, cells were washed twice in PBS. Samples were covered with staining solution and incubated overnight (12-16h) at 37°C (no CO₂). Images were captured by bright field microscopy.

2.7 Quantitative RT-PCR

Total RNA from cultured cells was extracted with the RNeasy Mini Kit (QIAGEN). cDNA was prepared with an iScript kit (BioRad) and PCR was carried out with SYBR Green (BioRad). The following primers were used in this study:

Cyclin A: Forward 5'-CGCTGGCGGTACTGAAGTC-3' and Reverse 5'-AAGGAGGAACGGTGACATGC-3',
IL-6: Forward 5'-AACCTGAACCTTCCAAAGATGG-3' and Reverse 5'-TCTGGCTTGTTCTCACTACT-3',
MMP2: Forward 5'-CCGTCGCCCATCATCAAGTT-3' and Reverse 5'-CTGTCTGGGGCAGTCCAAAG-3',
IL-8: Forward 5'-ACTGAGAGTGATTGAGAGTGGAC-3' and Reverse 5'-AACCCTCTGCACCCAGTTTTTC-3',
PAI-1: Forward 5'-GCTTGTCCAAGAGTGCATGGT-3' and Reverse 5'-AGGGCTGGTTCTCGATGGT-3',
Rb: Forward 5'-GCCTCTCGTCAGGCTTGAG-3' and Reverse 5'-TCATCTAGGTCAACTCGTGCAA-3',
SIRT1: Forward 5'-GCAGATTAGTAGGCGGCTTG-3' and Reverse 5'-GCTGGTGGAACAATTCCTGT-3',
p14: Forward 5'-GGCCCTCGTGCTGATGCTAC-3' and Reverse 5'-TGGAGCAGCAGCAGCTCCGC-3',
p15: Forward 5'-GGACTAGTGGAGAAGGTGCG-3' and Reverse 5'-GGGCGCTGCCCATCATCATG-3',
p16: Forward 5'-CACCGAATAGTTACGGTCGG-3' and Reverse 5'-GCACGGGTCGGGTGAGAGTG-3',
p18: Forward 5'-GGGGACCTAGAGCAACTTAC-3' and Reverse 5'-GTAGCAGTCTCCTGGCAATC-3',
p19: Forward 5'-

CTCAACCGCTTCGGCAAGAC-3' and Reverse 5'-
GGACTGGTACCGGAGGTGTC-3'. *GAPDH*: Forward 5'-
GAGTCAACGGATTTGGTCGT-3' and Reverse 5'-
TTGATTTTGGAGGGATCTCG-3' was used as an internal control. Analysis was performed with the delta-delta Ct method.

2.8 Immunofluorescence (IF)

Cells were cultured on 8-well chamber-slides and fixed with methanol at -20°C for 10 min. Samples were incubated overnight at 4°C with primary antibodies diluted in 1% bovine serum albumin PBS. Fluorescently labeled secondary antibodies were applied for 1 hr at room temperature. Cells were counterstained with DAPI. A Nikon Eclipse 80t microscope and SPOT camera were used for analyzing and photographing the stained sections. The antibodies used included Ki-67 (AbCam), γ H2AX (Cell Signaling), p53BP (Cell Signaling), and pATM/ATR (Cell Signaling).

2.9 Immunohistochemistry (IHC)

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections with sodium citrate antigen retrieval, followed by visualization with the ABC Elite peroxidase kit and DAB substrate (Vector Labs) for detection of SIRT1 (Millipore). IHC results were semi-quantitatively analyzed using the Allred Score as previously described (Proia 2011).

2.10 Telomere Chromatin Immunoprecipitation and qPCR

ChIP assays on shControl, shBRCA1 and shSIRT1 HMECs were performed as previously described (Garcia Cao 2004). In brief, after crosslink and sonication, chromatin from 4x10⁶ cells were used per each immunoprecipitation with protein A/G Plus agarose beads (Santa Cruz Biotechnology, sc-2003) and the following antibodies: 5 µg of anti-histone H3 (#ab1791, Abcam), 5 µg of anti-H3K9 (#H9286, Sigma), 5 µg anti-histone H4 (#ab10158, Abcam), 5 µg of anti-H4K16Ac (#39167, Active Motif) or preimmune serum. The immunoprecipitated DNA was transferred to a Hybond N+ membrane using a dot blot apparatus. The membrane was then hybridized with a telomeric probe containing TTAGGG repeats. Quantification of the signal was performed with ImageJ software. The amount of telomeric DNA after ChIP was normalized to the total telomeric DNA signal respectively for each genotype (input), as well as to the H3 and H4 abundance at these domains, thus correcting for differences in the number of telomere repeats or in nucleosome spacing.

Chromatin immunoprecipitations on *BRCA1*^{mut/+} and WT HMECS were performed as previously described (Lee 2006), except that cross-linked nuclei were sonicated to 150–500 bp fragments in buffer containing 1% SDS, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1 mM PMSF, and complete protease inhibitors (Roche), and bound ChIP complexes were washed according to the Upstate/Millipore protocol, as previously described (Palacios 2010, Mulligan 2011). Antibodies used were: anti-SIRT1 (Cyclex Co. Ltd., Japan), anti-H4K16ac (Millipore, MA, USA) and anti-histone H3 (Abcam, UK). Quantitative PCR analysis of telomeric sequences was performed as described previously (Cawthon 2002), using forward primer (5'-CGGTTTGTGGTTGGGTTGGGTTGGGTTGGGTTGGGTT) and reverse primer

(5'- GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCC) at an annealing temperature of 60°C.

2.11 Chromosomal Metaphase Analysis

Cultures were checked for harvest on the third day after trypsinization, and 30 ul of colcemid (10 ug/ml Gibco) was added per 5 ml of culture medium. Cultures were incubated for 30 minutes at 37°C. Cells were detached from flasks with trypsin and the supernatant and cells were spun at 1,100 rpm for 5 minutes. The supernatant was discarded and replaced with 2:1 hypotonic solution (2 parts 0.075M potassium chloride to one part 0.6% sodium citrate). The cultures were incubated at 37°C for 20 minutes, and then fixed with several changes of fixative (methanol, acetic acid). Slides were prepared, treated with trypsin and stained with Wright's-Giemsa.

2.12 Telomere Length Assays

The overall telomere lengths for each experimental sample were determined relative to the reference DNA by comparing the difference in their ratios of the telomere copy number (T) to the single copy gene copy number (S) using quantitative PCR. This ratio is proportional to the mean telomere length (Cawthon 2002, Cawthon 2009). We used a modified qPCR assay for telomere sequence quantitation that is compatible with Applied Biosystems 7900 HT instrumentation. Each plate (384 wells on each plate) contained a set of standards spanning an 81-fold range prepared by serial dilution, and each sample was analyzed in triplicate. Two master mixes of PCR reagents were prepared, one with the telomere primers (telc and telg) and the other

with either the albumin pair (albd, and albu) or the beta-globin pair (hgbu, and hgbd). The final concentrations in each PCR reaction were 0.8X SYBR Green I Master Mix (Agilent Technologies), and 900nM of the telomere pair, or 900nM of the albumin pair, or 500nM of the beta-globin pair. The thermal cycling profile used was 15min at 95°C, 2 cycles of 15s at 94°C, 15s at 49°C, followed by 32 cycles of 15s at 94°C, 10s at 62°C, and 15s at 74°C with data acquisition. The plates were read at 74°C to minimize the interference from the telomere primer-dimers. The ABI software SDS version 2.0 was used to generate two standard curves from each plate, one for the telomere amplification, and the other for the single copy gene. The ratio (T/S) of the telomere copy number (T) to the single gene copy number (S) was generated for each experimental sample, and the value averaged across the triplicates, which provides the average telomere length for each experimental sample. The T/S ratios relative to the reference sample were generated using the comparative CT (cycle threshold) method (Cawthon 2002, Cawthon 2009).

2.13 Allele Specific Loss of Heterozygosity Studies

PCR primers were designed flanking the *BRCA1* mutations from the individuals in the study (187delAG, 2800delAA, 4184del4, 5385insC, 943ins10, and 4154delA). PCR products were treated with ExoSap-It (USB) and sequenced. Sequence traces in the forward and reverse direction were compared between control blood DNAs of individuals with these germline mutations and the different derivatives of primary human mammary epithelial cells from individuals with these mutations using DNASTAR 3.0 (www.dnastar.com). Loss was determined visually by

two reviewers and consisted of at least 30% difference between the two alleles compared to normal carrier ratios as described (Spearman 2008).

2.14 Quantitative Telomere Fluorescence in-situ Hybridization

For qFISH analysis on breast tissue samples, de-paraffinated sections were hybridized with a PNA-tel Cy3-labeled probe, and telomere length was determined as described ([Zijlmans 1997](#), [Gonzalez-Suarez 2000](#), [Samper 2000](#), [Muñoz 2005](#), Flores 2008). DAPI and Cy3 signals were acquired simultaneously into separate channels using a confocal ultraspectral microscope Leica TCS-SP5 and maximum projections from image stacks were generated for image quantification.

For image acquisition we used a new tool for intelligent screening named “matrix screening remote control (MSRC)” developed at CNIO. The MSRC application manages a first fast scan with low-resolution settings, generating one image per sample of the whole tissue and later localizes the areas of interest, extracting their coordinates and surface area. With the spatial information, the MSRC application interacts with the microscope and load high-resolution settings, scanning automatically just the areas of interest.

Quantitative image analysis of telomere fluorescence intensity was performed on confocal images using the Definiens Developer Cell software (Definiens Developer XD). The DAPI image was used to define the nuclear areas that were separated by a Cellenger-Solution. After defining the nuclear areas a predefined Ruleset was used for the quantification of telomere fluorescence intensity (Cy3 image). The fluorescence values for each section were exported to GraphPad Prism,

and graphs were generated. The total number of telomeric spots scored for each genotype is shown. Student t-test was used for statistical analysis.

2.15 *BRCA1*^{mut/+} Gene expression analysis, GSEA and network analysis

Gene Set Enrichment Analysis (GSEA) was applied to previously published gene expression data collected on cultured proliferating primary human mammary epithelial cells isolated from *BRCA1*-mutation carriers (N=6) or age-matched WT (N=6) (GSE19383, Bellacosa *et al.*, 2010). Two-sided T tests were run on the gene sets and the top 2000 genes from each set were ranked. Gene Set Enrichment Analysis (GSEA) was performed as described previously (Subramanian *et al.*, 2005). Gene networks were constructed from our previously published gene expression data collected on freshly isolated human mammary epithelial cells isolated from *BRCA1*-mutation carriers (N=4) or age-matched WT (N=4) (GSE25835, Proia *et al.*, 2011). Important hubs were identified using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Mountain View, CA) based on differentially expressed genes between *BRCA1*^{mut/+} and WT patients (n = 701 genes).

CHAPTER 3.

RESULTS

3.1 *BRCA1*^{mut/+} HMECs exhibit increased genomic instability and telomere dysfunction.

Loss of ability to properly maintain genetic information and loss of control over propagation of those mutations is one of the critical steps in tumorigenesis (Vogelstein and Kinzler, 1993; Weinberg, 2007). A hallmark of BRCA1-deficient cells is increased genomic instability that is thought to be due to the central role of BRCA1 in mediating the steps between sensing and repairing the DNA damage, as well as coupling these processes to the cell-cycle machinery (Huen *et al.*, 2010; Roy *et al.*, 2012). Therefore, BRCA1 appears to be involved in an array of pathways essential for genomic maintenance such as homologous recombination, double strand break repair, S-phase, G2/M, and spindle checkpoints, as well as in centrosomal regulation (Zhang and Powell, 2005). Furthermore, an essential role of BRCA1 in these DNA damage response (DDR) processes has been demonstrated in various cells-types (usually in a setting of biallelic *BRCA1* inactivation) of human as well as mouse origin (Cao *et al.*, 2002; Greenberg *et al.*, 2006; Pathania *et al.*, 2011).

Recent studies suggest that considerable genomic abnormalities can be seen in breast epithelium from *BRCA1*-mutation carriers as well as genetically engineered BRCA1-haploinsufficient mammary epithelial cells, prior to any evidence of BRCA1-loss. These studies reported increase in gene copy number gains and losses (such as p53, PTEN, c-MYC etc.) as well as deficiencies in error-free DNA damage repair (Konishi *et al.*, 2011; Baldeyron *et al.*, 2002; Rennstam *et al.*, 2010). However, whether other BRCA1-haploinsufficient cells-types exhibit increased genomic instability has not been examined. In addition, the mechanism of how precisely BRCA1-haploinsufficiency leads to breakdown of DNA damage repair functions and thereby increased genomic instability has not been addressed in these studies.

Therefore, to analyze these phenotypes further in a cell-type and tissue-specific context and to address the mechanism that might be responsible for increased genomic instability in BRCA1-haploinsufficient cells, we set out to examine the DNA damage and genomic instability in primary human mammary epithelial cells (HMECs), dermal epithelial cells (HDEs) and fibroblasts isolated from disease-free breast (HMF) and skin (HDF) tissues of women with or without deleterious mutations in *BRCA1*, (*BRCA1*^{mut/+} and WT, respectively; Table 3.1).

Table 3.1 List of *BRCA1*^{mut/+} patient samples used in this study. Table contents include the type of tissue, patient age, mutation status, and type of analyses performed on a specific sample.

Sample ID	Tissue Type	Patient Age	Mutation (y/n)	Specific Mutation	Analysis
616	Proph.Mastec-Brca1	40	yes	exon13ins	qFISH
617	Proph.Mastec-Brca1	53	yes	BRCA1 5385insC	qFISH
627	Proph.Mastec-Brca1	53	yes	BRCA1 187 delAG	qFISH
628	Proph.Mastec-Brca1	40	yes	BRCA1 5385 insC	qFISH
629	Proph.Mastec-Brca1	44	yes	BRCA1 2800 delAA	qFISH, LOH, GC, P/R, TELPCR, MET
634	Proph.Mastec-Brca1	39	yes	BRCA14184del4	qFISH, LOH, TELPCR, MET
635	Proph.Mastec-Brca1	35	yes	BRCA1 5385 insC	qFISH, LOH, GC, P/R, TELPCR, MET
642	Proph.Mastec-Brca1	36	yes	BRCA1 187 delAG	qFISH, LOH, GC, P/R, TELPCR, MET
643	Proph.Mastec-Brca1	47	yes	BRCA1C61G	qFISH
650	Proph.Mastec-Brca1 Skin	33	yes	BRCA1 943ins10	LOH, GC, P/R, TELPCR
651	Proph.Mastec-Brca1 Skin	47	yes	delexon14intron14	GC, P/R, TELPCR
652	Proph.Mastec-Brca1 Skin	46	yes	BRCA1 4154delA	LOH, GC, P/R, TELPCR

qFISH=In vivo telomere length, LOH= LOH analysis; GC= growth curves; P/R= protein and RNA analysis; TELPCR= telomere length PCR MET=metaphase spreads

3.1.1 Proliferating *BRCA1*^{mut/+} HMECs exhibit increased DNA damage

Induction of DNA damage response involves detection of DNA damage by MRN complex, which leads to direct ATM homodimer recruitment. Upon MRN-ATM interaction, ATM undergoes an intermolecular autophosphorylation (Ser1981, Ser367, Ser1893) resulting in dimer dissociation, kinase activation and phosphorylation of downstream substrates such as histone H2AX (γ H2AX) at the site of DNA damage (Bakkenist *et al.*, 2004). In addition, p53BP1 relocates to the sites of DNA damage where it becomes hyperphosphorylated due to ATM activation (Rappold *et al.*, 2001). Thus, in order to evaluate the levels of DNA damage and activity of DNA damage response machinery in WT and *BRCA1*^{mut/+} HMECs we examined the levels of these bonafide DNA damage markers. The number of γ H2AX and p53BP1 foci as well as the levels of substrates phosphorylated by ATM/ATR kinases were determined by immunofluorescence in proliferating cultures of WT and *BRCA1*^{mut/+} HMECs.

While WT HMECs on average had 2 γ H2AX (462 total) and 2 p53BP1 (369 total) foci per nucleus, *BRCA1*^{mut/+} HMECs on average had 4 γ H2AX (587 total) and 3 p53BP1 (490 total) foci per nucleus (Figure 3.1). In addition, ATM/ATR signaling cascade was activated in 33% of WT HMECs and 43% of *BRCA1*^{mut/+} HMECs (Figure 3.1). Therefore, we found that proliferating *BRCA1*^{mut/+} HMECs exhibited significantly higher levels of phosphorylated ATM/ATR substrates as well as γ H2AX and 53BP1 recruitment to DNA ($p=0.01$; $p=0.009$; $p=0.03$, respectively; Figure 3.1) than WT cells. This was observed across multiple patient-derived *BRCA1*^{mut/+} HMECs and across multiple *BRCA1* mutations (Table 3.1). These data indicate that *BRCA1*^{mut/+} HMECs suffer increased DNA damage and double-strand breaks compared to proliferating WT cells.

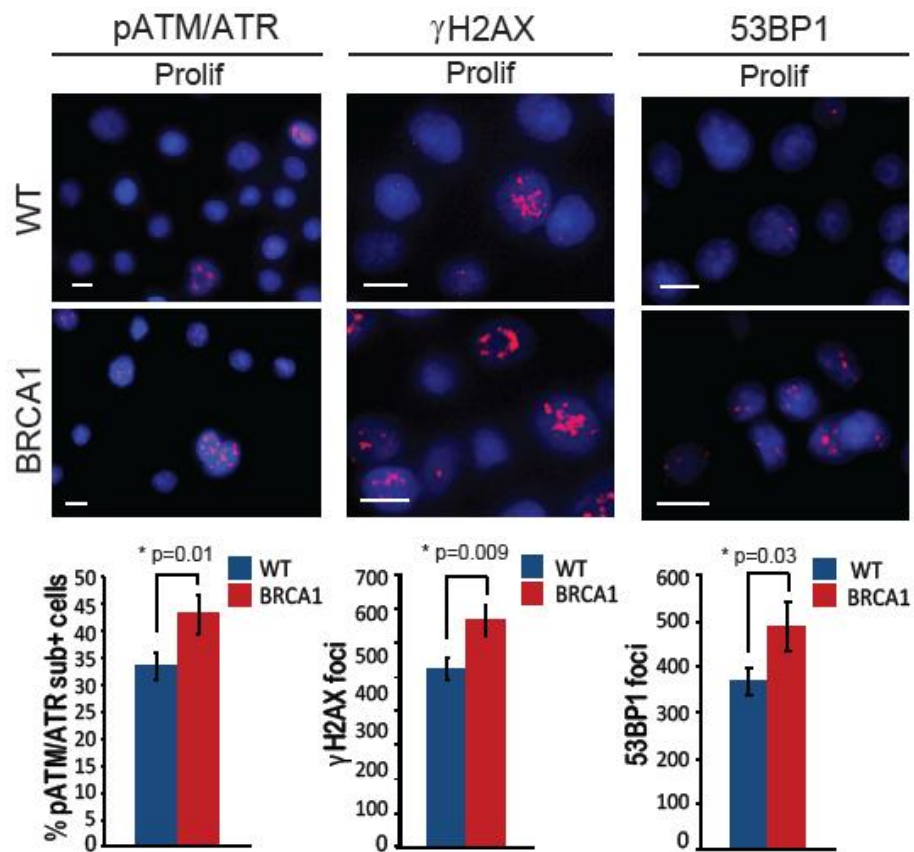


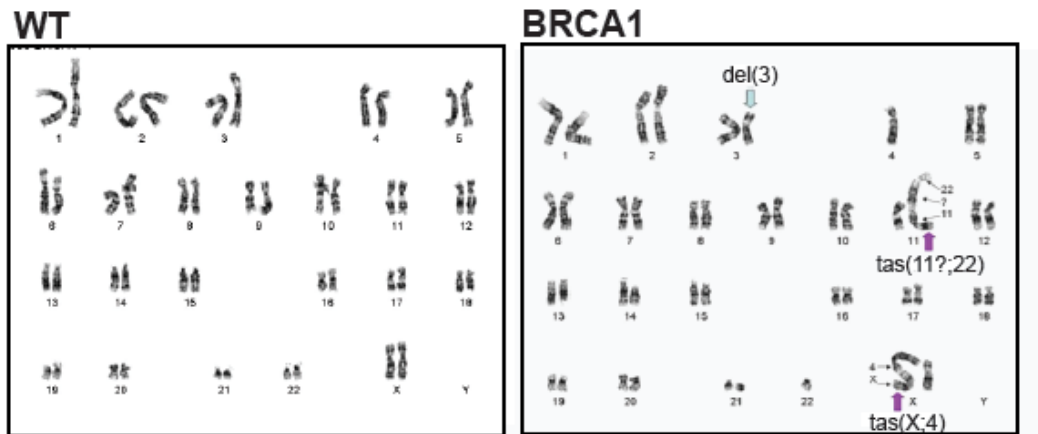
Figure 3.1. Proliferating $BRCA1^{+/mut}$ HMECs suffer increased DNA damage. Representative images of immunofluorescence (IF) staining for phospho-ATM/ATR substrates, γ H2AX foci, p53BP1 foci in proliferating (Prolif) WT and $BRCA1^{mut/+}$ HMECs. Graphs under the respective images represent the percent of cells positive for phospho-ATM/ATR substrates, as well as the average number of γ H2AX foci per WT nuclei (N=278) and BRCA1 nuclei (N=182), and the average number of p53BP1 foci per WT nuclei (N=262) and BRCA1 nuclei (N=187). Scale bar = 10 μ m.

3.1.2 Proliferating $BRCA1^{mut/+}$ HMECs exhibit increased genomic instability

Primary WT HMECs cultured over a long period of time (~100 days, > 20 PDs) begin to exhibit gross chromosomal abnormalities. The abnormalities accumulate rapidly and coincide with slowing of the proliferation rate (Romanov *et al.*, 2001). The types of chromosomal rearrangements commonly found in these cells include translocations, deletions, telomeric associations, polyploidy and aneuploidy – all pointing to telomeric dysfunction taking place in these cells (Romanov *et al.*, 2001, Soler *et al.*, 2005). It is well known that telomeric dysfunction can cause

chromosomal fusions, initiating breakage-fusion-bridge cycles that result in structural abnormalities and changes in the number of chromosomes (Feldser *et al.*, 2003). Thus, it was shown that late-passage WT HMECs exhibited increased genomic instability associated with telomere attrition, concomitant with activation of DNA damage response (specifically, p53 signaling pathway) (Romanov *et al.*, 2001, Soler *et al.*, 2005). Since *BRCA1*^{mut/+} HMECs displayed increased levels of DNA damage at early passages, we set out to examine the extent of any large scale DNA abnormalities in these cells by comparing the metaphase spreads of proliferating WT and *BRCA1*^{mut/+} HMECs.

Cytogenetic analysis indicated that proliferating cultures of WT HMECs were mostly diploid with an occasional tetraploid cell (Figure 3.2). In addition, one sample (WT-1) had a single, same translocation present in all cells probably because of clonal expansion of this HMEC population. *BRCA1*^{mut/+} HMECs, on the other hand, were mostly aneuploid due to frequent loss of entire chromosomes (Figure 3.2). Further, in several instances extra chromosomes were present (trisomy and tetraploidy data). In addition, the majority of cells in several samples of *BRCA1*^{mut/+} HMECs (BRCA-1 and -4) had a number of different translocations including frequent telomeric associations, indicative of telomeric dysfunction (Figure 3.2). In summary, these data indicate that proliferating *BRCA1*^{mut/+} HMECs display increased genomic instability in comparison to WT HMECs (same PDs), evidenced by the larger number of chromosomal aberrations including unbalanced translocations, telomeric fusions, and aneuploidy (Figure 3.2). In fact, it is fair to say that karyotypes of proliferating *BRCA1*^{mut/+} HMECs resemble karyotypes of late-passage WT HMECs.



Genotype	Diploid p=0.001	Translocations p=0.23	Tetraploidy p=0.29	Trisomy p=0.10	Aneuploid p=9.57E06	Total Abnormalities p=0.02
WT-1	9/12	12/12	3/12	0/12	0/12	12/12 (100%)
WT-2	19/20	0/10	1/19	0/20	0/10	1/20 (5%)
WT-3	18/18	0/18	0/18	0/18	0/18	0/18 (0%)
WT-4	20/20	1/20	0/20	0/20	0/20	4/20 (5%)
BRCA1-1	7/20	11/13	0/20	0/20	3/20	13/20 (65%)
BRCA1-2	1/7	0/7	2/7	3/7	4/7	7/7 (100%)
BRCA1-3	1/4	0/4	0/4	1/4	3/4	4/4 (100%)
BRCA1-4	0/20	20/20	0/20	0/20	4/20	20/20 (100%)

Figure 3.2. Proliferating *BRCA1*^{+/-mut} HMECs display increased genomic instability. Representative images and summary table of significant genetic and chromosomal events determined by karyotype analysis in proliferating WT and *BRCA1*^{mut/+} HMECs. In collaboration with Janet Cowan. Maja provided patient derived HMECs cultured for ~100 days. Janet performed karyotype analysis and metaphase spreads for these cells.

In addition to already mentioned telomeric dysfunction, several other processes can cause chromosomal abnormalities such as aberrant DNA replication, unrepaired DSBs, abnormal mitotic disjunctions and weak mitotic checkpoint. Since BRCA1 functions to prevent most of these processes in proliferating cells, it is not hard to imagine that one or more of these events are taking place in BRCA1-haploinsufficient cells, thus contributing to the overall decrease in genomic stability. The question is: which process should we go after first?

3.1.3 Telomere attrition rate is increased in *BRCA1*^{mut/+} HMECs

Studies in early 2000s found that BRCA1-deficient human and mouse cell lines frequently display short telomeres and high levels of end-to-end chromosome fusions, which might reflect dysfunctional telomeres (Al-Wahiby and Slijepcevic, 2005; McPherson *et al.*, 2006). Consistent with these reports, studies of BRCA1-knockdown in human mammary epithelial cell line (MCF10A) showed that these cells have increased numbers of chromatin bridges in anaphase, possibly reflecting an increase in telomere dysfunction (Cabuy *et al.*, 2008). Subsequently, a study of BRCA1 acute knockdown in breast cancer cell lines reported that BRCA1 can localize at telomeres (20-25%), associate with TRF1/2 and Rad50, and positively regulate 3' overhang length (Ballal *et al.*, 2009). Interestingly, upon DNA damage, BRCA1 disappears from telomeres (suggesting that it is not an integral part of the shelterin complex), and triggers modest but significant reduction in G-strand overhang length (Ballal *et al.*, 2009). Therefore, these studies imply that BRCA1 might play an important role in telomere biology.

Given the increase in chromosomal alterations, particularly in lesions associated with telomere-end fusions, we decided to compare telomere length and erosion rates in WT and *BRCA1*^{mut/+} HMECs. For this, we employed qPCR-based assay to measure telomere length in early- and late-passage WT and *BRCA1*^{mut/+} HMECs. Telomere erosion rate (TER) for each sample was calculated by the following formula: $TER = (\Delta \text{ telomere length} / \Delta \text{ PDs})$. Indeed, there was a ~4-fold increase in telomere erosion rates in *BRCA1*^{mut/+} HMECs compared to telomere erosion rates in WT cells ($p < 0.04$; Figure 3.3). This finding reveals that telomere stability is compromised in BRCA1-haploinsufficient HMECs and further suggests

that this premature telomere dysfunction increases genomic instability in $BRCA1^{mut/+}$ HMECs.

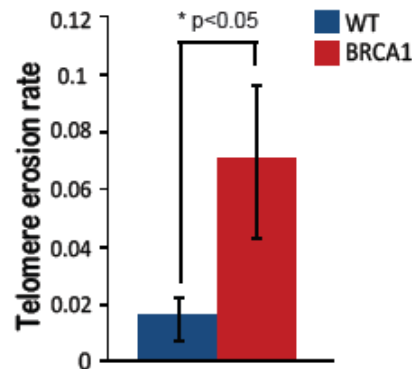


Figure 3.3 $BRCA1^{+/-mut}$ HMECs exhibit increased telomere attrition rate. Telomere erosion rate in WT (N=4) and $BRCA1^{mut/+}$ (N=4) HMECs. Telomere length was determined by qPCR in proliferating and agonescent/senescent WT and $BRCA1^{mut/+}$ HMECs. Telomere erosion rate was calculated by the following formula: $TER = (M2/M^* \text{ telomere length} - \text{Prolif. telomere length}) / PDs$. In collaboration with Krithika Ravichandran. Maja provided patient derived HMECs in culture for ~100 days and M2/M*. Krithika performed telomere length analysis in these cells.

3.1.4 GSEA corroborates the observed increase in DDR pathway activation in $BRCA1^{+/-mut}$ HMECs

In order to corroborate our findings that $BRCA1^{mut/+}$ HMECs exhibit increased DDR activity, we compared the expression of genes involved in DDR regulation by Gene Set Enrichment Analysis (GSEA) in proliferating WT and $BRCA1^{mut/+}$ HMECs. GSEA was applied to previously published gene expression data collected on cultured proliferating primary human mammary epithelial cells isolated from $BRCA1$ -mutation carriers (N=6) or age-matched WT (N=6) (GSE19383, Bellacosa *et al.*, 2010).

Consistent with the findings that DDR pathway is increasingly active in $BRCA1^{mut/+}$ HMECs, GSEA of global transcriptional profiling data revealed a significant enrichment of genes associated with DNA repair ($p < 0.0137$; Table 3.2),

homologous recombination ($p < 0.022$; Table 3.2), as well as genes involved in activation of ATR in response to replicative stress ($p < 0.049$; Table 3.2), and extension of telomeres ($p < 0.049$; Table 3.2). These data further support our observations that *BRCA1*^{mut/+} HMECs exhibit increased DDR, genomic instability and telomere dysfunction.

Table 3.2 GSEA pathway list

Name of the pathway	Gene Set SIZE	ES	NES	NOM p-val	FDR q-val
REACTOME_CELL_CYCLE_MITOTIC	261	-0.1822147	-3.333048	0	0
REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION	27	-0.494275	-3.0600283	0	2.95E-04
REACTOME_G2_M_TRANSITION	72	-0.2799382	-2.8140864	0	0.00105462
REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	33	-0.402977	-2.7753196	0	0.0012494
REACTOME_CENTROSOME_MATURATION	60	-0.2964273	-2.7085989	0	0.00188686
REACTOME_E2F_TRANSCRIPTIONAL_TARGETS_AT_G1_S	18	-0.5194901	-2.6574273	0	0.00293562
REACTOME_G2_M_CHECKPOINTS	38	-0.3559447	-2.5365891	0	0.00567763
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	27	-0.3674328	-2.2960405	0	0.01802512
REACTOME_MITOTIC_PROMETAPHASE	71	-0.2303958	-2.2924228	0	0.01653609
BIOCARTA_MCM_PATHWAY	18	-0.4495234	-2.2790813	0	0.01604816
KEGG_CELL_CYCLE	111	-0.1723867	-2.2049823	0	0.02107831
REACTOME_MITOTIC_M_M_G1_PHASES	134	-0.1573849	-2.1053176	0	0.03355169
REACTOME_G1_S_TRANSITION	94	-0.1818364	-2.066683	0	0.03683207
CHICAS_RB1_TARGETS_GROWING	221	0.27887425	4.7940974	0	0
CHICAS_RB1_TARGETS_SENESCENT	495	0.16796239	4.2921963	0	0
KAMMINGA_EZH2_TARGETS	39	0.41060108	3.059856	0	0
V\$E2F1_Q3	210	0.15255454	2.5253246	0	2.13E-04
TANG_SENESCENCE_TP53_TARGETS_DN	52	0.2647142	2.2747254	0	0.00351118
CHICAS_RB1_TARGETS_CONFLUENT	490	0.0820208	2.0581293	0.00194932	0.01106058
KUMAMOTO_RESPONSE_TO_NUTLIN_3A_DN	10	0.5047875	1.9616181	0.0020202	0.01498721
REACTOME_DNA_STRAND_ELONGATION	27	-0.2954367	-1.8379503	0.00567108	0.08739737
REACTOME_DNA_REPAIR	93	-0.1684125	-1.8715832	0.01372549	0.07727332
REACTOME_DNA_REPLICATION_PRE_INITIATION	72	-0.1729187	-1.751833	0.01724138	0.1213078
KEGG_HOMOLOGOUS_RECOMBINATION	27	-0.2745898	-1.6734208	0.02249489	0.15364626
REACTOME_SYNTHESIS_OF_DNA	84	-0.1630917	-1.7996148	0.02574257	0.09985348
REACTOME_M_G1_TRANSITION	59	-0.1824439	-1.6782709	0.0308642	0.15378681
REACTOME_CELL_CYCLE_CHECKPOINTS	99	-0.1440138	-1.663237	0.03571429	0.15834156
CHICAS_RB1_TARGETS_LOW_SERUM	76	0.16329645	1.6577407	0.04268293	0.07548876
REACTOME_EXTENSION_OF_TELOMERES	24	-0.2721803	-1.5520241	0.04930966	0.23302579

3.1.5 Increased DDR, genomic instability, and telomere attrition rate are *BRCA1*^{mut/+} HMEC-specific.

Loss of BRCA1 correlates with increased DNA damage and DDR activation regardless of the cell-type. There is growing evidence that BRCA1-haploinsufficiency also increases genomic instability. Furthermore, our data suggest that primary HMECs from *BRCA1*-mutation carriers exhibit increased DDR, genomic instability and telomere dysfunction. However, whether these DDR related phenotypes in BRCA1-haploinsufficient cells are tissue and cell-type specific has not been examined to date.

In order to determine whether increased DDR, telomere attrition, and genomic instability are features of BRCA1-haploinsufficiency in general, we examined γ H2AX foci, karyotypes, and telomere erosion rates in proliferating HMFs and HDEs from age-matched individuals, respectively (Figure 3.4). We found that proliferating WT and *BRCA1*^{mut/+} HMFs had similar numbers of γ H2AX foci (2) per nucleus (Figure 3.4A). Furthermore, cytogenetic analysis of proliferating WT and *BRCA1*^{mut/+} HMFs was consistent with previous reports, revealing overall few chromosomal rearrangements of no significant difference (Figure 3.4B). In addition, telomere length measurement and erosion rate analysis revealed no difference between WT and *BRCA1*^{mut/+} HDEs (p=0.324, Figure 3.5).

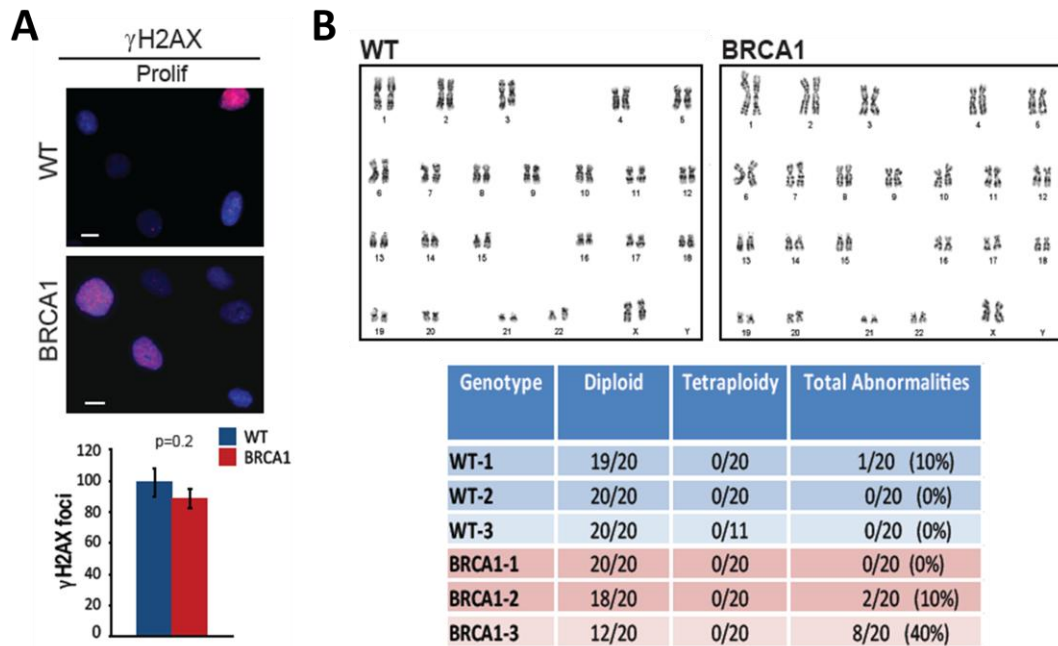


Figure 3.4. *BRCA1*^{mut/+} HMFs do not exhibit increased DDR and genomic instability. A) Representative images of IF staining for γ H2AX foci in WT and *BRCA1*^{mut/+} HMFs at day 100 in culture (same day in culture as proliferating HMECs). Graph under the images represents the average number of γ H2AX foci per WT nuclei (N=278) and BRCA1 nuclei (N=182). B) Representative images and summary table of significant genetic and chromosomal events determined by karyotype analysis in WT and *BRCA1*^{mut/+} HMFs at day 100 in culture (same day in culture as proliferating HMECs). Scale bar = 10 μ m. In collaboration with Janet Cowan. Maja provided patient derived HMFs cultured for ~100 days and assessed γ H2AX levels in these cells. Janet performed karyotype analysis and metaphase spreads in HMFs.

Therefore, in contrast to HMECs, we found no difference in DDR and chromosomal abnormalities between proliferating WT and *BRCA1*^{mut/+} HMFs, and HDEs did not exhibit a difference in telomere attrition rates. This suggests that increased DDR, telomere attrition and genomic instability are characteristic only to *BRCA1*^{mut/+} HMECs, and hence are cell and tissue type specific phenotypes.

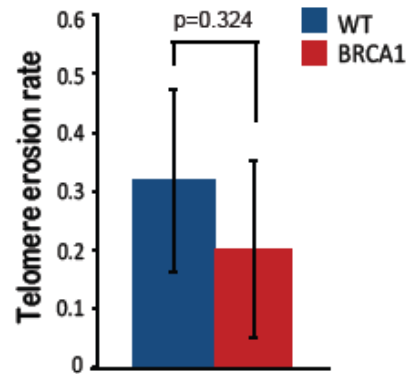


Figure 3.5. *BRCA1*^{mut/+} HDEs do not exhibit increased telomere erosion rate. Telomere erosion rate in WT (N=3) and *BRCA1*^{mut/+} (N=3) HDEs. Telomere length was determined by qPCR in proliferating and agonescent/senescent WT and *BRCA1*^{mut/+} HMECs. Telomere erosion rate was calculated by the following formula: TER = (M2/M* telomere length-Prolif. telomere length)/PDs. In collaboration with Krithika Ravichandran. Maja provided patient derived HDEs in culture for ~0 days and M0. Krithika performed telomere length analysis in these cells.

3.1.6 Ectopic telomerase expression attenuated genomic instability in *BRCA1*^{mut/+} HMECs.

It was shown that overexpression of the catalytic subunit of telomerase (hTERT) in primary cells results in telomere extension, enhanced genome stability, DNA repair and immortalization (Newbold *et al.*, 1982; Sharma *et al.*, 2003). One of the proposed mechanisms is that, in addition to telomere length regulation, hTERT also increases expression of DNA-repair-associated genes and the NTP pool levels, which leads to telomere stability and a decrease in spontaneous chromosome damage (Sharma *et al.*, 2003). In addition, hTERT overexpression along with p16/pRb signaling pathway inactivation can immortalize WT HMECs grown on plastic (Kiyono *et al.*, 1998). Therefore, by extending the mean telomere length, ectopic overexpression of hTERT can rescue telomere dysfunction in late-passage WT HMECs.

Given the findings that *BRCA1*^{mut/+} HMECs exhibit accelerated telomere dysfunction correlating with increased genomic instability, we reasoned that the expression of hTERT in these cells could rescue telomere erosion as well as genomic instability. As expected, overexpression of the catalytic subunit of telomerase (hTERT) in either WT or *BRCA1*^{mut/+} HMECs resulted in telomere elongation and cellular immortalization. Telomere length was measured by PCR-based assay, while cellular immortalization was determined by long-term passaging of WT and *BRCA1*^{mut/+} HMECs (> 50 PDs). Surprisingly, however, hTERT expressing *BRCA1*^{mut/+} HMECs exhibited telomeres that were 2-fold longer than WT cells. Furthermore, cytogenetic analysis revealed that there was no difference in the number of chromosomal abnormalities between WT and *BRCA1*^{mut/+} HMECs. More importantly, the number of chromosomal rearrangements associated with telomere erosion (i.e. telomeric associations) were attenuated in hTERT expressing *BRCA1*^{mut/+} HMECs (Figure 3.1F, G). These results indicate that hTERT expression is able to alleviate telomere dysfunction and telomere-associated genomic instability in *BRCA1*^{mut/+} HMECs.

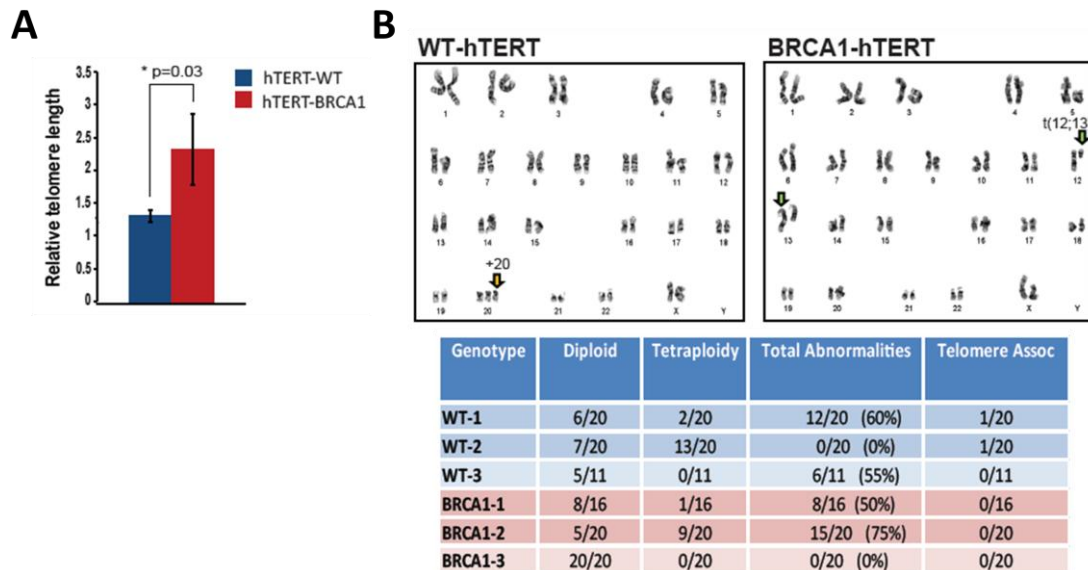


Figure 3.6. Ectopic hTERT expression attenuated genomic instability in *BRCA1*^{mut/+} HMECs. A) Average telomere length in hTERT immortalized WT (N=3) and *BRCA1*^{mut/+} (N=3) HMECs. B) Representative images and summary table of significant genetic and chromosomal events determined by karyotype analysis in immortalized WT and *BRCA1*^{mut/+} HMECs. In collaboration with Janet Cowan and Krithika Ravichandran. Maja generated hTERT immortalized HMECs. Krithika performed telomere length analysis in these cells. Janet performed karyotype analysis and metaphase spreads for hTERT immortalized HMECs.

3.1.7 Discussion of results

Cells with biallelic inactivation of BRCA1 have been extensively used to study the role of BRCA1 in DDR – a putative tumor suppressive function of BRCA1 (Cao *et al.*, 2003; Greenberg *et al.*, 2006; Pathania *et al.*, 2011). This was relevant because it was reflecting the idea that loss of BRCA1 was likely the driver of *BRCA1*-associated cancers and reflective of the status of BRCA1 in the majority of tumors with loss of BRCA1. This work led to the understanding that BRCA1's role in DDR (specifically events related to homologous repair of double strand breaks) is critical for all cell-types. However, this weakened the hypothesis that BRCA1 suppresses tumor growth solely through its role in DDR. A possibility that there are DDR-related

phenotypes in BRCA1-haploinsufficient cells has not been explored until recently. Here for the first time we show that primary BRCA1-haploinsufficient HMECs exhibit increased DNA damage response and genomic instability through rapid telomere erosion and dysfunction.

In order to determine whether increased DDR, genomic instability and telomere attrition, associated with BRCA1-haploinsufficiency are unique to HMECs, we examined whether these DDR-related phenotypes are present in other BRCA1-haploinsufficient cell-types. Thus, we looked for any evidence of increased DNA damage, telomere attrition rate and chromosomal abnormalities in proliferating HMFs and HDEs. Our analysis revealed that other cell-types did not exhibit any of the DDR-related phenotypes observed in HMECs. Collectively, this indicates that phenotypes associated with cellular transformation are specific to *BRCA1*^{mut/+} HMECs, and therefore might be important for the increased propensity for *BRCA1*^{mut/+} HMECs to undergo neoplastic transformation.

Finally, our goal was to find a mechanism that could be responsible for higher probability of cellular transformation in *BRCA1*^{mut/+} HMECs. Karyotype analysis of *BRCA1*^{mut/+} HMECs and telomere length measurements revealed that these cells exhibited increased telomere attrition and harbored increased numbers of chromosomal rearrangements associated with telomere dysfunction. This suggested that increased telomere dysfunction is responsible for greater genomic instability in these cells. In addition, overexpression of hTERT resulted in extended telomeres, which appeared to stabilize the genome in *BRCA1*^{mut/+} HMECs (as the number of chromosomal abnormalities was overall similar to WT cells expressing hTERT). To corroborate these data, it would be useful to compare the number of telomere dysfunction induced foci (TIFs) in proliferating WT and *BRCA1*^{+ /mut} HMECs. To

accomplish this levels of DSB γ H2AX or p53BP1, markers that colocalize with damaged telomeres, would be examined. The results of this experiment would strengthen the conclusion that increased genomic instability is mainly due to telomere dysfunction induced by accelerated telomere erosion in *BRCA1*^{+mut} HMECs.

Interestingly, in this study we observed that the ectopic hTERT expression in *BRCA1*^{mut/+} cells resulted in telomeres that are 2-fold longer than that in the WT HMECs. Ballal and colleagues have shown that BRCA1 knockdown in cell lines results in increased levels of hTERT expression and telomere length in cells, altogether suggesting that BRCA1 is involved in suppression of hTERT-dependent mechanisms of telomere lengthening. Therefore, it is possible that in *BRCA1*^{mut/+} HMECs processes involved in suppression of telomere lengthening are weakened (i.e. the cells are poised for telomere lengthening), and this becomes apparent with ectopic expression of hTERT. Taken together, these data revealed an important tumor suppressive function of BRCA1 that requires bi-allelic expression of BRCA1 involved in telomere stability in HMECs.

3.2 BRCA1-haploinsufficiency induces cell type-specific premature senescence.

Cellular senescence has emerged as an intrinsic mechanism to undergo growth arrest in response to various insults, and therefore represents an important barrier in early neoplastic transformation. It has been shown that senescence is induced in the context of many forms of cellular stress including increased DNA damage, telomere erosion, oncogene activation and most recently tumor suppressor loss (Berger *et al.*, 2011; Kuilman *et al.*, 2010; Young *et al.*, 2008). Senescence is commonly manifested by reduced proliferative capacity, changes in cellular morphology, and increased SA- β -galactosidase activity.

Previously, it has been demonstrated that BRCA1 deficiency in mice triggers senescence in mutant MEFs and embryos as well as premature aging in adult animals (Cao *et al.*, 2002). Premature senescence in this system is mediated via DNA damage dependent upregulation of p53 and p21, involved in G1-S cell cycle checkpoint regulation (Cao *et al.*, 2002). Furthermore, it has been shown that BRCA1 knockdown in primary human fibroblast can also induce premature senescence. This cell-cycle arrest was dependent on pRb activation due to increased p16 and p21 levels (in DDR-independent fashion). This group showed that premature senescence was mediated by increased association of BRG1 (a component of SWI/SNF chromatin remodeling enzyme) with chromatin and pRb resulting in increased SAHFs formation (Tu *et al.*, 2013). However, premature senescence in BRCA1-heterozygous cells has not been previously reported. Since *BRCA1*^{+mut} HMECs exhibited increased telomere erosion and dysfunction, accompanied by increased DNA damage levels, we hypothesized that these cells might undergo premature senescence. In order to examine the tissue and cell-type specificity of this phenotype we also monitored WT and *BRCA1*^{+mut} HDEs, HMFs and HDFs growth in culture.

3.2.1 *BRCA1*^{mut/+} HMECs encounter a premature proliferation barrier.

WT HMECs encounter two mechanistically distinct senescent-like barriers *in vitro* (Figure 3.7A). The first proliferative barrier, referred to as stasis or M0, is associated with classical p16/INK4a-dependent stress induced senescence and concomitant p53 pathway activation (Figure 3.7A, B) (Hammond *et al.*, 1984; Brenner *et al.*, 1998; Huschtscha *et al.*, 1998; Romanov *et al.*, 2001; Holst *et al.*, 2003; Garbe *et al.*, 2007; Garbe *et al.*, 2009). Cells at this stage exhibit typical morphological features of senescence and stain positive for SA- β -galactosidase

(Figure 3.7C). In addition, this senescence-like growth arrest does not appear to be associated with telomere shortening and genome instability. Cells that emerge from this barrier do so through downregulation of p16/INK4a and rapidly proliferate until they reach the second proliferative barrier referred to as agonescence or M2 (Figure 3.7A). Unlike senescence, M2 is induced through p53 pathway activation in response to DNA damage and genomic instability as a consequence of telomere attrition and dysfunction (Figure 3.7B). In addition, the apparent proliferative arrest observed during M2 is maintained through a balance of proliferation and apoptosis. Many cells at this stage, however, exhibit typical morphological features of senescence and stain positive for SA- β -galactosidase (Figure 3.7C) (Romanov *et al.*, 2001; Garbe *et al.*, 2009).

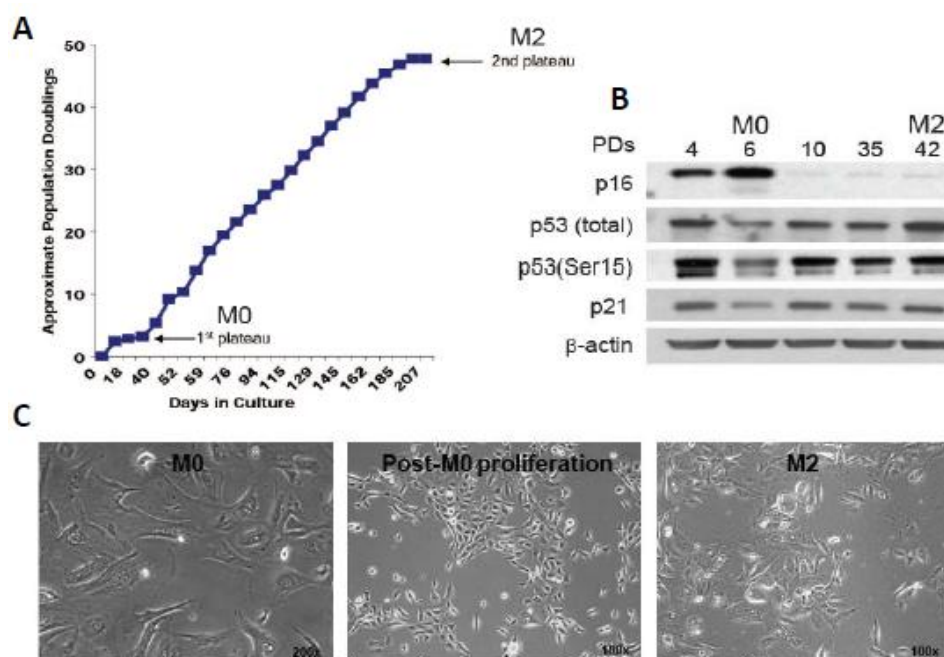


Figure 3.7. Patient derived HMECs activate p16 and p53 pathways. A) Representative growth curve of WT HMECs. M0 = Stasis, M2 = Agonescence B) Western blot analysis of p16^{INK4a}, total p53, p53 (Ser15), and p21 levels in WT and *BRCA1*^{mut/+} HMECs at indicated population doublings (PDs). C) Representative phase-contrast brightfield images of WT HMECs in M0, post-M0 proliferation, and M2. In collaboration with Patty Keller. Patty initiated growth curves of two WT patient samples and provided images of HMECs in M0 and post-M0 proliferation. Maja completed growth curves of four WT patient samples, performed WB analysis, and provided image of HMECs in M2.

Examination of *BRCA1*^{mut/+} and WT HMECs revealed similar growth kinetics and molecular responses in early cultures; both WT and *BRCA1*^{mut/+} HMECs entered into M0, induced p16/INK4a and p53 protein expression in a similar fashion (Figure 3.8A, B). Likewise, WT and *BRCA1*^{+/-mut} HMECs overcame M0 with similar frequencies and efficiencies, and both exhibited loss of p16/INK4a expression upon emergence from stasis (Figure 3.8A, B). However, while WT HMECs on average continued to proliferate for an additional ~44 population doublings (PDs), *BRCA1*^{+/-mut} HMECs stopped proliferating after ~31 PDs (Figure 3.8A). This premature growth arrest (M*) was observed across multiple patient-derived *BRCA1*^{mut/+} HMECs with different *BRCA1* mutations, and was observed in *BRCA1*^{mut/+} HMECs well before M2 in WT HMECs (p=0.004, Table 3.1). Notably, unlike M2, M* was characterized by significantly lower proliferation and apoptosis indexes, indicating senescence-like cell cycle arrest in *BRCA1*^{+/-mut} HMECs (Figure 3.8C, D; p<0.0001, p=0.002, respectively). In addition, cells in both M* and M2 displayed the senescent phenotype, characterized by enlarged, flattened morphology and positive staining for SA-β-galactosidase (Figure 3.8D).

3.2.2 Premature growth arrest is specific to *BRCA1*^{mut/+} epithelial cells.

In order to determine whether premature senescence is a feature unique to *BRCA1* haploinsufficient HMECs, we also examined the growth kinetics as well as p53 and p16/INK4a pathway activation in skin epithelial cells from age-matched individuals. Shortly after plating, WT HDEs enter a phase of exponential growth reaching up to 20-35PDs, after which they encounter a senescent-like proliferative barrier. This stage is associated with classical p16/INK4a-dependent stress induced senescence – a proliferative barrier mechanistically similar to M0 in HMECs. Also

like M0, this senescence-like growth arrest is not associated with telomere shortening and genome instability (Dickson *et al.*, 2000). Furthermore, cells at this stage exhibit senescence associated morphological changes and SA- β -galactosidase positivity. Unlike HMEC though, keratinocytes are unable to spontaneously overcome M0. In addition, senescent keratinocytes do not reattach efficiently when subcultured, thus cells are progressively lost at subsequent passages (Dickson *et al.*, 2000).

Similar to HMECs, *BRCA1*^{+/-mut} HDEs also underwent rapid premature growth arrest with typical features of senescence compared to WT HDEs (Avg PD=7 \pm 2.5 vs. Avg PD=17 \pm 4, respectively; Figure 3.9A). This was also observed across multiple patient-derived *BRCA1*^{mut/+} samples and across multiple *BRCA1* mutations (p=0.01, Table 3.1). In addition, consistent with classical p16/INK4a-dependent stress induced senescence M0, HDE induced p16/INK4a as they approached senescence (Figure 3.9B).

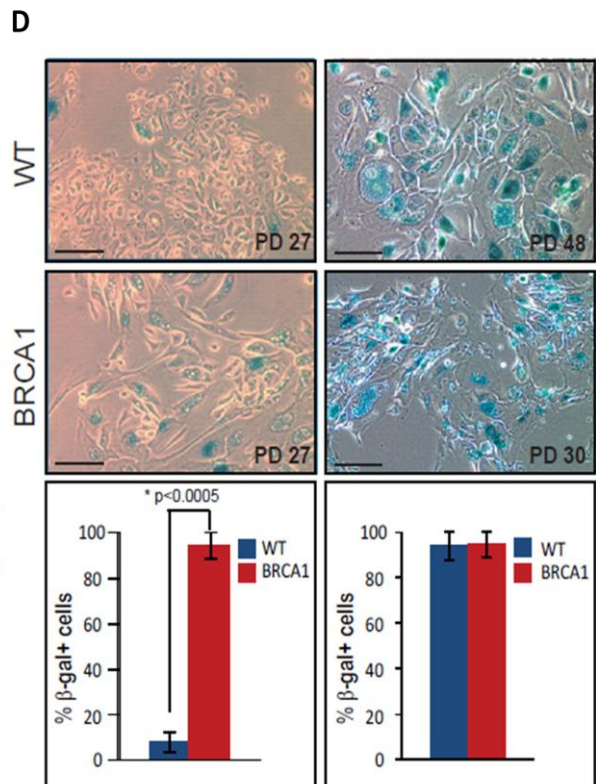
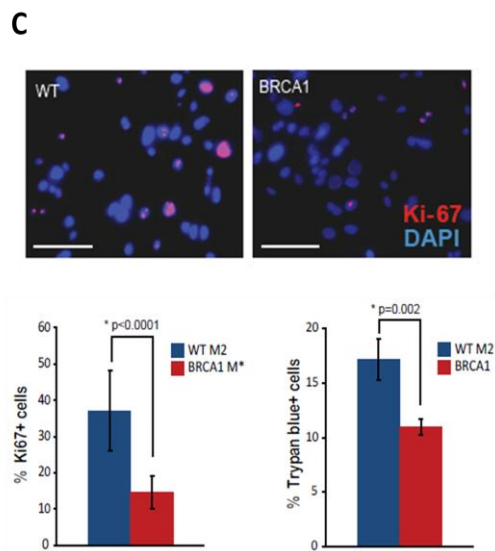
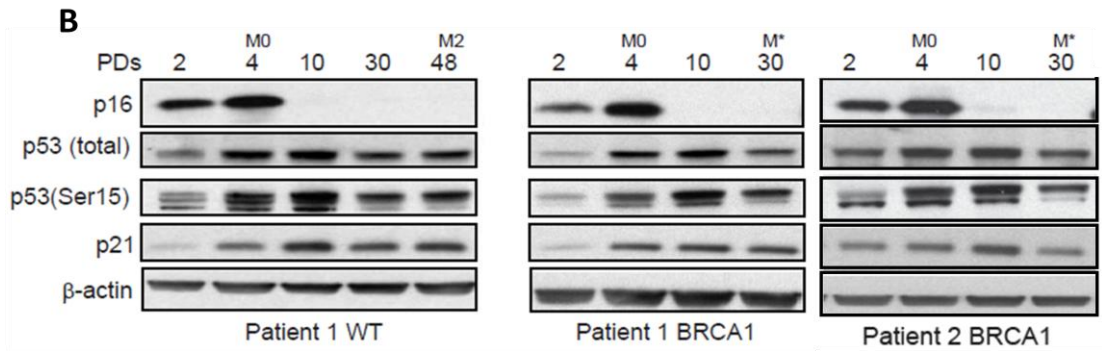
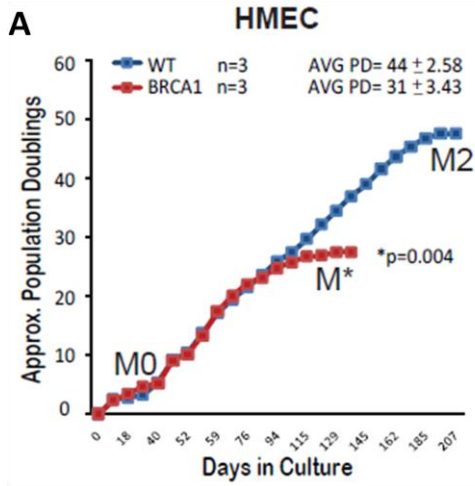


Figure 3.8. (On previous page.) *BRCA1*^{mut/+} HMECs encounter a premature proliferation barrier. A) Representative growth curves of WT (N=3) and *BRCA1*^{mut/+} (N=3) HMECs. B) Western blot analysis of p16^{INK4a}, total p53, p53 (Ser15), and p21 levels in WT and *BRCA1*^{mut/+} HMECs at indicated population doublings (PDs). M0 = Stasis, M2 = Agonescence (WT HMECs), M* = Premature growth-arrest (*BRCA1*^{mut/+} HMECs). C) Images of Ki67 positive cells determined by IF staining in M2 WT and M* *BRCA1*^{mut/+} HMECs. Quantification of Ki67 positive cells by IF staining in M2 WT and M* *BRCA1*^{mut/+} HMECs. Quantification of Trypan blue positive cells in M2 WT and M* *BRCA1*^{mut/+} HMECs. D) Brightfield images of SA β -galactosidase staining and quantification of positive cells at selected PDs in WT and *BRCA1*^{mut/+} HMECs. Scale bar = 100 μ m.

In order to determine whether premature senescence is a feature of BRCA1-haploinsufficiency in general, we also examined the growth kinetics as well as p53 and p16/INK4a pathway activation in fibroblasts from age-matched individuals. HMF and HDF behave like typical primary fibroblasts in culture, where a phase of long exponential growth (20-50PDs) is followed by slow growth arrest. This stage is also termed replicative senescence or M1. Mechanistically, M1 is associated with telomere attrition, increased p16 expression and p53 activation. In addition, M1 stage in fibroblasts is associated with typical senescence associated morphological changes and SA- β -galactosidase positivity (Romanov *et al.*, 2001).

In contrast to epithelial cells, WT and *BRCA1*^{mut/+} mammary and skin fibroblasts underwent comparable population doublings in culture, after which cells became SA β -galactosidase positive and stopped dividing (Figure 3.9 C, D and F, G). In addition, consistent with classical replicative senescence M1, skin and mammary fibroblasts induced p53 and p16/INK4a as they approached senescence (Figure 3.9 E, H).

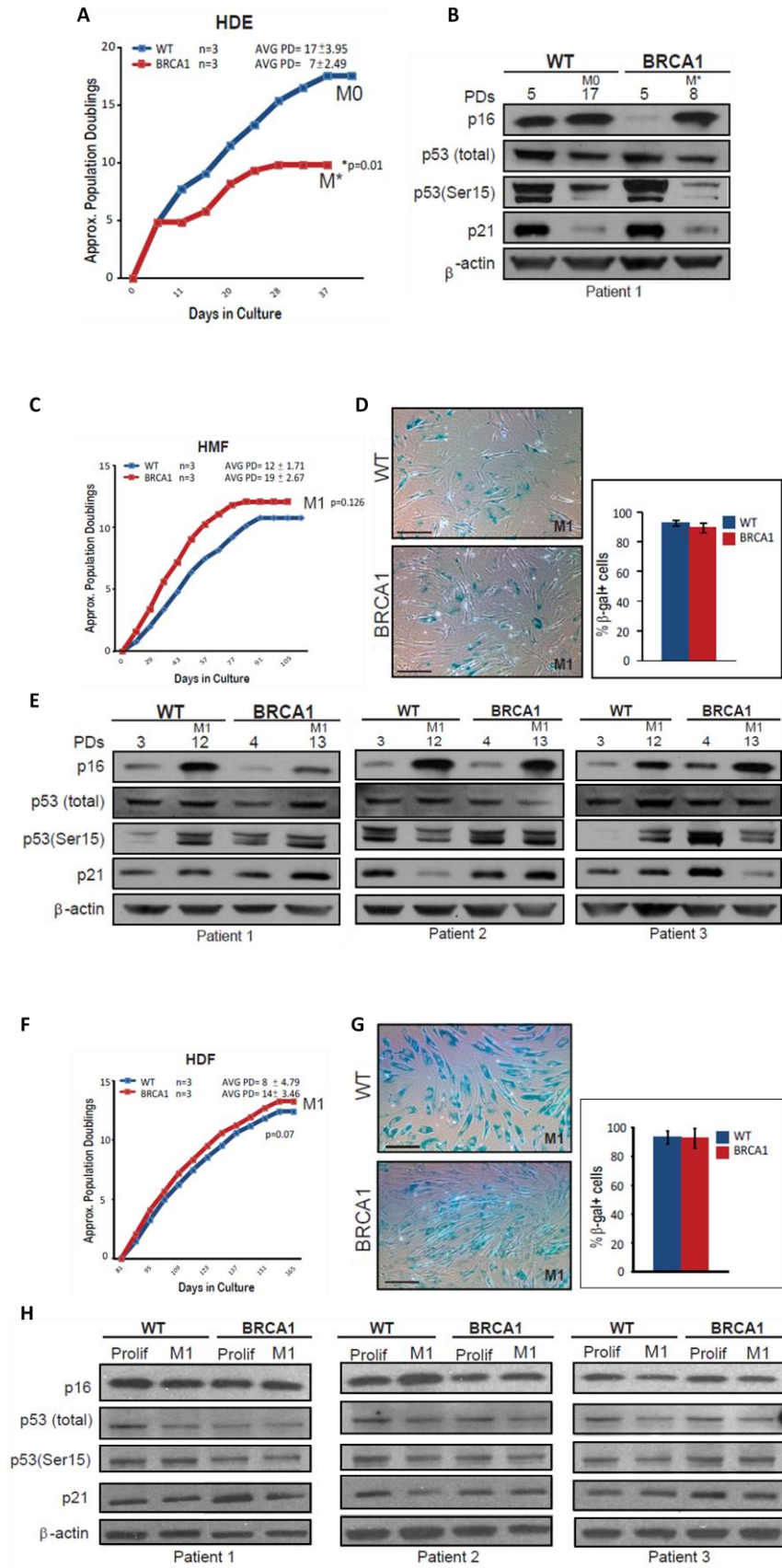


Figure 3.9. (On previous page) Premature growth arrest is specific to *BRCA1*^{mut/+} epithelial cells. A) Representative growth curves of WT (N=3) and *BRCA1*^{mut/+}(N=3) HDEs. B) Western blot analysis of p16^{INK4a}, total p53, p53 (Ser15), and p21 levels in WT and *BRCA1*^{mut/+} HDEs at indicated population doublings (PDs). M0 = Stasis. C) Representative growth curves of WT (N=3) and *BRCA1*^{mut/+}(N=3) HMFs. D) Brightfield images of SA β -galactosidase staining and quantification of positive cells at selected PDs in WT and *BRCA1*^{mut/+} HMFs. E) Western blot analysis of p16^{INK4a}, total p53, p53 (Ser15), and p21 levels in WT and *BRCA1*^{mut/+} HMFs at indicated population doublings (PDs). M1 = Senescence. F) Representative growth curves of WT (N=3) and *BRCA1*^{mut/+}(N=3) HDFs. G) Images and quantification of SA- β -galactosidase positive cells using β -galactosidase detection assay in senescent (M1) WT and *BRCA1*^{mut/+} HDFs. H) Western blot analysis of p16^{INK4a}, total p53, p53 (Ser15), and p21 levels in proliferating and M1 WT (N=3) and *BRCA1*^{mut/+} (N=3) HDFs. Scale bar = 100 μ m.

3.2.3. No evidence of LOH in *BRCA1*^{mut/+} epithelial cells.

Several lines of evidence have shown that LOH of tumor suppressor genes (e.g *VHL*, *PTEN*, *NF1*, or *BRCA1*) can lead to the induction of premature senescence programs (Berger *et al.*, 2011, Kuilman *et al.*, 2010, Young *et al.*, 2008). In addition, it was also demonstrated that in co-operation with a cell-cycle checkpoint inactivation, this senescence barrier is bypassed (one example is *BRCA1* and p53). LOH is frequently found in *BRCA1*-associated cancers and in tissues of *BRCA1*-mutation carriers, indicating that *BRCA1*-haploinsufficient cells have increased propensity to lose *BRCA1* allele. Given that *BRCA1*^{+/-mut} HMECs exhibited increased genomic instability and premature senescence, we decided to examine whether premature senescence in these cells occurred due to *BRCA1* LOH. We included *BRCA1*^{+/-mut} HDEs in the analysis, since these cells also senesced prematurely.

PCR-based Sanger sequencing method was used to interrogate the individual *BRCA1* mutation sites for LOH in *BRCA1*^{+/-mut} HMECs and HDEs. The following *BRCA1* mutation sites were analyzed: 187delAG, 2800delAA, 4184del14, 5385insC, 943ins10, and 4154delA (Table 3.1). This analysis revealed that both WT and mutant

alleles were present in both proliferative and senescent cells (Figure 3.10). These findings indicate that loss of the remaining WT allele was not responsible for premature senescence. Thus, haploinsufficiency for BRCA1 results in the engagement of a novel premature senescence-like barrier in epithelial cells but not other cell-types (a process hereafter termed: haploinsufficiency-induced senescence (HIS)).

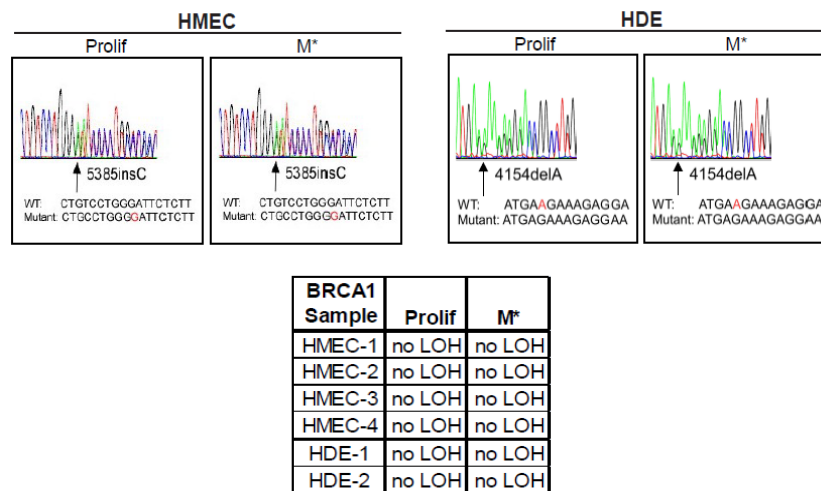


Figure 3.10. Premature senescence in $BRCA1^{mut/+}$ epithelial cells does not occur due to LOH. LOH analysis in proliferating (Prolif) and cell cycle-arrested (M^*) $BRCA1^{mut/+}$ HMECs and HDEs using PCR-based Sanger sequencing method. In collaboration with Amanda Toland. Maja provided gDNA extracted from proliferating and arrested (M^*) $BRCA1^{+/mut}$ epithelial cells. Amanda performed the LOH analysis.

3.2.4. Discussion of results

Proper BRCA1 function is essential for cell viability, since BRCA1-loss in otherwise healthy cells induces apoptosis or premature senescence. This holds true for all cell-types, regardless whether they are of human or mouse origin. On the other hand, $BRCA1^{+/-}$ cells do not appear to exhibit any phenotypes associated with premature senescence. In this study we show for the first time that epithelial cells, but

not fibroblasts, derived from breast and skin tissues of *BRCA1*-mutation carriers spontaneously undergo premature senescence in culture. This suggests that the premature senescence phenotype is specific to *BRCA1*-haploinsufficient human epithelial cells.

Our data also revealed a notable temporal difference between senescent *BRCA1*-haploinsufficient HMECs and HDEs. *BRCA1*-haploinsufficient HDEs senesced prior to M0, while *BRCA1*-haploinsufficient HMECs behaved similarly to WT cells prior to M0, and instead senesced well before M2. Why did premature senescence manifest in HMECs after M0? HMECs and HDEs have considerably different growth pattern in culture; therefore, it is possible that the events leading up to M0 (such as p16 upregulation, p53 activation, etc.) slightly differ between HMECs and HDEs. Furthermore, cultured HMECs have an intrinsic ability to overcome M0, which is not commonly observed in HDEs. Since it has been shown that overcoming M0 in HMECs is accompanied by considerable chromatin remodeling and changes in gene expression, it is quite likely that one of the differences between HMECs and HDEs at M0 is due to this inherent chromatin plasticity, which is present in HMECs but absent in HDEs. Also, it is possible that certain chromatin remodeling enzymes are expressed in HMECs but not in HDEs. Thus, HMECs might not be as sensitive to p16 upregulation as they seem to be able to undergo methylation of p16 promoter during M0, while HDEs seem to be “locked” in this phase.

Since several studies showed that premature senescence correlates with loss of tumor suppressor gene, we examined whether premature senescence in epithelial cells of *BRCA1*-mutation carriers was due to LOH of *BRCA1* allele. Sequencing analysis across *BRCA1* mutation sites indicated that both *BRCA1* alleles were present in all proliferating or senescent *BRCA1*^{+/-mut} epithelial cells. Thus, we concluded that the

observed senescence is associated with haploinsufficiency rather than loss of tumor suppressor gene, a novel form of senescence we have termed HIS.

Because *BRCA1* LOH is frequent in *BRCA1*-associated cancers, it is thought that *BRCA1* behaves like a classical tumor suppressor gene. Thus, according to the Knudson's "two hit hypothesis" loss of *BRCA1*WT allele was thought to be an early and rate limiting step in progression of these cancers (Fackenthal and Olopade, 2007). So, how do we reconcile the observed findings concerning *BRCA1* in human tumors with studies showing that *BRCA1* deletion and *BRCA1*-haploinsufficiency result in premature senescence? So far, several groups, including us, have reported that considerable genomic abnormalities accumulate in *BRCA1*-haploinsufficient cells. This, combined with the fact that LOH in *BRCA1*-associated cancers seems to be a stochastic process affecting the WT as well as mutant *BRCA1* allele, suggests that early events in progression of *BRCA1*-associated cancers involve mutations and inactivation of other tumor-suppressor genes and cell-cycle checkpoints such as PTEN and p53, pRb, and that *BRCA1* LOH is most likely a late event. In other words, cell-cycle checkpoint inactivation may be required for cells to survive loss of *BRCA1* WT allele.

3.3. Characterization of *BRCA1* haploinsufficiency-induced senescence.

After nearly four decades of research on senescence, we have come to appreciate that cultured cells can encounter different types of senescence-like growth arrests such as M0, M1, M2, OIS etc. The main reason for distinction here is that each proliferative barrier is induced by a different mechanism. While some are induced by stress in culture (M0) or increased DNA damage (OIS), others reflect a balance between proliferation and apoptosis rate (M2). Classical replicative senescence (M1),

on the other hand, is induced in response to progressive telomere shortening associated with p53 and pRb activation. Regardless of the senescence-inducing mechanism, cells in all of these different growth arrested stages exhibit some if not all of the commonly observed phenotypes of classical senescence (Kuilman *et al.*, 2010).

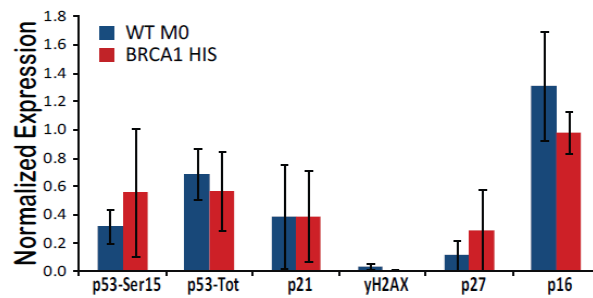
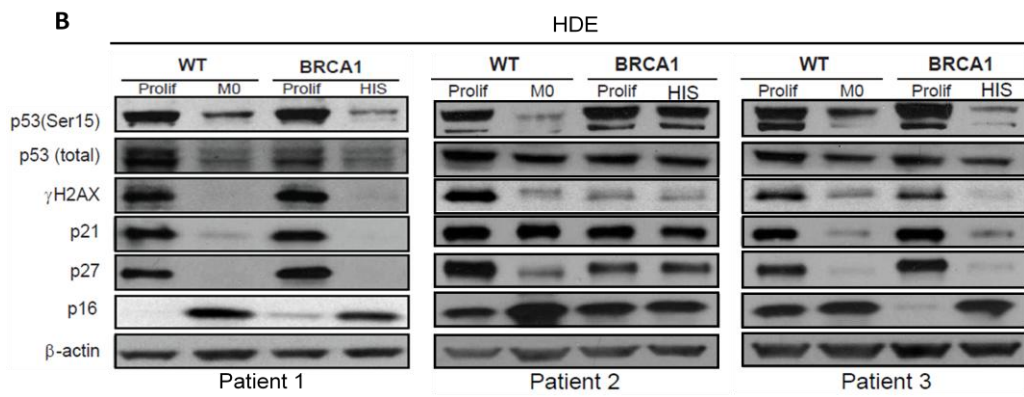
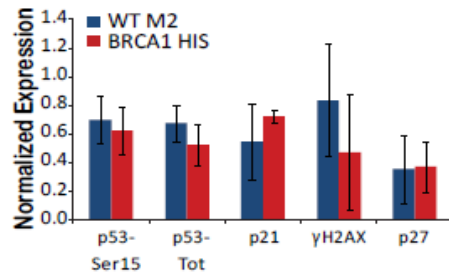
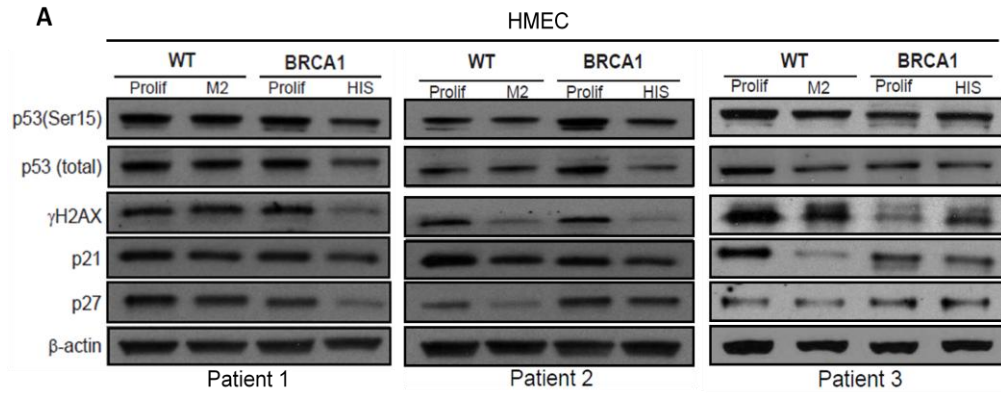
HIS in *BRCA1*^{+mut} HMECs occurred long before M2 in WT cells, while HIS in *BRCA1*^{+mut} HDEs was induced much sooner than M0 in WT cells. In addition, we also found that proliferating *BRCA1*^{+mut} HMECs suffered increased DNA damage prior to HIS, which then might be triggering premature senescence. Thus, in order to understand the mechanisms responsible for induction of HIS we decided to examine the activity of signaling pathways linking DNA damage repair with cell-cycle regulation (i.e. p53 and pRb) in *BRCA1*^{+mut} HMECs and HDEs. In addition, to further distinguish between M2 in WT HMECs and HIS in *BRCA1*^{+mut} HMECs, we compared the gene expression levels of some of the established SASFs.

3.3.1 HIS does not correlate with increased activity of p53 signaling pathway

The importance of p53 in induction of senescence has been well established (Itahana *et al.*, 2001, Fujita *et al.*, 2009). This protein coordinates signaling between DNA damage- and telomere dysfunction- sensing kinases (ATM/ATR) and the cell-cycle machinery (pRb). Thus, in response to various types of DNA damage ATM/ATR, in addition to γ H2AX, phosphorylates p53 at Ser15. This stabilizes nuclear p53, and increases its DNA binding and transcriptional activity. Among other genes, p53 induces the expression of cell-cycle inhibitor p21^{Cip1/Kip1}, which inhibits the activity of cyclin dependent kinases CDK4, CDK6 and CDK2. This, in turn, prevents pRb hyperphosphorylation and cell-cycle progression (reviewed by Sherr

and McCormick, 2002). Since it has been shown that senescence in BRCA1-deficient mouse embryonic fibroblasts or human cells can be induced in response to excessive DDR through a p53-dependent pathway (Cao *et al.*, 2003, Tu *et al.*, 2011), we hypothesized that observed increase in DDR in *BRCA1*^{+/-mut} HMECs results in corresponding increase in p53 activity that is responsible for induction of premature senescence. Since *BRCA1*^{+/-mut} HDEs senesced prematurely, we inspected the activity of p53 pathway in these cells as well. The levels of phosphorylated ATM/ATR substrates, as well as γ H2AX and p53BP1 foci, and the activity of p53 signaling pathway (phosphorylated p53 (Ser15), total p53, γ H2AX, p21 and the related family member p27) were evaluated in proliferating and senescent WT and *BRCA1*^{+/-mut} HMECs and HDEs.

As expected, westernblot analysis showed that the levels of critical components of DDR and p53 pathway activation, such as phosphorylated p53 (Ser15), total p53, γ H2AX, p21, p27 were elevated, but comparable between proliferating WT and *BRCA1*^{+/-mut} HMECs and HDEs (Figure 3.11A, B). In contrast, we found no increase in p53 pathway activity, phosphorylated ATM/ATR substrates, γ H2AX and p53BP1 in senescent *BRCA1*^{+/-mut} HMECs or HDEs (Figure 3.11A, B). In addition, expression levels of p14/ARF, an important regulator of p53 stability, did not differ between WT or *BRCA1*^{+/-mut} HMECs (Figure 3.12). Collectively, these data suggested that there was no preferential induction of the p53 pathway in BRCA1-heterozygous cells leading to HIS. In addition, the number of cells with phosphorylated ATM/ATR substrates (p=0.003), and γ H2AX foci (p<0.0001) were significantly reduced in HIS *BRCA1*^{mut/+} HMECs in comparison to M2 WT HMECs, suggesting that engagement of the DDR at HIS is suppressed and differs from that in M2 (Figure 3.11C).



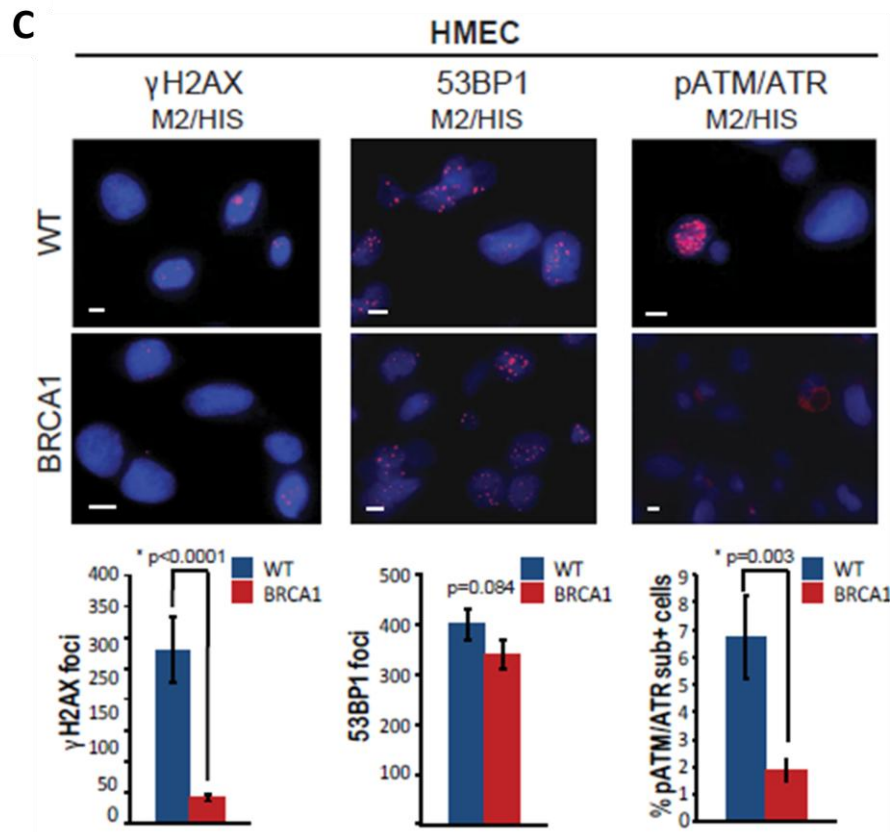


Figure 3.11. HIS does not correlate with increased activity of p53 signaling pathway. A) Western blot analysis of p53 (Ser15), total p53, γ H2AX, p21 and p27 levels in proliferating (Prolif) and M2/HIS WT and *BRCA1*^{mut/+} HMECs. Bar graph represents quantification of p53 signaling pathway determined by western blotting in M2 WT (N=3) and HIS *BRCA1*^{mut/+} (N=3) HMECs. B) Western blot analysis of p53 (Ser15), total p53, γ H2AX, p21, p27 and p16 levels in proliferating (Prolif) and M0/HIS WT and *BRCA1*^{mut/+} HDEs. Bar graph represents quantification of p53 signaling pathway determined by western blotting in M0 WT (N=3) and HIS *BRCA1*^{mut/+} (N=3) HDEs. C) Representative images of IF staining for phospho-ATM/ATR substrates, γ H2AX foci, 53BP1 foci in M2 WT and HIS *BRCA1*^{mut/+} HMECs. Graphs under the respective images represent the percent of cells positive for phospho-ATM/ATR substrates, and the average number of γ H2AX foci per WT nuclei (N=51) and BRCA1 nuclei (N=78), as well as the number of 53BP1 foci per WT nuclei (N=79) and BRCA1 nuclei (N=99).

3.3.2. *HIS* does not correlate with increased expression of *INK4s*

pRb can be activated in the context of increased expression of other cyclin-dependent kinase inhibitors such as p15/INK4b, p18/INK4c and p19/INK4d. INK4 family members share ankryn-like repeats and specifically associate with CDK4 and CDK6. Quantitative RT-PCR (qRT-PCR) analysis of p15/INK4b, p18/INK4c and p19/INK4d expression levels revealed no difference between senescent WT or *BRCA1*^{+mut} HMECs (Figure 3.12). This suggests that pRb activation, and thereby *HIS* in *BRCA1*^{+mut} HMECs, is not induced through expression of INK4 cell-cycle inhibitors.

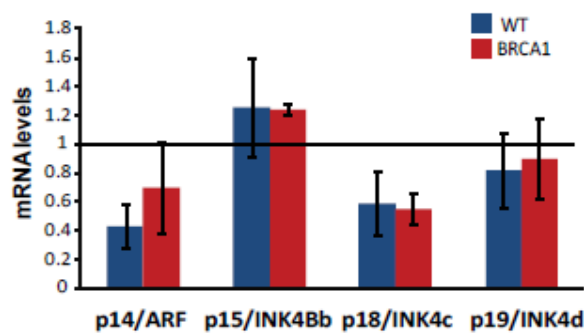


Figure 3.12. *HIS* does not correlate with increased expression of *INK4s*. mRNA levels of p14/ARF, p15/INK4b, p18/INK4c, and p19/INK4d in M2 WT and *HIS BRCA1*^{mut/+} HMECs. The values were determined by qRT-PCR and normalized to proliferating cells (represented by line set at 1).

3.3.3. *HIS* is mediated by active pRb signaling pathway

As a master regulator of G1-S transition and cell-cycle progression, pRb signaling pathway plays an important role in senescence induction (reviewed by Sherr and McCormick, 2002). Activity of pRb is regulated by various post-translational modifications including acetylation and phosphorylation (Ngyen *et al.*, 2004). In complex with cyclins, cyclin-dependent kinases phosphorylate pRb.

Hyperphosphorylation of pRb induces a conformational change of the protein, resulting in dissociation of pRb from E2F transcription factors (Lundberg and Weinberg, 1998). This, in turn, relieves the repression of E2F target genes, many of which are cyclins and cell-proliferation related genes. In contrast, CDK inactivation or increased acetylation of pRb inhibits pRb phosphorylation, resulting in hypophosphorylated pRb, repression of E2F target genes and permanent arrest of the cell-cycle (Chicas *et al.*, 2010). Since senescence can also be mediated by activation of the pRb pathway, we assessed the levels of pRb phosphorylation and E2F target genes (cyclin A and cyclin E) in HMECs and HDEs.

As expected, westernblot analysis showed that the components of pRb signaling pathway such as phosphorylated pRb at Ser795, total pRb, and E2F target gene Cyclin A were elevated, but comparable between proliferating WT and *BRCA1*^{+/-mut} HMECs and HDEs (Figure 3.13A, B). In addition, westernblot analysis indicated that, despite similar levels of total pRb, levels of phosphorylated pRb (Ser795) were reduced in senescent *BRCA1*^{+/-mut} HMECs compared to WT HMECs (Figure 3.13A, B). Also, levels of cyclin A were significantly decreased in senescent *BRCA1*^{+/-mut} HMECs compared to WT HMECs (Figure 3.13A). This further supports the notion that, in HMECs, HIS is distinct from M2. Premature senescence in HDEs was also associated with decreased levels of pRb phosphorylation and cyclin A expression (Figure 3.13B). Collectively, these data suggests that HIS in BRCA1-haploinsufficient epithelial cells is mediated by pRb – dependent cell-cycle arrest.

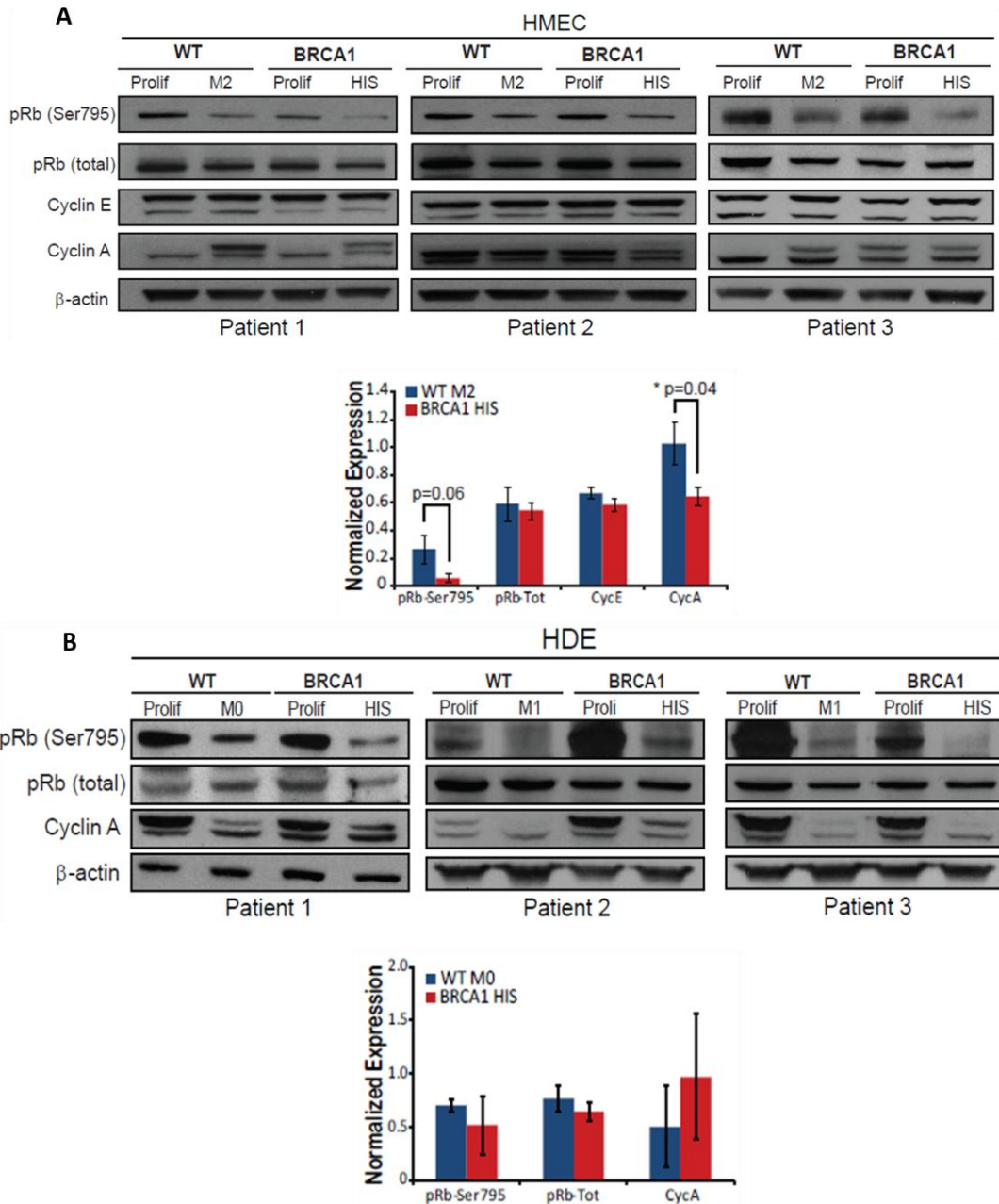


Figure 3.13. HIS is mediated by active pRb signaling pathway. A) Western blot analysis of phospho-pRb (Ser795), total pRb, Cyclin E and Cyclin A levels in proliferating (Prolif) and M2/HIS WT and *BRCA1*^{mut/+} HMECs. Bar graph represents quantification of phospho-pRb (Ser795), total pRb, Cyclin A levels determined by western blotting in M2 WT (N=3) and HIS *BRCA1*^{mut/+} (N=3) HMECs. B) Western blot analysis of phospho-pRb (Ser795), total pRb, Cyclin E and Cyclin A levels in proliferating (Prolif) and M0/HIS WT and *BRCA1*^{mut/+} HDEs. Bar graph represents quantification of phospho-pRb (Ser795), total pRb, Cyclin A levels determined by western blotting in M0 WT (N=3) and HIS *BRCA1*^{mut/+} (N=3) HDEs.

3.3.4 GSEA corroborates the observed increase in pRb pathway activation in $BRCA1^{+/mut}$ HMECs

In order to corroborate our findings that $BRCA1^{mut/+}$ HMECs exhibit increased pRb pathway activity, we compared the expression of genes involved in pRb pathway activation and senescence by Gene Set Enrichment Analysis (GSEA) in WT and $BRCA1^{mut/+}$ HMECs. GSEA was applied to previously published gene expression data collected on cultured proliferating primary human mammary epithelial cells isolated from $BRCA1$ -mutation carriers (N=6) or age-matched WT (N=6) (GSE19383, Bellacosa *et al.*, 2010).

Consistent with our data, GSEA of gene expression data from $BRCA1^{mut/+}$ HMECs revealed a significant enrichment of various pRb target genes, including those associated with senescence ($p < 10^{-4}$; Table 3.2), E2F1-regulated genes ($p < 10^{-4}$; Table 3.2) as well as genes down-regulated in senescent cells lacking p53 activity ($p < 10^{-4}$; Table 3.2). These data further support our observations that $BRCA1^{mut/+}$ HMECs exhibit pRb-mediated cell-cycle arrest resulting in premature senescence.

3.3.5 Expression of SASFs is increased in senescent $BRCA1^{+/mut}$ HMECs

Even though senescent cells remain viable and metabolically active, the proteins they express and secrete are quite different from proliferating cells (Shelton *et al.*, 1999). In fact, recent characterization of senescence associated secretory factors (SASFs) provided a detailed picture of this molecular signature of senescence, concluding that there are unprecedented similarities with inflammatory response as seen in wound healing (Coppe JP *et al.*, 2008). Globally, SASFs can be divided into several categories: interleukins (such as IL-6, IL-7, IL-1a and b), chemokines (such as

IL-8, GROs, MCPs, MIPs, and Eotaxins), growth factors (such as amphiregulin, EGF, bFGF, HGF, VEGF, angiogenin, SDF-1, and IGFBPs), proteases and regulators (MMPs, TIMPs, PAIs, and Cathepsins), as well as soluble/non-soluble factors (such as ICAMs, FasL, uPAR, PGE2, ROS, fibronectin, collagens, and laminin) (reviewed in Coppe *et al.*, 2010). In addition, SASFs phenotype in senescent cells strongly correlates with severe DNA damage (such as telomere dysfunction or oncogenic stress), whereas cell-cycle arrest in the absence of DNA damage does not seem to associate with increased expression of SASFs (Rodier *et al.*, 2009). Furthermore, it is thought that such profound changes in gene expression profile in senescence are brought about due to considerable chromatin remodeling that takes place in senescent cells (i.e. through SAHFs) (Zhang *et al.*, 2003).

Unlike WT HMECs, *BRCA1*^{mut/+} HMECs encounter premature senescence associated with cell-cycle arrest and increased DNA damage and telomere dysfunction. To extend our findings that HIS in *BRCA1*^{mut/+} HMECs is distinct from M2 in WT HMECs, we decided to compare the levels of SASFs in these cells (specifically, IL-6, IL-8, MMP-2, and PAI-1). qRT-PCR analysis of SASFs expression levels revealed that IL-6 and MMP-2 (but not IL-8 or PAI-1) were increased in senescent *BRCA1*^{+ /mut} HMECs compared to M2 WT HMECs (Figure 3.14) further implying that HIS in *BRCA1*^{+ /mut} HMECs is distinct from M2.

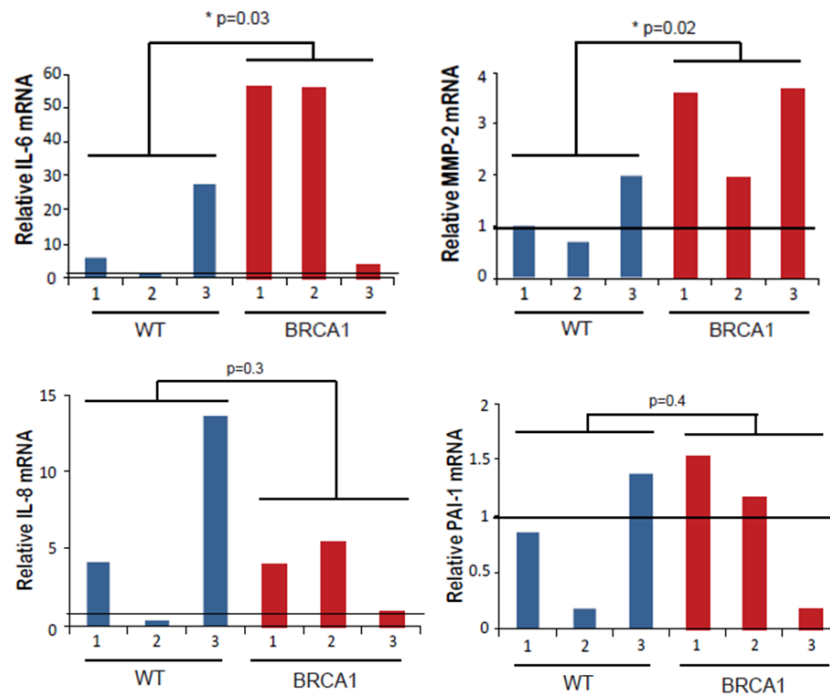


Figure 3.14. Expression of SASFs is increased in senescent $BRCA1^{+/mut}$ HMECs. mRNA levels of SASFs: IL-6, MMP2, IL-8 and PAI-1 in M2 WT and HIS $BRCA1^{mut/+}$ HMECs. The values were determined by qRT-PCR and normalized to proliferating cells (represented by line set at 1).

3.3.6 pRb knockdown extends the proliferation of $BRCA1^{+/mut}$ HMECs

Next, in order to show that senescence in $BRCA1^{+/mut}$ HMECs was in fact mediated by active pRb, we decided to knockdown pRb in these cells. In general, pRb knockdown in cultured cells resulted in proliferation extension beyond senescence, which led to further telomere attrition that triggers p53 dependent crisis or apoptosis (Newbold, 2002; Shay and Wright, 2005). Considering that HIS occurred in the absence of increased p53 activity, we were also interested in analyzing whether pRb knockdown would induce p53 activation in $BRCA1^{+/mut}$ HMECs. Therefore, functionality of p53 signaling pathway was also examined in control and pRb KD $BRCA1^{+/mut}$ HMECs.

Lenti-viral mediated shRNA was used to knockdown pRb in proliferating *BRCA1*^{mut/+} HMECs. Compared to control *BRCA1*^{mut/+} HMECs, knockdown of pRb led to an increase in replicative potential (Figure 3.15A). This indicated that pRb activity was likely one of the main mediators of premature senescence. Moreover, cytogenetic analysis of control and pRb KD *BRCA1*^{mut/+} HMECs revealed that bypassing HIS led to further increase in genomic instability associated with telomere-end fusions (p=0.01, Figure 3.15C). Interestingly, *BRCA1*^{mut/+} HMECs forced to proliferate as a result of pRb knockdown eventually arrested (Figure 3.15A). We found that this growth arrest was associated with elevated levels of all components of the p53 signaling pathway (phosphorylated p53 (Ser15), total p53, p21, p27) (Figure 3.15B). These data indicated that accumulation of additional genomic abnormalities in *BRCA1*^{+/-mut} HMECs was concomitant with p53 pathway activation resulting in growth arrest.

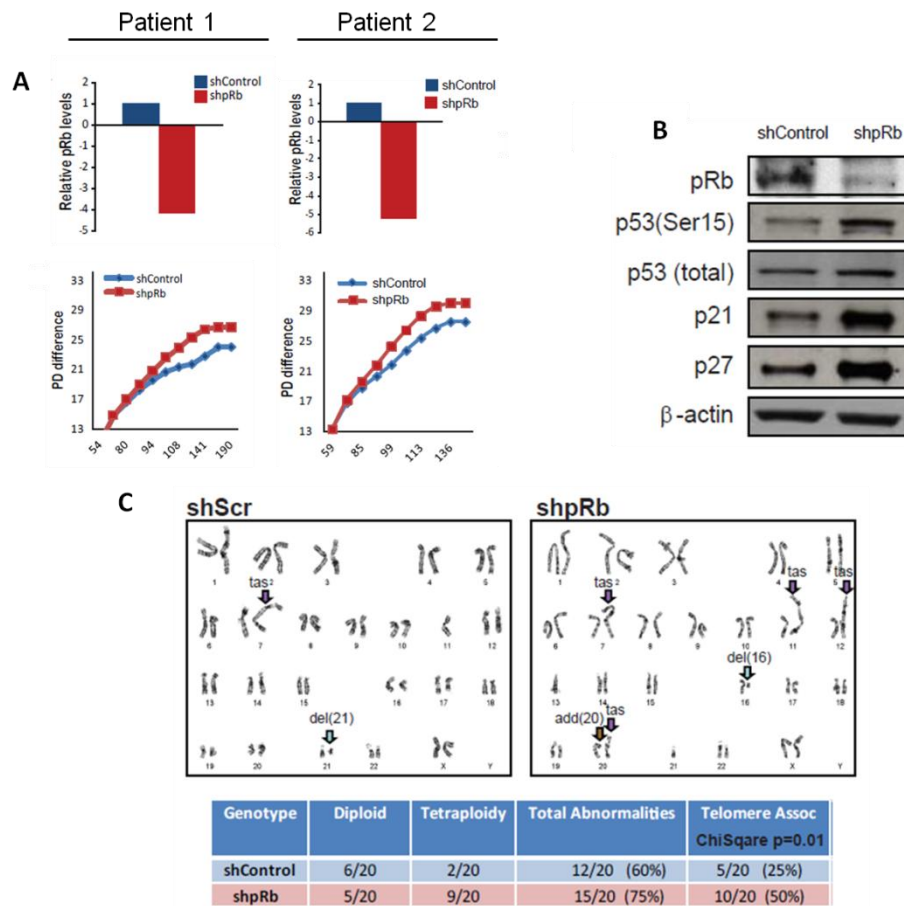


Figure 3.15. pRb knockdown extends the proliferation of $BRCA1^{+/mut}$ HMECs. A) pRb knockdown in proliferating $BRCA1^{mut/+}$ HMECs (mRNA levels). Representative growth curve of control (shScr) and shpRb $BRCA1^{mut/+}$ HMECs. B) Western blot analysis of pRb, p53 (Ser15), total p53, p21 and p27 levels in growth-arrested shScr (control) and shRb $BRCA1^{mut/+}$ HMECs. C) Representative images and summary table of significant genetic and chromosomal events determined by karyotype analysis in proliferating shScr (control) and shpRb $BRCA1^{mut/+}$ HMECs. In collaboration with Janet Cowan. Maja generated $BRCA1^{+/mut}$ HMECs with pRb knockdown and performed growth curve and WB analysis. Janet performed karyotype analysis and metaphase spreads for these cells.

3.3.7. Discussion of results

Agonescence (M2) in WT HMECs is associated with p53 pathway activation in response to DNA damage and genomic instability as a consequence of telomere attrition and dysfunction (Romanov *et al.*, 2001). Even though some cells in M2

exhibit typical morphological features associated with growth arrested senescence and SA- β -galactosidase positivity, a special feature of this proliferative barrier is that other non-senescent proliferating cells are counterbalanced by apoptosis resulting in plateau in the growth curve. Here we show that HIS is a distinctive proliferative arrest in comparison to M2 by several criteria. (1) We previously observed that cells undergoing HIS exhibited typical morphological features associated with senescence and SA- β -galactosidase positivity well before M2. (2) Levels of both proliferation and apoptosis were significantly reduced in HIS in comparison to M2 suggesting that cells undergo cell-cycle arrest. (3) pRb phosphorylation and cyclin A expression are decreased in *BRCA1*^{+/-mut} HMECs and HDEs, corroborating that cell-cycle arrest in HIS is mediated by pRb signaling pathway activation. (4) pRb knockdown extended the proliferative capacity of *BRCA1*^{+/-mut} HMECs, which is in contrast to M2 where inhibition of p53 is necessary to extend proliferative capacity. Finally, in comparison to M2 HIS correlated with increased DDR, telomere attrition, genomic instability, and SASFs expression. Taken together, these data suggest that HIS is a molecularly distinct senescence-like barrier compared to the other forms of senescence previously reported.

In contrast to the well-established role of p53 in DNA damage induced senescence or BRCA1 loss-induced senescence, HIS did not correlate with increased activation of p53 signaling pathway. We observed no difference in p53 signaling pathway in proliferating *BRCA1*^{+/-mut} HMECs when compared to *BRCA1* WT cells, even though *BRCA1*^{+/-mut} HMECs displayed increased DDR and genomic instability. In addition, we also did not see p53 activation in senescent *BRCA1*^{+/-mut} HMECs even though the number of p53BP1 foci per cell, labeling persistent DNA damage, increased in comparison to proliferating *BRCA1*^{+/-mut} HMECs. In fact, in two out of

three senescent *BRCA1*^{+/-mut} HMECs there was a noticeable decrease in total p53 levels. Consistent with this, when we examined the activity of p53 pathway in HMECs with BRCA1 knockdown, we found that the levels phospho- and total- p53 levels were decreased, as well (Figure 3.16). Interestingly, we found that p53 pathway was activated in *BRCA1*^{+/-mut} HMECs upon pRb knockdown in addition to an increase in additional genomic abnormalities. These data in human cells are in stark contrast to mouse cells, where BRCA1 biallelic inactivation robustly activates p53 signaling early on, leading to premature senescence (resulting in premature organism aging), as well as cell apoptosis (resulting in blunted branching/growth of mammary epithelium in mice with conditional BRCA1 knockout in mammary gland). Two reasons might be contributing to this observed “p53 unresponsiveness” to DNA damage in BRCA1-haploinsufficient HMECs: one possibility is that the DNA damage threshold that activates p53 pathway is increased in *BRCA1*^{+/-mut} HMECs. This idea has been proposed by Bartek *et al.* (2007) stating that in healthy cells activation of DNA damage response depends on the quality of damage as well as the quantity. Also, the need to engage DDR seems to be different between different tissues. In addition, germline mutations in DDR genes may very well weaken the response itself, raising the threshold of p53 activation to DNA damage to a higher level. Therefore, it is also possible that BRCA1-haploinsufficiency in epithelial cells raises the threshold of p53 activation to DNA damage to a higher level. Thus, only when DNA damage reaches catastrophic levels, such as in case of pRb inactivation, p53 pathway becomes engaged in BRCA1-haploinsufficient cells. This is potentially a very dangerous situation, because it suggests that many small and large scale mutations might go undetected in the cell for a long time, increasing the possibility of cellular transformation. One way to test this would be to compare DDR and activation of p53

signaling pathway in WT and *BRCA1*^{+mut} HMECs to various DNA damaging agents (UV, IR, actinomycin D, etc.) at various concentrations and/or dosages. Another possibility is that BRCA1 is involved in regulation of p53 stability in human cells. It is known that BRCA1 and p53 form a complex that plays an important role in p53-dependent gene expression (Ouchi *et al.*, 1998; Zhang *et al.*, 1998). Since phospho- and total-p53 levels decrease noticeably upon BRCA1 knockdown in WT HMECs, it is possible that BRCA1-p53 interaction is necessary for stability and retention of p53 in the nucleus. Also, in BRCA1-haploinsufficient cells the number of BRCA1-p53 complexes might be reduced as well. One way to test this would be to compare the localization of p53 or co-localization of p53 and BRCA1 in WT and *BRCA1*^{+mut} HMECs before and after DNA damage.

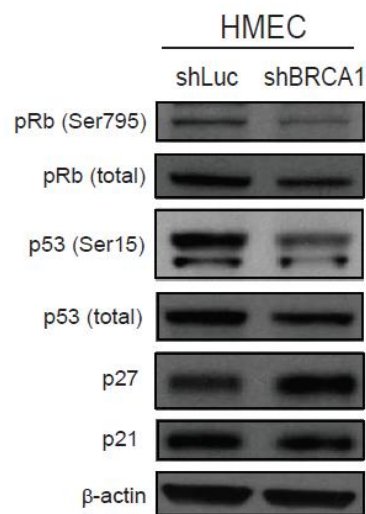


Figure 3.16. BRCA1 knockdown in HMECs induces premature senescence. Western blot analysis of pRb (Ser795), total pRb, p53 (Ser15), total p53, p21 and p27 levels in growth-arrested shLuc (control) and shBRCA1 HMECs. Levels of pRb phosphorylation at Ser 795 were decreased. Surprisingly, levels of p53 (Ser15) and total p53 were decreased, as well. This indicates that premature senescence induced by inhibition of BRCA1 expression in HMECs did not correlate with increased activity of p53 signaling pathway, which is similar to *BRCA1*^{+mut} HMECs undergoing HIS. In contrast to *BRCA1*^{+mut} HMECs, p27 seem to be upregulated in these cells, which might be inducing cell-cycle arrest. In collaboration with Mona Gauthier. Mona supplied cell pellets of shLuc and shBRCA1 HMECs. Maja performed the WB analysis.

Another intriguing observation regarding DDR in *BRCA1*^{+/-mut} HMECs is that the number of γ H2AX foci and levels of phosphorylated substrates by ATM/ATR are decreased at HIS. This could be due to impaired DDR in *BRCA1*^{+/-mut} HMECs, corroborating the disengagement of p53 signaling pathway discussed above. This would suggest that BRCA1 is required for persistent DDR activity through ATM/ATR- γ H2AX-p53 pathway. However, it could also be a reflection of different proliferative barriers occurring in cultured WT and *BRCA1*^{+/-mut} HMECs (agonescence vs. senescence, respectively). In this study we found that unlike WT HMECs at M2, prematurely arrested *BRCA1*^{+/-mut} HMECs display most hallmarks of senescence: telomere attrition, pRb pathway activation, E2F target gene repression, and SAFSs expression. Additionally, Bakkenist and colleagues (2004) found that pre-senescent human fibroblast (passage 68) display ATM/ATR- γ H2AX-p53 activation in response to telomere-dysfunction induced stress, however, fully senescent fibroblasts (passage 71) completely extinguish this pathway. They concluded that constitutive activation of DDR pathway is not necessary to maintain senescent state. Therefore, decreased number of γ H2AX foci and levels of phosphorylated substrates by ATM/ATR (and perhaps even decreased levels of total-p53) at HIS might just as well be a consequence of cell being fully senescent further supporting our observations that *BRCA1*^{+/-mut} HMECs undergo premature senescence.

3.4. In *BRCA1*^{mut/+} HMECs SIRT1 regulates HIS through acetylation of pRb and histone H4K16.

Since *BRCA1*^{+/-mut} HMECs undergo premature senescence associated with accelerated rate of telomere attrition and increased telomere dysfunction, we

speculated that this is due to aberrant regulation of telomere stability in these cells. BRCA1 induces expression of SIRT1, a class III NAD-dependent deacetylase involved in telomere length maintenance (Anastasiou and Krek, 2006; Wang *et al.*, 2008). SIRT1 is a human homologue of Sir2 (silent information regulator 2), a nutrient-responsive enzyme whose overexpression results in extension of the life span in wide range of lower eukaryotes, including yeast, worms and flies. Experimental evidence suggests that Sir2 promotes longevity by establishing and maintaining telomeric heterochromatin, as well as mediating the beneficial effects of caloric restriction by modulating IGF signaling pathway (Anastasiou and Krek, 2006). Similarly, the function of mammalian SIRT1 has been implicated in chromatin remodeling, transcription, DNA damage repair, apoptosis/cell survival and differentiation. It has been reported that SIRT1 deacetylates histones to regulate gene expression, protect telomere ends, and maintain heterochromatin structure (Anastasiou and Krek, 2006; Mulligan *et al.*, 2011). Specifically, SIRT1 mediates deacetylation of histone H1K26, H3K9, H3K56 and H4K16 during cellular aging on telomeric and subtelomeric regions. Thus, loss of SIRT1 leads to loss of histones, shorter telomeres, and genomic instability (Garcia-Cao *et al.*, 2004, Dang *et al.*, 2009). Also, SIRT1 seems to be directly involved in genomic maintenance since it regulates the function of NBS1 (a component of DNA damage sensor complex MRN) and has been found at the sites of DNA damage where it promotes repair (Yuan *et al.*, 2007). Consistent with these reports, it was found that SIRT1 inhibition results in increased genomic instability, telomere dysfunction and premature senescence (Oberdoerffer *et al.*, 2008; Palacios *et al.*, 2010). Therefore, we reasoned that misregulation of SIRT1 in *BRCA1*^{mut/+} HMECs might result in increased histone acetylation leading to telomere dysfunction and increased genomic instability.

SIRT1 also targets nonhistone proteins, including p53, pRb and several FOXO family members to promote cell survival and inhibit senescence or apoptosis (Anastasiou and Krek, 2006). Indeed, pRb phosphorylation on multiple residues can be regulated by deacetylation events catalyzed by SIRT1 in pRb–SIRT1 complexes (Wong and Weber, 2007). In addition, several recent studies have shown that SIRT1 protein expression decreases during replicative senescence and that there is a negative correlation between levels of SIRT1 and SA-β-galactosidase activity (Langley *et al.*, 2002, Ota *et al.*, 2007, Huang *et al.*, 2008). The cell-cycle arrest in these settings was associated with both decreased pRb phosphorylation and increased pRb acetylation (Huang *et al.*, 2008). Given the lack of differences in the expression levels of cell cycle inhibitors in *BRCA1*^{+/-mut} HMECs despite the reduction in pRb phosphorylation and activity, we reasoned that misregulation of SIRT1 in *BRCA1*^{mut/+} HMECs might also result in modifications of pRb acetylation leading to induction of HIS.

Therefore, to address the mechanism that might be responsible for HIS and to analyze it in a cell-type and tissue-specific context in BRCA1-haploinsufficient cells, we first set out to examine SIRT1 levels in HMECs, HDEs, HMFs and HDFs.

3.4.1 SIRT1 levels are decreased in *BRCA1*^{mut/+} HMECs

A recent study showed that BRCA1-deficiency correlates with decreased levels of SIRT1 and that SIRT1 expression is regulated by BRCA1 in breast epithelial cell line (Wang *et al.*, 2008). However, whether *BRCA1*^{mut/+} HMECs have decreased SIRT1 levels and whether this is related to premature senescence phenotype has never been examined before. Westernblot analysis showed that there was a notable decrease in SIRT1 levels in proliferating *BRCA1*^{mut/+} HMECs in comparison to WT cells (Figure 3.16). Furthermore, SIRT1 levels were significantly reduced in senescent

BRCA1^{mut/+} HMECs in comparison to M2 WT HMECs (p=0.019; Figure 3.16). This indicates that *BRCA1*^{mut/+} HMECs indeed have decreased levels of SIRT1. Consistent with previous reports linking SIRT1 and senescence, these data also suggest that premature senescence in *BRCA1*^{mut/+} HMECs correlates with decrease in SIRT1 levels.

Since SIRT1 is ubiquitously expressed, it is not known whether BRCA1 also regulates SIRT1 levels in other cells and tissues (Michishita *et al.*, 2005). In order to determine if other BRCA1-haploisufficient cells have decreased levels of SIRT1, we also examined SIRT1 levels in proliferating and senescent HDEs, HMFs and HDFs. Westernblot analysis revealed that the levels of SIRT1 in senescent *BRCA1*^{mut/+} HDEs, HMFs or HDFs did not differ from those found in senescent WT cells of the same tissue origin (Figure 3.17). These findings indicate that the decrease in SIRT1 was a feature unique to *BRCA1*^{mut/+} HMECs, and also suggest that regulation of SIRT1 levels by BRCA1 is HMEC-specific.

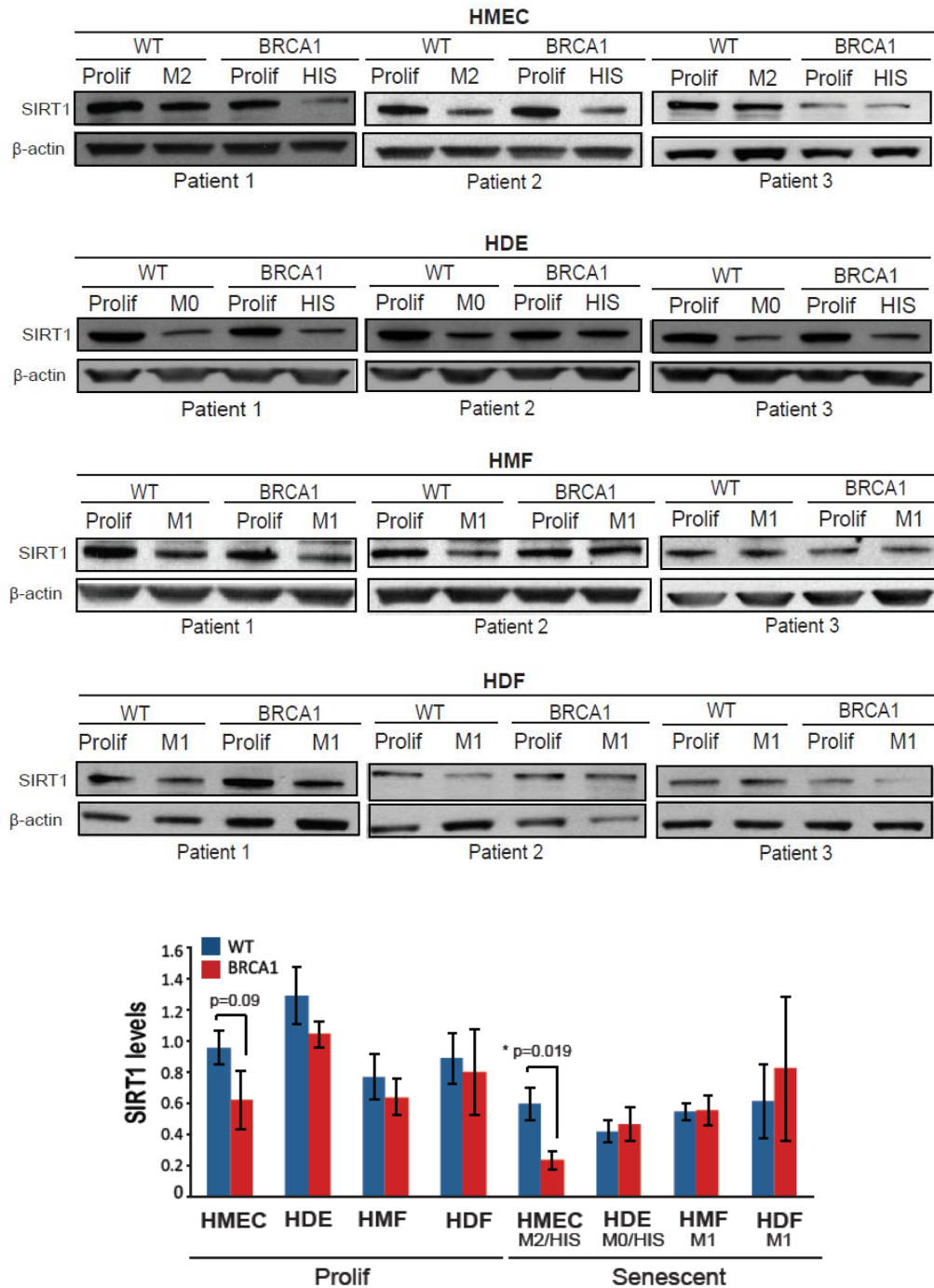


Figure 3.17. SIRT1 levels are decreased in $BRCA1^{mut/+}$ HMECs. Western blot analysis of SIRT1 levels in proliferating (Prolif) and growth-arrested (M2/HIS) WT (N=3) and $BRCA1^{mut/+}$ (N=3) HMECs, proliferating (Prolif) and senescent (M0/HIS) WT (N=3) and $BRCA1^{mut/+}$ (N=3) HDEs, as well as proliferating (Prolif) and senescent (M1) WT (N=3) and $BRCA1^{mut/+}$ (N=3) HMFs and HDFs. Bar graph represents quantification of SIRT1 levels determined by western blotting in proliferating and senescent HMECs, HDEs, HMFs and HDFs.

3.4.2. BRCA1 regulates SIRT1 levels in HMECs

Wang and colleagues (2008) reported that BRCA1 regulates SIRT1 transcription by binding to 1354-1902bp region of SIRT1 promoter. Therefore, to determine if SIRT1 transcription was altered in *BRCA1*^{mut/+} HMECs, we compared SIRT1 mRNA levels in WT and *BRCA1*^{mut/+} HMECs. Surprisingly, qRT-PCR analysis revealed no difference in SIRT1 mRNA levels between WT and *BRCA1*^{mut/+} HMECs (Figure 3.18A). However, in WT HMECs where BRCA1 expression was attenuated through lentiviral-mediated short hairpin inhibition, SIRT1 mRNA and protein levels were markedly decreased (Figure 3.18B). These data indicate that, in addition to transcriptional regulation, BRCA1 might also be involved in post-transcriptional regulation of SIRT1 levels, perhaps by modifying SIRT1 stability.

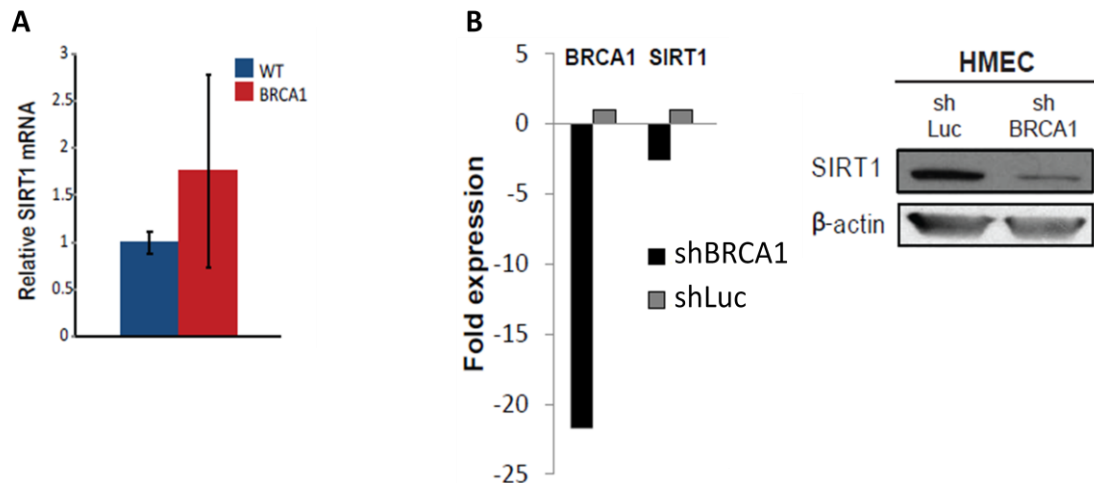


Figure 3.18. BRCA1 regulates SIRT1 levels in HMECs. A) mRNA levels of SIRT1 in WT and *BRCA1*^{mut/+} HMECs. The values were determined by qRT-PCR and normalized to GAPDH. The graph represents fold expression in *BRCA1*^{mut/+} HMECs compared to WT cells. B) mRNA levels of SIRT1 in shLuc (control) and shBRCA1 HMECs. The values were determined by qRT-PCR and normalized to GAPDH. The graph represents fold expression in shBRCA1 HMECs compared to shLuc (control) cells. SIRT1 levels in shLuc (control) and shBRCA1 HMECs determined by western blotting. In collaboration with Mona Gauthier. Mona supplied cell pellets of shLuc and shBRCA1 HMECs. Maja performed qRT-PCR and WB analysis in WT and *BRCA1*^{+/-mut} HMECs as well as in shLuc and shBRCA1 HMECs.

3.4.3. SIRT1 knockdown induces premature senescence in WT HMECs

In order to show that decrease in SIRT1 levels induces premature senescence, we used lenti-viral mediated shRNA to inhibit SIRT1 expression in WT HMECs. Down-regulation of SIRT1 (mRNA and protein) levels in these cells resulted in cell cycle arrest and morphological changes associated with senescence (Figure 3.19). This suggests that decreased levels of SIRT1 induce premature senescence in HMECs, and is consistent with phenotype observed in *BRCA1*^{mut/+} HMECs (and BRCA1 KD HMECs).

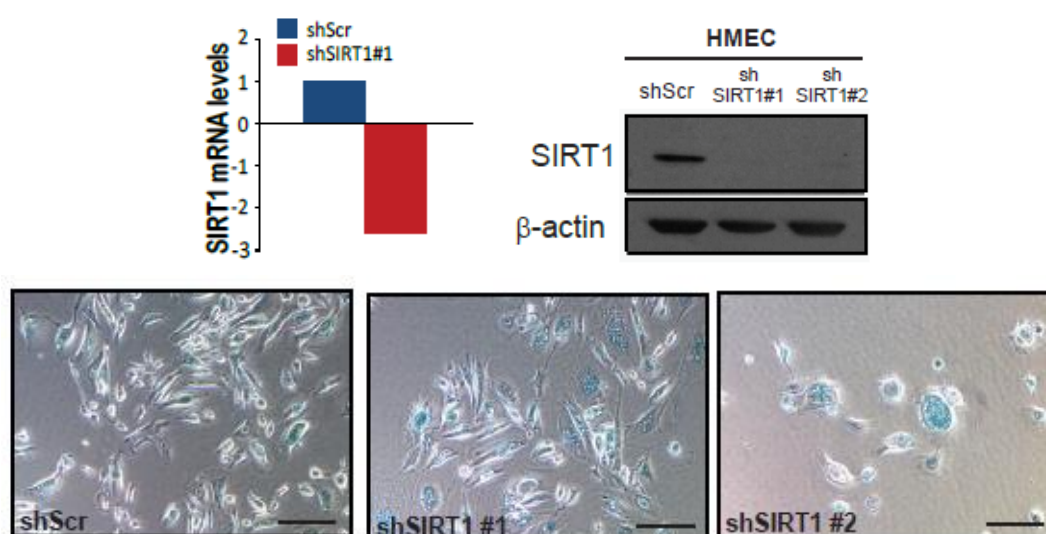


Figure 3.19. SIRT1 knockdown induces premature senescence in WT HMECs. Relative mRNA levels of SIRT1 in shScr and shSIRT1 HMECs. The values were determined by qRT-PCR and normalized to GAPDH. The graph represents fold expression in shSIRT1 HMECs compared to shScr (control) cells. SIRT1 levels in shScr (control) and shSIRT1 HMECs (two different hairpin knockdowns included) determined by western blotting. Representative images of SA- β -galactosidase staining in shScr (control) and shSIRT1 HMECs (two different hairpin knockdowns included).

3.4.4. Inhibition of SIRT1 and BRCA1 expression results in increased Ac-pRb levels

Next, we wanted to examine how decreased SIRT1 expression might induce cell-cycle arrest and premature senescence in HMECs. Cell-cycle arrest is induced by active pRb in hypophosphorylated and acetylated state. Decreased acetylation of pRb exposes residues for phosphorylation, which initiates the process of pRb inactivation and cell-cycle progression (Nguyen *et al.*, 2004). Wong and Weber (2007) showed that SIRT1 and pRb form a complex, in which SIRT1 acts as a potent deacetylase for pRb. Furthermore, they showed that overexpression of SIRT1 resulted in significant reduction of pRb acetylation, whereas contact inhibition or gene knockdown of SIRT1 produced accumulation of acetylated pRb. Given this, we examined if decreased expression of SIRT1 results in increased levels of acetylated pRb in HMECs.

Because antibodies against Ac-pRb are unavailable, total pRb was immunoprecipitated from whole cell lysates of control, SIRT1 KD and BRCA1 KD HMECs. Westernblots were probed with antibodies raised against acetylated-lysine residue (anti-Ac-Lys). We found that Ac-pRb levels increased following knockdown of BRCA1 or SIRT1 in HMECs (Figure 3.20A). Furthermore, when we probed westernblots of whole cell lysates of control, SIRT1 KD and BRCA1 KD HMECs with anti-Ac-Lys we found that acetylation levels of a number of other proteins increased following knockdown of BRCA1 or SIRT1 (Figure 3.20B). Collectively, these data indicate that a decrease in SIRT1 expression in HMECs correlates with increased Ac-pRb resulting in cell-cycle arrest and premature senescence.

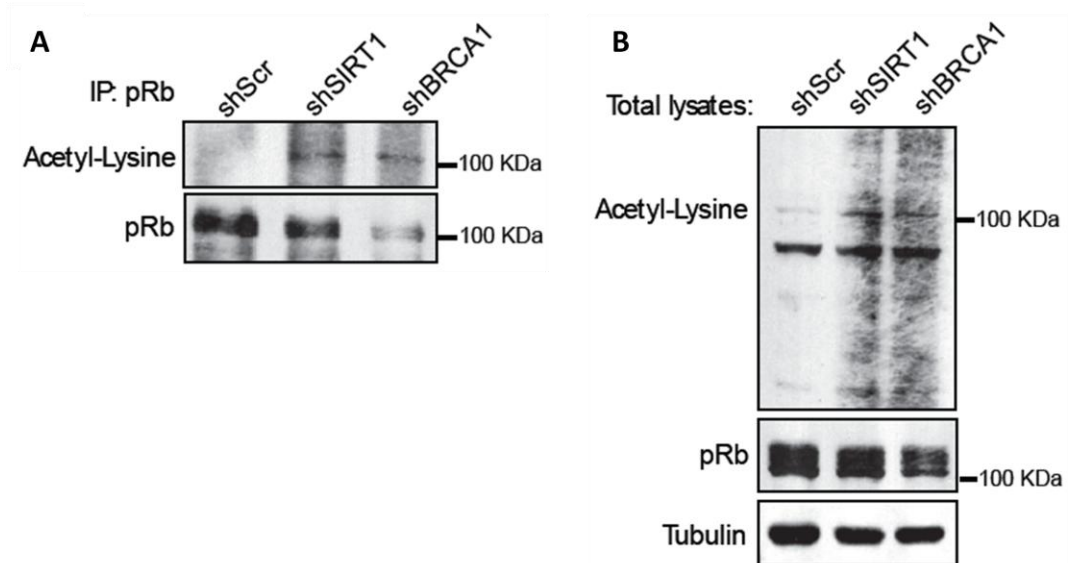


Figure 3.20. Inhibition of SIRT1 and BRCA1 expression results in increased Ac-pRb levels. A) Acetyl-pRb (Acetyl-Lysine blot) and total-pRb levels in shScr (control), shSIRT1 and shBRCA1 HMECs determined by pRb IP followed by western blot. B) Western blot analysis of Acetyl-Lysine, total-pRb, and tubulin (loading control) levels in total cell lysates from shScr (control), shSIRT1 and shBRCA1 HMECs. In collaboration with Nelson Brown. Maja generated shSIRT1 and shBRCA1 knockdowns in HMECs and provided cell pellets. Nelson performed pRb IP and WB analysis.

3.4.5. *SIRT1* occupancy at telomeres is decreased in *BRCA1*^{mut/+} HMECs

In their extensive study of the role of SIRT1 in mammalian telomere biology, Palacios and colleagues (2010) showed that SIRT1 binds to telomeric repeats. There, it attenuates telomere fragility by decreasing aberrations associated with replication fork stalling and breakage of telomeres. In addition, they showed that SIRT1 acts as a positive regulator of telomere length and protects telomere function by negatively modulating histone acetylation, and concomitantly increasing heterochromatic marks. Moreover, Vaquero *et al.* (2007) also showed that SIRT1 directly promotes heterochromatin formation by recruiting and deacetylating SUV39H1, a histone methyltransferase responsible for accumulation of repressive histone marks at telomeres.

Given that *BRCA1*^{mut/+} HMECs had decreased SIRT1 levels and increased telomere dysfunction, we hypothesized that these cells have decreased SIRT1 occupancy at telomeres as well. In order to assess this, chromatin immunoprecipitation (ChIP) was carried out in WT and *BRCA1*^{mut/+} HMECs. SIRT1 levels at telomeres were found to be significantly reduced in *BRCA1*^{mut/+} compared to WT HMECs (p=0.017; Figure 3.21). This suggests that increased telomere attrition in *BRCA1*^{mut/+} HMECs correlates with decreased SIRT1 abundance at telomeres.

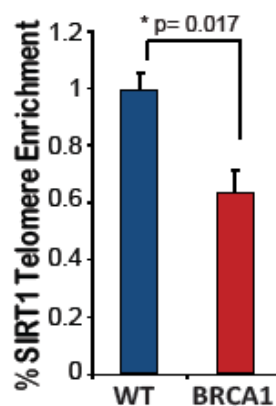


Figure 3.21. SIRT1 occupancy at telomeres is decreased in *BRCA1*^{mut/+} HMECs. ChIP analysis of SIRT1 abundance at telomeres in WT and *BRCA1*^{mut/+} HMECs. Data are presented as average of 3 WT and 3 BRCA1 patient samples \pm SEM. Percent enrichment of SIRT1 in *BRCA1*^{mut/+} HMECs was normalized to WT HMECs. In collaboration with Peter Mulligan. Maja provided WT and *BRCA1*^{+mut} HMECs. Peter performed the ChIP analysis.

3.4.6. Inhibition of SIRT1 and BRCA1 expression results in increased global and telomeric acetylation of H4K16 levels

Recent studies have shown that telomeres and subtelomeric regions are enriched in epigenetic marks characteristic of heterochromatin: HP1, trimethylated H3K9 and H4K20, and contain low levels of acetylated H3 and H4 (Blasco, 2007). Inhibition of master regulators involved in propagation of repressive marks correlates with loss of telomere-length control (Garcia-Cao *et al.*, 2004). These studies imply

that chromatin modifications are important regulators of telomere stability. SIRT1 modifies histones through deacetylation of K26 on histone H1, K9 and K56 on histone H3, and K16 on histone H4 (Palacios *et al.*, 2010; Mulligan *et al.*, 2011). It has been shown that, in this manner, SIRT1 can regulate expression of target genes as well as chromatin structure of subtelomeric and telomeric regions. In particular, SIRT1 decreases acetylation of H4K16 and H3K9 to preserve telomeric heterochromatin and prevent telomere shortening and dysfunction (Palacios *et al.*, 2010; Mulligan *et al.*, 2011).

Since SIRT1 levels were decreased at telomeres in *BRCA1*^{mut/+} HMECs, we reasoned that the levels of H4K16 and H3K9 acetylation are increased in these cells. Therefore, global and telomere-specific levels of Ac-H4K16 and Ac-H3K9 in HMECs in which BRCA1 or SIRT1 expression was inhibited was assessed. Histone acid extraction and westernblot analysis of global histone acetyl-marks indicated that the levels of Ac-H4K16 were markedly increased in shBRCA1 and shSIRT1 HMECs compared to control cells (Figure 3.22A). In addition, ChIP analysis of telomere-specific histone acetyl-marks also revealed that the levels of Ac-H4K16 were significantly increased in shBRCA1 and shSIRT1 HMECs compared to control cells (Figure 3.22B). No significant changes were observed in the levels of global or telomere-specific Ac-H3K9 (Figure 3.22A, B). Taken together, these findings indicate that upon reduction of SIRT1 global and telomere-specific H4K16 acetylation is increased, likely leading to telomere fragility in HMECs.

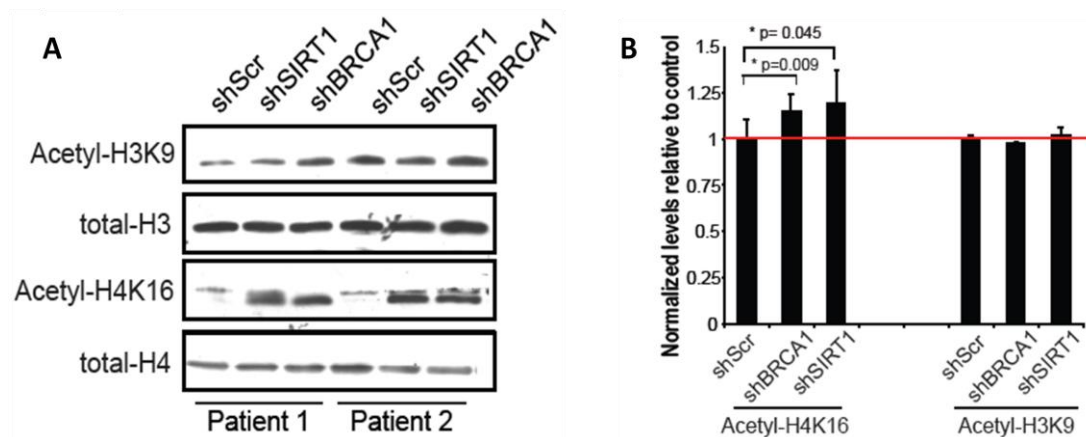


Figure 3.22. Inhibition of SIRT1 and BRCA1 expression results in increased global and telomeric acetylation of H4K16 levels. A) Western blot analysis of Acetyl-H3K9, total H3, Acetyl-H4K16, and total H4 levels in shScr (control), shSIRT1 and shBRCA1 HMECs from two patient samples. B) ChIP analysis of Acetyl-H4K16 and Acetyl-H3K9 abundance at telomeres in shScr (control), shSIRT1 and shBRCA1 HMECs from two patient samples (results were averaged). Percent enrichment of Acetyl-H4K16 and Acetyl-H3K9 in shSIRT1 and shBRCA1 HMECs was normalized to shScr (control) cells. In collaboration with Adam Skibinski and Paula Martinez. Maja generated shSIRT1 and shBRCA1 HMECs. Adam performed the histone WB analysis, while Paula performed the ChIP analysis.

3.4.7. Features of BRCA1-haploinsufficiency in breast tissue specimens

Consistent with increased chromosomal abnormalities, unbalanced translocations and the aneuploidy we observed in *BRCA1*^{+/-mut} HMECs *in vitro*, recent *in vivo* analysis of disease-free human breast tissues from *BRCA1*-mutation carriers has revealed similar genomic and chromosomal aberrations, as well as multipolar mitoses and abnormal centrosomes (Baldeyron *et al.*, 2002; Rennstam *et al.*, 2010; Konishi *et al.*, 2011; Martins *et al.*, 2012). To determine whether other features of BRCA1-haploinsufficiency including telomere erosion, increased DDR, SIRT1 misregulation and HIS are also present *in vivo*, we examined disease-free breast tissue specimens from *BRCA1*-mutation carriers for telomere length, γ H2AX levels, SIRT1

expression, and evidence for pRb pathway activation. Telomere length was measured by Q-FISH with a telomeric probe in WT (N=21) and *BRCA1*^{+/-mut} (N=9) tissues. Telomeres were indeed significantly shorter in breast epithelial cells within lobules of *BRCA1*^{mut/+} breast tissues compared to lobules of WT breast tissues (p=0.003, Figure 3.23A). This finding is of particular significance given that the cellular precursors to breast cancers reside within lobules. In addition, although breast epithelium from WT tissues was associated with overall shorter telomeres compared to breast stromal fibroblasts in WT tissues (Figure 3.23B, p=0.04), this association was not observed in *BRCA1*-mutation carriers indicating that mechanisms regulating telomere length are altered *in vivo*. Furthermore, we examined if decreased telomere length in *BRCA1*^{mut/+} breast tissue specimens correlated with activated DDR. Using IHC we stained WT and *BRCA1*^{mut/+} tissue sections for γ H2AX. *BRCA1*^{mut/+} breast epithelium stained negative for γ H2AX, suggesting that DDR was not activated (Figure 3.23C). Moreover, using IHC we examined SIRT1 expression and distribution in the breast tissue. Interestingly, we found that SIRT1 is mainly localized in the nuclei of luminal breast epithelial cells. Comparison of SIRT1 expression in WT and *BRCA1*^{mut/+} breast tissue specimens indicated that SIRT1 levels were significantly reduced in luminal cells within lobules of *BRCA1*^{mut/+} breast tissues compared to their WT counterparts (p=9.15x10⁻⁹; Figure 3.23D), which was consistent with the lower levels of SIRT1 observed *in vitro*.

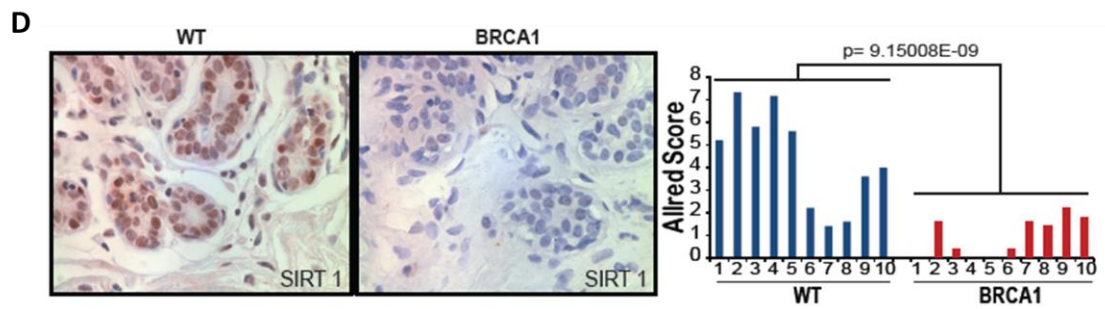
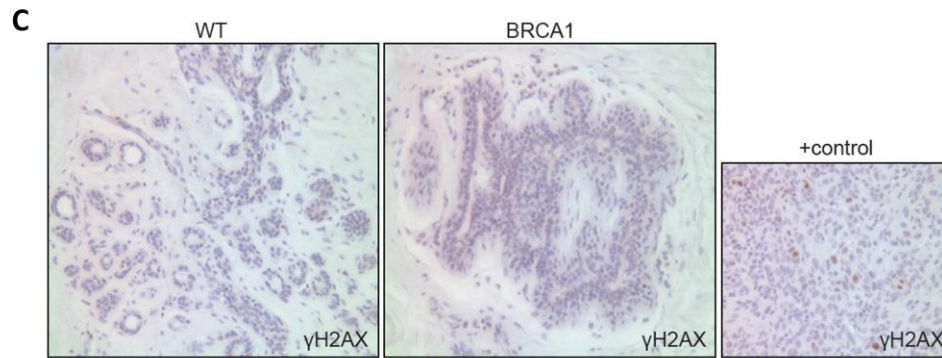
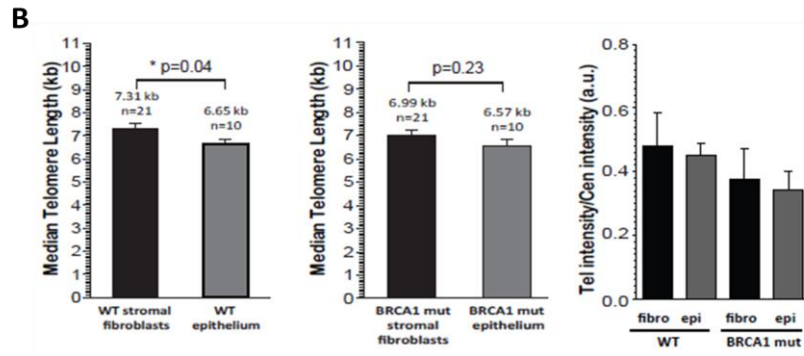
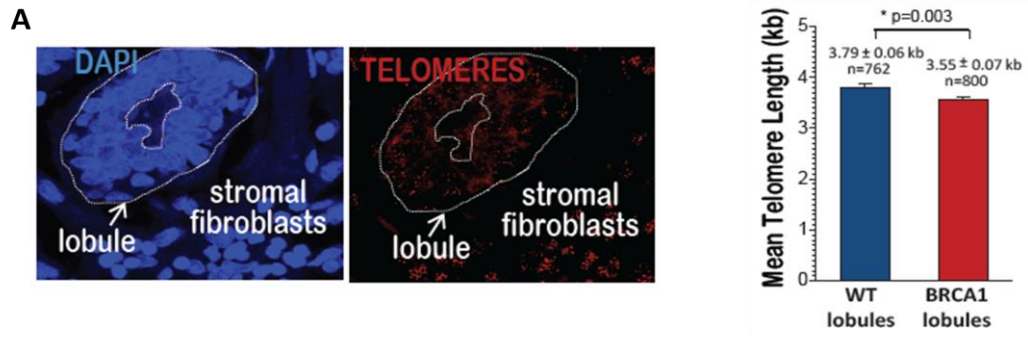
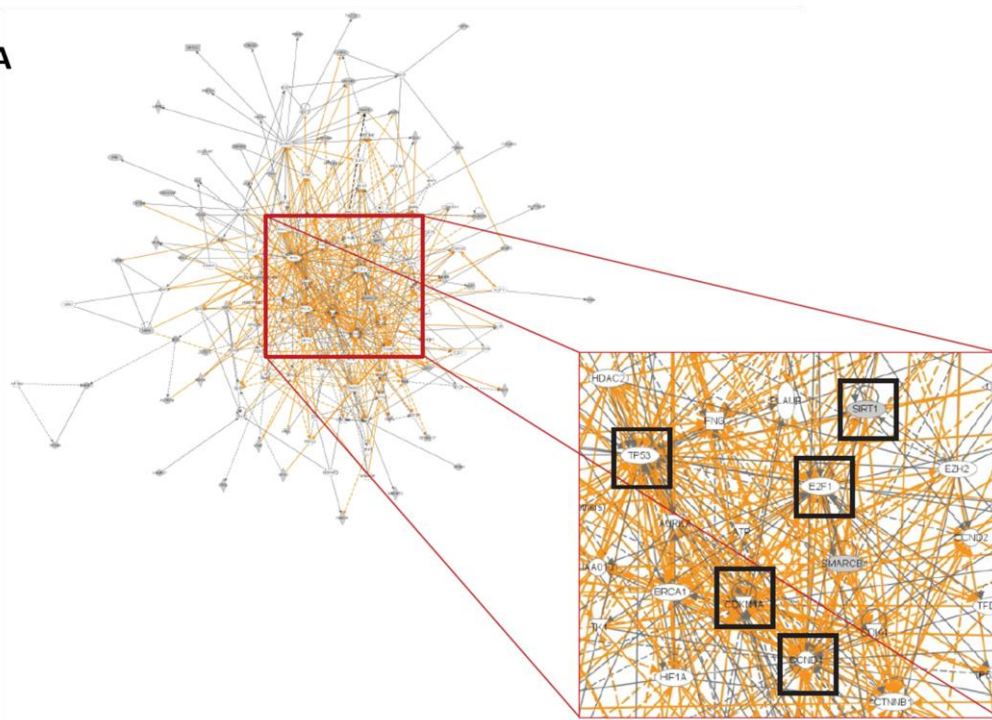


Figure 3.23. (on previous page) Features of BRCA1-haploinsufficiency in breast tissue specimens. A) Representative images and mean telomere length (kb) determined by qFISH in WT lobules (N=762 cells) and *BRCA1*^{mut/+} lobules (N=800 cells). B) Median telomere length (kb) determined by qFISH in stromal fibroblasts from WT (N=21) and *BRCA1*^{mut/+} (N=10) disease-free patient tissues. C) Representative images of IHC staining of γ H2AX in breast tissue sections of WT (N=3) and *BRCA1*^{mut/+} (N=3) patients. Breast cancer tissue sections were used as positive control for γ H2AX staining. Positive control D) Images of IHC staining and quantification of SIRT1 levels in epithelial cells from WT (N=10) and *BRCA1*^{mut/+} (N=10) breast tissues. Allred score methodology was used to measure SIRT1 antibody staining. In collaboration with Mercedes Gallardo. Mercedes performed telomere qFISH analysis, while Maja performed γ H2AX and SIRT1 staining of patient tissue sections.

Finally, we queried our gene expression data collected from freshly isolated breast epithelial cells from WT (N=4) and *BRCA1*-mutation carriers (N=4) to determine whether evidence of DDR and HIS pathway activation could be observed *in vivo* (Proia *et al.*, 2011). Consistent with *in vitro* findings, Ingenuity Pathway Analysis revealed that in addition to double strand break repair, and mismatch repair, ATM signaling ($p=5.83 \times 10^{-3}$) and DNA methylation ($p=3.58 \times 10^{-2}$) were significantly enriched in *BRCA1*^{mut/+} tissues. Moreover, comprehensive network analysis using Ingenuity Gene Network Analysis revealed 25 significant networks as major regulators in epithelial cells from *BRCA1*-mutation carriers, 12 of which formed an overlapping network with central nodes consisting of SIRT1, cyclin D1, CDKN1A, and p53 (Figure 3.24A, B). Interestingly, additional networks involving cellular stress, metabolism, and autophagy were also enriched *in vivo* in *BRCA1*^{mut/+} tissues, consistent with the role of these pathways in regulating autophagy and senescence in response to DNA damage and chronic apoptotic stress (Esteve *et al.*, 2010; Tang and Wong, 2011; Brown *et al.*, 2012; Salem *et al.*, 2012; Singh *et al.*, 2012).

A



B

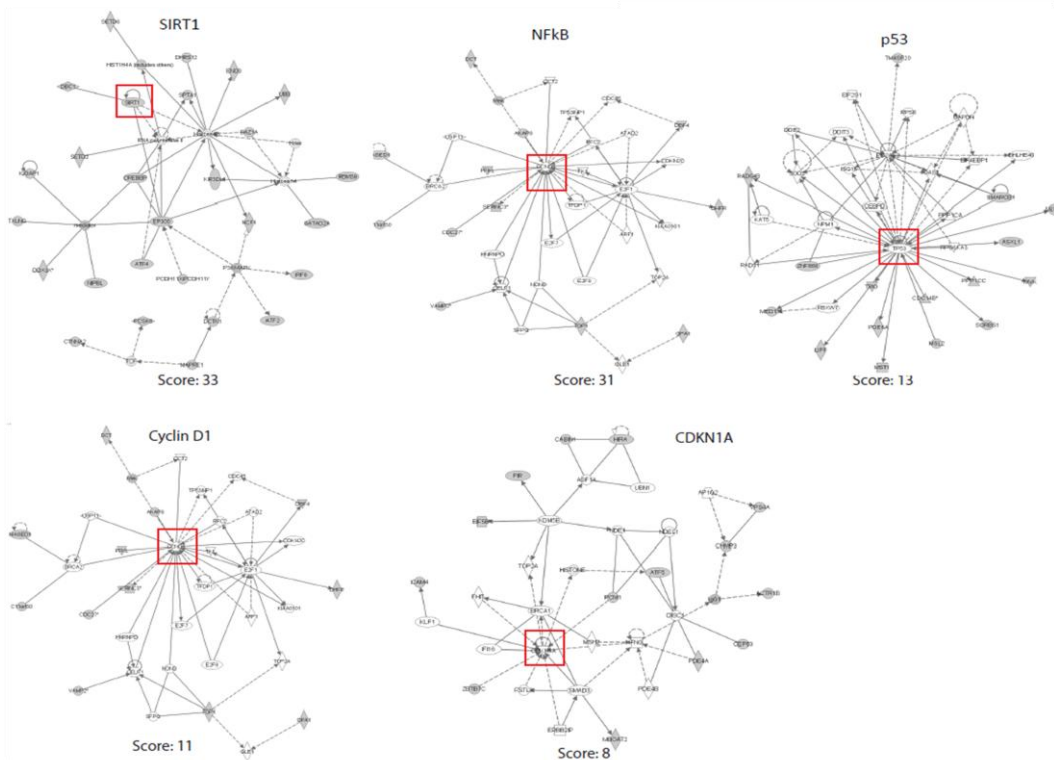


Figure 3.24. (on previous page) Pathway analysis of mammary epithelial cells *in vivo* from *BRCA1*-mutation carriers. A) Ingenuity Pathway Analysis identified 25 significant gene networks from differentially expressed genes from freshly isolated HMECs, as previously described (Proia *et al.*, 2011, and as described in Materials and Methods). A gene network of 12 overlapping central nodes was constructed from this analysis. Genes colored in grey represent genes differentially expressed in *BRCA1*^{mut/+} tissues vs WT tissues. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges with dashed lines show indirect interaction, while a continuous line represents direct interactions. B) The most significant gene networks identified involved central nodes in SIRT1, Rb, p53, and NFkappaB pathways. Charlotte Kuperwasser performed Ingenuity Pathway Analysis.

3.4.8. Discussion of results

We have found that HIS is mediated by pRb-dependent cell-cycle arrest and specifically in *BRCA1*^{mut/+} HMECs, it associates with increased telomere erosion and genomic instability. In order to address why *BRCA1*^{mut/+} HMECs have an increased susceptibility to telomere dysfunction we looked for candidates that link BRCA1 to pathways involved in telomere maintenance. We found that BRCA1 regulates expression of SIRT1, a deacetylase whose enzymatic versatility ranges from propagation of heterochromatin at telomeres to cell-cycle regulation. Thus, we deemed it to be likely that altered SIRT1 levels in *BRCA1*^{mut/+} HMECs might be responsible for increased telomeric attrition and premature senescence induction. Although one study showed that BRCA1-null cells exhibit low levels of SIRT1 (Wang *et al.*, 2008), here we report for the first time that SIRT1 is specifically misregulated in *BRCA1*^{mut/+} HMECs, but not other cells of *BRCA1*-mutation carriers. Likewise, we also explored for the first time what might be the consequence of SIRT1 misregulation in HMECs in general.

Here we show that the decrease in SIRT1 levels was unique to *BRCA1*^{mut/+} HMECs, as other BRCA1-haploinsufficient cells exhibited levels similar to WT cells. Our data suggest that BRCA1 regulates SIRT1 levels in *BRCA1*^{mut/+} HMECs via post-

transcriptional mechanism, likely involved in SIRT1 stability. When SIRT1 expression is inhibited in HMECs, it leads to induction of premature senescence. Furthermore, this premature senescence phenotype is associated with accumulation of acetylated pRb leading to pRb mediated cell-cycle arrest, a feature consistent with premature senescence in *BRCA1*^{mut/+} HMECs. In addition, decrease in SIRT1 levels in *BRCA1*^{mut/+} HMECs was accompanied by low SIRT1 occupancy at telomeres. Since inhibition of SIRT1 expression in HMECs also leads to increased acetylation of global and telomeric histones, we speculate that SIRT1 decrease in *BRCA1*^{mut/+} HMECs results in loss of telomeric heterochromatin increasing telomere fragility and genomic instability. In sum, these findings imply that BRCA1-haploinsufficiency in HMECs, but not in other cell types examined, is associated with misregulation of SIRT1, leading to accumulation of Ac-H4K16 and Ac-pRb, and thereby resulting in telomere erosion, genomic instability, and pRb-dependent HIS.

In this study we also examined whether any of the *in vitro* characterized phenotypes of BRCA1-haploinsufficiency also existed in breast tissues from *BRCA1*-mutation carriers. Consistent with our findings in *BRCA1*^{mut/+} HMECs, we found shorter telomeres, decreased SIRT1 expression and increased activation of pathways involved in DDR and cell-cycle inhibition in breast epithelial cells from *BRCA1*-mutation carriers. Collectively, these findings suggest that breast epithelial cells in *BRCA1*-mutation carriers are poised for the rapid development of cancer due to misregulation of SIRT1, leading to telomere dysfunction, genomic instability, and pRb and p53 pathway activation.

To corroborate these data further it would be necessary to show causation between BRCA1 and regulation of SIRT1 stability. In order to examine turnover of SIRT1 in *BRCA1*^{mut/+} HMECs, we would treat these cells with MG132 to inhibit the

proteasomal protein degradation pathway. We would expect to see accumulation of SIRT1 levels in *BRCA1*^{mut/+} HMECs following MG132 treatment, which would indicate that decreased SIRT1 levels in *BRCA1*^{mut/+} HMECs are due to increased proteasomal degradation of this protein. In addition, to get a better appreciation of how SIRT1 deregulation is affecting chromatin remodeling in HMECs, it would be useful to examine the levels of acetylated SUV39H1 as well as trimethylated H3K9 and H4K20, all associated with heterochromatin formation at telomeres, in WT and *BRCA1*^{mut/+} HMECs as well as in shSIRT1 and shBRCA1 HMECs. We would expect to see decreased levels of acetylated SUV39H1 and trimethylated H3K9 and H4K20 at telomeres in all cells with decreased SIRT1 levels. This would be consistent with our observation that SIRT1 and BRCA1 inhibition leads to increased H4K16 acetylation. In addition, it would be interesting to determine if decreased SIRT1 levels alter gene expression profile in HMECs and which genes SIRT1 targets in these cells. For this it would be necessary to use a weaker shRNA constructs that inhibits SIRT1 expression (<50%) but do not induce rapid premature senescence. Another possibility is to try this in an immortalized cell line such as MCF10A where inhibition of SIRT1 expression might not have an effect on telomere stability.

Given these conclusions, a key question emerges: how might BRCA1 regulate SIRT1 stability in HMECs? A glance at what is known about regulation of SIRT1 does not provide any clear connections to BRCA1. It has been shown that SIRT1 activity and function can be regulated by post-translational modifications such as phosphorylation, methylation, SUMOylation, nitrosylation and through protein-protein interactions (reviewed in Revollo and Li, 2013). SIRT1 can be phosphorylated by cyclin B/cyclin-dependent kinase 1 (CDK1), C-Jun N-terminal kinase (JNK)1, Casein kinase (CK)2, prosurvival dual specificity tyrosine phosphorylation-regulated

kinases DYRK1A/DYRK3, and cyclic AMP/protein kinase A (cAMP/PKA) (Nasrin *et al.*, 2009; Guo *et al.*, 2010; Kang *et al.*, 2009; Gerhart-Hines *et al.*, 2011) . Phosphorylation of SIRT1 seems to be concomitant with stressful cellular conditions. It is thought that this modification, along with SUMOylation, promotes the nuclear localization of SIRT1 and enhances its affinity towards acetylated p53, acting as a prosurvival signal and protecting the cell from stress-induced apoptosis (reviewed in Revollo and Li, 2013). In addition, SIRT1 interacts with active regulator of SIRT1 (AROS) which enhances the activity of SIRT1 towards p53 (Kim *et al.*, 2007). In contrast to AROS, interaction with deleted in breast cancer 1 (DBC1) disrupts the conformation essential for SIRT1 activity thereby inhibiting affinity of SIRT1 towards p53, FOXO1 and FOXO3 (Kim, *et al.*, 2008). Taking in consideration that both BRCA1 and SIRT1 can be found at similar locations such as chromatin, telomeres, and DNA damage sites, it is possible that BRCA1 can regulate stability of SIRT1 by directly binding to SIRT1. Perhaps SIRT1 is a component of one of BRCA1 complexes. In order to address this SIRT1 immunoprecipitation (IP) would be performed followed by westernblot assessment of BRCA1 levels bound to SIRT1. It would be informative to examine colocalization of SIRT1 and BRCA1 in cells by immunofluorescence (IF) in HMECs. Colocalization of SIRT1 and BRCA1 could also be analyzed in presence or absence of DNA damage, since it was found that SIRT1 undergoes major redistribution along the chromatin upon DNA damage and that it associates with MRN complex as well as Rad51 (Oberdoerffer *et al.*, 2008). In addition, it would be interesting to examine SIRT1 and BRCA1 occupancy on chromatin as well as at telomeres. We would expect to see decreased interaction of BRCA1-SIRT1 in *BRCA1*^{+mut} HMECs.

CHAPTER 4.

DISCUSSION

4.1. In search of tumor suppressive function of BRCA1

BRCA1 has been of great interest to the scientific community ever since its discovery in the early 1990s in connection with families that have a high incidence of breast and ovarian cancers. Over the past 20 years we have made tremendous progress in understanding the function of BRCA1 gene products. We have uncovered the complex and ubiquitous role of BRCA1 in DNA damage repair, and additionally have uncovered BRCA1's involvement in chromatin organization, gene transcription, protein stability and cell-division (Zhang and Powell, 2005). We have learned that the versatility of BRCA1 comes from its ability to interact with a number of different proteins, to act as a scaffold for various complexes, and to regulate post-translational modifications of many binding partners including its own (Huen *et al.*, 2010; Clark *et al.*, 2012; Roy *et al.*, 2012). However, although the number of studies on BRCA1 is ever-increasing, one question still remains: which one of these functions is necessary for BRCA1 tumor suppression in a tissue-specific manner?

BRCA1 is defined as a chromatin-interacting E3 ubiquitin ligase that is involved in the homologous recombination type of DNA damage repair – a process deemed to be error-free compared to the alternative non-homologous end-joining (Clark *et al.*, 2012). Since all tumor cells with BRCA1- dysfunction display genomic instability associated with impaired DDR machinery, it is thought that this particular function of BRCA1 is critical for tumor suppression (Silver and Livingston, 2012). However, the issue with this idea is that genomic maintenance is essential for the viability of all cells, which implies that loss of BRCA1 function, and thereby genome stability, would lead to tumor formation in multiple tissues. Therefore, this hypothesis fails to address why cancers associated with germline mutation of BRCA1 preferentially develop in breast and ovarian cells and not in other tissues.

What then is so special about BRCA1 in breast and ovarian tissues? Addressing this problem over the past 20 years has proved to be very challenging. Several ideas have emerged as possible explanations for tissue specific tumor suppression such as: breast and ovarian epithelium specifically require BRCA1 for DDR while other epithelial cells can compensate using other pathways, or breast and ovarian cells might exhibit delay in apoptosis or a different rate of LOH compared to other tissues (Monteiro, 2003). However, none of these ideas have been tested yet. Furthermore, modeling human disease associated with BRCA1-haploinsufficiency in mice has been particularly difficult. While deletion of BRCA1 is embryonic lethal, BRCA1-heterozygous mice do not develop tumors spontaneously. Since this implies that there is considerable difference in the function of human and mouse BRCA1, doubts have been raised whether studying BRCA1 function in mouse mammary epithelium is even suitable (Drost and Jonkers, 2009; Dine and Deng, 2012).

Our recent findings that BRCA1-haploinsufficiency in breast epithelial cells results in incomplete differentiation leads us to postulate that in addition to DNA damage repair, BRCA1 must have other functions that are specific to breast and ovarian tissues. With this in mind, we designed this study to compare the effect of BRCA1-haploinsufficiency in breast and skin tissues from *BRCA1* WT and *BRCA1*^{+/-mut} healthy patients. For the purposes of this study, epithelial cells and fibroblasts from skin tissues were suitable counterparts since they are not associated with BRCA1 cancers and are easily obtainable after the reduction mammoplasties and prophylactic mastectomies. Thus, in order to address the tissue- and cell-type specificity of the BRCA1-haploinsufficiency phenotype, epithelial cells as well as fibroblasts were isolated from breast and skin tissues and cultured. With this unique

approach, our goal was to identify tissue- and cell-type specific function of BRCA1 that is associated with predisposition to breast cancer.

In this study we found that BRCA1-haploinsufficient epithelial cells, but not fibroblasts, undergo premature senescence which we named haploinsufficiency induced senescence or HIS (Figure 1). The finding that the epithelial cells suffer increased DNA damage and appear to be more sensitive to decreased levels of BRCA1 when compared to fibroblasts suggests that epithelial cells mostly rely on BRCA1-directed homologous recombination (HR) pathway to repair double strand breaks. Therefore, HIS probably exists as a response to genetic insults that accumulate in epithelial cells due to inefficient DNA damage repair. It is possible that fibroblasts do not undergo HIS because they are able to compensate for weakened HR pathway by utilizing other DNA damage repair mechanisms such as non-homologous end joining. This finding is consistent with other studies reporting differences in DDR between epithelial cells and fibroblasts (D'Errico *et al.*, 2007). Furthermore, the difference between epithelium and fibroblasts with regards to their response to BRCA1-haploinsufficiency is interesting because it is consistent with the observation that germline mutations in BRCA1 do not predispose to soft tissue sarcomas, but rather carcinomas.

Epithelial cells undergoing HIS displayed typical phenotypes of senescence such as: morphological changes, SA- β -galactosidase positivity, increased SASF expression, and cell cycle arrest mediated by active pRb signaling pathway. Surprisingly, given the well-established role of p53 in mediating growth arrest in response to BRCA1 loss, HIS in epithelial cells did not correlate with increased p53 signaling pathway. Furthermore, as the name of this senescence alludes, HIS did not associate with loss of BRCA1 heterozygosity. More importantly, we identified that

the molecular mechanism inducing HIS differed between breast and skin epithelial cells, providing the first molecular evidence of the tissue and cell-type specific difference in BRCA1 function. HIS in HMECs was manifested by decreased SIRT1 levels which correlated with increased telomere erosion, whereas HIS in HDEs was not associated with this phenotype. Furthermore, we also found that SIRT1 was less abundant in telomeric regions of chromatin in BRCA1-haploinsufficient HMECs. These data reveal that BRCA1 is involved regulation of SIRT1 levels as well as maintenance of telomere stability specifically in HMECs and further suggest that BRCA1 plays an important role in chromatin remodeling in HMECs. Given these findings, we hypothesized that decreased SIRT1 levels lead to telomere fragility and increased genomic instability associated with telomere-end fusions, ultimately resulting in cell-cycle arrest. Indeed, when we tested the consequences of inhibiting SIRT1 in HMECs, we found that it induced an increase in histone acetylation (specifically, H4K16) globally as well as at telomeres. Loss of telomeric heterochromatin is associated with telomere attrition and increased telomere dysfunction leading to rapid genomic instability (Palacios *et al.*, 2010), which were some of the phenotypes found in *BRCA1*^{+/-mut} HMECs. In addition, SIRT1 inhibition in HMECs also induced premature senescence and cell-cycle arrest due to increased levels of pRb acetylation, which is in agreement with our finding that active pRb signaling mediated HIS in *BRCA1*^{+/-mut} HMECs. Taken together, we have identified an important role of BRCA1-SIRT1 interaction in telomere heterochromatin maintenance in HMECs. Furthermore, we showed that this interaction is weakened in BRCA1-haploinsufficient state, which may contribute to greater likelihood of transformation in these cells (Figure 4.1).

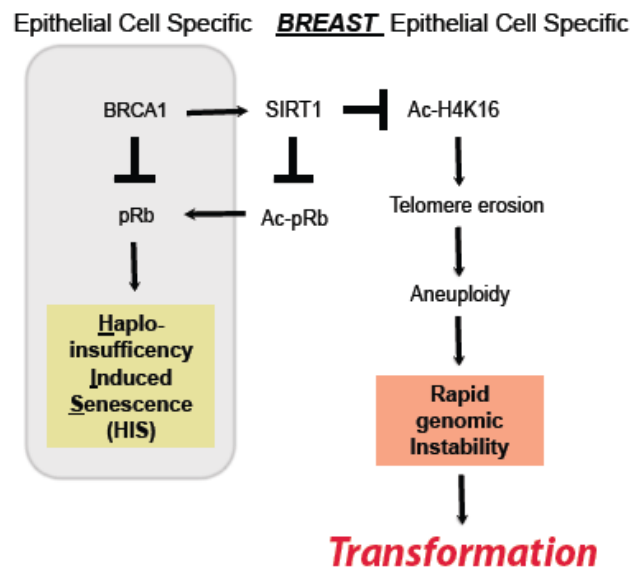


Figure 4.1. Model of tissue and cell type-specific response to BRCA1-haploinsufficiency. WT BRCA1 in epithelial cells represses HIS, which is mediated by active pRb signaling pathway. In breast epithelial cells BRCA1 regulates SIRT1 which: 1) inhibits the activation pRb pathway and thereby HIS, and 2) inhibits the increase in histone acetylation that may promote telomere fragility and rapid genomic instability leading to increased propensity to undergo neoplastic transformation.

In this study we show that HIS in HMECs displays some general characteristics of senescence, such as pRb mediated cell-cycle arrest, that are in common with other forms of senescence (M1, M0, OIS, TSLIS) (Table 4.1). HIS appears to be most similar to M1, because both senescence stages display decreased levels of SIRT1, telomere attrition, telomere attrition-associated DDR and SASFs (d'Adda di Fagagna *et al.*, 2003, Rodier *et al.*, 2009). However, p53 signaling pathway activation and SAHFs formation are features of M1 that are not found in HIS. In comparison to OIS, HIS is similar with respect to increased levels of DNA damage in cells and SASFs secretion (Nakamura *et al.*, 2008). However, in contrast to OIS, increased DDR in HIS did not associate with engagement of p53 signaling pathway. Furthermore, increased DDR is the only other feature shared between TSLIS and HIS, since HIS did not correlate with active p53 signaling pathway, SAHFs formation or loss of BRCA1 heterozygosity (Berger *et al.*, 2011, Kuilman *et*

al., 2010, Young *et al.*, 2008). Finally, decrease in SIRT1 levels is the only other phenotype shared by HIS and M0, since HIS was not induced in response to stress in culture and was not associated with activation of p53 pathway (Dickson *et al.*, 2000). Therefore, in comparison to other forms of senescence we found that HIS in HMECs is mechanistically distinct proliferative barrier and therefore is a novel type of senescence.

Table 4.1. Phenotypes shared between HIS and other forms of senescence.

Phenotype	M1	M0	OIS	TSLIS
p16	no	no	no	no
p53	no	no	no	no
DDR	yes		yes	yes
telomere attrition	yes			
pRb	yes	yes	yes	yes
stress in culture		no		
p38			?	
SASFs	yes		yes	
SAHFs	no		no	no
TSL				no
SIRT1	yes	yes		

Fields marked with “+” represent similarities, while fields marked with “-” represent differences with between HIS and the other types of premature senescence. Fields marked “?” have not been assessed. Fields that are in gray either have not been assessed or have not been shown to correlate with specific type of senescence.

This study has revealed an important role for pRb in suppression of cellular replication and neoplastic transformation in BRCA1 haploinsufficient cells, which is further supported by the high incidence of *RBI* loss or mutations in human breast cancers with inactivated BRCA1 (Hu *et al.*, 2009; Stefansson *et al.*, 2011; Jonsson *et al.*, 2012). Our findings indicating that *BRCA1*^{mut/+} HMECs exhibit even greater

genomic instability and telomeric fusions following forced proliferation beyond HIS suggests that a second non-proliferative barrier is likely triggered in response to excessive DNA damage response and must be overcome for neoplastic transformation. Indeed, the increased genomic instability and elevated p53-dependent responses following pRb inhibition in tissues from *BRCA1*-mutation carriers is consistent with the role p53 likely plays upon loss of pRb during cancer progression. This notion is further supported by the observation that p53 is also frequently mutated or lost in *BRCA1*-associated breast cancers (Konishi, *et al.*, 2011; Jonsson *et al.*, 2012) and in *BRCA1*-deficient murine cells that overcome senescence (Cao, *et al.*, 2003). Senescence is not a foolproof mechanism to prevent neoplastic transformation, as it has been shown to be bypassed following loss of p53 and pRb (Shay and Wright, 2005). Thus, these and other functionally related mutational events or hTERT re-expression are likely to overcome or bypass HIS leading to rapid neoplastic transformation.

Our finding that *BRCA1*-haploinsufficiency, but not deficiency, leads to ways to overcome HIS in HMECs is in support of the idea that LOH might not be an obligatory first step in *BRCA1*-associated tumor progression and that inactivation of cell-cycle checkpoints might precede an LOH event. This presumably would help cells survive *BRCA1* LOH. In support of this is observation in a recent study of genetically engineered single-allelic *BRCA1* mutation in spontaneously immortalized mammary epithelial cell lines where it was reported that *BRCA1*-null cells could not be generated. The authors speculated that those cells cannot survive in the presence of intact cell-cycle checkpoints (Konishi *et al.*, 2011). Furthermore, analysis of *BRCA1* tumors revealed that PTEN and p53 mutations preceded *BRCA1* LOH, and that LOH is frequently heterogeneous (Martins *et al.*, 2012). This implies that *BRCA1* LOH is

dependent on abrogation of other tumor suppressive networks and that wildtype *BRCA1* allele still seems to be preserved in many tumor cells. Collectively, this suggests that partial loss of *BRCA1* function is more tumorigenic for HMECs than a complete loss of this tumor suppressor gene. This evokes a concept termed “obligatory haploinsufficiency” that has been recently coined for other tumor suppressor genes such as *PTEN* that do not seem to behave according to Knudson’s “two hit” hypothesis (Berger *et al.*, 2011). Knudson’s two hit paradigm is exemplified by *pRb* where loss of two alleles of this tumor suppressor gene leads to cancer. Obligatory haploinsufficiency, on the other hand, refers to phenomenon where tumorigenesis is more likely to occur due to decreased levels of tumor suppressor gene because complete loss of this gene leads to activation of a fail-safe mechanism within the cell. Thus, we think that *BRCA1* also belongs to this group of tumor suppressor genes.

Tissue-specific tumor suppression is not only limited to *BRCA1*. Loss of other tumor suppressor genes such as *pRb* and *APC* (adenomatous polyposis coli) also display tissue preference in tumor spectrum (Hu, 2009). While inactivation of *pRb* in the germline mainly predisposes to retinoblastoma and osteosarcoma, mutations in *APC* are associated with colorectal cancer. Studies have shown that *pRb* promotes cell-cycle exit and terminal differentiation in retinal precursor cells as well as osteoprogenitors (Chen *et al.*, 2004; Gutierrez *et al.*, 2008). In addition, it has been shown that *APC* is important for differentiation and removal of intestinal cells (Cordero *et al.*, 2009). Thus, inactivation of these genes increases a pool of multipotent cells that may be susceptible to additional transforming events leading to cancer. These studies suggest that *pRb* and *APC* are not simply controlling the proliferation and/or death rate of cells but are also relevant for pathways involved in

proper development and differentiation of these tissues. Therefore, it appears that tissue-specific tumor suppression is a complex process.

Recently, we reported that haploinsufficiency for BRCA1 in disease-free breast epithelial cells from *BRCA1*-mutation carrier tissues also results in altered phenotypes and the misexpression of several genes involved in the establishment and/or maintenance of chromatin structure and concomitant defects in proper differentiation programs (Proia *et al.*, 2011). Consistent with these findings, haploinsufficiency for BRCA1 in breast epithelial cells results in altered epigenetic histone modifications both globally and at telomeres. These findings suggest that mutation in a single copy of *BRCA1* is sufficient to induce a mutator phenotype driven by genetic and epigenetic events activating a novel form of senescence, which can be bypassed either through reactivation of hTERT or loss of pRb. Since mutation or loss of p53 and pRb pathways are obligate events in the pathogenesis of *BRCA1*-associated breast cancers, this previously unrecognized function of BRCA1 haploinsufficiency offers insights into the evolution of cancer in a tissue-specific manner associated with *BRCA1*-mutation carriers. Indeed our studies show that of the cell and tissue types examined here, it is only in HMECs that BRCA1 haploinsufficiency leads to both pRb-dependent senescence and the means to overcome it through changes in telomere stability induced by loss of SIRT1. Future studies evaluating whether HIS is observed in the context of other tumor suppressor genes and whether it is associated with cell-type specific predisposition to cancer will be needed. This will help broaden the knowledge about tumor suppressor genes beyond their generalized division into the “gatekeepers” and “caretakers” as well as improve our understanding of the requirements for neoplastic transformation in a tissue and cell-type specific manner.

4.2. Future directions

Observations in this study generated ideas and questions that would be interesting to pursue and explore further:

1. What are the underlying reasons for other types of chromosomal abnormalities in *BRCA1*^{mut/+} HMECs?

Cytogenetic analysis revealed that, in addition to increased telomeric-end fusions, proliferating *BRCA1*^{mut/+} HMECs suffered frequent gain or loss of chromosomes. A large number of aneuploid cells in cultures of *BRCA1*^{mut/+} HMECs indicates that these cells might exhibit mitotic checkpoint abnormalities. Indeed, several reports implicate BRCA1 in control of spindle-assembly checkpoint (Wang et al, 2004; Chabalier *et al.* 2006). It has been shown that loss of BRCA1 or BRCA1 downregulation in mouse and human cell-lines resulted in decreased expression of several key spindle checkpoint components: Mad2 and BubR1 (Wang *et al.*, 2004; Chabalier *et al.*, 2006). Both of these proteins are potent inhibitors of APC/C (anaphase promoting complex/cyclosome) activity, which is required for ubiquitination and degradation of cyclin B and the subsequent inactivation of Cdk1. Thus, inhibition of BRCA1 in these cell lines leads to an increased proportion of premature sister-chromatids separations after spindle damage (induced by either nocodazole or paclitaxel) (Wang *et al.*, 2004; Chabalier *et al.*, 2006). Given these data, it would be interesting to test whether abnormalities in mitotic checkpoint in BRCA1-haploinsufficient HMECs are also due to decreased levels of Mad2 and BubR1 and increased activity of APC/C. Also, it would be worthwhile to determine if these cells are resistant to nocodazole and paclitaxel treatment. In addition, these

phenotypes should be examined in other BRCA1-haploinsufficient cells. If abnormalities with mitotic checkpoint are specific to HMECs then that would further provide molecular evidence of a tissue and cell-type specific difference in BRCA1 function.

2. Is HIS occurring in other BRCA1-haploinsufficient epithelial cells?

In this study we have examined the phenotypes associated with BRCA1-haploinsufficiency in epithelial cells and fibroblasts from breast and skin tissues. Future studies should examine if HIS occurs in epithelial cells from ovarian tissue, as well. Also, future studies should address the mechanism that leads to HIS in these epithelial cells. A recent report has shown that ovarian epithelial cells from *BRCA1*-mutation carriers also accumulate DNA double-strand breaks that lead to premature ovarian aging (Titus *et al.*, 2013). Therefore, it would be important to determine whether SIRT1-dependent telomere erosion, genomic instability and HIS are also features of BRCA1 haploinsufficient ovarian epithelial cells. These studies will strengthen the association of BRCA1-SIRT1 function in telomere stability and HIS with the cell-type specific predisposition to cancer.

3. Would p53 and pRb inactivation immortalize *BRCA1*^{+/-mut} HMECs?

Previously it was demonstrated that p53 inhibition in WT HMECs results in proliferation beyond M2, but these cells ultimately encountered crisis and massive apoptosis due to DNA damage and telomere dysfunction (Garbe *et al.*, 2007). On very

rare occasions it has been observed that WT HMECs can spontaneously immortalize after M2. Here we observed that HIS in *BRCA1*^{+/-mut} HMECs is mainly mediated through pRb activation. Furthermore, we showed pRb knockdown in *BRCA1*^{+/-mut} HMECs resulted in extended proliferative capacity and further increase in genomic instability. However, these cells eventually encountered growth arrest associated with robust activation of p53 signaling pathway. It would be interesting to examine whether *BRCA1*^{+/-mut} HMECs would undergo growth arrest or spontaneous immortalization if both p53 and pRb pathways were inactivated. Given the extent of genomic instability and mutations that could spontaneously occur in these cells in the absence of p53 and pRb, it is possible that these cells would immortalize (Endo et al., 1990, Shay et al, 1991).

4. Would *BRCA1*^{+/-mut} HMEC require fewer additional events for neoplastic transformation than *BRCA1* WT cells?

Previous studies have shown that genetic and biochemical alterations required for transformation of primary HMECs to malignant cells include expression of SV40 large T antigen, small t antigen, the telomerase catalytic subunit and oncogenic H-*ras* (Hahn *et al.*, 1999; Elenbaas *et al.*, 2000, Zhao *et al.*, 2003). Since *BRCA1*^{+/-mut} HMECs exhibit increased genomic instability, we hypothesize that fewer genetic and biochemical alterations would be necessary to transform these cells in comparison to WT HMECs. We propose to start by testing whether expression of SV40 early region would lead to rapid transformation of primary *BRCA1*^{+/-mut} HMEC, since this combination alone does not result in transformation of WT HMECs in our hands

using HIM (human in mouse) model. If introduction of SV40 early region is not sufficient to transform *BRCA1*^{+/-mut} HMEC, then the experiment would be repeated with one or two additional combinations of transforming oncogenes such as oncogenic *H-ras*.

5. Would *Terc*^{-/-} background promote tumorigenesis in *BRCA1*^{+/-} mice?

Studying BRCA1 function in tissue-specific tumor suppression in mice has been challenging. *BRCA1*^{+/-} mice do not develop spontaneous mammary and/or ovarian tumors. Furthermore, deletion of BRCA1 full-length isoform in mice induces premature senescence as early as embryonic stage. Also, conditional deletion of BRCA1 in mouse mammary epithelial cells does not result in accelerated tumor formation. Rather, mammary tumors form at a low frequency and late in life in these mice. Only on the background of additional genetic mutations such as p53 heterozygosity is mammary tumorigenesis observed (Drost and Jonkers, 2009; Dine and Deng, 2012). Since our studies have revealed that BRCA1 might be playing an important role in telomere stability in human cells, we hypothesize that the challenges of recapitulating features of human *BRCA1* mutation in mice are likely due to the important differences in telomere biology between mice and humans. Unlike human cells, mouse cells have longer telomeres and have endogenously active telomerase. In order to evaluate the role of telomerase in mammalian development, cell growth and tumor formation a line of mice was developed with germline deletion of the gene encoding the telomerase RNA component *Terc*. In late generations (5 to 6) these mice exhibit some of the phenotypes associated with aging in humans such as shortened telomeres, gray hair, decreased life-span, and increased incidence of spontaneous

malignancies (Blasco *et al.*, 1997). Therefore, we would like to test the idea that *BRCA1*^{+/-} mice crossed on to the *Terc*-null background for several generations might become prone to tumor formation well before *Terc*^{-/-} mice. In order to test this, Ben Dake is currently breeding *BRCA1*^{+/-} mice with *Terc*^{-/-} mice. Beginning with third generation of this cross, tumor formation and survival of *BRCA1*^{+/-}, *Terc*^{-/-}, and *BRCA1*^{+/-}*Terc*^{-/-} mice will be monitored. Mammary tumors that form will be collected and analyzed for features found in human *BRCA1*-associated breast cancers such as expression of hormone receptors and basal cytokeratins, proliferation, BRCA1 LOH, p53 mutations, and genomic instability.

4.3. Conclusion

In conclusion, we compared epithelial cells and fibroblasts from breast and skin tissues of WT and *BRCA1*^{mut/+} patients to address the issue of BRCA1's tissue-specific tumor suppressive function. We have identified a novel type of senescence (HIS) that is specific to BRCA1-haploinsufficient epithelial cells. Furthermore, we found that the molecular mechanism involved in induction of HIS differed between breast and skin epithelial cell, which provided the first evidence of the tissue and cell type specific difference in BRCA1 function. Collectively, these data provide a framework for future studies of BRCA1 tumor suppressive function in a cell- and tissue- specific manner.

CHAPTER 5.

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CHAPTER 6.

APPENDIX