Loss of BRCA1 in Normal Human Mammary Epithelial Cells Induces a Novel Mechanism of Senescence

by

Noor Herah Salman

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Department of Medical Biophysics
University of Toronto

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Noor H. Salman
Masters of Science
Department of Medical Biophysics
University of Toronto
2011

Abstract

Early events in BRCA1-associated tumorigenesis remain poorly understood. To understand the immediate consequences of BRCA1 loss of function, we modeled BRCA1 loss of function \textit{in vitro} using normal primary human mammary epithelial cells (HMEC). We have found that in HMEC, loss of BRCA1 results in a novel type of senescence. Loss of BRCA1-induced senescence is not associated with DNA damage or p53 upregulation. We find that p53 protein levels are down regulated due to proteasome-mediated degradation. Although p53 levels are down regulated, we find that BRCA1 loss induced expression of a number of p53-dependent anti-oxidant genes. In particular we uncovered that SESN2, a p53 downstream target gene, inhibits loss of BRCA1 induced ROS and activates autophagy. In contrast to human fibroblasts, we found that loss of BRCA1 induced senescence is p53 independent, and can occur in the absence of ROS upregulation and autophagy induction.
Acknowledgments

As of today, September 30th 2011, my research graduate training has taken full 3 years and 1 month to complete. The phase of compilation and writing of this thesis was unexpectedly difficult. The hardship was because not the concepts that I had studied were hard to describe, instead it was due to looking back and analyzing all the mistakes that I had made during my research training. The only reason I was able to pull through mistake after mistake was the support of the people around me. Amidst my mistakes studded data were a few successes detailed in this thesis, which would have never been possible without the guidance and support of my colleagues.

First and foremost, I must thank my supervisor, Dr. Mona Gauthier for providing unconditional support throughout my graduate term. If I were to list all the things for which I am to acknowledge Mona, there would be no room left for this thesis to be written. Mona not only gave me the opportunity to work in her lab but also to work on, what I think to be, the best project in the lab. As a mentor in life science, her efforts have been exemplary. From her I received supervision, guidance, and on one too many occasions rigorous bashings in matters of both life and science. What Mona has done is carved my mind in a manner no one else could have and shaped the person that I now am. For this I can never be grateful enough.

I am very fortunate to have more than one mentor during my training. This thesis has been in large part with Jennifer Cruickshank, a collaborator/mentor. In many ways, I have come to see Jen as a role model. I have benefitted from Jen’s experience and knowledge in many ways. Throughout the past three years, whenever I had any questions during bench work, in preparation of presentations, and even in the writing of this thesis, I could always interrupt Jen. To help me, she has always put her work on hold and given all attention to me. This thesis tells an interesting story because I got a lot of help from Jen. I have become a big fan of Jen forever.

My fellow graduate student, Rania Chehade, has also contributed important pieces of data that in this thesis. Rania and I have worked together, literally side by side for almost years. I have thoroughly enjoyed her company. I have savored her excitement about science and also the food she would so frequently shared. Rania’s presence made my doing science lively. Bernie Martin, our lab manager, has also assisted me from the first day that I joined. From Bernie I
learned the fundamentals of tissue culture and many other things in the lab. Members of our collaborating lab, Sophia George, Sanjeev Shaw, and graduate student Anca Milea, have helped with trouble shooting and scientific discussions on numerous occasions. Their presence in our lab meetings and in our lab has flavored and enriched my experience. I have benefitted from them in many ways. Brenda Cohen, in our neighboring lab, helped pretty much ‘stand on my feet’ when I first started. I would frequently go to her with questions, reagents, and help with trouble shooting. Both of her students and my class mates, Julia Izrailit and Pavel Goldvasser, have provided good advice whenever needed and great company throughout. I have loved working with our present undergrad student Nymisha Chilukuri and our past summer students, Dan Myran and Lesley Leung. I got the privilege to spend the most time with Nymisha, and it has been a great pleasure. To all of these people I owe special thanks for sharing my joys and making my gloomy days bright.

My committee members, Dr. Senthil Muthuswamy, Dr. Linda Penn, and Dr. Nadeem Mughol, have always constructively critiqued during my committee meeting, which has been helpful. Dr. Hal Berman, our collaborator, has been my unofficial mentor. He has always had an open door policy about going to him for help on matters ranging from PCR problems, life related resolutions and most importantly career counseling. Help in these areas is not always available to all graduate students. I have been very fortunate to have Hal help during my training.

These past three years have been unpredictable ride. As I leave graduate school and go on to a still unknown next step in life, more than anything else, I will always remember the people who stood by me during my hard and happy days in the lab. Of course, my family has taken the lead in allowing me the time to do this thesis. In particular, my parents supported me during the first half of school and my husband, Muhammad, during the second half of it. I am blessed to have family and friends like mine.
Statement of Contribution

Figure 2. Isolation of all HMECs from mammary tissue was done by Jennifer Cruickshank and Bernard Martin.

Figure 3A. Propagation and infection of cells and rtPCR of BRCA1 was done by Jennifer Cruickshank.

Figure 4A, B, D. Propagation and infection of cells with control and shBRCA1 was done with Dr. Gauthier. The growth curve, cell cycle and cell death analysis was also done with Dr. Gauthier.

Figure 5B, C. β-galactosidase staining and quantification was done with Dr. Gauthier.

Figure 6. Propagation, infection of cells with control and shBRCA1, and β-galactosidase was done with Dr. Gauthier.

Figure 7B. Cell cycle blots were done with Dr. Gauthier.

Figure 8 B, C, D, E. rtPCR of p53 was done by Jennifer Cruickshank. Cell culture, infection, and β-galactosidase and IF were done with Dr. Gauthier.

Figure 9B. Cell culture and Western blot were done with Dr. Gauthier.

Figure 12 A, B. Cell culture and Western blots were done with Dr. Gauthier. Comet assay was done by Jennifer Cruickshank.

Figure 13. ROS analysis was done Jennifer Cruickshank.

Figure 14 B, C. ROS analysis was done by Jennifer Cruickshank and trypan blue analysis was done with Dr. Gauthier.

Figure 16C. ROS analysis was done by Rania Chehade.
Figure 17 C, D. Beclin1 rtPCR was done by Rania Chehade and microarray analysis was done by Nymisha Chilukuri.

Figure 20 A, C. Beclin1 rtPCR and ROS analysis was done by Rania Chehade.

Figure 21. Cell culture, Western blots, growth curves and β-galactosidase assays were done with Dr. Gauthier.

Figure 22. Cell culture, Western blots, and growth curves were done with Dr. Gauthier.

Figure 23 B, C, D. Cell culture, infection, β-galactosidase staining, and ROS analysis was done by Jennifer Cruickshank.

Figure 24. Cell culture, infection, and β-galactosidase assays were done by Rania Chehade.

Table 8. Microarray analysis was done by Dr. Gauthier.
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Loss of BRCA1 results in senescence

Loss of BRCA1 induced senescence is unique to normal epithelial cells

HMECs display unique senescence-associated phenotypes

Senescence is associated with p53 down regulation

Loss of BRCA1 induces p53 down regulation

p53 down regulation correlates with cytoplasmic shuttling

p53 down regulation occurs through a proteasome mediated degradation

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## Abbreviations

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<tr>
<td>AMPK</td>
<td>protein kinase, AMP-activated, beta 1 non-catalytic subunit</td>
</tr>
<tr>
<td>ARHGAP5</td>
<td>Rho GTPase activating protein 5</td>
</tr>
<tr>
<td>ATG13</td>
<td>ATG autophagy related 13 homolog</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
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<tr>
<td>ATR</td>
<td>Ataxia Telangiectasia and Rad3 related</td>
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<tr>
<td>BECN1</td>
<td>Beclin 1, autophagy related</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-raf murine sarcoma viral oncogene homolog B1</td>
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<td>BRCA1</td>
<td>Breast Cancer 1, early onset</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast Cancer 2, early onset</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CALD1</td>
<td>Caldesmon 1</td>
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<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
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<td>CHK1</td>
<td>CHK1 Checkpoint homolog</td>
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<td>CHK2</td>
<td>CHK2 Checkpoint homolog</td>
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<td>CK17</td>
<td>Keratin 17</td>
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<td>CK18</td>
<td>Keratin 18</td>
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<td>CK5/6</td>
<td>Keratin 5/6</td>
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<td>cKIT</td>
<td>v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog</td>
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<td>COL4A1</td>
<td>Collagen, type IV, alpha 1</td>
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<td>CRM1</td>
<td>Exportin 1 (CRM1 homolog, yeast)</td>
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<td>CXCR2</td>
<td>Chemokine (C-X-C motif) receptor 2</td>
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<td>DCF</td>
<td>Dichlorofluorescein</td>
</tr>
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<td>DCFDA</td>
<td>Dichlorodihydrofluorescein Diacetate</td>
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<td>DCIS</td>
<td>Ductal Carcinoma In Situ</td>
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<td>DDR</td>
<td>DNA Damage Response</td>
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<tr>
<td>DI</td>
<td>Deionized</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DRAM</td>
<td>DNA-damage Regulated Autophagy Modulator 1</td>
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<td>E2F</td>
<td>E2F transcription factor</td>
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<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>ENC1</td>
<td>Ectodermal-Neural Cortex 1</td>
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<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
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<td>Abbreviation</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>GPX3</td>
<td>Glutathione Peroxidase 3</td>
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<td>GSE22</td>
<td>Genetic Suppressor Element 22</td>
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<td>HBOC</td>
<td>Hereditary Breast and Ovarian Cancer</td>
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<tr>
<td>HER2</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 2</td>
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<td>HMEC</td>
<td>Human Mammary Epithelial Cells</td>
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<td>HMOX1</td>
<td>Heme Oxygenase (decycling) 1</td>
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<td>hRASV12</td>
<td>v-Ha-ras Harvey rat sarcoma viral oncogene homolog</td>
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<tr>
<td>hTERT</td>
<td>human Telomerase Reverse Transcriptase</td>
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<td>IDC</td>
<td>Invasive Ductal Carcinoma</td>
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<td>IF</td>
<td>ImmunoFlorescence</td>
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<td>IGFB1</td>
<td>Insulin-like Growth Factor Binding protein 1</td>
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<td>IL8</td>
<td>Interleukin 8</td>
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<td>INFγ</td>
<td>Interferon Gamma</td>
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<td>iPSC</td>
<td>induced Pluripotent Stem Cells</td>
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<td>KD</td>
<td>Knock Down</td>
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<td>LC3</td>
<td>microtubule-associated protein 1 Light Chain 3</td>
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<td>LFS</td>
<td>Li-Fraumeni Syndrome</td>
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<td>LOH</td>
<td>Loss Of Heterozygosity</td>
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<td>MEF</td>
<td>Mouse Embryonic Fibroblasts</td>
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<td>MEGM</td>
<td>Mammary Epithelial Cell Medium</td>
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<td>MKI67</td>
<td>antigen identified by monoclonal antibody Ki-67</td>
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<td>MRN</td>
<td>Mre11, Rad50 and Nbs1</td>
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<td>mTOR</td>
<td>mammalian Target Of Rapamycin</td>
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<td>MYC</td>
<td>v-myc myelocytomatosis viral oncogene homolog (avian)</td>
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<td>NMF</td>
<td>Normal Mammary Fibroblasts</td>
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<td>NQO1</td>
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<td>NRF2</td>
<td>Nuclear Factor (erythroid-derived 2)-like 2</td>
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<td>OCT4</td>
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<td>OIS</td>
<td>Oncogene Induced Senescence</td>
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<td>p16</td>
<td>cyclin-dependent kinase inhibitor 2A</td>
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<td>cyclin-dependent kinase inhibitor 1A</td>
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<td>p53</td>
<td>Tumor Protein p53</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCR</td>
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<td>Retinoblastoma</td>
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<td>Reduction Mammaplasty</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>Replicative Senescence</td>
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<td>reverse transcriptase Polymerase Chain Reaction</td>
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<td>SASP</td>
<td>Senescence Associated Secretory Phenotype</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>Sestrin 2</td>
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<td>sh</td>
<td>short hairpin</td>
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<td>short hairpin Luciferase</td>
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Chapter 1: Introduction

Breast cancer

With 1 in 9 women expected to develop breast cancer and 1 in 29 to die of it, breast cancer is the most common cancer occurrence in Canadian women (after non-melanoma skin cancer) and the second most common cause of cancer death. This year alone (2011) in Canada, an estimated 23,400 women will be diagnosed with breast cancer and 5,100 women will not survive it [1]. Although the mortality rate of breast cancer has gone down over the past 40 years, a large number of women, when diagnosed at late stage, are still detrimentally affected by the disease [2].

The most unique aspect of breast cancer biology is that both molecular and morphological tumor heterogeneity is appreciated. Within the disease, there are many types that are morphologically distinct, of which ductal carcinoma is the most prevalent; [3]. Within this particular type, there are many distinctly classified molecular sub-types [4]. Upon a breakdown of the mortality rates stratified by each sub-type of breast cancer, it was found that the rates are skewed towards a few sub-types [5]. This feature not only provides the sub-type classification prognostic power, but also raises the question of how this disease arises in its multiple forms. Despite great advances in understanding breast cancer and its management, the series of early events that occur in a cell to give rise to any specific type of breast cancer remains unknown.

Sporadic breast cancer

Breast cancer predominantly occurs sporadically, i.e. randomly in a population. Although there are a few risk factors associated with the disease, the causal mechanistic relationship of these risk factors with the disease is actively being studied. Like most other cancers, breast cancer development is a complex, multi-step process. Over the past several years, a number of studies have focussed on understanding and characterizing invasive breast cancer.
Molecular Sub-types of Invasive Ductal Carcinoma:

The use of whole genome expression array experiments and hierarchical clustering of breast tumor samples has revealed that invasive ductal carcinoma (IDC) can be subdivided into at least 4 distinct molecular sub-types. Tumors of each sub-type cluster together and have a gene expression signature (of several thousand genes), which distinguishes them from tumors of other sub-types [4, 6-9]. The distinguishing features of IDC sub-types are the expression of differentiation genes, oncogenes, and proliferation genes. An important benefit of this classification is that it has prognostic significance [5, 9, 10], making it clinically applicable (Fig.1).

**ER positive sub-types: Luminal A and Luminal B tumors**

The sub-type of IDC with the longest disease free survival and best prognosis is Luminal A and second to it is Luminal B. They are called luminal because the tumor cells from these sub-types show markers of differentiated luminal cells of the normal breast. For example, both sub-types express the estrogen receptor (ER) and are said to be ER positive. However, they are distinguished by expression of the progesterone receptor (PR) and by expression of a number of cell cycle regulatory proteins [11, 12]. Luminal A (but not Luminal B) shows expression of progesterone receptor, while the majority of Luminal B tumors are devoid of PR expression [13, 14]. Luminal B tumors are highly proliferative and show over expression of proliferation-associated genes such as MKI67 (Ki67) [12].

**ER negative sub-types: HER2 positive and basal-like tumors**

The two sub-types associated with poor prognosis are Her2 expressing tumors and tumors that show expression of basal epithelial makers and are hence, referred to as basal-like tumors. Neither of these sub-types expresses ER and are said to be ER negative. The Her2 sub-type is named as such because these tumors have amplification of the HER2 gene [15]. In the past decade, the use of Herceptin, a monoclonal antibody developed to inhibit HER2 function, has significantly increased the disease free survival of these patients [16, 17].

Basal-like tumors have unique characteristics. About 86% of ‘basal’ tumors present with a histological grade of 3, meaning that the tumor tissue has very little similarity with the normal
tissue from which it originates and hence, displays a lack of differentiation [18]. They also show evidence of necrosis and lymphocytic infiltrate [18]. Immunohistochemical, these tumors stain negative for ER, PR, and HER2, and express normal basal cell cytokeratins such as CK 5/6 and 17 [19]. Roughly, half of basal tumors stain positive for EGFR and a third for c-KIT expression [19].

A key feature of basal-like tumors is the high frequency of point mutations in p53. About 30% of breast cancers have p53 mutations and the majority of these are found in basal-like tumors. Over 90% of basal-like tumors acquire p53 mutations compared to 13% of Luminal A [5, 20]. Sequencing of the p53 gene has revealed that most basal-like tumors carry missense p53 mutations, which when detected by IHC show an absence of p53 protein accumulation [20, 21]. The explanation for the high rate of these mutations in basal-like tumors is so far unresolved [20]. It is speculated that loss of p53 function might be an early event in breast cancer. One reason supporting this evidence is that people who inherit germ line mutations in p53, predominantly develop breast cancer [22]. Since p53 mutations occur so frequently in basal-like tumors, it is thought that p53 loss may be necessary for these tumors to develop. The loss of p53 function correlates with highly proliferative tumors which are initially chemosensitive; [23-25] however, patients commonly develop disease recurrence [24]. Several long term studies have shown that women with basal-like breast cancer have a higher recurrence rate and lower overall survival than women with other sub-types of breast cancer [23, 26].

Studies have been done to investigate if there is a precursor state of invasive basal-like tumors. One such report demonstrated that a large percentage of patients with basal-like breast cancer also show the presence of Ductal Carcinoma In Situ (DCIS) [27]. DCIS is a non-invasive lesion, in which pre-malignant cells remain contained within the basement membrane. When immunostaining was done on regions of DCIS in samples that had invasive basal cancer, it revealed the same immunophenotype of basal-like tumors. The DCIS regions were negative for ER, PR, and HER2 expression, and positive for expression of CK 5/6, 17, and EGFR [27]. Another study found that 6% of DCIS cases show features of basal-like IDC, including triple
Figure 1: Sub-types of invasive breast cancer. Kaplan-Meier analysis of time to development of distant metastasis stratified according breast cancer sub-type [9]. Data and patient cohort from van’t Veer et. al. [10]
negativity and positive staining for basal cytokeratins [28]. In an elaborate study, Gauthier et. al. demonstrated that women with DCIS lesions expressing hallmarks of basal-like tumors and high levels of p16 and Ki67, were at increased risk of developing subsequent invasive tumors of the basal-like sub-type [29, 30].

How basal-like tumors originate remains debatable. There is data showing a molecular resemblance between basal-like tumors and normal embryonic stem cells. Genes that are up-regulated in stem cells and those that drive induced pluripotency, such as Oct4 and Sox2, show an increased expression by microarray analysis in basal-like tumors [31]. Whether basal-like tumors arise from de-differentiation of fully differentiated cells (by a mechanism similar to induced pluripotent stem cells (iPSC)) or from mammary progenitor stem cells remain unclear.

**Familial breast cancer**

Although the majority of breast cancer is sporadic, a small percentage of breast cancer (about 15%) occurs due to cancer syndromes caused by inherited gene mutations (see Table 1). The cancer syndromes described below predispose women to cancers of several different organs, including breast.

**Li Fraumeni syndrome:**

Hereditary mutations in p53 are highly penetrant and show an autosomal dominant pattern. People who inherit germ line mutations in p53 develop Li Fraumeni syndrome (LFS) [32]. Although LFS patients develop multiple cancers, the most frequent occurrence is that of early onset breast cancer. More than 30% of all LFS tumors develop in the breast [33, 34].

**Cowden’s syndrome:**

Similar to p53, hereditary mutations in the tumor suppressor gene PTEN also show an autosomal dominant pattern and cause Cowden’s syndrome [35, 36]. Patients with Cowden’s syndrome develop multiple hamartomas, which are benign lesions. As with LFS patients, Cowden’s syndrome patients also develop early onset breast lesions more frequently than
lesions in any other organ [37]. About 75% of Cowden’s patients develop benign breast disease, which increases their risk of developing malignant disease by 25-50% [37].

**Peutz-Jeghers syndrome:**

Like p53 and PTEN, hereditary mutations in the tumor suppressor gene STK11 also result in an autosomal dominant cancer syndrome called Peutz-Jeghers syndrome (PJS) [38]. This syndrome is characterized by multiple gastrointestinal polyps. However, the most commonly developed malignancy in patients with PJS is breast cancer [39], accounting for more than 30% of all cancers that occur in PJS patients. As with LFS and Cowden’s disease, breast cancer in PJS patients occurs as an early onset disease.

**ATM heterozygous Mutations:**

All of the known mutations that increase the risk of breast cancer occur in autosomal dominant genes except for mutations in ATM, which are autosomal recessive. Homozygous deletion of ATM predisposes to malignancy, particularly lymphoma or leukemia. Heterozygote carriers of ATM also have an increased risk of developing breast cancer [40].

**Hereditary Breast Ovarian Cancer:**

More than 80% of all hereditary breast cancer is caused by Hereditary Breast Ovarian Cancer (HBOC) syndrome [41]. HBOC occurs due to highly penetrant, autosomal dominant genetic mutations in BRCA1 [42] or BRCA2 [43]. Among the women who inherit BRCA1 mutations, approximately 50 - 80% will develop breast cancer and 40% will develop ovarian cancer [41]. Among the women who inherit BRCA2 mutations, approximately 40% will develop breast cancer and 20% will develop ovarian cancer [41]. Although mutations in both BRCA1 and BRCA2 increase the risk for breast cancer, the tumors that develop are distinct for each gene. The breast tumors that occur in BRCA1 mutation carriers are predominantly of the basal-like sub-type [9, 44] while tumors arising from BRCA2 mutations are predominantly of the Luminal B molecular sub-type [45]. Like basal-like breast cancer, BRCA1-associated tumors are ER, PR, and HER2 negative and harbor frequent p53 mutations. In contrast to basal-like tumors, the spectrum of p53 mutations in BRCA1-related tumors is different from those in non-BRCA1 mutated basal-like tumors [21]. BRCA1 related tumors have non-sense p53
mutations that results in a truncated protein and accumulation of p53 protein. Despite the
different spectrum of p53 mutations, in murine models, p53 loss of function appears necessary
for tumor development in cells that have lost BRCA1 function [46-48].

Table 1. Breast cancer sub-types in as they occur in breast cancer syndrome.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Penetrance</th>
<th>Tumor Sub-type</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>BRCA 1</td>
<td>high</td>
<td>Basal</td>
<td>Sorlie et al. PNAS 2003</td>
</tr>
<tr>
<td>BRCA 2</td>
<td>high</td>
<td>Luminal B</td>
<td>Melchor et al. Ontogeny 2008</td>
</tr>
<tr>
<td>p53</td>
<td>high</td>
<td>Basal?</td>
<td>Gonzales et al. JCO 2009</td>
</tr>
</tbody>
</table>

Not only is breast cancer the most common sporadically occurring disease, it is apparent
that the mammary gland is more vulnerable to tumorigenesis than any other gland in the body in
cancer syndrome patients. We think that this may be due, in part, to different or more lenient
early barriers to tumorigenesis in mammary cells as compared to those from those of other cell
types.

Early events in malignant transformation

The events that increase the risk of malignant transformation may occur through a
combination of cell type and mutation of origin. Given that there are many cell types in the
normal breast, and that there are also multiple molecular sub-types of breast cancer, the question
arises whether a particular cell type gives rise to a specific tumor sub-type. The early events that
occur and the susceptibility for malignant transformation may be different for the distinct
molecular sub-types of breast cancer. The cell of origin hypothesis predicts that distinct cell
types give rise to distinct tumor molecular sub-types, while the mutation of origin hypothesis
states that mutations in key pathways dictate molecular sub-type. It is likely that the
combination of the cell type and the mutation increases the risk for malignant transformation.
Bypass of barriers required for transformation:

Regardless of whether the cause of a particular molecular sub-type is due to cell type or mutation of origin, tumor cells of all sub-types must bypass a number of intrinsic barriers. The bypass of some but not all barriers does not necessarily lead to transformation. For example, it has been shown that human pre-malignant lesions bypass growth barriers as they commonly show increased mitotic indices. However, unlike malignant disease, pre-malignant lesions commonly show intact apoptotic barriers [49].

Normal cells must acquire several biological alterations to become transformed cells. A cell’s transition from normal to malignant is a multistep process in tumorigenesis. It has been established by *in vitro* experiments that loss of tumor suppressor function combined with oncogenic activation and immortalization will transform normal cells into tumor cells [50]. When these cells are implanted into a mouse recipient, they commonly give rise to a tumors [51]. It is important to recognize that human cells are more resistant to transformation compared to mouse cells [52]. When experiments were done *in vitro*, normal human cells were found to require multiple events for successful transformation compared with murine cells. Several groups have shown that immortalized mouse fibroblasts can be transformed by oncogenic RAS alone [53, 54], while immortalized human fibroblasts require both RAS and MYC [51] or RAS and p53 deregulation for transformation [50].

Normal human mammary epithelial cells (HMEC) have also been immortalized. Unlike fibroblasts, the activation of hTERT alone does not immortalize epithelial cells. Combined expression of hTERT and loss of p16 are required for HMEC immortalization [55]. In the setting of hTERT activation and deregulation of RB/p16, HMEC can be transformed by expression of oncogenic RAS [56, 57]. This data implies that epithelial cells may be more resistant to immortalization as compared to fibroblasts.

**Barriers: Apoptosis and Senescence**

Both tumor suppressor dysfunction and oncogene activation are required for transformation. Independently, tumor suppressor inactivation or oncogene activation results in
either apoptosis or cellular senescence. Both are mechanisms to limit the propagation of potentially damaged cells.

**Apoptosis**: Apoptosis is a definitive barrier in tumorigenesis and is therefore disabled in tumor cells. It has been argued that since apoptosis kills damaged cells, it *should* be the mechanism of choice, instead of growth arrest. To address this question, it has been demonstrated that the threshold of DNA damage is the most probable deciding factor. If the threshold of damage is greater than some cell intrinsic level, cells will initiate an apoptotic program. Likewise, if the damage is relatively low, then the cells initiate a senescence program [58].

**Senescence**: Although senescence limits the propagation of damaged cells, it also promotes cell survival, which may allow for the selection of clones that have bypassed senescence and therefore, increases the risk of transformation. Senescent cells display distinct hallmarks which are discussed below [59].

**Morphology**: The morphology of cells is distinguishably altered as they undergo senescence. Senescent cells increase in size and become 5-10 times larger than their original size. Their shape also changes and they become flattened. These changes do not occur immediately; rather, the cells alter gradually over the course of a few days and then, maintain this enlarged phenotype [59]. It can be speculated that even though the cells do not divide and increase in number, protein synthesis continues, resulting in the increased size [60]. Increased metabolic activity results in the formation of vacuoles and the nucleus of senescent cells becomes enlarged. Reports have documented chromatin remodeling in senescent cells. Narita, et. al. showed that senescence associated heterochromatic foci (SAHF) are a hallmark of senescence [61]. These heterochromatic regions are compact with hypermethylated regions of silenced DNA. [62]. As cells senesce, proliferation genes are silenced and DNA hypermethylation results in an irreversible growth arrest [62].

**Cell cycle arrest**: Cell cycle arrest is the most distinguishing feature of senescence. The mechanism of senescence has been well studied at the molecular level. Nearly all cells become senescent either through the p53 or p16/RB pathway or through both pathways. p53 activation plays a central role in senescence induction. The immediate downstream effector of p53 senescence induction is transcription of p21 [63], a CDK inhibitor that prevents the CDKs from
binding with cyclins, thus preventing cell division [64]. p16 maintains RB in its active form, bound to E2F, preventing E2F from transcribing proliferation genes [65]. Activated RB also mediates the formation of senescence-associated heterochromatin foci [61]. Upregulation of p16 can occur through multiple mechanisms including inhibition of upstream regulators like BMI1 [59]. When BMI is down-regulated, p16 levels are increased and as a consequence, senescence is induced [66].

*DNA damage response activation:* p53 stabilization and activation occurs in response to upregulation of DNA damage response proteins. DNA breaks are sensed by the MRN complex, which activates ATM [67] (when double stand breaks occur) and ATR [68] (when single strand breaks occur) by phosphorylation. Phosphorylated ATM then activates CHK1 [69], CHK2 [70] and p53 [67], while phosphorylated ATR activates CHK1 [71] and p53 [68], also by phosphorylation. ATM also inhibits Mdm2, thereby inhibiting p53 degradation and favouring p53 stabilization [72].

*Reactive Oxygen:* Reactive oxygen species (ROS) has been shown to be a key factor that contributes to senescence induction. When oxidative stress was measured in replicative senescent human fibroblasts, a several fold increase in oxidative stress was found in senescent cells as compared to young cells [73]. Culturing cells at reduced oxygen levels such as 3% O₂ results in prolonged lifespan [73]. Recent data has shown that p53 is required to mediate mitochondrial changes and ROS production is required for the induction of RAS induced senescence [74]. The current understanding of the role of ROS in senescence induction is that increased mitochondrial activity results in increased ROS, leading to increased DNA damage which then results in the activation of p53 and the induction of senescence.

*Autophagy:* Autophagy has been shown to play a role in senescence [75]. Autophagy is a process by which damaged cellular components and organelles are sequestered in double membrane structures called autophagosomes and are transported to lysosomes for degradation [76]. Autophagy not only removes waste from cells, but also allows cells to recycle macromolecules and generate energy during times of stress and starvation. However, unregulated autophagy results in excessive digestion of cellular components and can lead to cell death. This feature of autophagy gives it a tumor suppressive property.
During senescence, autophagy provides amino acids and simple forms of lipids to be reused to maintain cell viability. Due to this role of autophagy in cell survival, it has also been shown to have oncogenic properties. Rapidly proliferating tumor cells use autophagy to derive nutrients and energy through catabolism [77].

The main regulators of autophagy are mTOR and p53. Classically, mTOR inhibits autophagy by inhibiting ULK1 [78, 79]. mTOR phosphorylates ULK1, preventing it from forming a complex with ATG13, which is necessary for autophagy initiation. AMPK, a well studied inhibitor of mTOR [80] also triggers autophagy directly activating ULK1 [81, 82]. The regulation of autophagy by p53 is unique. p53 nuclear and cytoplasmic localization within the cell differentially regulates autophagy. Nuclear p53 induces autophagy while cytoplasmic p53 inhibits it. Nuclear p53 transcribes autophagy regulatory genes such as DRAM [83] and mTOR inhibitory genes such as SESN2 [84]. Since autophagy is upregulated in senescent cells, it can be hypothesized that these genes should also be upregulated in senescent cells. In contrast, cytoplasmic levels of p53 inhibit autophagy. Cytoplasmic p53 inhibits AMPK, a positive regulator of autophagy, which in turn activates mTOR, a negative regulator of autophagy [85].

**Secretory phenotype:** Recent data has shown that once cells become senescent, they secrete a variety of biologically active molecules. This has been termed the Senescence Associated Secretory Phenotype (SASP) [86]. Expression array studies [87] and antibody arrays [88] have revealed numerous secreted factors released by senescent cells. These include diverse interleukins, chemokines, and growth factors that have been shown to modulate the microenvironment. For example, the SASP due to oncogenic BRAF induced senescence was capable of altering the behavior of neighboring cells. In particular, IGFBP7, IL8 and CXCR2 secreted by oncogene induced senescent cells induced senescence and apoptosis in neighboring cells [89-91].

**Inducers of Senescence:**

There are several stressors that are capable of inducing senescence. These include mitochondrial dysfunction, oxidative stress, DNA damaging agents that cause irreparable DNA damage, aberrant microenvironment, and chromatin disruption among others. The most studied
types of senescence are those induced by Replicative Senescence (RS) and pre-mature Oncogene Induced Senescence (OIS).

Primary cells have a finite life span or a set number of replicative cycles in cell culture before they senesce [92]. This is called replicative senescence. It was shown that telomere length shortens with each replicative cycle [93] and telomere erosion beyond a critical threshold results in DNA damage [94]. In response to telomere erosion, DNA Damage Response proteins (DDR) activate p53, which induces senescence. p53 appears to play a causal role in senescence [95] as mutant p53 results in extended lifespan and allows for bypass of senescence [96].

Activation of oncogenes or loss of tumor suppressor genes results in pre-mature senescence. Induction of pre-mature senescence due to over-expression of oncogenic RAS in normal cells results in cellular hyper-proliferation and the collapsing of replication forks. This is detected as DNA damage by the cell resulting in DDR up regulation, activation of p53, and senescence induction [97]. Similarly, loss of tumor suppressor genes, such as NF1, also results in senescence as a consequence of DNA damage [98]. This work led to the establishment of the paradigm that senescence induction is mediated through DDR and activation of p53. To date, over-expression of a number of oncogenes and loss of tumor suppressor genes have been shown to induce senescence [99, 100].

**Senescence-associated phenotypes: Similarities and differences**

Although oncogene activation or loss of tumor suppressor function in normal cells results in shared senescence associated phenotypes, the mechanism of senescence induction is different. The shared senescence phenotypes include irreversible growth arrest and senescence-associated morphological changes (large, flat, and vacuolated cells). All senescent cells thus far analyzed also produce a secretory phenotype [86]. Increased oxidative stress is another shared feature among all senescent cells studied to date [101]. Autophagy has only been described in oncogenic RAS-induced senescence [75].

The mechanism of senescence onset can be specific to different inducers of senescence. One very well studied example is the regulation of p53 protein levels. The role of p53 in senescent cells has been shown to be both context dependent and cell type dependent. The levels
of p53 protein are differentially expressed in human fibroblasts, in response to different inducers of senescence. Activation of oncogenes and silencing of certain tumor suppressors results in senescence correlated with an increased expression of p53 [98, 102, 103]. However, in human fibroblasts, when senescence is induced by telomere shortening, p53 protein levels are not increased [95, 104-106]. Furthermore, DDR proteins are not universally up regulated in senescent cells. For example, loss of PTEN-induced senescence does not result in DNA damage or activation of a DNA damage response [107]. Another inducer-specific difference is the secreted factors released by senescent cells. All senescent cells have a secretory phenotype; however, the molecules that are secreted are not necessarily the same. One example is that of interferon gamma (INF γ) which is only secreted by fibroblasts undergoing oncogenic RAS-induced senescence and not by cells undergoing RS or irradiated senescent cells [88, 108]. These findings suggest that although senescent cells share several features, there are inducer and/or cell type specific differences that may influence the mechanisms by which cells bypass this barrier to tumorigenesis.

**Consequences of senescence: anti-tumorigenic versus pro-tumorigenic:**

Senescence is an anti-tumorigenic mechanism that inhibits the growth of damaged cells. Although senescence has been well described in cultured cells, the *in vivo* relevance of senescence has been questioned. Recent seminal reports have established that senescence does occur *in vivo* and limits the propagation of damaged cells as previously shown in experimentally cultured cells [98, 102, 109-115]. In both mouse and human models of tumorigenesis, genotoxic stress (oncogene activation or loss of TSGs) results in pre-malignant disease that correlates with the induction of senescence. Lesions that have progressed to malignancy show an absence of senescence markers, suggesting that bypass of senescence is required for cellular transformation [98, 102, 109-115].

Senescent cells can also play pro-tumorigenic role. This pro-tumorigenic role is largely mediated by senescence-associated secreted factors that promote cell survival and the growth of neighbouring cells. *In vitro*, senescent cells were first shown to increase the invasiveness of pancreatic cancer cell lines [116]. In another study, immortalized, but non-tumorigenic MCF10A cells, co-cultured with senescent fibroblasts were more proliferative and underwent
malignant transformation in a mouse model [117, 118]. There have now been several reports of senescent cells altering the micro-environment in a pro-tumorigenic manner [88-90].

**BRCA1 Tumorigenesis**

**BRCA1 function:**

BRCA1 is identified as a breast cancer susceptibility gene mapped by genetic linkage analysis to chromosome 17q12-21 [119]. Initially, several studies demonstrated that BRCA1 was involved in DNA repair and interacts with and acts upstream of RAD51 [120-122]. This lead to the understanding that BRCA1 is a DNA damage repair protein. Later reports demonstrated that BRCA1 can also sense DNA damage and plays a role in DNA damage response signaling [123]. BRCA1 has been shown to have a cell cycle regulation role as well. Loss of BRCA1 has shown to result in defective G2-M checkpoint [124] and functional BRCA1 has been demonstrated to be a co-activator of p53 [125, 126]. Recently, BRCA1 has also been shown to serve an antioxidant function as well [127, 128]. As research has advanced in studying BRCA1, accumulating evidence suggests that BRCA1 is an important multifunctional protein.

**Mouse Models of BRCA1 tumorigenesis:**

BRCA1 tumorigenesis has been studied extensively using several mouse models. Complete knock out of BRCA1 results in embryonic lethality in the mouse [47]. This parallels the fact that there are no human reports of BRCA1 homozygous deletion cases. Conditional knock down of BRCA1 targeted to the mammary gland does not result in breast tumor development, suggesting that additional events are required for transformation [129]. Inactivation of p53 in combination with conditional inactivation of BRCA1 in the mammary gland does allow for mammary tumor formation [129]. However, these tumors appeared after a long latency period, suggesting that p53 inactivation alone does not recapitulate early disease onset in women with BRCA1 mutations [48]. Studies have shown that combined knock down of BRCA1 with other tumor suppressor genes such as CHK2 also results in the formation of breast tumors [130]. The efficiency of tumor formation in these mice is reduced, such that 50% of mice develop mammary tumors and only after a long latency. Similarly, combined knock down of BRCA1 and ATM results in mammary tumors after long latency in a small percentage (10%) of
mice [131]. Knock down of BRCA1 has also been combined with 53BP1 inactivation. Interestingly, 53BP1 inactivation rescues embryonic lethality due to BRCA1 loss, but these mice do not develop mammary tumors [132]. (See Table 2 for a summary of known mediators of BRCA1-associated murine mammary tumorigenesis.)

Table 2. Mouse models of BRCA1 tumorigenesis

<table>
<thead>
<tr>
<th>Driver</th>
<th>Co-driver</th>
<th>Latency/frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1-/+</td>
<td>-</td>
<td>no tumor</td>
<td>YM Jing et al. Ontogeny 2007</td>
</tr>
<tr>
<td>BRCA1 -/-</td>
<td>-</td>
<td>no tumor</td>
<td>X Liu et al PNAS 2007</td>
</tr>
<tr>
<td>BRCA1 -/-</td>
<td>p53 -/-</td>
<td>long/80%</td>
<td>X Liu et al PNAS 2007</td>
</tr>
<tr>
<td>BRCA1 -/-</td>
<td>CHK2 -/-</td>
<td>long/50%</td>
<td>McPherson et al. Gen &amp; Dev 2004</td>
</tr>
<tr>
<td>BRCA1 -/-</td>
<td>ATM -/-</td>
<td>long/10%</td>
<td>Liu Cao et al. EMBO 2006</td>
</tr>
<tr>
<td>BRCA1 -/-</td>
<td>53BP1 -/-</td>
<td>no tumor</td>
<td>Liu Cao et al. Cell 2009</td>
</tr>
</tbody>
</table>

Human studies of BRCA1 tumorigenesis:

Although loss of BRCA1 function has a very high correlation with breast cancer incidence, there is relatively little known about the early events in BRCA1-associated tumorigenesis. Tumors that develop in mammary tissue from BRCA1 mutation carriers all show LOH at the BRCA1 locus, strongly suggesting that LOH is a causal event for tumor development [133-135]. Evidence suggests that loss of heterozygosity (LOH) in BRCA1 heterozygous mammary epithelial cells may be an early initiating event. Tissue samples from histologically normal mammary tissue from disease-free BRCA1 mutation carrier women and from normal regions of mammary tissue adjacent to carcinoma in BRCA1 carriers show an increased frequency of LOH compared with samples from non-BRCA1 mutation carriers. However, the consequences of loss of BRCA1 in normal mammary cells remains underexplored.

Rationale:

In this study, we proposed to advance our understanding of the consequences of loss of BRCA1 in normal mammary epithelial cells. We think it is necessary to unveil the early events that occur following loss of BRCA1. If we have the knowledge of how BRCA1 related breast
cancer develops in the early stages, then this may provide that can be used for early diagnostics, targeted therapeutics, and possibly even prevention. The central aim of starting this project was to investigate the consequences of loss of BRCA1 for the purpose of understanding breast cancer etiology.

Hypotheses:

1. Loss of BRCA1 results in the induction of senescence.

2. BRCA1 loss induced senescence involves DNA damage, p53 activation, ROS up-regulation and autophagy induction.

3. BRCA1 loss induced senescence is maintained by p53, ROS and autophagy.
Chapter 2: Materials and Methods

Cell Culture

Isolation and propagation of HMECs in Culture

The HMEC system is a well established model for studying early events in breast carcinogenesis, first described in 1980 [136, 137]. We obtain fresh reduction mammoplasty (RM) tissue from consenting patients undergoing breast reduction or reconstruction. Briefly described, we process the tissue first by mechanical disruption, followed by overnight enzymatic digestion at 37°C using 50-100mL digestion media, which contains RPMI + 10% Fetal Bovine Serum (FBS) (Hyclone, Fisher Scientific) + 1% Penicillin/Streptomycin/Fungizone (pen/strep; Invitrogen) + 0.8mg/ml collagenase type I (Sigma) + 0.36mg/ml hyaluronidase (Sigma). The following day, the digested mix is spun down and washed 3 times with RPMI + 10% FBS (Hyclone) + 1% pen/strep (Invitrogen). After the final wash, the digested tissue pellet is resuspended in 15mL RPMI and filtered through a pre-wetted 150 um filter first, then through a 50µm filter. The final filtrate contains single cell fibroblasts and the residue on both the filters contains cell clusters called organoids. These organoid structures are enriched in terminal ductal lobules. The organoids are collected and cryofrozen in freezing media. The freezing media is RPMI + 20% FBS + 10% DMSO (Sigma). When thawed and plated in culture, primary epithelial cells are encouraged to proliferate in a serum-free defined media. HMECs were cultured in serum-free media MEGM (Mammary Epithelial Cell Basal Medium) from Clonetics (Catalog # CC-3150). Growth supplements were also obtained from Clonetics and were added separately; these include BPE, hEGF, Hydrocortisone, Insulin, and Gentamicin sulphate amphotericin. All cell culture experiments were conducted in 21% O₂ with 5% CO₂ at 37°C. In experiments where indicated, HMECs were grown in 3% O₂ with 5% CO₂ at 37°C.

Additional Cell Lines: MCF10A cells were obtained from American Type Culture Collection (ATCC) and grown in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 5% horse serum (Invitrogen), 20 ng/ml epidermal growth factor (Sigma), 0.5 µg/ml hydrocortisone (Sigma), 100 mg/ml cholera toxin (Sigma), 10 µg/ml insulin (Sigma) and 1% antibiotic/antimycotic (Invitrogen) [138]. IMR90 cells were obtained ATCC and grown in
DMEM F12 supplemented with 10% FBS (Hyclone). NMF were isolated from the filtrate of processed RM specimens and were also grown in DMEM F12 with 10% FBS. 184A1 were obtained from ATCC and cultured in MEGM.

**Proliferation analysis**

Growth kinetics was determined as follows. HMECs were typically trypsinized into 6-well tissue culture plates one day post-infection. Control HMECs were seeded at a density of 1 X 10^4 cells/well while experimental HMECs were seeded at 5 X 10^4 cells/well. Media was changed every day and cells were counted every 2-3 days using a hemocytometer. To count, cells were first washed with 1 X PBS, trypsinized using 1 mL 0.05% trypsin (Invitrogen) for approximately 5 min and collected following trypsin neutralization using PBS containing 2% FBS. Approximately 15µL of cell suspension was used to determine the number of cells per ml. All samples were counted in triplicates. Cells counts were used to calculate population doublings as follows:

\[
\text{LOG} \left( \frac{\text{# of cells counted}}{\text{# of cells seeded}} \right) \times \text{LOG} (2)
\]

**Analysis of apoptosis**

Apoptosis was determined by flow cytometry. Annexin V, a marker of early apoptotic cell, conjugated with Fluorescence Isothiocyanate (FITC) was used in combination with Propidium Iodide (PI), a marker of late apoptotic and necrotic cells. 5 X 10^5 cells were trypsinized from each plate and suspended in cold binding buffer to a volume of 500µL in a microfuge tube, as per manufacturer instructions (Calbiochem). 1.25µL of annexin was added 5 X 10^5 cells in each microfuge tube and incubated for 15 min at room temperature in dark. This was followed by centrifugation and re-suspension in binding buffer. 10µL PI was added and samples were stored on ice in the dark until analyzed. Flow cytometry was performed on a FACS Calibur machine at the Princess Margaret Hospital. FITC is detected at 518nm and PI fluoresces at 620nm. FITC was plotted on the x-axis and PI on the y-axis. Data was analyzed using Flowjo analysis software.
Engineering of stable cell lines

Retroviral and lentiviral vectors

Retroviral vectors: pBabe control vector and pBabe-H-RAS\textsuperscript{V12} containing a puromycin resistance cassette was obtained from Dr. Weinberg through Addgene distribution (http://www.addgene.org/1768/).

Lentiviral vectors: We designed and generated short hairpin RNA expressing vectors against BRCA1 #1, BRCA2, and SESN2 #2 using pLKO construct and following Addgene instructions. Short hairpins against BRCA1 #2, #3, #4, #5, p53, and BECN1 were provided by Dr. Jason Moffat. Control vectors, scrambled short hairpin (shScr), shLuciferase (shLuc), and an empty vector (LKO), were also provided by Dr. Moffat.

Transfection of cells for lentivirus and retrovirus production: Lentiviral transfections were carried out in 293T cells (ATCC). Fugene 6 (Roche) was used as the transfection reagent for DNA delivery into the cells. For transfection of a 10cm plate, 5µg lentiviral plasmid DNA, 4µg packaging vector (pCMV), and 2µg envelope vector (pMD) was used in the transfection reaction in 4mL DMEM H21 (high glucose) 10% FBS media. Cells were incubated for 4-6 hours at 37°C with 5% CO\textsubscript{2} and then, 6mL of additional media was added. Media was replaced with 10mL fresh DMEM H21 5% FBS media after 24 hours and virus was collected at 48 hours and 72 hours and filtered through a 0.45µm filter. Retrovirus was generated using Phoenix A cells. For a 10cm plate, Fugene 6 and 10µg retroviral vector DNA were used in the transfection reaction; retroviral collection was performed as described for lentivirus collection above.

Generation of stable HMEC cell lines: For knockdown (KD) experiments, cells were incubated with lentivirus for 4-6 hours in a total volume of 4 mL (1mL virus + 3mL media + 4µL of 4µg/µL polybrene (Sigma)) in a 10cm plate, after which this infection media was replaced with fresh MEGM media. For retroviral infection, cells were incubated in a total volume of 4mL (10cm plate) for 4-6 hours (2mL virus + 2mL media + 4µL of 4µg/µL polybrene) after which 6mL media was added to the plate and cells were incubated overnight. On day 1 post infection, selection media containing 2µg/mL puromycin or 3µg/mL blasticidin was applied and was used to select for stable lines expressing the construct of interest.
Table 3. Short hairpin sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Target Sequence</th>
<th>Provider/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LKO</td>
<td>Empty vector</td>
<td>Moffat Lab</td>
</tr>
<tr>
<td>shLUC</td>
<td>ACGCTGAGTACTTCGAAATGT</td>
<td>Moffat Lab</td>
</tr>
<tr>
<td>shScr</td>
<td>AGGTAAAGTCGCCCTCG</td>
<td>Addgene Plasmid 1864</td>
</tr>
<tr>
<td>shBRCA1 #1</td>
<td>GCTCCTCTCCTCTTCTCATGGTT</td>
<td>Designed in lab</td>
</tr>
<tr>
<td>shBRCA1 #2</td>
<td>TCACAGTGTCCTTTTATGTA</td>
<td>Moffat Lab</td>
</tr>
<tr>
<td>shBRCA1 #3</td>
<td>GCCTACAAGAAAGTAGAGGAT</td>
<td>Moffat Lab</td>
</tr>
<tr>
<td>shBRCA1 #4</td>
<td>GAGAATCCTAGAGATACTGAA</td>
<td>Moffat Lab</td>
</tr>
<tr>
<td>shBRCA1 #5</td>
<td>GCCCACCCTAATTGTACTGAAT</td>
<td>Designed in lab</td>
</tr>
<tr>
<td>BRCA2 #1</td>
<td>AAGCTCCACCCTATAATTCTG</td>
<td>Schoenfeld et al. MCB 2004</td>
</tr>
<tr>
<td>BRCA2 #2</td>
<td>AATCAGCTGGCTTCAACTCCA</td>
<td>Li et al. Oncogene 2006</td>
</tr>
<tr>
<td>shBECN1 #1</td>
<td>CCGTGGAATGGAATGAGATT</td>
<td>Moffat Lab</td>
</tr>
<tr>
<td>shp53</td>
<td>GTCCAGATGAAGCTCCCAGAA</td>
<td>Designed in lab</td>
</tr>
<tr>
<td>shSESN2 #1</td>
<td>AAGACCATGGCTACTGCTGTA</td>
<td>Budanov et. al. Science 2004</td>
</tr>
<tr>
<td>shSESN2 #2</td>
<td>AACTCAGCGAGATCAACAAGT</td>
<td>Designed in lab</td>
</tr>
</tbody>
</table>

Pharmacological treatment of HMECs

*MG132*: Stable cell lines of HMEC-LKO and HMEC-shBRCA1 were treated with 10µM MG132 in culture media for 4 hours on day 2 post infection. After the treatment, cells were washed with PBS and media was replaced. Control lines were treated with equal volume of DMSO.
**Leptomycin B**: HMEC-LKO and HMEC-shBRCA1 were incubated with 25nM leptomycin B in culture media from day one of infection until collection on day 5 post-infection.

**Doxorubicin**: Where indicated, HMEC-LKO and HMEC-shBRCA1 were incubated with 0.5µM doxorubicin or DMSO solubilized in culture media for 6 hours. Cells were collected after 6 hours of doxorubicin exposure or allowed to recover in regular media for an additional 24 h prior to collection.

**Senescence Associated β-galactosidase activity**

Cells were seeded in triplicate in a 24 well plate one day after infection with the gene of interest and cultured until ready for β-galactosidase staining as indicated. Culture media was aspirated from each well and cells were washed with 1mL 1X PBS 3 times for 2 minutes each time with gentle shaking. After the final wash, PBS was aspirated and replaced with 500µL fix solution. Cells were incubated with the fix solution (2% Formaldehyde, 0.2% Glutaraldehyde, in 1X PBS) for 5 minutes at room temperature with gentle shaking. Fix solution was aspirated and cells were washed once with 1mL 1X PBS. 500µL of β-galactosidase staining solution (Table 4) was added to each well and the cells were incubated at 37°C in the absence of CO₂ for 5-7 hours. After the incubation, cells were washed with 1X PBS and analyzed in PBS. For analysis, 3 pictures of different fields of view were taken from each well and a minimum of 100 cells were counted from each picture. The total number of cells and the number of cells that stained positive with β-galactosidase were counted and the percentage of positive cells was calculated and plotted in a histogram.

**Table 4. β-galactosidase staining solution**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Volume per rxn</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-gal</td>
<td>1mg/mL</td>
<td>25µL</td>
</tr>
<tr>
<td>Citric Acid/Na₂HPO₄</td>
<td>40mM</td>
<td>100µL</td>
</tr>
<tr>
<td>Potassium ferrocyanide</td>
<td>5mM</td>
<td>25µL</td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td>5mM</td>
<td>25µL</td>
</tr>
<tr>
<td>NaCl</td>
<td>150mM</td>
<td>15µL</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2mM</td>
<td>1µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>500µL</td>
</tr>
</tbody>
</table>
Western blot analysis

**Total protein isolation:** NP40 cell lysis buffer (20mM Tris, 150mM NaCl, 0.1mM EDTA, 20mM NaF, 1% NP40) was used for protein isolation. 10µL of NP40 lysis buffer was added per 1 X 10⁴ cells to re-suspend the frozen cell pellet. Resuspended cells were incubated on ice for 10 min and then pipetted gently to mix. The samples were pelleted by centrifugation at 14,000 RPM for 30 min at 4°C and the supernatant (protein lysate) was frozen at -80°C until further use.

**Protein quantification:** Protein concentration was determined using the Bradford assay [139]. Bradford reagent was diluted 1:5 with Deionized (DI) water. 200µL of diluted Bradford reagent was aliquoted into each well of a 96 well plate. Protein samples were assessed in triplicate. Colormetric analysis was determined using Coomassie Brilliant Blue dye (Bradford reagent Bio-Rad) in which, 1µL of protein sample was added and the absorbance was measured at A595. Bovine Serum Albumin (BSA) was used as a protein standard. Once protein concentrations were determined, 6X sample buffer (0.5M Tris, 30% glycerol, 10% SDS, 5% β-mercaptoethal, 0.04% Bromophenol blue) was used to dilute the protein lysates down to a final concentration of 3µg/µL.

**SDS-PAGE:** BioRad gel casting apparatus was used to cast all polyacrylamide minigels. The resolving solution (1.5M Tris pH 8.8 (stock), 12% Acrylamide, 0.1% SDS, 0.1% APS, and 0.4% TEMED) was prepared, poured and left to polymerize for 20-30 min. The stacking gel (1M Tris pH 6.8 (stock), 4% Acrylamide, 0.1% SDS, 0.1% APS, and 0.4% TEMED) was prepared, poured, a 10 well comb was inserted and the gel was allowed to polymerize for at least 1 hour. Protein lysates were boiled for 5-6 min, allowed to cool for about 3-4 min and then loaded in each well. After loading all the samples, the wells were topped up with running buffer (10X = 0.25M Tris, 2.5M glycine, 1% SDS). The plates were then taken out of the casting stand and assembled in the gel running apparatus and the gels were run at 110V for roughly 1 hour 30 min in running buffer. Once gel electrophoresis was completed, the gels plates were disassembled very carefully, the stacking gel was removed, and the gels were equilibrated in transfer buffer with gentle shaking for 10 min at room temperature.
Western blot protocol: The separated proteins were transferred to PVDF membrane (BioRad). First, the PVDF membrane was submerged in methanol for 30sec and then transferred to transfer buffer (25mM Tris, 192mM glycine, and 10% methanol) for 10 min. An immunoblot sandwich was made in a tray filled with transfer buffer. The transfer cassette was place in the tray, followed by a pad, a filter paper, the gel, the membrane, a second filter paper and then a pad. The transfer cassette was latched properly and then placed in the tank with an ice pack in it and filled with transfer buffer. The proteins were transferred at 100V for 1 hour. After the transfer, the membrane was blocked in blocking buffer (5% milk in 0.1% TTBS (1X TBS+ 0.1% Tween)) for 1 hour and probed with primary antibody as indicated. The membrane was then washed 3 times with 0.1% TTBS (10X = 0.25M Tris, 80g NaCl, 2g KCl, pH 7.4 in 1L, and 0.1% Tween) and then incubated with the secondary antibody, after which it was washed again 3 times with 0.1% TTBS. ECL+ (GE) solution was applied to the membrane as per manufacturer instructions. The chemiluminescent signal was visualized using scientific imaging film (Kodak).
Table 5. Antibodies

<table>
<thead>
<tr>
<th>Antibody (provider)</th>
<th>Dilution factor</th>
<th>Incubation time</th>
<th>2\textsuperscript{nd}ary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin (Santa Cruz)</td>
<td>1:10,000</td>
<td>3 hr minimum</td>
<td>Mouse</td>
</tr>
<tr>
<td>AMPK (Cell Signaling)</td>
<td>1:1000</td>
<td>Overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>53BP1 (Cell Signaling)</td>
<td>1:1000</td>
<td>Overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>BRCA1 (Santa Cruz)</td>
<td>1:100</td>
<td>48 hours</td>
<td>Mouse</td>
</tr>
<tr>
<td>LC3 (MBL)</td>
<td>1:750</td>
<td>Overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>RB (BD Pharmagen)</td>
<td>1:200</td>
<td>Overnight</td>
<td>Mouse</td>
</tr>
<tr>
<td>p15 (Neomarkers)</td>
<td>1:200</td>
<td>Overnight</td>
<td>Mouse</td>
</tr>
<tr>
<td>p16 (BD Bioscience)</td>
<td>1:300</td>
<td>Overnight</td>
<td>Mouse</td>
</tr>
<tr>
<td>p21 (Cell Signaling)</td>
<td>1:1000</td>
<td>Overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pAMPK (Cell Signaling)</td>
<td>1:750</td>
<td>Overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pATM (Cell Signaling)</td>
<td>1:100</td>
<td>Overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pCHK2 (Cell Signaling)</td>
<td>1:200</td>
<td>Overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pH2AX (Upstate)</td>
<td>1:100</td>
<td>Overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>RAS\textsuperscript{V12} (Santa Cruz)</td>
<td>1:1000</td>
<td>Overnight</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>
Reverse Transcription PCR

**Total RNA isolation and quantification:** Total RNA was isolated using Trizol (Invitrogen) following manufacturer’s instructions. 1µL of isolated RNA was run on 2% agarose gel to determine RNA integrity. RNA concentration was measured using NanoDrop (Thermo Scientific).

**Polymerase Chain Reaction:** 1µg RNA was used in a total reaction of 20 µL for rtPCR. Manufacturer instructions were followed for the synthesis of cDNA using iScript kit (Bio-Rad). The concentration of cDNA was measured using NanoDrop and all samples were diluted in DEPC water to a final concentration of 500ng/µL. 1µg of cDNA was used for semi-quantitative PCR. The PCR conditions are listed in Table 6.

**Agarose gel electrophoresis:** PCR amplified products were separated through a 2.5% agarose gel (ONBIO) in 1X TAE buffer (50X = 1M Tris, 28.6mL Acetic acid + 0.5M EDTA). DNA ladder (Invitrogen) was always loaded in the first lane and the PCR product in the following lanes. Electrophoresis was done at 90V for 35-40 minutes and the gel was analyzed in the Gel Doc imaging system (Bio-Rad).

**rtPCR primers:** Where indicated, primer sequences were determined by previously published reports; otherwise they were designed using Primer 3, as indicated in table 7. Primers were synthesized by Eurofins MWG Operon (EMO). Lyophilized primers that were received from EMO were dissolved with DI water to generate a 100µM stock. For PCR, 10µM concentration of all primers was used.
### Table 6. PCR conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Annealing temp °C</th>
<th># of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>57</td>
<td>25</td>
</tr>
<tr>
<td>BECN1</td>
<td>61</td>
<td>29</td>
</tr>
<tr>
<td>BRCA1</td>
<td>57.3</td>
<td>28</td>
</tr>
<tr>
<td>BRCA2</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>GPX3</td>
<td>60</td>
<td>32</td>
</tr>
<tr>
<td>HMOX1</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>NQO1</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>NRF2</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>SESN2</td>
<td>60</td>
<td>28</td>
</tr>
<tr>
<td>SOD2</td>
<td>57</td>
<td>32</td>
</tr>
<tr>
<td>TP53I3</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td>TP53INP1</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td>TXNRD1</td>
<td>60</td>
<td>32</td>
</tr>
<tr>
<td>p53</td>
<td>50</td>
<td>30</td>
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</table>
### Table 7. Sequences of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>TCCTTCCTGGGCATGGAG</td>
<td>AGGAGGAGCAATGATCTTGATCTT</td>
<td>[140]</td>
</tr>
<tr>
<td>BECL1</td>
<td>TCCAGGAACTCACAGCTCCA</td>
<td>GCTGTTGGGCACTTTTCTGTTG</td>
<td>Designed</td>
</tr>
<tr>
<td>BRCA1</td>
<td>TATTTCGGAGGTGCTGTTTAC</td>
<td>CTAAAAAACCCCAACCACTATCCC</td>
<td>[141]</td>
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<tr>
<td>BRCA2</td>
<td>CAAGCAAGATGATGTTTCCCTGC</td>
<td>AGAACTAAAGGCTGTTGTTGATGC</td>
<td>Wu et. al. 2003</td>
</tr>
<tr>
<td>GPX3</td>
<td>ATGACTGACGTTCCCCGAGAAG</td>
<td>GTCACATTCGGCGTGTTTGA</td>
<td>Designed</td>
</tr>
<tr>
<td>HMOX1</td>
<td>TCTCTTGCTGCTCTCTCTTA</td>
<td>ACTCAGGGCTTTGGGAGGTT</td>
<td>Designed</td>
</tr>
<tr>
<td>NQO1</td>
<td>AAAACACTGCCCCCTTTTGGG</td>
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<td>Designed</td>
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<tr>
<td>NRF2</td>
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<tr>
<td>SESN2</td>
<td>TGCTCTCTTCTCTGACAGTT</td>
<td>CCTCTCTCTCTCTAGCACC</td>
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</tr>
<tr>
<td>SOD2</td>
<td>TTCAATAAGGAAGGGGGCAACA</td>
<td>ACACATCAATCCCAGCAGT</td>
<td>Designed</td>
</tr>
<tr>
<td>TP53I3</td>
<td>TAGCCGTGCACTTTGACAAG</td>
<td>ATGCCTCAAGCTCCCAATG</td>
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</tr>
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<td>TP53INP1</td>
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<td>TGAGCAGAAGCACTCAAGAAGA</td>
<td>Designed</td>
</tr>
<tr>
<td>TXNRD1</td>
<td>GCAGCACTGAGTGCTGAAAAA</td>
<td>TGCCCTCAATTGCTCTCTCCT</td>
<td>Designed</td>
</tr>
<tr>
<td>p53</td>
<td>CCATGGCCATCTACAAGGAG</td>
<td>AGGGTGAAAATATCTCC</td>
<td>Designed</td>
</tr>
</tbody>
</table>
Comet assay

*Slide preparation:* The comet assay was performed as previously described [142]. Briefly, microscope slides were coated with 1% Normal Melting Agarose (NMA) (ONBIO) in DI. 1 X $10^4$ cells in 10µL mixed with 75µL of 0.5% Low Melting Point Agarose (Sigma) solubilized in PBS was added to the NMA coated slide and protected by a coverslip. The slides were placed on a tray resting on ice until the agarose layer hardened. Cells were lysed by incubating the slides in lysing solution (2.5mM NaCl + 100mM EDTA + 1mM Tris) at 4°C for 2 hours.

*Electrophoresis:* Slides were placed in gel box filled with 1X TAE buffer for 20 minutes before electrophoresis. Electrophoresis was performed at 24 volts for 30 minutes in cold room. The slides were then stained with SYBR Green I (Invitrogen) for about 30 minutes with gentle agitation at room temperature. The slides were visualized with a fluorescent microscope (Zeiss).

Flow cytometry

ROS was measured by probing for Di-Chloro-Fluorescein (DCF) levels using flow cytometry. A 250 µM solution of CM-H$_2$DCFDA (Invitrogen) was prepared by resuspending the CM-H$_2$DCFDA powder in DMSO prior to each experiment. Media was aspirated from the plate and adherent cells were washed twice with 1X PBS. Adherent cells were incubated with 250 nM of CM-H$_2$DCFDA at 37°C for 30 minutes. After the incubation with CM-H$_2$DCFDA, the cells were washed with 1X PBS, trypsinized and resuspended in cold 1X PBS and kept in dark until ready for flow cytometry analysis. A FACS Calibur flow cytometer was used for measuring DCF levels and FlowJo software was used for data analysis.

Immunofluorescence (IF)

*Sample preparation:* HMECs were seeded on coverslips in a 24 well plate and were infected with LKO and shBRCA1. IF was done on control cells on the day of infection and on HMEC-shBRCA1 every post-infection until day 6. The samples were prepared by washing the cells with 1X PBS and then fixing with 3% paraformaldehyde (PFA) for 10 minutes at 4°C. The cells were then washed twice with 10mM glycine in PBS. To permeabilize, the cells were incubated with 1mL 0.2% Triton X-100 for 5 minutes at room temperature (RT), after which they were washed twice with glycine/PBS.
**Antibodies:** p53 primary antibody was used from Santa Cruz. The secondary antibody was Alexa Fluor 488 Goat anti-Mouse IgG1 from Invitrogen.

**Immunocytochemistry (ICC):** To block, cells were incubated with 2% BSA (in PBS) for 30 minutes at RT. The primary antibody, 1:200 of p53, was then applied to the cells for 1 hour at RT. The cells were then washed twice with glycine/PBS, for 10 minutes each wash. The cells were incubated with 1:200 secondary antibody for 1 hour at RT and then washed with glycine/PBS twice for 10 minutes. After incubation with antibodies, the cells were stained with 1:1000 Hoechst 33342 for 10-15 minutes at RT and then washed with PBS containing 0.05% tween for 10 minutes at RT. Coverslips were mounted on slides with 2 µL mounting media/coverslip (Vector Laboratories) and nail polish was used to seal the edges of the coverslips. Cells were visualized with a fluorescent microscope (Zeiss). Nuclei were visualized using DAPI (Invitrogen) at 1:1000 dilution.

**Microarray analysis**

**Sample preparation:** HMECs were infected with LKO and shBRCA1 and cultured in media containing 2µg/µL puromycin. The cells were harvested on day 6 post infection and cell pellets were snap frozen.

**RNA isolation:** RNA was isolated using the column extraction methods. Columns were obtained from the RNeasy Qiagen kit. Manufacturer’s instructions were followed for RNA isolation.

**Bioanalyzer:** Agilent 2100 Bioanalyzer was used to determine RNA integrity of the samples. Agilent RNA 600 Nano kit containing microfluidic chips were used and manufacturer’s instructions were followed.

**Microarray center:** Affymetrix whole genome U1332A arrays were used. Samples were sent for array profiling at the UHN Microarray Facility.
Electron microscopy (EM)

Sample preparation: HMEC-LKO and HMEC-shBRCA1 were seeded on Thermonox plastic cover slips in a 24 well plate and on day 1 post-infection. Cells were cultured for 6 days post-infection.

Mt Sinai lab center: Sample preparation and analysis for EM was done at the Advanced Bioimaging Centre.
Chapter 3: Results

Loss of BRCA1 in normal mammary epithelial cells induces cellular senescence

Modeling BRCA1 loss of function

To study consequences of loss of BRCA1, we chose to perform our experiments using normal Human Mammary Epithelial Cells (HMEC) from disease free-reduction mammoplasty tissue (RM). In vitro propagation of primary HMECs is an established model for studying early events in breast carcinogenesis, first described in 1980 [136, 137]. As previously described, reduction mammoplasty tissue is obtained from consenting patients undergoing breast reduction or reconstruction (Fig. 2A). Live tissue is first mechanically disrupted followed by overnight enzymatic digestion with hyaluronidase and collagenase. Digested tissue is filtered through a series of 2 meshes. The filtrate is enriched for mesenchymal cells including immune cells, fibroblasts and endothelial cells. Tissue clusters of organoids larger than the meshes (50µm) are enriched for mammary epithelial cells. When these organoids are plated, primary mammary epithelial cells are encouraged to proliferate in a serum-free defined media. Proliferating early passage epithelial populations represent both luminal and basal cells, as indicated by expression of luminal markers such as CK18 and basal markers such as CK17 (Fig. 2B). As the cells are passaged in culture they are pre-dominantly CK17 positive and show markers of basal progenitor cells such as CD49+ and EpCam- (data not shown).

In culture, HMECs grow for approximately 10 population doublings before reaching a p16-dependent growth arrest [143]. This growth plateau has been termed stasis. After 30 to 45 days, approximately 1 in 10^6 cells escape stasis via p16 promoter hypermethylation [144]. These cells proliferate for an additional 30-60 population doublings until reaching a second senescence growth plateau due to telomere erosion [145]. The second growth plateau has been called agonescence [146] and can be bypassed by reactivation of human telomerase [147]. The natural lifespan of HMECs in culture is unique in that senescence induction occurs twice through two mechanistically different pathways.

To mimic BRCA1 loss of function in normal mammary epithelium, we genetically targeted down regulation of BRCA1. A lentiviral construct, pLKO, harboring a short hairpin
Figure 2. Isolation and propagation of human mammary epithelial cells (HMEC).

A. Schematic representation of how breast tissue is processed in the laboratory to generate primary non-immortalized, non-tumorigenic cell lines.

B. Immunofluorescence staining with cytokeratin 17 (red), cytokeratin 18 (green) and DAPI (blue) in pre-senescent primary mammary epithelial cells generated from normal breast tissue.
targeting BRCA1 mRNA was designed in the lab (shBRCA1 #1). Four additional lentiviral short hairpins against BRCA1 were also used to validate results where indicated and were generously provided by Dr. Moffat (TRC). HMECs stably expressing each short hairpin were selected by propagation in culture media containing 4µg/ml puromycin. RNA and protein was isolated 5 days post-infection. BRCA1 mRNA expression was determined by rtPCR and protein levels were determined by Western blot analysis (Fig. 3A). All five short hairpins showed successful down regulation of BRCA1 mRNA. BRCA1 protein levels were also down regulated in cells prepared from HMEC-shBRCA1 #1 (Fig. 3B).

Chappuis et. al. [148] first reported that tumors that occur in BRCA1 mutation carriers are hypersensitive to platinum therapy. There is increasing evidence from both in vitro and in vivo studies that loss of BRCA1 renders increased sensitivity to carboplatin [122, 149]. To determine if using lentiviral knock down of BRCA1 can recapitulate the functional effect of loss of BRCA1, we treated MDA-MB-231 cells stably expressing shBRCA1 with carboplatin. Lentiviral down regulation of BRCA1 in MDA-MB-231 resulted in increased sensitivity to carboplatin treatment compared to treated LKO control cells (Nagao and Gauthier, unpublished data). This result confirmed that down regulation of BRCA1 functionally recapitulates BRCA1 loss of function.

**Loss of BRCA1 results in growth arrest**

We determined the consequences of loss of BRCA1 in HMECs by first assessing growth kinetics following BRCA1 down regulation. To do this, we analyzed population growth by calculating population doublings over time. Cells were seeded in 6 well plates and then infected after 3 days with control HMEC-LKO and with a short hairpin to BRCA1. The cells were then counted every 3 days. Three separate growth curves with LKO controls were performed and all 5 previously tested short hairpins to BRCA1 were used to generate 8 lines in total. Cells were manually counted and population doublings were calculated.

We found that loss of BRCA1 resulted in an immediate population growth arrest as compared with control cells (Fig. 4A). We assessed the population growth of two different controls, empty vector LKO and shLuciferase (shLuc). Cells were infected with each control
Figure 3. Lentiviral mediated down regulation of BRCA1.

A. rtPCR of BRCA1 and B-actin following stable lentiviral integration of shBRCA1 #1

B. Western blot of BRCA1 and B-actin following stable lentiviral integration of shBRCA1 #2 through #5
Figure 4. Loss of BRCA1 results in growth arrest in the absence of cell death.

A. Population doublings were calculated for vector control (LKO) cells and cells expressing 5 different short hairpins against BRCA1.

B. Population doublings for HMEC stably expressing LKO or shLuciferase.

C. Cell cycle analysis of HMEC-LKO (black) and HMEC-shBRCA1#1 (grey) following BrdU labeling, PI staining and flow cytometry analysis. The percent cells in G1, S and G2 phases of the cell cycle are shown.

D. Analysis of cell death of HMEC-LKO (black) and HMEC-shBRCA1 #1 (grey) following Annexin V staining and flow cytometry. The percent cells staining positive for Annexin V staining is shown.
and then allowed to undergo selection. On day 5 post-infection, cells were seeded in 6 well plates and counted. We found that both HMEC-LKO and HMEC-shLuc have similar growth kinetics (Fig. 4B). We next determined if the observed population growth arrest in the HMEC-shBRCA1 was due to cell cycle arrest or apoptosis. To determine the cell cycle profile following loss of BRCA1, HMEC-shBRCA1 and HMEC-LKO, on day 5 post-infection were pulsed with 10uM BrdU for 4 hours, and stained with PI. BrdU and PI content were analyzed by flow cytometry. We found that only 1% of cells with loss of BRCA1 showed DNA in the S-phase compared to 16% of LKO control cells (Fig. 4C). The remaining DNA was distributed between G1 and G2 phases of the cell cycle. The growth kinetics and BrdU/PI analysis demonstrated an absence of proliferation in HMEC-shBRCA1 cells. We next determined if BRCA1 loss affected cell death by Annexin V and Propidium Iodide (PI) staining. Quantification of Annexin V fluorescence by flow cytometry demonstrated insignificant death in response to loss of BRCA1 (Fig. 4D). All this data supports that BRCA1 loss induces arrest.

**Loss of BRCA1 results in senescence**

A hallmark of senescence is irreversible growth arrest in the absence of cell death. *In vitro*, senescent cells adopt characteristic morphological features which include becoming large, flat, and vacuolated. Upon a close examination of the morphological appearance of HMEC following BRCA1 down regulation, we observed the cells were large, flat and vacuolated (Fig. 5A). To determine if loss of BRCA1 in HMEC induces senescence, we performed β-galactosidase staining, a standard assay for senescence. β-galactosidase is a lysosomal enzyme whose enzymatic activity can be detected by monitoring the cleavage of X-gal substrate, in a colorimetric assay, with cells of interest [150]. In proliferating cells, β-galactosidase activity can be visualized at pH 4. In senescent cells, β-galactosidase significantly increases and can also be visualized at pH 6, a level at which β-galactosidase activity is undetected in proliferating and quiescent cells [151]. This allows for selective positive staining of senescent cells.

On day 5 post-infection, we found that cells with loss of BRCA1 stained strongly positive for β-galactosidase activity (Fig. 5B). Quantification of the positive staining was done by counting 3 fields of a minimum of 100 cells per field that were positive (blue) or negative (no
Figure 5. Loss of BRCA1 results in senescence.

A. Phase contrast images of control cells (HMEC-LKO) and HMEC-shBRCA1. The scale bar is 100µm.

B. β-galactosidase staining (blue) on HMEC-LKO and HMEC-shBRCA1 5 days post-infection.

C. Quantification of β-galactosidase positive stained cells in HMEC-LKO and HMEC-shBRCA1 5 days post-infection.
stain) and then calculating % positive cells (Fig. 5C). 80% of HMEC-shBRCA1 cells stained positive for β-galactosidase compared to 8% of control HMEC-LKO cells. Taken together, our data has demonstrated that loss of BRCA1 in primary human mammary epithelial cells induces senescence.

Loss of BRCA1 induced senescence is unique to normal epithelial cells

We established that loss of BRCA1 induces senescence in primary mammary epithelial cells. To determine if loss of BRCA1-induced senescence is unique to HMEC, we down regulated BRCA1 in 3 primary cell lines and in 2 immortalized, non-tumorigenic cell lines. HMECs and NMFs (Normal Mammary Fibroblasts) were isolated and propagated in the lab from RM specimens. PrECs (Prostate Epithelial Cells) were obtained from Clonetics. IMR90 (lung embryonic cells), 184A1 (immortalized mammary cells), and MCF10A (immortalized mammary cells) cell lines were obtained from ATCC. All cell types were infected with control LKO and shBRCA1 and allowed to pass through puromycin selection. β-galactosidase staining was performed on day 5 post-infection. Cell morphology of all cell lines was analyzed and images were captured 5 days post-infection. In response to loss of BRCA1, all the primary cell lines (HMEC, PrEC, NMF, and IMR90) displayed morphological features of senescence: cells were large, flat, vacuolated, non-refractive and displayed increased β-galactosidase activity (Fig. 6). Immortalized cell lines, 184A1 and MCF10A, did not acquire senescence morphology, nor did they show increased β-galactosidase activity. To determine if these cell lines were sensitive to senescence induction, 184A1 and MCF10A were treated with 1µM doxorubicin for 2 hours and allowed to recover for 72 hrs. β-galactosidase staining was performed and both cell lines showed increased staining (Fig. 6B). These findings strongly suggest that primary cells senesce in response to loss of BRCA1 while immortalized cells, even though have maintained the senescence barrier, are refractive to BRCA1 loss senescence induction.

HMEC display unique senescence-associated phenotypes

1) Senescence is associated with p53 down regulation

Loss of BRCA1 induces p53 down regulation
Figure 6. Loss of BRCA1 induced senescence is unique to normal primary cells.

A. Phase contrast images of control LKO and shBRCA1-expressing HMEC (Human Mammary Epithelial Cells), PREC (primary human prostatic epithelial cells), NMF (normal human mammary fibroblasts), IMR90 (normal human lung embryonic fibroblasts), 184A1 (immortalized non-tumorigenic human mammary epithelial cells), and MCF10A (immortalized non-tumorigenic human mammary epithelial cells) with corresponding quantification of β-galactosidase staining. The scale bar equals 100µm. The micrographs and β-galactosidase staining were performed 5 days post-infection.

B. β-galactosidase quantification 184A1 and MCF10A control (Ctrl) and 1µM doxorubicin (Dox) treated cells. Cells were treated for 2 hr and then cultured for 3 days before β-galactosidase staining was performed.
Figure 7. Loss of BRCA1 results in the down regulation of p53 protein levels.

A. rtPCR of BRCA1 and B-actin in HMEC-LKO (control) and HMEC-shBRCA1.

B. Western blot analysis of p16, p15, p53, p21 and B-actin.

C. Western blot analysis of p53 and B-actin in HMEC-LKO and HMEC stably expressing 4 different BRCA1 short hairpins (shBRCA #2 through #5).
p53 and RB are the two dominant pathways known to regulate senescence induction [63, 66]. It has been shown that p53 stabilization precedes RB hypo-phosphorylation during senescence induction and is sufficient for senescence to occur [152]. p53 protein upregulation is considered a central event in senescence induction. Stabilization of p53 results in transcriptional upregulation of a number of downstream targets including, p21 [125], also known to be essential for senescence induction [153]. RB hypo-phosphorylation is maintained through increased p16 levels [154]. We sought to determine the expression levels of cell cycle inhibitors in HMEC in response to loss of BRCA1.

Control HMEC-LKO and HMEC-shBRCA1 were harvested 5 days post-infection. RNA from these samples was isolated and rtPCR for BRCA1 and B-actin was done to confirm KD of BRCA1 (Fig. 7A). Protein was isolated from these samples and western blots were done to probe for major cell cycle proteins (Fig. 7B). We found that in HMEC, loss of BRCA1 resulted in increased p15. p16 protein levels are absent because we have used variant HMECs that have escaped senescence by p16 hypermethylation. These observations are consistent with previous findings [103, 155]. In contrast, we found that p53 and p21 levels were surprisingly down regulated. Previous studies using MEF show that loss of BRCA1 induced senescence is associated with p53 and p21 upregulation [156]. To ensure that down regulation of p53 in response to loss of BRCA1 was not due to an off target effect, we down regulated BRCA1 using 4 additional short hairpins (shBRCA1 #2 through #5) and harvested cells 5 days post infection. Protein lysates were probed for p53 and B-actin by western blot (Fig. 7C). We were able to reproduce the results that were generated using the first short hairpin, further validating that p53 protein levels are down regulated in response to loss of BRCA1. Down regulation of p53 in a senescent cell, particularly in response to loss of BRCA1, is a novel observation. To further understand our finding, we sought to elucidate how p53 was being down regulated in loss of BRCA1 senescent cells.

$p53$ down regulation correlates with cytoplasmic shuttling

Our analysis of p53 protein levels was performed 5 days following lentiviral expression of shBRCA1, when senescence is maximally achieved. To determine the kinetics of p53 down regulation during senescence, we measured p53 protein levels every 24 hours for 5 days.
Figure 8. p53 down regulation is concurrent with the induction of senescence phenotype and correlates with cytoplasmic shuttling.

A. HMEC-LKO collected at day 5, HMEC-shBRCA collected every 24 hrs for 5 days (D1,D2,D3,D4,D5) were probed for BRCA1 and B-actin by rtPCR and for p53 and B-actin by western blot.

B. Quantification of β-galactosidase staining of HMEC-LKO cells and successive HMEC-shBRCA1 cells from D1 of shBRCA1 expression through D5.

C. rtPCR for p53 and B-actin in HMEC-LKO and HMEC-shBRCA1 cells collected at day 5.

D. Phase contrast (with scale bar equals 50µm) and p53 immunofluorescence images of HMEC-shBRCA1 captured on Day 0 of shBRCA1 expression through Day 5.

E. Quantification of nuclear (N) and nuclear/cytoplasmic (N/C) localization of p53 immunofluorescence from D0 through D5 following shBRCA1 infection.
following lentiviral infection of cells with shBRCA1. rtPCR of BRCA1 and B-actin was done to confirm KD in HMEC-shBRCA1. We observed a gradual decrease in BRCA1 levels that was maximally achieved by day 3 (Fig. 8A). Similarly, we observed that p53 protein levels consistently decreased over time and corresponded with increasing levels of β-galactosidase activity and the appearance of senescence morphology (Fig. 8B, D). It is intriguing that in cells with loss of BRCA1, p53 levels are consistently down regulated prior to the onset of senescence. Down regulation of p53 could occur either at the transcriptional level with reduced p53 mRNA or it could occur at the post-translational level through p53 protein degradation [157, 158]. To determine if the down regulation of p53 is due to transcriptional regulation, we measured p53 mRNA levels. We did rtPCR for p53 and B-actin on HMEC-shBRCA1 and HMEC-LKO at D5 post-infection. We found no difference in p53 mRNA levels (Fig 8C), indicating that p53 down regulation following BRCA1 loss occurs in a transcriptionally independent manner.

Nuclear localization is essential for p53 transcriptional activity of cell cycle regulatory proteins [159, 160]. When p53 is sequestered into the cytoplasm and is in its un-phosphorylated form [161], it gets ubiquitinated by MDM2 and degraded [162]. As such, we determined if down regulation of p53 occurs at the post-translational level. We hypothesized that p53 would have to be mislocalized into the cytoplasm for post-translational modification to occur. To determine the p53 sub-cellular localization during senescence induction, we performed p53 immunofluorescence (IF) from Day 0 through Day 5 of BRCA1 KD (Fig. 8D). IF images along with IF quantification (Fig. 8E) shows that as the cells senesce, p53 is shuttled to the cytoplasm. p53 mislocalization in response to senescence induction or loss of BRCA1 has not been previously demonstrated. We concluded from these results that p53 down regulation occurs concurrently with cytoplasmic shuttling and senescence induction.

**p53 down regulation occurs through a proteosome mediated degradation**

We have so far uncovered that p53 levels are down regulated in response to loss of BRCA1 and that this down regulation occurs in parallel with cytoplasmic shuttling of p53 (Fig. 7, 8). Ubiquitination of p53 has been shown to trigger p53 nuclear export and protein
Figure 9. Loss of BRCA1 results in post-translational down regulation of p53

A. HMEC-LKO and HMEC-shBRCA1 cells treated with 25nM of leptomycin B and collected on D1, D3, and D5 were probed for p53 and B-actin by Western blot.

B. HMEC-LKO and HMEC-shBRCA1 cells treated with 10uM MG132 for 4 hrs were probed for p53, p21, and B-actin by Western blot.
degradation [163]. To determine if cytoplasmic shuttling is required for p53 degradation, we inhibited nuclear export in our cells by treating them with Leptomycin B. Leptomycin B inhibits the nuclear export of proteins by inhibiting CRM1 (re-named Exportin 1), which is an essential nuclear export protein [164]. CRM1 (Chromosome Region Maintenance 1) was first identified in yeast [165] and later its human homolog was found to bind to a protein and move between the nuclear pores and nucleoplasm suggesting that the human homolog might be a nuclear transport factor [166]. Leptomycin B works by inhibiting the binding of CRM1 with the nuclear export signal (NES) of proteins [164, 167].

HMEC-LKO and HMEC-shBRCA1 cells were incubated with 25nM Leptomycin B one day post-infection and collected on D1, D3, and D5. Protein lysates were prepared from all 6 samples. Western blots probing for p53 and B-actin showed that in untreated HMEC-shBRCA1 cells, p53 levels were down regulated, while leptomycin B treatment abrogated p53 protein down regulation (Fig. 9A). This supports our hypothesis that in response to loss of BRCA1, p53 is shuttled to the cytoplasm for degradation.

To determine if down regulation of p53 is mediated through proteasome degradation, we measured p53 levels following exposure to the proteasome inhibitor, MG132. MG132 is a potent, cell permeable inhibitor that blocks the proteolytic activity of 26S proteasome [168]. Protein lysates were prepared from cells incubated with 10µM of MG132 for 4 hours and probed for p53, p21, and B-actin by western blot (Fig. 9B). p53 and p21 levels in untreated HMEC-shBRCA1 cells were down regulated as seen previously. However, in MG132 treated HMEC-shBRCA1 cells, p53 and p21 levels were significantly elevated. Thus, proteasome inhibition by MG132 treatment abrogates p53 down regulation, suggesting that p53 down regulation in response to BRCA1 loss is mediated post-translationally through a proteasome mediated mechanism.

*p53 down regulation in response to loss of BRCA1 induced senescence is unique to epithelial cells*

Our observations that loss of BRCA1 in human epithelial cells induces p53 protein down regulation is in contrast to previous reported studies using MEFs [156]. To elucidate the discrepancy between these results, we sought to determine if p53 regulation in response to
BRCA1 loss is cell type dependent. We induced down regulation of BRCA1 in 2 different primary fibroblast cell lines, IMR90 and NMFs. BRCA1 was down regulated in these cells using our lentiviral short hairpin against BRCA1. Cells were cultured in DMEM with 10% serum and harvested on Day 5 post-infection. Protein was isolated to determine the expression level of BRCA1 and cell cycle proteins. Similar to our observations in HMEC, we found that in response to BRCA p16 and p15 protein levels were upregulated (Fig. 10). In contrast, the expression of p53 and p21 were increased in response to loss of BRCA1 (Fig.10). By comparing the responses to loss of BRCA1 in fibroblasts and epithelial cells, we have uncovered that the down regulation of p53 is unique to epithelial cells.

**p53 down regulation in response to loss of BRCA1 in epithelial cells is not unique to loss of BRCA1**

To determine if p53 protein down regulation in response to loss of BRCA1 in epithelial cells is unique to loss of BRCA1 induced senescence, we measured p53 protein levels in HMEC undergoing replicative senescence and premature senescence. HMECs were allowed to reach replicative senescence by serial passaging. Premature senescence was induced in two ways. The first, by overexpression of oncogenic hRAS\textsuperscript{V12} through stable retroviral integration. Second, by down regulation of BRCA2 by stable integration of a lentiviral short hairpin against BRCA2. Stable HMEC lines over expressing hRAS\textsuperscript{V12} and HMECs with stable KD of BRCA2 were generated and analyzed. Replicative senescent HMEC (HMEC-RS), HMEC-hRAS\textsuperscript{V12}, and HMEC-shBRCA2 all acquired a senescent cell morphology by day 5 post-infection (Fig. 11A) and showed significant upregualtion of β-galactosidase staining (Fig. 11B). Western blot analysis demonstrated that p53 protein levels were down regulated in all senescent HMECs: replicative senescent HMEC (Fig. 11C), hRAS\textsuperscript{V12} overexpressing HMEC (Fig. 11D), and BRCA2 KD HMEC (Fig. 11E). We found that p21 levels remained unchanged in these senescent cells. We concluded that the down regulation of p53 occured in response to senescence in epithelial cells and was independent of the inducer of senescence.

2) **Senescence occurs in the absence of DNA damage**

DNA damage has been previously demonstrated to be important for senescence induction and has been shown to occur either through telomere shortening (RS) or over
Figure 10. p53 down regulation in response to BRCA1 is unique to epithelial cells

NMF and IMR90 cells were infected with stable lentiviral constructs of control LKO and shBRCA1 and harvested 5 days post-infection. NMF-LKO and NMF-shBRCA1, and IMR90-LKO and IMR90-shBRCA1 were probed for BRCA1, p16, p15, p53, p21, and B-actin by Western blot.
Figure 11. Replicative and premature senescence in HMEC results in p53 down regulation.

A. Micrograph images with scale bar of 100µm of HMEC-control cells, replicative senescent HMEC, HMEC-hRAS\textsuperscript{V12}, and HMEC-shBRCA2.

B. Quantification of β-galactosidase staining of HMEC-control cells, replicative senescent HMEC, HMEC-hRAS\textsuperscript{V12}, and HMEC-shBRCA2 on Day 5 post-infection.

C. Western blots of HMEC-proliferating cells (control) and HMEC-replicative senescent probing for p53, p21, B-actin.

D. Western blots of HMEC-pBabe(control) and HMEC-hRAS\textsuperscript{V12} probing for RAS, p53, p21, and B-actin.

E. rtPCR of HMEC-LKO and HMEC-shBRCA2 probing for BRCA2. Western blots of p53 and B-actin.
Figure 12. Loss of BRCA1 induced senescence is independent of DNA damage.

A. Western blot analysis of p53, pATM, pCHK2, pH2AX, 53BP1, and B-actin in HMEC-LKO and HMEC-shBRCA1.

B. Comet assay to probe for damaged DNA in HMEC-LKO, HMEC-shBRCA1 cells, and of control and etoposide treated HeLa cells.

C. HMEC-LKO and HMEC-shBRCA1 cells were treated with 0.5uM of doxorubicin for 6 hours with or without recovery for 24 hours and were probed for pCHK2, p53, p21, and B-actin by Western blot.
expression of oncogenes. When telomere erosion reaches a critical length, a DNA damage response is induced to trigger terminal replication or replicative senescence [94]. Expression of oncogenes such as RAS cause hyper-proliferation of cells, resulting in DNA adducts that also trigger a DNA damage response to limit the propagation of damaged cells [97]. DNA Damage Response (DDR) proteins such as CHK2 [69, 70] and ATM [169] phosphorylate p53 to inhibit proliferation and induce senescence. p53, CHK2 and ATM have all been demonstrated to be necessary for senescence as loss of any of these proteins render cells refractive to senescence in response to oncogenic RAS [97, 170]. Fig. 7 demonstrates that loss of BRCA1-induced senescence is associated with down regulation of p53, thus raising the possibility that DDR may not play a central role in premature senescence in HMEC.

To determine if DNA damage is activated in response to loss of BRCA1, we performed Western blot analysis of DNA damage response proteins. Protein lysates from HMECs with loss of BRCA1 and vector control were probed for p53, DDR proteins, and B-actin (Fig. 12A). p53 protein levels were down regulated in response to loss of BRCA1. DDR proteins, pATM, pCHK2, p-H2AX, and 53BP1, were all surprisingly down regulated in response to loss of BRCA1 (Fig. 12A). To determine if the down regulation of DDR proteins was due the absence of DNA damage, we examined the extent of DNA damage using the comet assay. In this assay, cells with damaged DNA form a smear on the gel referred to as comet tail. HeLa cells treated with etoposide did form comet tails. HMEC-shBRCA1 did not show comet tails, suggesting the absence of DNA damage in response to loss of BRCA1 (Fig. 12B).

The down regulation of DDR proteins promoted us to determine if cells with BRCA1 loss of function remain responsive to DNA damage. HMEC-LKO and HMEC-shBRCA1 were exposed to the double-strand break inducing agent, doxorubicin, and were either harvested 6 hr after treatment or were allowed to recover from doxorubicin exposure for an additional 24 hr in regular media. Protein lysates were prepared and Western blots were done probing for pCHK2, p53, and B-actin. In HMEC-LKO, we observed a robust upregulation of pCHK2, p53, and p21 (Fig. 12C) in response to doxorubicin exposure. HMEC-LKO treated with doxorubicin and allowed to recover for 24 hours showed levels of pCHK2 and p53 similar to those in untreated cells, suggesting that DNA repair had taken place. Doxorubicin treatment of HMEC-shBRCA1 cells showed a similar pattern of upregulation of pCHK2 and p53. pCHK2 and p53 levels also
Figure 13. ROS upregulation is unique to loss of BRCA1 induced senescence.

A. ROS levels were determined by DCF fluorescence in HMEC-LKO and HMEC-shBRCA1. Corresponding quantification is expressed as fold increase over control. Significance was calculated using paired Student t-test.

B. ROS levels were determined by DCF fluorescence in HMEC-pBabe and HMEC-HRAS<sup>V12</sup>. Corresponding quantification is expressed as fold increase over control.

C. ROS levels were determined by DCF fluorescence in proliferating HMEC (prolif.) and replicative senescent HMEC (RS). Corresponding quantification is expressed as fold increase over control.
returned to baseline after 24 hr recovery. Interestingly, p21 protein levels were not up-regulated in loss of BRCA1 treated cells suggesting BRCA1 may coordinately regulate p53 downstream genes. These findings support the work of others that demonstrate BRCA1 physically interacts with p53 and acts as a transcriptional co-regulator [126, 171]. Upregulation of p53 in response to doxorubicin treatment indicates that despite down regulation of pCHK2 and p53 in response to loss of BRCA1, cells remain responsive to exogenous damage.

3) Senescence is associated with ROS deregulation

*ROS deregulation is senescence inducer specific*

It has been previously demonstrated that ROS upregulation plays a causal role in both hRAS\textsuperscript{V12} induced senescence [172] and replicative senescence [73]. When cells were cultured in 1% O\textsubscript{2} or treated with scavengers of hydrogen peroxide, expression of oncogenic hRAS\textsuperscript{V12} was unable to trigger senescence [172]. Similarly, cells grown in 3% O\textsubscript{2} achieved 50% more population doublings than those grown in 20% O\textsubscript{2} [73]. Cells cultured at lower O\textsubscript{2} levels show diminished ROS, reduced DNA damage and show a prolonged life span compared to control cells [73].

We sought to determine if cellular senescence alters re-dox regulation in HMEC. Senescence was induced in HMECs by serial passaging cells in culture, by down regulation of BRCA1 and by over expression of oncogenic hRAS\textsuperscript{V12}. ROS levels were detected by DCF (dichlorofluorescein) fluorescence and were measured using flow cytometry. DCF is permeable to cell membranes and non-fluorescent in its reduced form (DCFDA). DCFDA oxidization to DCF results in fluorescence that can be quantified by flow cytometry analysis. We found that inducers of senescence differentially modulated ROS levels. Loss of BRCA1 resulted in upregulation of ROS (Fig. 13A). In contrast, replicative senescence and senescence induced by over expression of hRAS\textsuperscript{V12}, resulted in down regulation of ROS levels (Fig. 13B, C). Previous reports have consistently demonstrated that replicative and premature senescence is associated with an increase in ROS levels [73]. These conclusions were generated from studies using primary murine or human fibroblasts. Our data suggests that ROS regulation may be cell type dependent, in addition to senescence inducer specific.
Figure 14. p53 quenches ROS levels in response to BRCA1 loss.

A. Western blots probing for p53, p21 and B-actin in HMEC and HMEC-GSE22 treated with 0.5µM Doxorubicin (Dox) for 6 hours.

B. Western blots probing for p53, p21, and B-actin in HMEC-GSE-LKO and HMEC-GSE-shBRCA1.

C. ROS levels measured by DCF florescence in HMEC-LKO, HMEC-shBRCA1, HMEC-GSE-LKO and HMEC-GSE-shBRCA1. DCF fluorescent levels are expressed as fold change compared to control cells. Significance was calculated using paired Student t-test.

D. Trypan blue analysis of HMEC-LKO, HMEC-shBRCA1, HMEC-GSE22, and HMEC-shBRCA1-GSE22.
p53 regulates ROS in response to loss of BRCA1

Recent studies have described p53 as having both a pro-oxidant and an anti-oxidant role. The pro-oxidant role of p53 can be observed in response to exogenous damage. For example, under severe stress p53 stabilization leads to transcription of pro-oxidant genes such as Bax and PUMA resulting in ROS accumulation and induction of senescence or apoptosis [173-175]. The antioxidant function of p53 has been primarily described in unstressed cells under basal conditions where p53 transcribes antioxidant genes to reduce ROS [176]. In other words, when p53 protein levels are reduced or at basal levels, it serves an antioxidant function.

Given that BRCA1 can co-regulate p53 target genes, we determined if p53 is involved in ROS regulation in response to BRCA1 loss [126]. p53 was inactivated by GSE22. GSE22 (Genetic Suppressor Element #22) is a cDNA fragment encoding a dominant negative peptide that corresponds to the C-terminal portion of the p53 protein, which regulates sequence specific DNA binding [177]. Thus, expression of GSE22 results in a stable, but functionally inactive form of p53. We show increased p53 levels and lower levels of p21 in HMEC-GSE22 compared to control cells (Fig. 14A). HMEC-GSE22 treated with 5μM Doxorubicin for 6 hours also fails to transcriptionally upregulate the p53 downstream target gene p21 compared with control HMEC (Fig. 14B). To determine the role of p53 in the regulation of ROS following BRCA1 loss, HMECs expressing stable integration of GSE22 were infected with LKO and shBRCA1. Protein lysates were prepared from HMEC-GSE22-LKO and HMEC-GSE22-shBRCA1. Western blots were done probing for p53, p21, and B-actin to confirm inactivation of p53 by GSE22. DCF levels were measured to determine the role of p53 in the regulation of ROS in the context of loss of BRCA1. We found that BRCA1 loss in HMEC-GSE22 led to a 3-fold increase in ROS levels compared to control GSE-LKO cells (Fig 14B). This increase was significantly higher than that observed in the presence of wild-type p53 (1.7 fold increase in ROS levels). These data demonstrate that in the absence of p53, ROS is significantly up-regulated and suggests that p53 acts to reduce ROS accumulation in response to loss of BRCA1. To determine the consequences of elevated ROS in the absence of p53 and BRCA1, we measured cell viability by trypan blue exclusion. We found that HMEC-GSE22-shBRCA1 show a significant increase in trypan blue staining compared with HMEC-shBRCA1 (Fig. 14C). These observations suggest that p53 plays a role in cell survival following BRCA1 loss by maintaining ROS levels below a threshold that induces cells death.
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Table 8. p53 target genes are up regulated in response to loss of BRCA1
p53 responsive antioxidant genes are up regulated in response to BRCA1 loss

Our findings that p53 regulates ROS in response to BRCA1 suggests loss of BRCA may induce a p53-dependent anti-oxidant program. We have found that p53 remains responsive to exogenous damage in the absence of BRCA1 (Fig. 12C). This indicates that even though p53 protein levels are down regulated in cells with loss of BRCA1, p53 may maintain transactivation capacity. To determine if loss of BRCA1 induces a unique p53-dependent transcriptional program, we analyzed p53-dependent genes in presence and absence of BRCA1. Whole genome Affymetrix expression array was done on HMEC-LKO and HMEC-shBRCA1 cells at the UHN Microarray facility. We identified p53 target genes previously published [178] and cross-referenced them to our microarray data. Among the 122 published p53 target genes, 32 genes were up-regulated more than 2 fold in HMEC lacking expression of BRCA1 (Table 8). The list of p53 target genes that are up regulated in loss of BRCA1 cells have a variety of known functions. These include: upregulation genes such as PCDH7 [179], which functions in cell adhesion and genes responsible for cytoskeleton regulation, such as ARHGAP5 [180] and CALD1. We also observed increased levels of genes involved in differentiation, namely, ENC1 and COL4A1. Of interest, we found several antioxidant genes among the genes that were significantly up-regulated, including SESN2, TP53I3, TP53INP1, HMOX1, and SOD2. SESN2 (Sestrin 2) was up regulated in response to loss of BRCA1 by 5.3 fold. SESN2 has also been shown to be a p53 target, [176] antioxidant gene [181]. TP53I3 (Tumor Protein 53 Inducible Protein 3) and TP53INP1 (Tumor Protein p53 Inducible Nuclear Protein 1) are also a known p53 target antioxidant genes known to be involved in apoptosis [182, 183]. In response to loss of BRCA1, TP53I3 is up-regulated 4.5 fold and TP53INP1 2.49 fold. HMOX1 (Heme Oxygenase (decycling) 1) expression was stimulated by a factor of 8.3 in cells with loss of BRCA1 compared to control cells. HMOX1 is a heme catabolizing enzyme that has previously shown to be p53-dependant [184] and has an antioxidant function [185]. SOD2 (Superoxide Dismutase 2), a well studied mitochondrial antioxidant gene, is also p53 dependent [186] and was up regulated in response to loss of BRCA1. These data suggest that BRCA1 loss results in transcriptional upregulation of several p53-dependent antioxidant genes.

We validated our microarray data by rtPCR and found increased mRNA expression of SESN2, TP53I3, TP53INP1, HMOX1, and SOD2 in response to BRCA1 loss (Fig 15). In order to validate that the increased expression of the antioxidant genes was dependent on p53, we
Figure 15. p53 dependent genes are up regulated in response to loss of BRCA1.

Western blots probing for p53 and actin in HMEC-LKO, HMEC-shBRCA1, and HMEC-shBRCA1-shp53 followed by rtPCR of antioxidant genes and B-actin in HMEC-LKO, HMEC-shBRCA1, and HMEC-shBRCA1-shp53.
measured mRNA expression of several genes in the absence of p53 and BRCA1. Three lines were generated: HMEC-LKO, HMEC-shBRCA1, and HMEC-shp53-shBRCA1. Cells were harvested 5 days post-infection, protein was isolated and Western blots were performed to confirm KD of p53 (Fig. 15). p53 was down-regulated in loss of BRCA1 cells and further down regulated in HMEC-shp53-shBRCA1 cells, indicating effective KD of p53. We found that HMOX1, SESN2, SOD2, TP53I3, and TP53INP1 mRNA expression levels were down regulated in the absence of p53, thereby confirming the role of p53 in transcriptional upregulation of these genes in response to BRCA1 loss. We also analyzed the expression of a number of other antioxidant genes shown to be up-regulated in response to BRCA1 loss in our microarray analysis, but not dependent on p53. These include GPX3, NQO1, TXNRD1 and NRF2. We find only GPX3 and NQO1 were transcriptionally up-regulated. We concluded that BRCA loss induces upregulation of several p53 dependent antioxidant genes that function to maintain ROS levels below an intrinsically defined threshold that induces apoptosis.

**SESN2 regulates ROS in response to BRCA1 loss**

To determine if SESN2, one of the most highly upregulated antioxidant genes in HMEC-shBRCA1, regulates stress induced ROS, we measured ROS levels in HMEC in response to BRCA1 loss in the absence of SESN2. Lentiviral short hairpins to BRCA1 and control LKO with blasticidin resistance were generated in the (designated shBRCA1(b) and LKO(b)). In this manner we were able to select for shBRCA1 and LKO with blasticidin and for shSESN2 and LKO with puromycin (designated shSESN2(p) and LKO(p)). Cells were double infected with all combinations of control LKO, shBRCA1, and shSESN2. One day after infection, cells were cultured in media containing 2µg/mL puromycin + 3µg/mL blasticidin. Four lines were generated: HMEC-LKO(b)-LKO(p), HMEC-LKO(p)-shBRCA1(b), HMEC-LKO(b)-shSESN2(p), and HMEC-shBRCA1(b)-shSESN2(p). On day 5 post-infection, cells were harvested and RNA was isolated to test KD. rtPCR was done for BRCA1, SESN2, and β-actin and confirmed KD (Fig. 16A). The morphology of cells with combined loss of BRCA1 and SESN2 showed evidence of senescence (Fig. 16B) and stained positive of β-galactosidase (data not shown). We measured DCF levels in all four lines and found that ROS levels were increased in response to loss of BRCA1 alone and SESN2 alone (Fig. 16C). Combined down regulation of
Figure 16. Sestrin 2 regulates ROS in response to BRCA1 loss.

A. rtPCR of BRCA1 and SESN2 in HMEC-LKO-LKO, HMEC-LKO-shBRCA1, HMEC-LKO-shSENS2, and HMEC-shBRCA1-shSESN2.

B. Phase contrast images with scale bar of 100µm of HMEC-LKO-LKO, HMEC-LKO-shBRCA1, HMEC-LKO-shSENS2, and HMEC-shBRCA1-shSESN2 on day 5 post-infection.

C. Quantification of DCF fluorescence measured by flow cytometry in HMEC-LKO-LKO, HMEC-LKO-shBRCA1, HMEC-LKO-shSENS2, and HMEC-shBRCA1-shSESN2.
BRCA1 and SESN2 showed a 4.5 fold increase in ROS levels. These data suggest SESN2 plays an important role in regulating ROS in cells following loss of BRCA1.

4) Loss of BRCA1 results in autophagy

Autophagy is a process involving the degradation of cellular components into macromolecules which can be recycled to allow for cell survival under conditions of stress. Autophagy was recently shown to play an important role in senescence induction and maintenance as the absence of an essential autophagy gene, ATG5, resulted in the delay of hRAS\textsuperscript{V12} induced senescence [187]. Microscopic analysis of HMEC-shBRCA1 cells shows an increase in the number of peri-nuclear vacuoles, a characteristic feature of cells undergoing autophagy. This observation led us to examine if autophagy was induced in loss of BRCA1 senescent cells. Electron microscopy (EM) allows for ultra structural imaging and is an accurate method for detecting autophagosomes [188]. Cells infected with shBRCA1 or LKO, were allowed to pass through puromycin selection and on Day 6 post-infection the cover slips on which the cells were grown were sent to Mount Sinai Laboratory Medicine for sample preparation and EM analysis. We detected abundant autophagosomes in HMEC-shBRCA1 compared to HMEC-LKO (Fig. 17A). The HMEC-shBRCA1 cells showed a striking phenotype; there were densely embedded with vacuoles, whereas the control cells had very few of these vacuoles. The different stages of the autophagosomes could not be distinguished; however, several autophagic vacuoles contained partially degraded cytoplasmic material, indicative of active autophagy. The EM photographs identified autophagy induction in response to loss of BRCA1. To further confirm autophagy induction, we measured other autophagy hallmarks such as LC3 lipidation and mRNA expression of autophagy regulatory genes. HMECs were infected with LKO and shBRCA1 and cells were harvested every 24 hours for 5 days following down regulation of BRCA1. RNA was isolated to confirm BRCA1 KD by rtPCR for BRCA1 and B-actin (Fig. 17B). Protein was isolated and probed for the autophagy marker, LC3 (microtubule-associated protein 1 Light Chain 3). LC3 plays an essential role in autophagosome formation [189] and has two forms, LC3-I and LC3-II [190]. The LC3 precursor gets cleaved forming LC3-I, which is a soluble form that localizes to the cytosol [190]. During autophagy initiation, LC3-I gets lipid conjugated to form LC3-II [191], which is very stable and is localized to the autophagosome membrane and is, hence used as a marker of autophagy [190, 192].
Figure 17. Loss of BRCA1 results in the induction of autophagy.

A. EM micrographs of HMEC-LKO and HMEC-shBRCA1 on day 6 post-infection.
B. rtPCR of BRCA1 and B-actin in HMEC-LKO and HMEC-shBRCA1 cells collected every 24 hours for 5 days post-infection. Western blots for LC3 in HMEC-LKO and HMEC-shBRCA1 cells collected every 24 hours for 5 days post-infection.
C. rtPCR of BRCA1, BECL1, and B-actin in HMEC-LKO and HMEC-shBRCA1 collected on day 5 of infection.
D. Data from expression array showing fold increase in expression of autophagy regulatory genes in cells with loss of BRCA1
Figure 18. Loss of BRCA1 results in p53 dependent autophagy induction.

A. Schematic diagram showing p53 induction of autophagy by suppression of mTOR.

B. HMEC cells expressing stable integration of GSE22 were infected with LKO (or shLuc) and shBRCA1 and then harvested after day 5 of infection. Western blots probing for p53, p21, AMPK, pAMPK, B-actin and LC3 and its corresponding B-actin in HMEC-LKO, HMEC-shBRCA1, HMEC-GSE22-LKO, HMEC-GSE22-shBRCA1.
observed a consistent increase in LC3-II levels following down regulation of BRCA1 (Fig. 17B). To further validate that autophagy is induced, we determined the expression levels of other autophagy regulatory genes. We found that BECN1 expression was increased in loss of BRCA1 cells (Fig. 17C). We also found 8 other autophagy regulatory genes that showed increased expression in cells with loss of BRCA1 (Fig. 17D). These genes include the autophagy initiating gene, ULK1 [193], which showed a 7 fold increase in expression and ULK2, the ortholog of ULK1, which showed a 2 fold increase in expression [194]. SQSTM1(p62) is required for the degradation of proteins by autophagy [195], and was increased by 4.5 fold in cells with loss of BRCA1. DRAM encodes a lysosomal membrane protein that is essential in p53 modulated autophagy [83]. DRAM expression was also increased 2 fold in cells with loss of BRCA1. ULK1, SQSTM1, and DRAM are all major regulators of autophagy that have increased expression in cells with loss of BRCA1.

**Loss of BRCA1 induced autophagy is p53 and SESN2 dependent**

The role of p53 in autophagy is unique as it is known to be capable of both inducing [83, 196] and inhibiting autophagy, depending on the context [85]. A schematic of how p53 is known up regulate autophagy is depicted in Fig.18A. To determine if the induction of autophagy that we have observed in loss of BRCA1 cells is p53 dependent, we inactivated p53 using GSE22. Already established HMEC-GSE and HMECs were infected with control LKO and shBRCA1 to generate the following cell lines: HMEC-LKO, HMEC-shBRCA1, HMEC-GSE22-LKO, and HMEC-GSE22-shBRCA1. Western blot analysis confirmed p53 inactivation by stable integration of GSE22 (Fig. 18B). LC3 levels were increased in response to loss of BRCA1 as observed in figure 16B. However in the absence of p53, LC3 protein levels were decreased and were not upregulated in response to BRCA1 loss (Fig. 18B). These data suggest loss of BRCA1-induced autophagy is mediated at least in part through p53. Previous studies have shown p53 regulates autophagy through AMPK/mTOR signaling [196, 197]. We sought to determine if p53 regulation of autophagy is mediated through AMPK/mTOR signaling following BRCA1 loss. We probed for total AMPK protein and phospho-AMPK and found that loss of BRCA1 resulted in a significant increase in phospho-AMPK levels; this increase was
Figure 19. Loss of SESN2 results in the down regulation of autophagy proteins.

A. rtPCR of SESN2 and B-actin in HMEC-LKO and HMEC-shSESN2.

B. Western blots probing for AMPK, pAMPK, LC3, and B-actin in HMEC-LKO and HMEC-shSESN2.
found to be p53 dependent (Fig. 18B). Recent studies have shown the p53 target, SESN2, regulates AMPK through phosphorylation and promotes AMPK/TSC1/2 complex formation, thus inhibiting mTOR [198]. To determine if p53 induced autophagy is SESN2 dependent we determined phospho-activation of AMPK and LC3 accumulation in the absence of SESN2. We found that both total AMPK protein levels and phospho-AMPK levels were down regulated in response to loss of SESN2 (Fig. 19). We probed for LC3 and found that it was also down regulated in response to loss of SESN2 (Fig. 19). The down regulation of both pAMPK and LC3 demonstrate that in the absence of SESN2 autophagy is not induced and supports a model by which loss of BRCA1 induced autophagy is mediated through SESN2.

**Autophagy does not inhibit ROS in response to loss of BRCA1**

Our previous findings show elevated ROS levels in response to BRCA1 loss are maintained within a physiological range that allows for cell survival through a p53-SESN2 dependent process (Fig. 14B, 16C). Since SESN2 also regulates autophagy, (Fig. 19) we sought to determine if autophagy induction also regulates ROS following BRCA1 loss of function. We hypothesized that if autophagy inhibits ROS in cells with loss of BRCA1, down regulation of the essential autophagy regulatory gene, BECN1, would result in increased ROS levels. We performed double infections with short hairpins targeting BRCA1 and BECN1 in HMECs. We used shBRCA1 with blasticidin (b) resistance and shBECN1 with puromycin (p) resistance to allow us to select for cells harboring both constructs. Cells were cultured in media containing 2µg/mL puromycin and 3µg/mL blasticidin. Four stable cell lines were generated: HMEC-LKO(b)-LKO(p), HMEC-LKO(p)-shBRCA1(b), HMEC-LKO(b)-shBECN1(p), and HMEC-shBRCA1(b)-shBECN1(p). We confirmed KD of BRCA1 and BECN1 by rtPCR (Fig. 20A). Combined loss of BRCA1 and BECN1 resulted in a morphology consistent with cellular senescence, while the cellular phenotype of HMEC with only BECN1 down regulation was small and round (Fig. 20B). DCF fluorescence showed ROS levels were increased in HMEC-LKO(p)-shBRCA1(b) compared to control cells (Fig. 20C). We also found ROS levels were increased in cells with loss of BECN1. In cells in which both BRCA1 and BECN1 were down regulated, we did not observe a further increase in ROS compared to cells with loss of BRCA1 or BECN1 alone; ROS levels were decreased by about 30% in HMEC-shBRCA1(b)-
Figure 20. Loss of autophagy does not result in upregulation of ROS.

A. HMEC were double infected with shBRCA1 together with shBECN1 and harvested on day 5 of infection. rtPCR of BECN1, BRCA1, and B-actin in cells expressing two short hairpins.

B. Phase contrast images with scale bar of 500µm taken of day 5 of infection of HMEC-LKO-LKO, HMEC-LKO-shBRCA1, HMEC-LKO-shBECN1, and HMEC-shBRCA1-shBECN1.

C. Quantification of DCF fluorescence measured by flow cytometry in of HMEC-LKO-LKO, HMEC-LKO-shBRCA1, HMEC-LKO-shBECN1, and HMEC-shBRCA1-shBECN1. Significance was calculated using paired Student t-test.
shBECN1(p). We had expected that inhibiting autophagy would result in increased ROS and so this was a surprise. This data suggests that in response to loss of BRCA1, autophagy does not inhibit ROS.

**Bypass of loss of BRCA1-induced senescence does not occur through the known regulators of senescence induction**

Previous studies have established that senescence induction is p53 and/or RB dependent, regardless of the inducer of senescence. In MEFs, loss of BRCA1 induced senescence is bypassed in the absence of p53 [156]. In mice with conditional KD of BRCA1, KD of p53 is required for bypass of senescence and tumor formation [129]. In both mouse and human fibroblasts, oncogene-induced senescence is also bypassed by the inactivation of p53 [103]. The role of RB inactivation in the bypass of OIS is restricted to MEFs as OIS is not rescued by RB inactivation in human fibroblast cell lines. We have found that senescence induction in response to loss of BRCA1 is different in epithelial cells as compared to fibroblasts when it comes to p53 expression (Fig. 10). We have also found that p53, even though down-regulated, is functional in loss of BRCA1 senescent cells (Fig. 1, 8). We sought to determine the role of RB and p53 in senescence induction by BRCA1 loss. HPV16-E7 gene of the human papilloma virus binds to RB allowing the release of E2F transcription factors, thereby inhibiting RB function [199]. Stable lines were generated expressing HMEC-E7 and HMEC-E7-shp53. Both these lines were then infected with shBRCA1 and LKO. As a control HMEC-LKO and HMEC-shBRCA1 lines were also generated. This gave us 6 lines: HMEC-LKO, HMEC-shBRCA1, HMEC-E7-LKO, HMEC-E7-shBRCA1, HMEC-E7-shp53, and HMEC-E7-shp53-shBRCA1. Protein was isolated from all of these lines and Western blots were done to confirm down regulation of BRCA1, RB, and p53 (Fig. 21A). Western blots for the control lines, HMEC-LKO and HMEC-shBRCA1, are presented separate from the Western blots of experimental lines. BRCA1 protein expression was absent in cells expressing shBRCA1. Similarly, RB expression was also absent in all lines expressing E7. Since we used pre-senescent cells in this experiment, all cells expressed p16 levels. Cells expressing shp53 showed reduced expression of p53. All cells with loss of BRCA1 (HMEC-shBRCA1, HMEC-E7-shBRCA1, and HMEC-E7-shp53-shBRCA1) displayed
Figure 21. Loss of BRCA1-induced senescence in HMEC is independent of p53 and RB.

A. Western blots of BRCA1, Rb, p16, p53, p21, and B-actin in (starting from left) HMEC-LKO and HMEC-shBRCA1. (Continued in the next column) HMEC-pBABE, HMEC-E7, HMEC-LKO-E7, HMEC-E7-shBRCA1, HMEC-E7-shp53, and HMEC-E7-shp53-shBRCA1.

B. Phase contrast images of HMEC-LKO, HMEC-LKO-E7, HMEC-E7-shp53, HMEC-shBRCA1, HMEC-shBRCA1-E7, HMEC-shBRCA1-E7-shp53 cells on day 5 post-infection.

C. Growth kinetics of all the cell lines calculated by population doublings over time.

D. Quantification of β-galactosidase positive stained cells in all of the cell lines on day 5 post-infection.
senescent cell morphology compared to their respective controls (HMEC-LKO, HMEC-E7-LKO, and HMEC-E7-shp53) (Fig. 21B). We then determined population doublings of all cell lines by counting cell number every 3 days. We found that BRCA1 down regulation in presence or absence of RB and/or p53 resulted in an immediate population growth arrest (Fig.21C). This demonstrates that loss of BRCA1 induced growth arrest is both p53 and RB independent. To determine if the growth arrest in these lines was due to senescence, we performed β-galactosidase staining. We found increased β-galactosidase staining in all cells with loss of BRCA1, including cells with prior RB inactivation and p53 down regulation (Fig. 20D). However, we also observed increased cell death and relatively reduced β-galactosidase activity in response to combined loss of p53, RB, and BRCA1. Contrary to the paradigm that senescence cannot be induced in the absence of p53, we found that loss of BRCA1 induced senescence in HMECs is both p53 and RB independent.

The data demonstrating that even with combined loss of RB and p53, loss of BRCA1 induced senescence is not bypassed was very astonishing. We sought to evaluate if p53 is required for loss of BRCA1 induced senescence or if p53 is cell type specific and required only for senescence induction in fibroblasts. To do so, we down regulated BRCA1 along with p53 in two different fibroblast lines: NMF and IMR90. The cell lines that were generated were LKO, shBRCA1, LKO-shp53, and shBRCA1-shp53 for both NMF and IMR90. Cells from all of these lines were collected and protein lysates were made. Western blots probing for p53, p21, and B-actin confirmed effective down regulation of p53 in response to the short hairpins (Fig.22A). To determine if these cells senesced in the absence of p53 and BRCA1, we first assessed proliferation rates. Both NMF-shBRCA1 and IMR90-shBRCA1 showed an immediate growth arrest, indicative of senescence (Fig. 21B). Control LKO and shp53 cell lines continued to proliferate. Cells with combined loss of BRCA1 and p53 also continued to proliferate, clearly indicating that senescence was not induced in the absence of p53. This is in agreement with previously published data showing that loss of BRCA1 induced senescence is p53 dependent [156].
Figure 22. Loss of BRCA1-induced senescence in NMF and IMR90 is p53 dependent.


B. Growth kinetics of all IMR90 and NMF in the presence or absence of BRCA1 were determined by calculating population doublings over time.
Loss of BRCA1 results in senescence independent of ROS upregulation

To determine if reduced ROS has an effect on senescence induction, we down regulated BRCA1 in HMECs while culturing cells in reduced oxygen conditions. HMEC-LKO and HMEC-shBRCA1 cells were cultured in 3% O₂ or 21% O₂ for the course of the experiment. rtPCR confirmed BRCA1 down regulation under both conditions (Fig. 23A). ROS levels were determined by DCF fluorescence. As previously demonstrated, when cultured in 21% O₂, HMEC-shBRCA1 showed increased ROS compared to HMEC-LKO, and showed a large, flat cell morphology with increased β-galactosidase expression (Fig. 23B, C, D). In contrast, when cells were cultured in reduced O₂ conditions, ROS levels were significantly reduced and were not increased in response to BRCA1 loss (Fig. 23B). Despite reduced ROS levels, the cells with loss of BRCA1 cultured in 3% O₂ also exhibited the large, flat, senescent cell morphology and stained positive for β-galactosidase expression (Fig. 23C, D). These data demonstrate that oxidative stress is not necessary for senescence induction in response to loss of BRCA1.

Loss of BRCA1 results in senescence independent of autophagy

To determine if loss of BRCA1 induced senescence is dependent on autophagy, we down regulated BRCA1 in the absence of autophagy and then performed β-galactosidase staining. We down regulated BRCA1 together with BECN1 by performing double infections with a short hairpin targeting each gene. As previously described, we used shBRCA1 with blasticidin resistance and shBECN1 with puromycin resistance to allow for selection of cells harboring both constructs. rtPCR confirmed KD of BRCA1 and BECN1 in these lines (Fig. 24A). Cell morphology and β-galactosidase expression showed that BRCA1 loss in presence or absence of BECN1 resulted in cellular senescence (Fig. 24B, C). As previously shown, cells with loss of BRCA1 showed more that 75% positivity in β-galactosidase assays. Cells with combined loss of BECN1 and BRCA1 demonstrated more that 50% positivity in B-galactosidase, suggesting that BECN1 loss does not inhibit senescence, but may delay senescence onset.
Figure 23. Loss of BRCA1-induced senescence is independent of ROS.

A. rtPCR of BRCA1 and B-actin in HMEC-shLuc and HMEC-shBRCA1 cultured in 21% O₂ or 3% O₂.

B. ROS levels measured by DCF fluorescence and analyzed by flow cytometry in HMEC-shLuc and HMEC-shBRCA1 in 21% O₂ and 3% O₂.

C. Phase contrast images with scale bar of 100µm of HMEC-shLuc and HMEC-shBRCA1 in 21% O₂ and HMEC-shLuc and HMEC-shBRCA1 in 3% O₂ captured on day 6 after infection.

D. Images of β-galactosidase staining of HMEC-shLuc and HMEC-shBRCA1 in 21% O₂ and 3% O₂ captured on day 6 after infection.
Figure 24. Loss of BRCA1-induced senescence is not dependent on autophagy.

A. rtPCR of BECN1, BRCA1, and B-actin in cells expressing HMEC-LKO-LKO, HMEC-LKO-shBRCA1, HMEC-LKO-shBECN1, and HMEC-shBRCA1-shBECN1. Cells were double infected with shBRCA1 together with shBECN1 and harvested on day 5 of infection.

B. Phase contrast images with scale bar of 100µm were taken on day 5 on infection of HMEC-LKO-LKO, HMEC-LKO-shBRCA1, HMEC-LKO-shBECN1, and HMEC-shBRCA1-shBECN1.

C. Quantification of β-galactosidase staining in HMEC-LKO-LKO, HMEC-LKO-shBRCA1, HMEC-LKO-shBECN1, and HMEC-shBRCA1-shBECN1.
Chapter 4: Discussion

Senescence has been demonstrated to be a tumor suppressive mechanism in transgenic mouse models of carcinogenesis and mouse xenograft models using human cell lines. Activation of oncogenes or loss of TSGs in primary cells launches fail-safe mechanisms to limit the propagation of damaged cells. The role of senescence in human carcinogenesis is best described in studies that have examined the distribution of senescence markers in normal, pre-malignant and malignant lesions. Studies of prostate, lung, skin, and breast [29, 109, 111, 113] have all found that markers of senescence are commonly found in pre-malignant lesions compared with normal and malignant tissues. These studies have reinforced that mechanisms that allow cells to bypass senescence are necessary for malignant transformation. Since the physiological inducers of senescence are unique to each cell type, the mechanism of senescence bypass may be equally unique.

In this study we used an in vitro model to study the consequences of BRCA1 loss of function in human mammary epithelial cells. We find that loss of BRCA1 induces cellular senescence that is restricted to normal cells. Premalignant and malignant mammary epithelial cells are refractive to senescence induction by loss of BRCA1. This is not because these cells are not capable of senescence induction as doxorubicin treatment of breast cancer cell lines induces senescence (data not shown). We have hypothesized that this data could signify two different scenarios in terms of BRCA1-associated tumorigenesis. In the first scenario, BRCA1 loss of function is not the first event in carcinogenesis and does not occur in cells that have acquired events that render them refractive to senescence induction by BRCA1 loss. The second possibility is that BRCA1 loss of function is the first event, leading to cellular senescence, and these senescent cells must acquire additional event(s) that promote escape of senescence. In either scenario, the need to understand the immediate consequences of BRCA1 loss in normal cells is essential. In the process of uncovering these immediate consequences, we find that loss of BRCA1 induces a novel mechanism of cellular senescence.
The role of DNA damage and p53 in BRCA1-loss induced senescence

Classic studies have established that DNA damage induction and consequent p53 upregulation are essential for senescence induction. It was for this reason that we examined these factors in our model.

DNA damage response

Typically, the DNA damage response (DDR) is the major mechanism of senescence induction. Both RS and oncogene induced senescence (OIS) occur in response to DDR activation upregulation [94, 97]. In RS, when telomeres become critically short due to DNA replication, the DDR is initiated, resulting in p53 stabilization and transcriptional upregulation of p53 target genes [94]. In OIS, hyper-proliferation results in stalled replication forks, which results in replication induced DDR upregulation, again leading to p53 stabilization and transactivation [97]. We have found that loss of BRCA1 uncouples DNA damage from senescence induction. We observed that BRCA1 loss does not cause cell hyper-proliferation and DNA damage. This suggests that BRCA1 loss induced senescence does not rely on DNA replication, but instead, results in an immediate cell cycle arrest and progressive accumulation of β-galactosidase activity that is maximally achieved in 5 days. Although the DDR is not engaged in response to loss of BRCA1, we find these cells are equally responsive to exogenous stress as compared to control cells.

Although the majority of oncogenes studied so far induce senescence due to DNA damage, senescence induction by tumor suppressors may occur in the absence of DNA damage. In addition to our findings, senescence induced by loss of VHL or PTEN do not require DNA damage [107, 200]. Senescence in response to loss of VHL occurs through RB activation and is DNA damage independent [200]. Loss of PTEN also results in DNA damage independent senescence but is accompanied with p53 upregulation [107]. Senescence in the absence of DNA damage may also be cell type dependent. A recent publication demonstrated that HMEC OIS (hRASV12) could also occur in the absence of DNA damage [201]. Since RAS-induced senescence in mesenchymal cells is classically associated with DNA damage, these data question the role of DNA damage during senescence in epithelial cells.
p53 regulation

p53 is a well known tumor suppressor that is exquisitely sensitive to cellular stress. It acts as a barometer for the type and intensity of stress. For example, in response to oncogene activation (e.g. RAS) and moderate DNA damage, p53 induces senescence [58, 103]. While in response to intense damage, p53 transcribes pro-apoptotic genes to induce apoptosis [58]. Senescence typically results in p53 upregulation due to DNA damage, which activates the DDR. CHK2, when activated by phosphorylation [69], stabilizes p53 leading to increased p53 protein levels which leads to senescence induction.

We find that in loss of BRCA1 induced senescence in HMECs, p53 levels are down-regulated and p53 is not required for loss of BRCA1 induced senescence. This was in stark contrast to previously published reports whereby loss of BRCA1 in MEFs results in p53-dependent senescence [156] and the combined inactivation of BRCA1 and p53 gives rise to tumors in the mouse mammary gland [48]. We also observed that down regulation of p53 was not unique to BRCA1 loss. OIS (via over expression of hRAS\textsuperscript{V12}), loss of BRCA2, and even RS was accompanied with the down regulation of p53 protein levels in HMECs. Since these data were in opposition to that previously published using mesenchymal cells, we compared senescence induction in fibroblasts to that in epithelial cells. We demonstrated that in response to BRCA1 loss, upregulation of p53 does occur in fibroblasts, both in IMR90 and normal mammary fibroblasts, whereas in epithelial cells p53 is downregulated. We conclude that the role of p53 in response to cellular stress may be cell type dependent. During the writing of this thesis, the novel observation that p53 protein levels are down regulated in senescent HMEC was reported by another group. Similar to our findings, Cipriano et. al. have also found reduced p53 protein levels in response to hRAS\textsuperscript{V12} induced senescence. These results further highlight important differences between epithelial and mesenchymal cells in response to cellular stress.

In response to BRCA1 loss, we found that p53 is shuttled to the cytoplasm and targeted for proteasome mediated degradation. We demonstrated by immunocytochemistry that as the senescence phenotype is induced, p53 is gradually shuttled to the cytoplasm. The levels of p53 protein also gradually decrease over 5 days and this decrease occurs simultaneously with p53 mislocalization. Despite the reduced levels of p53 in response to BRCA1 loss, p53 is still
transcriptionally active. Several p53 target antioxidant genes, including SESN2, HMOX1, TP53INP1, and TP53I3 are upregulated in response to loss of BRCA1. Forced inactivation of p53 inhibited loss of BRCA1 induced upregulation of these antioxidant genes, suggesting that p53 plays a central role in BRCA1 loss-induced antioxidant upregulation.

Our finding that antioxidant genes are upregulated in response to BRCA1 loss in HMEC is in opposition to previous studies. BRCA1, when upregulated, has been shown to induce the transcription of antioxidant genes and to protect against oxidative stress [127]. In the absence of BRCA1, antioxidant genes were found to be downregulated [127] and ROS levels were increased [128]. Authors of both of these studies used MEF isolated from BRCA1 knock-out mice in addition to the breast cancer cell line, MCF7, to conduct their experiments. We think that a possible reason why our results are in opposition to those previously published by others is due to differences in cell type specificity. Our study utilized normal epithelial cells and we and others have already shown key differences between senescence mechanisms in epithelial versus fibroblast cells. This may account for the differences between MEF and HMEC, in addition to species differences.

Although loss of BRCA1 results in senescence in the absence of p53, it appears that p53 plays a role in maintaining senescence. We have found that in the absence of p53, cells with loss of BRCA1 acquire increased levels of ROS and show increased cell death. We hypothesize that p53 at lower levels upregulates antioxidant genes to quench ROS levels. This results in maintaining ROS levels below a threshold that would otherwise induce cell death in response to loss of BRCA1.

**The role ROS and autophagy in response to loss of BRCA1**

Autophagy induction and ROS regulation are both not only associated with senescence but are also important processes in tumor formation. In this study we have been able to explore the role of both these processes as they relate to senescence.

**ROS regulation**

The upregulation of ROS plays an integral role in senescence induction. The role of ROS in senescence was first appreciated when cells cultured at lower O$_2$ showed increased lifespan
Since then, a number of reports have documented increased ROS levels in response to oncogene activation [74, 172] and have shown that senescence can be averted if ROS levels are inhibited [73, 202]. We have found that ROS is differentially regulated in HMECs in an inducer specific manner. In HMECs undergoing replicative senescence or RAS-induced senescence, ROS levels were surprisingly downregulated as compared to control cells, while ROS levels were increased in response to loss of BRCA1 induced senescence. Others have also reported that BRCA1 loss in MEFs is associated with increased levels of ROS [127, 128]. These findings were attributed to down regulation of antioxidant gene expression in response to BRCA1 loss. In HMECs, we find that loss of BRCA1 induces upregulation of p53 dependent antioxidant genes that appear to play an important role in quenching ROS levels to maintain cell viability. The loss of p53 or SESN2 in combination with loss of BRCA1 resulted in increased cell death, presumably due to deleterious ROS levels. Upregulation of an antioxidant program is likely initiated in response to increased ROS levels. The mechanism that results in ROS upregulation following BRCA1 loss remains unknown. Increased ROS may be due to increased mitochondrial metabolism. Recent studies using cancer cell lines and MEFs have demonstrated that RAS-induced tumorigenesis increases mitochondria metabolism and as a consequence, leads to increased oxidative stress that is required for anchorage independent growth [74, 203]. Although we have observed an increase in mitochondria numbers by EM, we have not quantified our findings or probed with mitochondria specific markers. Understanding the consequences of BRCA1 on mitochondria metabolism requires further study.

Persistent ROS accumulation may increase the susceptibility of cells to genomic instability, leading to increased predisposition to tumorigenesis. This could be one explanation for the increased risk of tumor development due to BRCA1 loss of function. Human mammary epithelial cells that acquire other forms of aberrations (such as oncogenic RAS), maintain reduced levels of ROS, thereby decreasing the chances of genomic instability. Perhaps, it is the unique consequence of ROS upregulation in response to loss of BRCA1 that drives BRCA1 related tumor formation.

**Autophagy induction**

Autophagy has been shown to play a role in senescence [75]. Autophagy not only removes wastes from cells and recycles cellular components, but is also an adaptation
mechanism during times of stress and starvation as it generates energy. We have found that loss of BRCA1 results in the induction of autophagy. We were also able to uncover the mechanism by which autophagy is induced. As described above, p53 levels are down regulated in response to loss of BRCA1, resulting in the upregulation of the antioxidant gene, SESN2. It has recently been found that SESN2 serves an antioxidant independent function as well. SESN2 phosphorylates AMPK and forms a complex with AMPK, TSC1, and TSC2 to indirectly inhibit mTOR [198], thus activating autophagy [84]. We have found that in HMECs, autophagy is induced in a p53 dependent manner through SESN2. In cells with loss of BRCA1, reduced levels of p53 upregulate SESN2, resulting in the phosphorylation of AMPK and the induction of autophagy. In the absence of SESN2, the induction of autophagy in these cells is abrogated.

Autophagy is known to play both a tumor suppressive and an oncogenic role. On the one hand, it has been demonstrated that autophagy is an important mechanism in senescence and can act as a tumor suppressor [204, 205]. On the other hand, autophagy induction has been shown to promote cancer cell survival in several studies [206]. We have found that in the absence of autophagy, loss of BRCA1 induced senescence is delayed, but not bypassed, indicating that autophagy is not absolutely required for senescence. Since autophagy has a tumor promoting function, we think that perhaps combining autophagy activation with additional mutation events that result in bypass of loss of BRCA1 induced senescence may lead to tumorigenesis.

**Significant findings**

Our most striking finding was that loss of BRCA1 induced senescence is not dependent on p53, RB, ROS upregulation, or autophagy induction. This demonstrates that BRCA1 loss results in a novel type of senescence that is mechanistically different from that which has been previously described. We think that although p53 is not required for the induction of loss of BRCA1 senescence, it might be required for bypass along with additional events. A recently published report demonstrated that in HMECs, RAS induced senescence was p53 independent, but could be bypassed with the down regulation of p53 together with TGFβ inhibition [201].

We think that our results regarding SESN2 provide important links between p53, ROS, and autophagy. Since SESN2 plays a dual role of inhibiting ROS and inducing autophagy in response to loss of BRCA1, we think that we have uncovered an important element in
senescence induction. Inhibition of SESN family members has been shown to be associated with mutagenesis in response to expression of oncogenic RAS [207]. The expression of SESN genes was able to reduce ROS levels in response to oncogenic RAS and decrease the rate of tumorigenesis. We hypothesize that loss of SESN2 function might be an additional event that drives BRCA1 tumorigenesis.

Conclusion

In summary, we find that loss of BRCA1 results in a down regulation of p53 and an upregulation of ROS and autophagy (Fig. 25). We have mechanistically linked the p53-downstream gene, SESN2, to the regulation of ROS and autophagy in response to BRCA1 loss. Although ROS levels appear to be quenched by SESN2, more work needs to be done to identify how ROS gets upregulated in response to loss of BRCA1. Although p53 is seemingly central in our model, it is not required for the induction of senescence since senescence can be induced in its absence. It does, however, function in maintaining the senescence phenotype since β-galactosidase expression is diminished in its absence. It remains to be explored if coupling loss of p53 and autophagy would be sufficient for bypass of BRCA1 loss-induced senescence. In summary, we conclude that loss of BRCA1 induced senescence in HMECs is unique from previously described phenotypes of senescence. It is, therefore essential to further explore the underlying causes of senescence induction and the events that result in its bypass.

Figure 25. Summary of the consequences of BRCA1 loss in normal mammary epithelial cells.
References


