IDENTIFICATION OF GENES INDUCED BY BRCA1 IN BREAST CANCER CELLS

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I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

IDENTIFICATION OF GENES INDUCED BY BRCA1 IN
BREAST CANCER CELLS

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Ph.D. in Molecular Biology and Genetics

Supervisor: Assist. Prof. Işık G.Yuluğ

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Inherited mutations of the BRCA1 gene predispose to cancer of the breast, ovaries and other organs. The BRCA1 protein product is implicated in the maintenance of chromosomal integrity as BRCA1-deficient cells display gross chromosomal rearrangements. Chromosomal instability in BRCA1-deficient cells is related to inappropriate DNA double-strand break repair. The role of the BRCA1 gene in the maintenance of chromosomal integrity is linked to a number of biological properties of its protein product including transcriptional regulation. The aim of this study is to identify genes that are regulated by BRCA1. Initial attempts to overexpress BRCA1 in breast cancer cells with the tightly-regulated ecdysone inducible system did not result in the desired levels of BRCA1 protein and ectopic BRCA1 expression was therefore performed by using the constitutive expression vector. In this study, we have identified genes whose expression levels are upregulated as a result of BRCA1 overexpression in MCF7 breast carcinoma cells by using the suppression subtractive hybridisation (SSH) method. Differential screening, sequencing and homology search studies showed that BRCA1 overexpression in breast cancer cells leads to transcriptional upregulation of distinct classes of genes encoding proteins involved in cellular processes such as DNA repair, chromosome assembly and segregation, signal transduction, RNA surveillance, ubiquitin-mediated proteolysis, amino acid transport, RNA metabolism and glucose metabolism. This study is the first to report BRCA1-induced genes in breast carcinoma cells with the SSH technique. The identified genes in this study may provide new insights into the tumour suppressor functions of BRCA1.

ÖZET

MEME KANSERİ HÜCRELERİNDE BRCA1 TARAFINDAN İNDÜKLENEN GENLERİN TANIMLANMASI

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BRCA1 geninin kalıtsal mutasyonları meme, rahim ve diğer organlarda kansere yatkınlığa yol açar. BRCA1-eksik hücrelerde yeni kromozomal düzenlenmeler görüldüğünden BRCA1 protein ürününün kromozomal bütünlüğün devam ettirilmesinde görevli olduğu düşünülmektedir. BRCA1-eksik hücrelerdeki kromozomal dengesizlik DNA çift-ipliğinin yanlış tamiriyle ilişkilidir. BRCA1 geninin kromozomal bütünlüğün sağlanmasındaki rolü protein ürününün transkripsiyonel düzenleme dahil bir kaç biyolojik özelliğine bağlıdır. Bu çalışmanın amacı BRCA1 tarafından düzenlenen genlerin tanımlanmasıdır. BRCA1'i meme kanseri hücrelerinde sıkı-düzenlenen "ecdysone" ile indüklenebilen bir sistemle fazla ifade etmek için yapılan ilk çalışmalar, istenen düzeyde BRCA1 proteini vermemiştir ve bu nedenle ektopik BRCA1 ifadesi konstitütif ekspresyon vektörü kullanılarak yapılmıştır. Bu çalışmada MCF7 meme kanseri hücrelerinde BRCA1'in fazla ifade edilmesi sonucu ifade düzeyleri artan genler "Suppression Subtractive Hybridisation" (SSH) metodu kullanılarak tanımlanmıştır. Ayrımsal tarama, dizileme ve homoloji araştırma çalışmaları meme kanseri hücrelerinde BRCA1'in fazla ifade edilmesinin DNA tamiri, kromozom yapımı ve ayrılması, sinyal iletimi, RNA tarama, ubikitin aracılıklı proteoliz, amino asit taşınımı, RNA metabolizması ve glukoz metabolizması gibi hücresel olaylarda yer alan proteinleri kodlayan farklı gen gruplarının ifadelerinde artışa yol açtığını göstermiştir. Bu çalışma meme karsinom hücrelerinde SSH tekniği kullanılarak BRCA1 tarafından indüklenen genleri gösteren ilk çalışmadır. Bu çalışmada tanımlanan genler BRCA1'in tümör baskılayıcı görevlerine yeni bir bakış açısı sağlayabilir.

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ABBREVIATIONS

Ad1	Adaptor 1
Ad2R	Adaptor 2R
APS	Ammonium persulphate
BASC	BRCA1 Associated Genome Surveillance Complex
BBS	Bes buffered saline
Bp	Base pair
BRCA1	BReast CAncer susceptibility gene 1
BRCA2	BReast CAncer susceptibility gene 2
BRCT	BRCA1 Carboxyl Terminus
BSA	Bovine serum albumin
cAMP	Cyclic adenosine mono phosphate
cDNA	Complementary DNA
Ci	Curie
CIAP	Calf intestinal alkaline phosphatase
Cpm	Counts per minute
dCTP	Cytosine deoxyribonucleotide
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ds	Double strand
DOC	Deoxycholate
DSB	Double strand break
EDTA	Ethylenediaminetetra-acetic acid
ER	Estrogen receptor
EtBr	Ethidium bromide
FCS	Fetal calf serum
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
H_2O_2	Hydrogen peroxide

HDAC	Histone deacetylase
HBS	Hepes buffered saline
HR	Homologous recombination
h	Hour
IP	Immunoprecipitation
IPTG	Isopropyl β-D-thiogalactopyronoside
IR	Ionising radiation
IRIF	Ionising radiation induced foci
IVTT	In vitro transcription-translation
kb	Kilobase
LB	Luria-Bertani medium
LOH	Loss of heterozygosity
M	Molar
mA	Muristerone A
µg	Microgram
mJ	Milijoule
min	Minute
ml	Mililiter
µl	Microliter
mm	milimeter
mM	Milimolar
MOPS	3-(N-morpholino)propane-sulphonic acid
mRNA	Messenger RNA
N	Normal
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
NHEJ	Nonhomologous end joining
Oligo(dT)	Oligodeoxythymidylic acid
pA	Ponasterone A
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen

PCR	Polymerase chain reaction
pmol	Picomole
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
Sec	Second
ss	Single strand
SSH	Suppression subtractive hybridization
TBS	Tris buffered saline
TCR	Transcription coupled repair
TEMED	N,N,N',N'-tetramethylenediamine
U	unit
UV	Ultraviolet
V	Volt
v/v	volume for volume
xg	gravity
X-gal	5-bromo-4-chloro-3-indonyl-β-D-galactosidase
ZBRK1	Zinc binding and BRCA1-interacting protein with a KRAB domain

CHAPTER 1. INTRODUCTION

Breast cancer, which is the most common female malignancy, is a major cause of death in middle-aged women and its incidence is increasing (Parkin and Coleman, 1990). Breast cancer affects almost one million women worldwide at any given time. One in eight American women will develop breast cancer at some point in their lifetimes, and of these women approximately 30% will develop the metastatic form which is ultimately fatal (Bowcock, 1999).

The breast epithelium undergoes distinct developmental programs during puberty and pregnancy. During puberty, in particular, rapid proliferation of breast tissue occurs. Lobules of the breast are clonal (Kordon and Smith, 1998) and the progeny of this proliferative burst are retained within the breast lobule. In this way, breast epithelial cells have the potential to retain “memory” of genetic alterations that occurred earlier in breast development. In contrast, some other epithelia that are characterized by rapid proliferation, such as the intestinal epithelium, shed cells continuously.

1.1 Risk factors for breast and ovarian cancer

It is well understood that one of the most important risk factors for developing breast cancer is a family history of the disease. However, many other nongenetic risk factors contribute to disease etiology. Besides sex and country of birth, increasing age is an important risk factor. Other suggested risk factors are influences of hormones. Early age at menarche, late menopause, and late first full-term pregnancy, all confer increased risk for breast cancer. Even the prenatal environment might be of importance. It has been shown repeatedly that estrogen exposure is directly associated

with risk for developing breast cancer. High body-mass index after menopause, high alcohol consumption and some dietary factors increase the risk (Martin and Weber, 2000). Exposure to ionising radiation is a well-known risk factor for different kinds of malignancies; young women who received mantle radiation for Hodgkin's lymphoma have a markedly increased risk for developing breast cancer (Wolden *et al.,* 1998). All these mentioned nongenetic factors increase the risk by about 1.5- to three-fold.

Multiple epidemiological studies have reported that a family history of breast cancer is a reproducible predictor of breast cancer risk (Lynch *et al.,* 1981; Ottman *et al.,* 1986). The risk conferred by having relatives with breast cancer varies with closeness of kinship, numbers of affected relatives and the age of onset. A more than two-fold increase in risk for women with one first-degree relative with early onset breast cancer has been reported in many studies (Arver *et al.,* 2000). Having more than one close relative with breast cancer, or cases of bilateral disease among relatives, seem to confer even higher risks.

Families with three or more close relatives with breast cancer are, in the literature, classified as 'breast cancer families'. One of the earliest descriptions of a breast cancer family was written in 1866 by the French surgeon P. Broca in the publication '*Traite des tumeurs'*. Numerous pedigrees from families with apparent inherited breast cancer susceptibility have been reported (Lindblom *et al.,* 1993; Lynch *et al.,* 1978) and it was noticed early on that ovarian cancer was frequent in many of these families (Go *et al.,* 1983). Other features found in breast cancer families are early age of onset and bilateral disease (Lynch, 1990; Lynch *et al.,* 1978).
In ovarian cancers, about 10% of cases have a hereditary basis. A family history of ovarian cancer is the strongest risk factor for developing the disease. Germline mutations in *BRCA* genes appear to account for most hereditary ovarian cancers. DNA repair gene mutations (in HNPCC families) account for a much smaller fraction of cases (Lynch *et al.,* 1998).

1.2 Genes implicated in breast cancer

BRCA1, BRCA2, p53, STK11/LKB1, PTEN, MSH2/MLH1, ATM are the genes implicated in formation of hereditary breast cancer. Besides, *CYP1A1, GSTs, NAT2* and androgen receptor are some of the low penetrance breast cancer susceptibility genes.

BRCA1 and BRCA2: Familial breast and ovarian cancer predisposition syndromes have long been recognized. Their genetic bases have become clear with the cloning of two major disease susceptibility genes *BRCA1* and *BRCA2* (Hall *et al.,* 1990; Miki *et al.,* 1994; Wooster *et al.,* 1995; Wooster *et al.,* 1994). Each has characteristics of a tumour suppressor gene; inheritance within affected families follows an autosomal-dominant pattern of inheritance and loss of heterozygosity (LOH) at the relevant gene locus is seen in familial tumours with retention of the disease-predisposing allele (Collins, 1995; Gudmundsson *et al.,* 1995; Neuhausen and Marshall, 1994). The spectrum of disease-associated mutations includes frequent truncating mutations and less frequent missense mutations. Although LOH is frequently at the *BRCA1* or *BRCA2* locus in sporadic breast cancer, the retained allele is almost wild-type (Futreal *et al.,* 1994; Lancaster *et al.,* 1996). Thus, in contrast to the casual role of *BRCA1* gene mutation in the hereditary syndrome, *BRCA1* gene mutation in sporadic breast and ovarian cancer seldom conforms to Knudson's model for tumour suppressor genes (Knudson, 1971). Cancer risk in *BRCA* gene mutation carriers may be increased modestly in other organs, but, highly penetrant, early-onset, site specific cancer is restricted to the breast and ovary.

p53: *p53* germline mutations are thought to be a rare cause of breast cancer, except in the setting of Li-Fraumeni syndrome. The first documentation of Li-Fraumeni syndrome was in 1969, in which four families with children with soft tissue sarcomas were found to have an excess of sarcomas in other relatives. In addition these families exhibited an excess of early onset breast cancer and other cancers such as childhood leukemia, adrenocortical carcinoma and brain cancer (Li and Fraumeni, 1969). In 1990, the presence of germline *p53* mutations in approximately half of the families with classic Li-Fraumeni syndrome was reported (Malkin *et al.,* 1990). In women

with germline *p53* mutations who survive childhood cancers, it is estimated that 50% will have developed breast cancer by the age of 50 (Easton *et al.*, 1993b).

STK11/LKB1: Peutz-Jeghers syndrome is caused by germline mutations in *STK11/LKB1*, a serine-threonine kinase located on chromosome 19q13.3 (Jenne *et al.*, 1998). Peutz-Jeghers syndrome is characterized by hamartomatous polyps in the small bowel and pigmented macules of the buccal mucosa, lips, fingers and toes. In spite of the early-onset breast cancer that can be seen in patients with Peutz-Jeghers syndrome, mutations in *STK11/LKB1* do not appear to play an important role in sporadic breast cancers, based on the very low prevalence of mutations in the population.

PTEN: Cowden syndrome is a rare autosomal dominant predisposition to both benign and malignant neoplasms. Breast cancer develops in 20-30% of carrier women. Other tumours seen among patients with Cowden syndrome include adenomas and follicular cell carcinomas of the thyroid gland, polyps and adenocarcinomas of the gastrointestinal tract and ovarian cysts and carcinoma (Hanssen and Fryns, 1995). Cowden syndrome is caused by germline mutations in the *PTEN* gene (*MMAC1/TEP1*). PTEN, a tumour suppressor gene on 10q23.3, is a dual-specifity phosphatase (Steck *et al.*, 1997). *PTEN* does not play a role in familial breast cancer, apart from its role in Cowden syndrome.

MSH2/MLH1: Muir-Torre syndrome is defined by the presence of sebaceous gland tumours and visceral malignancy. It is inherited in an autosomal dominant fashion with high penetrance because of mutations in the same genes associated with hereditary nonpolyposis colorectal cancer (HNPCC) (Schwartz and Torre, 1995). The most common malignancy in this syndrome is colorectal cancer, seen in 50% of patients, but breast cancer occurs in approximately 25% of women carriers. As in HNPCC, microsatelite instability is observed in the tumours of patients with Muir-Torre syndrome. Mutational analysis has shown that the mutations are predominantly in *MSH2*.

ATM: Ataxia-telangiectasia (AT) is a complex, autosomal recessive disorder characterized by cerebellar ataxia, telangiectasia, immunodeficiency, radiation

sensitivity and cancer predisposition caused by homozygous mutations in the *ATM* gene. Epidomiologic studies have shown that AT carriers might have an increased risk for breast cancer, and this observation was consistent with the recent finding that ATM phosphorylates BRCA1 protein upon DNA damage (Li *et al.*, 2000).

Low penetrance breast cancer susceptibility alleles: Low-penetrance susceptibility alleles, sometimes called “modifier genes”, are defined as polymorphic genes with specific alleles that are associated with an altered risk for disease susceptibility. Further studies are needed to clarify the role of all the following genes in breast cancer as rewieved by Martin and Weber (2000):

The **P450 gene CYP1A1** encodes aryl hydrocarbon hydroxylase, which is the primary catalyst in the conversion of estradiol to hydroxylated estrogen. A reduced estrogen level is protective for developing breast cancer, whereas increased estrogen exposure can increase the risk for developing breast cancer. Alterations in the activity of aryl hydrocarbon hydroxylase could therefore plausibly lead to a change in the levels of estrogen and could ultimately affect breast cancer risk.

The **glutathione S-transferases** (**GSTs**) constitute a family of genes that encode for enzymes that catalyze the conjunction of reactive chemical intermediates to soluble glutathione conjugates to facilitate clearance. Inability to metabolise carcinogens may increase breast cancer risk.

N-acetyltransferase (**NAT2**) is an important component of the carcinogen metabolism pathway and polymorphisms in the *NAT2* gene are associated with an altered rate of metabolism of carcinogens.

The effect of CAG microsatelite found in exon 1 of **androgen receptor** gene has been investigated for its role in breast cancer penetrance. BRCA1 mutation carriers who carry at least one androgen receptor allele with more than 29 CAG repeats were diagnosed with breast cancer statistically significantly earlier than women with shorter CAG repeats in their androgen receptor genes, suggesting that pathways

involving androgen signalling may affect the penetrance of BRCA1-associated breast cancer (Rebbeck *et al.*, 1999).

1.3 Mechanisms of BRCA-mediated breast and ovarian cancer formation

Relationship between mutation events and formation of breast or ovarian carcinomas are summarized in Figure 1. Estrogen mediated proliferation of breast and ovarian epithelial cells and the distinctive genomic context (high percentage of repetitive sequences) of the *BRCA* genes are critical components of this pathway. During puberty estrogen stimulates breast epithelial cells to proliferate. Among somatic mutations that appear in these rapidly dividing cells, alterations of *BRCA* genes are likely to be relatively frequent because of the density of repeats in these genes. Inactivation of the second allele in such a cell would generally result in cell death. Tumorigenesis would require inactivation of both alleles in a cell capable of escaping cell cycle checkpoints. Unlike inherited disease, inactivation of genes critical to cell cycle checkpoints could occur prior to inactivation of the second allele. Therefore, BRCA1-mediated sporadic tumorigenesis would be less likely. Also, because more mutational events are required, tumours occur later in a woman's life.

Various genetic mechanisms may be responsible for somatic inactivation of both alleles by generation of large deletions of *BRCA1* and *BRCA2* in both inherited and somatic tumours. Deletion of a chromatin loop containing all or a large portion of *BRCA* genes may be mediated by homologous recombination between repeat sequences. Repeat mediated loss of chromatin formed at different points in the cell cycle will yield deletion of different sizes. If this process occurs in both copies of the *BRCA1* region, deletions including the *BRCA* gene will overlap but not be identical. If genomic deletions overlap, they may incorrectly appear to define one region of LOH. This hypothesis may explain the large deletions observed in *BRCA1* tumours (Welcsh and King, 2001).

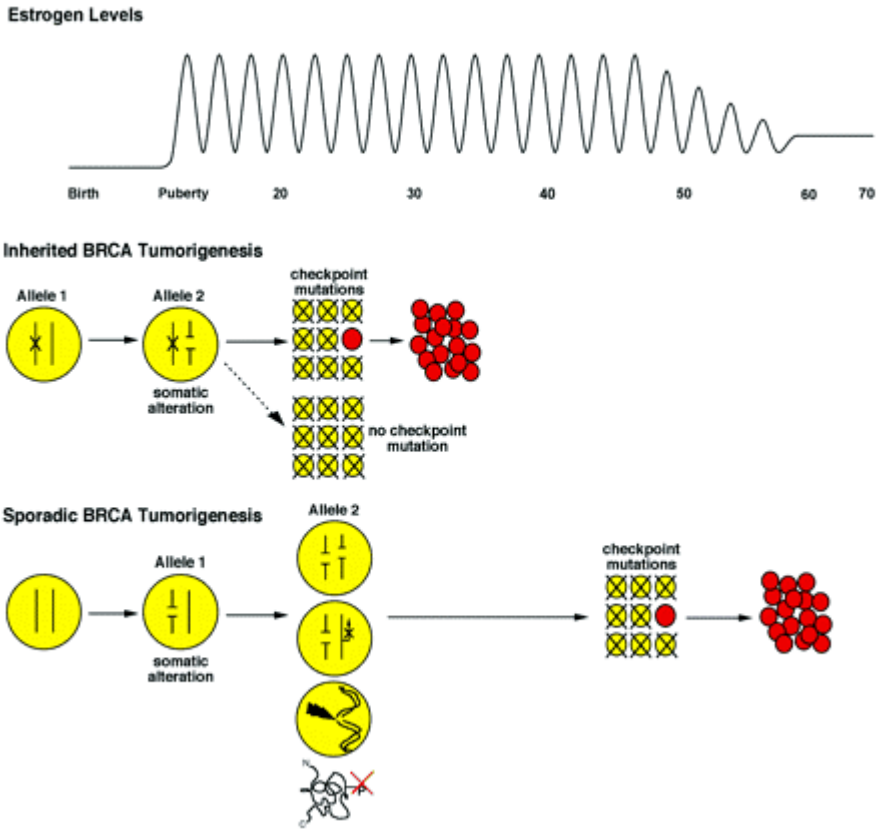


Figure 1: Relationship between mutation events and formation of breast or ovarian cancers. (Welcsh and King, 2001).

Estrogen levels in women are cyclic from menarche until menopause when peak estrogen levels decrease. In response to estrogen at puberty and pregnancy, breast epithelial cells proliferate. Among women with a germline mutation in *BRCA1* or *BRCA2*, somatic inactivation of the remaining allele will result in repair-deficient cells. These cells will be unable to repair damaged DNA in the following cell cycle and will ultimately die due to activation of critical cell cycle checkpoints. The rare repair-deficient cell (shown in red) that escapes death by checkpoint may acquire mutations at other sites including critical checkpoint genes, ultimately resulting in tumour formation. In inherited *BRCA* tumorigenesis, somatic inactivation of the second *BRCA* allele likely occurs early in a young woman's life, leading to early onset of breast cancer. In women without a *BRCA* germline mutation (sporadic BRCA tumorigenesis), somatic alteration of one allele of *BRCA1* or *BRCA2* in breast epithelial cells is likely to occur during estrogen-mediated proliferation, due to the density of repetitive elements in these genes. Inactivation of a second *BRCA* allele in a cell would generally result in cell death.

Alternatively it has been proposed that inactivation of a *BRCA* allele could result in an overall decrease in BRCA function (haplo-insufficiency). Presence of only one *BRCA* allele is clearly sufficient for normal growth and development, because persons heterozygous for *BRCA* mutations have normal phenotypes apart from their cancer predisposition. However under conditions of cellular stress in breast and ovarian epithelium, caused by estrogen-stimulated proliferation, it is possible that even a modest decrease in BRCA function in cells with one somatically inactivated allele could increase the risk for additional cancer promoting mutations.

Third, transcriptional silencing may inactivate *BRCA* alleles, by loss of proteins that positively regulate their expression or by an increase in negative regulatory proteins. Table 1 summarizes the known BRCA1 regulatory proteins, and the nucleotide sites at which they function if the regulation is direct:

BRCA1 regulatory protein	**Binding domain on BRCA1 (L78833)**	**Reference**
Brn-3b	-400 to –1 (2944-3344)	(Budhram-Mahadeo *et al.,* 1999)
GA binding protein α/β	-204 to –159 (3140-3185)	(Atlas *et al.,* 2000)
RB-E2F	-23 to 15 (3321-3339)	(Wang *et al.,* 2000a)
p53	indirect	(Arizti *et al.,* 2000)
TGFβ (requires RB)	?	(Satterwhite *et al.,* 2000)
Id4	indirect	(Beger *et al.*, 2001)

Table 1: BRCA1 regulatory proteins

Among the BRCA1 regulatory proteins, Id4 (inhibitor of DNA binding 4) inversely regulates *BRCA1* expression (Beger *et al.*, 2001). Overexpression of Id4 (and concomitant reduction of *BRCA1* expression) is associated with anchorage-independent growth, a critical characteristic of tumour cells. The relationship between Id4 and BRCA1 may be important for breast cancer because estrogen reduces Id4 expression, hence increasing expression of *BRCA1*. Conversely, breast epithelial cells that are no longer responsive to estrogen may overexpress Id4, with consequent reduction of BRCA1 expression. If this occurs in a cell that has lost one *BRCA1* allele through somatic inactivation, these cells would then be BRCA1 deficient.

Transcriptional silencing of one *BRCA1* allele could be accomplished through methylation of CpG islands, which are often found in the regulatory region of many genes. The 5' regulatory region of the *BRCA1* gene contains a TATA-less promoter with high cytosine-guanine content, making BRCA1 an excellent candidate for CpG methylation. In 5-10% of sporadic breast tumours, *BRCA1* transcription is silenced by the methylation of CpG residues, which leads to absence of protein expression (Esteller *et al.,* 2000). In a recent report, the *BRCA1* promoter was methylated in 15% of sporadic ovarian tumours (Baldwin *et al.,* 2000).

BRCA1 might be inactivated post-transcriptionally by failure of phosphorylation or other posttranslational modifications. Phosphorylation of BRCA1 is required for normal functioning of BRCA1. The roles of ATM, ATR and Chk2 in phosphorylating BRCA1 in response to DNA damage are likely to be most critical to carcinogenesis.

1.4 BRCA1

Hereditary breast cancer families account for only 5-10% of all breast cancer cases. Mutations in *BRCA1* are responsible for nearly all of the hereditary breast and ovarian cancer families and up to 40-50% of families with hereditary breast cancer only (Easton *et al.,* 1993a). *BRCA2* is strongly linked to hereditary breast cancer in both males and females (Wooster *et al.,* 1995; Wooster *et al.,* 1994). Together BRCA1 and BRCA2 account for the majority, although not all, of hereditary breast cancer cases.

1.4.1 Mutational spectrum of BRCA1

Since the isolation of *BRCA1*, more than 700 sequence variations have been identified. Initially eight disease-associated mutations were described within the gene (Futreal *et al.,* 1994; Miki *et al.,* 1994), followed shortly by an increasing number of mutations (Castilla *et al.,* 1994; Friedman *et al.,* 1994; Simard *et al.,* 1994). Most are frameshift mutations, but several missense mutations are known to alter protein

function. Mutational spectrum of *BRCA* genes is summarized in Table 2. Most known mutations are listed in the Breast cancer Information Core (BIC) and are available on the Internet (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic). Several founder mutations have been identified in *BRCA1*.

Gene	***BRCA1***	***BRCA2***
Mutation type		
Frameshifts	70%	68%
Nonsense mutations	20%	12%
Splice site	5%	7%
Missense	5%	13%

Table 2: Mutation types in BRCA1 and BRCA2 (Bowcock, 1999)

1.4.2 BRCA1 gene and protein structure

BRCA1 gene is localized on chromosome 17q21 and spans 100 kb genomic sequence. *BRCA1* is a large gene with a total mRNA of 5711 base pairs, divided into 24 exons, of which 22 are coding. Exon 1 is not coding and exon 4 is an Alu repetitive motif. The coding region begins in exon 2. Exon 11 constitutes more than 60% of the coding region, while most of the other exons are relatively small (Miki *et al.*, 1994). The gene encodes a nuclear phosphoprotein of 220 kDa (Chen *et al.*, 1996a) and smaller splice variants have been described (Wang *et al.*, 1997). Full-length BRCA1 protein is localized in the nuclear foci of epithelial cells (Wilson *et al.*, 1999) while a splice variant, BRCA1 delta 11b, might be cytoplasmic (Thakur *et al.*, 1997).

Functional domains have been identified in BRCA1 (Figure 2). The most phylogenetically conserved region is the C3HC4 zinc binding RING-finger domain at the amino terminus of the protein (Chen *et al.*, 1996a; Saurin *et al.*, 1996). Several other human proteins with zinc finger motifs have suggested this domain to be a region of protein-protein interactions and such motifs are found in a number of regulatory proteins. In addition, BRCA1 has two nuclear localization signals in exon

11 (Chen *et al.*, 1995; Miki *et al.*, 1994). The "BRCT" (BRCA1 Carboxyl-terminal) domain is an evolutionary region in BRCA1 (Callebaut and Mornon, 1997; Koonin *et al.*, 1996). This region resembles the transactivation domain of a number of transcription factors (Abel *et al.*, 1995) and contains two BRCT domains, which are situated in tandem at amino acids 1646-1736 and 1760-1855. BRCT motifs have been described in a number of proteins such as 53BP1, RAD9, RAD4, Crb2 and RAP1, that have function in cell cycle control and DNA damage repair pathways (Callebaut and Mornon, 1997; Koonin *et al.*, 1996), consistent with recent data suggesting an important role for BRCA1 in cellular responses to DNA damage.

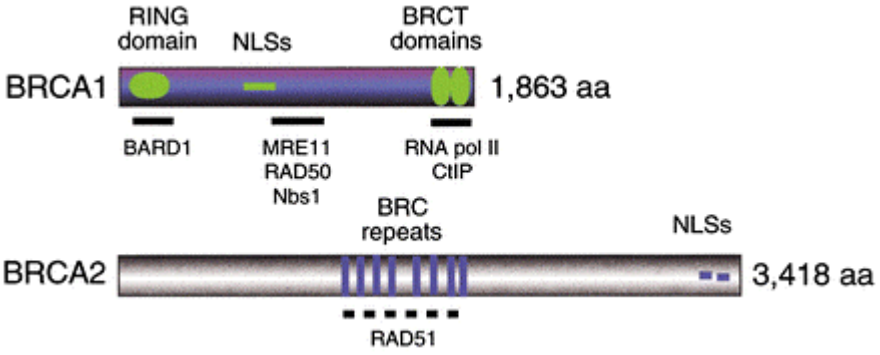


Figure 2: Features of the human BRCA Proteins (Venkitaraman, 2002) BRCA1 contains an N-terminal RING domain, nuclear localization signals (NLSs), and two C-terminal BRCT domains of ~110 residues (also found in several proteins with functions in DNA repair or cell cycle control). BRCA2 contains eight repeats of the ~40 residue BRC motifs. Six of the eight motifs in human BRCA2 can bind directly to RAD51 when expressed *in vitro*.

1.4.3 Cellular expression of BRCA1

In normal cells BRCA1 is a nuclear protein. BRCA1 protein expression is reduced or absent in most sporadic, advanced (grade III) ductal breast carcinomas (Wilson *et al.,* 1999). Nuclear localization signals have been identified on BRCA1 (Thakur *et al.,* 1997), which interact with importin-α, a subunit of the nuclear transport signal receptor.

BRCA1 mRNA and protein are prefentially expressed during the late G1-early S phase of the cell cycle (Gudas *et al.,* 1996). In mitotic cells, BRCA1, BRCA2 and RAD51 interact and co-localize in a punctate pattern in the nucleus during the S phase of the cell cycle, whereas in meiotic cells all three proteins associate with unsynapsed axial elements along developing synaptonemal complexes (Chen *et al.,* 1998b; Scully *et al.,* 1997b). BRCA1 function is regulated by phosphorylation; it is hyperphosphorylated during the late G1 and S phases by an endogenous kinase activity from S1497 and T967 residues, minimally composed of the CDK2-cyclin complex that controls the G1-S transition, with dephosphorylation occurring at the M phase (Ruffner *et al.,* 1999). A function of BRCA1 at the G1-S transition might be to arrest cell cycle progression by binding hyperphosphorylated retinoblastoma protein (Aprelikova *et al.,* 1999).

BRCA1 might also regulate the G2-M checkpoint by controlling the assembly of mitotic spindles and the appropriate segregation of chromosomes to daughter cells. Mouse embryonic fibroblasts carrying a targeted deletion of Brca1 exon 11 had an intact G1-S checkpoint but were unable to arrest at the G2-M checkpoint (Xu *et al.,* 1999). In a significant fraction of cells centrosomes were amplified, resulting in abnormal chromosomal segregation and aneuploidy. Proteins that regulated the G2-M checkpoint, including p53 and pRB, localize to centrosomes. BRCA1 also localizes to centrosomes during mitosis and interacts with γ tubulin, a component of the centrosome (Hsu and White, 1998). Mutant BRCA1 might induce genetic instability by disrupting centrosome duplication.

BRCA1 and BRCA2 are expressed ubiquitously with the highest levels in the thymus and testis (Hakem *et al.,* 1996; Miki *et al.,* 1994). In developing mouse embryos, both genes are highly expressed in rapidly dividing, differentiating tissues and most notably during mammary epithelial proliferation and differentiation (Rajan *et al.,* 1997; Rajan *et al.,* 1996). In the mammary gland the expression of both messages is developmentally regulated and is induced during puberty and pregnancy and reduced during lactation.

1.4.4 BRCA1-deficient cells and animal models

To understand how the loss of *BRCA1* leads to breast cancer formation, mouse genetic models for *Brca1* mutation have been established. This work has revealed that *Brca1* homozygous deletions are lethal at early embryonic day E6.5 (Hakem *et al.,* 1996; Liu *et al.,* 1996; Ludwig *et al.,* 1997). Gowen *et al* generated a distinct mutation in *Brca1* and observed that embryos survived until E13.5 and exhibited defects in neural development, including anencephaly and spina bifida (Gowen *et al.,* 1996). Another group generated a mouse model with targeted deletion of Brca1 exon 11 and the resulting embryo died at E12-E18.5 (Xu *et al.,* 1999). Collectively these findings imply a role for the *BRCA1* gene product in growth and/or differentiation during mouse embryogenesis.

Both BRCA1 and BRCA2-deficient cells are characterized by cumulative chromosomal abnormalities including chromosomal breaks, aberrant mitotic exchanges and aneuploidy (Lee *et al.,* 1999; Xu *et al.,* 1999). *BRCA1* and *BRCA2* deficient mice harbor inactivating mutations in p53 and mitotic checkpoint genes (Lee *et al.,* 1999; Xu *et al.,* 1999). Mutational inactivation of p53, which governs the G1/S cell cycle checkpoint, may thus circumvent the growth arrest that is normally induced upon DNA damage and also inhibit p53-mediated apoptosis, thereby permitting the survival of cells with severe chromosomal damage. Consistently, the embryonic lethality associated with *brca1*-null mutations can be partially rescued by targeted deletion of *p53* or *p21* (Hakem *et al.,* 1997). On the other hand, inactivation of mitotic checkpoint genes could bypass mitotic arrest and permit aberrant chromosomes to segregate into progeny cells.

BRCA1-deficient HCC1937 cells, *BRCA1*-null embryonic stem (ES) cells and *Brca1*-exon 11 deletion MEF (fibroblast) cells are all characterized by radiation hypersensitivity. Increased sensitivity to the radiomimetic agent methyl methane-sulfonate (MMS) and ionising radiation (IR), but not to ultraviolet (UV) radiation, has also been observed in *BRCA1*-deficient cells (Gowen *et al.*, 1998; Scully *et al.*, 1999; Xu *et al.*, 1999; Zhong *et al.*, 1999). Reintroduction of a wild type *BRCA1* allele, but not clinically validated *BRCA1* missense mutant alleles, can complement the MMS and IR sensitivity of *BRCA1*-deficient cells, suggesting that cellular response to DNA damage is compromised in breast cancer patients carrying BRCA1 mutations (Scully *et al.*, 1999; Zhong *et al.*, 1999)

1.4.5 BRCA1 and p53

One of the key checkpoint genes for BRCA1-mediated tumorigenesis is *p53*, which is the most commonly mutated gene in many common human cancers. There are interesting parallels between p53 and BRCA1. BRCA1, like p53, is a cell cycle regulated nuclear phosphoprotein and has also been implicated in DNA damage response and repair pathways. In addition BRCA1 interacts with some of the major proteins involved in DSB repair and HR and participates in TCR. Both p53 and BRCA1 are post-translationally altered by phosphorylation in response to DNA damage. p53 regulates BRCA1 expression in response to stress conditions (Arizti *et al.*, 2000). BRCA1 associates with the C-terminus of wild-type p53 (Ouchi *et al.*, 1998) and stimulates transcription from p53-responsive promoters; whereas tumour associated mutants of BRCA1 are deficient in this coactivating activity (Somasundaram *et al.*, 1999; Zhang *et al.*, 1998). p53 coimmunoprecipitates with BRCA1. Interaction domains of p53 on BRCA1 were identified both at the N-terminus, overlapping the Rad50 binding domain, and in the second BRCT motif at the C-terminus. While BRCA1 is a potent enhancer of p53-mediated transcription, BRCA1 mediated stabilization of wild type p53 protein occurs through transcriptional activation of the $p14^{ARF}$ gene and effects on the phosphorylation of p53 through the presence of BRCA1. Indeed, cells with deletions of exon 11 of BRCA1 are defective in the rapid stabilization of p53 following DNA damage (Xu *et al.*, 2001). It is likely that BRCA1 participates in p53 stabilization *in vivo*. Like $p14^{ARF}$, p53 has been

shown to repress the transcription of BRCA1, acting in a negative feedback loop of its own stabilization (Arizti *et al.*, 2000; MacLachlan *et al.*, 2000).

Evidence from conditional knockout mice suggests that loss of *BRCA1* in mammary epithelial cells leads to incomplete proliferation, apoptosis and tumours at a low frequency (Xu *et al.*, 1999). In these mice additional heterozygous mutations in *p53* leads to many more mammary tumours, most of which have lost the remaining *p53* allele. Tumours in patients with germline *BRCA1* or *BRCA2* mutations are frequently associated with somatic mutations of *p53*. In the proposed model, mutant p53 would inactivate a cell cycle checkpoint and lead to uncontrolled growth and invasive growth. Cells that have successfully escaped death by checkpoint will probably accumulate multiple mutations. Amplification of MYB oncogene and reduction of anti-apoptotic gene Bcl-2 are characteristics of most breast tumours from *BRCA1* mutation carriers (Freneaux *et al.*, 2000; Kauraniemi *et al.*, 2000).

A recent report indicated that BRCA1 selectively coactivates the p53 transcription factor towards the genes that direct DNA repair and cell cycle arrest but not towards those that direct apoptosis (MacLachlan *et al.*, 2002). Because BRCA1 overexpression stabilizes wild-type p53 but does not lead to apoptosis in most cell lines, the selectivity of BRCA1 for p53 dependent target gene activation has been analyzed using arrays containing known targets. It has been found that BRCA1-stabilized p53 regulates transcription of DNA repair and growth arrest genes, while p53 stabilized by DNA damaging agents induces genes involved in apoptosis.

1.5 Functions of BRCA1

Cancer susceptibility gene mutations fall into two general classes (Kinzler and Vogelstein, 1997). Genes whose mutation or altered expression relieves normal controls on cell division, death or lifespan, promoting the outgrowth of cancer cells, have been termed “gatekeepers”. Those whose disruption causes genome instability, increasing the frequency of alterations in gatekeeper genes, work instead as “caretakers”. BRCA genes are essential for preserving chromosome structure,

suggesting that, in their role as tumour suppressors, they behave as caretakers, suppressing genome instability (Figure 3).

BRCA1 may function as a scaffold in the assembly of a multi-protein complex, which is important in maintenance of genomic integrity by functioning in gene transcription, chromatin remodelling, checkpoint activation, DNA damage repair and transcription-coupled DNA-damage repair. Some of the functions in which the BRCA1 protein has been implicated are summarized in Figure 4. Figure 5 shows the putative roles of BRCA1 based on reported interactions. BRCA1 interacting proteins are summarized in Table 3. BRCA1 performs its tumour suppressor function in many ways:

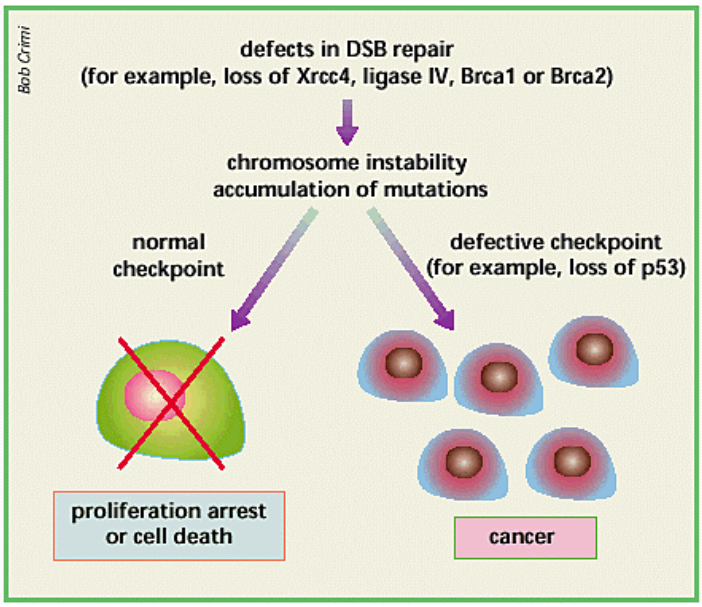


Figure 3: Repair genes as caretakers of the genome. (Khanna and Jackson, 2001)

When cells lack one of the repair proteins, they fail to repair the DSBs correctly. This leads to genetic instability, gross chromosomal rearrangements and accumulation of mutations. These events then trigger cell-cycle checkpoints resulting in permanent growth arrest or death of affected cells. If the checkpoints are inactivated by mutations, this leads to tumorigenesis.

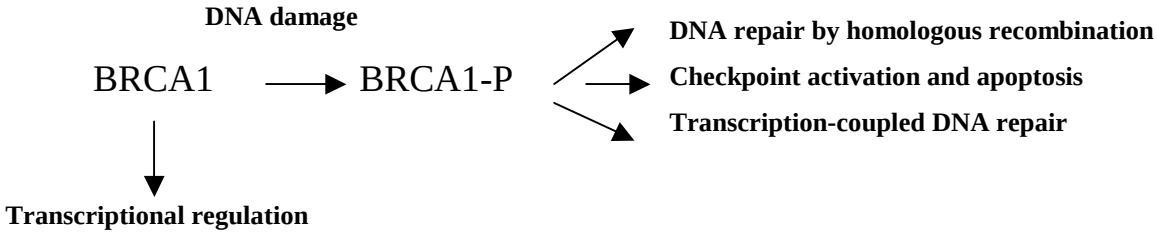


Figure 4: Multiple cellular functions of BRCA1

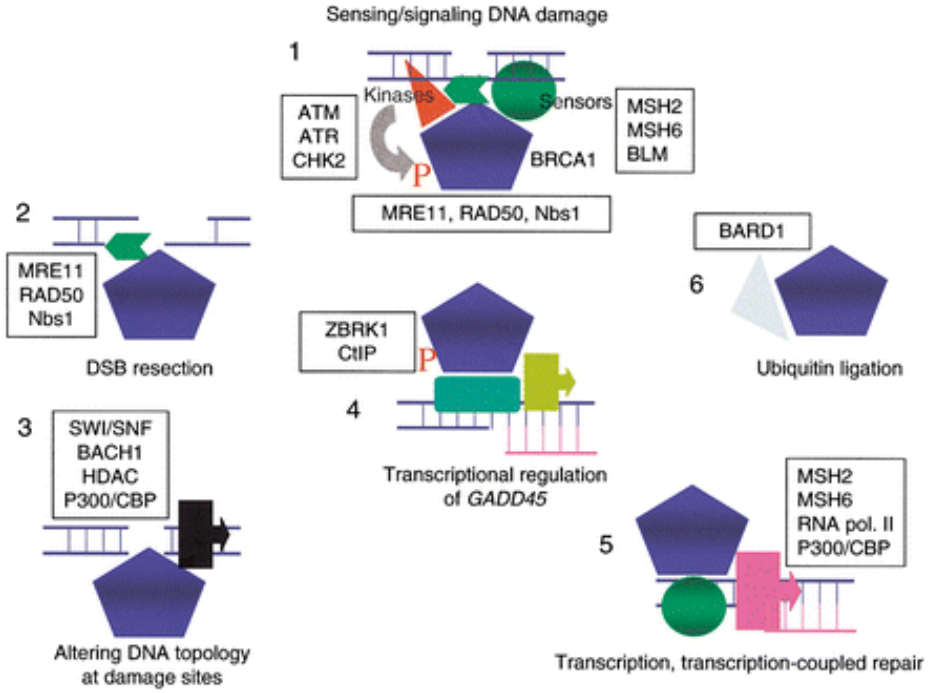


Figure 5: Putative roles of BRCA1 (Venkitaraman, 2002)

BRCA1 works as a signal processor (1) during DNA damage responses in complex with proteins that bind to aberrant DNA structures (sensors), and the kinases that signal their presence. Phosphorylation of BRCA1 may be essential for local functions [control of DSB resection (2), altering DNA topology (3)] near a DNA lesion, as well as for distant functions such as transcriptional control of checkpoint genes (4) (e.g., GADD45) or targets of estrogen receptor signalling, or transcription-coupled DNA repair (5) BRCA1 works with BARD1 (6) works as an ubiquitin ligase

Table 3: BRCA1 interacting proteins (Modified from Welcsh and King, 2002**)**

BRCA1 interacting protein or complex	Function of interacting protein	Interacting domain on BRCA1 (aa)	Reference
RAD51	DSB repair	exon 11 (758-1064)	Scully et al., 1997
RAD50	DSB repair	exon 11 (341-748)	Zhong et al., 1999
BRCA2	DSB repair	BRCT domain (1314-1863)	Scully et al., 1997
BASC (ATM, BLM, MSH2, MSH6, MLH1, RCF)	Mismatch repair	BRCA1 part of complex	Wang et al., 2000
ATP-MSH2	Mismatch repair	?	Wang et al., 2000
H2AX	Signals DNA damage	?	Paull et al.,2000
p53	transcription factor, tumor suppressor	Exon 11 and BRCT domain (244-500 and 1760-1863)	Zhang et al., 1998;Ouchi et al., 1998; Chai et al., 1999
pRB	cell cycle regulator, tumor suppressor	exon 11 and BRCT domain (304-394 and 1536-1863)	Aprelikova et al., 1999
c-Myc	transcription factor, oncogene	N-terminus and exon 11 (175-303 and 433-511	Wang et al., 1998
ZBRK1	transcription factor, represses GADD45	exon 11 (341-748)	Zheng et al., 2000
ATF	transcription factor	RING (1-101)	Houvras et al., 2000
STAT1	signal transducer, transcriptional activation	exon 11 (502-802)	Ouchi et al.,2000
E2F	transcription factor, cell cycle regulator	N-terminus (1-76)	Wang et al., 1997
RNA Pol II Holoenzyme (hRPB10a;hRPB2)	transcription	BRCT domain (1650-1800)	Scully et al., 1997; Schlegel et al.,2000
RNA helicase A	component of RNA PolII holoenzyme	BRCT domain (1650-1800)	Anderson et al., 1998
BACH1	DNA helicase	BRCT domain (1650-1800)	Cantor et al., 2001

Estrogen receptor	ligand responsive transcription factor	N-terminus (1-300)	Fan et al., 1999;2001
Androgen receptor	ligand responsive transcription factor	exon 11 and BRCT domain (758-1064 and 1314-1863)	Yeh et al., 2000
CtIP	binds CtBP; transcriptional co-repressor	BRCT domain (1651-1863)	Li et al., 1999; Yu et al.,1998; Wong et al.,1998
p300/CBP	transcriptional co-activator	RING and BRCT domain (1-303 and 1314-1863)	Pao et al.,1998
SWI/SNF	chromatin remodelling complex	exon 11 (260-553)	Bochar et al., 2000
HDAC l and 2	Histone deacetylation, chromatin remodelling	BRCT domain (1563-1863)	Yarden and Brody, 1999
Centrosome (p53, pRB, Nm23)	chromosome segregation	BRCA1 part of complex	Hsu and White, 1998
BRAP2	cytoplasmic retention	NLS (303-701)	Li et al.,1998
Vasolin containing protein (VCP)	ATPase	exon 11 (303-625)	Zhang et al.,2000
BARD1	Ubiquitination? Polyadenylation via CstF-50?	RING (1-101)	Wu et al.,1996
BAP1	Deubiquitinating enzyme	RING (1-101)	Jensen et al., 1998
Importin a	nuclear transport	NLS (303-701)	Chen et al., 1996

1.5.1 BRCA1 in damage signalling

An important step in the cellular response to DNA damage is to transduce damaged signals to downstream effectors involved in the arrest of cell cycle and repair of damaged DNA (figure 6). In mammalian cells, several kinases such as, ATM, ATR, DNA-PK, Chk1 and Chk2 are activated in response to DNA damage. BRCA1 becomes hyperphosphorylated in response to treatment of cells with a variety of DNA damaging agents including UV, hydroxyurea, mitomycin C, MMS, IR, H_2O_2 and adriamycin (Chen *et al.,* 1996a; Li *et al.,* 1999; Scully *et al.,* 1997b). *In vitro* and *in vivo* phosphorylation sites in BRCA1 have been identified. The data from these studies have suggested that phosphorylation of BRCA1 by these kinases is necessary for BRCA1-mediated DNA damage response and multiple kinase activities are responsible for the DNA damage-induced hyperphosphorylation of BRCA1. Table 4 shows the *in vivo* phosphorylation sites on BRCA1. In addition to ATM, ATR and Chk2, DNA-PK have been shown to phosphorylate BRCA1 *in vitro* (Lim *et al.,* 2000)

BRCA1 kinases	***In vivo* phosphorylation sites**	**Reference**
ATM	S1189, S1457, S1524, S1542	(Li *et al.,* 2000)
ATR	S1423	(Tibbetts *et al.,* 2000)
Chk2	S988	(Lee *et al.*, 2000)

Table 4: BRCA1 modifying proteins

Besides its roles in maintenance of genomic integrity, chromatin remodelling, and transcriptional regulation, BRCA1 has also been implicated in cell cycle/checkpoint control. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. BRCA1 regulates the expression of both Wee1 kinase, an inhibitor Cdc2/Cyclin B kinase and 14-3-3 family of proteins that sequesters phosphorylated Cdc25C and Cdc2/cyclin B kinase in the cytoplasm, leading to a conclusion that BRCA1 regulates key effectors that control the G2/M checkpoint and is therefore involved in regulating the onset of mitosis (Yarden *et al.,* 2002).

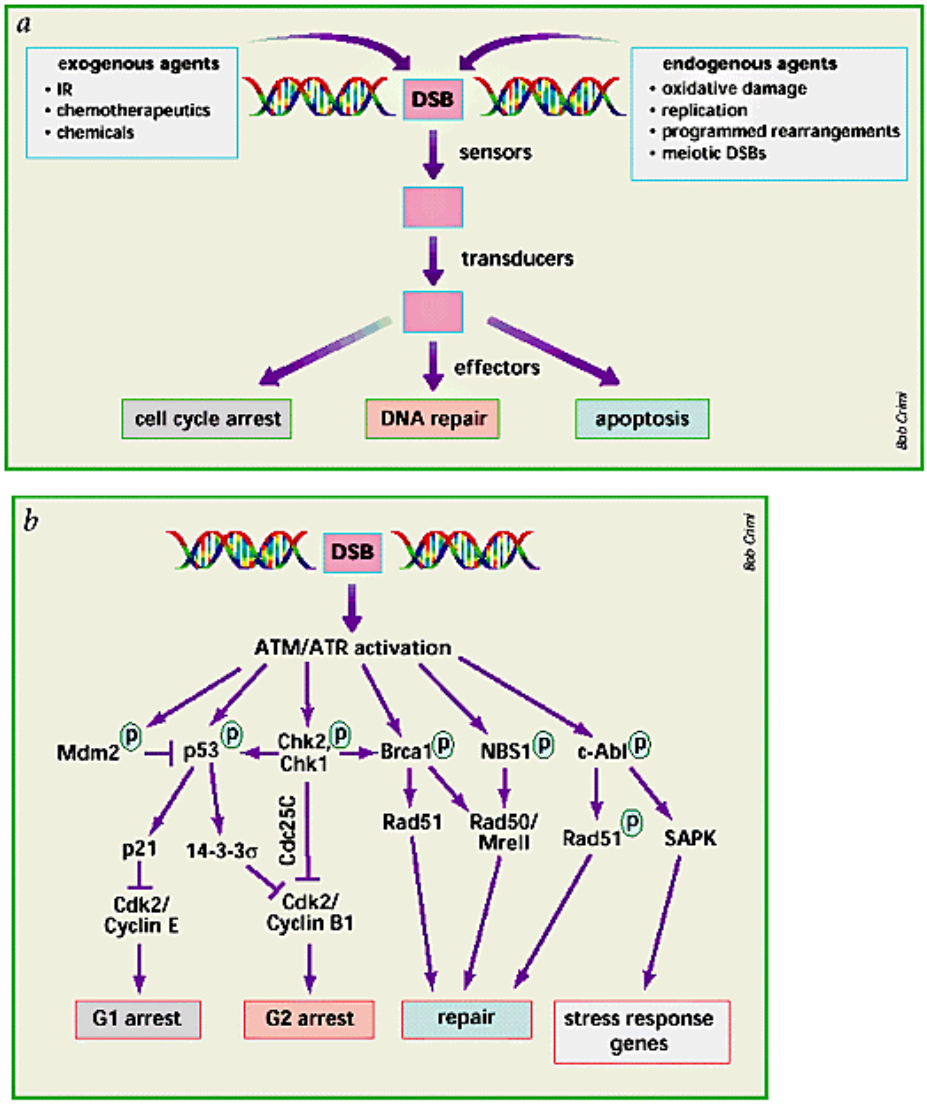


Figure 6: Signaling of DSBs. (Khanna and Jackson, 2001)

a, The general organization of the DNA-damage response pathway. The presence of DSBs is recognized by a sensor, which transmits the signal to a series of downstream effector molecules through a transduction cascade to activate signaling mechanisms for cell-cycle arrest and induction of repair, or cell death if the damage is irreparable. **b**, A central role for ATM in the cellular response to DSBs. ATM is activated in response to DSBs. Activated ATM signals the presence of DNA damage by phosphorylating targets involved in cell-cycle arrest, DNA repair and stress response. p21 inhibits the activity of cdk2/cyclinE and 14-3-3σ inhibits the activity of cdc2/cyclin B and effecting cell-cycle arrest. c-Abl activates stress-activated protein kinase (SAPK) for transcriptional regulation of stress-response genes.

Phosphorylation of BRCA1 is apparently important for a proper DNA damage response. However, it remains unclear how phosphorylation modulates activities of BRCA1. Since BRCA1 forms a complex with RAD50/MRE11/NBS1 and colocalizes to DNA damage sites following ionizing radiation, it has been speculated that BRCA1 is involved in double strand break repair (Cortez *et al.*, 1999; Lee *et al.*, 2000). Alternatively, phosphorylation of BRCA1 may influence the transcriptional regulation activities of BRCA1 leading to cell cycle checkpoint. ATM phosphorylates CtIP *in vivo* and *in vitro* following IR treatment (Li *et al.*, 2000). This ATM dependent phosphorylation is required for dissociation of the CtIP/CtBP corepressor complex from BRCA1 and subsequently leads to relieving BRCA1 mediated repression of GADD45 transcription and p21 expression (Li *et al.*, 2000). These studies suggest that CtIP mediates one of the functional links between ATM and BRCA1 in DNA damage signaling pathways.

1.5.2 BRCA1 in chromatin remodelling

BRCA1 plays an important role in the cellular response to double strand breaks. Within minutes of DNA damage, the histone H2A family member H2AX becomes extensively phosphorylated and forms foci at break sites. BRCA1 is recruited to these foci several hours before other factors such as RAD50 and RAD51, suggesting that BRCA1 and H2AX initiate repair by modifying local chromatin structure, thereby allowing DNA repair proteins access to damaged sites (Paull *et al.*, 2000). In support of this role for BRCA1 in response to DNA damage, it has been shown that BRCT domains of BRCA1 bind double strand breaks (Yamane *et al.*, 2000).

BRCA1 interacts with the SWI/SNF chromatin remodelling complex (Bochar *et al.*, 2000), with regulators of histone acetylation/deacetylation (Pao *et al.*, 2000; Yarden and Brody, 1999), with a novel helicase BACH1 (Cantor *et al.*, 2001) and RecQ homolog encoded by the Bloom's syndrome gene, BLM helicase (Wang et al., 2000b). How these interactions may assist DSB repair is currently speculative. Chromatin changes mediated by histone modification may make DNA more accessible by the helicases and repair machinery.

1.5.3 BRCA1 in DNA repair

BRCA1 appears to participate in the cellular DNA damage response at multiple stages. In normal cells, responses to DNA damage include sensing damaged DNA, transducing DNA damage signals, relocating repair machinery to damage sites, completing a repair process and coordinating cell cycle progression with the DNA repair process. Accumulating evidence suggests that BRCA1 functions not only in association with the DNA repair machinery, but also in DNA damage induced cell cycle checkpoint control. BRCA1 may additionally regulate the expression of genes involved in DNA damage repair and also directly participates in the repair process itself. Finally phosphorylation of BRCA1 upon DNA damage implies a role for BRCA1 in DNA damage-induced signal delay.

BRCA1 interacts with the RAD50/MRE11/NBS1 protein complex containing the mammalian homologs of yeast molecules known to participate in double strand break repair, comigrants to sites marked by phospho-H2AX (Zhong *et al.,* 1999). The equivalent of this complex in yeast, the Rad50/Mre11/Xrs2 complex functions in both nonhomologous end joining (Ivanov *et al.,* 1992) and homologous recombinational repair of DNA double strand breaks (Bressan *et al.,* 1999). Rad50/Mre11/Xrs2 complex has been shown to be involved in other cellular processes including chromatin configuration and telomere maintenance (Chamankhah *et al.,* 2000; Gerecke and Zolan, 2000; Ohta *et al.,* 1998). It has been proposed that Rad50/Mre11/Xrs2 is responsible for end processing of double strand breaks (Tsubouchi and Ogawa, 1998). In support with this idea, recombinant MRE11 proteins and purified human RAD50/MRE11/NBS1complexes exhibit exonuclease and endonuclease activities (Paull and Gellert, 1998). MRE11 encodes a nuclease activity, which resects flush DSB ends to generate ssDNA tracts. Under certain *in vitro* conditions, BRCA1 can inhibit this activity of MRE11 (Paull *et al.,* 2001), regulating the length and presumably the persistence of ssDNA generation at sites of DNA breakage. It has been proposed that RAD50 may be a chromatin-associated protein and participate in chromatin structural reconfiguration (Alani *et al.,* 1989). NBS1 is the product of the gene mutated in Nijmegen Break Syndrome (Carney *et al.,* 1998).

BRCA1 appears to interact with the RAD50/MRE11/NBS1 complex directly through RAD50 (Zhong *et al.,* 1999). Similar to the formation of the RAD50/MRE11/NBS1 complex, the association of BRCA1 with this complex does not change in response to DNA damage. Rather, the nuclear partitioning of this BRCA1-containing complex changes and BRCA1 forms ionising radiation-induced foci (IRIF), which is also a characteristic of RAD50, MRE11 and NBS1 (Maser *et al.*, 1997).

Besides RAD50/MRE11/NBS1, other components involved in DNA damage repair, such as MSH2, MSH6, MLH1, ATM and BLM have been found to reside in a large BRCA1-containing DNA repair complex (Wang et al., 2000b). In addition, DNA replication factor C and PCNA (proliferating cell nuclear antigen) were also found in this complex. This complex has been proposed to present a BRCA1-associated genome surveillance complex (BASC) since many of its constituent proteins individually recognize distinctly abnormal DNA structures such as double strand breaks, base pair mismatches and stalled replication forks. Therefore the association of BRCA1 with these proteins suggests that BRCA1 may also participate in the resolution of aberrant DNA structures that occur during DNA replication or when DNA replication is stalled (Wang et al., 2000b). Consistent with this notion, a previous observation that BRCA1 foci at S phase disperse in response to DNA damage or replication blocks, and relocalize to PCNA-containing structures (Scully *et al.,* 1997a), suggestive of a role for BRCA1 in replicational DNA repair.

BRCA1 has also been shown to interact and colocalize with RAD51 (Chen *et al.,* 1998b; Scully *et al.,* 1997b). Eukaryotic RAD51 proteins are homologs of bacterial RecA and are required for recombination during mitosis and meiosis and for recombinational repair of DNA double strand breaks. In response to DNA damage, BRCA1 undergoes distinctive phosphorylation that differs from the phosphorylation during G1-S transition (Scully *et al.,* 1997a). The association of RAD51 and BRCA1 is supported by the observation that BRCA1 foci disperse during the S phase, and relocalize to PCNA-containing structures in response to UV-treatment or replication block by hydroxyurea (Scully *et al.,* 1997b). BRCA1 has also been observed in IR-induced RAD51 foci; however such foci are distinct from those comprising BRCA1 and the RAD50/MRE11/NBS1 complex (Zhong *et al.,* 1999). While the

RAD50/MRE11/NBS1 complex has been proposed to function in end processing, an early step in both homologous recombination and non-homologous end joining based repair of DNA double strand breaks, Rad51 is involved in strand exchange, a later step in homologous recombination. Homologous recombination is defective in BRCA1-deficient cells, but non-homologous end joining is unaffected (Wang *et al.*, 2001). The biological implications underlying the dual participation of BRCA1 in two distinct steps of homology based recombinational DSB remain to be resolved.

On the other hand, besides DSB repair BRCA1 specifically enhances the global genomic repair pathway, independent of p53, and can induce p53 independent expression of the nucleotide excision repair genes *XPC* (a gene defective in xeroderma pigmentosum group C), *DDB2* (a gene defective in xeroderma pigmentosum group E cells and the p48 protein) and *GADD45* (gene involved in growth arrest and DNA damage). (Hartman and Ford, 2002). This work has suggested a mechanism for the effect of BRCA1 on global genome repair involving transcriptional regulation of nucleotide excision repair genes. BRCA1 can activate *GADD45* promoter independent of p53, supporting the notion that BRCA1 activates *GADD45* and maintains global genome repair in p53 deficient cells. This finding pointed to an important role for BRCA1 in DNA repair and the maintenance of genomic stability suggesting a specific mode of action for BRCA1 in DNA damage response pathways.

Transcription coupled repair: Brca1-deficient mouse embryonic stem cells have been shown to be defective in the ability to carry out transcription-coupled repair (TCR), a process in which DNA damage is repaired more rapidly in transcriptionally active DNA than in the genome as a whole (Gowen *et al.*, 1998). DNA damage induced by UV as well as oxidative damage caused by IR or H_2O_2 can be repaired by TCR. It has been demonstrated that Brca1-deficient cells are defective in TCR of oxidative DNA damage, but not in TCR of UV-induced DNA damage. Consistent with this observation, these Brca1-deficient cells are hypersensitive to oxidative DNA damage. Presently, it is not clear whether BRCA1 itself participates directly in TCR or, alternatively, whether, it functions as a transcription factor essential for the expression of genes whose products are required for TCR of oxidative damage (Gowen *et al.*, 1998).

1.5.4 BRCA1 as a modulator of gene transcription

A role for BRCA1 in transcriptional regulation was initially indicated by the identification of an acidic domain near the carboxyl-terminus of BRCA1 with an inherent transactivation function that is sensitive to cancer predisposing mutations (Chapman and Verma, 1996; Chen *et al.*, 1996a; Monteiro *et al.*, 1996). This segment of BRCA1 polypeptide is rich in acidic amino acid residues, a character found in the activation domain of many transcription factors. When fused to a heterologous DNA-binding domain, the carboxyl terminal of BRCA1 was observed to exhibit strong transcriptional activity in mammalian cells and this activity was completely abolished by familial breast cancer derived BRCA1 mutations (Chapman and Verma, 1996; Chen *et al.*, 1996a; Monteiro *et al.*, 1996). The presence of an autonomous transactivation function within BRCA1, coupled with the absence of demonstrable sequence-specific DNA-binding activity, has led to the hypothesis that BRCA1 functions as a co-regulator of transcription.

BRCA1 interacts with several transcription factors such as p53, CtIP, c-myc, ZBRK1, ATF, E2F and signal transducer STAT1 (Zheng *et al.*, 2000) and modulates their activity. In these studies, it appears that BRCA1 can activate transcription in some cases and repress transcription in others. For example, BRCA1 can bind to p53 and enhance its transcription activity at the promoter of $p21^{WAF/CIP}$ or BAX (Ouchi *et al.*, 1998; Somasundaram *et al.*, 1997; Zhang *et al.*, 1998). Furthermore BRCA1-induced cell cycle arrest is dependent on functional p21. A recent study identified the transactivation of cyclin dependent kinase inhibitor $p27^{Kip1}$ by BRCA1 (Williamson *et al.*, 2002).

BRCA1-mediated transcriptional activation

The initiation of RNA polymerase II transcription represents a critical step for gene expression regulation within the cell. Gene-specific activators function to stimulate the rate of transcription initiation largely through the recruitment of either chromatin remodelling activities and/or the general transcription machinery in order to override nucleosome-mediated promoter repression and assemble transcription-

competent pre-initiation complexes, respectively. BRCA1 may mediate gene-specific transcriptional activation via both of these recruitment steps.

BRCA1 could play a role in the recruitment of chromatin remodelling activities. BRCA1 has been shown to interact directly or indirectly with chromatin modifying activities including p300 (Pao *et al.,* 2000), hBRG1 (Bochar *et al.,* 2000) and BRCA2 (Chen *et al.,* 1998b), which itself is associated with histone deacetyltransferase activity. BRG1 is a component of SWI/SNF chromatin remodelling complex. It has recently been shown that BRCA1 carboxyl terminal transactivation domain, when targeted to chromatin via a heterologous DNA-binding domain, can alter chromatin structure (Hu *et al.,* 1999). Significantly, the cancer-predisposing mutations that abolish its transcriptional activation function also abrogate the ability of this domain to effect chromatin remodelling.

BRCA1 is likely to function in transcriptional activation involving RNA polymerase II holoenzyme recruitment. BRCA1 was found to be associated and co-purified with the RNA polymerase complex and interactions have been demonstrated with constituent holoenzyme components, including RNA helicase A (RHA) (Anderson *et al.,* 1998; Schlegel *et al.,* 2000; Scully and Livingston, 2000). CBP/p300 (Pao *et al.,* 2000) and RNA polymerase II itself from its subunits hRBP2 and hRBP10a (Schlegel *et al.,* 2000). The ability of BRCA1 to bind to RNA helicase A has been shown to be essential for the transactivation activity of BRCA1. CBP/p300 has been found to interact with BRCA1 and stimulates BRCA1-directed transcription activation (Pao *et al.,* 2000). These observations implicate a model to account for transcriptional activation by BRCA1: gene-specific activators, by interacting with BRCA1, recruit the RNA polymerase II holoenzyme complex onto target promoters in order to effect an increase in the transcription rate of genes under their control.

BRCA1-mediated transcriptional repression

The carboxyl terminus of BRCA1 not only binds transcriptional coactivators but also transcriptional corepressors such as CtIP/CtBP complex and histone deacetylases (HDACs) (Li *et al.,* 1999; Yarden and Brody, 1999). Interestingly familial breast cancer derived mutations that compromise its transactivation activity also abolish the binding of BRCA1 to CtIP and HDACs.

BRCA1 has been shown to repress c-Myc-mediated transcriptional activation from synthetic promoters carrying c-Myc response elements as well as natural c-myc responsive CDC25A promoter (Wang *et al.,* 1998). The negative effect of BRCA1 on c-myc mediated transactivation could derive from trans-repression involving inhibition of either Myc-Max heterodimer formation or DNA binding by Myc-Max heterodimers. This BRCA1-mediated transcriptional repression can be correlated with its inhibition of c-myc mediated cellular transformation, thus providing one potential mechanism for BRCA1-mediated tumour suppression (Wang *et al.,* 1998).

BRCA1 inhibits the transactivation activity of estrogen receptor (Fan *et al.,* 1999). This BRCA1-mediated inhibition of estrogen receptor transactivation takes place by the interaction of p300 and ER-α. Cofactors p300 and CBP modulate the ability of BRCA1 protein to inhibit ER- α signaling. It has also been suggested that, BRCA1 inhibition of ER- α activity may be, in part, attributable to the down regulation of p300 (Fan *et al.,* 2002). The estrogen signaling pathway controls multiple aspects of breast and ovarian cell growth, differentiation and homeostasis. Furthermore, estrogen itself is a distinct etiological factor in breast and ovarian cancer. By affecting hormone response pathways, BRCA1 may regulate growth or differentiation in a cell-type specific manner.

BRCA1 mediated transcriptional repression has also been implicated in silencing the DNA-damage inducible *p21* and *GADD45* genes in their uninduced states. BRCA1 can mediate sequence specific transcriptional repression through its selective recruitment by a novel DNA-binding transcription factor ZBRK1 (zinc finger and BRCA1-interacting protein with a KRAB domain). BRCA1 has been shown to mediate ZBRK1-directed repression through a ZBRK1 binding site identified in intron 3 of the *GADD45* gene, thus providing a potential mechanistic link between the activities of BRCA1 in gene-specific transcription control, the cellular DNA damage response and the maintenance of genome integrity (Zheng *et al.,* 2000). ZBRK1 binds to the specific sequence GGGxxxCAGxxxTTT. This motif has been identified in the previously identified BRCA1 downstream targets (Harkin *et al.,* 1999; MacLachlan *et al.,* 2000) such as *GADD153, Ki-67, Bax, p21 EGR1,*

amphiregulin prothymosin, *TIMP-1, TIMP-2,* and *Topo IIa*. BRCA1 mediated control of p21 and GADD45 gene transcription may contribute to its role in cell cycle checkpoint, since p21 and GADD45 have been implicated in DNA-damage induced G1/S and G2/M checkpoint control, respectively (Brugarolas *et al.,* 1995; Deng *et al.,* 1995; el-Deiry *et al.,* 1993; Wang *et al.,* 1999).

The association of BRCA1 with CtIP/CtBP or HDACs suggests a more direct role for BRCA1 in active repression and thus provides an alternative explanation for the negative effect of BRCA1 on transcription.

Human CtBP was initially identified as an adenovirus E1A C-terminal interacting protein capable of attenuating E1A-mediated transcriptional activation and tumorigenesis (Schaeper *et al.,* 1995). CtBP serves as corepressor for a variety of DNA binding transcriptional repressors. Each of these CtBP-interacting transcriptional repressors harbor a conserved amino acid sequence motif, PLDLS, that was originally identified in E1A and that specifies the association of each with CtBP. The same sequence motif present in CtBP-interacting transcriptional repressors also specifies the interaction of CtBP with CtIP, a protein initially identified by virtue of this interaction (Schaeper *et al.,* 1995). Meanwhile, several groups have identified the interaction between CtIP and BRCA1 (Li *et al.,* 1999; Wong *et al.,* 1998; Yu *et al.,* 1998). CtIP was found to link the corepressor CtBP to BRCA1 (Li *et al.,* 1999). Interestingly, a second tumour suppressor, the retinoblastoma (Rb) protein and one of its associated family members, p130, also interact with CtIP (Meloni *et al.,* 1999). Its specific interaction with two distinct tumour suppressor proteins implies a fundamental role for CtIP in tumour suppression.

Another distinct corepressor complex which BRCA1 interacts, is the histone deacetylase complex (Yarden and Brody, 1999). BRCA1 has been shown to associate at least four components of the histone deacetylase complex, HDAC1, HDAC2, RbAp46 and RbAp48. This observation suggests that BRCA1-mediated transcriptional repression may derive, at least in part, from active recruitment of HDACs.

Current experimental observations are consistent with several alternate possibilities by which BRCA1 mediates transcriptional repression. First, BRCA1 corepression could involve targeted chromatin remodelling; BRCA1 could, by virtue of its direct recruitment of HDAC complexes, alter the chromatin structure of its target genes into a repression-favored status. Alternatively, BRCA1 could affect remodelling via its interaction with the CtIP-CtBP corepressor complex. Or, the association of BRCA1 with the RNA polymerase II holoenzyme may support a distinct mechanism for corepression by which BRCA1 directly targets either the general transcription machinery or interacting coactivators.

1.5.5 Other potential functions of BRCA1

BRCA1 associates with BAP1, which is a de-ubiquitinating enzyme, suggesting a role for BRCA1 in ubiquitin-dependent protein degradation. The potential role of BRCA1 in post-transcriptional RNA processing may coordinate with its role in transcriptional regulation. Like other RING proteins BRCA1/BARD1 complex functions as an E3 ubiquitin ligase of undetermined specifity (Hashizume *et al.,* 2001; Ruffner *et al.*, 2001). Cancer predisposing mutations within the BRCA1 RING domain abolishes its ubiquitin ligase activity (Ruffner *et al.,* 2001). The ubiquitin ligation function of BRCA1 is poorly understood. The protein encoded by FANCD2 mutated in Fanconi's anemia, a disease characterized by chromosomal instability and cancer predisposition, localizes with BRCA1 to focal sites after DNA damage (Garcia-Higuera *et al.,* 2001). Redistribution is dependent upon monoubiquitination of FNACD2, although it is not known whether the BRCA1/BARD1 complex can perform this modification.

The BRCA1 binding protein, BARD1, can associate with polyadenylation factor CstF50 and inhibit polyadenylation (Kleiman and Manley, 1999). BARD1 was found colocalized with BRCA1 during S-phase but not in G1 (Jin *et al.,* 1997). Therefore BRCA1-BARD1 complex may also play a role in gene transcription at the level of polyadenylation of mRNA.

1.6 Identified BRCA1 regulated targets

Recent studies utilizing gene expression profiling methodologies have revealed that ectopic overexpression of BRCA1 can induce a diverse array of genes implicated in cell growth control, cell cycle regulation, DNA replication and repair. As BRCA1 protein levels increase between mid-S and G2 phases of the cell cycle (Chen *et al.,* 1996b), it is possible that BRCA1 overexpression strategies may stimulate the status of BRCA1 during these physiological periods and thereby provide at least a limited window onto the spectrum of target genes under its transcriptional control.

In general, several gene expression-profiling methodologies are used to assess the transcriptional activity in the cells. A cell responds to its changing environment in many ways, including regulated shifts in gene transcription. Experiments are often designed to monitor altered gene expression of the gene of interest in response to a stimulus. In addition, enforced expression or inhibition of a gene may induce a biological response that illuminates the activity of the gene product. Several overexpression studies have been extensively used in order to identify the shifts in patterns of gene expression such as tight regulation of the expression of the gene of interest (tetracycline or ecdysone inducible system) or expression of the gene of interest under modified strong viral promoter (CMV promoters). Once the components of the inducible system are stably introduced into the cell, tightly regulated expression systems are preferred because the expression of the gene of interest is controlled in terms of induction of the gene with a small molecule that is not toxic to mammalian cells. Because overexpression of many genes may result in apoptosis in cells, the use of inducible systems also overcome this problem. Reporter genes (ß-galactosidase or luciferase) with these expression systems are also used to elucidate promoter functioning.

As gene expression studies alone will not elucidate the function of the encoded protein, expression-profiling methodologies are used to analyse the transcriptional response of the cells to the changing environment.

Expression profiling describes a group of technologies that simultaneously assess the transcriptional activity of a large number of genes. Recent technological developments have made it possible to rapidly evaluate hundreds or even thousands of genes for their transcriptional responses in biological and pathological situations. These methods assess alterations in steady-state levels of mRNA. The strengths and the weaknesses of some the techniques are summarized in Table 5:

Method	***Gene discovery***	***Gene coverage***	***Sensitivity***	***Labor***	***Set-up cost***
EST sequencing	Yes	Low	Low	Medium	Low[a]
SAGE	Yes	Medium	Low	Medium	Low[a]
Real-time Q-PCR	No	Low	High	Low	Medium
Differential display	Yes	Low	High	Medium	Low
Subtractive hybridization	Yes	Low	High	Medium	Low
cDNA microarrays	Yes[b]	High	Medium	High[c]	High
Oligo microarrays	No	High	Medium	Low[d]	High[d]

Table 5: Summary of methods for gene expression profiling. Seven methods for gene expression analysis are compared. Gene discovery refers to the capability of the method to identify new genes. Gene coverage indicates the number of genes that are reasonably handled by the technique. Sensitivity is an assessment of the technique to detect rare mRNAs. Also summarized are the labor and the initial set-up costs required to complete a typical experiment.

[a] Assumes adequate DNA sequencing capacity is available.

[b] Considers arraying anonymous genes from cDNA libraries.

[c] Considers labor to fabricate and utilize arrays.

[d] Assumes the purchase of prefabricated arrays.

(Modified from Stanton, 2001)

In this thesis study, subtractive hybridization was used to identify the transcriptionally regulated targets of BRCA1 since it is sensitive, allows detection of low abundance transcripts, allows discovery of new genes and is cheaper when compared to other techniques. Several other studies have aimed to identify the

BRCA1 regulated genes by other methodologies. This is the first study to investigate the downstream targets of BRCA1 by the SSH technique.

In order to identify a series of downstream targets of BRCA1, Harkin *et al* initially established an osteosarcoma cell line with tightly regulated BRCA1 expression with the tetracycline inducible system (Harkin *et al.,* 1999). High-density oligonucleotide arrays were used to analyze gene expression profiles at various times following BRCA1 induction. Among many genes, they have identified *GADD45* as one of the major targets of BRCA1 (Table 6) and showed the p53 independent activation by BRCA1, which suggested the JNK/SAPK pathway for BRCA1-induced apoptosis. With the same tetracycline inducible system, but this time with a breast carcinoma cell line, Mullan *et al* have again identified *GADD45* as the major target of BRCA1 by using oligonucleotide arrays (Mullan *et al.,* 2001).

Table 6: List of BRCA1 upregulated genes (Harkin *et al.,* 1999)

Description	**Fold induction by northern**
BRCA1	60
GADD45	35
EGR1	10
Tristetraproline (TTP)	5
Gem GTPase	5
Br140	4
Unidentified cDNA (R12810)	4
PM-scl 75	3
ATF3	3
ARD1	3
Hepatic leukemia factor	3
Amphiregulin	3
TR3 Orphan receptor (NAK1)	3
TNFα-inducible gene A20	2
Fibroblast activating protein α	2
IL4 receptor α	2
EST (R41997)	2
EST (H81220)	2
KIAA0055	2

Later on MacLachlan *et al*, have overexpressed BRCA1 by using an adenovirus vector and high density cDNA array screening of colon, lung and breast cancer cells and have identified several genes affected by BRCA1 expression in a p53-independent manner including DNA damage response genes and genes involved in cell cycle control (MacLachlan *et al.*, 2000). Notable changes were observed as the induction of *GADD45* and *GADD 153* genes and reduction of cyclin B1 expression.

Table 7: List of BRCA1 downstream genes (MacLachlan *et al.*, 2000)

Induced genes		Repressed genes	
Gene	Fold induction	Gene	Fold reduction
CDC34[a]	2.14	GRB2 adaptor	2.05
PCTAIRE1[a]	1.59	grb10	5.50
cdk4	2.18	PIN1[a]	5.30
p21$^{WAF1/Cip1}$[a]	1.68	Cyclin B1[a]	1.50
PCNA[a]	3.40	Cytokeratin 4	1.23
Cytokeratin 2E	1.23	Cytokeratin 5	1.30
RhoA[a]	3.42	BAX[a]	3.80
MET[a]	2.70	BAK[a]	3.70
Superoxide dismutase[a]	1.10	Frizzled-related[a]	4.30
IGFBP2	2.08	Frizzled homolog	8.30
IGFBP4	3.30	Dishevelled homolog	2.00
Ku70[a]	2.01	RAR rxr-β	3.30
Ku60[a]	3.40	Collagen type VIII α-1	4.30
Yeast rad6 homolog[a]	2.62	Zyxin	1.29
Gadd 153[a]	1.76	CD59	2.80
Gadd 45[a]	2.65	Collagen type XVI α-1	1.20
DNA Topo II α isozyme	2.56	Semaphorin V	1.31
DNA Topo I	1.32	CD9[a]	1.17
Lunatic fringe	1.73	β-actin	1.04
TIMP-1[a]	2.03		
TIMP-2	1.34		
αCatenin[a]	3.20		
p21RAC1	1.83		
Nucleoside kinase A[a]	1.64		
Nucleoside kinase B	1.63		
EB1 (APC binding protein)	1.21		
rho GDP dissociation inhibitor 1	1.76		
Glyceraldehyde-3-phosphate dehydrogenase	1.01		

Aprelikova *et al* have analyzed Brca1-deficient mouse embryonic stem cells, using oligonucleotide arrays to characterize the BRCA1 regulated genes (Table 8) (Aprelikova *et al.*, 2001). They have found that loss of BRCA1 results in decreased expression of stress response genes and 14-3-3σ, a major G2/M checkpoint control gene. BRCA1 induced transcription of the endogenous 14-3-3σ was in a p53 dependent manner.

Table 8: Genes downregulated in Brca1 -/- cells (Aprelikova *et al.*, 2001)

Function	Gene name
G2/M control	14-3-3 σ
Stress response	DNAJ protein homolog 2
	Heat shock protein 86
Transription	Id3
	AP-2.2
	EST (S-II TF related) (D00925)
	ATP-dependent RNA helicase
	HMGi-C
	DNA replication 30 kD subunit RPA
	ADP ribosylation factor 1
Cytoskeleton/ contractyle	Cardiac muscle alpha actin
	Skeletal muscle alpha actin
	Extra-embryonic endodermal cytokeratin
	Tropomyosin isoform 2
	Alpha tubulin isotype M
	Vimentin
	SM22 alpha
	Vascular smooth muscle alpha actin
	Spectrin alpha chain
	NF68 neurofilament 68 kD protein
	ROCK-2 (Rho-associated protein kinase
Signal transduction	FGFR1
	FGF binding protein
	FGF inducible gene 14
	PLC-alpha
	Pw1 zink finger protein
	Signal transducing adaptor STAM
	Rit (Ras related protein

	Wnt-3*
	Creatin kinase B
Protein synthesis and degradation	Translation initiation factor 2alpha
	Eukaryotic initiation factor 4A-like, CIF5.10
	Eukaryotic initiation factor 4A-like, NUK-34
	QK1 (Quaking type 1)
	Translation initiation eif-3, p110
	Seryl-tRNA synthetase
	Multifunctional aminoacyl-tRNA synthetase
	Tryptophan-tRNA ligase
	Aspartate aminotransferase
	Protease nexin II
	26S protease regulatory subunit 7 (MSS1)
	ATPase 26S proteasome subunit 4
	ATPase (lysosomal)
Membrane protein	Annexin V
	Annexin III
ECM component	Latexin
Differentiation	Lethal(1) discs large-1 tumor suppressor
	Anhydrase isozyme II
Unknown	EST U70674
	EST U58494
	EST M29325

Welcsh *et al* have developed an embryonal kidney epithelial cell line with stable ecdysone inducible expression of BRCA1 in modest levels, and then used microarray technology to identify the BRCA1 regulated genes: estrogen responsive genes MYC and cyclin D1, which are overexpressed in many breast tumours; STAT1 and JAK1, key components of the cytokine signal transduction pathway; the extracellular matrix protein laminin 3A; Id4, an inhibitor of DNA-binding transcriptional activators, which in turn negatively regulates BRCA1 expression; and the prohormone stanniocalcin, expression of which is lost in breast tumours (Table 9) (Welcsh *et al.*, 2002).

Table 9: Genes with altered expression following induction of BRCA1 (Welcsh *et al*, 2002). Fold change in expression following BRCA1 induction has been indicated.

Genes up-regulated at least 2-fold		
PBP	Progesterone-binding protein	2.1
XIST	X inactivation-specific transcript	2.0
JAK1	Janus kinase 1	2.4
H1F0	H1 histone family member 0	4.6
SEPP1	Selenoprotein plasma protein 1	3.2
TSNAX	Translin-associated factor X	2.2
MGAT2	UDP-*N*-acetylglucosamine	2.3
STC1	Stanniocalcin 1	*
CALD1	Caldesmon 1	2.6
ID4	Inhibitor of DNA binding 4	2.1
SEC23A	Yeast coat protein complex homolog	4.6
HSEC10L1	*Saccharomyces cerevisiae* Sec10 homolog	2.0
STAT1	Signal transducer/activator of transcription 1	2.5
VAMP3	Vesicle-associated membrane protein 3	2.3
FSTL1	Follistatin-like 1	2.6
EXTL2	ER-localized transmembrane glycoprotein	3.6
CCNG2	Cyclin G2	2.8
CITED2	CBP/p300 transactivator 2	2.1
ZNF138	Zinc-finger protein 138	2.2
IDH1	Isocitrate dehydrogenase 1	2.7
RDX	Radixin	3.1
LIPA	Lipase A	2.0
TFPI2	Placental tissue factor pathway inhibitor 2	2.6
GALNT3	N-acetylgalactosaminyltransferase 3	4.2
ZNF148	Zinc-finger protein 148	2.0
IL16	Interleukin 16	3.5
Clone 23654		2.7
ADD1	Adducin 1	2.3
COP9	COP9 subunit 3	2.0
NFYC	Nuclear transcription factor Y, ϒ	2.1
RRM2	Ribonucleotide reductase subunit 2	3.0
WRB	Tryptophan-rich basic protein	2.0
PPP1R1A	Protein phosphatase 1, regulatory subunit 1A	3.2
CLTB	Clathrin, light polypeptide B	2.1
KCNK1	Inward rectifier potassium channel	4.6
MAP2K1	Mitogen-activated protein kinase kinase 1	2.0

KIF2	Kinesin heavy chain 2	2.4
LIMS1	LIM and senescent cell antigen-like domain 1	2.5
ENPP2	Ectonucleotide phosphodiesterase 2	2.2
P2RX1	Ligand-gated ion channel receptor P2X	*
LAMA3	Laminin, α-3	2.5
TITF1	Thyroid transcription factor 1	2.2
ELANH2	Protease inhibitor 2	2.0
HTATIP	HIV tat-interacting protein	2.0
MICA1	MHC complex, class I-related gene A	2.3
APP	Amyloid β-A4 precursor protein	2.3
TCRA	T-cell antigen receptor, α-subunit	2.3

Genes down-regulated at least 2-fold

HRC1	HRAS-related cluster 1	2.7
NDRG1	NMYC downstream-regulated gene 1	2.1
CCND1	Cyclin D1	3.2
RCD8	Autoantigen RCD8	2.7
BOP1	Block of proliferation 1	2.0
SLC19A1	Folate transporter	3.1
NUP214	Nucleoporin 214 KD	2.1
HSF1	Heat-shock transcription factor 1	3.5
MYC	MYC oncogene	4.2
RXRA	Retinoid X receptor-α	2.0
SLC6A10	Solute carrier family 6, member 10	2.4
CHD4	Chromatin helicase DNA-binding protein 4	2.3
GCN5L2	Control of amino acid synthesis homolog 2	2.0
SKIV2L	Superkiller viralicidic activity 2	3.0
EIF4G1	Eukaryotic translation initiation factor 4γ	2.4

1.7 Strategy

Several lines of evidence suggest that BRCA1 has a role in transcriptional regulation. The aim of this study was to identify the genes that are upregulated by BRCA1 and therefore contribute to a better understanding of its cellular functions.

The expression profile of control MCF7 cells was compared with MCF7 cells ectopically expressing BRCA1 by performing Suppression Subtractive Hybridization (SSH). A subtracted cDNA library was generated in order to identify novel BRCA1-induced genes as candidate mediators of tumour suppression by BRCA1.

The strategies used in this study were as follows:

Generation inducible BRCA1 expression in breast carcinoma cells

The MCF-7 breast carcinoma cell line was selected to overexpress the *BRCA1* gene in this study since it has a very low endogenous BRCA1 expression level and maintains wild-type p53. One allele of *BRCA1* is intact but the second allele has been lost within the 2 Mb genomic region of chromosome 17q21 containing *BRCA1* gene (Kachhap *et al.,* 2001).

- First we aimed to generate a stable cell line in which the BRCA1 expression could be controlled by an inducible system. To this aim, the full-length *BRCA1* cDNA was subcloned into the eukaryotic expression vector pIND, which is a component of the ecdysone inducible system. Three different sources of full-length *BRCA1* cDNA were used to generate pIND.BRCA1 construct, since the first two *BRCA1* cDNA sources obtained in the early studies were later reported to have some discrepancies.
- Second, the MCF7 cells were transfected with pVgRXR regulatory plasmid which is another component of ecdysone inducible system to generate cells stably keeping the pVgRXR plasmid. PVgRXR plasmid works together with pIND response plasmid to induce the *BRCA1* gene, which was cloned into pIND plasmid.

- Third, pVgRXR plasmid containing MCF7 cells were transiently transfected with pIND.lacZ reporter construct or with pIND.BRCA1 construct and treated with ecdysone analog ponasterone A or muristerone A to induce the expression of reporter or BRCA1 gene. The cells were then tested for the induced reporter gene or BRCA1 expression level with various methods.
- We also used U2OS osteosarcoma cells which were stably transfected with pVgRXR plasmid to introduce pIND.BRCA1 constructs and induce the cells as described above.

This strategy did not produce desirable inducible BRCA1 expression system. The results of this strategy are explained in the results section (3.1 and 3.2).

Ectopic expression of BRCA1 in MCF7 breast carcinoma cells

- The second strategy to obtain high level expression of BRCA1 was to transiently transfect the MCF7 cells with pCMVmycBRCA1 plasmid, which has a strong CMV promoter to express BRCA1 gene in mammalian cells.
- The control MCF7 cells were transfected with the pCMVmyc control plasmid lacking BRCA1 insert.
- BRCA1 or control plasmid transfected cells were examined for BRCA1 protein and RNA level by Western and Northern blot analysis respectively.

RNA isolation for cDNA subtraction

DNA-free mRNA samples were prepared from BRCA1 or control plasmid transfected cells.

Generating forward subtracted cDNA library

- Suppression Subtractive Hybridization (SSH) technology was used to identify genes that are differentially expressed in BRCA1 transfected MCF7 cells.
- SSH was performed with double strand tester (MCF7/pCMVmycBRCA1) and driver (MCF7/pCMVmyc) cDNAs (forward subtraction).

- Reverse subtraction was also performed where tester cDNA derived from pCMVmyc transfected MCF7 cells and driver cDNA derived from pCMVmycBRCA1 transfected MCF7 cells.
- The resulting differentially expressed cDNAs from forward subtraction were ligated into pGEM-T Easy vector to generate a cDNA library enriched for the BRCA1 upregulated genes.

Screening and analysis of the differentially expressed clones

- The forward subtracted cDNA library clones were analyzed by dot blot hybridization and differential screening.
- The cDNA clones which showed more than five-fold increase were selected for sequencing analysis.
- The partially sequenced cDNA inserts were subjected to homology search with current databanks.
- Further confirmations of upregulation of the candidate genes were performed by Western blot, Northern blot and semi-quantitative RT-PCR analyses.

The results of this strategy are explained in the results section (3.2.4, 3.3 and 3.4).

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 General reagents

The general laboratory chemicals were supplied from Sigma Chemical Co. (St. Louis, U.S.A), Merck (Darmstadt, Germany), Stratagene (Heidelberg, Germany), Carlo Erba (Rodano, France), Delta Kim. San. (Istanbul, Turkey) and MBI Fermentas (Germany).

2.1.2 Nucleic Acids and Proteins

1 kb DNA ladder, 100 bp DNA Ladder Plus, and 100 bp DNA Ladder and *Hae*III-digested *ϕX174* were used as DNA molecular weight size markers in this study and supplied from MBI Fermentas, Gibco-BRL and Sigma. Ultrapure deoxyribonucleotides were from Pharmacia (Vienna, Austria) and MBI Fermentas. Protein size markers were from Bio-Rad (U.S.A) and New England Biolabs (U.K.).

2.1.3 Oligonucleotides

The oligonucleotides used in polymerase chain reactions were synthesized in the Beckmann Oligo 1000M DNA Synthesizer (Beckmann Instruments Inc. CA, U.S.A) at Bilkent University, Department of Molecular Biology and Genetics

(Ankara, Turkey) or supplied from Iontek Inc. (Bursa, Turkey) or Oswell DNA services (Southampton, U.K.).

2.1.4 Enzymes

Restriction endonucleases and *Taq* DNA polymerases were supplied by MBI Fermentas, New England Biolabs, Stratagene or Promega. Sequenase was from USB (United States Biochemicals, Cleveland, U.S.A). Ribonuclease A was from Sigma.

2.1.5 Bacterial strains

The characteristics of the bacterial strains used in this study were as follows:

DH5α: F-, (f80dÊ(lacZ) M15), *rec*A1, *end*A1, *gyr*A96, *thi*1, *hsd*R17, (r-km-k), supE44, *rel*A1, *deo*R, Ê(lacZYA-ar gF) U169

JM109: *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_K-, m_K+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), (F´, *tra*D36, *pro*AB, *lac*I^qZΔM15)(8)

Bacterial strains were stored at –70°C in LB medium containing 50% (v/v) glycerol for long-term storage. Recombinant clones were stored under the same conditions in media supplemented with appropriate antibiotics. Strains were maintained as isolated colonies on LB agar plates at 4°C for short-term storage.

2.1.6 Plasmids

pcDNA3.myc.BRCA1 was a gift from Dr. F.J. Calzone (Amgen Center, CA, U.S.A.). pRc.CMV.BRCA1 construct was a gift from Dr. Daniel Haber (Harvard Medical School, Boston, U.S.A). pCR3.BRCA1 plasmid was a gift from Dr. Barbara Weber (University of Pennsylvania Cancer Center, Pennsylvania, U.S.A.). pIND and pIND.p53 vectors were gifts from Dr. Kannan Karuppiah and Dr. David Givol (Weizmann Institute of Science, Rehovot, Israel). pCMV.myc, pCMV.myc.BRCA1,

pIND, pIND.lacZ, pVgRXR vectors were gifts from Dr. Tim Crook (Ludwig Institute for Cancer Research, London, U.K.).

2.1.7 Nucleic acid and protein transfer materials

Hybond N, Hybond N^+ nylon membranes and Immobilen P transfer (PVDF) membrane were from Amersham (U.K.). 3MM filter paper was from Whatman International Ltd. (Madison, U.S.A).

2.1.8 Radioisotopes

(α-^{32}P)dCTP (800 Ci/mmol and 3000 Ci/mmol) and (α-^{35}S)dATP (1000 Ci/mmol) were from Amersham and Perkin Elmer/NEN (Belgium).

2.1.9 Sequencing reagents

Sequenase version 2.0 DNA sequencing kit was obtained from USB. Sequencing reactions were run on denaturing acrylamide gels made from the components of Ultrapure sequagel (concentrate and diluent) from National Diagnostics (Atlanta, U.S.A).

2.1.10 Tissue culture reagents and cell lines

Dulbbecco's Modified Eagle's Medium (DMEM), fetal calf serum, L-glutamine, penicillin/streptomycin, calcium and magnesium-free phosphate buffered saline (PBS) were obtained from Sigma and Gibco-BRL. Electroporation cuvettes were supplied from Bio-Rad Inc.. The Ecdysone-Inducible Mammalian Expression System, ponasterone A and muristerone A were from Invitrogen Corporation (CA, U.S.A).

Antibiotics used for selection in medium: Zeocin was from Invitrogen Corporation (CA, U.S.A) and Cayla (France), geneticine was from Promega (CA, U.S.A), hygromycin was from Boehringer Mannheim, and puromycin and tetracycline were from Sigma.

MCF-7 is a human breast carcinoma cell line, HBL-100 is a human breast epithelial cell line, U2OS is a human osteosarcoma cell line (see Appendix B for the characteristics of these cell lines). U2OS/RXR cell line was a gift from Dr. Tim Cook (Ludwig Institute for Cancer Research, London, U.K.) and stably transfected with pVgRXR plasmid. UBR60-bcl2 cell line was a gift from Dr. Paul Harkin (Queen's University of Belfast, Ireland), which expresses BRCA1 under the control of tetracycline-regulated promoter, has been previously described (Harkin *et al*., 1999).

2.1.11 Kits

Trireagent was from Sigma (U.S.A). PolyA spin mRNA isolation kit was from New England Biolabs Inc. (MA, U.S.A). MessageClean kit was from GenHunter Corporation (MA, U.S.A). RevertAid first strand cDNA synthesis kit was from MBI Fermentas and cDNA synthesis kit was from Boehringer Mannheim. PCR-Select cDNA Subtraction kit, PCR-Select Differential Screening kit, Advantage cDNA PCR kit were from Clontech laboratories (CA, U.S.A). Midiprep kit, PCR purification kit and QiaexII gel purification kit were from Qiagen (Germany). Quick spin columns were from Roche and Hexa-Label plus kit was from MBI Fermentas. pGEM-T Easy vector system and TNT Coupled Reticulocyte Lysate System were from Promega (WI, U.S.A). ECL western blotting detection reagent was from Amersham (U.K.). Big Dye terminator cycle sequencing kit was from Perkin Elmer (CA, U.S.A). Sequenase Version 2.0 DNA sequencing kit was from Amersham.

2.1.12 Antibodies

BRCA1 (C-20), BRCA1 (D-20) antibodies and anti-mouse HRP conjugated secondary antibody were obtained from Santa Cruz Biotechnology (CA, U.S.A). BRCA1 (Ab1, MS110) and MSH2 (Ab1) antibodies were from Oncogene Research Products (U.K.). BRCA1 (5HU) and cytokeratin 18 (JAR13 clone) antibodies were gifts from Dr D. Bellet (IGR, France). Myc (9E10) antibody was a gift from Dr. Tim Crook (Ludwig Institute for Cancer Research, London, U.K.). p53 (9E4B1) antibody was obtained from Bilkent University, Department of Molecular Biology and Genetics. Annexin V was from B.D. Pharmingen (U.S.A).

2.2 Solutions and Media

2.2.1 General solutions

1XTAE: 40 mM Tris-acetate, 2 mM EDTA, pH 8.0

1XTBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3

TE: 10 mM Tris, 1 mM EDTA, pH 8.0

10 mM dNTP: 100 µl of four different kinds of dNTP (from 100 mM stock) were mixed in 1 ml double distilled water. Working concentration was 100 µM in a typical PCR reaction.

Ethidium bromide: 10 mg/ml in water (stock solution), 30 ng/ml (working solution)

5x agarose gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol, 1 mM EDTA

Denaturing solution: 6 N NaOH (stock solution), 0.6 N NaOH (working solution)

Neutralization solution: 0.5 M Tris-HCl, pH 7.5

Solutions for plasmid DNA isolation:

Solution I: 50 mM glucose, 25 mM Tris pH: 8.0, 10 mM EDTA

Solution II: 0.2 N NaOH, 1% SDS

Solution III: 3 M potassium acetate, pH 4.8

Solutions for DNA isolation from tissue culture cells:

Lysis buffer: 10 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% SDS with 0.5 mg/ml proteinase K

Lysis mix: 0.05 M EDTA pH 8.0, 1 % sarcosyl, 200 μg/ml proteinase K

2.2.2 Microbiological media and antibiotics

LB broth: 1% tryptone, 0.5% yeast extract, 1% NaCl

LB agar plates: LB+2% agar

SOB: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl

SOC: SOB+ 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose

Transformation buffer: 10 mM K.PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7

IPTG: 0.5 M solution in water, filter sterilized and stored at –20°C in the dark (working solution 2.5 mM)

X-Gal: 250 mg/ml solution in DMF (stock solution)
6.5 mg/ml (working solution)

Ampicillin: 100 mg/ml solution in water sterilized by filtration and stored at –20°C.(working solution 100 μg/ml)

Kanamycin: 100 mg/ml solution in water, sterilized by filtration and stored at –20°C (working solution 30 μg/ml)

2.2.3 Tissue culture solutions

Growth medium: DMEM supplemented with 10% fetal calf serum, 1 mM glutamine 50 mg/ml penicillin/streptomycin

PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.H_2O$, 1.4 mM KH_2PO_4, pH 7-7.4

Freezing solution: 10% DMSO, 20% FCS, 70% DMEM

2xBBS: 50 mM BES pH: 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4

1xHBS: 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM HEPES, pH 6.95

Zeocin: 50 mg/ml in water, sterilized by filtration and stored at –20°C

Geneticin (G418): 50 mg/ml in PBS, sterilized by filtration and stored at –20°C

Puromycin: 1 mg/ml in DMEM, sterilized by filtration and stored at –20°C

Tetracyclin: 0.01 gr tetracycline was dissolved in 10 ml of 70% ethanol.

2.2.4 RNA solutions

DEPC ddH_2O: 1 ml DEPC was mixed with one liter of double distilled water, stirred at 37°C overnight and autoclaved.

Premix solution: 50% formamide, 6% formaldehyde and 1xMOPS

10XMOPS: 200 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0

RNA loading buffer: 50% glycerol in 1xMOPS, 0.12% bromophenol blue

20XSSC: 3M NaCl, 0.3 M Na_3.citrate.$2H_2O$, pH 7.0

Hybridization buffer: 0.5 M phosphate buffer pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA, 10 μg/ml salmon sperm DNA and yeast total RNA

2.2.5 Protein extraction and western blotting solutions

RIPA buffer: 10 mM Tris.Cl pH: 8.0, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1% NaDOC, protease inhibitor mix

Bradford stock solution: 17.5 mg Coomassie brilliant blue in 4.75 ml ethanol and 10 ml phosphoric acid, final volume up to 25 ml with ddH_2O

Bradford working solution: 0.75 ml 95% Ethanol, 1.5 ml phosphoric acid, 1.5 ml Bradford stock solution, final volume up to 25 ml with ddH_2O

Acrylamide-Bisacrylamide solution: 29 gr acrylamide, 1 gr bisacrylamide final volume up to 100 ml with ddH_2O and stored in dark bottle.

10% APS: 0.1 gr APS dissolved in 1 ml ddH_2O.

5x loading buffer: 62.5 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS, 15% glycerol, and 0.001% bromophenol blue

5x running buffer: 125 mM Tris, 1.25 M glycine, 0.5% SDS; pH 8.3

Wet transfer buffer: 6 g Tris, 28.8 g glycine, 1 ml 10% SDS and 20% methanol, final volume up to 1 lt with dH_2O

Semi-dry transfer buffer: 2.91 g Tris, 1.465 g glycine, 1.875 ml 10% SDS, 100 ml methanol, final volume up to 500 ml with dH_2O

TBS-T: 0.1 M Tris, 0.5 M NaCl, 0.5% Tween-20

Blocking solution: TBS-T and 3% milk powder

10xZ buffer: 0.6 M $Na_2HPO_4.7H_2O$, 0.4 M NaH_2PO_4, 0.1 M KCl, 0.01 M $MgSO_4.7H_2O$, 0.5 M β-mercaptoethanol; pH 7.2

ONPG: 4 mg/ml O-nitrophenyl β-D-galactopyranoside

2.2.6 Immunostaining solutions

Annexin V binding buffer: 0.01 M Hepes pH: 7.4, 0.14 M NaCl, 0.25 mM $CaCl_2$

Hoechst stain: 100 mg/ml stock solution in water (300 μg/ml working solution)

2.3 General methods

2.3.1 Transformation of *E.coli*

Two different calcium chloride mediated methods were used to prepare competent E.coli strains for transformation.

Method I (Ausubel *et al*., 1991): 500 µl of DH5α glycerol stock was inoculated into 5 ml of LB medium and cells were grown at 37^{o}C, shaking at 200 rpm to an optical density (OD) of 0.4-0.6 at 590 nm. 1.5 ml of culture was centrifuged at 13000 rpm for 1 min at 4^{o}C and suspended in 500 µl of ice cold 50 mM $CaCl_2$. After 30 min incubation on ice, the cells were centrifuged and the pellet was suspended in 100 µl of ice cold 50 mM $CaCl_2$. 1 µl of plasmid DNA (1 ng/µl) was added to the cells and incubated on ice for 30 min. The cells were heat shocked at 42^{o}C for 90 seconds and then incubated on ice for 2 min. 1 ml of LB medium was added onto the cells and incubated at 37^{o}C for 1 h to allow the expression of antibiotic resistance gene before plating the cells. After incubation, the transformation mixture was plated on LB agar plates containing 100 µg/ml ampicillin. Plates were incubated overnight at 37^{o}C to select the recombinant clones carrying the plasmid of interest.

Method II (Inoue *et al*., 1990): JM109 cells were grown in SOB at 18°C, shaking at 200-250 rpm until OD_{590}=0.5-0.6. They were then centrifuged for 10 min at 3000 rpm at 4°C. Cells were resuspended in 1/3 of the initial volume of ice-cold transformation buffer and incubated on ice for 10 min. After 10 min centrifugation, cells were again resuspended in 1/12.5 initial volume of ice-cold transformation buffer. DMSO was added to a final concentration of 7%. After incubating 10 min on ice, supercompetent cells were distributed to tubes, rapidly freezed by immersing in liquid nitrogen and stored at –80°C. For transformation, cells were rapidly thawed on ice and plasmid DNA was added onto 200 µl of supercompetent cells. After 30 min incubation on ice, cells were heat-shocked for 30 seconds at 42°C and were put back on ice. 800 µl SOC was added and the cells were incubated at 37°C with vigorous

shaking for 1 h. The transformation mixture was then plated on LB-agar plates containing the appropriate antibiotic and the plates were incubated at 37^{o}C overnight.

2.3.2 Plasmid DNA preparation

Small-scale isolation of plasmid DNA (mini-prep)

This protocol is based on the alkaline lysis method (Birnboim and Doly, 1989) with minor modifications.

The recombinant bacterial clone containing the plasmid of interest was grown in 5 ml LB medium containing appropriate the antibiotic at 37^{o}C overnight while shaking at 200 rpm. 1.5 ml of grown culture was centrifuged at 13000 rpm for 2 min. After removing supernatant, the pellet was resuspended in 100 µl of ice-cold solution I and kept at room temperature for 5 min. Freshly prepared 200 µl of solution II was added and the contents were mixed by inverting the tube very gently and then placed on ice for 5 min. Bacterial chromosomal DNA and cell debris were precipitated by adding 150 µl of solution III. The mixture was kept on ice for 5 min and centrifuged for 5 min at 13000 rpm at 4^{o}C to remove the cellular debris. Supernatant was transferred into a new tube and mixed with 800 µl of ice-cold absolute ethanol and the mixture was incubated at -20^{o}C for 20 min. Plasmid DNA was recovered by centrifugation at 13000 rpm for 10 min at room temperature. The pellet was washed with 300 µl 70% ethanol and centrifuged at 10500 rpm for 10 min at room temperature. Ethanol was aspirated and the pellet was air-dried for 10 min at room temperature. The pellet was resuspended in 20-30 µl sterile ddH_2O containing 10 µg/ml RNaseA. The sample was stored at 4^{o}C for short-term or -20^{o}C for long-term U.S.Age. This method yields approximately 1-1.5 µg of plasmid DNA.

Large-scale isolation of plasmid DNA (midi-prep)

For large-scale preparation of pure plasmid DNA, the Qiagen 100 plasmid isolation kit was used by following the manufacturer's instructions. This procedure yields approximately 60-150 µg of plasmid DNA for 100 ml initial LB culture.

2.3.3 Restriction enzyme digestion of plasmid DNA

Restriction enzyme digestions were routinely performed in 15 µl -50 µl reaction volumes containing 1 µg -10 µg plasmid DNA. Typically 1 unit enzyme was used per µg of plasmid DNA. Reactions were carried out with the appropriate reaction buffer, according to the manufacturer's recommendations.

Digestion of plasmid DNA with two or three different restriction enzymes was performed in the same reaction buffer to provide the optimal condition for all the restriction enzymes.

2.3.4 Agarose gel electrophoresis of DNA

DNA fragments were fractionated by horizontal gel electrophoresis by using 1xTAE or 1xTBE buffers, and 5xgel loading buffer.

DNA fragments less than 1 kb were generally separated on 1-2% agarose/EtBr gels, while those greater than 1 kb (up to 11.5 kb) were separated on 0.8% agarose/EtBr gels.

Agarose was completely dissolved in 1xTAE (or 1xTBE) electrophoresis buffer to the required percentage and ethidium bromide was added to a final concentration of 30 ng/ml. The samples were loaded onto agarose gel with 1/5 volume of loading buffer. The gel was run in the appropriate buffer (1xTAE or 1xTBE) at different voltage and time settings depending on the size and the fragment separation required at room temperature.

Nucleic acids were visualized under ultraviolet light (long wave, 340 nm) and different DNA size markers were used in order to estimate the fragment sizes. The DNA size markers used in this study were as follows:

Gibco-BRL 1 kb ladder:
12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2.0, 1.6,1.0, 0.5, 0.4, 0.3 and 0.2 kb.

MBI 1 kb ladder:
10, 8, 7, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.75, 0.5, 0.25 kb

MBI DNA Ladder Mix::
10, 8, 7, 6, 5, 4, 3.5, 2, 1, 1.2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 kb

MBI 100 bp DNA Ladder Plus:
3, 2, 1, 1.2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 kb

MBI 100 bp DNA Ladder:
1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 kb

*Hae*III digested ϕX174 (Sigma):
1.3, 1.1, 0.9, 0.6, 0.3 kb

2.3.5 Extraction of DNA fragments from agarose gel

DNA fragments were extracted from TAE agarose gels by using the QIAEX II gel extraction kit according to the manufacturer's instructions.

Gel purification with the QIAEX II kit yields 60-70% recovery of DNA fragments between 1.0 kb to 6.0 kb in 10-20 μl volumes.

The DNA fragments used as probes were prepared by using the same kit. The PCR products or the restriction enzyme digested DNAs were digested subjected to agarose gel electrophoresis. The location of the DNA fragment of interest was determined under the UV light and the fragment was excised using a scalpel blade. The gel slice was transferred into an eppendorf tube and the fragment was purified by using the gel extraction kit.

2.3.6 Radioactive labelling of DNA

Purified cDNA inserts were labelled with the HexaLabel Plus Kit according to the manufacturer's recommendations. Briefly, 100 ng DNA template was mixed with 10 µl 5x reaction buffer and the volume was adjusted to 40 µl with ddH_2O. This mixture was incubated in a boiling water bath for 5-10 min. Following cooling down on ice 3 µl Mix C, 6 µl [α-^{32}P]-dCTP and 1 µl (5U) Klenow fragment exo$^-$ were added to the tube and incubated for 10 min at 37°C. Following the addition of 4 µl dNTP, the mixture was incubated for 5 min at 37°C. The reaction was stopped by the addition of 1 µl 0.5 M EDTA. Unincorporated dNTPs were removed from the labelled DNA with G-50 sephadex chromatography by using Quick Spin columns. Briefly the column was centrifuged at 1100xg for 2 min and the eluted buffer was discarded. Labelled DNA sample was applied to the center of the column bed and the column was centrifuged at 1100xg for 4 min. The eluent contained the purified probe. The purity of the probe was checked by capillary paper electrophoresis (in 1 M Na_2HPO_4) and the activity of the probe was detected by counting on the scintillation counter.

2.3.7 DNA isolation from tissue culture cells

Method I: 200 µl of trypsinized cells were pelleted and lysed in 500 µl of lysis buffer. This mixture was incubated at 37^{o}C overnight. The next day 1 ml isopropanol was added to each tube and mixed. After centrifuging for 5-10 min, the pellet was washed with 70% ethanol. The pellet was dissolved in 30-50 µl TE or ddH_2O.

Method II: Trypsinized cells were pelleted and resuspended in 1 ml of lysis mix. After incubation at 55^{o}C overnight, DNA was extracted with an equal volume of phenol and then phenol:chloroform (1:1), precipitated with ethanol and washed with 70% ethanol, air-dried and dissolved in 30-50 µl TE or ddH_2O.

2.3.8 Amplification of DNA by Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed to amplify the desired DNA fragments from genomic or plasmid DNAs or from cDNAs using the thermal cyclers GeneAmp PCR system 9600 or 2400 (Perkin Elmer). Unless otherwise stated, all reactions were performed with either 100 ng genomic or 10 ng plasmid DNAs by using 1 unit Taq DNA Polymerase supplied with its 10x reaction buffer with the final concentrations of the following components: 1 pmol/μl forward and reverse specific primers, 100 μM dNTP and 1.5 mM $MgCl_2$. Table 10 shows the primers used in this study and PCR conditions are given in the appropriate places in the following sections.

The cDNA clones that became available from differential screening experiments were amplified by using T7-Sp6 universal primers. These amplifications were performed to analyze the presence or absence of cDNA inserts and the length of the cDNAs in the positive clones.

Table 10: List of primers pairs used in the study with Genbank accession numbers and expected PCR product sizes

Gene/vector/ Acc #	Primer pair	Product size
RXR/ Invitrogen	**TC222-F**: 5'- AGT TGA CCA GTG CCG TTC CG -3' **TC223-R**: 5'- CTG CTC CTC GGC CAC GAA GTG -3'	361bp
pIND-BRCA1	**EcF:** 5'- CTC TGA ATA CTT TCA ACA AGT TAC -3' **TC31-R:** 5'- ACC CTG ATA CTT TTC TGG -3'	1103 bp
pIND/ Invitrogen	**EcF:** 5'- CTC TGA ATA CTT TCA ACA AGT TAC -3' **EcR:** 5'- TAG AAG GCA CAG TCG AGG -3'	variable due to insert size
pGEMT-Easy/ Promega	**T7:** 5'- TAA TAC GAC TCA CTA TAG GG-3' **Sp6:** 5'- GAT TTA GGT GAC ACT ATA G-3'	variable due to insert size
GAPDH/M33197	**GAPDH F**: 5'-GGC TGA GAA CGG GAA GCT TGT CAT-3' **GAPDH R:** 5'-CAG CCT TCT CCA TGG TGG TGA AGA-3'	250 bp
BRCA1/U14680	**BRCA1 F4** 5'– AAC TTA GAA CAG CCT ATG GG –3' **BRCA1 R4** 5'– TTG CTC CTC CAC ATC AAC AA –3'	1217 bp
GADD45/L24498	**GADD45 F** 5'– ATG ACT TTG GAG GAA TTC TCG GCT –3' **GADD45 R** 5'– TCA CCG TTC AGG GAG ATT AA–3'	497 bp
pCMV-BRCA1	**PCMV F** 5'– GCG TGT ACG GTG GGA GGT CTA–3' **TC 31 (R)** 5'- ACC CTG ATA CTT TTC TGG -3'	1200 bp
Actin/X00351	**ACTIN F** 5'- GTG GGG CGC CCC AGG CAC CA -3' **ACTIN R** 5'- CTC CTT AAT GTC ACG CAC GAT TTC -3'	550 bp
RAD21/NM_006265	**RAD21 F:** 5'-TCC TCA GCA GGT AGA GCA GAT-3' **RAD21 R**: 5'-TCC AGG TGT TGC GAT GAT GTC-3'	406 bp
MSH2/XM_034901	**MSH2 F:** 5'-TGA TCT TCG TTC TGA CTT CT-3' **MSH2 R**: 5'-CGT CAT TAG GAA TAA ATG CAA-3'	641 bp
GNAS/ BC002722	**GNAS F:** 5'-AGC AAC AGC GAT GGT GAG AAG-3' **GNAS R**: 5'-CAT CCT CAG GAG TAG TGT AGC-3'	727 bp
MAC30/XM_031536	**MAC30 F**: 5'-TCT ACT TCC TCA GGC ACA TCC-3' **MAC30 R:** 5'-AAC CGT TCA TGC AAA GTC TCA-3'	376 bp

Amplification of zeocin resistance gene sequence from RXRIII clones

361 bp of zeocin resistance gene of pVgRXR was amplified with TC222 (RXR forward) and TC223 (RXR reverse) primers. Isolated genomic DNAs from the RXRIII clones were used as template for this reaction. The aim of this experiment was to detect the presence of the pVgRXR plasmid in the pVgRXR transfected MCF-7 cells. The sequences of the primers are shown in Table 10 and the PCR conditions were as follows;

Initial denaturing	94°C	5 min	
Denaturing	94°C	30 sec	30 cycles
Annealing	60°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	

5 µl of 25 µl PCR products were run on 1.5% agarose gel to observe the 361 bp expected product size.

Amplification of pIND.myc.BRCA1 sequence from U2OS cells

Genomic DNA was isolated from U2OS cells that stably express pIND.myc.BRCA1. PCR was carried out with EcF and TC31 primers (Table 10) to detect the presence of the plasmid DNA in these clones. EcF primer was for pIND plasmid, which was localized at the 5' end of the multiple cloning site, and TC31 primer was for exon 11A of BRCA1.

PCR conditions were as follows;

Initial denaturing	94°C	5 min	
Denaturing	94°C	30 sec	30 cycles
Annealing	55°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	

5 µl of 25 µl PCR products were run on 1% agarose gel for detection of the 1103 bp length expected product size.

First strand cDNA synthesis and Reverse Transcriptase-PCR (RT-PCR)

The cDNAs were prepared from total RNA using RevertAid First Strand cDNA Synthesis kit according to the manufacturer's instructions. Briefly, 4 µg of total RNA was mixed with 1 µl of oligo (dT) primer and incubated at 70°C for 5 min. Then 4 µl 5x reaction buffer, 1 µl ribonuclease inhibitor and 2 µl 10 mM dNTP were added and incubated at 37°C for 5 min. Following addition of 1 µl (200U/µl) RevertAid M-MuLV reverse transcriptase, the mixture was incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min.

The quality of first strand cDNA was initially tested by using gene specific primers by RT-PCR amplification. 1/40 volume of cDNA preparation was used for amplification. GAPDH primer pair was designed in such a way as to amplify 250 bp product from genomic DNA and 150 bp from cDNA, which enables detection of genomic DNA contamination in the RNA preparation.

PCR was also carried out with the following sequence specific primers in order to ensure the removal of plasmid DNA contamination from the cDNA preparations of DnaseI treated mRNAs before cDNA subtraction procedure. BRCA1 specific primers (F4-R4) amplify 1217 bp, actin F and R primers amplify 550 bp, GADD45 F and R primers amplify 497 bp and the pCMVmyc F and TC31 primer pair amplifies 1200 bp fragment. PCR conditions for GAPDH, F4-R4, GADD45, actin and pCMVmyc-TC31 amplification were as follows:

Initial denaturing	94^{o}C	5 min	
Denaturing	94^{o}C	30 sec	30 cycles
Annealing	55^{o}C	30 sec	
Extension	72^{o}C	30 sec-1 min	
Final extension	72^{o}C	10 min	

Semi-quantitative Reverse Transcriptase-PCR

The first strand cDNA product was amplified using the oligonucleotides that were designed according to the sequencing data and the gene structure that became available from this study.

Further PCR studies were performed with cDNA preparations yielding equal amounts of GAPDH amplification products. Briefly, the appropriate cycles for each set gene specific primers was detected by performing a 14-33 cycles PCR and taking an aliquot at the end of each cycle, which enables the determination of the optimum cycle not saturated for the amplified DNA fragment and the difference in expression between the two samples under comparison. After cDNA equalization with GAPDH amplification, all PCR reactions were carried out under the following conditions (Except for MSH2 for which the annealing temperature 60°C):

Initial denaturing	94^{o}C	3 min	
Denaturing	94^{o}C	30 sec	23 cycles for RAD21, 25 for MSH2, 19 for GNAS and 23 for MAC30
Annealing	55^{o}C	30 sec	
Extension	72^{o}C	1 min	
Final extension	72^{o}C	5 min	

All RT-PCR results were repeated at least 3 times.

Signal intensities were monitored by densitometric analysis using Multi-Analyst Image Software (Bio-Rad). Each product was normalised against GAPDH.

2.3.9 DNA sequencing

Manual DNA sequencing

The sequencing reactions were carried out using the dideoxy chain termination method (Sanger *et al*., 1997). T7 Sequenase version 2.0 DNA sequencing kit was used to sequence pIND.BRCA1 and pIND.HA.BRCA1 vectors by using the GenomyxLR

Programmable DNA Sequencer according to the manufacturer's instructions. EcF and EcR primers were used for pIND vector sequencing.

Automated DNA sequencing

Sequencing reactions were performed on double-stranded plasmid templates (pGEMT-Easy) with T7 and SP6 universal primers. Sequencing reactions were carried out with the Big Dye terminator cycle sequencing kit and analyzed with ABI 377 DNA Sequencer machine.

2.3.10 Computer analysis of DNA sequences and homology search

Restriction endonuclease maps of the plasmid DNAs were analyzed with the Webcutter program (designed by Max Heiman, 1995) freely available for public use at http://www.firstmarket.com/firstmarket/cutter.

Oligonucleotides for PCR and sequencing were designed with the primer programs "Primer Designer-Version 2.0" (Scientific and Educational Software) and "Amplify" (Bill Engels, 1992, University of Wisconsin, Genetics, Madison, U.S.A).

Nucleotide sequences were analyzed with the Genbank/EMBL and Expressed Sequence Tag (EST) databases. Homology search of the sequenced clones that came out from this study was mainly performed by BLAST search, available for public use at http://www.ncbi.nlm.nih.gov/Blast.

2.3.11 Extraction of total RNA from tissue culture cells

Total RNA was isolated with TRI Reagent from tissue culture cells. Cells were lysed in 1 ml Trireagent and the lysates were passed through a 21-gauge needle several times. After 5 min incubation at room temperature 0.2 ml chloroform was added per ml of Trireagent used. Tubes were shaked vigorously by hand for 15 seconds and incubated at room temperature for 2-3 min. Following centrifugation at 12000xg for 15 min at 4°C, the aqueous phase was transferred to a new tube and 0.5

ml isopropanol was added per ml of Trireagent. After incubation for 10 min at room temperature, total RNA was recovered by centrifugation at 12000xg for 10 min at 4°C. The pellet was washed with 75% ethanol, mixed and centrifuged at 7500xg for 5 min. Air-dried pellet was dissolved in ddH_2O and stored at –80°C.

2.3.12 Extraction of poly(A)$^+$ RNA and removal of DNA contamination

Polyadenylated RNA (poly (A)$^+$ RNA) was isolated from total RNA with the PolyA Spin mRNA isolation kit according to the manufacturer's instructions. 1 mg of total RNA from tissue culture cells yielded approximately 20 µg poly (A)$^+$ RNA.

The isolation of clean and intact mRNA is essential in order to obtain full-length cDNAs. The preparation should be free of DNA contamination before starting to the SSH protocol.

The Message Clean Kit was used to remove DNA contamination from the RNA samples according to the manufacturer's recommendations. 20 µg poly (A)$^+$ RNA was mixed with 5.7 ml 10x reaction buffer and 10 U DnaseI and the final volume was adjusted to 56.7 µl with ddH_2O. This mixture was incubated for 30 min at 37°C. Phenol:chloroform extraction was performed to ensure the removal of protein contamination and DnaseI from the RNA samples. Following ethanol precipitation the RNA samples were dissolved in DEPC ddH_2O. The integrity of the mRNA preparation was checked by running on denaturing formaldehyde agarose gel. Purified mRNA appeared as a smear from 0.5-12 kb with weak ribosomal RNA bands at approximately 1.9 and 4.5 kb as expected.

2.3.13 Northern Blotting

Sample running and transfer

20-30 µg of total RNA samples were denatured in premix solution at 55-60°C water bath for 15 min. Samples were loaded on 1% formaldehyde gel with loading

buffer containing ethidium bromide. The gel was run overnight at 50 V in 1xMOPS buffer. Before transfer, the gel was washed in ddH_2O 5 times each for 5 min, and gel and nylon membrane (Hybond N^+) were equilibrated in 10xSSC. After overnight transfer, the membrane was auto-crosslinked by using Stratalinker under 120 mJ and baked at 80°C for 2 h to reverse the formaldehyde reaction and fix the RNA.

Hybridization

The membrane containing the RNA samples was prehybridized in hybridization buffer for 1 h at 65°C. The membrane was then hybridized overnight at 65°C in hybridization buffer containing at least 10^7 cpm [α-^{32}P] dCTP-labelled DNA probes per membrane. Membranes were sequentially washed in 2x SSC and 0.5% SDS, 1x SSC and 0.5% SDS, 0.2x SSC and 0.5% SDS at 68°C and the filters were exposed to X-ray film with an intensifying screen at -70°C.

2.3.14 Protein extraction from tissue culture cells

Medium was aspirated from the tissue culture plates and the cells were rinsed twice with ice cold PBS. 1 ml ice cold PBS was added to the cells and the cells were collected by scraping from the plates. The cells were pelleted by centrifugation at 5000 rpm for 5 min. The cell pellets were lysed with RIPA buffer and incubated on ice for 30 min. After centrifugation at 13000 rpm for 30 minutes at 4°C, the supernatant was either stored at -80^{o}C for future use or immediately loaded onto SDS-PAGE gel.

After the cell lysates were prepared, their concentrations were detected by Bradford assay. 5 µl of the samples were diluted with 95 µl deionised water and then 1 ml of Bradford working solution was added to the samples and mixed well. After 5 minutes, OD was measured at 595 nm versus blank reagent. Known concentrations of BSA were prepared as a standard and the same procedure was performed. After reading at OD_{595}, samples and standard values were plotted; unknown concentrations were read from the standard curve.

2.3.15 Immunoprecipitation

Immunoprecipitation of BRCA1 was performed from cellular lysates and *in vitro* transcribed-translated radioactive labelled BRCA1 protein.

Immunoprecipitation of BRCA1 from cellular lysates

Cells were lysed in RIPA buffer and the amount of protein was detected by Bradford assay. 500 µg prewashed protein A was added to 1000 µg cellular lysate in a final volume was 500 µl. Pre-immune rabbit serum was added at a final dilution of 1:1000. Tubes were left overnight on shaker at cold room. Next day, after 10 min centrifuge at 2500 rpm, the supernatant was transferred into a clean tube, and 500 µg prewashed protein A was again added into the lysate. After leaving 2 h on shaker at the cold room, the samples were centrifuged and the supernatant was transferred to a new tube. 2 µg of the antibodies were added to the lysates and incubated for 6 h at the cold room on shaker. 250 µg of protein A+G mix (1:1, v/v) was added to the tubes and left 1 h at cold room on shaker. After centrifugation, the beads were washed 3 times in 1 ml RIPA buffer, and a final 10 min centrifuge was carried out. The beads were resuspended in 50 µl loading buffer and denatured for 10 min at 100°C. Then samples were loaded on 5% SDS-PAGE. The remaining protocol was the same as western blotting.

Immunoprecipitation of BRCA1 from IVTT product

pcDNA3.myc.BRCA1 and pCR3.BRCA1 plasmids, which have T7 promoter, were used for *in vitro* transcription/translation assay. Promega's TNT Coupled Reticulocyte Lysate Systems kit was used according to the manufacturer's instructions with minor modifications. The protocol for TNT lysate coupled transcription/translation reaction was as follows:

TNT rabbit reticulocyte lysate	25 µl
TNT reaction buffer	2 µl
T7 polymerase	1 µl
Amino acid mixture minus methionine	1 µl
$^{35}\alpha$-S methionine (1000Ci/mmol) at 10 mCi/ml	4 µl
Rnasin (ribonuclease inhibitor, 40 u/µl)	1 µl
DNA template (pcDNA3.myc.BRCA1/ pCR3.BRCA1)	1 µg
Nuclease free H_2O	to final volume 50 µl

The tubes were incubated at 25°C for 2.5 h. Afterwards the samples were either immunoprecipitated with BRCA1 antibodies or directly loaded on gel. The gel was then dried and left for autoradiography.

The final volume of the IVTT product was completed to 100 µl with RIPA and 1.5 µg of antibodies was added onto the lysate. Samples were left overnight on ice. 500 µg of prewashed protein A was added to the samples and left 1 h at the cold room on shaker. After washing the beads 3 times in 1 ml RIPA, the beads were denatured for 10 min at 100°C with loading buffer. The samples were run on 5% gel, the gel was dried and left for autoradiography.

2.3.16 Western Blotting

Performing SDS-PAGE

Preparation of 5% acrylamide gel (40 ml) was as follows;

H_2O	23.4 ml
30% Acrylamide mix	6.8 ml
1.5 M Tris, pH: 8.8	10 ml
10% SDS	0.4 ml
1.5 ml APS	0.4 ml
TEMED	32 µl

Preparation of 5% stacking gel (10 ml) was as follows;

H_2O	6.8 ml
30% Acrylamide mix	1.7 ml
1.0 M Tris, pH: 6.8	1.25 ml
10% SDS	0.1 ml
1.5 ml APS	0.1 ml
TEMED	10 µl

Equal amounts of proteins were denatured in 5x loading buffer at 95^{o}C for five minutes prior to loading on gel. Gels were run at 80-100 V during stacking gel and 120-150 V during resolving gel in running buffer. The run was stopped as bromphenol blue came to the end of the gel, and the gel was prepared for western blotting.

10% resolving gels were used for p53, MSH2 and cytokeratin 18 western blots. For BRCA1 western blots 5% resolving gel was used.

Transfer

BRCA1 immunoblotting was performed with wet transfer, while p53, MSH2 and cytokeratin 18 immunoblots were performed with semi-dry transfer procedures.

Wet transfer: Immobilen P transfer (PVDF) membrane was washed with methanol for 1-2 min, then rinsed several times with water and kept in wet transfer buffer. Gel and immunoblot papers were also kept in transfer buffer for equilibration for 5-10 min. Two immunoblot papers (prewet by transfer buffer), membrane, gel and two wet immunoblot papers were placed on transblotter. Transfer was performed overnight at 0.75 A at –5°C. After transfer, the membrane was air-dried. The size of the prestained molecular weight marker was 205, 121, 74, 47 kDa (Bio-Rad).

Semi-dry transfer*:* All the transfer protocol was the same as the wet transfer except for the use of semi-dry transfer buffer and transfer conditions. Semi-dry transfer was performed at 12V for 30 min at room temperature.

The membrane was kept in blocking solution for 2 h at room temperature or overnight at 4^{o}C. The membrane was washed (3 times, 5 min each) with TBS-T washing solution. Primary antibody (final concentration; 1 μg/ml) was added to the blocking solution and the membrane was left in primary antibody for 2 h to overnight at 4^{o}C on shaker. After washing three times with washing buffer, the membrane was left in secondary antibody for 1 h. After washing three times again, the membrane was exposed to ECL kit reagents according to the manufacturer's instructions and autoradiography was carried out.

2.3.17 Annexin V and Hoechst staining

Method I: Growing culture cells were washed two times with ice cold PBS and then resuspended in annexin V binding buffer. Cells were incubated for 20 min in the dark with annexin V by diluting 5 μl of annexin V in 100 μl binding buffer per coverslide.

Method II: Without changing the medium, annexin V was added to the growth medium in 10xbinding buffer, and cells were kept in the incubator for 30 minutes. The supernatants were collected with a gentle washing with PBS, cells were counted, centrifuged and the pellets were fixed on cover slides by cytospin by centrifuging for 5 minutes at 500 rpm. Later on, the cells grown on coverslides and the fixed cells in two methods were subjected to Hoechst staining, which stains the nucleus, as described in the following. The procedure was performed under dim light and the samples were kept away from direct light exposure. The coverslides were washed with binding buffer and fixed in 1 ml 90% cold ethanol for 30 min. After washing with PBS, Hoechst staining was performed for 5 min in the dark. The coverslides were extensively washed with H_2O and mounted onto 80% glycerol droplet.

2.3.18 PhosphorImager quantification

The dot blot and northern blot hybridization signals were analyzed by using a PhosphorImager (Bio-Rad). Northern blots and dot blots were exposed to phosphor

screen and the screen was scanned using Multi-Analyst software which is designed for analysing digitised images. After the scanned image was displayed, a rectangular area was drawn on the image of the region that needed to be analyzed and each signal's intensity was quantified. Multi-analyst software was also used for the quantification of semi-quantitative RT-PCR product intensities and analysing the changes in gene expression.

2.4 Construction of BRCA1 expressing eukaryotic expression vectors

During this study three different vectors containing full-length *BRCA1* cDNA were used for subcloning into pIND eukaryotic expression vector:

Vector	***Subcloned into***	***Cloning sites***
pcDNA3.myc.BRCA1 (Calzone F.J)	pIND.myc.BRCA1	*BamH*I-*Xba*I
pCR3.BRCA1 (B.Weber)	pIND.BRCA1	*Hind*III-*Not*I
pRC.CMV.BRCA1 (D.Haber)	pIND.HA.BRCA1	*Hind*III-*Not*I

Principle of Ecdysone-Inducible System

The Ecdysone-Inducible Mammalian Expression System was used in order to generate a stable breast carcinoma cell line where the BRCA1 gene expression can be controlled.

This system is based on the molting induction system of *Drosophila*, but has been modified for inducible expression in mammalian cells. The system uses the steroid hormone ecdysone analog, muristerone A or ponasterone A, to activate expression of the gene of interest via a heterodimeric nuclear receptor.

The Ecdysone-Inducible Mammalian Expression System includes:

-An inducible expression plasmid, pIND, for expression of the gene of interest that contains 5 modified ecdysone response elements (E/GREs) upstream of a minimal heat shock promoter and the multiple cloning site.

-A second plasmid, pVgRXR, which encodes the receptor subunits.

-Ponasterone A/muristerone A for inducing expression.

-A control expression plasmid containing the *lac*Z gene, that when cotransfected with pVgRXR expresses β-galactosidase upon induction with ponasterone A (Figure 7).

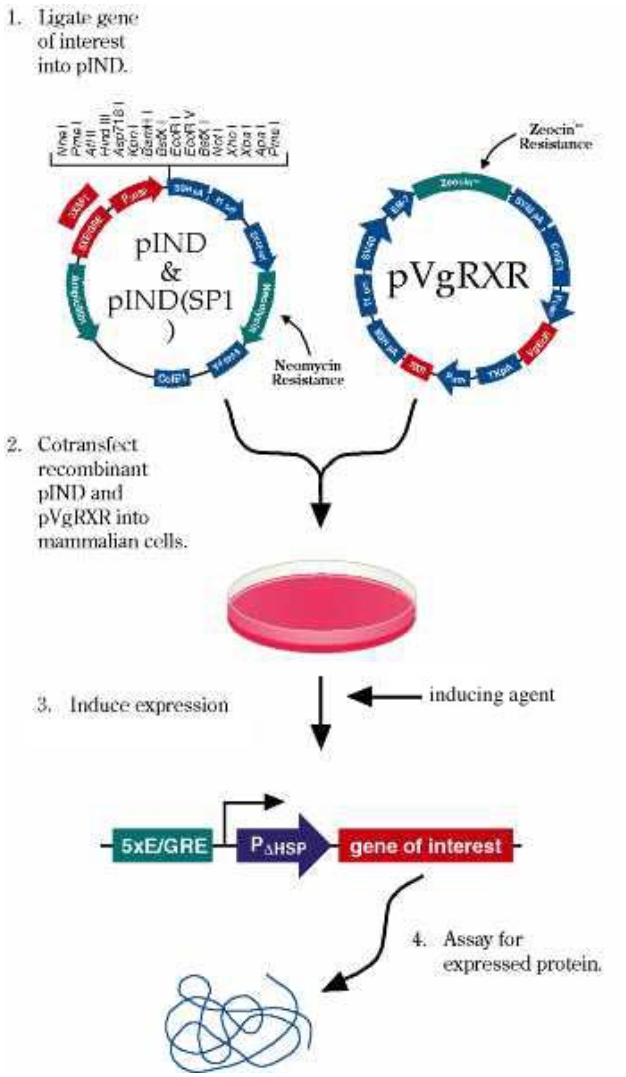


Figure 7: Principle of ecdysone inducible system pVGRXR plasmid constitutively synthesizes a heterodimer of the ecdysone receptor (VgEcR) and the retinoid X receptor (RXR) which binds a hybrid ecdysone response element (E/GRE) in pIND vector upstream of the gene of interest, in the presence of the synthetic analogs of ecdysone, ponasterone A or muristerone A.

2.4.1 Construction of pIND.myc.BRCA1

pcDNA3.myc.BRCA1 vector was used as the source of full-length BRCA1 cDNA. This vector contains two myc epitopes at the N-terminus of BRCA1, and its start codon is at the 5' end of myc epitopes. myc.BRCA1 in the pcDNA3 (Figure 8A) was subcloned into pIND (Figure 8B).

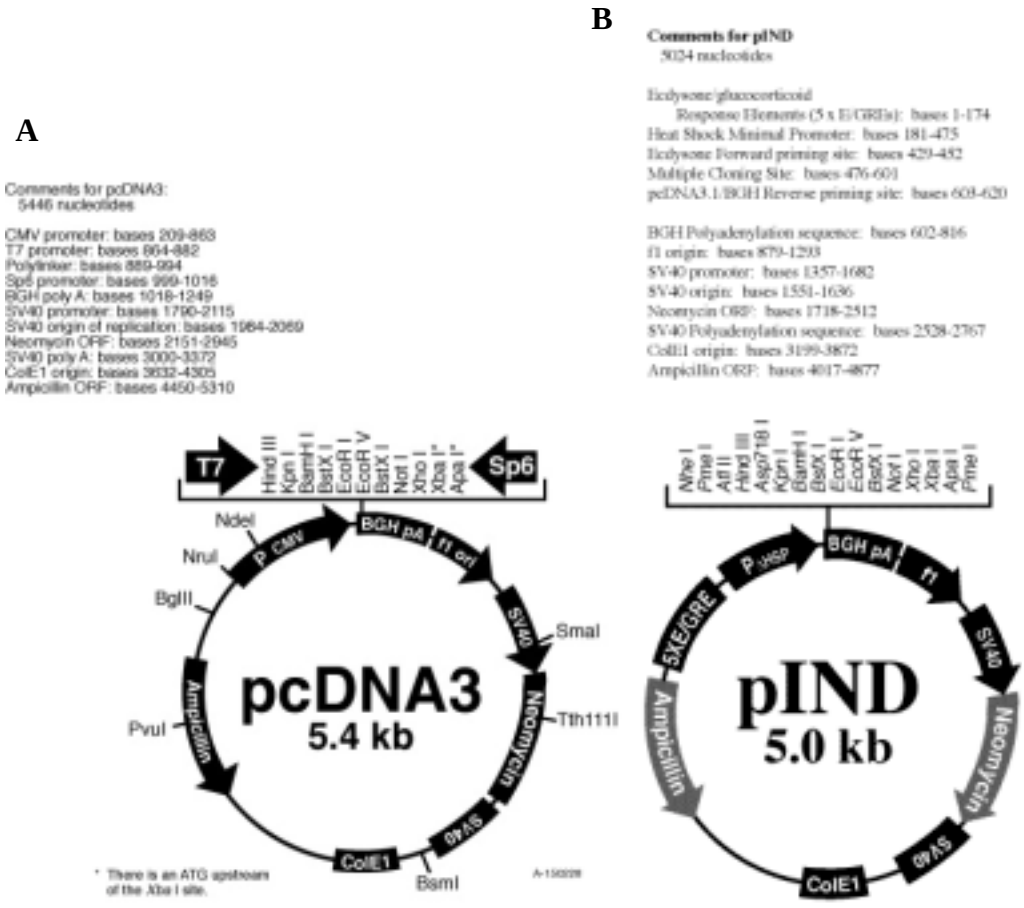


Figure 8: **Multiple cloning sites and properties of vectors** **(A)** pcDNA3 (Invitrogen) and **(B)** pIND (Clontech).

First pcDNA3.myc.BRCA1 was digested with *BamH*I-*Xba*I. Recognition sites of *BamH*I were at 907th and 4996th nucleotides on pcDNA3.myc.BRCA1 (11118 bp),

while *Xba*I recognises at 6655th position. After double digestion with *BamH*I-*Xba*I, the following fragments were expected;

*BamH*I- *Xba*I	5370 bp
*BamH*I-*BamH*I	4089 bp
*BamH*I-*Xba*I	1659 bp

4089 bp and 1659 bp fragments were extracted from the gel. 3 μg pIND vector was also digested with 5 U of each enzyme for 5 h in MBI yellow buffer at 37^{o}C and then extracted from the gel.

1659 bp fragment of myc.BRCA1 was ligated into *BamH*I-*Xba*I digested pIND, transformed into *E.coli* and plasmid DNA was prepared. Resulting construct - pIND+1.6 kb myc fragment- was digested with *BamH*I and extracted from the gel. Following treatment with CIAP the fragment was extracted from gel again. The last step was ligation of this *BamH*I digested/CIAP treated pIND+1.6 kb construct and 4089 bp fragment of BRCA1. The final construct was pIND.myc.BRCA1, which is 10.71 kb.

In order to check the orientation of the ligation products, a double digestion with *Xho*I-*Xba*I was performed in Stratagene assay buffer 3. So if the fragment was correctly ligated into vector the expected fragments after digestion were 5.6 kb and 5.1 kb, but if the orientation was wrong, 9.1 kb and 1.6 kb fragments were expected.

2.4.2 Construction of pIND.BRCA1

pCR3.BRCA1 vector was the second source of full-length *BRCA1* cDNA. Multiple cloning site and properties of this vector is shown in Figure 9. Subcloning of full-length *BRCA1* from pCR3 into pIND was performed by plasmid killing strategy. 5.6 kb full length *BRCA1* cDNA was in 5 kb pCR3 vector between *Hind*III-*Not*I sites in the pCR3.BRCA1 construct. It was very difficult to gel extract the 5.6 kb *BRCA1* fragment after *Hind*III-*Not*I double digest since vector and insert sizes were very close. *Msp*I cuts pCR3 vector 21 times and cuts its ampicillin resistance gene 5 times,

but this enzyme does not cut BRCA1 sequence. In order to get the intact *Hind*III-*Not*I digested 5.7 kb BRCA1 fragment without gel extraction, pCR3.BRCA1 was digested with *Hind*III-*Not*I-*Msp*I with 2xMBI Tango buffer. Meanwhile, pIND vector was digested with *Hind*III-*Not*I. This strategy eliminated gel extraction steps of the desired fragments before ligation reaction. After digestion the products were checked on agarose gel and double digested pIND vector was treated with alkaline phosphatase in order to prevent self-ligation. Last step was to ligate both *Hind*III-*Not*I digested BRCA1 and pIND and to transform the final construct into the DH5α strain.

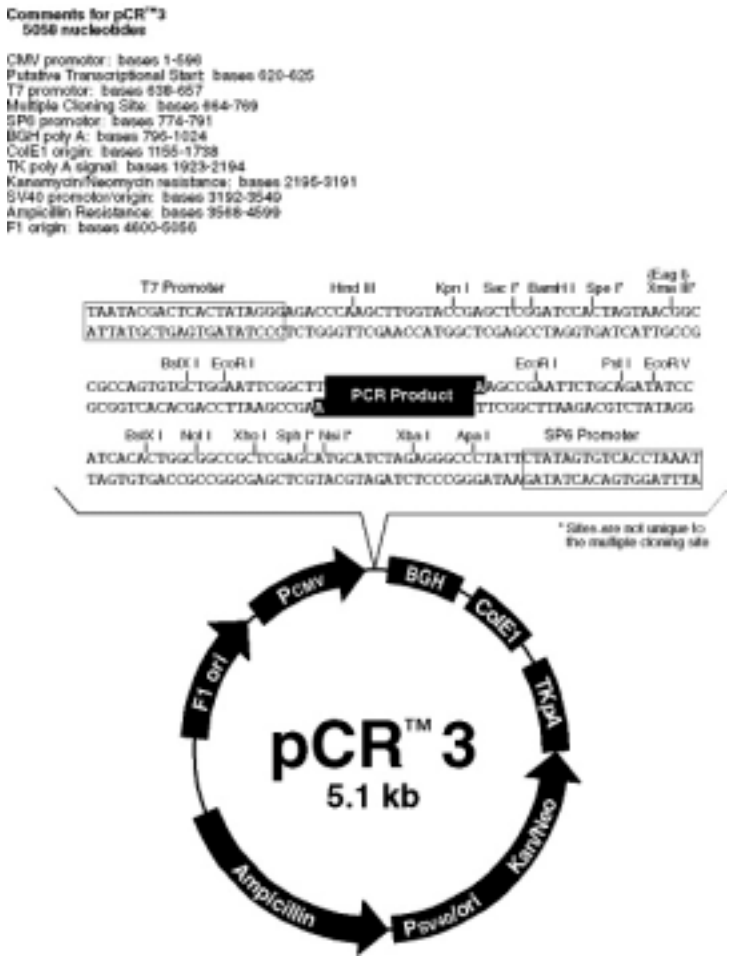


Figure 9: **Multiple cloning sites and properties of pCR3 vector (Invitrogen).**

2.4.3 Construction of pIND.HA.BRCA1

pRc/CMV.BRCA1 was the third source of full-length BRCA1 cDNA. 5.7 kb full length *BRCA1* cDNA was in pRc/CMV vector between *Hind*III-*Not*I sites with a HA tag at the 5' end. It was very difficult to distinguish the 5.7 kb BRCA1 fragment after *Hind*III-*Not*I double digest, because pRc/CMV vector was 5.5 kb in size (Figure 10). So pRc/CMV.BRCA1 was digested with *Hind*III-*Not*I-*Pvu*I (all New England Biolabs with Assay buffer 3). *Pvu*I cuts pRc/CMV vector once but does not cut BRCA1 sequence. Therefore the 5.7 kb BRCA1 fragment was gel extracted and ligated into 5 kb *Hind*III-*Not*I digested and purified pIND expression vector. The ligation reaction was performed overnight on ice and this mixture was transformed into the JM109 strain. The final construct was named as pIND.HA.BRCA1 since there is a HA tag at the 5' end of the BRCA1 sequence.

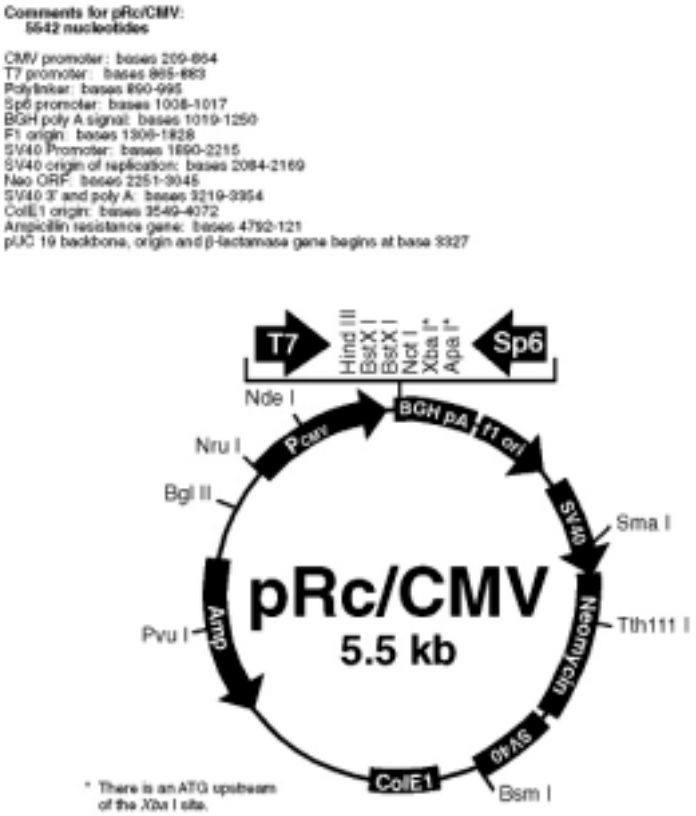


Figure 10: **Multiple cloning sites and properties of pRc/CMV vector** (Invitrogen).

2.5 Tissue Culture Techniques

2.5.1 Growth conditions of cell lines

All cell lines used in this study were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 1 mM glutamine and penicillin/streptomycin (50 mg/ml) and appropriate selective antibiotic.

U2OS/RXR cell line was stably transfected with pVgRXR plasmid and always grown in selective growth medium containing 100 μg/ml zeocin. UBR60-bcl2 cell line, which expresses BRCA1 under the control of tetracycline-regulated promoter, has been previously described (Harkin *et al*., 1999). Briefly, UBR60-bcl2 cells were maintained in growth medium, which contained 500 μg/ml geneticine (G418), 1 μg/ml puromycin, 200 μg/ml hygromycin and 1 μg/ml tetracycline. Upon tetracycline withdrawal the cells express the BRCA1 gene (Tet-off system, Clontech Laboratories Inc.).

The cells were incubated at a 37^{o}C incubator with an atmosphere of 5 % CO_2 in air. The cells were passaged before reaching confluence. The growth medium was aspirated and the cells were washed once with PBS. Trypsin solution was added to the petri dish to remove the monolayer cells from the surface. Fresh medium was added and the cell suspension was pipetted gently to disperse the cells. The cells were transferred to new petri dishes using different dilutions (from 1:2 to 1:10) depending on requirements.

All media and solutions used for tissue culture were kept at 4^{o}C and warmed to 37^{o}C before use.

2.5.2 Determination of cell sensitivity to selective system

Before generating stable pVgRXR expressing MCF7 breast carcinoma cell line, zeocin sensitivity of MCF7 has been determined by performing a kill curve. $1x10^6$ cells were plated in 100 mm plates and these cells were grown in DMEM supplemented with 800, 700, 600, 500, 400, 300 μg zeocin/ml for 3 weeks. Selection medium was changed every 2 days. Within the first week, cells stopped growing in zeocin containing medium. After 3 weeks cells were left with medium without zeocin for colony formation for one week. At the end of these 4 weeks, plates were stained with 2% trypan blue. First the medium was aspirated and replaced with 95% ethanol, then stained with 2% trypan blue solution for 10 min to stain the colonies. The plates carefully washed with water in order to get rid of excess stain. Colonies, which retained blue dye, were counted and as a conclusion, 400 μg/ml zeocin seemed to be enough for selection after transfection.

2.5.3 Cryopreservation of cell lines

Exponentially growing cells were harvested by trypsinisation and neutralised by adding growth medium. The cells were counted and precipitated at 1500 rpm for 5 min. The pellet was suspended in freezing solution at a concentration of $4x10^6$ cells/ml/vial. The tubes were left at -70^{o}C overnight. The next day, the tubes were transferred into the liquid nitrogen storage tank.

When frozen stocks were recovered from liquid nitrogen, the tubes were thawed rapidly at a 37^{o}C waterbath for 1 min. As the solution thawed, the cells were transferred into a 15 ml falcon tube and 10 ml DMEM was added gradually. The sample was centrifuged at 1500 rpm for 5 min. The supernatant was aspirated and the cell pellet was resuspended with 5 ml DMEM and transferred into a 100-mm petri dish.

2.5.4 Transfection of mammalian cells

2.5.4.1 Electroporation of mammalian cells

The cells were plated a day before the electroporation to obtain 60-90% confluency on the day of transfection. Before electroporation the cells were harvested by trypsinisation and washed twice with ice-cold calcium-magnesium free PBS. Harvested cells were counted with a haemocytometer and centrifuged at 1500 rpm for 5 min. The pellet was resuspended in ice cold PBS at a density of 4-15x10^6 cells/800 µl PBS /cuvette according to the experiment. 20-40 µg of the supercoiled plasmid DNA was added to the cell suspension, mixed well and transferred into a 0.4 cm electroporation cuvette. The cuvettes were incubated on ice for 10 min and then placed into the BioRad Gene Pulser chamber. The cells were electroporated at 950 µF, 0.22 kV/cm (t=19-22 ms). The cuvettes were then incubated on ice for 10 min and the cells were transferred into a tube containing growth medium. The cells were plated into one or two 100-mm plates according to the aim of the experiment

2.5.4.2 Calcium phosphate mediated transfection of mammalian cells

$CaPO_4$ transfection for generating a stable cell line

Exponentially growing MCF-7 cells were trypsinized and plated at a concentration of 5x10^5 cells/100 mm plate a day before transfection. The cells were incubated overnight in 10 ml growth medium. Next day, the medium was changed with 9 ml of fresh medium before transfection. 30 µg of pVgRXR plasmid DNA was mixed with 1.25 ml of 1xHBS and then with 62 µl of 2.5 M $CaCl_2$. This mixture was incubated for 20 min at room temperature. The precipitate was mixed gently and added onto the cells drop by drop. The cells were incubated at 37^{o}C under 5% CO_2 for 6 h. After the incubation, the medium was aspirated, 3 ml of 15% glycerol in 1xHBS was added to cells and distributed gently on the cells for 90 sec to shock the cells. The solution was removed and the cells were then washed twice with calcium-magnesium free PBS. 10 ml of growth medium was added to the cells and they were left for overnight incubation.

24 h after transfection the pEGFP transfected cells were observed to determine the efficiency of transfection and the MCF-7 / pVgRXR cells were expanded.

The cells were then passaged into four 150 mm plates 48 h after the transfection and the selection medium containing 500 μg/ml zeocin was applied to produce stable clones. Zeocin selection was applied for six weeks. Some independent colonies were picked and expanded in tissue culture selective medium. The clones were transfected with the reporter plasmid, pIND.lacZ to observe pVgRXR expression by using β-galactosidase assay.

$CaPO_4$ transfection for transient expression (Chen and Okayama, 1987)

Exponentially growing MCF-7 or U2OS cells or RXRIII clones were trypsinized and plated at $5x10^5$-$1x10^6$ cells/100 mm plate a day before transfection. The cells were incubated overnight in 10 ml growth medium. Next day, the medium was replaced with 9 ml of fresh medium. 20-30 μg of plasmid DNA was mixed with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml 2xBBS. This mixture was incubated 10-20 min at room temperature. $CaPO_4$.DNA precipitate was added drop by drop onto the cells and the plate was mixed gently. Plates were incubated either at 35^{o}C under 3% CO_2 or at 37^{o}C under 5% CO_2 for 15-24 h. Afterwards, this medium was removed and the cells were rinsed twice with growth medium and incubated at 37^{o}C under 5% CO_2. U2OS cells and RXRIII clones were expanded into two plates and induced with muristerone A or ponasterone A.

2.5.4.3 β-galactosidase assay

In order to find the best pVgRXR expressing MCF7 RXRIII clone, pIND.lacZ was transiently transfected into these clones and also into U2OS cells as control, which already stably expresses pVgRXR plasmid. RXRIII clones and U2OS control cells were transfected with pIND.lacZ vector. 24 h after the transfection the cells were splitted into two plates. One plate was induced with 10 μM muristerone A or

ponasterone A, while the other plate was left uninduced. 48 h after transfection -72 h for RXRIII clones- the cells were harvested for β-galactosidase assay, which detects the amount of RXR gene induction and the expression of lacZ gene from the reporter plasmid pIND.lacZ. Figure 11 shows the properties of the vectors pVgRXR and pIND.lacZ, which are the main components of the ecdysone inducible expression system.

β-galactosidase assay was performed as follows: Medium was aspirated from the plates and cells were rinsed with ice cold PBS. 750 µl ice cold PBS was added to the cells and the cells were scraped. The cells were pelleted by centifugating. The cell pellets were lysed in 200 µl of 5x reporter lysis buffer (Promega) or in RIPA buffer for 30 min. 24 µl of 10xZ buffer, 25 µl lysed cell extract, 40 µl ONPG and 151 µl H_2O were mixed and incubated at 37°C for 1 h. The reaction was stopped with 760 µl 1 M Na_2CO_3 (pH 12). Blank was the same mixture without cell lysate. Optic densities of the samples were read at 420 nm.

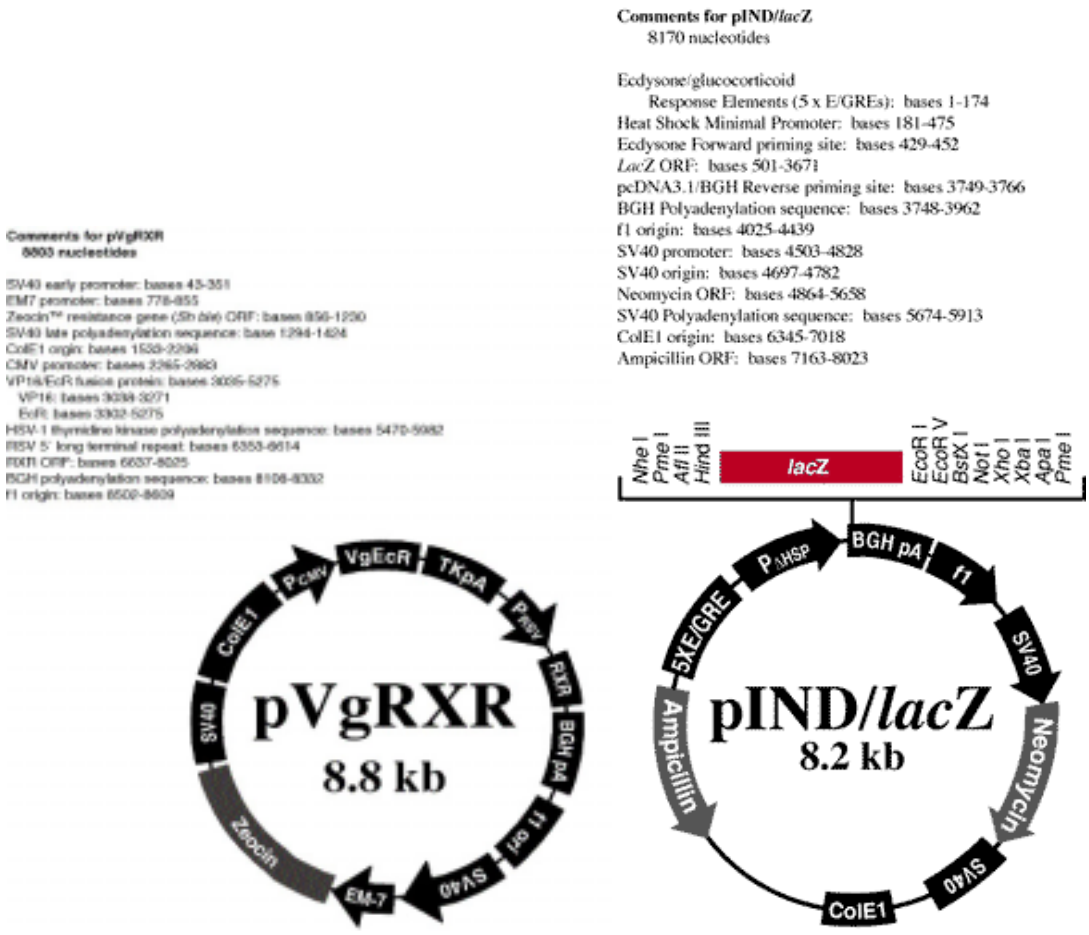


Figure 11: **Properties of the vectors pVgRXR and pIND.lacZ** (Clontech)

2.6 Subtractive Hybridization

Subtractive hybridization is a powerful technique to compare two populations of mRNA and obtain genes that are expressed in one population but not in the other. Both mRNA populations are converted to cDNA. The cDNA population, which contains the specific (differentially expressed) transcripts is named as "**tester**" and the reference cDNA population is named as "**driver**". Tester and driver cDNAs are hybridized and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester, but are absent from the driver mRNA. Clontech PCR-Select cDNA Subtraction method is based on selective amplification of differentially expressed sequences. Figure 9 summarizes the principle of suppression subtractive hybridization technique. First, cDNA is synthesized from poly A^+ RNA from the two types of population under comparison. The tester and driver cDNAs are digested with *Rsa*I, a four base cutting restriction enzyme that yields blunt ends. The tester cDNA is then subdivided into two portions and each is ligated with a different cDNA adaptor. The ends of the adaptor do not have a phosphate group so only one strand of each adaptor attaches to the 5' ends of the cDNA. The two adaptors have stretches of identical sequence to allow annealing of the PCR primer once the recessed ends have been filled in.

During first hybridization, an excess driver is added to each sample of tester. The samples are then heat denatured and allowed to anneal, generating the type **a**, **b**, **c**, and **d** molecules in each sample. The concentration of high- and low-abundance sequences is equalized among the type **a** molecules because reannealing is faster for the more abundant molecules. At the same time, the ss type **a** molecules are significantly enriched for differentially expressed sequences, as cDNAs that are not differentially expressed form type **c** molecules with the driver.

During the second hybridization, the two primary hybridization samples are mixed together without denaturing. Only the remaining equalized and subtracted cDNAs can reassociate and form new type **e** hybrids. These new hybrids are ds tester molecules with different ends, which correspond to the sequences of adaptors 1 and 2R. Fresh denatured driver cDNA is added to further enrich fraction **e** for differentially expressed sequences.

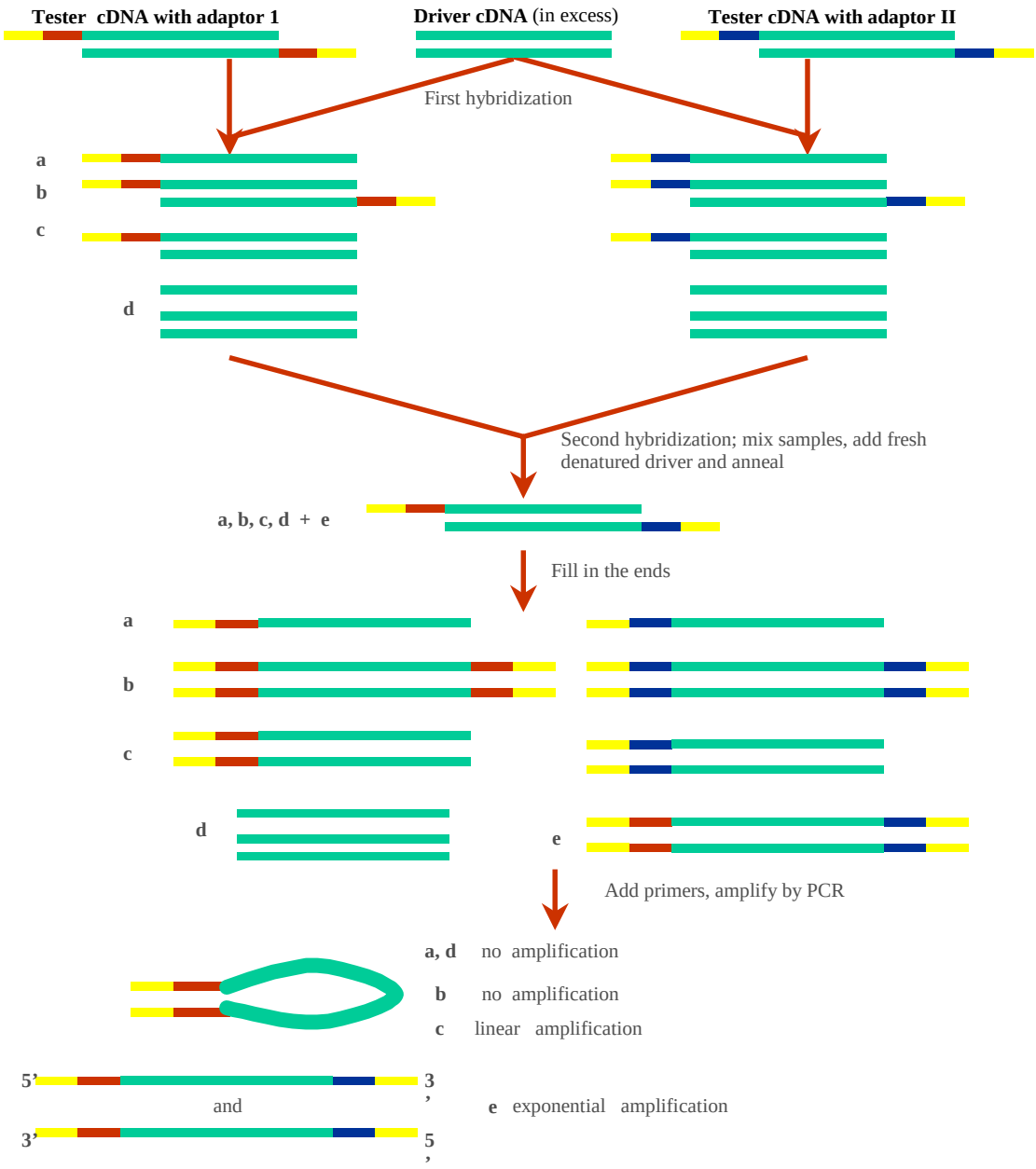


Figure 12: Principle of suppression subtractive hybridization

After filling in the ends by DNA polymerase, the type **e** molecules -the differentially expressed tester sequences- have different annealing sites for the nested primers on their 5' and 3' ends.

The entire population of molecules are then subjected to PCR to amplify the desired differentially expressed sequences. During PCR, type **a** and **d** molecules are

missing primer-annealing sites, thus cannot be amplified. Due to the suppression PCR effect, most type **b** molecules form a pan-like structure that prevents their exponential amplification. Type **c** molecules have only one primer-annealing site and can only be amplified linearly. Only type **e** molecules, which have two different adaptors, can be amplified exponentially. These are the equalized, differentially expressed sequences. Next a secondary PCR amplification is performed using nested primers to further reduce any background PCR products and to enrich for differentially expressed sequences. The cDNAs can then be directly inserted into a T/A cloning vector for identifying the differentially expressed sequences by sequencing and hybridization analysis. The PCR mixture can also be used as a hybridization probe to screen cDNA libraries.

2.6.1 Double strand cDNA synthesis

First and second strand cDNA synthesis were performed with Boehringer Mannheim cDNA synthesis kit. During first strand synthesis, the oligo (dT) primer included in the PCR-Select ^{TM} cDNA subtraction kit, was used.

cDNA synthesis primer 5'–TTTTGTACAAGCTT$_{30}$N$_1$N–3'

Each of 3.5 μg MCF-7 transfected pCMVmyc (cDNA2) and pCMVmycBRCA1 (cDNA1) poly (A)$^+$ RNA and control mRNA (cDNA3) from the subtraction kit was used to synthesize ds-cDNA. Briefly, each RNA was mixed with 1.7 μl oligo(dT) and incubated at 70°C for 2 min. 4 μl first strand buffer, 1 μl Rnase inhibitor, 2 μl dNTP mix, 2 μl [α-^{32}P]-dCTP (20 μCi final) and 2 μl AMV-reverse transcriptase were added and the final volume was adjusted to 20 μl with deionised water. The mixture was incubated at 42°C for 1 h and 40 μl second strand synthesis buffer II, 1 μl RnaseH, 34 μl H_2O and 5 μl DNA polymerase I were added to tubes. The tubes were always kept on ice and mixed thoroughly. The tubes were incubated at 12°C for 1 h, 22°C for 1 h and 65°C 10 min. Following addition of 4 μl T4 DNA polymerase the samples were incubated at 37°C for 10 min. Reactions were stopped

by the addition of 5 µl 20xEDTA/Glycogen mixture supplied within the subtraction kit.

After this step, all the protocol for cDNA subtraction was performed as recommended in Clontech PCR-Select cDNA subtraction kit with minor modifications.

Phenol:chloroform extraction was carried out with ds-cDNAs. During this protocol, the pellet was checked with a Geiger counter to ensure the presence of the pellet. The precipitate was dissolved in 50 µl of H_2O.

2.6.2 *Rsa*I digestion

44 µl each of cDNA1, cDNA2 and cDNA3 were digested with 15 U of *Rsa*I at 37°C for 2 h. In order to analyze the efficiency of *Rsa*I digestion, 2.5 µl undigested and 5 µl *Rsa*I digested cDNAs were run on a 1% ethidium bromide containing agarose gel in 1XTAE buffer. *RsaI* digested cDNAs were subjected to phenol:chloroform extraction. During this protocol, the pellet was checked with a Geiger counter. The precipitate was dissolved in 5.5 µl of ddH_2O.

2.6.3 Adaptor ligation

Preparation of experimental cDNAs: 1 µl from 5.5 µl *Rsa*I digested experimental cDNAs (cDNA1 and cDNA2) were diluted in 5 µl of ddH_2O.

2 µl (3 ng/µl) of *Hae*III digest of *ϕX174* DNA was diluted with 38 µl of sterile ddH_2O.

1 µl from 5.5 µl *Rsa*I digested control skeletal muscle cDNA was mixed with 5 µl of the diluted *ϕX174* DNA/ *Hae*III DNA.

Adaptor ligation: 2 µl of diluted tester cDNAs were mixed with either adaptor 1(10 µM) or adaptor 2R (10 µM) and 2 µl of 5xligation buffer and 1 µl T4 DNA ligase (400 U/µl).

Tester 1.1**:** cDNA1 ligated to adaptor 1, ***Tester 1.2****:* cDNA1 ligated to adaptor 2,
Tester 2.1: cDNA2 ligated to adaptor 1, ***Tester 2.2****:* cDNA2 ligated to adaptor 2,
Tester 3.1*:* cDNA3 ligated to adaptor 1, ***Tester 3.2****:* cDNA3 ligated to adaptor 2

In a fresh tube 2 µl of tester 1.1 and 2 µl of tester 2.2 were mixed. 1 µl from this mixture was diluted in 1 ml of H_2O and this mixture was named ***unsubtracted tester control*** **(1-c)**. The same setup was repeated for the other samples and controls 2-c and 3-c were prepared. After aliquoting the samples for preparation of unsubtracted tester controls the tubes were incubated at 16°C overnight for adaptor ligation. The ligation reaction was stopped by the addition of 1 µl 20xEDTA/Glycogen mix. Adaptor ligated tester cDNAs and unsubtracted tester controls were ready for ligation efficiency analysis.

Ligation efficiency analysis**:** 1 µl of each ligated tester cDNA was diluted into 200 µl ddH_2O. PCR setup was as follows:

Component	Tube# 1	2	3	4
Tester 1.1 or 2.1 or 3.1	**2**	**2**	**-**	**-**
Tester 1.2 or 2.2 or 3.2	**-**	**-**	**2**	**2**
G3PDH 3' primer (25 pmole/µl)	**1**	**1**	**1**	**1**
G3PDH 5' primer (25 pmole/µl)	**-**	**1**	**-**	**1**
PCR Primer 1 (25 pmole/µl)	**1**	**-**	**1**	**-**
Total volume	**4**	**4**	**4**	**4**

G3PDH 5' Primer 5'–ACCACAGTCCATGCCATCAC–3'
G3PDH 3' Primer 5'–TCCACCACCCTGTTGCTGTA–3'

2.5 µl of 10x buffer (MBI), 1.5 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTP were added to each tube bringing the final volume to 20 µl. After hot start, 5 U Taq polymerase (MBI) was added to each tube in 5 µl H_2O. PCR condition was as follows:

Hot start	75°C	1 min (Taq polymerase was added)	
Initial denaturing	75°C	5 min	
Denaturing	94°C	30 sec	30 cycles
Annealing	60°C	30 sec	
Extension	72°C	2 min	
Final extension	72°C	5 min	

5 µl from each reaction was analyzed on a 2% agarose gel run in 1xTAE buffer.

2.6.4 First Hybridization

First hybridization setup was as follows for tester 1.1 and 1.2. The same setup was repeated for testers 2.1, 2.2, 3.1, 3.3.

Component	**Hyb. sample 1**	**Hyb. Sample 2**
*Rsa*I digested driver cDNA	1.5	1.5
Adaptor 1 ligated tester 1.1	1.5	-
Adaptor 2R-ligated tester 1.2	-	1.5
4X hybridization buffer	1	1
Total volume	4 µl	4 µl

After overlaying the samples with mineral oil, the tubes were incubated in a thermal cycler at 98°C for 1.5 min. After hybridization at 68°C for 8 h the samples were subjected to second hybridization immediately.

2.6.5 Second Hybridization

1 µl driver cDNA, 1 µl 4xhybridization buffer and 2 µl H_2O were mixed; 1 µl of this mixture was put in a tube and one drop of mineral oil was added. This diluted driver sample was denatured at 98°C for 1.5 min. Hybridization samples 1 and 2 were simultaneously mixed with this freshly denatured driver. After incubation at 68°C overnight, 200 µl of dilution buffer was added to the tube and mixed. The tubes were incubated at 68°C for 7 min. The samples were stored at –20°C.

2.6.6 PCR amplification

1, 2 and 5 µl from each 1:200 diluted subtracted experimental cDNAs and 1:1000 diluted unsubtracted tester control cDNAs were used for first round PCR amplification in order to enrich the differentially expressed sequences after second hybridization.

Templates were amplified with PCR primer 1 with 5 U Taq polymerase (MBI) in 25 µl PCR reaction with the following conditions:

PCR primer 1 5'–CTAATACGACTCACTATAGGGC-3'

Initial denaturing	75°C	1 min (Taq polymerase added)	
Denaturing	94°C	30 sec	30 cycles
Annealing	66°C	30 sec	
Extension	72°C	1.5 min	
Final extension	72°C	5 min	

8 µl of primary PCR products were analyzed on 2% agarose gel.

3 µl of each primary PCR product was diluted in 27 µl of H_2O. 1 µl of this diluted primary PCR product mixture was used for secondary PCR. These primary

PCR products were amplified with nested PCR primer 1 and nested primer 2R with 5 U Taq polymerase (MBI) in 25 µl PCR reaction with the following conditions:

Nested PCR primer 1 5'–TCGAGCGGCCGCCCGGGCAGGT–3'
Nested PCR primer 2R 5'–AGCGTGGTCGCGGCCGAGGT–3'

Initial denaturing	75°C	1 min	
Denaturing	94°C	30 sec	15 cycles
Annealing	68°C	30 sec	
Extension	72°C	1.5 min	
Final extension	72°C	5 min	

8 µl each of secondary PCR products were analyzed on 2% agarose gel.

2.6.7 Subtraction Efficiency test

Secondary PCR products of subtracted tester cDNAs and unsubtracted tester controls were diluted in 10-fold ddH_2O. 1 µl of each template was amplified with G3PDH 3' and G3PDH 5' primers with 5 U taq polymerase (MBI) in 30 µl PCR reaction with the following conditions:

Denaturing	94°C	30 sec	18-23-28-33 cycles
Annealing	60°C	30 sec	
Extension	68°C	2 min	5 µl removed from tubes after each 5 cycles

Samples were run on 2% agarose gel.

2.6.8 Cloning and pre-screening of forward subtracted library

pGEM-T Easy Vector system was used for cloning of the forward subtracted library into TA vector. Secondary PCR products of forward subtracted library were ligated into pGEM-T Easy vector (Figure13), which allows blue/white selection. Briefly 3 µl of secondary PCR product was mixed with 1 µl pGEM-T vector, 5 µl 2x ligation buffer and 1 µl T4 DNA ligase. The ligation reaction was performed at 4°C overnight. Ligation products were transformed into supercompetent JM109 according to the manufacturer's recommendations and each transformation was plated onto two 150 mm LB agar plates containing ampicillin, IPTG and X-Gal.

The white colonies were picked up and grown in LB medium containing 100 µg/ml ampicillin and the glycerol stocks were prepared. The bacterial culture was directly used as template for PCR amplification of cDNA inserts with T7-SP6 primers. Amplified PCR products were run on 2% agarose gel and analyzed.

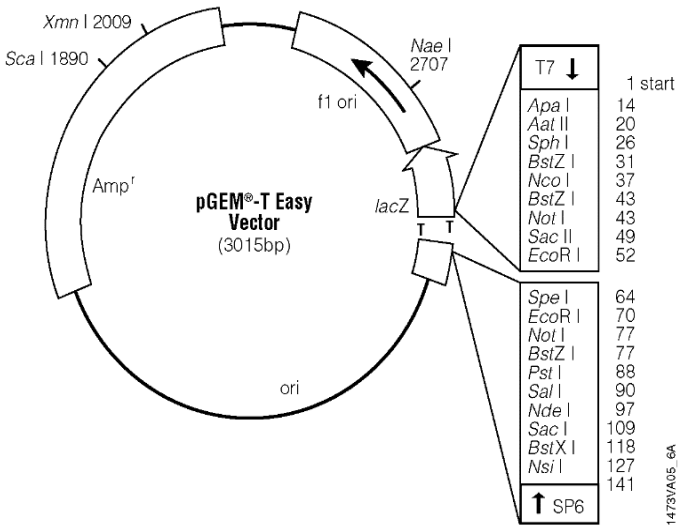


Figure 13: Map of pGEM-T Easy vector (Promega)

2.7 Differential Screening

PCR-Select Differential Screening Kit was used according to the manufacturer's recommendations with minor modifications.

2.7.1 Preparation of cDNA dot blots of forward subtracted library

Equal volumes of PCR products of forward subtracted library (amplified with T7/Sp6 primers) and freshly diluted 0.6 N NaOH were mixed in a 96 well plate for denaturing the DNA. 1.5 µl of each mixture was blotted onto a nylon membrane (Hybond N^+) in duplicate. The blots were neutralized for 2-4 min in 0.5 M Tris-HCl and washed in ddH_2O. The DNA was crosslinked to the membrane with Stratalinker under 120 mJ.

2.7.2 Probe preparation for dot blots

40 µl each of forward and reverse subtracted secondary PCR products were purified (PCR purification kit, Qiagen) and ethanol precipitated with NH_4OAc. The pellets were dissolved in 28 µl H_2O. 3 µl restriction buffer 1 and 1.5 µl *RsaI* were added to each tube. 3 µl of this restriction digest sample was mixed with 25 ng of control plasmid (pIND has 5 *RsaI* digestion sites). The tubes were incubated for 1 h at 37°C. Without stopping the reactions, 3 µl each of undigested cDNA, 3 µl each of digested cDNA and 3 µl each of digested cDNA, digested plasmid and also uncut plasmid were run on 2% agarose gel. After analysing the results, 1 µl of *SmaI* was added to the tubes and incubated for 1 h at room temperature. 61 µl H_2O, 10 µl restriction buffer 2 and 1 µl *EagI* were added to the tubes. Following incubation for 1 h at 37°C the digested adaptors were separated from the cDNA by using the Qiagen PCR purification kit. The volume of each sample was adjusted to 50 µl.

2.7.3 Random primer labelling of cDNA probes

50 ng (in 9 µl H_2O) of each adaptor-free digested forward and reverse subtracted probes were denatured by heating for 8 min at 95°C and cooled on ice. 3 µl reaction buffer (α-dCTP), 2 µl random primer mix, 5 µl (α-^{32}P)-dCTP (3000 Ci/mmol), and 1 µl Klenow enzyme (exo$^-$) were added and incubated at 37°C for 30 min. After the reactions were terminated by the addition of 5 µl stop solution, unincorporated dNTPs were purified from the labelled probes by using Quick spin columns as described.

2.7.4 Hybridization of the arrays with the subtracted cDNAs

For each membrane 50 µl of 20xSSC and 50 µl blocking solution were mixed and boiled for 5 min. After cooling down on ice, the mixture was combined with 5 ml of hybridization solution and the membranes were prehybridized for 2-4 h with continuous agitation at 65°C. Hybridization probe was prepared by mixing 50 µl of 20xSSC, 50 µl blocking solution and 10^7 cpm [α-^{32}P] dCTP-labelled purified probe. After boiling this mixture for 5 min, it was mixed with 5 ml hybridization solution and the membranes were prehybridized overnight with continuous agitation at 65°C. Membranes were sequentially washed in 2x SSC and 0.5% SDS, 1x SSC and 0.5% SDS, 0.2x SSC and 0.5% SDS at 68°C and the filters were exposed to X-ray film with an intensifying screen at -70°C.

2.7.5 Analysis of differential screening results

Initial screening of cDNA dots on arrays was analyzed by exposing the membranes to phosphorimager to measure the intensity of the signals. After first screening the clones showing more than five fold increase in signal intensity were selected and the differential screening procedure was repeated two more times. The signal intensities were normalized to actin signal intensities on each array; the ratio of actin values on each forward and reverse subtracted probe labelled arrays were used to

normalize the values. The average of three values for every dot was calculated. The clones that showed a dramatic increase in expression were selected for sequencing.

CHAPTER 3. RESULTS

3.1 Generating stable pVgRXR expressing MCF7 breast carcinoma cell line

The MCF7 breast carcinoma cells contain one copy of BRCA1 gene and display low expression of this gene when compared to other cell lines. These cells also represent the cellular environment of breast tissue. Studying the genes that are regulated by the BRCA1 gene in this cell line can be expected to give a better understanding of their biological mechanism. We therefore wanted to generate a MCF7 breast cancer cell line where the BRCA1 gene expression level can be controlled. In order to achieve this aim the ecdysone inducible mammalian expression system was used.

The first step in this system was to obtain MCF7 cells stably transfected with pVgRXR plasmid.

Determination of effective zeocin concentration

pVgRXR vector which expresses the heterodimeric ecdysone receptor contains the zeocin resistance gene. Mammalian cells lacking this gene are sensitive to zeocin which is a toxic agent.

Prior to the transfection experiment, control experiments were set up to determine the minimal concentration of zeocin required to prevent growth of the MCF7 breast carcinoma cell line. Various zeocin concentrations (800, 700, 600, 500, 400, 300 μg/ml) were used and a concentration of 500 μg/ml was found to be effective in eliminating all MCF7 cells by 7 days.

Transfection for generating stable pVgRXR expressing MCF7 breast carcinoma cell line was performed as described in the methods section 2.5.4. 48 hr

after transfection, the selection medium containing 500μg/ml zeocin was applied to the cells.

Two weeks after applying selection medium to the zeocin resistant cell clones, 96 clones were picked up into 96 microwell plates. 10 days later the growing colonies were splitted into 12 well plates and one week later to 100 mm plates. 52 MCF7/RXRIII clones were viable and grown.

Genomic DNA from these 52 clones were isolated. The presence of zeocin resistance gene encoded by plasmid pVgRXR in these clones were detected by using RXR-F and RXR-R primers for PCR reaction. 33 out of 52 clones were zeocin sequence positive after PCR reaction. Figure 14 shows the results of PCR reaction of some MCF7/RXRIII clones.

As expected pVgRXR plasmid DNA and U2OS cell line which carry the pVgRXR plasmid stably gave the 361 bp band and control MCF7 cells did not show any bands. The positive clones were RXRIII 1,2,5,6, 8, 9, 13, 20, 21,22,23, 24, 25, 26, 27, 28, 32, 33, 34, 35, 36, 38, 39, 40, 44, 45, 46, 47, 48, 49, 50, 51 and 52. All 33 positive clones were used for transfection with the pIND.lacZ reporter plasmid in order to determine the induction level of LacZ reporter gene.

β-galactosidase assay results

The selected MCF/RXRIII clones were transfected with pIND.lacZ reporter plasmid to test whether the zeocin-selected clones were really stably transfected with the pVgRXR plasmid. After the reporter plasmid transfection, the cells were induced with 10 mM muristrone A and the expression of lacZ gene was determined by the β-galactosidase assay, as described in the methods section. 23 out of 33 MCF7/RXRIII clones were tested and Table 12 shows the results of these assays.

MCF7 and U2OS cells were also transfected with pEGFP plasmid to determine the transfection efficiency at each experiment.

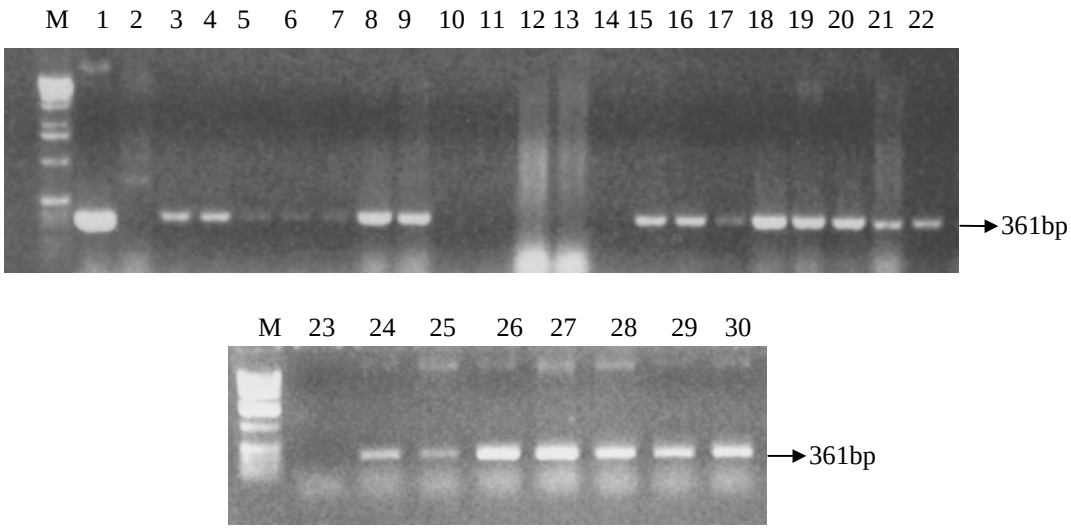


Figure 14: Analysis of pVgRXR transfected MCF7 cells. The genomic DNA was used from RXRIII clones for PCR reaction to determine the presence of the zeocin gene. The amplification product was 361bp. Lanes **1**: pVgRXR plasmid as a positive control, **2:** MCF7 cells, **23**: negative control, **24**: U2OS cells as a positive control, Lanes **3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 25, 26, 27, 28, 29, 30** are RXRIII clones 1- 2- 5- 6- 8- 9- 13- 15- 16- 17- 18- 19- 20- 21- 22- 23- 24- 25- 26- 27-28- 32- 33-34- 35-36 respectively.

U2OS cells were also transfected with pIND.lacZ in each experiment because it stably expresses pVgRXR plasmid and were used as positive control during transfection experiments. Table 11 shows the results of β-galactosidase assay of pIND.LacZ transfected U2OS cells used as a positive control. Different transfection protocols and cell lysis buffers were tried to analyze the induction level. The best induction was obtained with protocol 4, but this method requires the usage of a large number of cells. Protocol 5 was therefore selected to analyze the RXR/MCF7 clones for ß-galactosidase assay.

Table 12 shows the results of ß-gal assay of MCF7/RXR clones after transfection with the reporter plasmid pIND.lacZ with protocol 5 in Table 1. Although there was induction in U2OS/RXR cells, no induction was observed in the MCF7/RXR clones.

These results indicate that although all the clones were selected with effective killing concentration of zeocin antibiotic and were positive for pVgRXR plasmid transfection, they were not stably transfected with the plasmid. The positive result of the PCR analysis might be due to the detection of the free plasmid present in the cells rather than the plasmid integrated into the genome of the cells or the cells may have become resistant to zeocin with an unknown mechanism. These MCF7/RXR clones were not used for further experiments. We decided to continue to the experiments with the U2OS/RXR cells.

Table 11: Results of the β-gal assay of U2OS cells after transfection with pIND.lacZ reporter plasmid.

OD at 420 nm			
-mA	**+ mA**	**Induction fold**	**Protocol**
0.1462	1.7548	12	1
0.0375	0.8531	22	2
0.1574	0.9355	6	2
0.0385	1.6889	44	3
0.0114	1.1189	98	4
0.0162	1.4341	88	5
0.0515	0.1780	19	6

1) $CaPO_4$ transfection with HeBS, reporter lysis buffer
2) Electroporation, reporter lysis buffer
3) Electroporation, RIPA buffer
4) Electroporation, reporter lysis buffer, lysis time was increased
5) $CaPO_4$ transfection with BBS, RIPA buffer, lysis time was increased to two hours
6) $CaPO_4$ transfection with BBS, reporter lysis buffer

Table 12: Results of the β-gal assay of MCF7/RXR clones after transfection with pIND.lacZ.

	OD at 420 nm	
RXR Clone #	**-mA**	**+ mA**
1	0.0420	0.0299
2	0.0554	0.0621
5	0.0603	0.0660
6	0.1980	0.1590
8	0.0451	0.0304
9	0.0500	0.0632
13	0.0687	0.0441
20	0.0682	0.0708
21	0.0377	0.0676
22	0.0471	0.0743
23	0.0708	0.0589
24	0.0875	0.0883
25	0.0462	0.0615
26	0.1056	0.0795
27	0.0320	0.0349
28	0.0260	0.0343
32	0.0311	0.0384
33	0.0212	0.0226
34	0.1381	0.1235
35	0.0958	0.0899
36	0.0380	0.0309
38	0.0382	0.0418
39	0.0242	0.0342
40	0.0651	0.0632
44	0.1002	0.0869
45	0.0491	0.0527
46	0.0276	0.0499
47	0.1421	0.1405
48	0.0622	0.0512
49	0.0289	0.0729
50	0.0621	0.0872
51	0.1595	0.1733
52	0.0203	0.0525
U2OS/RXR	0.0550	0.9321

3.2 Construction and analysis of eukaryotic expression vectors

Three different full-length *BRCA1* cDNA sources were used for construction of pIND.BRCA1 as mentioned in the methods section 2.4, because at the beginning of this study the full length BRCA1 cDNA was not available to whole community and the discrepancies in some of the cDNA constructs were reported later. *BRCA1* cDNA sources and resulting constructs used during this study were as follows:

Vector (Source)	**Subcloned into**	**Cloning sites**
pcDNA3.myc.BRCA1 (F.J. Calzone)	pIND.myc.BRCA1	*BamH*I-*Xba*I
pCR3.BRCA1 (B.Weber)	pIND.BRCA1	*Hind*III-*Not*I
pRC.CMV.BRCA1 (D.Haber)	pIND.HA.BRCA1	*Hind*III-*Not*I

All full-length BRCA1 cDNAs were subcloned into pIND vector in order to express high amount of BRCA1 transcript in a controllable manner. The resulting constructs were analyzed after cloning into pIND vector with various methods for the appropriate expression of full-length BRCA1 protein.

3.2.1 Construction and analysis of eukaryotic expression vector pIND.myc.BRCA1

3.2.1.1 *In vitro* transcription-translation and immunoprecipitation assay results of pcDNA3.myc.BRCA1

The first source for the full-length BRCA1 cDNA, pcDNA3.myc.BRCA1, was provided from Dr. Calzone. It was partially sequenced from 3' end 5'- ends and *in vitro* transcription-translation assay was performed as described in the methods section 2.3.15. It was important to show that this full-length *BRCA1* cDNA was translated into expected protein product. Figure 15 shows the result of IVTT and immunoprecipitation assays performed with pcDNA3.myc.BRCA1 construct.

Following the IVTT assay, radiolabeled IVTT product was immunoprecipitated with the antibodies 5HU (for BRCA1) and 9E10 (for myc epitope) in order to check the specifity of BRCA1 and myc antibodies that were going to be used for future western blot experiments.

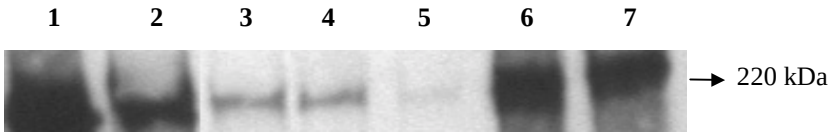


Figure 15: *In vitro* transcription-translation and immunoprecipitation assays of pcDNA3.myc.BRCA1 construct. Lane 1: IVTT product of pcDNA3.myc.BRCA1, Lane 2: IVTT-IP product of pcDNA3.myc.BRCA1 immunoprecipitated with 9E10, Lane 3: IVTT-IP product of pcDNA3.myc.BRCA1 immunoprecipitated with 5HU, Lane 4: Beads only of lane 2, Lane 5: Beads only of lane 3, Lane 6: Primary supernatant of lane 2 after binding to protein A, Lane 7: Primary supernatant of lane 3 after binding to protein A sepharose.

The band in the first lane corresponds to 220 kDa protein according to the molecular weight marker which means that pcDNA3.myc.BRCA1 *in vitro* transcribed-translated into expected BRCA1 protein product. When this IVTT protein product immunoprecipitated with myc and BRCA1 antibodies, although 5HU and 9E10 antibodies specifically recognized IVTT product of BRCA1, the bands were not very strong. This indicated that either the antibodies may be too weak or there may be a problem with protein A sepharose beads since a high percentage of the radioactive protein product was found unbound in the primary supernatant (lanes 6 and 7).

The results of this experiment show that the expected size of the BRCA1 protein product can be obtained from this construct. Therefore pCDNA3.myc.BRCA1 construct was used as a source of BRCA1 cDNA and cloned into pIND vector.

3.2.1.2 Construction of eukaryotic expression vector pIND.myc.BRCA1

Full length *BRCA1* cDNA with 2 myc epitopes at the 5' end, was subcloned from pcDNA3 vector into pIND vector. To achieve this, the first step was to perform BamHI-XbaI double digestion with both pIND vector and pcDNA3.myc.BRCA1 construct (Figure 16).

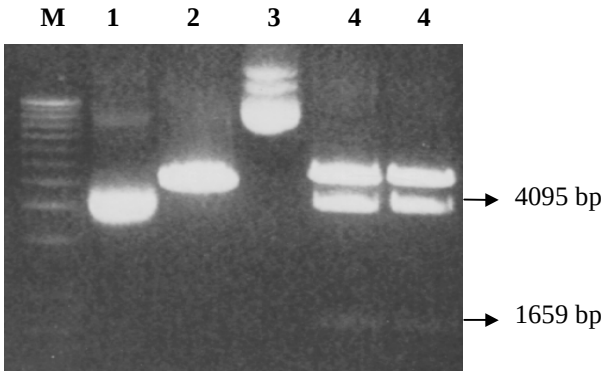


Figure 16: BamHI-XbaI double digestion profiles of pIND and pcDNA3.myc. BRCA1. Lanes **M**: 1 kb ladder, Gibco-BRL, **1**: undigested pIND, **2:** BamHI-XbaI digested pIND, **3**: undigested pcDNA3.myc.BRCA1, **4:** BamHI-XbaI digested pcDNA3.myc.BRCA1.

BamHI-XbaI double digested 5 kb pIND fragment from lane 2 and 4095 bp BamHI-BamHI and 1659 bp BamHI-XbaI fragments from lanes 4 were extracted. As a first step BamHI-XbaI digested pIND and 1.65 kb bands were extracted from the gel, ligated with vector, transformed into *E.coli* strain and miniprep DNAs of five colonies were purified. The resulting ligation colonies were named pIND+1.6kb myc. pIND+1.6 kb myc miniprep DNAs were digested with BamHI-XbaI in order to confirm that 1.6 kb insert had been ligated. Figure 17 shows the control BamHI-XbaI digestion profile of four of these colonies.

As observed on the gel photo in Figure 17, all pIND+1.6 kb myc colonies were positive for the 1.6 kb fragment, so BamHI digested pIND+1.6 kb myc colony number 1 from lane 5 was used to isolate the 6.6 kb band and the band was cut and purified by gel extraction procedure.

This 6.6 kb BamHI digested vector was used for ligation with the 4 kb BamHI fragment of BRCA1 from pcDNA3.myc.BRCA1.

Before ligation BamHI digested pIND+1.6 kb myc vector was treated with CIAP in order to minimize the self ligation of the vector without insert.

Figure 18 shows the vector and insert concentrations before the ligation reaction.

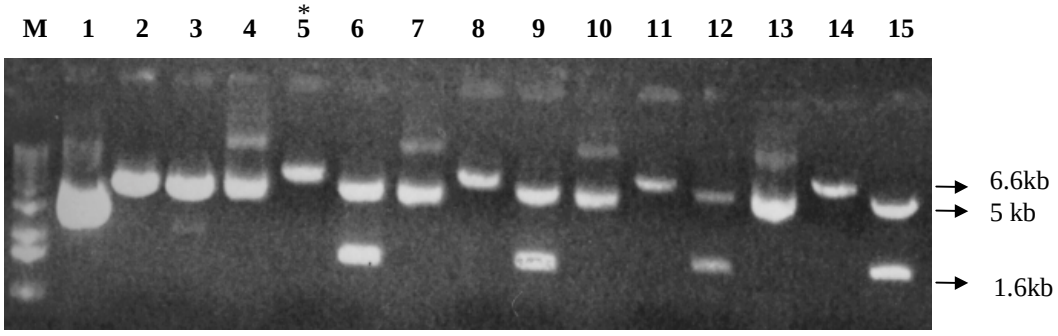


Figure 17: BamHI-XbaI double digestion profile of pIND+1.6 kb myc colonies.
M: Marker1 kb ladder, Gibco-BRL, Lanes **1:** undigested pIND, **2:** BamHI digested pIND, **3:** BamHI-XbaI digested pIND; **4, 7, 10, 13:** undigested pIND+1.6 kb myc colonies 1,2,3,4 respectively; **5, 8, 11, 14:** BamHI digested pIND+1.6 kb myc colonies 1,2,3,4 respectively; **6, 9, 12, 15:** BamHI- XbaI double digested pIND+1.6 kb myc colonies 1,2,3,4 respectively.

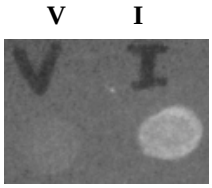


Figure 18: Determination of DNA concentrations by using the ethidium bromide containing agarose gels. The BamHI digested pIND+1.6 kb myc (vector) fragment and 4 kb BamHI DNA fragment (insert) were dotted on the agarose and the relative DNA intensities were determined under ultraviolet light. (Vector, **V:** 1 µl of BamHI digested CIAP treated pIND+1.6 kb myc, Insert, **I:** 1 µl of BamHI digested 4 kb BRCA1 fragment).

The 4kb BamHI BRCA1 fragment and the pIND+1.6kb vector containing 1.6 kb BRCA1 fragment were ligated and transformed into E.coli strain as described in the methods section. The next day 23 colonies were picked up from the plate and miniprep DNAs were prepared. There was no colony formation on the control plate.

Ligation products were double digested with XbaI-XhoI to control the orientation of the inserted fragment during the ligation and the expected 5.75 kb and 5 kb fragments after this digestion were observed in 11 out of 33 clones. These clones were named pIND.myc.BRCA1/1-2-5-6-9-10-11-13-15-16-23. Figure 19 shows the double digest profiles of two of the insert positive clones (lanes 6,8) and one wrong oriented clone (clone 4, lane10). Expected fragments after XbaI-XhoI double digestion of pcDNA3. myc.BRCA1 are 5.6 and 5.5 kb which was used as control for digestion (lane 4).

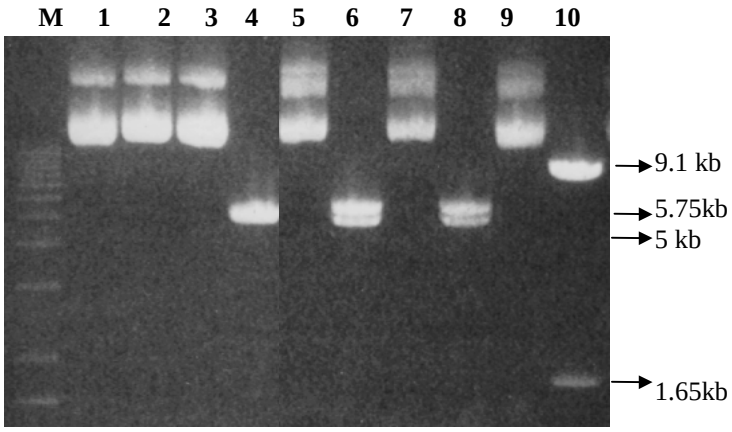


Figure 19: XbaI-XhoI double digestion profiles of pIND.myc.BRCA1 colonies.
M: 1 kb ladder, Gibco-BRL**;** Lanes **1:** undigested pcDNA3 myc.BRCA1; **2:** XbaI digested pcDNA3 myc.BRCA1; **3:** XhoI digested pcDNA3 myc.BRCA1; **4:** XbaI-XhoI double digested pcDNA3 myc.BRCA1; **5, 7, 9:** undigested pIND.myc.BRCA1/ 1-2-4; **6, 8,10:** XbaI-XhoI double digested pIND.myc.BRCA1/ 1-2-4.

3.2.1.3 Generating a U2OS cell line that stably expresses pIND.myc.BRCA1

The pIND.myc.BRCA1 construct carries an antibiotic selection marker neomycin gene, which enables the selection of the mammalian cells carrying this construct with neomycin analog G418. Before generating U2OS cell line that stably expresses the pIND.myc.BRCA1, G418 antibiotic sensitivity of U2OS was determined. $5x10^5$ cells were plated on 60 mm plates, and were grown in DMEM supplemented with 800, 700, 600, 500, 400, 300 µg/ml G418 for 3 weeks. The minimum effective concentration of G418 killing all the cells was found to be 500 µg/ml. This concentration was used to select the U2OS cells after transfection with pIND.myc.BRCA1 construct.

A day before transfection U2OS cells were seeded on 60-mm dishes at a cell concentration $2.5x10^5$ in each plate. The BBS mediated transfection method was used to transfect the cells with the construct as described in the methods section.
The cells were selected in 500 µg/ml G418 for five weeks and then maintained in 100 µg/ml zeocin. A total of 96 putative positive cell clones for pIND.myc.BRCA1 were selected.

Genomic DNAs were isolated from the 96 pIND.myc.BRCA1 expressing clones. PCR was performed with EcF-TC31 primers for confirming the presence of pIND.myc.BRCA1 plasmid in the selected cell clones. 56 of the clones were positive for the presence of the 1103 bp expected fragment. Figure 17 shows the result of the PCR reactions as an example of the positive clones 7,17,18,19 and 20 for pIND.myc.BRCA1 plasmid.

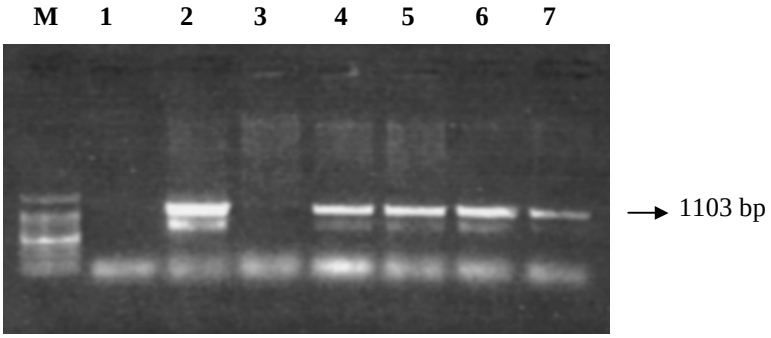


Figure 20: EcF-TC31 amplification results of pIND.myc.BRCA1 colonies.
M: ΦX 174/HinfI marker, MBI, Lanes **1:** negative control**; 2,3,4,5,6,7** are clones, 7,16, 17, 18, 19, 20 respectively

3.2.1.4 Analysing the induction of BRCA1 expression in pIND.myc.BRCA1 clones

The next step was to analyse some of these positive U2OS clones for the induction of the expression of the BRCA1 gene by inducing the cells with 10 µM muristerone A. These cell clones were grown, expanded and induced with 10 µM muristerone, A which was added into the culture medium. The cells were collected 24 hours after the transfection and lysed for Western blot analysis.

14 clones were analyzed with 5HU (BRCA1 ab), 4 with 9E10 (myc ab) and 7 with MS110 (BRCA1 ab).

Figures 21 and 22 shows the results of western blot analysis. Although there seems to be a slight induction in some of the samples, some of them showed a decrease, making the result unsatisfactory.

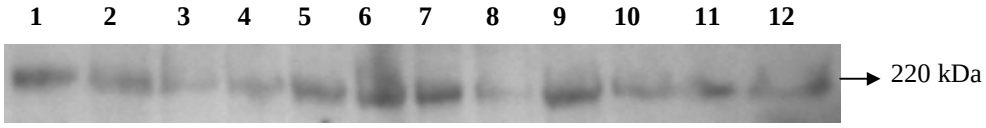


Figure 21: Western blot result of stable U2OS cell clones for pIND.myc.BRCA1 construct analyzed with 5HU. 400 µg of the cellular lysates were loaded into each well and the blot was hybridized with 5HU BRCA1 antibody Lanes **1:** HBL-100, **2:** U2OS, **3:** Transient transfection of U2OS cells pA (-), **4:** Transient transfection of U2OS cells pA(+)**, 5:** clone 43 pA(-) **6:** clone 43 pA(+), **7:** clone 66 pA(-), **8:** clone 66 pA(+), **9:** clone 70 pA(-), **10:** clone 70 pA(+), **11:** clone 81 pA(-), **12:** clone 81 pA(+).

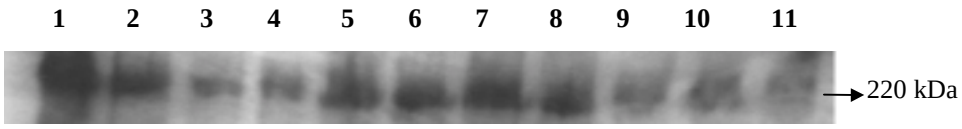


Figure 22: Western blot result of stable U2OS cell clones for pIND.myc.BRCA1 construct analyzed with MS110. 400 µg of the cellular lysates were loaded into each well and the blot was hybridized with MS110 BRCA1 antibody. Lanes **1:** HBL-100, **2:** clone 7 pA(-), **3:** clone 7 pA(+), **4:** clone 22 pA(-), **5:** clone 22 pA(+), **6:** clone 26 pA(-), **7:** clone 26 pA(+), **8:** clone 28 pA(-), **9:** clone 28 pA(+), **10:** clone 29 pA(-), **11:** clone 29 pA(+).

The first source of the full length BRCA1 cDNA was pcDNA3.myc.BRCA1 from Dr. Calzone. Although the first source of BRCA1 cDNA gave the expected 220kDa BRCA1 protein as a result of IVTT experiment, it was later released that this BRCA1 cDNA might contain some discrepancies. The stable transfection experiments with this BRCA1 cDNA cloned into pIND vector did not show significant increase for BRCA1 gene in the U2OS cells which contain RXR regulatory plasmid. Since the

cloning of BRCA1 cDNA into the pIND vector was successful, one possible explanation for not getting enough induction could be the following. The cell clones selected with G418 were not stably transfected with the pIND.myc.BRCA1 construct and the cells therefore did not actually keep the construct. Although the PCR analysis of the clones with EcF-TC31 primers for confirming the presence of pIND.myc.BRCA1 plasmid gave a positive result, at the time of the analysis the cells might still have contained the plasmid but could have degraded or kicked it out from the cells after some further passaging of the cells. This is not an unusual mechanism and has been observed by scientists who have done similar experiments.

Since the discrepancies for the full length BRCA1 cDNA was released, these clones were not pursued further to analyse if they really stably contained the pIND.myc.BRCA1 construct, which could have been analyzed by the Southern blot method.

3.2.2 Construction and analysis of eukaryotic expression vector pIND.BRCA1

3.2.2.1 *In vitro* transcription and translation-Immunoprecipitation assay result of pCR3.BRCA1

Since the experiments performed with the previous cDNA did not result in the induction of the BRCA1 protein in the U2OS cells, another source for the full length BRCA1 cDNA (pCR3.BRCA1) was used to clone the BRCA1 gene to pIND vector. The pCR3.BRCA1 construct was a gift from Dr. Barbara Weber.

pCR3.BRCA1 was the second source of BRCA1 cDNA. *In vitro* transcription and translation assay (IVTT) has been performed as described in the methods section to determine the integrity of this construct. It is important to show that this full-length BRCA1 cDNA is translated into protein product properly. Figure 20 shows the IVTT assay results of pCR3.BRCA1. Radiolabelled IVTT product was immunoprecipitated with the BRCA1 antibodies MS110, C-20, and D20 (Figure 23). The

immunoprecipitation assay was performed to test the efficient binding of the available BRCA1 antibodies to the BRCA1 protein.

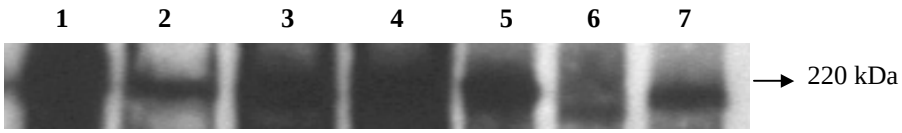


Figure 23: *In vitro* transcription and translation-Immunoprecipitation assay with the pCR3.BRCA1 construct. Lanes **1:** IVTT product of pCR3.BRCA1, **2:** IVTT-IP product of pCR3.BRCA1 immunoprecipitated with MS110, **3:** IVTT-IP product of pCR3.BRCA1 immunoprecipitated with C-20, **4:** IVTT-IP product of pCR3.BRCA1 immunoprecipitated with D-20, **5:** Primary supernatant of lane 2 after binding to protein A, **6:** Primary supernatants of lane 3 after binding to protein A, **7:** Primary supernatant of lane 4 after binding to protein A.

The band in the first lane corresponds to 220 kDa according to the molecular weight marker which means that pCR3.BRCA1 had translated into protein product properly. When this *in vitro* translated protein product immunoprecipitated with several BRCA1 antibodies, bands indicated that these antibodies specifically bind the radiolabelled protein product and a small percentage remained unbound.

3.2.2.2 Cloning of BRCA1 from pCR3.BRCA1 into pIND vector

Subcloning of full-length BRCA1 from pCR3.BRCA1 vector into pIND vector was performed by plasmid killing strategy as described in the methods section. In order to get the intact HindIII-NotI digested 5.7 kb BRCA1 fragment without gel extraction, pCR3.BRCA1 was digested with HindIII-NotI-MspI with 2xMBI Tango buffer. Meanwhile, pIND vector was digested with HindIII-NotI. After digestion the products were checked on agarose gel and double digested pIND vector was treated with alkaline phosphatase in order to minimize self-ligation.

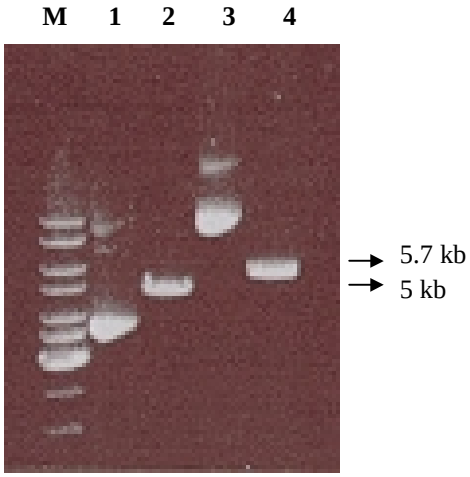


Figure 24: Digestion profiles of pCR3.BRCA1 and pIND. Lanes **M:** 1 kb ladder, MBI, Lanes **1:** undigested pIND, **2:** HindIII-NotI digested pIND, **3:** undigested pCR3.BRCA1, **4:** HindIII-NotI-MspI digested pCR3.BRCA1

After the control of the digestions on the gel, digested samples from lanes 2 and 4 were ligated in different vector:insert ratios and transformed into *E. coli* strain as described in the methods section. Next day 50 colonies out of about 1500 were picked up from the plates with different vector/insert ratios and DNA was isolated from the colonies. There was no colony formation in the control plate. The DNA samples were first digested with EcoRI in order to eliminate the colonies smaller than 10 kb and self-ligated vector (Figure 25).

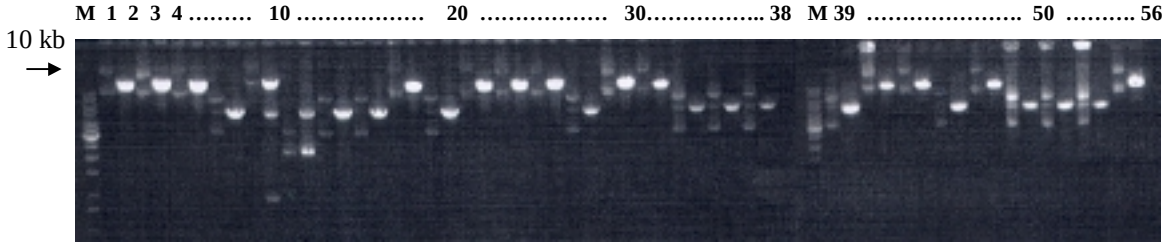


Figure 25: EcoRI digestion profiles of pIND.BRCA1 colonies. Lanes M: 1 kb ladder from MBI; Lanes **1,3,5,7,9,11,13,15,17,19,21,23,25,27,29,31,33,35,37,39,41,43,45,47,49,51,53,55:** undigested pIND.BRCA1 colonies 5.9,6.1-6.9,7.1-7.9,8.1-8.9; **2,4,6,8,10, 12,14,16,18,20,22,24,26,28,30,32,34,36,38,40,42,44,46,48,50,52,54,56**:EcoRI digested pIND.BRCA1 colonies 5.9,6.1-6.9,7.1-7.9,8.1-8.9 respectively.

Out of 50 colonies 18 of them were found to be bigger than 10 kb. 9 of them were digested with AvaI (Figure 26) and HindIII-NotI (Figure 27) for further characterization.

Expected sizes were as follows:

Vector	*Enzyme*	*Expected fragments (bp)*
pIND	AvaI	3524,1091,391
pIND.BRCA1(control)	AvaI	5833,3542,1085,92
pCR3.BRCA1	AvaI	10564,219
pIND.BRCA1 colonies	AvaI	5833,3542,1085,219
pIND	HindIII-NotI	4956,68
pIND.BRCA1(control)	HindIII-NotI	10552
pCR3.BRCA1	HindIII-NotI	5726,4986
pIND.BRCA1 colonies	HindIII-NotI	5726,4956

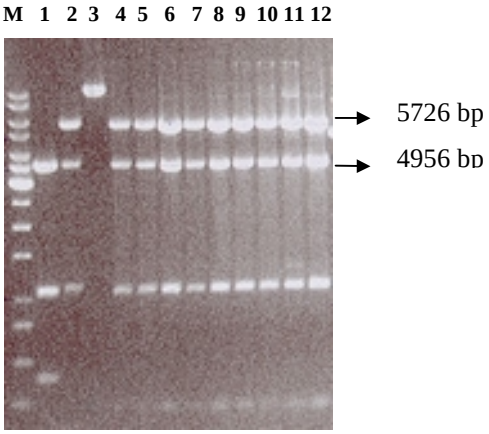


Figure 26: AvaI digestion profiles of pIND.BRCA1 colonies. Lanes **M:** 1 kb ladder, MBI; AvaI digestion profiles of colonies. Lanes **1:** pIND; **2:** pIND.BRCA1(control); **3:** pCR3.BRCA1; **4,5,6,7,8,9,10,11,12:** pIND.BRCA1 clones 4.4, 4.9, 5.9, 6.2, 7.2, 7.3, 7.5, 8.5, 8.9 respectively.

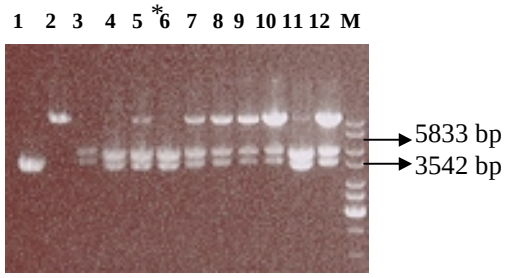


Figure 27: HindIII-NotI digestion profiles of pIND.BRCA1 colonies. M: 1 kb ladder, MBI; HindIII-NotI digestion profiles: Lanes **1:** pIND; **2:** pIND.BRCA1 (control); **3:** pCR3.BRCA1; **4,5,6,7,8,9,10,11,12:** pIND.BRCA1 clones 4.4, 4.9, 5.9, 6.2, 7.2, 7.3, 7.5, 8.5, 8.9 respectively.

According to the gel photo some digests were uncompleted but all 9 colonies gave the expected digestion patterns. Colony 5.9 was used for further studies.

3.2.2.3 Sequencing of eukaryotic expression vector pIND.BRCA1

pIND.BRCA1 construct, originated from colony 5.9, has been sequenced from 5' and 3' ends with the manual sequencing kit as described in the methods section. Figure 28 shows the sequencing result.

Sequencing result showed that *Hind*III-linker sequence, start codon and BRCA1 sequence were as expected but some mismatch was observed at the 3' end after the stop codon. BRCA1 sequence and stop codon were as expected but no matching was observed with the *Not*I site and linker sequence. The same results were observed after the sequencing reactions were repeated once more.

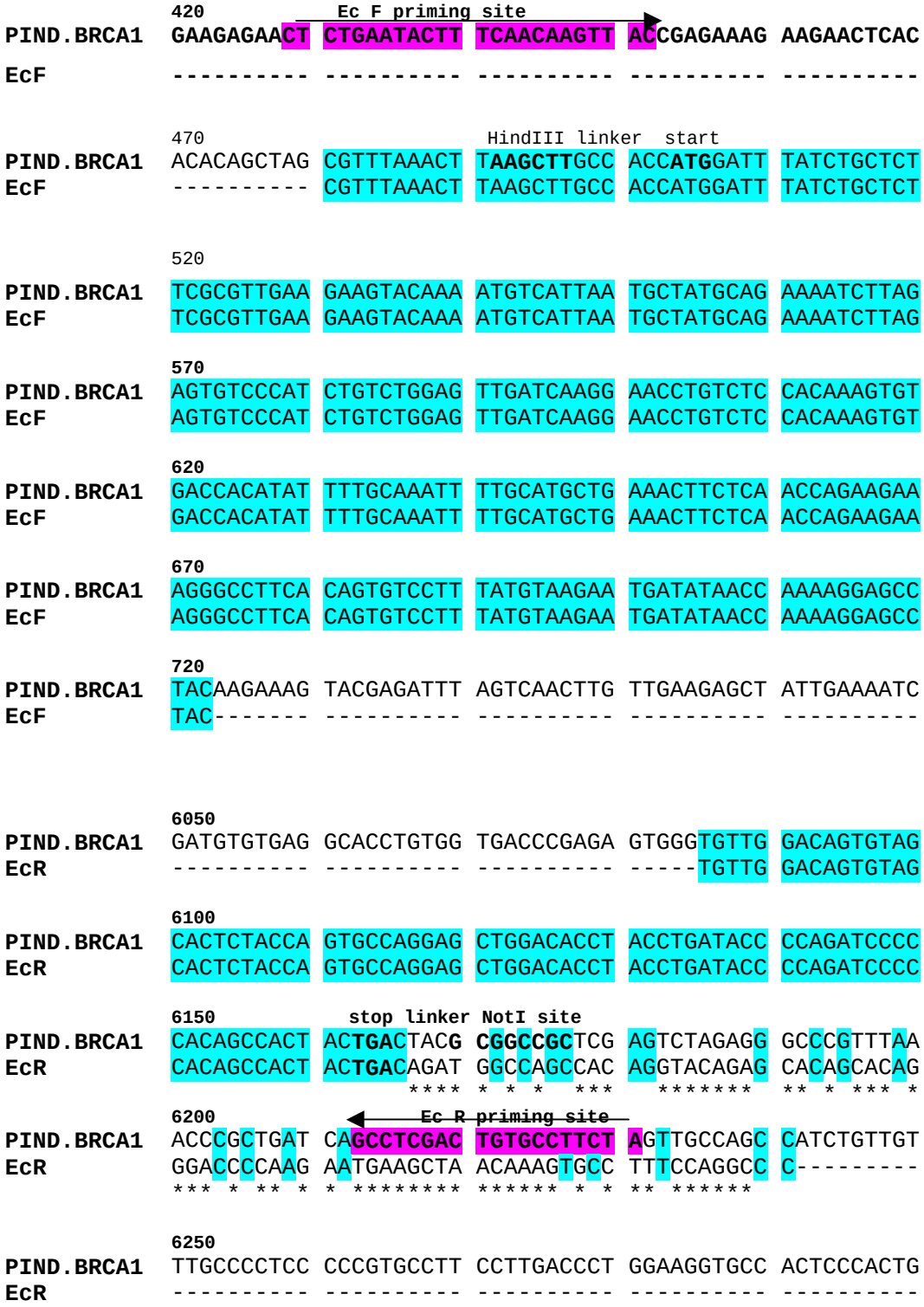


Figure 28: Sequencing result of pIND.BRCA1 Upper lines indicate the expected sequence, lower line is sequenced fragment. Blue highlight indicates the matching sequences. (***** : mismatch, -: no sequence read)

3.2.2.4 Analysing BRCA1 expression by transient transfection into U2OS cells

Expression of BRCA1 was analyzed by transfection of pIND.BRCA1 into the U2OS cell line. Although sequencing of the construct showed mismatch after stop codon, 4 transient transfections were made with pIND.BRCA1 and analyzed with MS110 BRCA1 antibody (data not shown) but no induction was observed.

Since the 3' end of the cloning site after the stop codon showed mismatches, during the preparation of the construct something must have gone wrong and affected the induction of this construct in U2OS cells.

At the same time, pCR3.BRCA1 and pCMV.myc.BRCA1 constructs carrying CMV promoters at the upstream of BRCA1, were also transfected into U2OS cell line independently and analyzed with western blot for BRCA1 expression by using MS110 BRCA1 monoclonal antibody (See appendix A for pCMV.myc.BRCA1 sequence and the restriction enzyme map). Seven independent transient transfection experiments with pCR3.BRCA1 and 8 transfections with pCMV.myc.BRCA1 were performed in order to analyse the BRCA1 expression level in U2OS cells (Figures 29 and 30).

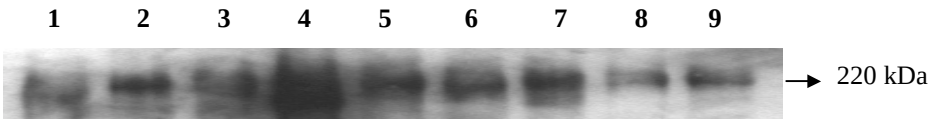


Figure 29: Western blot result of the cells transiently expressing BRCA1 in U2OS cells. The cells were transiently transfected with pCR3.BRCA1 and 200 µg of the cellular lysates were loaded into each well and the blot was analyzed with MS110 BRCA1 monoclonal antibody. Lanes **1**: HBL-100; **2**: U2OS cells only; **3, 6, 8:** mock transfection of U2OS cells; **4:** Transient transfection of U2OS cells with pCMV.myc.BRCA1; **5, 7, 9:** Transient transfection of U2OS cells with pCR3.BRCA1.

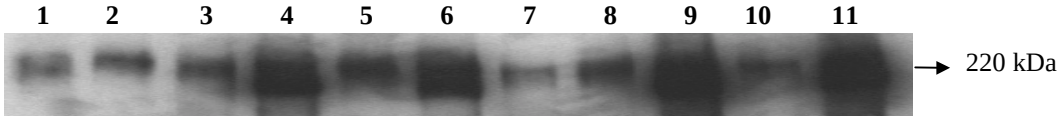


Figure 30: Western blot result of U2OS cells which were transiently transfected with pCMV.myc.BRCA1. In each lane equal amount **(**200 µg) of the cellular lysates was used and the blot was analyzed with MS110 BRCA1 antibody. Lanes **1:** HBL-100; **2:** U2OS; **3,7:** mock transfection of U2OS cells; **5, 8,10:** Transient transfection of U2OS cells with pCMV.myc vector; **4, 6, 9, 11:** Transient transfection of U2OS cells with pCMV.myc.BRCA1.

Western blot analysis showed that the BRCA1 expression was high in each of the independent transfections with these CMV promoter-containing BRCA1 constructs. The highest protein expression was observed with the pCMV.myc.BRCA1 construct.

MCF7 cells were also transiently transfected with pCMV.myc.BRCA1 and after electroporation the cells were harvested within different time intervals. The expression level of BRCA1 protein was analyzed with western blot by using MS110 BRCA1 monoclonal antibody (Figure 31).

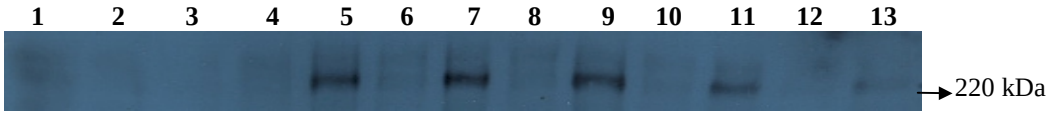


Figure 31: Western blot result of MCF7 cells which were transiently transfected with pCMV.myc.BRCA1. In each lane an equal amount (100 µg) of the cellular lysates was used and the blot was analyzed with MS110 BRCA1 antibody. Lanes **1:** HBL-100; **2:** MCF7; **3:** MCF7 mock transfection; **4, 6, 8, 10, 12:** MCF7 cells transfected with pCMV.mvc; **5, 7, 9, 11, 13:** MCF7 cells transfected with pCMV.mvc.BRCA1; **4-5:** harvested 12 hr after electroporation; **6-7:** cells harvested 15 hr after electroporation; **8-9;** harvested 18 hr after electroporation; **10-11:** cells harvested 21 hr after electroporation; **12-13:** cells harvested 24 hr after electroporation.

3.2.3 Construction and analysis of eukaryotic expression vector pIND.HA.BRCA1

3.2.3.1 Subcloning of BRCA1 from pRc/CMV.BRCA1 into pIND

pRc/CMV.BRCA1 was the third source of BRCA1. The full length 5.7 kb BRCA1 cDNA was cloned into 5.5kb pRc/CMV vector between HindIII-NotI sites with a HA tag at the 5' end of the start codon. Dr. Daniel Haber provided this construct and the whole BRCA1 gene was sequenced before using this construct. When the construct was double digested with HindIII-NotI, it was very difficult to distinguish the 5.7 kb BRCA1 fragment from the 5.5kb pRc/CMV vector. pRc/CMV.BRCA1 was therefore digested with HindIII-NotI-PvuI, which PvuI cuts pRc/CMV vector (5.5 kb) once but not BRCA1 sequence. The 5.7 kb BRCA1 fragment was extracted from the gel and ligated into 5 kb HindIII-NotI digested pIND expression vector (Figure 32).

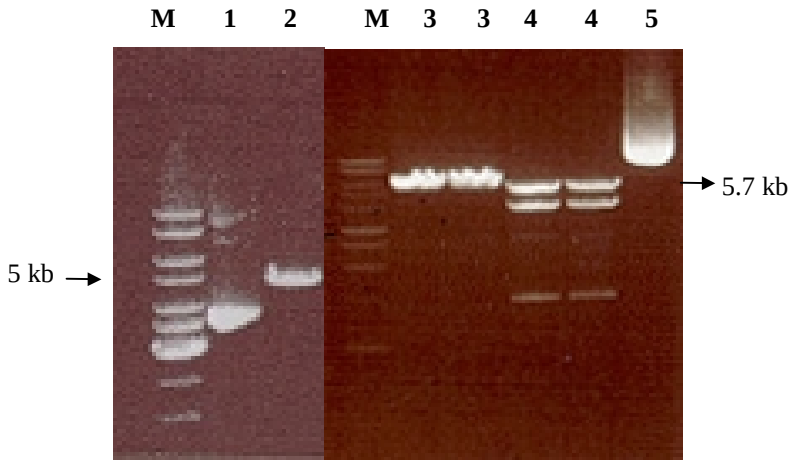


Figure 32: Restriction enzyme digestion profiles of pIND and pRc/CMV.BRCA1
Lanes **M:** 1 kb ladder, MBI, Lanes **1:** undigested pIND, **2:** HindIII-NotI digested pIND, **3:** HindIII-NotI digested pRc/CMV.BRCA1, **4:** HindIII-NotI-PvuI digested pRc/CMV.BRCA1, **5:** uncut pRc/CMV.BRCA1 .

The 5 kb pIND band from lane 2 and 5.7 kb BRCA1 band from lane 4 were gel extracted. Ligation reaction and transformation into supercompetent JM109 were performed as described in the methods section. The next day 10 colonies were picked up from plates and miniprep DNA samples were prepared. There was no colony formation in control plate.

In order to check whether ligation had taken place, the plasmid DNAs from the colonies were double digested with HindIII-NotI and HindIII-NotI-PvuI. The expected fragment sizes after HindIII-NotI digestion were 5.7 and 5 kb (Figure 33). DNA samples were also digested with HindIII-NotI-PvuI in order to observe the intact 5.7 kb BRCA1 band (Figure 34).

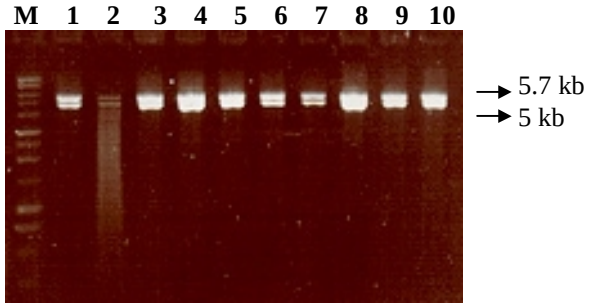


Figure 33: HindIII-NotI digestion profiles of pIND.HA.BRCA1 colonies. Lanes **M*:*** 1 kb ladder, MBI; Lanes **1, 2, 3, 4, 5, 6, 7, 8, 9, 10:** HindIII-NotI digestion profiles of pIND.HA.BRCA1 colonies 1.1, 1.2, 1.3, 1.4, 1.5, 2.1, 2.2, 2.3, 2.4, 2.5 respectively.

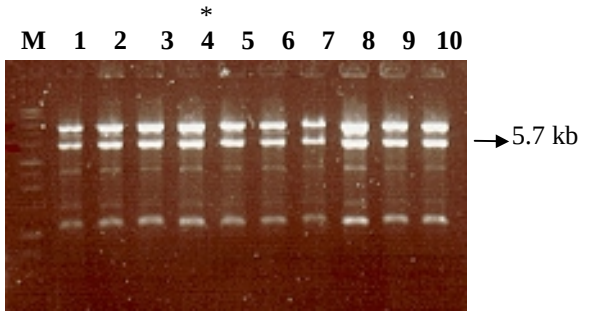


Figure 34: HindIII-NotI-PvuI digestion profiles of pIND.HA.BRCA1 colonies. Lanes **M:** 1 kb ladder, MBI**,** Lanes **1, 2, 3, 4, 5, 6, 7, 8, 9, 10:** HindIII-NotI-PvuI digestion profiles of pIND.HA.BRCA1 colonies 1.1, 1.2, 1.3, 1.4, 1.5, 2.1, 2.2, 2.3, 2.4, 2.5 respectively.

Colony 1.4 was used for sequencing and further studies and named pIND.HA.BRCA1.

3.2.3.2 Sequencing of pIND.HA.BRCA1

The 5’ and 3’ ends of pIND.HA.BRCA1 were sequenced by using the manual sequencing protocol as mentioned in the methods section. Figures 35 and 36 show sequencing data and Figure 37 summarizes the sequencing results.

The sequencing result showed that all pIND and BRCA1 fragments, HindIII, start codon, HA tag, stop codon and NotI sites were as expected.

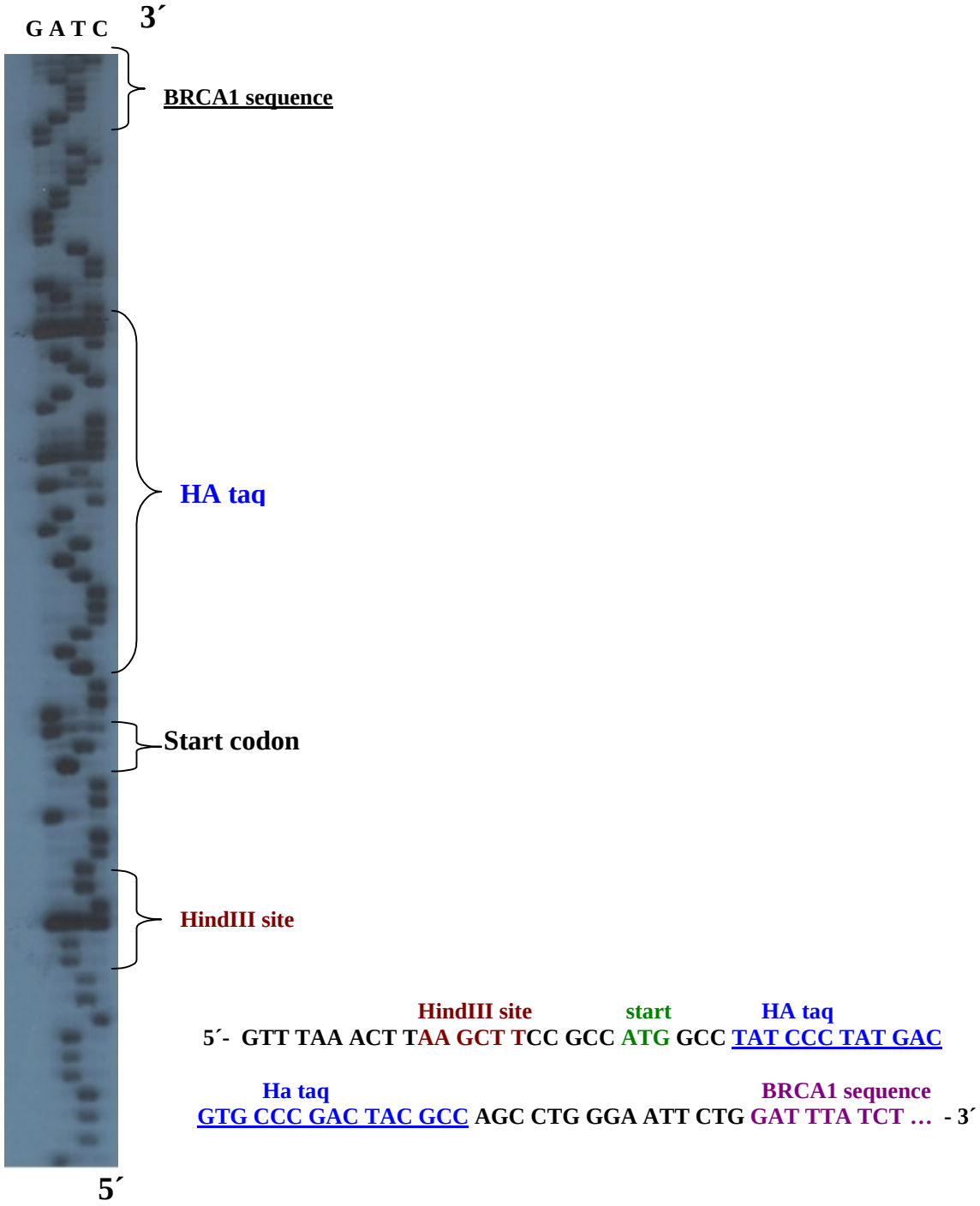


Figure 35: Sequencing data of pIND.HA.BRCA1 sequenced by EcF

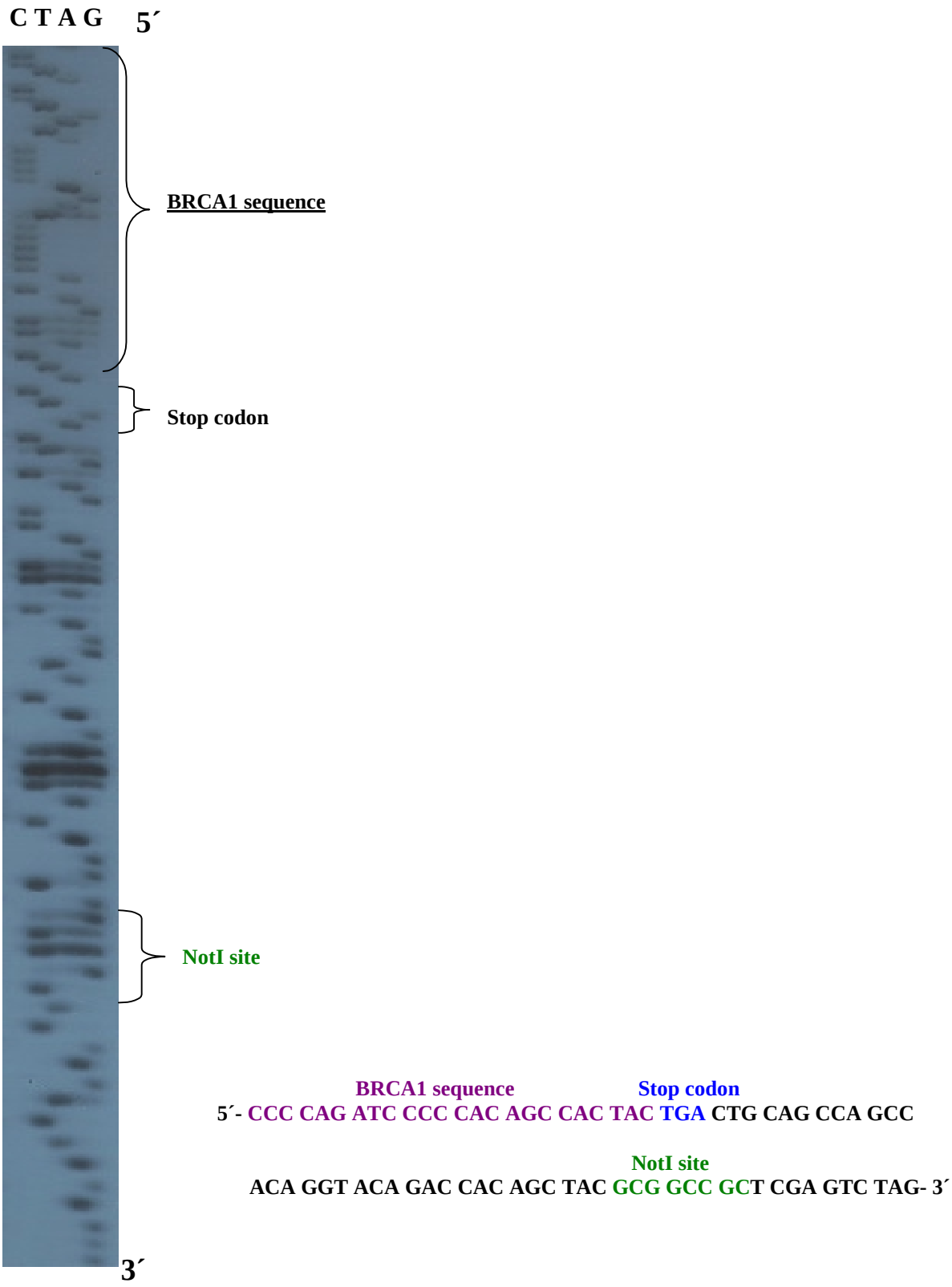


Figure 36: Sequencing data of pIND.HA.BRCA1 sequenced by EcR

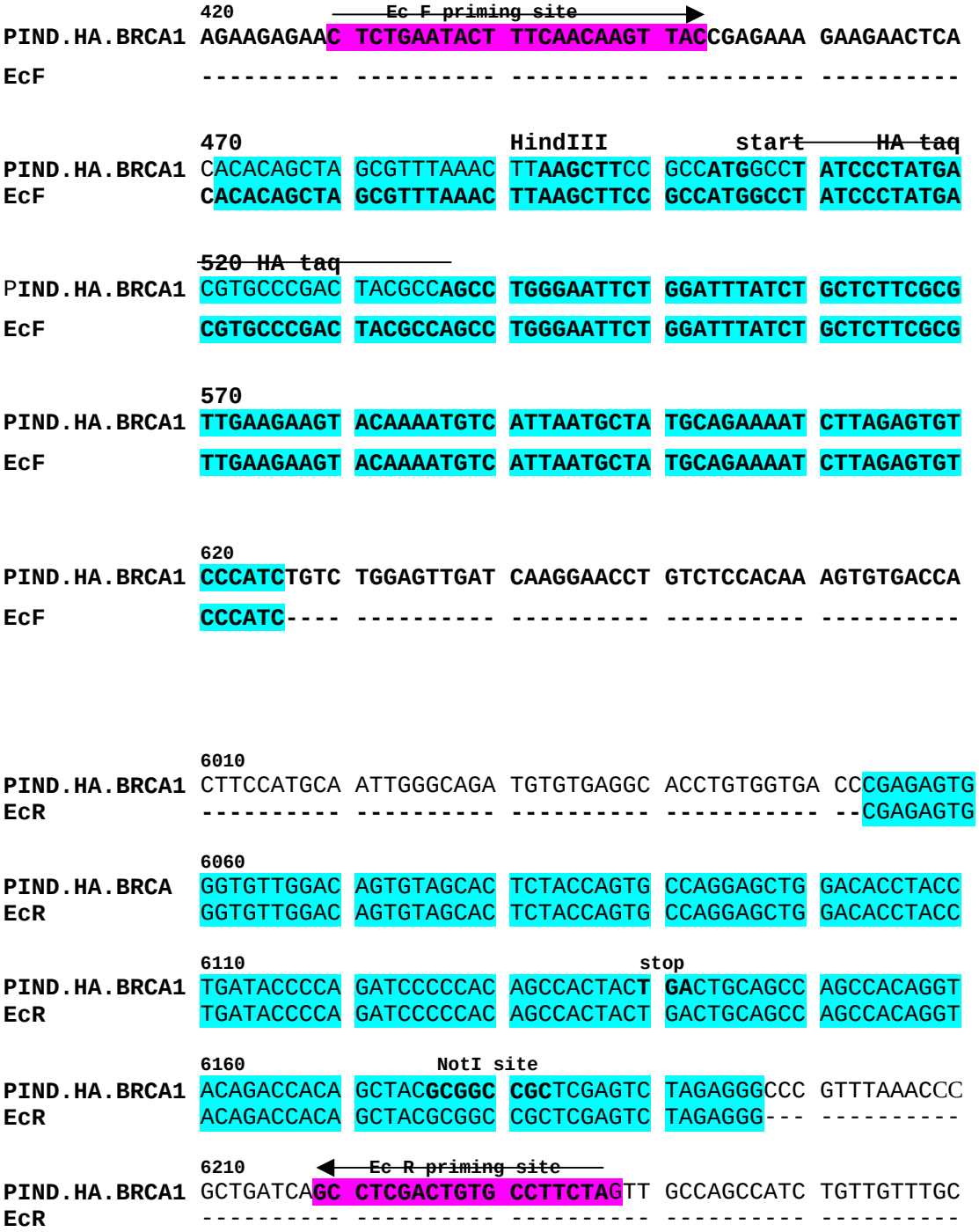


Figure 37: Sequencing results of pIND.HA.BRCA1: Upper lines indicate the expected sequence, lower line is the sequenced fragment. Blue highlight indicates the matching sequences. (***** : mismatch, -: no sequence read)

3.2.3.3 Analysing the induction of BRCA1 expression in transiently pIND.HA.BRCA1 transfected U2OS cells

The U2OS cells were transiently transfected with pIND.HA.BRCA1 and 24 hr after transfection the cells were induced with 10 µM ponasterone A. Six transient transfection experiments were carried out and induction of BRCA1 protein was analyzed by western blotting by using MS110 BRCA1 monoclonal antibody (Figures 38 and 39).

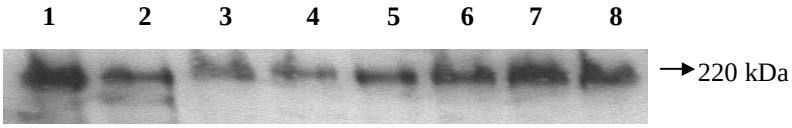


Figure 38: Western blot result of BRCA1 expressing cells that were transiently transfected with pIND.HA.BRCA1. 200 µg of the cellular lysates were loaded and analyzed with MS110 BRCA1 antibody. Lanes **1:** HBL-100; **2:** U2OS cells only; **3:** mock transfection; **4:** U2OS cells transfected with pRc/CMV.BRCA1; **5,7:** U2OS cells transfected with pIND.HA.BRCA1, ponasterone A (-); **6,8:** U2OS cells transfected with pIND.HA.BRCA1, ponasterone A (+).

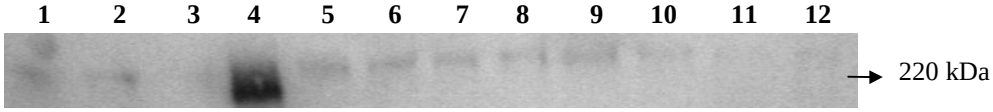


Figure 39: Western blot result of BRCA1 expressing cells that were transiently transfected with pIND.HA.BRCA1 and pCMVmycBRCA1. 50 µg of the cellular lysates were loaded and analyzed with MS110 BRCA1 monoclonal antibody. Lanes **1:** HBL-100; **2:** U2OS cells only; **3:** U2OS cells transfected with pCMVmyc; **4:** U2OS cells transfected with pCMV.myc.BRCA1; **5,7,9,11:** U2OS cells transfected with pIND.HA.BRCA1, ponasterone A (-); **6,8,10,12:** U2OS cells transfected with pIND.HA.BRCA1, ponasterone A (+).

There was no significant BRCA1 protein induction in the U2OS cells transfected with the pIND.HA.BRCA1 construct and induced with ponasterone A

(Lanes 6, 8, 10, 12) but the cells transfected with the pCMV.myc.BRCA1 construct showed a significant increase in the BRCA1 protein amount (Lane 4). One possibility was that the protein was induced but the induction level was too low to observe with the western blot technique. Therefore, immunoprecipitation was performed to increase the sensitivity of the protein level detection as described in the methods section. The U2OS cells were electroporated with pCMV.myc.BRCA1 as control or pIND.HA.BRCA1. The cells electroporated with pIND.HA.BRCA1 were either induced with ponasterone A or left uninduced. All cells were then harvested and lysed. The lysates were treated with 9E4B1 p53, C-20 BRCA1 and 9E10 myc antibodies and loaded on 5% polyacrylamide gel. The level of BRCA1 expression was detected by using MS110 BRCA1 monoclonal antibody (Figure 40).

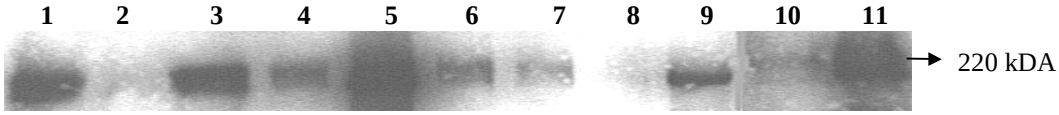


Figure 40: Immunoprecipitation results of the U2OS cells transiently transfected with pIND.HA.BRCA1 and pCMV.myc.BRCA1. The cells were immunoprecipitated with the indicated antibodies and the protein precipitates were blotted. The blot was treated with MS110 BRCA1 antibody.

1: HBL-100 cellular lysate (150 µg lysate)

2: HBL-100 cellular lysate immunoprecipitated with 9E4B1 (negative control)

3: HBL-100 cellular lysate immunoprecipitated with C-20 (positive control)

4: pCMV.myc transfected cells immunoprecipitated with C-20

5: pCMV.myc.BRCA1 transfected cells immunoprecipitated with C-20

6: pIND.HA.BRCA1 transfected cells immunoprecipitated with C-20, pA (-)

7: pIND.HA.BRCA1 transfected cells immunoprecipitated with C-20, pA (+)

8: pCMV.myc transfected cells immunoprecipitated with 9E10 (control)

9: pCMV.myc.BRCA1 transfected cells immunoprecipitated with 9E10 (control)

10: pCMV.myc transfected cells (150 µg lysate)

11: pCMV.myc.BRCA1 transfected cells (150 µg lysate)

The results from Figure 40 (lanes 6 and 7) showed that the pIND.HA.BRCA1 construct could not be induced by ponasterone A. The level of BRCA1 did not show a significant increase when compared to uninduced cells (lane 6).

This result was surprising since the construct was cloned correctly according to the sequencing results.

We then wanted to analyse the U2OS cell line integrity whether these cells really carried the regulatory plasmid RXR stably as indicated when we received the cells from another laboratory source. In order to analyse the integrity of the cells, we used a different gene, p53, cloned into pIND vector and transfected into U2OS cells.

3.2.3.4 Analysing the induction of p53 expression level in transiently transfected U2OS cells by using the pIND.p53 construct

The U2OS cells were transiently transfected with pIND.p53 and 24 hr after transfection the cells were induced with 10 µM ponasterone A. Two transient transfections experiments were carried out and induction of p53 protein was analyzed by western blot with 9E4B1 p53 antibody (Figure 41).

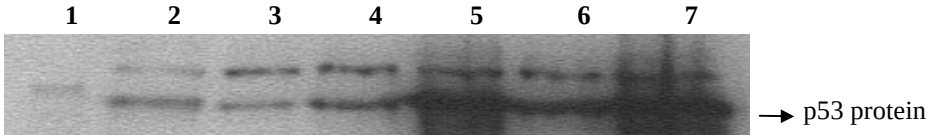


Figure 41: Western blot result of the cells transiently transfected with pIND.p53. 75 µg of cellular lysates were loaded on the gel and the blot was analyzed with 9E4B1 p53 antibody. Lanes **1:** 3 ug of pure p53 protein**; 2:** HepG2; **3:** mock transfection; **4, 6:** U2OS cells transfected with pIND.p53, ponasterone A (-); **5, 7:** U2OS cells transfected with pIND.p53, ponasterone A (+)

The pure p53 protein showed a bigger protein size because of six extra histidine tags present at its N terminus. 9E4B1 p53 antibody gives two bands at all times with western blots. The lower band corresponds to the p53 protein size. Figure 41, lanes 5 and 7 showed that p53 protein could be induced with the ecdysone inducible system in U2OS cells, which contains the RXR plasmid.

The last control experiment showed that there was nothing wrong with the U2OS cell line integrity. It is very difficult to explain why three different constructs we made with three different BRCA1 cDNA sources did not result in any induction with the ecdysone inducible system in U2OS cells.

We did not want to pursue this inducible system any further and decided to use pCMVmycBRCA1 construct to express BRCA1 protein at a high level in the MCF7 breast carcinoma cell line.

It was previously shown that pCMVmycBRCA1 could be used to express high levels of BRCA1 protein in both U2OS osteosarcoma and MCF7 breast carcinoma cell lines (Figures 30 and 31 respectively). The MCF7 cell line was used to express BRCA1 gene because this cell contains only one BRCA1 allele and displays a low level of BRCA1 expression.

3.2.4 Ectopic expression of BRCA1 in MCF7 cells by using pCMVmycBRCA1

It was previously reported that a high level of BRCA1 expression in the cells results in cell cycle arrest. It is therefore not possible to select an expression vector carrying the BRCA1 gene stably in the cells unless it is an inducible expression system. The MCF7 cells were transiently transfected with pCMVmycBRCA1 expression vector by using electroporation as a transfection method.

The MCF7 cells were transfected with the pCMVmycBRCA1 or pCMVmyc empty vector and the cells were collected 24 h after the transfection. The level of

ectopically expressed BRCA1 was determined by Northern and western blot analyses (Figure 42).

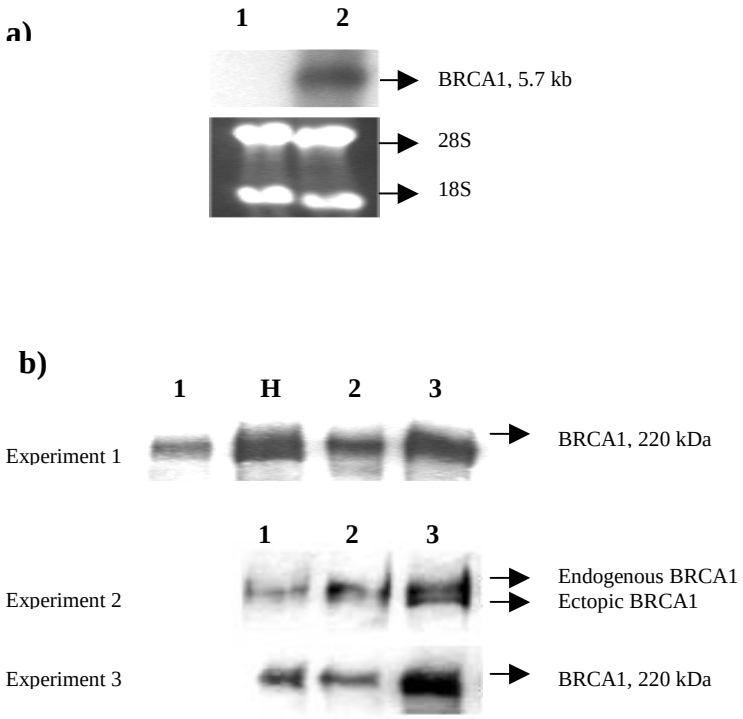


Figure 42: Ectopic expression of BRCA1 in MCF7 breast carcinoma cells.
(a) Northern blot data showing that BRCA1 mRNA which is undetectable in cells transfected with control plasmid (lane 1) is detected in cells transfected with pCMVmycBRCA1 plasmid (lane 2). Ethidium bromide staining shows equal RNA loading in each lane. **(b)** Western blot data showing that pCMVmycBRCA1 plasmid transfected cells express higher BRCA1 protein (lane 3) when compared to cells transfected with control plasmid (lane 2), and untransfected MCF7 cells (lane 1). HBL-100 cells, which express endogenous BRCA1 protein, were used as a positive control (H). Total protein extracts were prepared and analyzed for BRCA1 by western blot using Ab-1 antibody specific to BRCA1. Blots shown are representative of western blot analysis from six separate experiments.

Steady-state levels of BRCA1 mRNA and protein were substantially elevated 24 h after electroporation of MCF7 cells with pCMVmycBRCA1. When compared

with the cells transfected with control plasmid, pCMVmycBRCA1 transfected cells showed about 20-fold increase in the full-length BRCA1 mRNA (Figure 42a) and about 6-fold increase in the BRCA1 protein (Figure 42b). The results of three representative experiments are shown in Figure 42b. Both control MCF7 cells (lane 1) and the cells transfected with control plasmid without BRCA1 insert (lane 2) demonstrated endogenous BRCA1 protein. As shown in experiment 2 ectopically expressed BRCA1 protein level was similar to endogenous BRCA1 level, suggesting that transient transfection resulted in a significant increase in total BRCA1 levels in pCMVmycBRCA1-transfected MCF7 cells (lane 3). Taken together, these results indicated that it was possible to increase BRCA1 levels in MCF7 by transient transfection, without compromising cellular integrity.

Determining the transfection efficiency

The transfection efficiency of the MCF7 cells was determined by transfecting the MCF7 cells with the pEGFP plasmid under the same experimental conditions. pEGFP plasmid is an eukaryotic expression plasmid which carries a green fluorescent protein gene. This gene is expressed upon transfection of this plasmid to the mammalian cells and the protein product gives green fluorescence that can be observed under 525 nm wavelength fluorescence light.

Figure 43 shows a typical example of the transfected MCF7 cells with pEGFP plasmid. pEGFP plasmid was used in every transfection experiment to control the transfection efficiency in parallel to the other transfection experiments.

24 h after the pEGFP transfection the cells were trypsinized and counted under the fluorescent microscope. The transfection efficiency was approximately 40%.

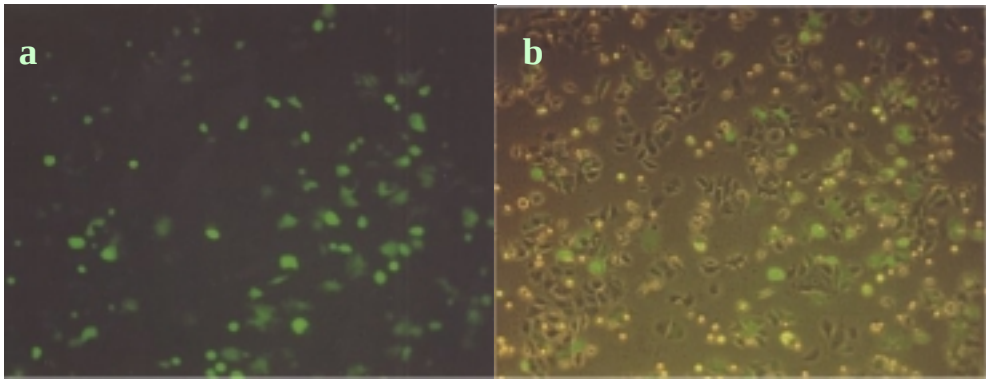


Figure 43: pEGFP transfected MCF7 breast carcinoma cells. The photograph was taken 24 h after transfection. Panels (a) and (b) show the cells in the same area under the microscope. a) The cells expressing GFP protein could be observed under 525 nm wavelength fluorescence light. b) pEGFP transfected and untransfected cells could be observed together under fluorescent and normal light.

AnnexinV staining

It was suggested that the BRCA1 expression also causes apoptosis. In order to understand whether the MCF7 cells expressing the BRCA1 gene could undergo apotosis, annexin V staining was performed. The pCMVmycBRCA1 and pCMVmyc vector transfected MCF7 cells were stained with annexin V 24 h after the transfection (Figure 44). Annexin V is an early marker for apoptosis. The MCF7 cells treated with ultraviolet light were used as positive control since UV induction causes cells to undergo apoptosis. BRCA1 overexpression in MCF7 cells did not result in apoptosis, compared to mock and vector only transfected samples. Thus at a transfection efficiency of 40%, no more than 5% of BRCA1 expressing cells were apoptotic.

Another annexinV staining assay was also performed in order to understand whether the apoptotic cells present in the tissue culture media of the transfected cells were due to apoptosis or electroporation procedure. Briefly, 24 h after transfection, annexin V was added to the growth medium without washing the cells, and cells were kept in the incubator for 30 minutes. The supernatants were collected, counted and the pellets were fixed on cover slides by cytospin. Hoechst staining was also performed

for both methods as described in the methods section in order to observe all the cells present on the slide (Figure 45).

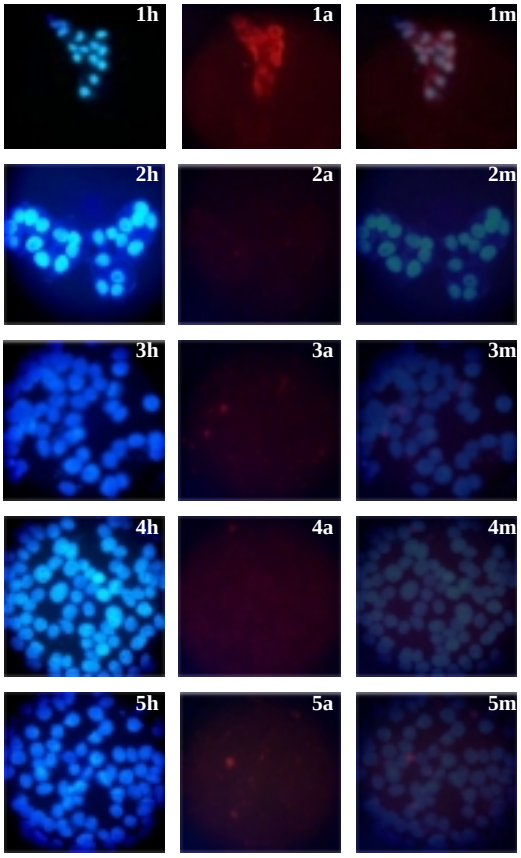


Figure 44: Annexin V staining with method I. Lanes **1**: 50 J/m^2 UV treated MCF7 cells, positive control; **2:** MCF7 cells, negative control, **3**: Mock-transfected MCF7 cells, **4**: pCMVmyc-transfected MCF7 cells; **5**: pCMVmycBRCA1-transfected MCF7 cells; **h**: Hoechst staining, **a**: Annexin V staining, **m**: Annexin V and Hoechst staining images were superimposed to create the merged image.

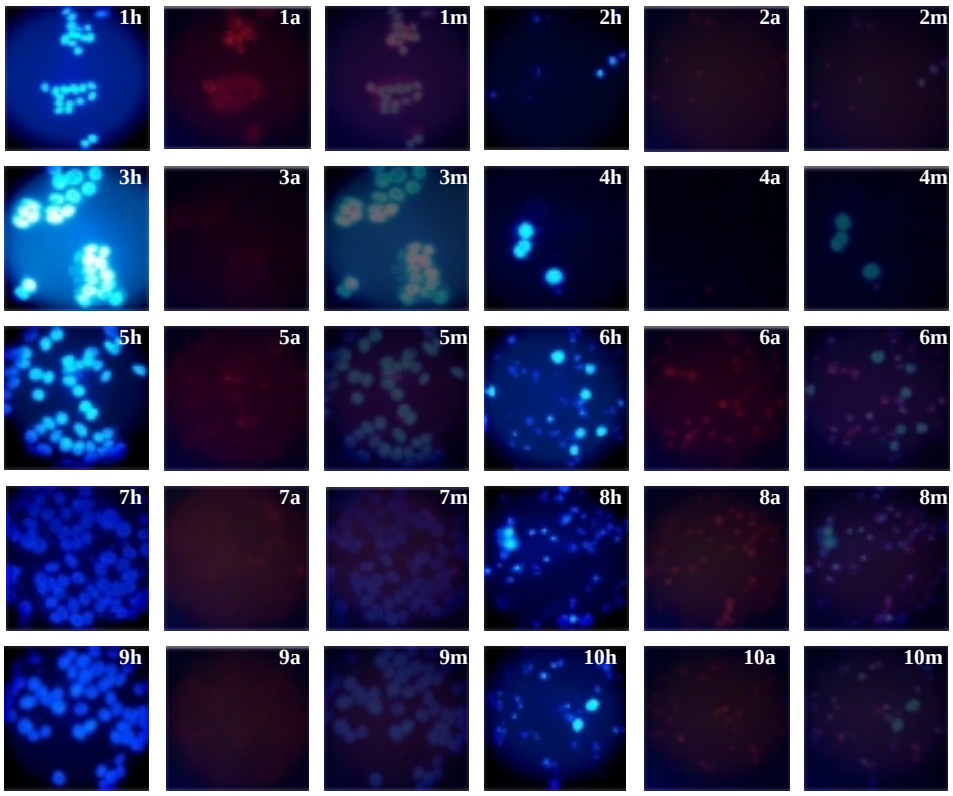


Figure 45: Annexin V staining with method II. Lanes **1**: 50 J/m^2 UV treated MCF7 cells, positive control; **2**: Supernatant of sample 1; **3**: MCF7 cells, negative control; **4**: Supernatant of sample 3; **5**: Mock-transfected MCF7 cells; **6**: Supernatant of sample 5; **7:** pCMVmyc transfected MCF7 cells; **8**: Supernatant of sample 7; **9**: pCMVmycBRCA1 transfected MCF7 cells; **10**: Supernatant of sample 9. **h**: Hoechst staining, **a**: Annexin V staining, **m**: AnnexinV and Hoechst staining images were superimposed to create the merged image.

Figure 45 shows the results of annexinV staining with method II. Samples 1, 3, 5, 7 and 9 are the cells that were grown on coverslides. Samples 2, 4, 6, 8 and 10 are the cells collected from the supernatants. Not more than 10 % of the cells were dead in the mock, empty vector and BRCA1 carrying vector transfected samples as calculated by counting the supernatants prior to collection. Furthermore there was no difference observed in the number of the dead cells between mock (6a) or empty vector (8a) and BRCA1 carrying vector (10a) transfected samples, suggesting that the dead cells were the result of electroporation. BRCA1 overexpression did not result in apoptosis (9a) in our system, which was also shown by the previous experiment (Figure 44).

3.3 Subtractive hybridization

Suppression subtractive hybridization (SSH) technology was used to generate a library of partial-length cDNAs representing differentially expressed mRNAs in BRCA1-overexpressing MCF7 cells. SSH combines subtractive hybridization with PCR to generate a population of PCR fragments enriched for sequences from genes expressed differentially. SSH-mediated cDNA enrichment allows the equalization of wide differences in the abundance of different transcript species. Consequently, differentially expressed transcripts of low abundance can be cloned (Diatchenko *et al*., 1996).

SSH was used to identify genes that are differentially expressed in BRCA1 transfected MCF7 cells. SSH was performed with the double strand as tester (MCF7/ pCMVmycBRCA1) and driver (MCF7/pCMVmyc) cDNAs (forward subtraction) by using PCR-Select cDNA Subtraction kit as described by the manufacturer with some modifications (Clontech).

Reverse subtraction was also performed where tester cDNA was derived from pCMVmyc transfected MCF7 cells and driver cDNA derived from pCMVmycBRCA1 transfected cells.

3.3.1 Preparation of DNA-free mRNA for subtractive hybridization

SSH requires high quality pure mRNA. mRNA was isolated from total RNA and treated with DNaseI in order to remove the DNA contamination as described in the methods section. Integrity of mRNA was checked by running on a formaldehyde gel. RT-PCR was also performed with these mRNA samples to control whether DNA contamination was still present. After the first strand cDNA synthesis, RT-PCR was performed with sequence and vector specific primers in order to control the presence or absence of plasmid and genomic DNA contamination. Figure 46 shows the results of these amplifications. BRCA1 primers (F4-R4) which are specific to BRCA1 gene, amplified 1217 bp, actin primers amplified 550 bp and GADD45 primers amplified 497

bp PCR products from cDNA preparations as expected in tubes containing the reverse transcriptase enzyme and also in the positive control tube which contained only pCMVmycBRCA plasmid as template. pCMVmyc F and TC31 primer pair amplifies 1200 bp fragment from the pCMVmycBRCA1 plasmid; amplification of this PCR product in the tube containing the plasmid, but not in the other tubes showed that plasmid DNA contamination was eliminated successfully.

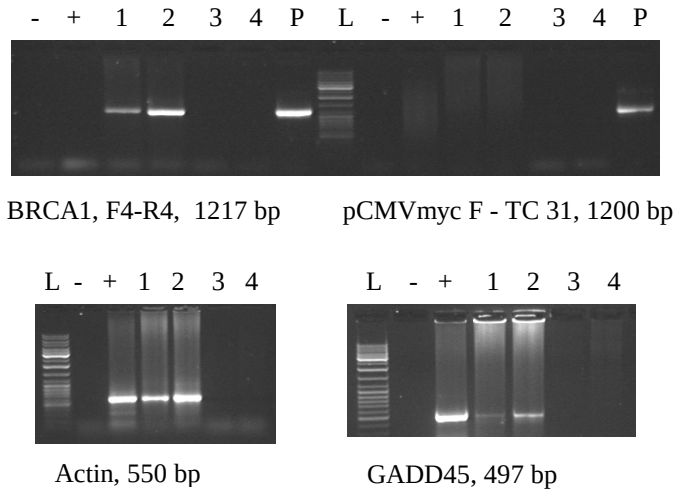


Figure 46: RT-PCR results with sequence specific primers from DnaseI treated mRNA samples. L: MBI DNA ladder mix, **+**: total RNA as positive control, **P**: pCMVmycBRCA1 plasmid, Lanes

1: MCF7 cells transfected with pCMVmyc (RT+),

2: MCF7 cells transfected with pCMVmycBRCA1 (RT+),

3: MCF7 cells transfected with pCMVmyc (RT-),

4: MCF7 cells transfected with pCMVmycBRCA1 (RT-).

3.3.2 ds cDNA synthesis

Tester and driver ds cDNAs were prepared from the two mRNA populations. 3.5 μg of mRNA was used to synthesize double strand cDNA as described in the methods section. Control mRNA provided in the kit was also used during SSH experiments for control. Forward, reverse and control subtractions performed with the double strand cDNAs were labelled as follows:

cDNA1: pCMVmycBRCA1 transfected MCF7 mRNA
cDNA2: pCMVmyc transfected MCF7 mRNA
cDNA3: Control mRNA from human skeletal muscle

Forward subtraction:
Tester*:* pCMVmycBRCA1 (cDNA1), ***Driver*****:** pCMVmyc (cDNA2)

Reverse subtraction:
Tester*:* pCMVmyc (cDNA2), ***Driver***: pCMVmycBRCA1 (cDNA1)

Control subtraction:
Tester*:* HaeIII digested ϕX174 cDNA + control cDNA from human skeletal muscle, **Driver***:* control cDNA from human skeletal muscle

3.3.3 RsaI digestion

Tester and driver ds cDNAs were separately digested to obtain shorter, blunt-ended molecules. Figure 47 shows the profiles of three cDNAs before and after RsaI digestions. The ds cDNA preparations appear as a smear from 0.5-10 kb. After RsaI digestion, the average cDNA size is smaller.

3.3.4 Adaptor ligation

Tester cDNAs of each forward, reverse and control subtractions were aliquoted into two and each aliquot ligated to adaptors 1 and 2R. These two adaptors provide different PCR primer annealing sites. This way, two tester populations from the same cDNA were created with different adaptors but driver cDNA had no adaptors.

Resulting tester cDNAs were named 1-1, 1-2, 2-1, 2-2, 3-1, 3-2. Ligation efficiency was analyzed by using internal control primers (G3PDH) and PCR primer 1 which is complementary to both adaptor sequences (Figure 48). Internal control G3PDH primer pair amplification resulted in amplification of 0.4 kb, while G3PDH 3´primer-PCR primer 1 pair amplified 0,75 kb fragment. This result showed that the adaptors were successfully ligated with cDNAs.

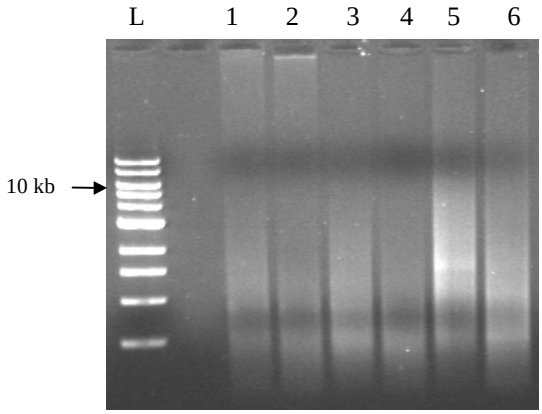


Figure 47: RsaI digestion of ds cDNAs

L :1 kb ladder (Gibco-BRL), Lanes **1,3,5**: ds cDNAs of cDNA1, cDNA2 & cDNA3, **2,4,6**: RsaI digested cDNA1, cDNA2 & cDNA3

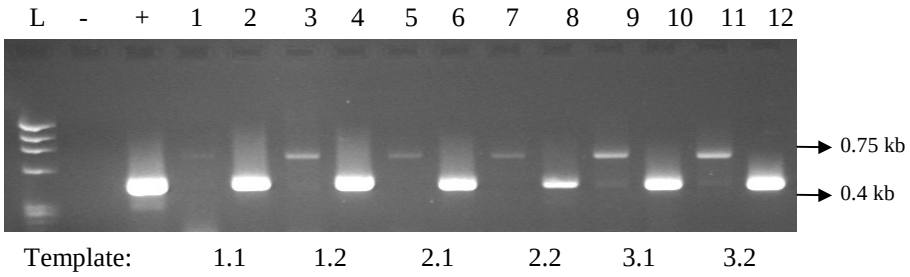


Figure 48: Analysis of ligation efficiency

L: HaeIII digested ϕX174 (Sigma), (1.3, 1.1, 0.9, 0.6, 0.3 kb); +: total RNA;

Lanes **1,3,5,7,9,11:** PCR with G3PDH 3´primer-PCR primer 1 (0,75 kb);

2,4,6,8,10,12: PCR with G3PDH 3´-5´ primers (0,4 kb).

3.3.5 First and second hybridizations for subtraction

During first hybridization, hybridization kinetics leads to equalization and enrichment of differentially expressed sequences. In the first hybridization, each tester (1-1, 1-2...) cDNA was hybridized with excess driver cDNA. Single strand cDNAs were enriched for differentially expressed sequences, as non-target cDNAs present in the tester and driver cDNA form hybrids. In the second hybridization, two samples from first hybridization were mixed together and excess driver cDNA was added. New hybrid molecules were formed which consists of differentially expressed cDNAs with different adaptors on each end that were ready for PCR.

3.3.6 Primary and secondary PCRs

Primary PCR led to exponential amplification of only differentially expressed sequences by using suppression PCR technology. Secondary PCR reduced the background and differentially expressed sequences were further enriched. Figure 49 shows results of the optimization of primary and secondary PCRs. Lanes 1-5 are primary PCR products, amplified by PCR primer 1. Lanes 6-10 are secondary PCR amplifications of these products in the same order. Amplifications were carried out with nested primers 1 and 2R.

After optimization of the PCR conditions, secondary PCR was performed with optimal PCR conditions. Primary and secondary PCRs were also performed with the reverse subtraction cDNAs (Figure 50). These secondary PCR products were used for subtraction efficiency test and generation of subtracted library construction.

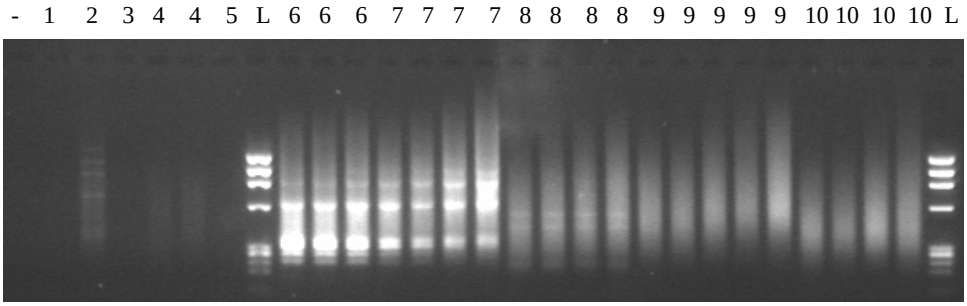


Figure 49: Optimization of primary and secondary PCR conditions

L : HaeIII digested ϕX174 (Sigma), (1.3, 1.1, 0.9, 0.6, 0.3 kb), Lanes **1,6**: PCR control subtracted cDNA, **2,7:** Subtracted control skeletal muscle cDNA, **3,8**: Unsubtracted control skeletal muscle cDNA, **4,9:** Forward subtracted experimental cDNA, **5,10:** Unsubtracted tester control

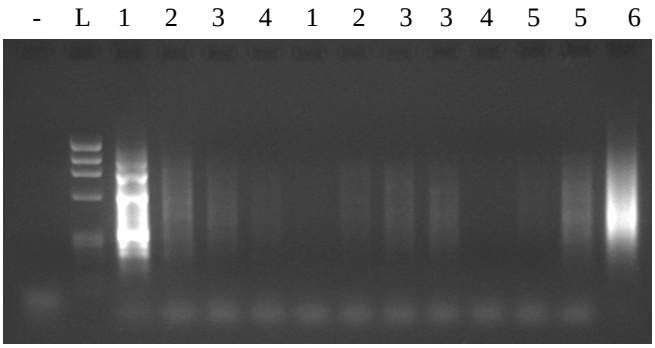


Figure 50: Secondary PCR results

L : HaeIII digested ϕX174 (Sigma), (1.3, 1.1, 0.9, 0.6, 0.3 kb), Lanes **1:** Subtracted control skeletal muscle cDNA, **2:** Unsubtracted control skeletal muscle cDNA, **3:** Forward subtracted experimental cDNA, **4:** Unsubtracted tester control, **5:** Reverse subtracted experimental cDNA, **6:** Unsubtracted tester control.

3.3.7 Subtraction efficiency test

In order to compare the abundance of known cDNAs before and after subtraction and to analyze the efficiency of subtraction, a quick PCR based assay was performed. PCR was performed for a non-differentially expressed gene (G3PDH) between the two RNA sources under comparison. Figure 51 shows the results of the subtraction efficiency test for the forward and reverse subtracted libraries. In the subtracted samples the G3PDH PCR product was observed 5-15 cycles later than the unsubtracted samples.

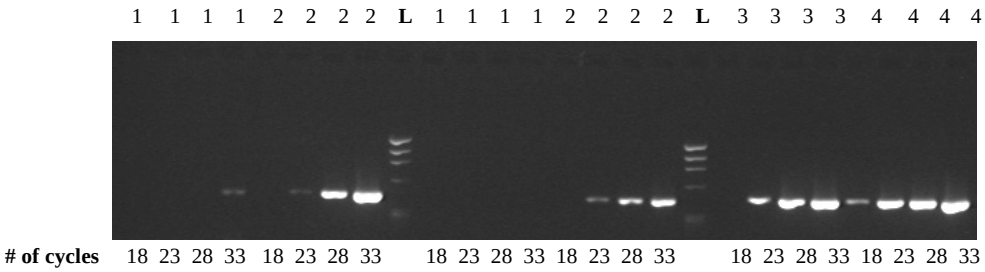


Figure 51: Analysis of subtraction efficiency test

L : HaeIII digested ϕX174 (Sigma), (1.3, 1.1, 0.9, 0.6, 0.3 kb), Lanes

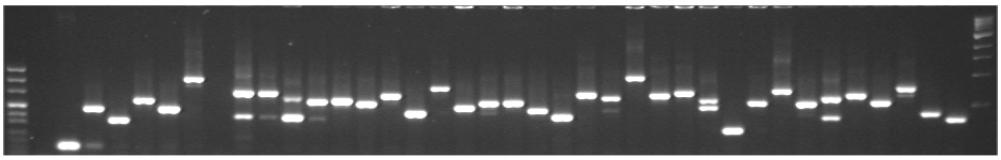
1: Forward subtracted experimental cDNA, **2:** Unsubtracted tester control for FS,

3: Reverse subtracted experimental cDNA, **4:** Unsubtracted tester control for RS

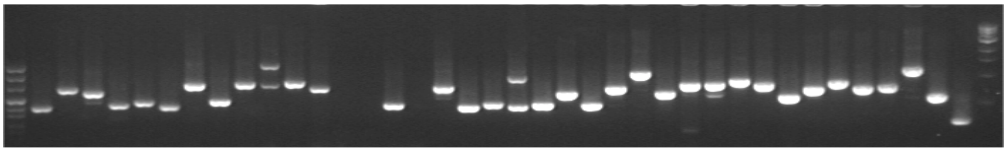
3.3.8 Cloning and pre-screening of forward subtracted library

Secondary PCR products of the forward subtracted library were cloned into the PGEM-T Easy vector system, which allows blue/white selection. More than 500 white colonies were picked up and used directly as template for PCR amplification of inserts with T7-SP6 primers. Figure 52 shows the amplified cDNA inserts of the white colonies picked up from the forward subtracted plate. The PCR products, which gave a single band, were used for further experiments.

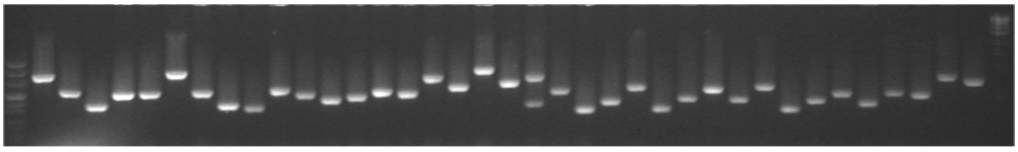
L1 - + 24.....59 L1



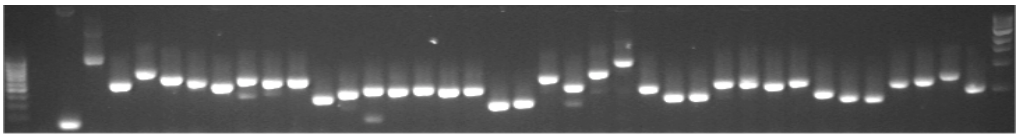
L1 60.....97 L1



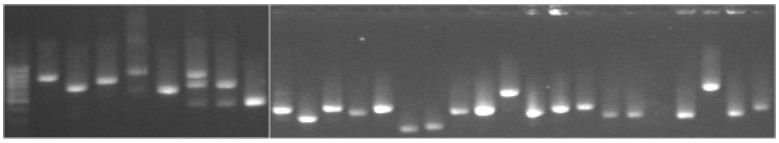
L1 98.....134 L2



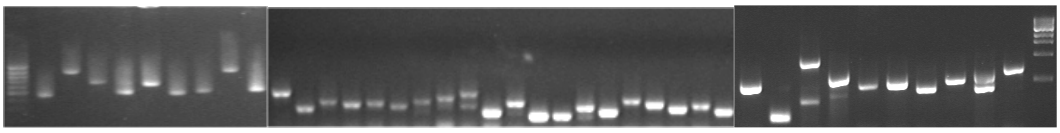
L1 - + 135.....172 L2



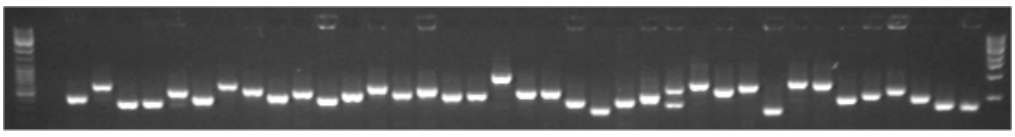
L1 173.....200



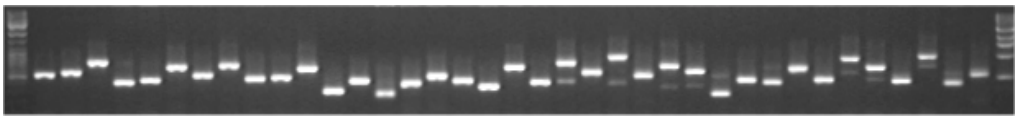
L1 201.....238 L2



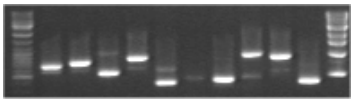
L1 - 239.....275 L2



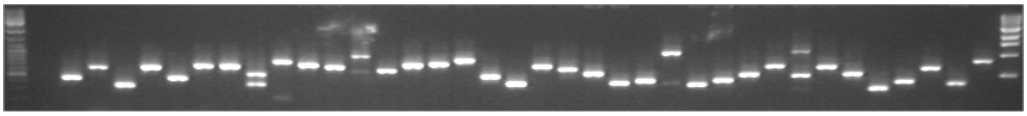
L1 276.....312 L2



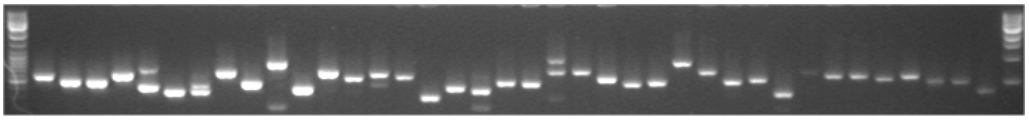
L1 313.....322 L2



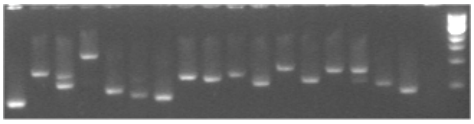
L1 - 323.....358 L2



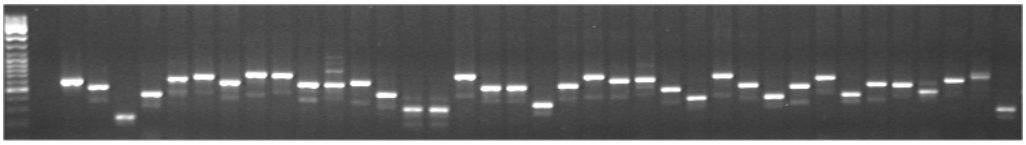
L2 359.....396 L2



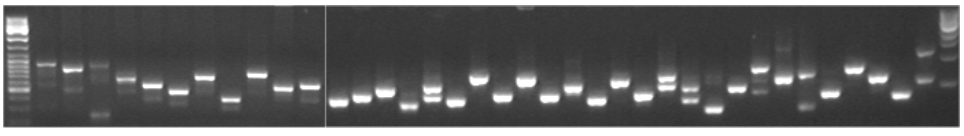
397.....414 L2



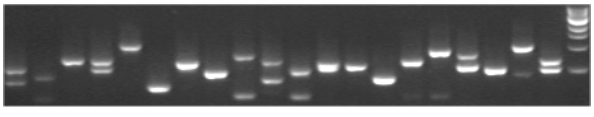
L1 - 415.....451



L1 452.....488 L2



489.....508 L2



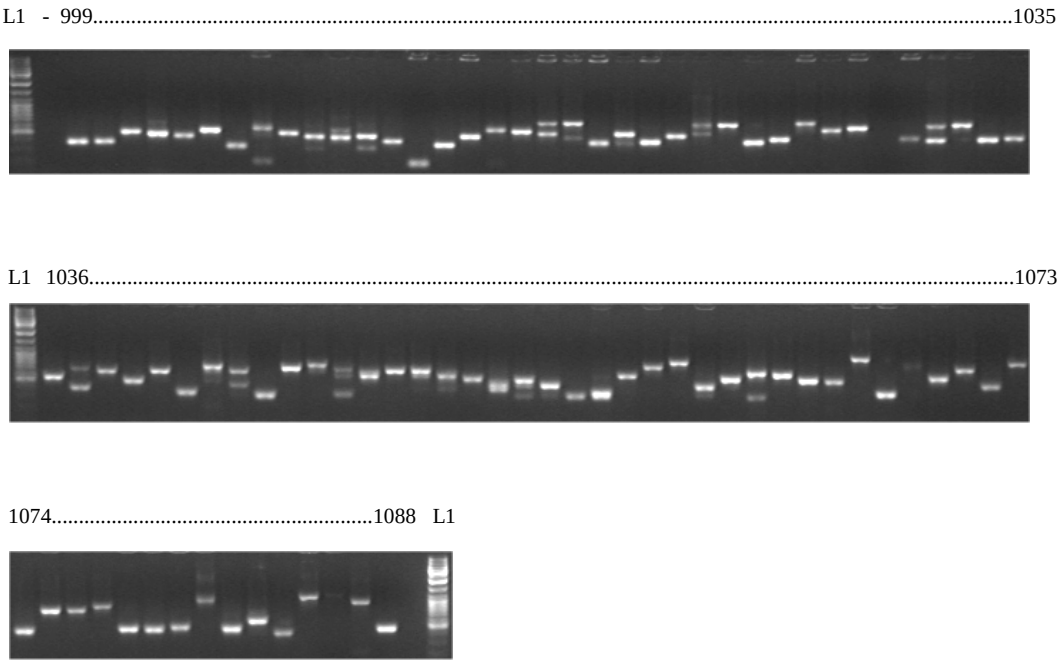


Figure 52: T7-Sp6 amplified cDNA inserts from white colonies of the forward subtracted library. Lanes **L1**: DNA Ladder mix, MBI; **L2**: 1 kb ladder, New England Biolabs; **-,+:** negative and positive controls for PCR; **Other lanes**: Colony numbers from 24 to 1088.

3.4 Differential screening of clones

Following SSH, the PCR-Select Differential Screening protocol was performed in order to identify differentially expressed sequences, minimise the false positives and reduce the background.

3.4.1 Arraying forward subtracted clones

Five hundred colonies were chosen and their PCR amplified cDNA inserts (Figure 52) were transferred into 96-well plates and denatured in equal amounts of

NaOH and 1.5 µl of each sample was dot-blotted onto 2 nylon membranes in an identical order as described in section 2.7.1. There were total of 6 pairs of hybridization membranes. G3PDH, Actin, BRCA1, GADD45, negative hybridization controls cDNA1 and cDNA2 were used as control cDNAs on arrays. PCR products of some of the control cDNAs are shown in Figure 53.

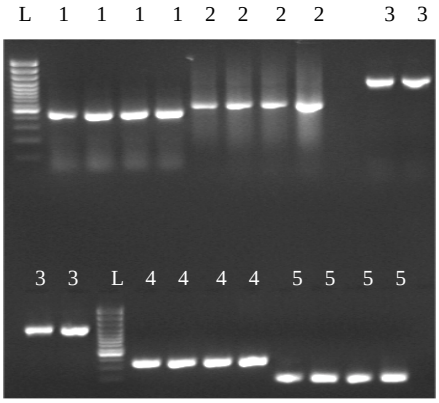


Figure 53: PCR products of control cDNAs
L: MBI, DNA ladder 100 bp plus, Lanes **1:** G3PDH**, 2:** Actin**, 3:**BRCA1, **4:** cDNA1**, 5:** cDNA2

3.4.2 Dot blot hybridization with subtracted cDNA probes

Preparation of probe sources

Each identical cDNA array pair was hybridized with [α-^{32}P]-dCTP labelled probes. One pair was hybridized with forward subtracted cDNA pool, and the other pair was hybridized with reverse subtracted cDNA pool. Figure 54 shows the secondary PCR products of forward and reverse subtracted library.

These secondary amplification products were then digested with *Rsa*I in order to get rid of the adaptor sequences on each end of cDNAs. This procedure was necessary for inhibiting the background, which might arise during hybridization of the adaptor sequences on each end of the PCR products on arrays and radiolabelled probes. Figure 55 shows the RsaI digested forward and reverse-subtracted probes with the digestion control plasmid pIND. Samples from lanes 2 and 5 were used as forward

and reverse probe sources respectively and [α-^{32}P]-dCTP labelled by using random primer labelling method.

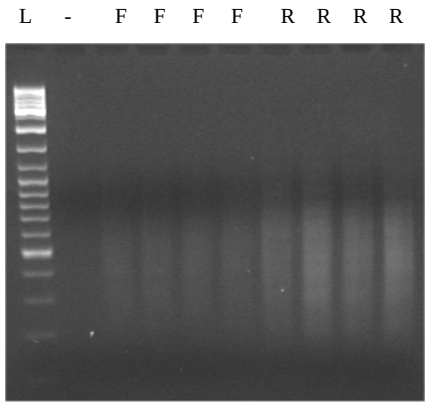


Figure 54: PCR products of forward and reverse subtracted library
L: DNA ladder mix, MBI, **F:** Forward subtracted library secondary PCR product, **R:** Reverse subtracted library secondary PCR product, (-) negative control for PCR.

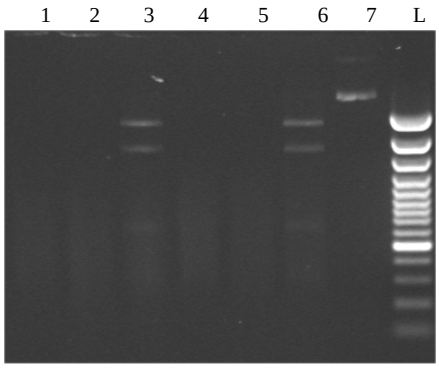


Figure 55: RsaI digestion of forward and reverse subtracted library PCR products **L:** MBI, DNA ladder 100 bp plus, Lanes **1:** Undigested forward subtracted library secondary PCR product, **2:** RsaI digested forward subtracted library secondary PCR product, **3:** RsaI digested forward subtracted library secondary PCR product and pIND, **4:** Undigested reverse subtracted library secondary PCR product, **5:** RsaI digested reverse subtracted library secondary PCR product, **6:** RsaI digested reverse subtracted library secondary PCR product and pIND, **7:** Undigested pIND plasmid.

Two identical cDNA blots were hybridized with either forward or reverse subtracted library probes with the same number of cpm for each pair (at least $2x10^7$ cpm/membrane). Dot blot hybridization was performed as described in the methods section. Figure 56 shows one pair of the six duplicate dot blots hybridized with forward and reverse subtracted cDNA probes.

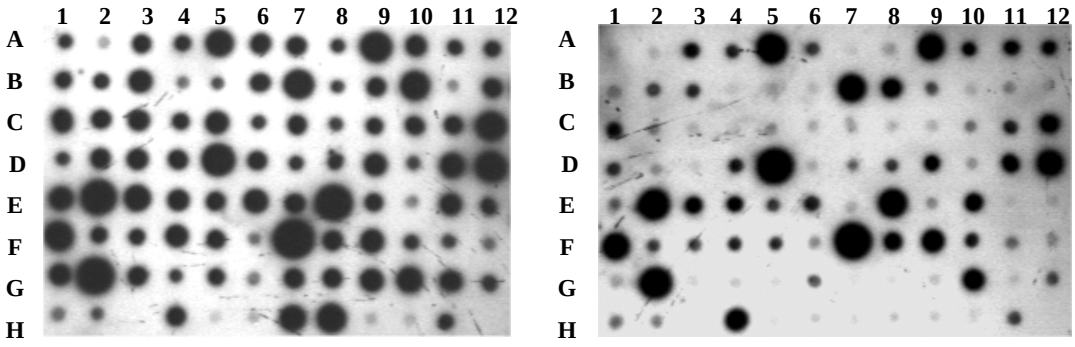


Figure 56: Differential screening of SSH-selected cDNA clones with forward (left panel) and reverse (right panel) subtracted probes. Array number 1: Rows **A-G**; test cDNA samples, Row **H**; control cDNA samples (**H1-H2**: GADD45, **H3**: negative PCR control, **H4**: GAPDH, **H5-H6**: cDNA1, **H7-H8**: BRCA1, **H9-H10**: cDNA2, **H11**: actin, **H12**: NaOH+water).

3.4.3 Analysis of differential screening results

After hybridizing 500 clones on 6 pairs of arrays, all the blots were exposed to X-ray films. The signal intensity of each dot was measured by phosphorimaging. Appendix C shows the phosphoimager values of each dot present in all 6 arrays. Figure 56 shows the array number 1. The dots corresponding to B10, E1, E11, G1, G9, G11 are examples of some of the cDNAs, which showed significant increase in signal intensity with forward (left panel) probe hybridization when compared to reverse (right panel) hybridization. On the other hand, control cDNAs, especially the negative hybridization controls (H5-6-9-10) did not give any signals as expected. Housekeeping genes like actin (H11) did not show any significant increase in expression as expected. Meanwhile, a very significant BRCA1 expression was

observed in samples H7-8 due to the enrichment of elevated expression of BRCA1 cDNA during SSH.

After first screening, 210 of the clones, which showed a significant increase were selected for further screening tests. They were again dotted on 3 sets of nylon membranes in pairs and this screening procedure was repeated two further times. These arrays have been named as arrays 7, 8 and 9. Figure 57 shows the dot blot hybridization of these 3 sets of arrays containing 210 cDNA inserts. Appendix D summarizes average of three values for the 210 selected clones after actin normalization.

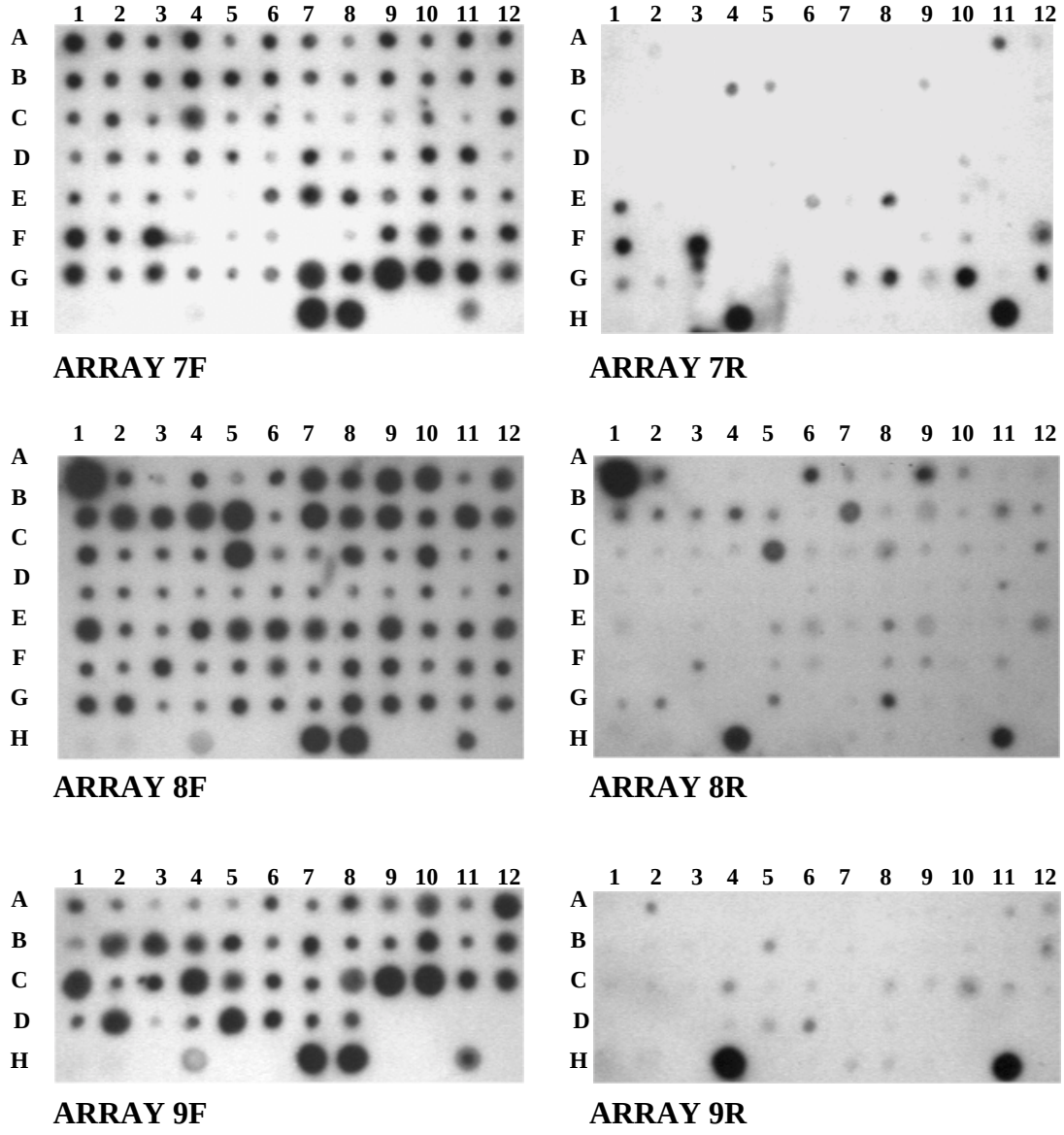


Figure 57: Differential screening of 210 selected clones after first screening

3.4.3.1 Sequencing, homology search and classification of BRCA1 upregulated genes

After actin normalization and analysing the average of three values for each dot (summarized in appendix D), clones which showed a robust increase in expression were subjected to DNA sequencing. Figure 58 shows the purified PCR products, which were selected for sequencing.

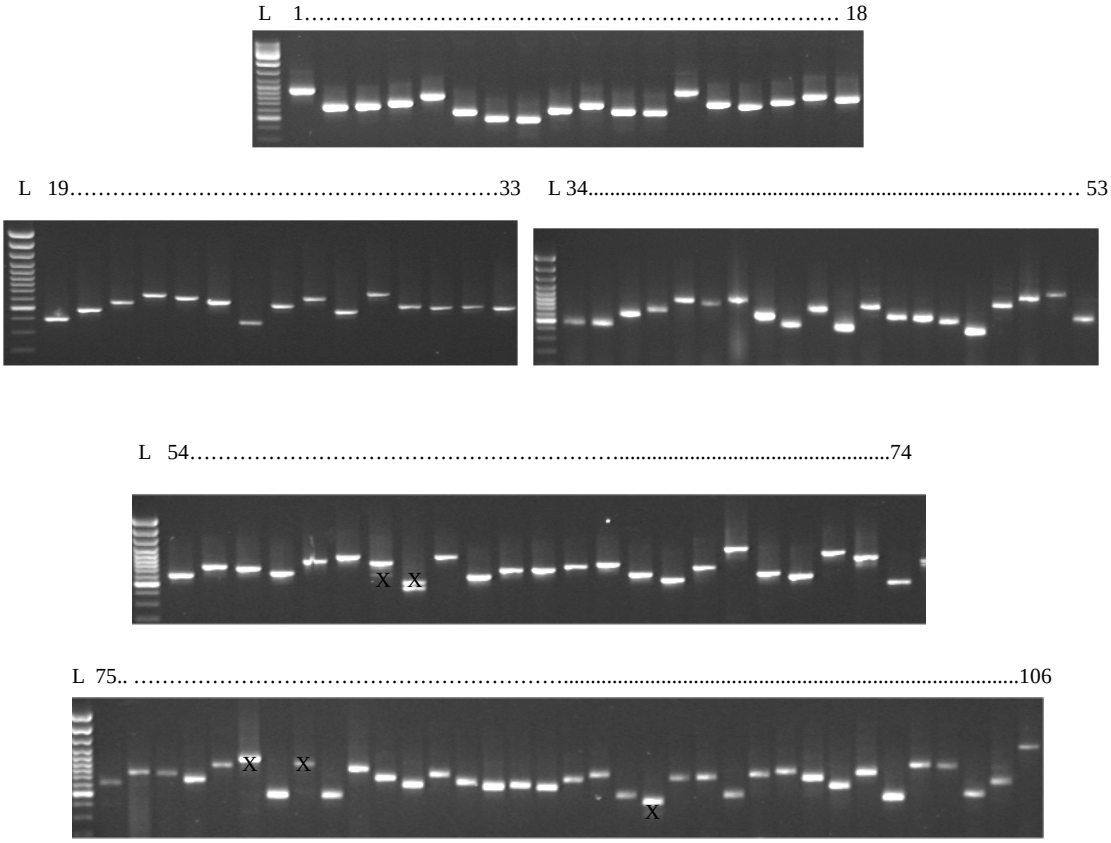


Figure 58: Purified PCR products selected for sequencing

L: MBI 100 bp DNA ladder plus, Lanes **1-18:** Clones 321, 324, 326, 352, 358, 359, 360, 361, 362, 370, 371, 381, 384, 390, 404, 406, 408, 410; **19-33:** Clones 59, 67, 131, 162, 234, 277, 293, 282, 326, 339, 406, 415, 424, 432, 446; **34-53**: Clones 67, 82, 107, 114, 133, 153, 190, 192, 259, 305, 311, 332, 415, 424, 438, 474, 1045, 1060, 1067, 1070; **54-74:** Clones 33, 48, 93, 163, 190, 218, 1060, 41, 55, 66, 89, 113, 114, 118, 153, 158, 174, 177, 268, 452, 1026; **75-106:** Clones 33, 48, 93, 121, 133, 259, 295, 314, 420, 421, 423, 426, 431, 434, 447, 458, 460, 470, 475, 1029, 1034, 1040, 1049, 1064, 1065, 1071, 1072, 1073, 1075, 1078, 1083, 1085.

Appendix E shows the sequencing results of selected clones.

Homology search was done with known gene sequences available at Genbank/EMBL and Expressed Sequence Tag (EST) databases, mainly by BLAST search. (http://www.ncbi.nlm.nih.gov/blast)

Appendix F shows a list of BRCA1 upregulated genes in this study as defined by differential screening and identified by homology search.

The analyzed clones in this study may be summarized as follows:

	Number
Analyzed putative positive clones after SSH	500
Confirmed by differential screening [a]	210
Number of clones sequenced [b]	83
>90% homology	60
known function	44
unknown function	16

[a] The cDNA clones which showed more than five-fold induction were selected for sequencing.
[b] Eight clones yielded poor sequence data. Twelve genes were represented more than once in the library.

Table 13 summarizes the classification of BRCA1 upregulated genes according to their functions. BRCA1-induced genes in breast cancer cells form several distinct functional classes. Of 60 BRCA1-upregulated genes shown in this table, 44 were associated to a known function or putative function. At least 6 of these genes were involved in transcriptional regulation, 4 in intermediate metabolism, 3 in ubiquitin-mediated proteolysis, 2 in chromatin organization, 2 in DNA repair, 2 in receptor mediated signalling and 2 in cytoskeletal organization. Others were involved in intermediate metabolism, RNA metabolism and surveillance, amino acid or ion transport, cell-cell and cell-ECM interaction, endosome or vesicle trafficking and N-glycan biosynthesis. The Cdc7 protein kinase, which is involved in the initiation of DNA synthesis, was also induced by BRCA1.

Table 13: Classification of BRCA1 upregulated genes

Fold induction values are according to differential screening results. Redundancy indicates the number of representation of a gene in the library.

* indicates the previously reported frequent LOH sites in breast cancers (Hirano *et al.*,2001).

Putative Function	Gene Name	Fold Induction	Redundancy	Accession#	Locus
Amino Acid Transport	Solute carrier family 38, member 2 (SLC38A2) (ATA2)	9	1	XM_028311	12q
Cell-Cell Interaction	CD24 antigen (small cell lung carcinoma cluster 4 antigen) (CD24)	29	1	XM_087865	6q21
Cell-ECM Interaction	Integrin, beta 1 (fibronectin receptor, beta polypeptide) (ITGB1),transcript variant 1E	6	1	NM_133376	10p11.2
Chaperon	Dystonia 1, torsion (autosomal dominant; torsin A)(DYT1)	11	1	NM_000113	9q34
Chromatin assembly/DNA repair and response	Anti-silencing function 1A (ASF1A)	13	2	AF279306	6q22.31
Chromatin structure	High-mobility group (nonhistone chromosomal) protein 1 (HMG1)	9	1	BC003378	13q12*
Cytoskeleton organization	Vinculin (VCL), transcript variant meta-VCL	7	1	NM_014000	10q22.1-q23
Cytoskeleton organization	Capping protein (actin filament) muscle Z-line, beta	14	1	BC008095	1p36.1*
DNA Repair	MutS homolog 2, colon cancer, nonpolyposis type 1 (E.coli) (MSH2)	9	1	XM_034901	2p22-p21
DNA Repair	RAD21 homolog (S. pombe) (RAD21)	11	1	NM_006265	8q24
Vesicle Trafficking	SEC22, vesicle trafficking protein (S. cerevisiae)-like 1	11	2	BC001364.	1q21.2-q21.3
Endosome-trafficking	Suppressor of K+ transport defect 1 (SKD1)	9	1	NM_004869	18q21.32-q21.33*
Intermediate Metabolism	Fructose-1,6-bisphosphatase 1 (FBP1)	9	1	NM_000507	9q22.3
Intermediate Metabolism	Galactose-4-epimerase, UDP- (GALE)	9	1	XM_032314	1p36-p35*
Intermediate Metabolism	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 9	9	1	BC007672	8q13.3
Intermediate Metabolism	Similar to cytochrome c oxidase subunit IV (LOC146478)	9	1	XM_085483	16q24.1
Ion Transport	ATPase, Na+/K+ transporting, beta 3 polypeptide (ATP1B3)	10	1	NM_001679.	3q22-q23
N-Glycan Biosynthesis	Dolichyl-phosphate N-acetylglucosaminephosphotransferase 1 (GlcNAc-1-P transferase)	6	1	BC008817	11q23.3*
Nuclear Envelope Protein	Thymopoietin (TMPO)	11	1	U18271	12q22
Cell cycle	G1 to S phase transition protein 1 (GSTP1)	7	1	XM_007816	16p13.1

Cell cycle	Retinoblastoma binding protein 2 (RBBP2)	9	1	XM_006998	12p11
Protein Kinase/DNA synthesis/Meiosis	Cdc7 (CDC7)	11	1	AF015592	1p22*
Receptor-mediated signalling	Erbb2 interacting protein (ERBB2IP) (ERBIN)	6	1	NM_018695	5q31
Receptor-mediated signalling	G protein alpha stimulating activity polypeptide 1 (GNAS)	13	1	BC002722	20q13.2-q13.3
Transcription Regulation	C3HC4-type zinc finger protein (LZK1)(Dif3 homolog)	8	1	NM_024835	17q12
Transcription Regulation	Activity-dependent neuroprotector (ADNP)	9	1	NM_015339	20q13.13-13.2
Transcription Regulation	Hypoxia-inducible factor 1, alpha subunit inhibitor HIF1AN (FIH-1)	7	1	XM_030426	10q24
Transcription Regulation	Sex comb on midleg-like 1 (Drosophila) (SCML1)	9	1	NM_006746	Xp22.2-22.1
Transcription Regulation	Kelch-like protein C3IP1 (C3IP1)	7	2	XM_086284	1q32.1
Mitochondrial stress response	D-prohibitin (Bap37)	8	1	AF178980	12p13
Ubiquitin-mediated proteolysis	Suppressor of G2 allele of SKP1, S. cerevisiae,homolog of (SGT1)	8	1	NM_006704	13q14.2*
Ubiquitin-mediated proteolysis	Ubiquitin protein ligase E3A, transcript variant 2 (UbE3A)	9	1	NM_000462	15q11-q13
Ubiquitin-mediated proteolysis	Proteasome subunit, beta type, 4 (PSMB4)	9	1	XM_047881	1q21
RNA surveillance	Regulator of nonsense transcripts 2 (RENT2), transcript variant 1	7	1	NM_080599.1	10p14-p13
RNA surveillance	5'-3' exoribonuclease 2 (XRN2)	8	1	XM_009601.3	20p11.2-p11.1
RNA surveillance	PI-3-kinase-related kinase SMG-1	11	1	AY014957.1	16p13.2
RNA synthesis/translation	Poly(A)-binding protein, cytoplasmic 1 (PABPC1)	8	3	XM_018280.1	8q22
RNA synthesis/translation	Sjogren syndrome antigen A2 (SSA2)	9	1	XM_001901.1	1q31
RNA translation	Ribosomal protein S6 (RPS6)	17	3	XM_015349.1	9p21*
RNA translation	Ribosomal protein L5 (RPL5)	6	1	XM_018498.1	1p
RNA translation	Ribosomal protein L3	11	1	BC008492	22q
RNA translation	Ribonuclease P, 40kD subunit (RPP40)	8	2	XM_004104.2	6
RNA translation	Elongation factor G1 (EFG1)	7	1	AF367998	3q25.1-26.2
RNA translation	Ribosomal protein, large, P0	10	1	BC005863	12q24.2
Unknown	KIAA0725 protein (KIAA0725)	12	1	XM_049445	8p11.21
Unknown	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B (ANP32B)	9	1	NM_006401	9q22.32
Unknown	Candidate tumor suppressor OVCA1 (OVCA1)	12	2	NM_080822	17p13.3*

Unknown	Hypothetical protein (MAC30), mRNA	17	2	XM_031536	17p11.1
Unknown	Similar to tumor metastasis-suppressor; longevity assurance (LAG1, S. cerevisiae) homolog 2	12	2	XM_065847	2q31.1
Unknown	DNA sequence from clone RP11-165J3 on chromosome 9	16	1	AL583839.1	9q22.1-22.33
Unknown	Hypothetical protein FLJ23375 (FLJ23375)	16	2	NM_024956	15q14
Unknown	Hypothetical protein MGC2714 (MGC2714)	8	1	NM_032299	11q22.1
Unknown	Hypothetical protein BC008322 (LOC92106)	8	3	NM_138381.1	3p24.3
Unknown	Hypothetical protein FLJ20060	12	1	XM_005467.4	16q13
Unknown	CG2277 gene product (LOC221294)	7	1	XM_166297.1	6q22.31
Unknown	Hypothetical protein MGC4767 (MGC4767)	10	1	XM_045844	12q24.31
Unknown	KIAA1007	6	2	XM_168026.1	7p11.2
Unknown	Clone FLB9213 PRO2474	11	1	AF130088	7
Unknown	CGI-48 protein (LOC51096)	13	1	NM_016001	17q21.33
Unknown	Homo sapiens cDNA FLJ31534	8	1	AK056096	9

3.4.3.2 Confirmation of BRCA1 induction of some of the candidate genes

It is important to confirm that the genes identified with differential screening will also show induction in MCF7 cells overexpressing BRCA1 with Northern and western blot analyses. Northern blot will not only confirm that the gene is upregulated by BRCA1 overexpression, but also show the true transcript size of the gene.

Several BRCA1-induced genes reported here are known to be involved in DNA repair (RAD21, MSH2); (Sonoda *et al*., 2001; Wang *et al*, 2000), and chromosomal structure maintenance (RAD21, CDC7, SGT1, HMG1); (Nasmyth *et al*., 2000; Lei *et al*., 2001; Kitagawa *et al*., 1999; Yamanaka *et al*., 2002). This observation strongly suggests that some of these BRCA1 target genes may mediate some of the effects attributed to BRCA1 for chromosomal stability. To further analyse their BRCA1-regulated expression, RAD21 and MSH2 expression in two different cell lines have been analyzed.

As shown in Figure 59, both RAD21 and MSH2 were upregulated following overexpression of BRCA1 in MCF7 cells. Furthermore, the same genes were also upregulated in the UBR60-bcl2 cell line following BRCA1 induction (Figure 59d). UBR60-bcl2 cells were modified to stably express BRCA1 with tetracycline inducible system (Harkin *et al*., 1999). In Figure 59a semi-quantitative RT-PCR amplification of RAD21 and MSH2 transcripts showed that pCMVmycBRCA1-transfected MCF7 cells (lane B) display higher levels of RAD21 and MSH2, when compared to pCMVmyc-transfected cells (lane V). Northern blot analysis confirms BRCA1-mediated induction of RAD21 expression in BRCA1 overexpressed MCF7 cells (Figure 59b). Western blot analysis of MSH2 protein levels confirms induced expression of MSH2 gene following BRCA1 overexpression in MCF7 cells (Figure 59c). Furthermore, following inducible BRCA1 (5,7 kb transcript size) expression in the UBR60-bcl2 cells, RAD21 (3,6 kb) and MSH2 (3,1 kb) transcripts also showed increased expression (Figure 59d). These observations confirm that, both RAD21 and MSH2 are indeed upregulated as a result of BRCA1 overexpression.

Another BRCA1-target gene was ERBIN, which encodes ERBB2/HER2-interacting protein (Borg *et al*., 2000). As shown in Figure 60, northern blot assays, using both MCF7 and UBR60-bcl2 cells, demonstrate that BRCA1 induces 2.5 and 3.5 fold induction of ERBIN (6,4 kb transcript size) expression respectively. GNAS encoding a G protein alpha stimulating activity polypeptide was also interesting for further consideration. Figure 60c shows that GNAS expression is also induced by BRCA1 (2.1 fold). OVCA1, which has been identified as a candidate tumour suppressor gene, was also studied. OVCA1 mRNA transcripts (1.1 kb and 2.3 kb species) levels were low in MCF7 cells and BRCA1 overexpression caused 2.4 fold induction of 1.1 kb and 1.7 fold induction of 2.3 kb transcripts (Figure 60a). MAC30, whose expression was reported to be decreased in meningiomas, schwannomas and neurofibromas, has two transcripts (Murphy *et al*., 1993). The 2.3 and 2.8 kb transcripts displayed 2.0 and 4.4 fold induction in MCF7 cells and 2.2 and 3.8 fold induction in UBR60-bcl2 by BRCA1, respectively (Figure 60).

These studies performed with UBR60-bcl2 cells that stably express BRCA1 at modest levels in an inducible manner confirm and validate our results obtained with a transient expression approach to identify potential BRCA1 target genes.

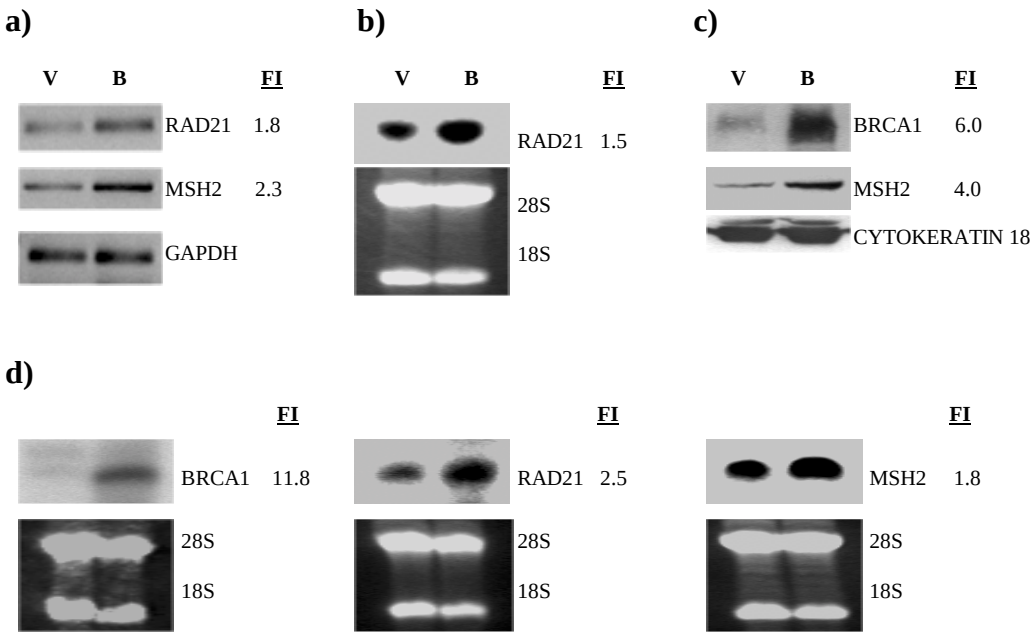


Figure 59: BRCA1-mediated upregulation of RAD21 and MSH2 DNA repair genes.

(a) Semi-quantitative RT-PCR amplification of RAD21 and MSH2. GAPDH was used as a control for equal template loading in PCR. **(b)** Northern blot analysis of total cellular RNA from MCF7 cells. Ethidium bromide staining shows equal RNA loading in each lane. **(c)** Western blot analysis of MSH2 and BRCA1 proteins in BRCA1 overexpressed MCF7 cells. Cytokeratin 18 was used for equal protein loading control. **(d)** Northern blot analysis from UBR60-bcl2 cells showing inducible BRCA1, RAD21 and MSH2 expressions following tetracycline withdrawal (lane –T) at 24 h. Ethidium bromide staining shows equal RNA loading and **"FI"** indicates fold induction in BRCA1-overexpressing cells.

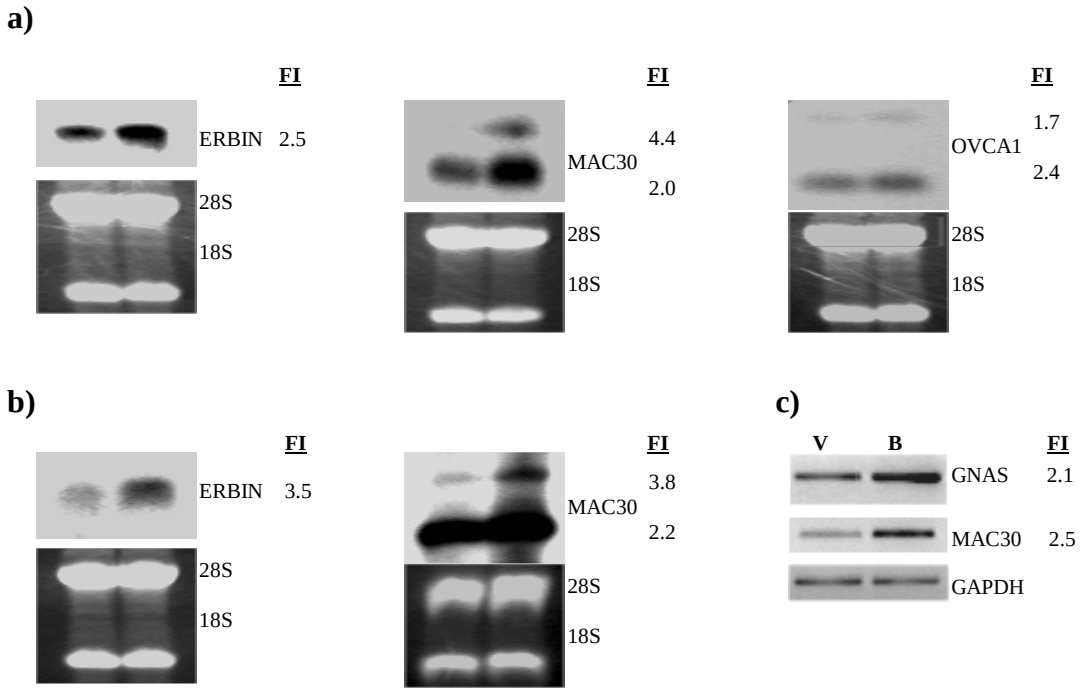


Figure 60: BRCA1-mediated upregulation of genes involved in cell signalling or tumour suppression.

(a) Northern blot analysis of total cellular RNA from MCF7 cells shows that the expression of ERBIN, OVCA1 and MAC30 genes are induced by BRCA1. **(b)** Northern blot analysis of total cellular RNA from UBR60-bcl2 cells with inducible BRCA1 expression shows that these cells overexpress ERBIN and MAC30 genes as a result of induction of BRCA1 gene expression upon tetracycline withdrawal (lane -T). **(c)** Semi-quantitative RT-PCR amplification of GNAS and MAC30 transcripts shows BRCA1-mediated induction of these genes in MCF7 cells. Ethidium bromide staining shows equal RNA loading and **"FI"** indicates fold induction in BRCA1-overexpressing cells.

CHAPTER 4. DISCUSSION

Generating ecdysone inducible BRCA1 expression in the cell lines

Germ-line mutations in the *BRCA1* tumour suppressor gene predispose carriers to cancer of the breast and ovaries and account for approximately 50% of inherited breast cancers (Miki *et al.*, 1994). *BRCA1* gene encodes a multifunctional protein involved in different cellular pathways including cellular response to DNA damage, cell cycle, growth suppression, apoptosis induction, ubiquitin ligation and transcription regulation (Venkitaraman, 2002). Therefore, the identification of genes whose expression is affected by *BRCA1* as aimed in this thesis work, may contribute to a better understanding of breast cancer suppression by this gene.

In this study, initial attempts were to generate an inducible and stable BRCA1 expressing breast carcinoma cell line. Ecdysone inducible system and its components were used to generate a tightly regulated BRCA1 expression. Three full-length *BRCA1* cDNAs from different sources were used for subcloning into pIND expression vector. The first step was to transfect MCF7 breast carcinoma cell line with the pVgRXR regulatory plasmid to generate a stable cell line; the next step was to introduce the expression plasmid pIND carrying BRCA1 cDNA and to induce BRCA1 expression with ecdysone analog ponasterone A or muristerone A. On the other hand regulatory plasmid pVgRXR harboring U2OS osteosarcoma cell line, pIND.p53 and pIND.lacZ plasmids were used as control during the experiments. After generation of stable pVgRXR expressing MCF-7 breast carcinoma cell line, selected clones were transiently transfected with pIND.lacZ and induced with mA, but induction of the reporter gene was not significant when compared to the pIND.lacZ transfected U2OS control cells. Therefore, U2OS cell line harboring pVgRXR plasmid was transfected with pIND.myc.BRCA1 plasmid to generate BRCA1

expressing clones and these clones were analyzed by western blot for induction of BRCA1 protein levels. Later on, U2OS cells were transiently transfected with pIND.BRCA1 and pIND.HA.BRCA1 plasmids and western blot analysis was performed with different BRCA1 specific antibodies. There were no significant increases in BRCA1 expression of the selected clones, compared to control, transiently pIND.p53 transfected U2OS cells. The inducible system has worked very well with U2OS cells with control plasmids pIND.lacZ and pIND.p53 but, in the case of BRCA1, a significant increase was not observed with pIND.BRCA1 constructs.

The first two BRCA1 cDNA sources, pcDNA3.myc.BRCA1 and pCR3. BRCA1 were *in vitro* transcribed-translated to control the translated BRCA1 protein product. The expected BRCA1 protein products were observed for both constructs. Then they were used for subcloning of BRCA1 into pIND vector. pIND.BRCA1 was partially sequenced from both 5' and 3' ends to make sure that the cloning sites were correct. The 3' end sequencing showed that there were some mismatched sequences in the vector site when compared to the expected vector sequence of the pIND.BRCA1 construct. Since the mismatch sequences were after the BRCA1 stop codon, not in the coding sequence of BRCA1 in pIND.BRCA1 construct, the pIND vector alone was also partially sequenced. The pIND vector sequence was correct and there was nothing wrong with the vector used for cloning, at least in the multiple cloning site.

Later on, pIND.HA.BRCA1 was prepared with different pIND vector and BRCA1 cDNA sources and with partial sequencing no mismatches was observed. pIND.HA.BRCA1 was transiently transfected into U2OS, but no significant increase was observed again. There might be two explanations for this result: When the cells were transfected with endogenous DNA, in some cases, they tend to hypermethylate the inducible promoters especially in the case of introducing a growth inhibitor gene. Harkin *et al,* have used tetracycline inducible system to overexpress BRCA1 (Harkin *et al.,* 1999) and have solved the hypermethylation problem with 5-azacytidine treatments of UBR60-bcl2 cells. Another explanation might be the minor induction of BRCA1 protein in our system. In some of the stable pIND.myc.BRCA1 expressing U2OS clones (Figure 21, clone 43; Figure 22, clones 22-26) a very slight increase was observed in BRCA1 expression, consistent with a recent report, showing the mild induction of BRCA1 by the use of ecdysone inducible system (Welcsh *et al.,* 2002).

With this system, they aimed to identify BRCA1 downstream targets, with modest BRCA expression levels, that would not induce apoptosis.

Ectopic expression of BRCA1 in MCF7 cells

Since the desired expression level of BRCA1 protein was not observed with the ecdysone inducible system, MCF7 cells were transiently transfected with pCMVmycBRCA1 plasmid that constitutively expresses BRCA1. Significant increases in BRCA1 mRNA transcript and protein levels were confirmed by northern and western blot analyses respectively. The transfection efficiency of the MCF7 cells was 40% as determined by the pEGFP reporter plasmid transfection in control MCF7 cells. In order to determine whether constitutive BRCA1 expression leads to apoptosis, the transfected cells were stained with an early apoptosis marker, annexin V. Under the experimental conditions used in this study, not more than 1-2% of the cells were apoptotic. Thus at a transfection efficiency of 40%, less than 5% of the BRCA1 expressing cells were apoptotic. This indicated that ectopically expressed BRCA1 was not significantly cytotoxic to MCF7 cells under test conditions. The lack of BRCA1 induced apoptosis in our experimental system is in agreement with recent findings indicating that BRCA1 directs a selective p53-dependent transcriptional response towards growth arrest and DNA repair targets (MacLachlan *et al*., 2002)

Identification and analysis of BRCA1 induced genes

Suppression Subtractive Hybridization (SSH) technology was used to generate a library of partial-length cDNAs representing differentially expressed mRNAs in BRCA1-overexpressing MCF7 cells. SSH combines subtractive hybridization with PCR to generate a population of PCR fragments enriched for sequences from genes expressed differentially. SSH-mediated cDNA enrichment allows the equalization of wide differences in abundance of different transcript species. Consequently, differentially expressed transcripts of low abundance can be cloned (Diatchenko *et al.*, 1996). SSH has some advantages over microarray analysis; it can theoretically detect differences in less abundant mRNAs.

By using this approach, 60 genes have been identified that are upregulated as a result of BRCA1 overexpression in breast cancer cells (Table 13). None of the genes identified in this study have been identified in previous reports (Aprelikova *et al.,* 2001; Harkin *et al.,* 1999; MacLachlan *et al.,* 2000; Mullan *et al.,* 2001; Welcsh *et al.,* 2002). This could be due to the fact that a breast cancer cell line was used to identify potential BRCA1 target genes by the SSH technique in our study.

In previous studies, Harkin *et al.* (Harkin *et al.,* 1999) have identified 20 BRCA1 target genes using microarray technology for screening of transcripts upregulated by ectopically expressed BRCA1 in the U2OS osteosarcoma cell line. Similarly, MacLachlan *et al*. (MacLachlan *et al.,* 2000) were able to identify 45 major targets in the SW480 colorectal cancer cell line. Aprelikova *et al*. (Aprelikova *et al.,* 2001) have identified 51 genes that are down-regulated in BRCA-/- mouse embryonic stem cells. Recently, Welcsh *et al* identified 62 genes that are targets of BRCA1 by using human embryonal kidney epithelial cells (Welcsh *et al.,* 2002). These studies have shown that DNA damage-responsive genes such as *GADD45* (Aprelikova *et al.,* 2001; Harkin *et al.,* 1999; MacLachlan *et al.,* 2000; Mullan *et al.,* 2001; Welcsh *et al.,* 2002), *GADD153, Ku70, Ku60* (MacLachlan *et al.,* 2000), and cell cycle control genes such as *14-3-3*σ (Aprelikova *et al.,* 2001) are upregulated by BRCA1.

In the present work, it has been shown that several DNA damage response (*RAD21, MSH2, ASF1A, CDC7*) and chromosomal structure maintenance (*RAD21, ASF1A, CDC7, SGT1, HMG1*) genes are upregulated targets of BRCA1. As BRCA1-deficient cells suffer from both DSB repair deficiency and chromosomal instability (Wang *et al.,* 2001), at least some of these defects may be attributed to inefficient expression of these target genes in the absence of functional BRCA1. The relevance of these genes in BRCA1 mediated tumour suppression may be related to their functions. RAD21 is a nuclear phosphoprotein that repairs double strand breaks and is a component of the cohesin complex that holds sister chromatids during mitosis (Chen *et al.,* 2002). MSH2 encodes a mismatch repair protein and has been shown to be a component of BASC (BRCA1-associated genome surveillance complex) (Wang *et al.,* 2000b). The histone chaperone anti-silencing function 1 (ASF1) has been implicated in efficient assembly of newly replicated and repaired DNA into chromatin (Mello *et*

al., 2002). It has also been proposed that in response to DNA replication blocks and DNA damage, Rad53 directs Asf1 to regulated nucleosome deposition and thereby links Rad53 to chromatin assembly (Hu *et al.,* 2001). Cdc7 kinase is one of the key regulators in the initiation of DNA replication in mammals. Genetic studies in yeast indicate additional roles of Cdc7 in meiosis, checkpoint responses, maintenance of chromosome structures and repair (Masai and Arai, 2002). SGT1 is required for assembly of kinetochore complex and also for both the G1/S and G2/M transitions in the cell cycle. It is also a subunit of the SCF ubiquitin ligase complex (Kitagawa *et al.,* 1999). HMGB1 (High Mobility Group Box protein) is a non-histone chromosomal protein and has role in V(D)J recombination as well as DSB repair. It stimulates DNA ligase IV/XRCC4 during non-homologous end joining of DSB repair (Yamanaka *et al.,* 2002). HMGB1 is one of the proteins that have been shown to interact with steroid hormone receptors, thus dramatically enhancing their binding to estrogen and progesterone responsive elements. Intertumoural variation of HMGB1 expression has been observed in breast cancers and because of its role in steroid receptor mediated gene expression HMGB1 can be considered to be one of the major players involved in mechanisms of steroid hormone-dependent growth of breast cancer cells (Flohr *et al.,* 2001).

Among BRCA1-target genes reported here several (such as *SGT1, UbE3A, PSMB4*) are involved in ubiquitin-mediated protein degradation. This may not be an unexpected finding based on the recent demonstration that BRCA1 itself (together with BARD1) functions as an E3 ubiquitin ligase (Hashizume *et al.,* 2001; Ruffner *et al.,* 2001; Venkitaraman, 2002). It may serve as a signaling event rather than proteosomal degradation, such as in DNA repair and or in regulating the BARD1-mediated inhibition of mRNA polyadenylation after DNA damage. The *UBE3A* gene encodes the E6-AP ubiquitin-protein ligase, which functions in protein ubiquitination and has been shown to be mutated in Angelman syndrome patients (Kishino *et al.,* 1997). PSMB4 is a subunit of PA28 proteasome activator and proteasome is responsible for degradation of short lived and misfolded cytosolic and nuclear proteins in cells (McCusker *et al.,* 1997). As suggested by Venkitaraman, the involvement of BRCA1 in ubiquitin-mediated protein degradation could help to explain the multiplicity of biological roles ascribed to this protein, including coordination of DNA repair-related events (Venkitaraman, 2002).

Interestingly, a variety of other genes involved in distant cellular processes such as intermediate metabolism, cell-cell interaction, receptor-mediated signaling, endosome trafficking, cytoskeletal organization and RNA surveillance and metabolism also appear to be upregulated by BRCA1 (Table 13). In this context, BRCA1 seems to act like another tumour suppressor gene, namely p53 which also upregulates a high number of genes involved in many different cellular processes (Balint and Vousden, 2001).

Related to its function in transcription regulation, a set of transcription related genes have been identified as BRCA1 downstream targets: *LZK1* encodes a C3HC4-type zinc finger protein, which is characteristic for protein-protein and protein-DNA interactions. This gene is also a likely ortholog of mouse dioxin inducible factor 3 (dif3). *ADNP* gene also has zinc finger and homeobox domains and is implicated in modulation of cell survival (Zamostiano *et al.,* 2001). C3IP1 has a kelch domain (beta propeller domain) involved in protein-protein interactions and some enzymatic activities. Kelch-like proteins have been found to interact with topoisomerase II (Xu *et al.,* 2002). HIF1AN has been found to interact and inhibit transcriptional activity of HIF1A and it has been suggested that it may interact either directly or indirectly with some histone deacetylases (Mahon *et al.,* 2001). Mammalian homologs of the PcG (Drosophila Polycomb group) are involved in the transcriptional repression of HOX genes. *SCML1* (sex comb on midleg like-1) appears to be a new member of this gene group and may play role in the control of embryonic development (van de Vosse *et al.,* 1998). These genes appear to act as transcriptional activators or repressors consistent with the finding that BRCA1 interacts with many transcriptional factors.

Another class of BRCA1 regulated genes were identified which have a role in RNA metabolism and surveillance. Some ribosomal genes and RNA metabolism genes such as poly(A)-binding protein (*PABPC1*), which makes a complex with the 3-prime poly(A) tail of eukaryotic mRNA, that is required for poly(A) shortening, translation initiation and mRNA stability (Deo *et al.,* 1999), were identified as BRCA1 targets. Interestingly several RNA surveillance genes such as *RENT2* (Mendell *et al.,* 2000), *XRN2* (Zhang *et al.,* 1999) and *SMG-1* (Yamashita *et al.,* 2001) have also been identified as BRCA1 upregulated genes in this study. Gene expression

requires the coordination and integration of multiple processes including transcription, splicing, polyadenylation, nucleocytoplasmic export, and translation of mRNAs. Premature termination codons in such a complex process or nonsense mutations introduced during DNA replication result in truncated proteins, which are toxic to cells. A mechanism, known as mRNA surveillance or nonsense-mediated mRNA decay (NMD) has evolved to recognize mRNAs containing premature termination codons and accelerate their degradation. Identification of NMD genes as BRCA1 target genes may suggest that BRCA1 may upregulate NMD process upon DNA damage leading to accelerated degradation of truncated proteins that are toxic to cells.

Of particular interest, several genes that have been identified here are among markers of a `poor prognosis` gene expression signature (*RAD21, ERBB2IP, UbE3A, FBP1, KIAA0725, TMPO, PSMB4, CD24, MSH2, ATP1B3*) identified by DNA microarray analysis of early primary breast tumours (van't Veer *et al*., 2002). Furthermore, Hirano *et al.,* have reported frequent LOH sites in breast cancers (Hirano *et al.*,2001) that correlates with the chromosomal locations of some of the genes (*HMG1, SKD1, GALE,* Capping protein muscle Z-line beta, GlcNAc-1-P transferase, *CDC7, SGT1, RPS6, OVCA1*) identified in this study. BRCA1-induced expression of six of these genes from this library was confirmed by additional experiments (semi-quantitative RT- PCR, northern blot or western blot) using MCF7 and UBR60-bcl2 cell lines. Therefore, a set of BRCA1 target genes that may be involved in BRCA1-mediated cellular processes as well as breast carcinogenesis has been identified in this study.

In relation to BRCA1-related breast/ovarian carcinogenesis, further analysis of the expression of several genes has been carried out. Among these BRCA1-target genes ERBIN and GNAS are involved in receptor-mediated signaling. ERBIN is an ERBB2/HER2-binding protein, which locates this receptor to the basolateral membrane (Borg *et al.,* 2000). Overexpression of ERBB2/HER2 is frequently observed in breast cancers and its overexpression is associated with poor tumour prognosis (Borg *et al.,* 1991). Although it is not known whether ERBIN is able to inhibit ERBB2/HER2 activity, its deregulation results in the mislocalisation of the receptor (Borg *et al.,* 2000). This raises the possibility that ERBIN deficit resulting from inactivation of BRCA1 could lead to a loss of epithelial homeostasis, and in

consequence pathological disorganisation in breast carcinoma. On the other hand, GNAS is a guanine nucleotide-binding protein that stimulates adenylyl cyclase. Activated GNAS leads to increase in cAMP and may regulate proliferation at multiple sites, which may be advantageous in regulating aggressive proliferative programs in metastatic cells. The active form of this protein leads to growth inhibition of breast cancer cells both *in vitro* and *in vivo* (Chen *et al.,* 1998a; Santore *et al.,* 2002). A third gene, namely OVCA1 that we show to be upregulated by BRCA1, was initially identified as a candidate ovarian tumour suppressor gene located on chromosome 17p13.3 (Schultz *et al.,* 1996). This locus displays frequent LOH in both ovarian and breast cancers (Hirano *et al.,* 2001; Lynch *et al.,* 1998a; Lynch *et al.,* 1998b; Lynch *et al.,* 1998c) and OVCA1 was reported to display reduced expression in ovarian and breast cancers. In addition, ectopic expression of OVCA1 causes a dramatic reduction in cell proliferation in association with accelerated cyclin D1 degradation (Bruening *et al.,* 1999). Even if additional studies are needed, these observations strongly suggest that BRCA1 may directly act as a tumour suppressor gene in breast cancer, for example through receptor-mediated regulation of cell proliferation.

In conclusion, in this study, 60 genes have been identified whose expression levels are upregulated as a result of BRCA1overexpression in breast carcinoma cells. The properties of several of these genes are consistent with putative tumour suppressor functions in breast neoplasia. It will now be important to construct a complete profile of BRCA1-regulated genes in order to achieve an integrated view of all the functional events regulated by BRCA1, and to assess how expression of these genes is affected by the BRCA1 status of cells.

CHAPTER 5. FUTURE PERSPECTIVES

The genes identified in this study as BRCA1 upregulated targets may help to explain the role of *BRCA1* in breast cancer suppression. The candidate genes have not been previously characterized. Further studies are therefore needed to increase the impact of this initial SSH screening.

One approach is to analyze the expression level of these genes in tumours and cell lines with known *BRCA1* and *p53* status, in order to understand whether the expression of these genes is regulated by BRCA1. At this point, the analysis of the normal-tumour samples for the expression of BRCA1 and the target genes is very important in order to confirm that BRCA1 protein deficiency in the tumour samples leads to impaired regulation of these genes. It was reported that BRCA1 could regulate the expression of many genes through p53-dependent or p53-independent pathways such as *p21* and *GADD45* (Somasundaram *et al*., 1997, Harkin *et al.*, 1999). In order to distinguish this, the regulation of a number of candidate genes can be analyzed in breast carcinoma cell lines with known *p53* and *BRCA1* status, such as MCF7 (wt-BRCA1/wtp53), MDA435 (wt-BRCA1/mt-p53 and HCC1937 (mt-BRCA1/mt-p53) by western and northern blot techniques.

BRCA1 can bind to DNA sequences in a non-specific manner, meaning the absence of demonstrable sequence-specific DNA binding activity. But BRCA1 can mediate sequence specific transcriptional repression through its selective recruitment by a novel DNA-binding transcription factor ZBRK1 (zinc finger and BRCA1-interacting protein with a KRAB domain). BRCA1 has been shown to bind to many transcription regulatory proteins and mediate ZBRK1-directed repression through a ZBRK1 binding site identified in intron 3 of the *GADD45* gene (Zheng *et al.,* 2000). ZBRK1 binds to the specific sequence GGGxxxCAGxxxTTT. This motif has been

identified in the regulatory regions of the previously identified BRCA1 downstream targets (Harkin *et al.*, 1999; MacLachlan *et al.*, 2000) such as *GADD153, Ki-67, Bax, p21, EGR1*, amphiregulin prothymosin, *TIMP-1, TIMP-2*, and *Topo IIa*. Since BRCA1 activates *GADD45* and *p21* via binding to the ZBRK1 transcriptional repressor, the regulatory and promoter regions of the candidate genes must be examined for BRCA1 responsive sites such as ZBRK1 binding site and whether BRCA1 indeed regulates the expression of the target genes. The BRCA1 responsive sites in the promoter and regulatory regions of these genes can be identified by using deletion-mapping strategies with the construction of reporter gene coding vectors.

Genes identified with an unknown function can be checked for BLAST search in the future because their functions may eventually be determined. Studies concerning the identification of the functions of these genes with unknown functions may bring a better understanding to BRCA1 mediated cancer suppression.

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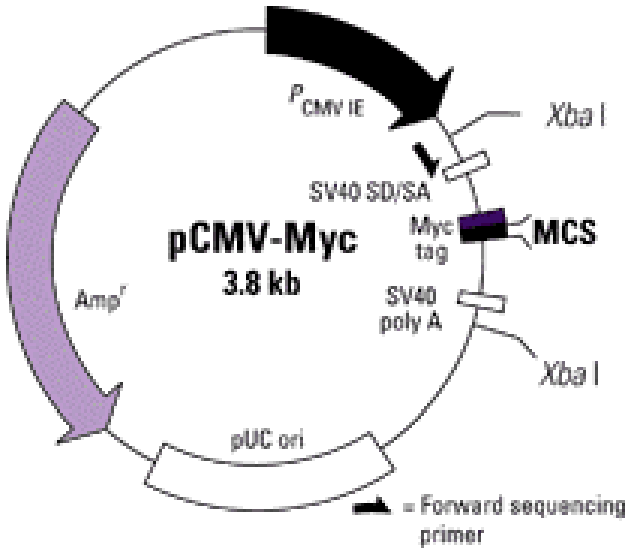
Appendix A: Map and sequence of pCMV.myc.BRCA1

Map and multiple cloning site of pCMV.myc vector (Clontech):



830 840 850 860 870
C ACC ATG GCA TCA ATG CAG AAG CTG ATC TCA GAG GAG GAC CTG CTT
Myc tag

880 890 900 910 920
ATG GCC ATG GAG GCC CGA ATT CGG TCG ACC GAG ATC TCT CGA GGT ACC GCG GCC GC
Sfi I[A,B] EcoR I[A,B] Sal I[B] Bgl II Xho I[A] Kpn I Not I

Appendix A: Map and sequence of pCMV.myc.BRCA1

SalI-NotI sites are highlighted (894-6509th nucleotides)

pCMV.myc.BRCA1

9380 base pairs

```
                SphI  PstI
                BbuI  Sse8387I
gagttcgagcttgcatgcctgcaggtcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgac base pairs
ctcaagctcgaacgtacggacgtccagcaatgtattgaatgccatttaccgggcggaccgactggcgggttgctg 1 to 75
                PaeI  SbfI
                        BspMI


               AatII                                                  AatII
ccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgg base pairs
ggggcgggtaactgcagttattactgcatacaagggtatcattgcggttatccctgaaaggtaactgcagttacc 76 to 150


                                                 NdeI
gtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgac base pairs
cacctcataaatgccatttgacgggtgaaccgtcatgtagttcacatagtatacggttcatgcgggggataactg 151 to 225
                                                 FauNDI


  AatII
gtcaatgacggtaaatggcccgcctggcattatgcccagtacatgaccttatgggactttcctacttggcagtac base pairs
cagttactgccatttaccgggcggaccgtaatacgggtcatgtactggaataccctgaaaggatgaaccgtcatg 226 to 300


      BstSNI
      SnaBI              NcoI
atctacgtattagtcatcgctattaccatggtgatgcggttttggcagtacatcaatgggcgtggatagcggttt base pairs
tagatgcataatcagtagcgataatggtaccactacgccaaaaccgtcatgtagttacccgcacctatcgccaaa 301 to 375
      BsaAI              Bsp19I
      Eco105I


                                              AccB1I
                                  AatII       BshNI
gactcacggggatttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggac base pairs
ctgagtgcccctaaaggttcagaggtggggtaactgcagttaccctcaaacaaaaccgtggttttagttgccctg 376 to 450
                                              BanI
                                              Eco64I


tttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtacggtgggaggtctatata base pairs
aaaggttttacagcattgttgaggcggggtaactgcgtttacccgccatccgcacatgccaccctccagatatat 451 to 525
```

```
        BanII
        Eco24I                          Esp3I
      EcoICRI Psp124BI               BpmI
agcagagctcgtttagtgaaccgtcagatcgcctggagacgccatccacgctgttttgacctccatagaagacac base pairs
tcgtctcgagcaaatcacttggcagtctagcggacctctgcggtaggtgcgacaaaactggaggtatcttctgtg 526 to 600
      Ecl136II                       GsuI
        SacI SstI                       BsmBI
        FriOI


               Bsp13I
               BspEI  XbaI     BsaWI
               MroI BsaWI   BamHI                                HpaI
cgggaccgatccagcctccggactctagaggatccggtactagaggaactgaaaaaccagaaagttaactggtaa base pairs
gccctggctaggtcggaggcctgagatctcctaggccatgatctccttgacttttttggtctttcaattgaccatt 601 to 675
               Kpn2I BseAI  BstI
               BsiMI
               AccIII

                           BstI
                        Psp5II   BsaWI
gtttagtctttttgtcttttatttcaggtcccggatccggtggtggtgcaaatcaaagaactgctcctcagtgga base pairs
caaatcagaaaaacagaaaataaagtccagggcctaggccaccaccacgtttagtttcttgacgaggagtcacct 676 to 750
                           PpuMI BamHI


                 SseBI                                                SacII
                 Eco147I                                              KspI Bsp12
                 StuI                                                 SstII
tgttgcctttacttctaggcctgtacggaagtgttacttctgctctaaaagctgcggaattgtacccgcgggccc base pairs
acaacggaaatgaagatccggacatgccttcacaatgaagacgagattttcgacgccttaacatgggcgcccggg 751 to 825
                 AatI                                                 Cfr42I
                 Pme55I                                               Sfr303I
                                                                       PspOMI

ApaI Bsp19I                                    MscI
    0I                                        CfrI
Eco24I                           Psp5II BspMI MluNI SfiI   EcoRI  SalI
accatggcatcaatgcagaagctgatctcagaggaggacctgcttatggccatggaggcccgaattcggtcgaca base pairs
tggtaccgtagttacgtcttcgactagagtctcctcctggacgaataccggtacctccgggcttaagccagctgt 826 to 900
 BanII                           PpuMI      EaeI Bsp19I            AccI
  FriOI                                       BalI
  NcoI                                         NcoI

          Bsp19I                                      VspI
HindIII   NcoI               SapI                     PshBI
agcttgccaccatggatttatctgctcttcgcgttgaagaagtacaaaatgtcattaatgctatgcagaaaatct base pairs
tcgaacggtggtacctaaatagacgagaagcgcaacttcttcatgttttacagtaattacgatacgtcttttaga 901 to 975
       BcgI                                               AsnI
                                                          AseI

                       BclI
                       Ksp22I
                BstXI BpmI                     DraIII
tagagtgtcccatctgtctggagttgatcaaggaacctgtctccacaaagtgtgaccacatattttgcaaatttt base pairs
atctcacagggtagacagacctcaactagttccttggacagaggtgtttcacactggtgtataaaacgtttaaaa 976 to 1050
                      GsuI
                        FbaI

     SphI
     BbuI
gcatgctgaaacttctcaaccagaagaaagggccttcacagtgtcctttatgtaagaatgatataaccaaaagga base pairs
cgtacgactttgaagagttggtcttctttcccggaagtgtcacaggaaatacattcttactatattggttttcct 1051 to 1125
     PaeI
```

```
                                        SapI
gcctacaagaaagtacgagatttagtcaacttgttgaagagctattgaaaatcatttgtgcttttcagcttgaca base pairs
cggatgttctttcatgctctaaatcagttgaacaacttctcgataacttttagtaaacacgaaaagtcgaactgt 1126 to 1200



caggtttggagtatgcaaacagctataattttgcaaaaaaggaaaataactctcctgaacatctaaaagatgaag base pairs
gtccaaacctcatacgtttgtcgatattaaaacgttttttccttttattgagaggacttgtagattttctacttc 1201 to 1275



               Van91I
               AccB7I
tttctatcatccaaagtatgggctacagaaaccgtgccaaaagacttctacagagtgaacccgaaaatccttcct base pairs
aaagatagtaggtttcatacccgatgtctttggcacggttttctgaagatgtctcacttgggcttttaggaagga 1276 to 1350
               Esp1396I
               PflMI



tgcaggaaaccagtctcagtgtccaactctctaaccttggaactgtgagaactctgaggacaaagcagcggatac base pairs
acgtcctttggtcagagtcacaggttgagagattggaaccttgacactcttgagactcctgtttcgtcgcctatg 1351 to 1425


                 AccI
             PshAI
aacctcaaaagacgtctgtctacattgaattgggatctgattcttctgaagataccgttaataaggcaacttatt base pairs
ttggagttttctgcagacagatgtaacttaaccctagactaagaagacttctatggcaattattccgttgaataa 1426 to 1500
             AatII



gcagtgtgggagatcaagaattgttacaaatcacccctcaaggaaccagggatgaaatcagtttggattctgcaa base pairs
cgtcacaccctctagttcttaacaatgtttagtggggagttccttggtccctactttagtcaaacctaagacgtt 1501 to 1575



                          Esp3I
aaaaggctgcttgtgaattttctgagacggatgtaacaaatactgaacatcatcaacccagtaataatgatttga base pairs
ttttccgacgaacacttaaaagactctgcctacattgtttatgacttgtagtagttgggtcattattactaaact 1576 to 1650
                          BsmBI



                    BsgI
acaccactgagaagcgtgcagctgagaggcatccagaaaagtatcagggtagttctgtttcaaacttgcatgtgg base pairs
tgtggtgactcttcgcacgtcgactctccgtaggtcttttcatagtcccatcaagacaaagtttgaacgtacacc 1651 to 1725
                    PvuII



agccatgtggcacaaatactcatgccagctcattacagcatgagaacagcagtttattactcactaaagacagaa base pairs
tcggtacaccgtgtttatgagtacggtcgagtaatgtcgtactcttgtcgtcaaataatgagtgatttctgtctt 1726 to 1800


                                        BlpI
                EcoRI                   CelII
tgaatgtagaaaaggctgaattctgtaataaaagcaaacagcctggcttagcaaggagccaacataacagatggg base pairs
acttacatcttttccgacttaagacattattttcgtttgtcggaccgaatcgttcctcggttgtattgtctaccc 1801 to 1875
                                        Bsp1720I
                                        Bpu1102I
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                                                                BsaMI
              BspLU11I                                  BglII   Mva1269I
ctggaagtaaggaaacatgtaatgataggcggactcccagcacagaaaaaaaggtagatctgaatgctgatcccc base pairs
gaccttcattcctttgtacattactatccgcctgagggtcgtgtctttttttccatctagacttacgactagggg 1876 to 1950
              AflIII                                            BsmI



tgtgtgagagaaaagaatggaataagcagaaactgccatgctcagagaatcctagagatactgaagatgttcctt base pairs
acacactctcttttcttaccttattcgtctttgacggtacgagtctcttaggatctctatgacttctacaaggaa 1951 to 2025



                      BsaMI
                      Mva1269I
ggataacactaaatagcagcattcagaaagttaatgagtggttttccagaagtgatgaactgttaggttctgatg base pairs
cctattgtgatttatcgtcgtaagtctttcaattactcaccaaaaggtcttcactacttgacaatccaagactac 2026 to 2100
                      BsmI

actcacatgatggggagtctgaatcaaatgccaaagtagctgatgtattggacgttctaaatgaggtagatgaat base pairs
tgagtgtactacccctcagacttagtttacggtttcatcgactacataacctgcaagatttactccatctactta 2101 to 2175



                              MscI
                              MluNI
                            EaeI         RcaI
attctggttcttcagagaaaatagacttactggccagtgatcctcatgaggctttaatatgtaaaagtgaaagag base pairs
taagaccaagaagtctcttttatctgaatgaccggtcactaggagtactccgaaattatacatttcactttctc 2176 to 2250
                            CfrI         BspHI
                              BalI



ttcactccaaatcagtagagagtaatattgaagacaaaatatttgggaaaacctatcggaagaaggcaagcctcc base pairs
aagtgaggtttagtcatctctcattataacttctgttttataaaccctttggatagccttcttccgttcggagg 2251 to 2325


     BfrI
     Vha464I
     MspCI
ccaacttaagccatgtaactgaaaatctaattataggagcatttgttactgagccacagataatacaagagcgtc base pairs
ggttgaattcggtacattgacttttagattaatatcctcgtaaacaatgactcggtgtctattatgttctcgcag 2326 to 2400
     AflII
     BspTI
     Bst98I
                                        SseBI    CvnI
                                   EcoNI Eco147I  AocI
                                BsaI     StuI     Bsu36I
ccctcacaaataaattaaagcgtaaaaggagacctacatcaggccttcatcctgaggattttatcaagaaagcag base pairs
gggagtgtttatttaatttcgcattttcctctggatgtagtccggaagtaggactcctaaaatagttctttcgtc 2401 to 2475
                                Eco31I   AatI     Eco81I
                                         Pme55I   Bse21I



atttggcagttcaaaagactcctgaaatgataaatcagggaactaaccaaacggagcagaatggtcaagtgatga base pairs
taaaccgtcaagttttctgaggactttactatttagtcccttgattggtttgcctcgtcttaccagttcactact 2476 to 2550



               RcaI
atattactaatagtggtcatgagaataaaacaaaaggtgattctattcagaatgagaaaaatcctaacccaatag base pairs
tataatgattatcaccagtactcttattttgttttccactaagataagtcttactctttttaggattgggttatc 2551 to 2625
               BspHI
```

```
aatcactcgaaaaagaatctgctttcaaaacgaaagctgaacctataagcagcagtataagcaatatggaactcg base pairs
ttagtgagctttttcttagacgaaagttttgctttcgacttggatattcgtcgtcatattcgttataccttgagc 2626 to 2700



aattaaatatccacaattcaaaagcacctaaaaagaataggctgaggaggaagtcttctaccaggcatattcatg base pairs
ttaatttataggtgttaagttttcgtggatttttcttatccgactcctccttcagaagatggtccgtataagtac 2701 to 2775



        SpeI
cgcttgaactagtagtcagtagaaatctaagcccacctaattgtactgaattgcaaattgatagttgttctagca base pairs
gcgaacttgatcatcagtcatctttagattcgggtggattaacatgacttaacgtttaactatcaacaagatcgt 2776 to 2850
        AclNI



gtgaagagataaagaaaaaaaagtacaaccaaatgccagtcaggcacagcagaaacctacaactcatggaaggta base pairs
cacttctctatttctttttttcatgttggtttacggtcagtccgtgtcgtctttggatgttgagtaccttccat 2851 to 2925




          BspMI   BpmI
aagaacctgcaactggagccaagaagagtaacaagccaaatgaacagacaagtaaaagacatgacagcgatactt base pairs
ttcttggacgttgacctcggttcttctcattgttcggtttacttgtctgttcattttctgtactgtcgctatgaa 2926 to 3000
                 GsuI


               HpaI      SexAI
tcccagagctgaagttaacaaatgcacctggttcttttactaagtgttcaaataccagtgaacttaaagaatttg base pairs
agggtctcgacttcaattgtttacgtggaccaagaaaatgattcacaagtttatggtcacttgaatttcttaaac 3001 to 3075



tcaatcctagccttccaagagaagaaaaagaagagaaactagaaacagttaaagtgtctaataatgctgaagacc base pairs
agttaggatcggaaggttctcttcttttttcttctctttgatctttgtcaatttcacagattattacgacttctgg 3076 to 3150

                                                                    Asp718I
                                                                    Acc65I
    BglII                                    BglII                  BshNI
ccaaagatctcatgttaagtggagaaagggttttgcaaactgaaagatctgtagagagtagcagtatttcattgg base pairs
ggtttctagagtacaattcacctctttcccaaaacgtttgactttctagacatctctcatcgtcataaagtaacc 3151 to 3225
                                                                    BanI
                                                                    AccB1I
                                                                    Eco64I

    KpnI
tacctggtactgattatggcactcaggaaagtatctcgttactggaagttagcactctagggaaggcaaaaacag base pairs
atggaccatgactaataccgtgagtcctttcatagagcaatgaccttcaatcgtgagatcccttccgtttttgtc 3226 to 3300
   SexAI


                            BsgI
aaccaaataaatgtgtgagtcagtgtgcagcatttgaaaaccccaagggactaattcatggttgttccaaagata base pairs
ttggtttatttacacactcagtcacacgtcgtaaacttttggggttccctgattaagtaccaacaaggtttctat 3301 to 3375



                                           HpaI
atagaaatgacacagaaggctttaagtatccattgggacatgaagttaaccacagtcgggaaacaagcatagaaa base pairs
tatctttactgtgtcttccgaaattcataggtaaccctgtacttcaattggtgtcagccctttgttcgtatcttt 3376 to 3450
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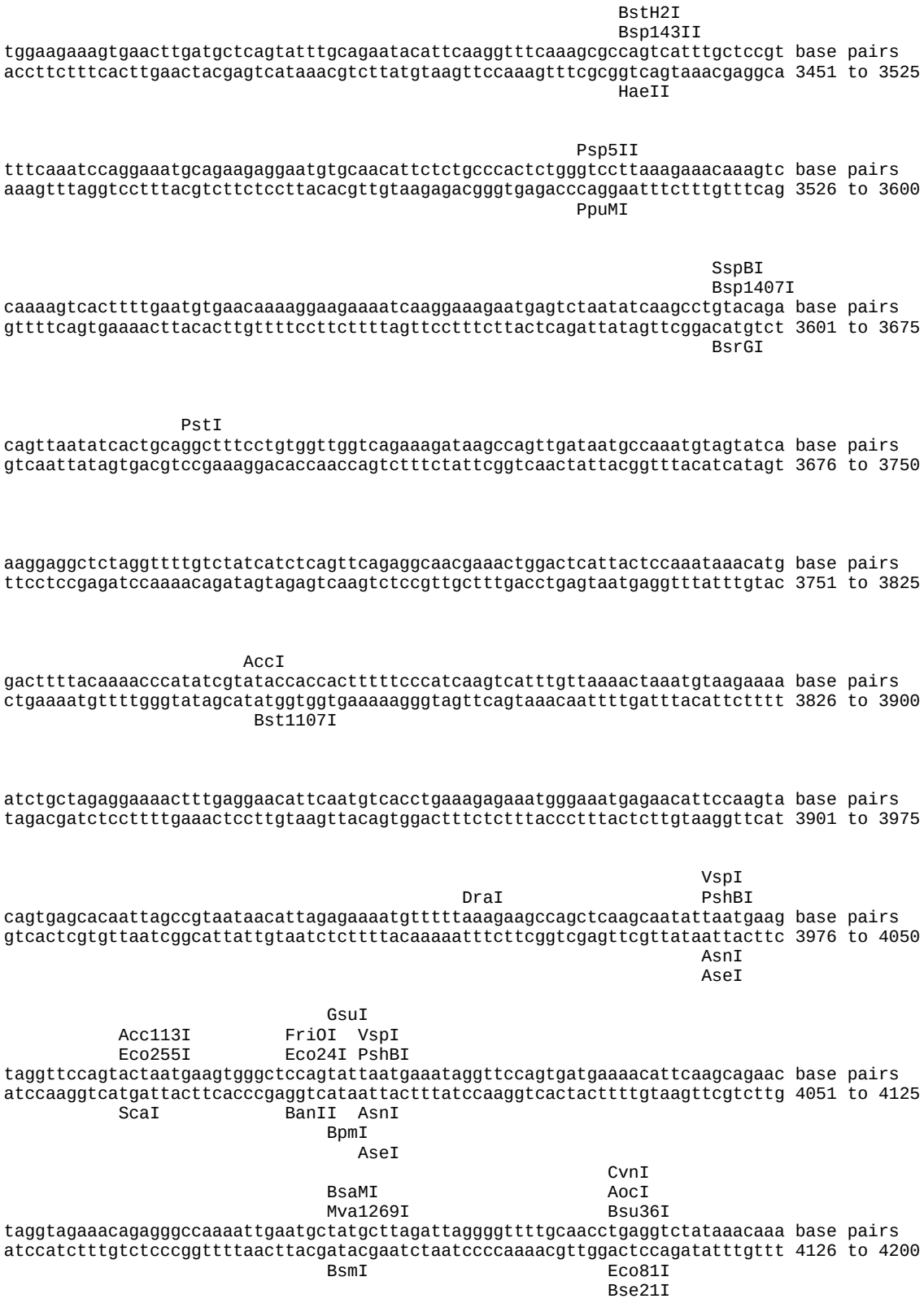
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Bsp143II
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HaeII
Psp5II
tttcaaatccaggaaatgcagaagaggaatgtgcaacattctctgcccactctgggtccttaaagaaacaaagtc base pairs
aaagtttaggtcctttacgtcttctccttacacgttgtaagagacgggtgagacccaggaatttctttgtttcag 3526 to 3600
PpuMI
SspBI
Bsp1407I
caaaagtcacttttgaatgtgaacaaaaggaagaaaatcaaggaaagaatgagtctaatatcaagcctgtacaga base pairs
gttttcagtgaaaacttacacttgttttccttctttagttcctttcttactcagattatagttcggacatgtct 3601 to 3675
BsrGI
PstI
cagttaatatcactgcaggctttcctgtggttggtcagaaagataagccagttgataatgccaaatgtagtatca base pairs
gtcaattatagtgacgtccgaaaggacaccaaccagtctttctattcggtcaactattacggtttacatcatagt 3676 to 3750
aaggaggctctaggttttgtctatcatctcagttcagaggcaacgaaactggactcattactccaaataaacatg base pairs
ttcctccgagatccaaaacagatagtagagtcaagtctccgttgctttgacctgagtaatgaggtttatttgtac 3751 to 3825
AccI
gacttttacaaaacccatatcgtataccaccactttttcccatcaagtcatttgttaaaactaaatgtaagaaaa base pairs
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Bst1107I
atctgctagaggaaaactttgaggaacattcaatgtcacctgaaagagaaatgggaaatgagaacattccaagta base pairs
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VspI
DraI PshBI
cagtgagcacaattagccgtaataacattagagaaaatgtttttaaagaagccagctcaagcaatattaatgaag base pairs
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AsnI
AseI
GsuI
Acc113I FriOI VspI
Eco255I Eco24I PshBI
taggttccagtactaatgaagtgggctccagtattaatgaaataggttccagtgatgaaaacattcaagcagaac base pairs
atccaaggtcatgattacttcacccgaggtcataattactttatccaaggtcactacttttgtaagttcgtcttg 4051 to 4125
ScaI BanII AsnI
BpmI
AseI
CvnI
BsaMI AocI
Mva1269I Bsu36I
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BsmI Eco81I
Bse21I

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gtcttcctggaagtaattgtaagcatcctgaaataaaaaagcaagaatatgaagaagtagttcagactgttaata base pairs
cagaaggaccttcattaacattcgtaggactttatttttcgttcttatacttcttcatcaagtctgacaattat 4201 to 4275

                                                                 Mph1103I
                                                                 EcoT22I
                                                              Ppu10I
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                                                                 NsiI
                                                                 Zsp2I

                                                SpeI
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                                                AclNI

                                           BlpI
                                           CelII
aaagttctgctgtttttagcaaaagcgtccagaaaggagagcttagcaggagtcctagccctttcacccatacac base pairs
tttcaagacgacaaaaatcgttttcgcaggtctttcctctcgaatcgtcctcaggatcgggaaagtgggtatgtg 4426 to 4500
                                           Bsp1720I
                                           Bpu1102I

           BstPI
           EcoO65I
           PspEI
atttggctcagggttaccgaagaggggccaagaaattagagtcctcagaagagaacttatctagtgaggatgaag base pairs
taaaccgagtcccaatggcttctccccggttctttaatctcaggagtcttctcttgaatagatcactcctacttc 4501 to 4575
           Eco91I
           BstEII
   SapI                                                 AccI
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tcgaagggacgaaggttgtgaacaataaaccatttcatttgttatatggaagagtcagatgatccgtatcgtggc 4576 to 4650



                                                                        PstI
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                                           EcoNI               PstNHI
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tggtccattataaccgtttccgtagagtccttgtagtggaatcactcctttgttttacaagacgatcgaacaaaa 4726 to 4800
 SexAI                                                  NheI

             BsgI                                 BamHI
cttcacagtgcagtgaattggaagacttgactgcaaatacaaacacccaggatcctttcttgattggttcttcca base pairs
gaagtgtcacgtcacttaaccttctgaactgacgtttatgtttgtgggtcctaggaaagaactaaccaagaaggt 4801 to 4875
                                                 BstI


                                BstXI
aacaaatgaggcatcagtctgaaagccagggagttggtctgagtgacaaggaattggtttcagatgatgaagaaa base pairs
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                                    SapI
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```

```
                     Esp3I
gtgagagtgaaacaagcgtctctgaagactgctcagggctatcctctcagagtgacattttaaccactcagcaga base pairs
cactctcactttgttcgcagagacttctgacgagtcccgataggagagtctcactgtaaaattggtgagtcgtct 5026 to 5100
                     BsmBI


                                  BpmI
gggataccatgcaacataacctgataaagctccagcaggaaatggctgaactagaagctgtgttagaacagcatg base pairs
ccctatggtacgttgtattggactatttcgaggtcgtcctttaccgacttgatcttcgacacaatcttgtcgtac 5101 to 5175
                                  GsuI


                                                        Psp5II BspMI
ggagccagccttctaacagctacccttccatcataagtgactcttctgcccttgaggacctgcgaaatccagaac base pairs
cctcggtcggaagattgtcgatgggaaggtagtattcactgagaagacgggaactcctggacgctttaggtcttg 5176 to 5250
                                                        PpuMI


                                                                              SseBI
                                                                              Eco147I
                                                                              StuI
aaagcacatcagaaaaagcagtattaacttcacagaaaagtagtgaataccctataagccagaatccagaaggcc base pairs
tttcgtgtagtctttttcgtcataattgaagtgtcttttcatcacttatgggatattcggtcttaggtcttccgg 5251 to 5325
                                                                              AatI
                                                                              Pme55I


                           PstI
tttctgctgacaagtttgaggtgtctgcagatagttctaccagtaaaaataaagaaccaggagtggaaaggtcat base pairs
aaagacgactgttcaaactccacagacgtctatcaagatggtcatttttatttcttggtcctcacctttccagta 5326 to 5400




ccccttctaaatgcccatcattagatgataggtggtacatgcacagttgctctgggagtcttcagaatagaaact base pairs
ggggaagatttacgggtagtaatctactatccaccatgtacgtgtcaacgagaccctcagaagtcttatctttga 5401 to 5475


                  BanII
                  Eco24I
                EcoICRI Psp124BI               PvuII
acccatctcaagaggagctcattaaggttgttgatgtggaggagcaacagctggaagagtctgggccacacgatt base pairs
tgggtagagttctcctcgagtaattccaacaactacacctcctcgttgtcgaccttctcagacccggtgtgctaa 5476 to 5550
                Ecl136II
                  SacI SstI
                  FriOI

                           BglII
tgacggaaacatcttacttgccaaggcaagatctagagggaacccccttacctggaatctggaatcagcctcttct base pairs
actgcctttgtagaatgaacggttccgttctagatctcccttggggaatggaccttagaccttagtcggagaaga 5551 to 5625
                              XbaI


                                    FriOI
                                    Eco24I          BsiI
ctgatgaccctgaatctgatccttctgaagacagagccccagagtcagctcgtgttggcaacataccatcttcaa base pairs
gactactgggacttagactaggaagacttctgtctcggggtctcagtcgagcacaaccgttgtatggtagaagtt 5626 to 5700
                                    BanII           BssSI


                     MfeI                           PvuII
cctctgcattgaaagttccccaattgaaagttgcagaatctgcccagagtccagctgctgctcatactactgata base pairs
ggagacgtaactttcaaggggttaactttcaacgtcttagacgggtctcaggtcgacgacgagtatgatgactat 5701 to 5775
                     MunI
```

```
                  BsrDI
ctgctgggtataatgcaatggaagaaagtgtgagcagggagaagccagaattgacagcttcaacagaaagggtca base pairs
gacgacccatattacgttaccttctttcacactcgtccctcttcggtcttaactgtcgaagttgtctttcccagt 5776 to 5850


                                                    BssSI
                                                   SspBI
            NcoI                                   Bsp1407I
acaaaagaatgtccatggtggtgtctggcctgaccccagaagaatttatgctcgtgtacaagtttgccagaaaac base pairs
tgttttcttacaggtaccaccacagaccggactggggtcttcttaaatacgagcacatgttcaaacggtcttttg 5851 to 5925
            Bsp19I                                 BsrGI
                                                    BsiI


accacatcactttaactaatctaattactgaagagactactcatgttgttatgaaaacagatgctgagtttgtgt base pairs
tggtgtagtgaaattgattagattaatgacttctctgatgagtacaacaatacttttgtctacgactcaaacaca 5926 to 6000


                                                                  BstPI Tth111I
                                                                  EcoO65I
                                                                  PspEI AtsI
gtgaacggacactgaaatattttctaggaattgcgggaggaaaatgggtagttagctatttctgggtgacccagt base pairs
cacttgcctgtgactttataaaagatccttaacgccctccttttacccatcaatcgataaagacccactgggtca 6001 to 6075
                                                                  Eco91I
                                                                  BstEII
                                                                        AspI


ctattaaagaaagaaaaatgctgaatgagcatgattttgaagtcagaggagatgtggtcaatggaagaaaccacc base pairs
gataatttctttcttttacgacttactcgtactaaaacttcagtctcctctacaccagttaccttctttggtgg 6076 to 6150









                                                                     BanII
                                                                     Eco24I
                                    BglII                          PspOMI
aaggtccaaagcgagcaagagaatcccaggacagaaagatcttcagggggctagaaatctgttgctatgggccct base pairs
ttccaggtttcgctcgttctcttagggtcctgtctttctagaagtcccccgatctttagacaacgatacccggga 6151 to 6225
                                                                  Bsp120I
                                                                     ApaI
                                                                     FriOI

                   XcmI                PvuII
tcaccaacatgcccacagatcaactggaatggatggtacagctgtgtggtgcttctgtggtgaaggagctttcat base pairs
agtggttgtacgggtgtctagttgaccttacctaccatgtcgacacaccacgaagacaccacttcctcgaaagta 6226 to 6300


                           MfeI            BsgI    BstXI
cattcacccttggcacaggtgtccacccaattgtggttgtgcagccagatgcctggacagaggacaatggcttcc base pairs
gtaagtgggaaccgtgtccacaggtgggttaacaccaacacgtcggtctacggacctgtctcctgttaccgaagg 6301 to 6375
                           MunI


                                BstPI Ama87I
      BstXI            AccB1I   EcoO65I
    MfeI               BshNI    PspEI Eco88I
atgcaattgggcagatgtgtgaggcacctgtggtgacccgagagtgggtgttggacagtgtagcactctaccagt base pairs
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    MunI               BanI     Eco91I BsoBI
                       Eco64I   BstEII BcoI
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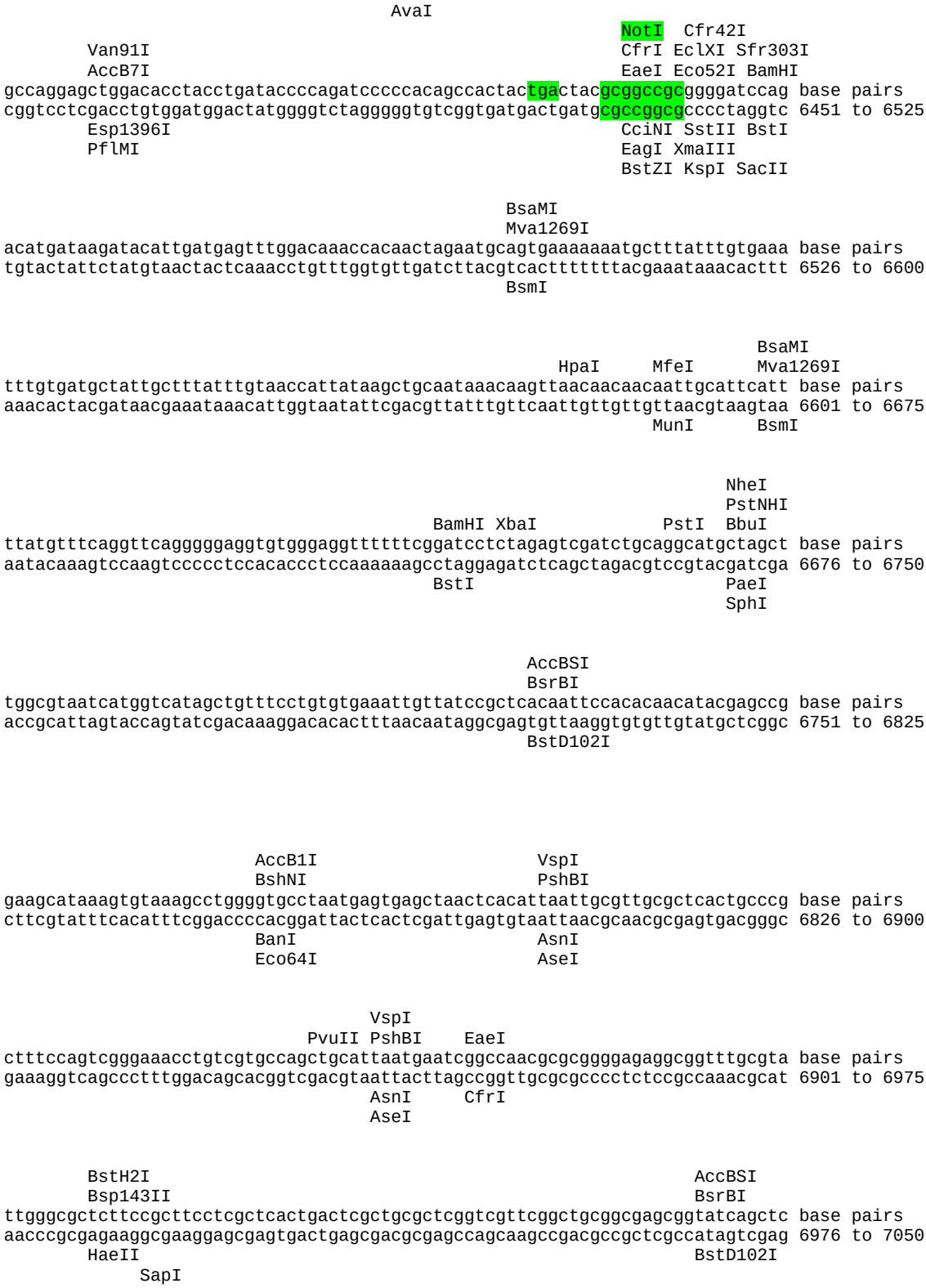
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PflMI EagI XmaIII
BstZI KspI SacII
BsaMI
Mva1269I
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BsmI
BsaMI
HpaI MfeI Mva1269I
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MunI BsmI
NheI
PstNHI
BamHI XbaI PstI BbuI
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BstI PaeI
SphI
AccBSI
BsrBI
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BstD102I
AccB1I VspI
BshNI PshBI
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BanI AsnI
Eco64I AseI
VspI
PvuII PshBI EaeI
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AsnI CfrI
AseI
BstH2I AccBSI
Bsp143II BsrBI
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HaeII BstD102I
SapI

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                                                    AflIII



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            DrdI
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       BsiI                       BsaWI
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       BssSI


   BstH2I                                                          Alw44I
   Bsp143II                                                        VneI
cgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacg base pairs
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   HaeII                                                           ApaLI


                                BsaWI
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ttgggggggcaagtcgggctggcgacgcggaataggccattgatagcagaactcaggttgggccattctgtgctga 7426 to 7500



tatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttga base pairs
atagcggtgaccgtcgtcggtgaccattgtcctaatcgtctcgctccatacatccgccacgatgtctcaagaact 7501 to 7575



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                        RcaI                                    DraI
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                        BspHI
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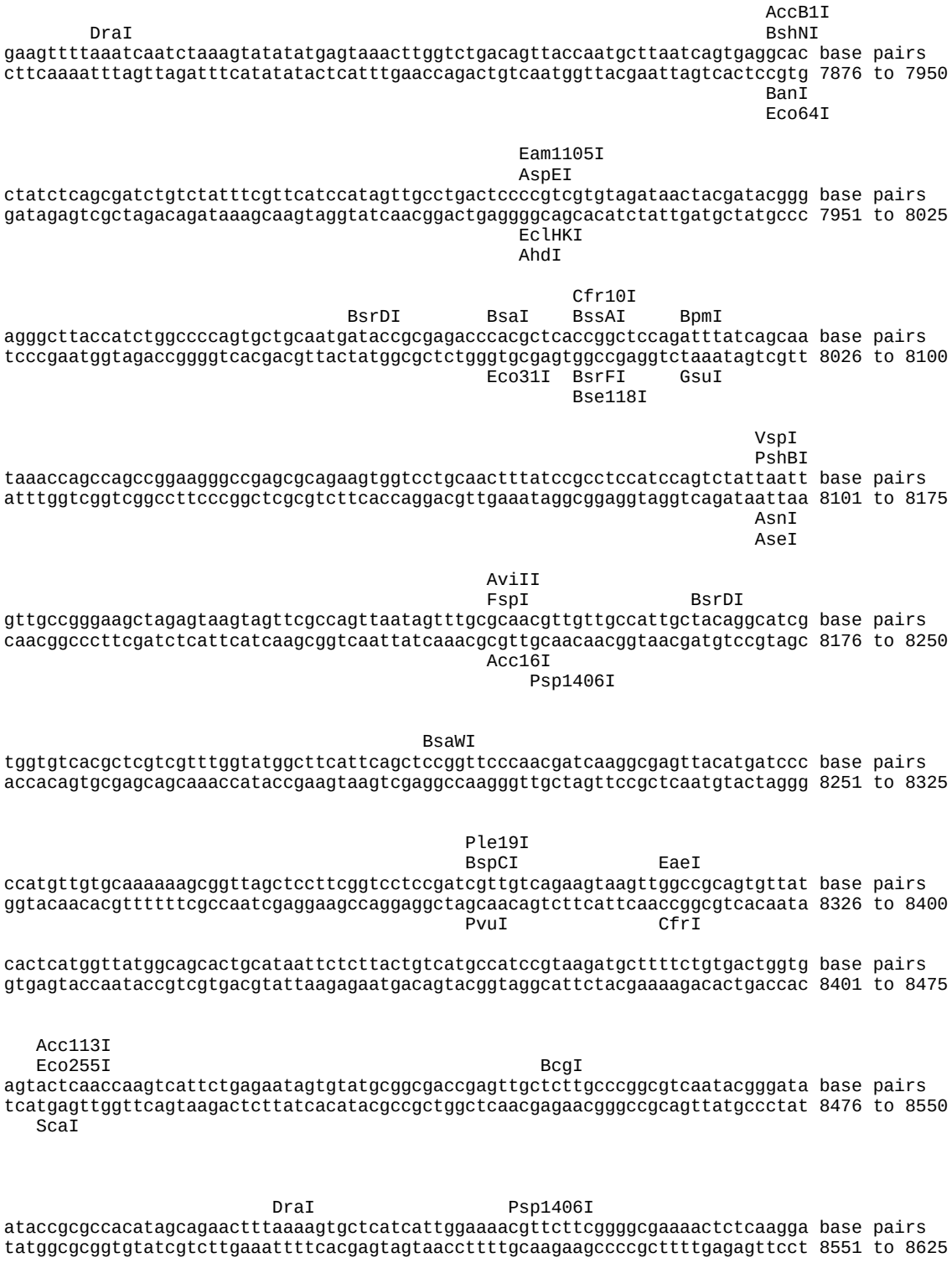
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DraI
BshNI
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BanI
Eco64I
Eam1105I
AspEI
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AhdI
Cfr10I
BsrDI
BsaI
BssAI
BpmI
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Eco31I
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GsuI
Bse118I
VspI
PshBI
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AsnI
AseI
AviII
FspI
BsrDI
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Psp1406I
BsaWI
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Ple19I
BspCI
EaeI
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PvuI
CfrI
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Eco255I
BcgI
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ScaI
DraI
Psp1406I
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```
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                                     Alw44I
                                     VneI
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                                     ApaLI
                                       BsiI


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                                                                 AccBSI
                                                        RcaI     BsrBI
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                                                        BspHI    BstD102I



                                                                   AatII
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            BspHI                               BssSI       BsmBI


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                                     BsmBI


      DrdI
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                                                                   Eco64I
           Alw44I                                                  BanI
           VneI   NdeI                                             KasI
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           ApaLI  FauNDI                                           BshNI
                                                                   AccB1I
                                                                    Nar

BbeI             AviII               Ple19I
EheI             FspI                BspCI                    PvuII
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 Bsp143II        Acc16I              PvuI
  BstH2I
 HaeII

                                                                     EaeI
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CfrI
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ccagt base pairs
ggtca 9376 to 9380

Table by Enzyme Name

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Acc16I	2	8220 9243	tgc/gca
Acc65I	1	3224	g/gtacc
AccB1I	6	431 3224 6398 6849 7946 9220	g/gyrcc
AccB7I	2	1292 6458	ccannnn/ntgg
AccBSI	3	6800 7041 8842	gagcgg
AccI	4	895 1444 3848 4632	gt/mkac
AccIII	1	617	t/ccgga
AclNI	2	2783 4398	a/ctagt
AflII	1	2330	c/ttaag
AflIII	2	1890 7105	a/crygt
AhdI	1	7998	gacnnn/nngtc
Alw44I	3	7419 8665 9162	g/tgcac
Ama87I	1	6412	c/ycgrg
AocI	2	2452 4183	cc/tnagg
ApaI	2	824 6223	gggcc/c
ApaLI	3	7419 8665 9162	g/tgcac
AseI	6	955 4042 4084 6876 6935 8170	at/taat
AsnI	6	955 4042 4084 6876 6935 8170	at/taat
Asp718I	1	3224	g/gtacc
AspEI	1	7998	gacnnn/nngtc
AspI	1	6071	gacn/nngtc
AtsI	1	6071	gacn/nngtc
AvaI	1	6412	c/ycgrg
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BalI	2	874 2208	tgg/cca
BamHI	5	630 708 4850 6518 6716	g/gatcc
BanI	6	431 3224 6398 6849 7946 9220	g/gyrcc
BanII	6	534 824 4077 5494 5663 6223	grgcy/c
BbeI	1	9224	ggcgc/c
BbuI	3	17 1055 6744	gcatg/c
BcgI	2	907 8525	cgannnnnntgc
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BcoI	1	6412	c/ycgrg
BfrI	1	2330	c/ttaag
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Bpu1102I	2	1847 4467	gc/tnagc
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BsaMI	5	1942 2049 4156 6573 6672	gaatgc
BsaWI	6	617 633 711 7311 7458 8289	w/ccggw
Bse118I	1	8078	r/ccggy
Bse21I	2	2452 4183	cc/tnagg
BseAI	1	617	t/ccgga
BsgI	4	1671 3330 4813 6344	gtgcag
BshNI	6	431 3224 6398 6849 7946 9220	g/gyrcc
BsiI	5	5679 5906 7283 8667 8974	ctcgtg
BsiMI	1	617	t/ccgga
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BsmI	5	1942 2049 4156 6573 6672	gaatgc
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Bsp120I	2	820 6219	g/ggccc
Bsp13I	1	617	t/ccgga
Bsp1407I	2	3668 5905	t/gtaca

Bsp143II	4	3509 6983 7353 9224	rgcgc/y
Bsp1720I	2	1847 4467	gc/tnagc
Bsp19I	5	326 827 875 910 5863	c/catgg
BspCI	2	8368 9264	cgat/cg
BspEI	1	617	t/ccgga
BspHI	5	2219 2567 7825 8833 8938	t/catga
BspLU11I	2	1890 7105	a/catgt
BspMI	4	26 868 2935 5238	acctgc
BspTI	1	2330	c/ttaag
BsrBI	3	6800 7041 8842	gagcgg
BsrDI	3	5795 8057 8239	gcaatg
BsrFI	1	8078	r/ccggy
BsrGI	2	3668 5905	t/gtaca
BssAI	1	8078	r/ccggy
BssSI	5	5679 5906 7283 8667 8974	ctcgtg
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BstEII	3	4512 6065 6407	g/gtnacc
BstH2I	4	3509 6983 7353 9224	rgcgc/y
BstI	5	630 708 4850 6518 6716	g/gatcc
BstPI	3	4512 6065 6407	g/gtnacc
BstSNI	1	306	tac/gta
BstXI	4	992 4908 6352 6381	ccannnnn/ntgg
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CelII	2	1847 4467	gc/tnagc
Cfr10I	1	8078	r/ccggy
Cfr42I	2	819 6515	ccgc/gg
CfrI	6	872 2206 6509 6944 8386 9373	y/ggccr
CvnI	2	2452 4183	cc/tnagg
DraI	4	4019 7864 7883 8575	ttt/aaa
DraIII	1	1024	cacnnn/gtg
DrdI	2	7213 9082	gacnnnn/nngtc
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EagI	1	6509	c/ggccg
Eam1105I	1	7998	gacnnn/nngtc
Ecl136II	2	532 5492	gag/ctc
EclHKI	1	7998	gacnnn/nngtc
EclXI	1	6509	c/ggccg
Eco105I	1	306	tac/gta
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EcoICRI	2	532 5492	gag/ctc
EcoNI	2	2437 4767	cctnn/nnnagg
EcoO65I	3	4512 6065 6407	g/gtnacc
EcoRI	2	887 1818	g/aattc
EcoT22I	1	4337	atgca/t
EheI	1	9222	ggc/gcc
Esp1396I	2	1292 6458	ccannnn/ntgg
Esp3I	5	566 1604 5047 8987 9041	cgtctc
FauNDI	2	200 9169	ca/tatg
FbaI	1	1000	t/gatca
FriOI	6	534 824 4077 5494 5663 6223	grgcy/c
FspI	2	8220 9243	tgc/gca
GsuI	6	563 998 2943 4081 5135 8088	ctggag
HaeII	4	3509 6983 7353 9224	rgcgc/y
HindIII	1	900	a/agctt
HpaI	4	666 3016 3422 6653	gtt/aac

KasI	1	9220	g/gcgcc
Kpn2I	1	617	t/ccgga
KpnI	1	3228	ggtac/c
Ksp22I	1	1000	t/gatca
KspI	2	819 6515	ccgc/gg
MfeI	4	5721 6328 6379 6662	c/aattg
MluNI	2	874 2208	tgg/cca
Mph1103I	1	4337	atgca/t
MroI	1	617	t/ccgga
MscI	2	874 2208	tgg/cca
MspCI	1	2330	c/ttaag
MunI	4	5721 6328 6379 6662	c/aattg
Mva1269I	5	1942 2049 4156 6573 6672	gaatgc
NarI	1	9221	gg/cgcc
NcoI	5	326 827 875 910 5863	c/catgg
NdeI	2	200 9169	ca/tatg
NheI	2	4788 6744	g/ctagc
NotI	1	6509	gc/ggccgc
NsiI	1	4337	atgca/t
PaeI	3	17 1055 6744	gcatg/c
PflMI	2	1292 6458	ccannnn/ntgg
Ple19I	2	8368 9264	cgat/cg
Pme55I	3	769 2443 5323	agg/cct
Ppu10I	1	4333	a/tgcat
PpuMI	4	702 861 3580 5231	rg/gwccy
PshAI	1	1440	gacnn/nngtc
PshBI	6	955 4042 4084 6876 6935 8170	at/taat
Psp124BI	2	534 5494	gagct/c
Psp1406I	2	8224 8597	aa/cgtt
Psp5II	4	702 861 3580 5231	rg/gwccy
PspEI	3	4512 6065 6407	g/gtnacc
PspOMI	2	820 6219	g/ggccc
PstI	5	23 3692 4722 5354 6738	ctgca/g
PstNHI	2	4788 6744	g/ctagc
PvuI	2	8368 9264	cgat/cg
PvuII	6	1671 5525 5754 6266 6929 9293	cag/ctg
RcaI	5	2219 2567 7825 8833 8938	t/catga
SacI	2	534 5494	gagct/c
SacII	2	819 6515	ccgc/gg
SalI	1	894	g/tcgac
SapI	5	930 1167 4578 4986 6988	gctcttc
SbfI	1	23	cctgca/gg
ScaI	2	4061 8478	agt/act
SexAI	3	3026 3227 4726	a/ccwggt
SfiI	1	880	ggccnnnn/nggcc
Sfr303I	2	819 6515	ccgc/gg
SnaBI	1	306	tac/gta
SpeI	2	2783 4398	a/ctagt
SphI	3	17 1055 6744	gcatg/c
Sse8387I	1	23	cctgca/gg
SseBI	3	769 2443 5323	agg/cct
SspBI	2	3668 5905	t/gtaca
SstI	2	534 5494	gagct/c
SstII	2	819 6515	ccgc/gg
StuI	3	769 2443 5323	agg/cct
Tth111I	1	6071	gacn/nngtc
Van91I	2	1292 6458	ccannnn/ntgg
Vha464I	1	2330	c/ttaag
VneI	3	7419 8665 9162	g/tgcac
VspI	6	955 4042 4084 6876 6935 8170	at/taat
XbaI	3	624 5582 6722	t/ctaga
XcmI	1	6245	ccannnnn/nnnntgg
XmaIII	1	6509	c/ggccg
Zsp2I	1	4337	atgca/t

APPENDIX B: Characteristics of cell lines

(MCF7, U2OS, HBL-100)

APPENDIX B: Characteristics of cell lines (MCF7, U2OS, HBL-100)

ATCC Number: HTB-22

Organism: Homo sapiens (human)

Designations: **MCF7**

Tissue: adenocarcinoma; mammary gland; pleural effusion

Age/Stage: 69 years

Gender: female

Ethnicity: Caucasian

Morphology: epithelial

Oncogene: wnt7h +

Karyotype: The stemline chromosome numbers ranged from hypertriploidy to hypotetraploidy, with the 2S component occurring at 1%. There were 29 to 34 marker chromosomes per S metaphase; 24 to 28 markers occurred in at least 30% of cells, and generally one large submetacentric (M1) and 3 large subtelocentric (M2, M3, and M4) markers were recognizable in over 80% of metaphases.
No DM were detected. Chromosome 20 was nullisomic and X was disomic.

FreezeMedium: Culture medium, 95%; DMSO, 5%

FluidRenewal: 2 to 3 times weekly

SubCulturing: Remove medium, add fresh 0.25% trypsin - 0.03% EDTA solution for 1 minute, remove and let the culture sit at room temperature for 5 to 10 minutes. Add fresh medium, aspirate and dispense into new flasks. Subculture every 6 to 8 days.

SplitRatio: A ratio of 1:3 to 1:6 is recommended

Receptors expressed: estrogen

Growth Properties: monolayer

Comments: The MCF7 line retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. Contains the Tx-4 oncogene. Growth of MCF7 cells is inhibited by tumor necrosis factor alpha (TNF alpha). Secretion of IGFBP's can be modulated by treatment with anti-estrogens.

ATCC Number: HTB-96

Organism: Homo sapiens (human)

Designations: **U2OS**

Age/Stage: 15 years

Gender: female

Ethnicity: Caucasian

Morphology: epithelial

Karyotype: (P11-46) hypodiploid to near tetraploid; (P111-118) modal numbers 34 to 37 and 64 to 67 with abnormalities including dicentrics, breaks, rings, and pulverizations plus acrocentric subtelocentric and minute markers

FreezeMedium: Culture medium, 95%; DMSO, 5%

FluidRenewal: 2 to 3 times weekly

SubCulturing: Remove medium, rinse with fresh 0.25% trypsin solution, remove trypsin and let the culture sit at room temperature (or at 37C) until the cells detach (about 10 minutes). Add fresh medium, aspirate and dispense into new flasks. Subculture every 6 to 8 days.

SplitRatio: A ratio of 1:3 to 1:6 is recommended

Receptors expressed: insulin-like growth factor I (IGF-I); insulin-like growth factor II (IGF-II)

Growth Properties: monolayer

Comments: J. Ponten and E. Saksela derived this line (originally 2T) in 1964 from a moderately differentiated sarcoma of the tibia of a 15 year old girl.

ATCC Number: HTB-124

Organism: Homo sapiens (human)

Designations: **HBL-100**

Tissue: mammary gland

Age/Stage: 27 years

Gender: female

Ethnicity: Caucasian

Morphology: epithelial

Tumorigenic: no (at passages below 25); yes, in nude mice at passage 35 and greater (tumorigenicity increases with increasing number of passages)

Karyotype: The stemline chromosome number is near triploid with the modal number of 67 chromosomes, and the 2S component occurring at 0.6%. Most chromosome complements consist of about 39 normal and 28 marker chromosomes. Markers such as 2q, 11q+, 11q, t(2q;12), t(2q;5q?), t(6p?;16), 16pt and many others are common to most metaphases. Normal chromosomes 11, 14, 15 and 16 are absent; 2, 12, 17 and 19 are monosomic, and the X is disomic. Double minutes (DM) were not detected.

FreezeMedium: Culture medium, 90%; glycerol, 10%

FluidRenewal: 2 to 3 times weekly

SubCulturing: Remove medium, rinse with fresh 0.25% trypsin, 0.02% EDTA solution, remove trypsin and let the culture sit at room temperature (or at 37C) until the cells detach (about 10 minutes). Add fresh medium, aspirate and dispense into new flasks. Subculture every 6 to 8 days.

SplitRatio: A ratio of 1:2 is recommended

Receptors expressed: estrogen; prolactin

Growth Properties: monolayer

Comments: HBL-100 is an epithelial cell line derived by E.V. Gaffney and associates from the milk of a nursing mother and obtained 3 days after delivery. Although there was no evidence of a breast lesion in the milk donor and she had no family history of breast cancer, the karyotype of the recovered cells was abnormal as early as passage 7. Tumorigenicity has been reported to increase with passage above passage 35. The cells contain a tamdemly integrated SV40 genome.

Appendix C: Phosphoimager values of 6 arrays

(500 clones)

Array numbers (1-6), position of the clones on arrays, and clone numbers are indicated.

F: Phosphoimager values of forward subtracted probe hybridized array

R: Phosphoimager values of reverse subtracted probe hybridized array

F long, R long, F/R long: Values of the same arrays exposed longer.

Average: Average values of short and long exposures

Appendix C: Phosphoimager values of 6 arrays (500 clones)

Array #	Position	Clone #	F	R	F/R	F long	R long	F/R long	Average
1	A1	**24**	1548,66	953,27	**1,62**	3252,71	2451,77	**1,33**	**1,48**
	A2	**25**	1009,25	777,04	**1,30**	2489,47	1706,65	**1,46**	**1,38**
	A3	**26**	3052,55	1217,11	**2,51**	6368,23	2635,04	**2,42**	**2,46**
	A4	**27**	2458,93	1138,24	**2,16**	4845,04	3281,44	**1,48**	**1,82**
	A5	**28**	11488,49	11464,24	**1,00**	21482,51	21653,73	**0,99**	**1,00**
	A6	**31**	3970,83	1187,75	**3,34**	8482,75	2946,56	**2,88**	**3,11**
	A7	**33**	3888,88	865,7	**4,49**	9127,14	3168,98	**2,88**	**3,69**
	A8	**34**	1727,59	870,51	**1,98**	4202,48	2556,39	**1,64**	**1,81**
	A9	**35**	18816,54	6441,36	**2,92**	34208,55	12099,38	**2,83**	**2,87**
	A10	**36**	4758,83	1374,67	**3,46**	10488,32	3195,05	**3,28**	**3,37**
	A11	**37**	2110,01	1639,85	**1,29**	4423,61	3712,62	**1,19**	**1,24**
	A12	**38**	2021,29	1604	**1,26**	4368,23	3816,01	**1,14**	**1,20**
	B1	**39**	2109,61	1125,44	**1,87**	4375,88	1694,1	**2,58**	**2,23**
	B2	**40**	1976,89	1025,57	**1,93**	4317,11	1410,65	**3,06**	**2,49**
	B3	**41**	6335,44	1044,88	**6,06**	12057,34	1642,93	**7,34**	**6,70**
	B4	**42**	1332,91	752,64	**1,77**	3024,82	1980,65	**1,53**	**1,65**
	B5	**43**	1419,52	933,24	**1,52**	3302,9	2311,16	**1,43**	**1,48**
	B6	**44**	3511,34	807,41	**4,35**	7202,19	1846,98	**3,90**	**4,12**
	B7	**45**	21230,76	8680,85	**2,45**	38797,67	15980,63	**2,43**	**2,44**
	B8	**46**	1531,89	2629,23	**0,58**	3226,75	5316,24	**0,61**	**0,59**
	B9	**47**	3319,04	838,19	**3,96**	6661,1	2245,07	**2,97**	**3,46**
	B10	**48**	14323,05	590,61	**24,25**	24933,52	1582,08	**15,76**	**20,01**
	B11	**50**	1249,62	770,23	**1,62**	2745,4	2091,57	**1,31**	**1,47**
	B12	**51**	3008,45	875,08	**3,44**	6624,29	2928,83	**2,26**	**2,85**
	C1	**52**	3740,87	1654,67	**2,26**	7476,66	2499,89	**2,99**	**2,63**
	C2	**53**	3277,08	999,98	**3,28**	6746,94	1273,43	**5,30**	**4,29**
	C3	**55**	5018,27	861,68	**5,82**	9853,51	1502,52	**6,56**	**6,19**
	C4	**56**	2641,56	1234,26	**2,14**	6249,89	3173,72	**1,97**	**2,05**
	C5	**57**	6834,85	1599,08	**4,27**	14259,1	2458,47	**5,80**	**5,04**
	C6	**58**	1576,07	855,42	**1,84**	3448,24	1987,98	**1,73**	**1,79**
	C7	**59**	3407,52	754,23	**4,52**	6908,55	2776,95	**2,49**	**3,50**
	C8	**60**	1465,13	723,42	**2,03**	3165,95	1660,01	**1,91**	**1,97**
	C9	**61**	2273,4	711,09	**3,20**	4838,75	1511,78	**3,20**	**3,20**
	C10	**62**	3144,95	715,06	**4,40**	6267,07	1524,04	**4,11**	**4,26**
	C11	**63**	3499,75	1187,26	**2,95**	7464,52	2326,93	**3,21**	**3,08**
	C12	**64**	10773,2	2770,74	**3,89**	20906,2	5139,38	**4,07**	**3,98**
	D1	**65**	1510,63	1444,39	**1,05**	3577,17	2129,36	**1,68**	**1,36**
	D2	**66**	3577,22	1120,87	**3,19**	7508,07	1445,48	**5,19**	**4,19**
	D3	**67**	4592,32	829,75	**5,53**	9389,58	1677,8	**5,60**	**5,57**
	D4	**68**	4446,49	2024,46	**2,20**	9472,67	4321,81	**2,19**	**2,19**
	D5	**70**	35403,01	35125,26	**1,01**	64663,77	61704,97	**1,05**	**1,03**
	D6	**71**	3355,81	1058,05	**3,17**	7039,21	2411,55	**2,92**	**3,05**
	D7	**74**	1733,92	955,78	**1,81**	4049,18	3783,63	**1,07**	**1,44**
	D8	**76**	2189,75	1272,89	**1,72**	5897,07	3318,81	**1,78**	**1,75**
	D9	**77**	5114,85	1800,48	**2,84**	10414,51	3050,65	**3,41**	**3,13**
	D10	**78**	1488,52	741,46	**2,01**	2990,47	1561,41	**1,92**	**1,96**
	D11	**80**	7437,93	1890,24	**3,93**	14246,7	3480,74	**4,09**	**4,01**
	D12	**81**	12929,53	7405,33	**1,75**	24233,83	13655,26	**1,77**	**1,76**
	E1	**82**	6026,38	1656,64	**3,64**	12349,18	3313,79	**3,73**	**3,68**
	E2	**83**	27234,54	15692,98	**1,74**	50217,52	28039,48	**1,79**	**1,76**
	E3	**84**	11185,21	2277,01	**4,91**	20723,94	4186,31	**4,95**	**4,93**
	E4	**85**	5398,15	1865,33	**2,89**	10439,44	3762,11	**2,77**	**2,83**
	E5	**86**	3794,69	1566,95	**2,42**	7217,83	4263,52	**1,69**	**2,06**
	E6	**87**	7626,15	1830,39	**4,17**	15407,55	4552,06	**3,38**	**3,78**

	E7	**89**	4597,16	1656,25	**2,78**	10800,08	2847,65	**3,79**	**3,28**
	E8	**90**	40438,86	14809,05	**2,73**	75754,38	23045,77	**3,29**	**3,01**
	E9	**91**	3117,39	996,8	**3,13**	6212,94	2158,6	**2,88**	**3,00**
	E10	**92**	1043,46	2171,32	**0,48**	2332,54	4153,53	**0,56**	**0,52**
	E11	**93**	4819,66	715,51	**6,74**	9428,83	1398,12	**6,74**	**6,74**
	E12	**94**	2191,89	806,46	**2,72**	3957,29	1480,6	**2,67**	**2,70**
	F1	**95**	10125,92	9700,65	**1,04**	19335,2	17630,78	**1,10**	**1,07**
	F2	**96**	2691,28	1401,92	**1,92**	5373,01	3943,23	**1,36**	**1,64**
	F3	**97**	2251,08	1233,76	**1,82**	4518,32	3805,25	**1,19**	**1,51**
	F4	**98**	5814,33	1330,75	**4,37**	11145,43	4673,49	**2,38**	**3,38**
	F5	**99**	2983,9	1360,96	**2,19**	6047,41	6374,26	**0,95**	**1,57**
	F6	**100**	2329,77	1814,11	**1,28**	5554,26	7837,03	**0,71**	**1,00**
	F7	**101**	66303	61225,94	**1,08**	125967,3	111106,9	**1,13**	**1,11**
	F8	**102**	4882,9	3535,5	**1,38**	9178,86	6048,07	**1,52**	**1,45**
	F9	**103**	6270,97	6804	**0,92**	12407,86	12251,67	**1,01**	**0,97**
	F10	**104**	1786,29	1431,37	**1,25**	3999,45	3022,98	**1,32**	**1,29**
	F11	**105**	1467,47	837,36	**1,75**	3165,7	1655,36	**1,91**	**1,83**
	F12	**106**	1065,49	746,63	**1,43**	2248,08	1496,85	**1,50**	**1,46**
	G1	**107**	4768,29	1629,82	**2,93**	9967,32	6259,73	**1,59**	**2,26**
	G2	**108**	28019,74	18034,24	**1,55**	52161,42	34741,07	**1,50**	**1,53**
	G3	**109**	3313,89	890,84	**3,72**	6676,84	3852,25	**1,73**	**2,73**
	G4	**110**	1470,48	878,05	**1,67**	2981,27	3090,74	**0,96**	**1,32**
	G5	**111**	2218,38	761,78	**2,91**	4512,64	2247,16	**2,01**	**2,46**
	G6	**112**	1429,75	1124,8	**1,27**	3131,72	2117,49	**1,48**	**1,38**
	G7	**113**	4837,25	1159,62	**4,17**	9245,16	1931,95	**4,79**	**4,48**
	G8	**114**	3819,02	790,38	**4,83**	7781,45	1459,36	**5,33**	**5,08**
	G9	**115**	5672,75	775,69	**7,31**	11408,14	1536,93	**7,42**	**7,37**
	G10	**116**	6616,37	4294,64	**1,54**	12539,59	7786,66	**1,61**	**1,58**
	G11	**118**	4455,38	641,56	**6,94**	8553,3	1361,78	**6,28**	**6,61**
	G12	**119**	1614,76	894,17	**1,81**	3501,78	1835,86	**1,91**	**1,86**
	H1	**GADD45**	1634,34	949,09	**1,72**	3143,84	1682,91	**1,87**	**1,80**
	H2	**GADD45**	1771,49	1010,25	**1,75**	3165,96	2079,91	**1,52**	**1,64**
	H3	**neg PCR**	856,3	822,75	**1,04**	1888,6	1670,88	**1,13**	**1,09**
	H4	**GADPH**	2632,19	4898,89	**0,54**	5237,3	9214,35	**0,57**	**0,55**
	H5	**cDNA1**	804,73	668,62	**1,20**	1771,64	1406,5	**1,26**	**1,23**
	H6	**cDNA1**	962,99	657,03	**1,47**	2308,54	1348,62	**1,71**	**1,59**
	H7	**BRCA1**	8962,31	606,93	**14,77**	17553,83	1337,15	**13,13**	**13,95**
	H8	**BRCA1**	13280,44	568,85	**23,35**	25351,56	1555,45	**16,30**	**19,82**
	H9	**cDNA2**	1033,68	594,18	**1,74**	2119,33	2081,33	**1,02**	**1,38**
	H10	**cDNA2**	939,14	647,71	**1,45**	1823,95	1857,97	**0,98**	**1,22**
	H11	**actin**	1642,91	976,47	**1,68**	3263,96	2079,39	**1,57**	**1,63**
	H12	**H2O**	659,81	497,42	**1,33**	2057,27	1248,01	**1,65**	**1,49**
2	A1	**120**	480,95	237,97	**2,02**	1397,12	627,1	**2,23**	**2,12**
	A2	**121**	5479,54	271,76	**20,16**	10852,34	682,27	**15,91**	**18,03**
	A3	**122**	1069,97	309,23	**3,46**	2331,06	767,68	**3,04**	**3,25**
	A4	**123**	1758	370,38	**4,75**	3821,2	975,56	**3,92**	**4,33**
	A5	**124**	1588,4	304,77	**5,21**	3286,5	894,02	**3,68**	**4,44**
	A6	**125**	1007,53	223,19	**4,51**	2578,91	610,7	**4,22**	**4,37**
	A7	**126**	1348,8	210,36	**6,41**	3447,04	531,47	**6,49**	**6,45**
	A8	**127**	1199,41	403,83	**2,97**	3005,32	831,91	**3,61**	**3,29**
	A9	**128**	1557,34	322,78	**4,82**	8425,74	635,45	**13,26**	**9,04**
	A10	**129**	1844,64	465,3	**3,96**	5062,52	1075,19	**4,71**	**4,34**
	A11	**130**	747,08	376,83	**1,98**	2204,74	1455,75	**1,51**	**1,75**
	A12	**131**	2111,46	408,69	**5,17**	5373,77	1056,28	**5,09**	**5,13**
	B1	**132**	827,9	186,92	**4,43**	2260,16	528,31	**4,28**	**4,35**
	B2	**133**	3790	234,85	**16,14**	7016,96	550,42	**12,75**	**14,44**
	B3	**134**	9450,9	3462,84	**2,73**	16158,32	6736,5	**2,40**	**2,56**
	B4	**135**	4906,27	4222,59	**1,16**	8741,52	8263,21	**1,06**	**1,11**
	B5	**136**	1798,71	441,61	**4,07**	3377,22	1197,14	**2,82**	**3,45**
	B6	**137**	1405,76	232,24	**6,05**	3062,99	608,56	**5,03**	**5,54**
	B7	**138**	1359,63	189,38	**7,18**	2968,34	480,24	**6,18**	**6,68**
	B8	**139**	986,53	259,71	**3,80**	2459,24	549,04	**4,48**	**4,14**
	B9	**140**	49974,8	17835,4	**2,80**	87909,53	32370,4	**2,72**	**2,76**
	B10	**141**	5726,61	1352,48	**4,23**	7168,27	2012,69	**3,56**	**3,90**

B11	**142**	6748,53	4165,73	**1,62**	11494,01	8128,83	**1,41**	**1,52**
B12	**143**	5626,08	1807,06	**3,11**	8481,63	3204,31	**2,65**	**2,88**
C1	**144**	680,47	269,84	**2,52**	1892	763,02	**2,48**	**2,50**
C2	**145**	1301,8	393,55	**3,31**	2650,6	858,07	**3,09**	**3,20**
C3	**146**	1095,46	331,94	**3,30**	2236,16	726,3	**3,08**	**3,19**
C4	**147**	2575,2	422,65	**6,09**	5145,31	886,66	**5,80**	**5,95**
C5	**148**	664,07	223,52	**2,97**	1517,7	552,17	**2,75**	**2,86**
C6	**149**	795,46	240,29	**3,31**	1966,57	570,05	**3,45**	**3,38**
C7	**150**	2049,98	326,89	**6,27**	4126,03	694,7	**5,94**	**6,11**
C8	**151**	611,91	213,93	**2,86**	1870,35	499,14	**3,75**	**3,30**
C9	**152**	1238,2	690,07	**1,79**	3156,25	902,64	**3,50**	**2,65**
C10	**153**	5358,37	638,29	**8,39**	10058,57	1186,43	**8,48**	**8,44**
C11	**156**	1793,22	512,62	**3,50**	3227,99	1063,78	**3,03**	**3,27**
C12	**157**	3884,78	1694,07	**2,29**	6141,31	3156,41	**1,95**	**2,12**
D1	**158**	5261,87	348,65	**15,09**	9481,25	946,89	**10,01**	**12,55**
D2	**159**	942,46	223,03	**4,23**	1639,66	527,78	**3,11**	**3,67**
D3	**160**	532,73	194,53	**2,74**	1252,99	512,42	**2,45**	**2,59**
D4	**161**	889,48	225,76	**3,94**	2065,73	550,06	**3,76**	**3,85**
D5	**162**	1909,7	310,65	**6,15**	3725,93	727,73	**5,12**	**5,63**
D6	**163**	5224,43	387,56	**13,48**	9775,73	860,63	**11,36**	**12,42**
D7	**164**	2656,1	254,75	**10,43**	5039,11	648,32	**7,77**	**9,10**
D8	**165**	4165,34	218,5	**19,06**	7861,17	594	**13,23**	**16,15**
D9	**166**	948,14	275,35	**3,44**	3877,54	642,33	**6,04**	**4,74**
D10	**167**	1291,04	935,02	**1,38**	3237,66	1644,72	**1,97**	**1,67**
D11	**168**	868,75	372,97	**2,33**	2089,23	727,33	**2,87**	**2,60**
D12	**169**	1721,1	464,94	**3,70**	3452,88	997,87	**3,46**	**3,58**
E1	**170**	3334,34	313,71	**10,63**	6536,69	889,96	**7,34**	**8,99**
E2	**171**	4286,37	436,95	**9,81**	7787,14	868,3	**8,97**	**9,39**
E3	**172**	1214,2	339,09	**3,58**	2322,13	783,84	**2,96**	**3,27**
E4	**173**	3525,36	689,71	**5,11**	6605,91	1705,53	**3,87**	**4,49**
E5	**174**	2887,38	385,53	**7,49**	5388,31	945,59	**5,70**	**6,59**
E6	**175**	2558,26	1584,07	**1,61**	4776,79	3073,44	**1,55**	**1,58**
E7	**176**	4932,14	1216,08	**4,06**	9173,18	2527,65	**3,63**	**3,84**
E8	**177**	1848,33	236,42	**7,82**	4697,94	828,69	**5,67**	**6,74**
E9	**179**	4587,43	470,86	**9,74**	10115,02	1083,29	**9,34**	**9,54**
E10	**180**	1820,2	365,34	**4,98**	3544,16	844,25	**4,20**	**4,59**
E11	**181**	762,02	286,1	**2,66**	1946,83	648,27	**3,00**	**2,83**
E12	**182**	1212,64	294,38	**4,12**	2029,11	929,34	**2,18**	**3,15**
F1	**183**	1356,12	187,29	**7,24**	2634,62	863,28	**3,05**	**5,15**
F2	**184**	469,31	304,81	**1,54**	1183,66	454,1	**2,61**	**2,07**
F3	**185**	934,04	734,37	**1,27**	1942,83	757,26	**2,57**	**1,92**
F4	**186**	390,17	374,29	**1,04**	917,33	1422,18	**0,65**	**0,84**
F5	**187**	594,63	824,42	**0,72**	1294,88	1896,24	**0,68**	**0,70**
F6	**188**	879,97	1090,25	**0,81**	1922,13	1925,13	**1,00**	**0,90**
F7	**189**	1457,12	2312,76	**0,63**	3086,15	4705,69	**0,66**	**0,64**
F8	**190**	5101,88	345,35	**14,77**	10607,91	1369,6	**7,75**	**11,26**
F9	**191**	21079,12	3352,63	**6,29**	35759,68	6663,67	**5,37**	**5,83**
F10	**192**	2500,53	321,47	**7,78**	3661,74	1059,49	**3,46**	**5,62**
F11	**193**	955,23	327,1	**2,92**	1844,73	794,88	**2,32**	**2,62**
F12	**194**	18073,74	4782,65	**3,78**	30562,91	9695,1	**3,15**	**3,47**
G1	**195**	320,48	183,59	**1,75**	926,01	1276,95	**0,73**	**1,24**
G2	**197**	592,51	237,84	**2,49**	1394,07	602,75	**2,31**	**2,40**
G3	**198**	1965,66	608,3	**3,23**	3778,18	605,14	**6,24**	**4,74**
G4	**199**	576,75	461,17	**1,25**	1248,26	1002,78	**1,24**	**1,25**
G5	**200**	718,45	396,56	**1,81**	1469,85	1046,78	**1,40**	**1,61**
G6	**211**	822,7	985,62	**0,83**	1782,41	974,4	**1,83**	**1,33**
G7	**212**	1490,81	803,24	**1,86**	3423,98	1878,59	**1,82**	**1,84**
G8	**213**	3105,26	301,59	**10,30**	5955,64	1479,66	**4,03**	**7,16**
G9	**214**	2854,34	407,78	**7,00**	3207,62	725,99	**4,42**	**5,71**
G10	**215**	2085,93	332,6	**6,27**	3195,81	727,28	**4,39**	**5,33**
G11	**216**	6671,44	5336,26	**1,25**	11555,52	10657,92	**1,08**	**1,17**
G12	**217**	3107,5	773,79	**4,02**	5881,44	1356,11	**4,34**	**4,18**
H1	**GADD45**	564,17	464,72	**1,21**	1331,31	1590,2	**0,84**	**1,03**
H2	**GADD45**	558,12	486,01	**1,15**	1304,47	1232,98	**1,06**	**1,10**
H3	**neg PCR**	231,26	438,99	**0,53**	753,6	677,53	**1,11**	**0,82**

	H4	**GADPH**	1036,56	4045,89	**0,26**	2107,35	7748,05	**0,27**	**0,26**
	H5	**cDNA1**	354,54	535,3	**0,66**	849,74	1499,63	**0,57**	**0,61**
	H6	**cDNA1**	347,89	1121,24	**0,31**	994,37	1294,07	**0,77**	**0,54**
	H7	**BRCA1**	2175,22	753,96	**2,89**	4756,15	2097,57	**2,27**	**2,58**
	H8	**BRCA1**	7796,33	298,25	**26,14**	14587,27	1408,4	**10,36**	**18,25**
	H9	**cDNA2**	1176,36	246,01	**4,78**	960,83	653	**1,47**	**3,13**
	H10	**cDNA2**	572,26	316,89	**1,81**	1007,94	642,7	**1,57**	**1,69**
	H11	**actin**	1680,57	3136,65	**0,54**	3387,46	6158,32	**0,55**	**0,54**
	H12	**H2O**	490,52	235,11	**2,09**	1181,82	717,26	**1,65**	**1,87**
3	A1	**218**	1523,71	367,18	**4,15**	3154,58	863,84	**3,65**	**3,90**
	A2	**219**	2023,97	1103,14	**1,83**	4148,98	2189,65	**1,89**	**1,86**
	A3	**232**	705,1	605,69	**1,16**	1629,56	1221,24	**1,33**	**1,25**
	A4	**233**	642,92	473,32	**1,36**	1502,28	987,28	**1,52**	**1,44**
	A5	**234**	1016,69	361,06	**2,82**	2171,15	785,72	**2,76**	**2,79**
	A6	**235**	472,63	307,69	**1,54**	1070,5	702,11	**1,52**	**1,53**
	A7	**236**	481,1	338,57	**1,42**	1123,31	760,87	**1,48**	**1,45**
	A8	**237**	1699,82	633,8	**2,68**	3381,94	1415,22	**2,39**	**2,54**
	A9	**238**	717,46	513,82	**1,40**	1739,22	1420,4	**1,22**	**1,31**
	A10	**239**	2906,75	1345,01	**2,16**	5872,16	2720,32	**2,16**	**2,16**
	A11	**240**	758,02	450,74	**1,68**	1619,52	1027,66	**1,58**	**1,63**
	A12	**241**	890,06	636,1	**1,40**	1781,5	1385,01	**1,29**	**1,34**
	B1	**242**	1292,06	877,54	**1,47**	2740,29	1928,32	**1,42**	**1,45**
	B2	**243**	1705,07	1185,93	**1,44**	3458,6	2374,43	**1,46**	**1,45**
	B3	**244**	1075,4	477,98	**2,25**	2373,15	1157,5	**2,05**	**2,15**
	B4	**245**	1559,33	849,59	**1,84**	3407,01	1832,45	**1,86**	**1,85**
	B5	**246**	2734,91	468,32	**5,84**	5615,55	1021,58	**5,50**	**5,67**
	B6	**247**	1261,78	749,74	**1,68**	2630,42	1670,4	**1,57**	**1,63**
	B7	**248**	1986,88	809,42	**2,45**	4004,41	1747,97	**2,29**	**2,37**
	B8	**249**	970,01	641,67	**1,51**	2268,85	1558,85	**1,46**	**1,48**
	B9	**250**	15046,64	6296,65	**2,39**	28597,18	12831,41	**2,23**	**2,31**
	B10	**251**	4085,51	1011,54	**4,04**	8358,32	1974,74	**4,23**	**4,14**
	B11	**252**	2130,82	1059,46	**2,01**	4357,8	2394,71	**1,82**	**1,92**
	B12	**253**	3973,64	1473,95	**2,70**	8116,1	3168,28	**2,56**	**2,63**
	C1	**254**	967,75	646,48	**1,50**	2132,25	1395,5	**1,53**	**1,51**
	C2	**255**	1462,01	1561,39	**0,94**	3147,74	3132,58	**1,00**	**0,97**
	C3	**256**	2617,19	857,24	**3,05**	6145,96	1924,51	**3,19**	**3,12**
	C4	**257**	10933,33	3163,21	**3,46**	20502,84	6241,67	**3,28**	**3,37**
	C5	**258**	1049,63	679,71	**1,54**	2165,94	1355,44	**1,60**	**1,57**
	C6	**259**	3236,43	634,48	**5,10**	6700,91	1446	**4,63**	**4,87**
	C7	**260**	883,44	493,27	**1,79**	1907,49	1167,98	**1,63**	**1,71**
	C8	**261**	3178,4	1917,71	**1,66**	6537,17	3928,85	**1,66**	**1,66**
	C9	**262**	3210,52	2409,65	**1,33**	6590,11	4788,36	**1,38**	**1,35**
	C10	**264**	21157,04	6298,73	**3,36**	39769,84	12478,15	**3,19**	**3,27**
	C11	**265**	15939,46	8237,41	**1,94**	30202,11	16349,97	**1,85**	**1,89**
	C12	**266**	13432,28	14357,1	**0,94**	25534,16	27813,9	**0,92**	**0,93**
	D1	**267**	627,68	397,6	**1,58**	1529,03	870,52	**1,76**	**1,67**
	D2	**268**	2524,51	394,91	**6,39**	5276,39	928,58	**5,68**	**6,04**
	D3	**269**	2399,18	720,2	**3,33**	5479,07	1797,06	**3,05**	**3,19**
	D4	**270**	18836,63	6199,39	**3,04**	35911,26	12163,6	**2,95**	**3,00**
	D5	**271**	1999,62	872,52	**2,29**	3927,45	1816,99	**2,16**	**2,23**
	D6	**272**	4252,35	4213,74	**1,01**	8726,1	8531,55	**1,02**	**1,02**
	D7	**273**	1382,08	770,07	**1,79**	2825,49	1654,6	**1,71**	**1,75**
	D8	**274**	1044,08	840,25	**1,24**	2167,88	1850,79	**1,17**	**1,21**
	D9	**275**	988,43	704,44	**1,40**	1950,5	1565,63	**1,25**	**1,32**
	D10	**276**	1345,51	662,07	**2,03**	2648,85	1409,11	**1,88**	**1,96**
	D11	**277**	2749,62	934,68	**2,94**	5452	1858,06	**2,93**	**2,94**
	D12	**278**	2351,86	1271,82	**1,85**	4747,88	2002,14	**2,37**	**2,11**
	E1	**279**	757,87	932,41	**0,81**	1717,05	1989,72	**0,86**	**0,84**
	E2	**280**	764	538,18	**1,42**	1782,91	1223,07	**1,46**	**1,44**
	E3	**281**	4459,39	1018,33	**4,38**	9239,7	2188,35	**4,22**	**4,30**
	E4	**282**	2141,25	626,08	**3,42**	4198,42	1534,64	**2,74**	**3,08**
	E5	**283**	10206,1	4913,54	**2,08**	19327,47	9838,19	**1,96**	**2,02**
	E6	**284**	1720,68	1087,96	**1,58**	3426,58	2302,95	**1,49**	**1,53**
	E7	**285**	4877,85	2430,38	**2,01**	9802,98	4913,47	**2,00**	**2,00**

	E8	**286**	2707,78	1270,78	**2,13**	5436,77	2689,84	**2,02**	**2,08**
	E9	**287**	999,28	753,06	**1,33**	2097,46	1818,16	**1,15**	**1,24**
	E10	**288**	1931,64	1120,92	**1,72**	3896,34	2448,85	**1,59**	**1,66**
	E11	**289**	1414,57	776,52	**1,82**	2816,28	1733,03	**1,63**	**1,72**
	E12	**290**	1977,51	1122,15	**1,76**	3895,52	2231,68	**1,75**	**1,75**
	F1	**291**	579,65	6037,73	**0,10**	1310,48	11512,76	**0,11**	**0,10**
	F2	**292**	571,75	629,49	**0,91**	1297,75	1274,93	**1,02**	**0,96**
	F3	**293**	1727,02	551,17	**3,13**	3524,73	1755,74	**2,01**	**2,57**
	F4	**294**	5385,59	1746,8	**3,08**	11016,33	3072,5	**3,59**	**3,33**
	F5	**295**	3185,27	662,65	**4,81**	6223,85	1584,27	**3,93**	**4,37**
	F6	**297**	948,61	574,91	**1,65**	2052,22	1341,64	**1,53**	**1,59**
	F7	**299**	1559,74	1487,12	**1,05**	3283,05	3216,22	**1,02**	**1,03**
	F8	**300**	11860,48	4375,25	**2,71**	22701,47	9084,47	**2,50**	**2,60**
	F9	**303**	1280,91	1382,83	**0,93**	2558,23	2923,2	**0,88**	**0,90**
	F10	**304**	1208,49	582,15	**2,08**	2449,37	1345,85	**1,82**	**1,95**
	F11	**305**	1075,84	532,05	**2,02**	2220,96	1162,3	**1,91**	**1,97**
	F12	**306**	1527,47	742,04	**2,06**	3087,52	1543,38	**2,00**	**2,03**
	G1	**307**	504,9	427,73	**1,18**	1042,69	1143,87	**0,91**	**1,05**
	G2	**308**	2196,31	2079,48	**1,06**	4386,68	4160,78	**1,05**	**1,06**
	G3	**309**	1218,46	466,21	**2,61**	2513,77	1232,71	**2,04**	**2,33**
	G4	**310**	1869,22	1003,88	**1,86**	3615,69	2227,46	**1,62**	**1,74**
	G5	**311**	828,36	521,5	**1,59**	1765,73	1216,55	**1,45**	**1,52**
	G6	**312**	1398,17	528,57	**2,65**	2839,06	1224,94	**2,32**	**2,48**
	G7	**313**	1730,57	464,34	**3,73**	3550,02	1141,16	**3,11**	**3,42**
	G8	**314**	3279,41	529,03	**6,20**	6431,47	1231,97	**5,22**	**5,71**
	G9	**315**	1397,42	691,62	**2,02**	2751,82	1507,34	**1,83**	**1,92**
	G10	**316**	1845,31	1311,42	**1,41**	3668,54	2705,14	**1,36**	**1,38**
	G11	**319**	1385,4	799,11	**1,73**	2758,95	1602,04	**1,72**	**1,73**
	G12	**320**	1107,15	598,14	**1,85**	2252,82	1258,47	**1,79**	**1,82**
	H1	**GADD45**	337	317,92	**1,06**	740,66	815,84	**0,91**	**0,98**
	H2	**GADD45**	455,98	342,03	**1,33**	962,02	873,52	**1,10**	**1,22**
	H3	**neg PCR**	363,98	280,41	**1,30**	785,29	927,15	**0,85**	**1,07**
	H4	**GADPH**	474,43	2893,85	**0,16**	962	5759,84	**0,17**	**0,17**
	H5	**cDNA1**	390,96	341,86	**1,14**	880,01	797,67	**1,10**	**1,12**
	H6	**cDNA1**	444,61	296,48	**1,50**	958,43	759,45	**1,26**	**1,38**
	H7	**BRCA1**	807,3	302,42	**2,67**	1688,11	718,62	**2,35**	**2,51**
	H8	**BRCA1**	764,42	305,1	**2,51**	1542,38	743,6	**2,07**	**2,29**
	H9	**cDNA2**	424,97	295,26	**1,44**	905,9	720,39	**1,26**	**1,35**
	H10	**cDNA2**	439,46	350,45	**1,25**	869,25	851,41	**1,02**	**1,14**
	H11	**actin**	910,13	1995,74	**0,46**	1845,26	3905,94	**0,47**	**0,46**
	H12	**H2O**	384,14	316,33	**1,21**	768,58	745,66	**1,03**	**1,12**
4	A1	**321**	6001,38	957,61	**6,27**				
	A2	**322**	1069,6	858,47	**1,25**				
	A3	**323**	3965,85	3431,85	**1,16**				
	A4	**324**	1157,52	490,46	**2,36**				
	A5	**325**	841,41	823,46	**1,02**				
	A6	**326**	3345,14	1397,66	**2,39**				
	A7	**327**	985,1	2217,21	**0,44**				
	A8	**328**	19445,38	25876,97	**0,75**				
	A9	**329**	25333,42	39019,93	**0,65**				
	A10	**331**	4162,46	1281,56	**3,25**				
	A11	**332**	4490,38	1934,85	**2,32**				
	A12	**333**	3054,28	6095,83	**0,50**				
	B1	**334**	3235,46	3158,95	**1,02**				
	B2	**335**	314,63	428,22	**0,73**				
	B3	**336**	1603,92	4778,12	**0,34**				
	B4	**337**	1734,44	3266,33	**0,53**				
	B5	**338**	2693,29	4487,75	**0,60**				
	B6	**339**	1877,24	761,72	**2,46**				
	B7	**340**	518,38	1135,04	**0,46**				
	B8	**341**	1118,62	1856,67	**0,60**				
	B9	**342**	1411,95	1543,59	**0,91**				
	B10	**343**	15256,37	17045,52	**0,90**				
	B11	**344**	1540,6	1798,56	**0,86**				

B12	**345**	1364,43	2375,03	**0,57**
C1	**346**	2025,52	319,26	**6,34**
C2	**347**	243,46	303,29	**0,80**
C3	**348**	519,45	314,73	**1,65**
C4	**349**	601,14	425,61	**1,41**
C5	**350**	695,8	1830,97	**0,38**
C6	**352**	952,6	307,57	**3,10**
C7	**353**	1734,5	829,42	**2,09**
C8	**354**	638,47	456,66	**1,40**
C9	**355**	790,1	442,52	**1,79**
C10	**356**	645,9	486,16	**1,33**
C11	**357**	1259,74	415,43	**3,03**
C12	**358**	4295,84	395,57	**10,86**
D1	**359**	1053,2	408,91	**2,58**
D2	**360**	530,11	304,39	**1,74**
D3	**361**	508,77	233,21	**2,18**
D4	**362**	2682,13	615,14	**4,36**
D5	**364**	460,65	419,54	**1,10**
D6	**365**	1022,92	367,94	**2,78**
D7	**366**	2362,38	3160,43	**0,75**
D8	**367**	443,42	421,84	**1,05**
D9	**369**	477,9	312,21	**1,53**
D10	**370**	2758,64	316,17	**8,73**
D11	**371**	2598,74	477,33	**5,44**
D12	**372**	2639,41	986,16	**2,68**
E1	**373**	317,28	516,13	**0,61**
E2	**374**	304,16	291,76	**1,04**
E3	**375**	254,54	215,41	**1,18**
E4	**377**	281,33	217,41	**1,29**
E5	**378**	439,99	235,62	**1,87**
E6	**380**	935,85	2833,76	**0,33**
E7	**381**	918,19	329,07	**2,79**
E8	**382**	514,27	226,63	**2,27**
E9	**383**	951,51	433,57	**2,19**
E10	**384**	962,22	221,71	**4,34**
E11	**385**	1701,03	285,14	**5,97**
E12	**386**	659,08	309,96	**2,13**
F1	**387**	484,1	508,22	**0,95**
F2	**388**	293,31	337,8	**0,87**
F3	**390**	553,88	192,8	**2,87**
F4	**391**	444,87	226,11	**1,97**
F5	**392**	451,43	214,76	**2,10**
F6	**393**	782,49	2625,72	**0,30**
F7	**394**	2350,89	1914,26	**1,23**
F8	**395**	411,14	364,79	**1,13**
F9	**396**	455,5	236,35	**1,93**
F10	**397**	313,29	227,22	**1,38**
F11	**398**	901,75	341,54	**2,64**
F12	**400**	1209,13	456,15	**2,65**
G1	**401**	313,66	263,05	**1,19**
G2	**402**	712,79	284,86	**2,50**
G3	**403**	267,37	263,05	**1,02**
G4	**404**	641,48	283,97	**2,26**
G5	**405**	279,64	253,9	**1,10**
G6	**406**	823,86	287,9	**2,86**
G7	**407**	1307,94	2308,29	**0,57**
G8	**408**	800,96	341,28	**2,35**
G9	**409**	517,19	261,31	**1,98**
G10	**410**	931,91	210,1	**4,44**
G11	**411**	1548,57	277,01	**5,59**
G12	**412**	1392,51	600,47	**2,32**
H1	**GADD45**	322,1	1374,51	**0,23**
H2	**GADD45**	296,63	2134,65	**0,14**
H3	**neg PCR**	194,92	741,75	**0,26**
H4	**GADPH**	297,73	6627,27	**0,04**

	H5	**cDNA1**	301,99	367,84	**0,82**				
	H6	**cDNA1**	297,13	305,15	**0,97**				
	H7	**BRCA1**	1488,84	326,23	**4,56**				
	H8	**BRCA1**	1440,33	342,23	**4,21**				
	H9	**cDNA2**	317,48	348,2	**0,91**				
	H10	**cDNA2**	319,14	569,14	**0,56**				
	H11	**actin**	701,47	2719,74	**0,26**				
	H12	**H2O**	639,71	455,19	**1,41**				
5	A1	**413**	594,67	342,4	**1,74**	1837,77	762,1	**2,41**	**2,07**
	A2	**415**	3465,18	865,76	**4,00**	9738,55	2039,12	**4,78**	**4,39**
	A3	**416**	48177,87	25493,52	**1,89**	88723,84	46532,48	**1,91**	**1,90**
	A4	**417**	1280,71	525,6	**2,44**	2023,28	961,18	**2,10**	**2,27**
	A5	**418**	1941,51	672,22	**2,89**	3611,47	1325,03	**2,73**	**2,81**
	A6	**419**	1892,28	1137,69	**1,66**	3672,13	2169,06	**1,69**	**1,68**
	A7	**420**	3385,16	310,83	**10,89**	6680,38	664,07	**10,06**	**10,48**
	A8	**421**	2047,92	316,36	**6,47**	4039,48	800,23	**5,05**	**5,76**
	A9	**422**	1312,87	335,46	**3,91**	2707,18	1310,13	**2,07**	**2,99**
	A10	**423**	4856,86	552,41	**8,79**	9752,36	2237,52	**4,36**	**6,58**
	A11	**424**	1883,7	463,83	**4,06**	3751,1	1296,98	**2,89**	**3,48**
	A12	**426**	3615,76	559,9	**6,46**	7422,46	1185,06	**6,26**	**6,36**
	B1	**427**	1361,27	344,82	**3,95**	2971,32	971,33	**3,06**	**3,50**
	B2	**428**	1770,21	631,14	**2,80**	3990,96	1695,36	**2,35**	**2,58**
	B3	**429**	1414,86	801,14	**1,77**	3616,17	1026,3	**3,52**	**2,64**
	B4	**430**	6725,08	1100,91	**6,11**	13034,82	2152,48	**6,06**	**6,08**
	B5	**431**	3197,48	379,01	**8,44**	6321,17	802,46	**7,88**	**8,16**
	B6	**432**	1127,68	273,46	**4,12**	2256,87	564,68	**4,00**	**4,06**
	B7	**433**	735,79	364,91	**2,02**	1545,87	736,46	**2,10**	**2,06**
	B8	**434**	1911,2	317,06	**6,03**	3851,58	666,55	**5,78**	**5,90**
	B9	**435**	3394,94	2390,47	**1,42**	7042	4483,42	**1,57**	**1,50**
	B10	**436**	8772,47	6699,98	**1,31**	16339,03	11552,17	**1,41**	**1,36**
	B11	**437**	3379,15	1012,23	**3,34**	6833,71	1608,68	**4,25**	**3,79**
	B12	**438**	1042,82	257,13	**4,06**	2110,41	557,11	**3,79**	**3,92**
	C1	**439**	984,76	470,44	**2,09**	2473,09	1126,1	**2,20**	**2,14**
	C2	**440**	9632,9	10322,44	**0,93**	17648,67	18932,6	**0,93**	**0,93**
	C3	**441**	2019,59	357,1	**5,66**	3995,13	741,59	**5,39**	**5,52**
	C4	**442**	1409,91	455,89	**3,09**	2962,95	1022,25	**2,90**	**3,00**
	C5	**443**	3218,08	500,89	**6,42**	6335,1	1045,8	**6,06**	**6,24**
	C6	**444**	1772,94	262,6	**6,75**	3458,8	511,97	**6,76**	**6,75**
	C7	**445**	1814,2	357,13	**5,08**	3597,24	698,21	**5,15**	**5,12**
	C8	**446**	3492,74	394,66	**8,85**	6974,21	784,73	**8,89**	**8,87**
	C9	**447**	5034,06	305,02	**16,50**	10174,02	629,78	**16,15**	**16,33**
	C10	**448**	766,19	347,66	**2,20**	1635,77	683,19	**2,39**	**2,30**
	C11	**449**	707,94	289,88	**2,44**	1572,05	667,84	**2,35**	**2,40**
	C12	**450**	3109,19	407,56	**7,63**	6208	854,04	**7,27**	**7,45**
	D1	**451**	490,69	350,25	**1,40**	1456,74	734,48	**1,98**	**1,69**
	D2	**452**	3237,01	528,75	**6,12**	6674,63	898,52	**7,43**	**6,78**
	D3	**453**	5148,38	312,36	**16,48**	9917,09	725,85	**13,66**	**15,07**
	D4	**455**	1775,38	1278,09	**1,39**	3474,33	2512,81	**1,38**	**1,39**
	D5	**456**	1082,01	627,7	**1,72**	2209	1259,41	**1,75**	**1,74**
	D6	**457**	700,91	244,52	**2,87**	1531,38	558,12	**2,74**	**2,81**
	D7	**458**	2197,7	239,87	**9,16**	4337,04	526,62	**8,24**	**8,70**
	D8	**459**	581,35	239,18	**2,43**	1415,61	498,85	**2,84**	**2,63**
	D9	**460**	4152,05	297,73	**13,95**	8482,02	686,64	**12,35**	**13,15**
	D10	**461**	2360,56	500,1	**4,72**	4646,06	1028,61	**4,52**	**4,62**
	D11	**462**	1414,92	696,54	**2,03**	2769,92	1415,74	**1,96**	**1,99**
	D12	**463**	577,26	267,9	**2,15**	1286,7	647,95	**1,99**	**2,07**
	E1	**464**	874,41	304,21	**2,87**	1991,09	698,46	**2,85**	**2,86**
	E2	**465**	485,09	298,25	**1,63**	1335,02	721,64	**1,85**	**1,74**
	E3	**466**	475,39	315,89	**1,50**	1216,35	765,08	**1,59**	**1,55**
	E4	**468**	1089,24	434,7	**2,51**	2518,35	1067,92	**2,36**	**2,43**
	E5	**469**	3389,98	753,56	**4,50**	6189,73	1974,91	**3,13**	**3,82**
	E6	**470**	2564,36	357,15	**7,18**	4487,22	921,89	**4,87**	**6,02**
	E7	**471**	1777,71	286,98	**6,19**	3309,49	693,21	**4,77**	**5,48**
	E8	**472**	682,62	250,69	**2,72**	1897,65	580,01	**3,27**	**3,00**

	E9	**473**	9665,28	1086,13	**8,90**	17985,71	2165,09	**8,31**	**8,60**
	E10	**474**	1859,38	324,43	**5,73**	3612,17	667,66	**5,41**	**5,57**
	E11	**475**	2935,33	404,44	**7,26**	5801,47	984,08	**5,90**	**6,58**
	E12	**476**	2377,49	524,38	**4,53**	4668,3	1445,01	**3,23**	**3,88**
	F1	**479**	1868,62	386,32	**4,84**	3442,28	899,47	**3,83**	**4,33**
	F2	**480**	2559,53	1748,01	**1,46**	4905,81	3368,81	**1,46**	**1,46**
	F3	**482**	3866,58	2199,91	**1,76**	7861,11	4467,34	**1,76**	**1,76**
	F4	**484**	2491,12	691,23	**3,60**	6633,24	1708,33	**3,88**	**3,74**
	F5	**485**	36648,14	31498,04	**1,16**	65760,16	59234,81	**1,11**	**1,14**
	F6	**486**	4926,54	1703,67	**2,89**	6328,93	2878,22	**2,20**	**2,55**
	F7	**487**	1116,24	837,92	**1,33**	1896,4	1531,18	**1,24**	**1,29**
	F8	**491**	1139,42	973,35	**1,17**	2357,73	1960,83	**1,20**	**1,19**
	F9	**493**	938,67	311,12	**3,02**	2009,5	673,98	**2,98**	**3,00**
	F10	**494**	945,57	363,06	**2,60**	2215,48	774,58	**2,86**	**2,73**
	F11	**495**	17546,85	3763,37	**4,66**	32092,33	7413,8	**4,33**	**4,50**
	F12	**496**	28411,03	11815,08	**2,40**	53042,84	21852,14	**2,43**	**2,42**
	G1	**500**	38391,81	8802,13	**4,36**	70218,97	16582,31	**4,23**	**4,30**
	G2	**501**	941,81	332,46	**2,83**	1959,48	740,75	**2,65**	**2,74**
	G3	**502**	564,67	304,5	**1,85**	2207,93	718,75	**3,07**	**2,46**
	G4	**503**	1558,96	348,45	**4,47**	5905,94	884,52	**6,68**	**5,58**
	G5	**504**	3159,15	878,72	**3,60**	8046,43	2025,62	**3,97**	**3,78**
	G6	**506**	2269,74	448,52	**5,06**	3614,74	1038,13	**3,48**	**4,27**
	G7	**507**	3722,58	723,5	**5,15**	7218,46	1299,5	**5,55**	**5,35**
	H1	**GADD45**	1244,91	645,61	**1,93**	1761,46	1810,54	**0,97**	**1,45**
	H2	**GADD45**	772,79	439,54	**1,76**	1230,57	922,83	**1,33**	**1,55**
	H3	**neg PCR**	372,55	270,26	**1,38**	1118,93	615,94	**1,82**	**1,60**
	H4	**GADPH**	1244,51	3334,95	**0,37**	2980,18	6506,96	**0,46**	**0,42**
	H5	**cDNA1**	724,67	357,41	**2,03**	2069,1	749,41	**2,76**	**2,39**
	H6	**cDNA1**	998,71	304,69	**3,28**	1842,09	705,85	**2,61**	**2,94**
	H7	**BRCA1**	4730,51	464,53	**10,18**	9521,14	966,73	**9,85**	**10,02**
	H8	**BRCA1**	7844,02	389,18	**20,16**	14655,55	767,52	**19,09**	**19,62**
	H9	**cDNA2**	588,53	265,08	**2,22**	1128,37	611,28	**1,85**	**2,03**
	H10	**cDNA2**	559,26	285,47	**1,96**	1077,46	623,29	**1,73**	**1,84**
	H11	**actin**	1080,13	2092,15	**0,52**	2168,45	4095,37	**0,53**	**0,52**
	H12	**H2O**	362,83	287,12	**1,26**	984,03	660,67	**1,49**	**1,38**
6	A1	**999**	446,31	266,09	**1,68**	1008,96	604,96	**1,67**	**1,67**
	A2	**1000**	437,2	278,16	**1,57**	943,75	620,45	**1,52**	**1,55**
	A3	**1001**	786	415,89	**1,89**	1675,78	867,19	**1,93**	**1,91**
	A4	**1002**	1760,84	1520,55	**1,16**	3671,14	3699,81	**0,99**	**1,08**
	A5	**1003**	653,75	463,35	**1,41**	1585,88	1431,29	**1,11**	**1,26**
	A6	**1004**	1579,35	704,07	**2,24**	3258,92	1428,68	**2,28**	**2,26**
	A7	**1005**	732,32	965,78	**0,76**	1911,79	2735,53	**0,70**	**0,73**
	A8	**1006**	1573,82	645,15	**2,44**	3269,82	2220,89	**1,47**	**1,96**
	A9	**1007**	1140,25	579,28	**1,97**	2354,46	1277,97	**1,84**	**1,91**
	A10	**1008**	756,31	468,62	**1,61**	1601,69	918,45	**1,74**	**1,68**
	A11	**1009**	1707,47	3763,24	**0,45**	3214,77	5763,78	**0,56**	**0,51**
	A12	**1010**	16440,83	37122,43	**0,44**	30576,12	64456,21	**0,47**	**0,46**
	B1	**1011**	2558,23	603,11	**4,24**	5078,04	1207,07	**4,21**	**4,22**
	B2	**1012**	421,81	294,36	**1,43**	970,37	640,83	**1,51**	**1,47**
	B3	**1013**	1200,78	649,83	**1,85**	2455,32	1093,68	**2,25**	**2,05**
	B4	**1014**	19331,93	22293,22	**0,87**	36307,74	40892,38	**0,89**	**0,88**
	B5	**1015**	2842,64	4324,56	**0,66**	5840,51	8552,05	**0,68**	**0,67**
	B6	**1016**	2295,22	1108,6	**2,07**	4298,47	1823,06	**2,36**	**2,21**
	B7	**1018**	28292,81	27411,94	**1,03**	52167,17	49944,89	**1,04**	**1,04**
	B8	**1019**	1115,08	1096,53	**1,02**	3005,53	2530,05	**1,19**	**1,10**
	B9	**1020**	2593,73	506,74	**5,12**	5275,49	1110,67	**4,75**	**4,93**
	B10	**1021**	1193,86	387,87	**3,08**	2501,41	796,15	**3,14**	**3,11**
	B11	**1022**	931,09	836,42	**1,11**	1935,82	1566,1	**1,24**	**1,17**
	B12	**1024**	1640,34	878,38	**1,87**	3212,14	1740,63	**1,85**	**1,86**
	C1	**1025**	1168,68	392,83	**2,98**	2357,85	800,41	**2,95**	**2,96**
	C2	**1026**	1629,9	411,44	**3,96**	3229,76	852,47	**3,79**	**3,88**
	C3	**1027**	1471,35	375,14	**3,92**	2948,1	781,2	**3,77**	**3,85**
	C4	**1028**	1469,25	639,88	**2,30**	2940,01	1314,86	**2,24**	**2,27**
	C5	**1029**	3297,1	465,02	**7,09**	6669,19	1041,35	**6,40**	**6,75**

C6	**1031**	1756,77	546,46	**3,21**	3368,09	1080,02	**3,12**	**3,17**
C7	**1033**	1988,93	780,82	**2,55**	4169,92	1615,25	**2,58**	**2,56**
C8	**1034**	2382,7	458,74	**5,19**	5089,23	1035,53	**4,91**	**5,05**
C9	**1035**	1474,94	423,26	**3,48**	3016,16	940,94	**3,21**	**3,35**
C10	**1036**	613,24	474,52	**1,29**	1419,48	986,52	**1,44**	**1,37**
C11	**1037**	4449,82	654,98	**6,79**	8401,09	1343,57	**6,25**	**6,52**
C12	**1038**	1473,24	3434,05	**0,43**	3218,79	6642,13	**0,48**	**0,46**
D1	**1039**	2023,47	290,97	**6,95**	4074,2	615,44	**6,62**	**6,79**
D2	**1040**	5852,05	287,14	**20,38**	11154,27	608,24	**18,34**	**19,36**
D3	**1041**	558,08	308,23	**1,81**	1218,98	682,75	**1,79**	**1,80**
D4	**1042**	2082,41	338,1	**6,16**	4180,25	739,24	**5,65**	**5,91**
D5	**1044**	689,14	474,32	**1,45**	1506,4	944,28	**1,60**	**1,52**
D6	**1045**	2014,49	335,2	**6,01**	3991,26	744,16	**5,36**	**5,69**
D7	**1046**	1165,21	348,85	**3,34**	2380,5	782,29	**3,04**	**3,19**
D8	**1048**	989,27	1706,67	**0,58**	2068,51	3309,68	**0,62**	**0,60**
D9	**1049**	10807,35	484,59	**22,30**	20405,36	1087,14	**18,77**	**20,54**
D10	**1050**	3094,15	466,9	**6,63**	6718,79	1050,84	**6,39**	**6,51**
D11	**1051**	1329,75	683,65	**1,95**	2701,66	1345,57	**2,01**	**1,98**
D12	**1052**	1991,62	649,95	**3,06**	3605,93	1306,2	**2,76**	**2,91**
E1	**1053**	2432,08	457,48	**5,32**	4911,86	947,35	**5,18**	**5,25**
E2	**1055**	3058,17	480,37	**6,37**	6210,32	981,72	**6,33**	**6,35**
E3	**1056**	485,53	367,95	**1,32**	1096,57	762,5	**1,44**	**1,38**
E4	**1057**	1234,42	524,98	**2,35**	2491,84	1085,98	**2,29**	**2,32**
E5	**1058**	841,02	420,56	**2,00**	1759,83	844,76	**2,08**	**2,04**
E6	**1059**	1464,01	838,37	**1,75**	2944,33	1682,78	**1,75**	**1,75**
E7	**1060**	1929,47	277,38	**6,96**	3798,98	670,41	**5,67**	**6,31**
E8	**1061**	1118,34	666,06	**1,68**	2231,81	1331,81	**1,68**	**1,68**
E9	**1062**	21631,52	9872,58	**2,19**	39848,27	18193,55	**2,19**	**2,19**
E10	**1064**	4025,9	612,69	**6,57**	9074,27	1685,03	**5,39**	**5,98**
E11	**1065**	4269,26	470,24	**9,08**	8612,32	1017,52	**8,46**	**8,77**
E12	**1066**	1069,87	1168,28	**0,92**	2356,48	2254,73	**1,05**	**0,98**
F1	**1067**	1879,36	270,01	**6,96**	3776,6	607,66	**6,21**	**6,59**
F2	**1068**	513,62	325,3	**1,58**	1110	735,91	**1,51**	**1,54**
F3	**1069**	1287,12	245,6	**5,24**	2571,68	556,12	**4,62**	**4,93**
F4	**1070**	1129,41	323,81	**3,49**	2294,19	694,06	**3,31**	**3,40**
F5	**1071**	8572,25	580,74	**14,76**	16256,52	1216,9	**13,36**	**14,06**
F6	**1072**	3908,11	561,64	**6,96**	7708,11	1175,4	**6,56**	**6,76**
F7	**1073**	2899,74	238,22	**12,17**	5656,66	628,07	**9,01**	**10,59**
F8	**1074**	894,76	509,57	**1,76**	1946,35	1066,11	**1,83**	**1,79**
F9	**1075**	2980,33	389,68	**7,65**	6136,32	950,47	**6,46**	**7,05**
F10	**1076**	2003,62	354,41	**5,65**	4474,4	812,49	**5,51**	**5,58**
F11	**1077**	1234,17	1436,18	**0,86**	2687,87	2718,2	**0,99**	**0,92**
F12	**1078**	3666,89	385,75	**9,51**	7576,4	919,17	**8,24**	**8,87**
G1	**1079**	862,24	257,97	**3,34**	1780,36	592,75	**3,00**	**3,17**
G2	**1080**	1040,68	276,09	**3,77**	2092,05	607,02	**3,45**	**3,61**
G3	**1081**	1370,22	929,59	**1,47**	2682,78	1758,36	**1,53**	**1,50**
G4	**1082**	1759,75	572,91	**3,07**	3468,01	1182,97	**2,93**	**3,00**
G5	**1083**	9026,92	1395,6	**6,47**	17045,83	2681,78	**6,36**	**6,41**
G6	**1084**	3034,25	652,95	**4,65**	6176,51	1391,42	**4,44**	**4,54**
G7	**1085**	3138,61	344,44	**9,11**	6272,49	790,59	**7,93**	**8,52**
G8	**1087**	2085,57	434,66	**4,80**	4186,39	953,07	**4,39**	**4,60**
G9	**1088**	1133,91	282,26	**4,02**	2381,16	639,2	**3,73**	**3,87**
H1	**GADD45**	936,53	409,08	**2,29**	1888,61	858,98	**2,20**	**2,24**
H2	**GADD45**	834,27	523,42	**1,59**	1713,31	1068,46	**1,60**	**1,60**
H3	**neg PCR**	414,89	394,25	**1,05**	928,9	850,77	**1,09**	**1,07**
H4	**GADPH**	1747,41	4176,2	**0,42**	3404,74	7662,31	**0,44**	**0,43**
H5	**cDNA1**	528,86	385,13	**1,37**	1373,96	915,97	**1,50**	**1,44**
H6	**cDNA1**	545,34	335,92	**1,62**	1296,46	754,74	**1,72**	**1,67**
H7	**BRCA1**	4906,62	296,68	**16,54**	9497,72	657,48	**14,45**	**15,49**
H8	**BRCA1**	10319,32	336,14	**30,70**	19420,54	765,8	**25,36**	**28,03**
H9	**cDNA2**	590,97	291,21	**2,03**	1526,08	617,81	**2,47**	**2,25**
H10	**cDNA2**	336,49	364,53	**0,92**	820,45	717,38	**1,14**	**1,03**
H11	**actin**	795,51	3925,06	**0,20**	1572,64	7621,25	**0,21**	**0,20**
H12	**H2O**	244,67	421,84	**0,58**	608,12	1047,07	**0,58**	**0,58**

Appendix D: Actin normalized average values of 210 clones

Array numbers (7, 8, 9), position of the clones on arrays, and clone numbers are indicated.

F/R: Phosphoimager value ratio of forward/reverse subtracted probe hybridized array for 3 repeats.

Average: Average values three repeated F/R subtracted probe hybridized array

Appendix D: Actin normalized average values of 210 clones

Array	Position	Clone #	F/R 1	F/R 2	F/R 3	Average
7	A1	**33**	4,49	15,32	5,53	**8,18**
	A2	**41**	6,06	9,39	4,41	**6,83**
	A3	**44**	4,35	10,61	3,86	**6,20**
	A4	**48**	24,25	18,48	7,82	**15,44**
	A5	**51**	3,44	7,92	4,63	**5,13**
	A6	**53**	3,28	11,03	5,87	**7,07**
	A7	**55**	5,82	10,29	7,22	**7,90**
	A8	**57**	4,27	7,30	8,14	**6,83**
	A9	**59**	4,52	14,90	10,66	**9,69**
	A10	**62**	4,4	10,23	6,16	**6,88**
	A11	**64**	3,89	5,73	6,85	**5,52**
	A12	**66**	3,19	10,40	6,95	**7,18**
	B1	**67**	5,53	9,80	6,18	**7,18**
	B2	**71**	3,17	8,24	6,38	**5,89**
	B3	**82**	3,64	12,41	8,22	**8,11**
	B4	**84**	4,91	8,90	7,76	**7,20**
	B5	**87**	4,17	7,88	6,88	**6,18**
	B6	**89**	2,78	12,04	10,29	**8,54**
	B7	**93**	6,74	9,20	10,64	**8,86**
	B8	**94**	2,72	7,76	6,18	**5,55**
	B9	**98**	4,37	7,82	9,42	**6,87**
	B10	**107**	2,93	11,48	8,46	**7,40**
	B11	**113**	4,17	12,71	9,27	**8,82**
	B12	**114**	4,83	13,78	12,21	**10,36**
	C1	**115**	7,31	4,57	8,05	**6,66**
	C2	**118**	6,94	6,17	8,58	**7,12**
	C3	**121**	20,16	5,21	6,78	**10,01**
	C4	**123**	4,75	9,92	7,93	**7,39**
	C5	**124**	5,21	4,93	6,25	**5,21**
	C6	**125**	4,51	6,99	6,04	**5,80**
	C7	**126**	6,41	5,32	6,63	**6,13**
	C8	**128**	4,82	5,07	5,52	**6,54**
	C9	**129**	3,96	6,04	7,12	**5,83**
	C10	**131**	5,17	10,23	7,15	**7,51**
	C11	**132**	4,43	6,24	6,20	**5,60**
	C12	**133**	16,14	14,10	8,63	**12,39**
	D1	**136**	4,07	2,81	5,76	**4,00**
	D2	**137**	6,05	4,04	4,61	**4,73**
	D3	**138**	7,18	3,91	7,16	**5,92**
	D4	**141**	4,23	5,30	8,90	**6,03**
	D5	**147**	6,09	4,94	5,61	**5,50**
	D6	**150**	6,27	4,16	8,40	**6,22**
	D7	**153**	8,39	9,47	9,99	**9,30**
	D8	**156**	3,5	5,47	9,99	**6,24**
	D9	**158**	15,09	7,59	11,73	**10,62**
	D10	**162**	6,15	11,40	11,13	**9,39**
	D11	**163**	13,48	13,71	9,21	**11,78**
	D12	**164**	10,43	8,76	9,19	**9,02**
	E1	**165**	19,06	2,05	4,48	**7,56**
	E2	**169**	3,7	3,24	6,52	**4,45**
	E3	**170**	10,63	4,48	5,67	**6,38**
	E4	**171**	9,81	4,16	5,50	**6,35**
	E5	**172**	3,58	4,19	5,70	**4,39**
	E6	**173**	5,11	5,74	7,05	**5,76**
	E7	**174**	7,49	12,73	7,33	**8,89**
	E8	**176**	4,06	5,36	10,32	**6,51**
	E9	**177**	7,82	11,46	15,67	**11,29**
	E10	**179**	9,74	12,16	12,27	**11,32**
	E11	**183**	7,24	9,79	14,14	**9,69**
	E12	**190**	14,77	13,70	25,17	**16,71**
	F1	**191**	6,29	4,07	0,86	**3,59**
	F2	**192**	7,78	5,71	7,01	**6,12**

	F3	**194**	3,78	3,48	3,01	**3,32**
	F4	**198**	3,23	6,59	8,37	**6,56**
	F5	**212**	1,86	5,95	10,77	**6,19**
	F6	**213**	10,3	7,78	17,34	**10,76**
	F7	**214**	7	7,20	7,33	**6,75**
	F8	**215**	6,27	8,24	16,22	**9,93**
	F9	**217**	4,02	13,84	15,82	**11,28**
	F10	**218**	4,15	17,40	11,33	**10,88**
	F11	**234**	2,82	13,91	15,69	**10,80**
	F12	**237**	2,68	6,07	15,70	**8,10**
	G1	**239**	2,16	6,62	5,85	**4,88**
	G2	**244**	2,25	4,62	8,40	**5,06**
	G3	**246**	5,84	5,04	10,76	**7,16**
	G4	**248**	2,45	5,34	9,21	**5,64**
	G5	**251**	4,04	3,63	11,47	**6,41**
	G6	**252**	2,01	8,53	16,47	**8,97**
	G7	**253**	2,7	10,91	18,52	**10,69**
	G8	**256**	3,05	7,27	8,90	**6,43**
	G9	**259**	5,1	25,51	9,33	**13,24**
	G10	**264**	3,36	8,83	10,57	**7,56**
	G11	**268**	6,39	31,96	23,66	**20,55**
	G12	**269**	3,33	4,77	6,92	**4,96**
	H1	**GADD45**		2,62	0,68	**1,65**
	H2	**GADD45**		1,97	0,64	**1,30**
	H3	**neg PCR**		1,16	1,24	**1,20**
	H4	**GADPH**		0,60	1,13	**0,86**
	H5	**cDNA1**		1,81	1,36	**1,58**
	H6	**cDNA1**		5,71	1,39	**3,55**
	H7	**BRCA1**		37,99	16,36	**27,18**
	H8	**BRCA1**		29,79	18,26	**24,03**
	H9	**cDNA2**		7,76	1,49	**4,62**
	H10	**cDNA2**		6,08	1,21	**3,64**
	H11	**actin**		1,01	1,00	**1,00**
	H12	**H2O**		5,16	1,24	**3,20**
8	A1	**270**	3,04	1,92	5,41	**3,44**
	A2	**273**	1,79	1,60	5,72	**3,02**
	A3	**276**	2,03	2,98	6,15	**3,70**
	A4	**277**	2,94	7,03	12,37	**7,45**
	A5	**278**	1,85	4,40	8,78	**5,10**
	A6	**281**	4,38	2,18	8,17	**4,88**
	A7	**282**	3,42	9,29	13,00	**8,46**
	A8	**293**	3,13	11,19	7,15	**6,97**
	A9	**294**	3,08	4,60	8,55	**5,50**
	A10	**295**	4,81	10,16	3,82	**6,12**
	A11	**304**	2,08	5,73	4,25	**3,98**
	A12	**305**	2,02	9,62	6,79	**6,13**
	B1	**306**	2,06	3,72	7,71	**4,49**
	B2	**309**	2,61	6,58	4,97	**4,63**
	B3	**312**	2,65	5,79	5,91	**4,73**
	B4	**313**	3,73	7,28	10,15	**6,95**
	B5	**314**	6,2	16,21	13,24	**11,72**
	B6	**320**	1,85	4,47	6,20	**4,16**
	B7	**321**	6,27	5,91	8,74	**6,97**
	B8	**326**	2,39	10,40	9,60	**7,46**
	B9	**331**	3,25	11,27	7,84	**7,45**
	B10	**332**	2,32	10,27	4,76	**5,78**
	B11	**339**	2,46	9,61	10,40	**7,49**
	B12	**342**	0,91	7,87	6,78	**5,19**
	C1	**346**	6,34	8,37	14,05	**9,59**
	C2	**352**	3,1	3,79	5,04	**3,98**
	C3	**355**	1,79	3,64	6,36	**3,93**
	C4	**358**	10,86	4,60	6,42	**7,30**
	C5	**359**	2,58	4,59	12,11	**6,43**
	C6	**360**	1,74	4,11	11,81	**5,89**
	C7	**361**	2,18	5,64	11,50	**6,44**
	C8	**362**	4,36	5,67	7,00	**5,67**
	C9	**370**	8,73	5,87	7,03	**7,21**
	C10	**371**	5,44	15,37	6,34	**9,05**
	C11	**381**	2,79	5,11	6,93	**4,94**

	C12	**383**	2,19	3,17	7,09	**4,15**
	D1	**384**	4,34	3,79	9,81	**5,98**
	D2	**385**	5,97	3,67	9,02	**6,22**
	D3	**398**	2,64	3,47	8,99	**5,03**
	D4	**400**	2,65	3,79	10,84	**5,76**
	D5	**402**	2,5	4,47	13,29	**6,75**
	D6	**404**	2,26	4,75	16,94	**7,98**
	D7	**406**	2,86	6,35	19,52	**9,58**
	D8	**408**	2,35	5,04	11,72	**6,37**
	D9	**410**	4,44	5,39	11,94	**7,25**
	D10	**411**	5,59	6,79	10,80	**7,73**
	D11	**412**	2,32	2,93	6,32	**3,86**
	D12	**415**	4	6,09	14,04	**8,17**
	E1	**420**	10,89	10,23	13,83	**11,51**
	E2	**421**	6,47	4,07	8,54	**6,12**
	E3	**422**	3,91	3,63	7,49	**4,70**
	E4	**423**	8,79	14,34	19,76	**13,56**
	E5	**424**	4,06	7,83	16,30	**9,20**
	E6	**426**	6,46	8,27	11,58	**8,73**
	E7	**427**	3,95	9,02	13,33	**8,62**
	E8	**430**	6,11	5,63	11,43	**7,71**
	E9	**431**	8,44	9,16	14,13	**10,48**
	E10	**432**	4,12	7,71	12,04	**7,94**
	E11	**434**	6,03	9,21	6,93	**7,35**
	E12	**437**	3,34	4,99	10,16	**6,31**
	F1	**438**	4,06	4,75	8,29	**5,65**
	F2	**441**	5,66	3,57	7,88	**5,66**
	F3	**443**	6,42	5,53	11,10	**7,62**
	F4	**444**	6,75	4,89	15,03	**8,89**
	F5	**445**	5,08	5,29	13,80	**8,07**
	F6	**446**	8,85	5,81	9,36	**8,01**
	F7	**447**	16,5	5,64	16,21	**12,73**
	F8	**450**	7,63	7,31	15,66	**10,14**
	F9	**452**	6,12	7,25	14,70	**9,58**
	F10	**453**	16,48	4,46	10,60	**10,04**
	F11	**457**	2,87	5,26	8,30	**5,45**
	F12	**458**	9,16	8,14	16,68	**11,17**
	G1	**460**	13,95	7,91	12,29	**11,12**
	G2	**461**	4,72	6,95	10,06	**7,21**
	G3	**464**	2,87	3,79	8,08	**4,91**
	G4	**468**	2,51	5,02	9,59	**5,68**
	G5	**469**	4,5	6,57	11,65	**7,35**
	G6	**470**	7,18	8,85	11,86	**8,91**
	G7	**471**	6,19	6,69	11,08	**7,75**
	G8	**473**	8,9	6,72	17,13	**10,82**
	G9	**474**	5,73	9,72	12,15	**9,15**
	G10	**475**	7,26	7,35	12,17	**8,70**
	G11	**476**	4,53	5,43	9,81	**6,37**
	G12	**479**	4,84	6,19	15,49	**8,67**
	H1	**GADD45**		1,54	1,12	**1,33**
	H2	**GADD45**		2,10	0,94	**1,52**
	H3	**neg PCR**		2,04	1,46	**1,75**
	H4	**GADPH**		0,68	1,37	**1,02**
	H5	**cDNA1**		2,40	1,78	**2,09**
	H6	**cDNA1**		3,14	1,47	**2,31**
	H7	**BRCA1**		25,91	23,61	**24,76**
	H8	**BRCA1**		27,64	13,24	**20,44**
	H9	**cDNA2**		2,91	2,36	**2,64**
	H10	**cDNA2**		2,06	1,96	**2,01**
	H11	**actin**		1,00	1,01	**1,00**
	H12	**H2O**		1,80	1,83	**1,81**
9	A1	**484**	3,6	10,63	5,14	**6,50**
	A2	**486**	2,89	5,39	3,56	**3,83**
	A3	**501**	2,83	7,33	2,84	**4,30**
	A4	**503**	4,47	9,50	4,60	**6,56**
	A5	**506**	5,06	10,57	2,61	**5,82**
	A6	**507**	5,15	16,31	4,90	**8,85**
	A7	**1004**	2,24	15,45	4,14	**7,28**
	A8	**1006**	2,44	20,60	5,49	**9,35**

A9	**1011**	4,24	12,05	6,80	**7,69**
A10	**1013**	1,85	12,99	4,73	**6,59**
A11	**1016**	2,07	6,48	8,68	**5,79**
A12	**1020**	5,12	21,42	5,69	**10,68**
B1	**1021**	3,08	7,29	4,38	**4,93**
B2	**1026**	3,96	17,59	4,98	**8,81**
B3	**1029**	7,09	21,59	9,82	**12,72**
B4	**1034**	5,19	17,27	4,38	**8,90**
B5	**1037**	6,79	14,08	3,75	**8,12**
B6	**1039**	6,95	12,24	3,34	**7,45**
B7	**1040**	20,38	23,94	5,76	**16,35**
B8	**1042**	6,16	14,14	4,07	**8,04**
B9	**1045**	6,01	13,11	6,92	**8,57**
B10	**1049**	22,3	26,93	11,33	**19,60**
B11	**1050**	6,63	8,91	5,17	**6,87**
B12	**1052**	3,06	11,69	8,44	**7,68**
C1	**1053**	5,32	23,69	7,87	**12,27**
C2	**1055**	6,37	7,97	6,04	**6,79**
C3	**1060**	6,96	17,88	6,30	**10,16**
C4	**1064**	6,57	25,54	8,33	**13,28**
C5	**1065**	9,08	15,20	7,32	**10,43**
C6	**1067**	6,96	13,36	6,09	**8,68**
C7	**1069**	5,24	13,32	5,68	**7,98**
C8	**1070**	3,49	15,11	7,45	**8,66**
C9	**1071**	14,76	47,96	24,99	**29,00**
C10	**1072**	6,96	29,41	7,70	**14,62**
C11	**1073**	12,17	16,00	5,87	**10,82**
C12	**1075**	7,65	17,99	8,09	**11,05**
D1	**1076**	5,65	8,12	10,25	**7,98**
D2	**1078**	9,51	27,03	12,17	**16,02**
D3	**1079**	3,34	5,76	6,48	**5,14**
D4	**1080**	3,77	8,07	6,33	**6,00**
D5	**1083**	6,47	28,70	14,02	**16,38**
D6	**1085**	9,11	15,06	8,29	**10,62**
D7	**1087**	4,8	12,73	7,36	**8,23**
D8	**1088**	4,02	12,52	10,99	**9,13**
E1	**GADD45**		3,12	0,99	**2,41**
E2	**GADD45**		3,96	0,86	**2,41**
E3	**neg PCR**		2,51	1,35	**1,93**
E4	**GADPH**		0,66	1,09	**0,87**
E5	**cDNA1**		3,88	1,47	**2,68**
E6	**cDNA1**		4,95	1,21	**3,08**
E7	**BRCA1**		49,42	21,33	**35,37**
E8	**BRCA1**		47,40	19,95	**33,68**
E9	**cDNA2**		5,51	1,27	**3,39**
E10	**cDNA2**		3,69	1,29	**2,49**
E11	**actin**		0,99	1,01	**1,00**
E12	**H2O**		2,82	1,54	**2,18**

Appendix E: Sequencing results of the clones

Adaptor numbers (ad1 and ad2R) have been indicated. The sequence after the adaptors was used for BLAST search.

Appendix E: Sequencing results of the clones

Clone 33 (ad1)

TCNCTACGCCCTGGGCCCGTACGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCGGGAAT
TCGATT**TCGAGCGGCCGCCCGGGCAGGT**CTGTAGGGATACTGCTTCATAAGCACCAGCA
GAACACCAAAGGAGACCATATGGGTGAAAGCAACCAGCACTGCCCTGGCGCTTCATAGGT
TCTTAGAGTTTTTATCTTTTACTTTCAGTCTAACACAGCACTGCCTGCTTTTTGTTTTTGTTG
CTTGGTTTGTTTTTTTCTTACCGTGTTCACCAAACTTGTGTCCAAATAGCTTTGGGCTGATG
CAAAAATATCTATGTGGAAGAGAAGAAGTTGTTCTCATGGAGGGCCTTCAGATGAGTGCT
ATAGACTCTCTAGGCAACTCCAAGAGGCTTCTCAAGCAGGGTGGGCAGTGAGAGCTGCTA
TGG

Clone 41 (ad1)

ATTTTTACGNCCTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCGGGAAT
TCGATT**TCGAGCGGCCGCCCGGGCAGGT**TCAATCTCCAACAACATGCCAGTGATTTTAC
CAGCAAGAGTAGGGTGCATGGCTTGAATAAGAGGAAACAGCCGTTCACCCAACATTTGCT
TTTGCTCTTGAGGAGGGGCAGATGCCAACATGGAAGCAGTCAAAGGTTCCTGGCCTTGTA
CATGAACAGCAGGCTGTTGCATTGTAACTTGTGGCTGTGCATTAAGATGTTGCTGAGGATT
GCGAACTCCTGCAGCATATTTATACTGTGGAACGGTGCGGACAGCAGGAGTAGCTGCGGC
GGCTGCAGCTGAGGACGTGGACCCATTGTCTGTGTGATGTGTTAGCAACACGCTGTGTTGA
CATGACTCGTGGAAC

Clone 48 (ad1)

NTTNTTACGACCTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCGGGAAT
TCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACTTTCTATGAGAAGCGTATGGCCACAGAA
GTTGCTGCTGACGCTCTGGGTGAAGAATGGAAGGGTTATGTGGTCCGAATCAGTGGTGGG
AACGACAAACAAGGTTTCCCCATGAAGCAGGGTGTCTTGACCCATGGCCGTGTCCGCCTG
CTACTGAGTAAGGGGCATTCCTGTTACAGACCAAGGAGAACTGGAGAAAGAAAGAGAAA
ATCAGTTCCGTGGTTGCATTGTGGATGCAAATCTGAGCGTTCTCAACTTGGTTATTGTAAA
AAAAGGAGAGAAGGATATTCCTGGACTGACTGATACTACAGTGCCTCGCCGCCTGGGCCC
CAAAAGAGCTAGCAGAATCCGCAT

Clone 55 (ad2)

ATTNTTGCGNCCTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATT
CGATT**AGCGTGGTCGCGGCCGAGGT**ACTTCAGATGAGGAAATCTTTATCTCACAAGATG
AAATACAGTCATTTATGGCTAATAACCAGTCTTTCTACAGCAATAGAGAACAATACCGAC
AGCATCTGAAGGAGAAATTTAATAAATACTGCCGGTTAAATGATCACAAGAGGCCCATTT
GTAGTGGCTGGTTGACAACGGCTGGAGCAAATTAAATAAATAAAATAGCTCTGTCTTTCA
ATGAAACACTCACGATGACTACTGCGCCTTCTCTTTCGAAAAACTCTTAATTTAGTGACTT
ATGGCAAAATTTTATCTTAAATCAATGTGATTCTTTCTTGTTTTGGGAGACGGGGAAGGTA
TCCTCATTAGTTCTTTCTT

Clone 59 (ad1)

GNNCTCGTNATTGCGGTCANCGTNNNCGCGNATGNTTNCCGGCCGCCATNGACGGCCGCG
GGCAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACACCAAGCATGTGGTAAGTTAGA
AATGACAGGAAAGTGCTCCCCAAAGCAGTGGGAGAGGCAATGTGGTCTCTNACATCCATT
CAATGTGCATTCTTTTTCCTGGAATATCTGTTACCCANCCTTCTCAGGAACAAGAACCAGG
ACGACGGGGAGTATGTGTCAAATAAGTNCCTCGGCCGAGACCACNCTAATCTCTAGTGAA
TTCGCGGCCGCTGCAGGTCGACCNTATGGNAAAGNTCCCAACGCGTTGGATGCATAGCTT
GAGTATTCTATANTGTACCTA

Clone 66 (ad2)

ATTNTTTCGNCCTGGGCCCCGACGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCGGGAA
TTCGATT**AGCGTGGTCGCGGCCGAGGT**GTCAGAATAAAGAGTCCAGCAAAGATGATAC
CAAAGAGGCAAAAGAATCTAAGGAGAATAAGGAGGTATCAAGTCCCGATGATTTGGAAC
TTGAGTTGGAGAATCTAGAAATTAATGATGCCACCTTAGAATTAGAGGGTGGAGATGAAG
CTGAAGATCTTACAAAGAAACTTCTTGATGAACAAGAACAAGAAGATGAGGAAGCCAGC
ACTGGATCTCATCTCAAGCTCATAGTAGATGCTTTCCTACAGCAGTTACCCAACTGTGTCA
ACCGAGATCTGATAGACAAGGCAGCAATGGATTTTTGCATGAACATGAACACAAAAGCAA
ACAGGAAGAAGTTGGTACGGGCN

Clone 67 (ad2)

TTCTNGACTACGCTTCTTGNNTCCCGACGCNCGCATGCTCCCGGCCGCCATGGACGGCCGC
GGGCAATTCGATT**AGCGTGGTCGCGGCCGAGGT**ACGAAAATGGGACCAGACACATGAA
CAGCCTGGCCTAAATGAACTCCATCAATCTTAACTGTGACAGAAGTAATGGAGGATAAGG
AAAAGGCCAAGACTCTGATGTGTGTTGAGTGAAGAAGTTTTTCTAGTGGNTCATGTTCACC
ACTANTATAANGGANTGATTTTTANNNGTGATAAAAACCTGGCCTTTCCTAANATCAATTN
TTNTGNGCACANNAGANTNGATCGAATNNATNNANCCNCCCTGCNNGGCNNCGNCCANTT
NNTACNTATTCNNGNACCNTGTANNNNGACCNNTGGGANACTTTGACGCC

Clone 82

TTTATGANANNGAANNGTGNATNTCTAACAGTGGTNNCANTTATNNCCGTATATGGNNCG
ATNGNGANNTATCTATATGAGTAGATGGNNCACNGACCNATAGTANTATGATNAAGTCTT
TNATATGAATGAAAATGCACCCGNTGGATTTTTTTCNCCCTCATNACATTAGCTTGTATAC
ACGTGGGAATTGAAAGGNTTGCATTTTCTTNACATAGTTCTTGCTATGATGACTGANGGAC
TTTTTGCCCTCAACAAATTNATCAAGAAAAACAGGCCAAGCANCATNTTCATATANNNCC
TAANATCNANATGNACTGTTCTGNTGAANTCTAATACNTTGTACCTGCCCGGNNTAANGT
NNNANNTCCTTNNGANTNTNCNNNTCNNTGCANNNNCCAAGCNGACTAGATGNNNNNAN
TTNANCNTTNCCNANTTCNCTATAGGCACTCTAAANNANNTNATNNNNTNTCA

Clone 89 (ad1)

NTCTGCGCCCTGNGTCCGTATTGCNGATGCTNCCGNCCGCCATGGNCGGCCGCGGGAATT
CGATT**TCGAGCGGCCGCCCGGGCAGGT**ACAGAAAGTGAAAATGCTGTTACAATCTCAG
TGTAACTGGTAGCCACAGACGGTTGACTCACCAATCACAACTATCCACGCTACAGCAAGA
ATCTTACACAAAAACCTAACAACGCTTACAATTTTGTTGGGGTGAAGTCCACAAGCTTGGT
GGTAGATTACCAAGAGGGACTACATGGAAGGAAGGCGGAATGTCACAGGAACTATTCTTT
GGCTCTTTGGGTGGNGGGGTGTCCTCGGGTTTGTATCACAGCTACCTTTTTTTTTTTTTAA
ACAGNTNCAAAATCCNTGANCAGNCCANCCAATACTGAACAGTTNTGTTTNTGCNGGTNA
TNT

Clone 93 (ad2)

TTTTTTCGACNTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCGGGAATTC
GATT**AGCGTGGTCGCGGCCGAGGT**ACAGGTTTCCTAAAGACAAAAAAAAAATGGAGGAA
TCTGTAAACCAAATGCAGCCACTGGATGAGAAGCAGATAGCCAATTCTCAGGATGGATAT
GTATGGCAAGTCACTGACATGAATCGACTACACCGGTTCTTATGTTTCGGTTCTGAAGGTG
GGACTTATTATATCAAAGAACAGAAGTTGGGCCTTGAAAATGCTGAAGCTTTAATTAGAT
TGATTGAAGATGGCAGAGGATGTGAAGTGATACAAGAAATAAAGTCATATAGTCAAGAA
GGCAGAACCACAAAGCAAGAGCCTATGCTCTTTGCACTTGCCATTTGTTCCCAGTGCTCCG
ACATAAGCACAAAACAAC

Clone 107

NGCAGACNNCTCCNTNCTATCCCGNTGCTNCNNTCCGTACTTGGNCNGATCGCGANANTT
TATATTTCGTGCGGCACGNCCGTACAGGTACATCCAAAAGTCTCAGTGTAATAGCAGGAC
CAAAATATTCTGTCAATCAGCTGACCATATACTTAATGACTCCTAAAATCTCGTGGACTTC
TAAGAAAGCGCCATGGCCTGTGCTGCTGTTATGATTCCTGGGTTGTTGCGGTGCTCTGTTG
GAGCCATCCGTATTGAGGCTGCGTCACTGAGATTGACACTCAGCACTTTGCGCCATCTTAC
TCTAACCAGCATAATGAAATCCAAAAGGAAAACTGATCACATGGAGAGAACTGCAAGTGT

CCTTCGACGGGAGAGCAACTTATTTCATCAAGGGAGAAAGCACTAACATCTGAGAAAGGA
AAGTGGACGAGCTCTTGTTACCCATCTAATGTACCTCGGCCGCGACACGCA

Clone 113 (ad1)

ATTTTTACGCCTGGGCCCGTNGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCG
ATT**TCGAGCGGCCGCCCGGGCAGGT**ACAAGAGCTGTTGGCTGTGTTAAGGTGGTGTTTA
TTGTTTCGTTAATTATCAAAGCAGCTTCCACAGGACACGGAACCTGAAATTTCTTGCAAAT
GACCACAATCAGAAAGAACACCATACACAACAAGGAAAGGCCACTGGTATATCCCAAAT
ATCCTAAATTTCTAAGCAGCGACAAAGGAAGAATGACCACCAATGACACCAACAGAACC
AAATAGTTCCCGTTCAGATACCACAATCCAGTTTTATCTTCAATGTTCTTAGNGCCTGGAN
CCCAAGGGNCTCTTATNTTTCTNTNNGAAGNGGGNTCTTGTATATCCCCANTGNTNANTNT
GNAAGNGCCCANA

Clone 114 (ad1)

TTNTTACGACCCTGGGTCCCGACGTCGCATGCCTCCCGGCCGCCATGGNCGGCCGCGGGA
ATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACCATCTTATTTCTTTTGGGGATTATACAC
CTCAGCCGCCTGAGATGGGGGTCAGCTCTTTATATAAAGGGAAACCAGACCAGGCCTAAA
GCCCACCCCTACCCTCACCCCCCCAATCCTCTCCTGAAACTTAAAAACAGTGGGAATATAG
GAAAGGGAACCAAATCTCATTAATTAATTGTTCTCCCCCATTACCCCACTGAATGAATGGC
CATACAGGCTAAGCTGAATAATGACGAAGTTGAAAGGACCAATACAGCCCCTTTTATAAG
GATTTTGAATGTTTTGCAAATGTATTGGCCCTGTGTTGTATTTTGTAGCCTTTTCCTGGGCT
TCAGCTCCCCTACT

Clone 118 (ad1)

TTTCGCNTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATT**T**
CGAGCGGCCGCCCGGGCAGGTACTCCCAAGGGAACGCTGTCCACCTCTATGAACTTCTG
AAGAAAATGACTGGCAAACCCGAACCCAAGGCTGTTTATTTTGGTGACAGCATGCATTCA
GATATTTTCCCAGCTCGTCACTATAGTAATTGGGAGACAGTCCTCATCCTGGAAGAACTCA
GAGGGGATGAAGGCACGAGGAGTCAGAGGCCTGAGGAGTCAGAGCCTCTAGAGAAGAAA
GGAAAATATGAGGGACCAAAAGCAAAACCTTTAAATACTTCATCTAAAAAATGGGGCTCT
TTTTTTATTGTTCAGTTTTGGGACTGGAAAATCAGAAGACTCCTTGGTTTATACATGGTCTT
GTAAGAGAATC

Clone 121 (ad2)

TTTTNTTNNCGNAAACCCNTTACTATAGGNTTNCNTACCNTCACGTATTGGGTNGATCGCC
GNCTAATCTNTATT**AGTCGTGGCCACNGACCGAGGT**ACCTTAACCACACACCCTACGAC
CAAGNAGAAATTANCAGTTGAGCAAAGATACAGACCAAATGCCTCTGGGAGATGGACTG
AAGCAGCTCCAAAGAAAGCTGTAAAAAAGTGACAAGAATTTGTTCTTCCACTTTGAGACT
GTTCGATTCAAACATGAGTCCAAGGCACGTATCCTTAAGAGGAGATGCTCTGCTGCTGTCT
CATTTGCCAGATTATCCTTCAGTTCCAGGCTTTCAACAGGATATGACTGGTTCATGCTCCA
TGATAGGAAAGGAATTAAAGTTTGAAGTCAGAGTGAATGGCCACAATGCCTGATGTTAGA
CTTTCGTAAGTCACCTTGTGA

Clone 133

TNGGCAGNNNCTACCTACTATCCCGACGTANNANTCCTATTGGNCCATACGACTCACTAT
ATGGNAANAGCNACTCNNTATAGGGACAGNNCANGGNCNTTATAATTTGGGCCTCAAGA
ATTGATAGTTGTCTCTTAACTGGTTCTGCTGAGGCGCACTCACAGCAGTGAGTGGAGGGTC
CTGGGAGGGCATGGATGACGGAGGCTGCCCCTAGAGGAGCTGGATGTAGGAGCAGAGAC
ATTTCTTGATCTTTCACTCTGCAAACTGGTCCAAGAACTTGAGGTAGGCCTGACGCTGGGG
TGCAGCTGCTGGAATGAAGTGGCCACCAGAGTGGGTGAGGGTGATGGCTCCGGGAAATTG
GCTGACCAGTTGCACACTCTCCTGAGAGGGGATGACTTTGTCAGTGTCCCCAAAAACATG
GAGCGAAGGCAATGACAAGGGCCTTTGCAGGATGGATTCTTGAA

Clone 153 (ad2)

ATTTNNTTCGACNTGGGCNCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAA
TTCGATT**AGCGTGGTCGCGGCCGAGGT**ACAGTCAGCTACTCATTTTAAAAAGGTTCGCG
GACCTTCCCGAGCTGATCCTAACCATCTTGTAGATGATCTGCTAACACCTTGCTCTCCAGG

TGACCCTGGTGCCATTGAAATGACATGGATGGATGTCCCTGGAGATAAACTTTTGGAGCC
AGTTGTTTCCATGTCGGATATGTTGCGGTCACTATCTAACACAAAACCTACAGTCAATGAA
CATGACTTGCTGAAATTAAAGAAGTTTACAGAAGATTTTGGTCAAGAAGGCTAAGCCAAA
GACAAGGAAGATGCCTACCATATGTATTCTTTCTTTCATAGATATTTTTGCTATTTGGATCG
CATTAATTGNTTCCA

Clone 158 (ad1)
NTNTTTACGNCCTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATT
CGATT**TCGAGCGGCCGCCCGGGCAGGT**CCACATTGCACTTTCCACATGCTTCAAACATC
ACCTTGTACAAGGATCAGTTACAGTTGTGGATTCGAGGCAACGTGAGTGCCTGTGCGAGG
TCCATCTTCATATTTGATGAAATGGATAAGATGCATGCAGGCCTCATAGATGCCATCAAGC
CTTTCCTCGACTATTATGACCTGGTGGATGGGGTCTCCTACCAGAAAGCCATGTTCATGTT
TCTCAGCAATGCTGGAGCAGAAAGGATCACAGATGTGGCTTTGGATTTCTGGAGGAGTGG
AAAGCGGAGGGAAGACATCAAGCTCAAAGACATTGAACACGCGTTGTCTGGTCGGTTTTC
AATAACAAGAACAGTGA

Clone 162 (ad2)
CCNGNACCNCNTNTTCACCGTCCCNCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAAT
TCGATT**AGCGTGGTCGCGGCCGAGGT**TGAAAACCTATATCCCTCCCAAAGGGGAGACA
AAAAAGAAGTTCAAGGATCCCAATGCACCCAAGAGGCCTCCTTCGGCCTTCTTCCTCTTCT
GCTCTGAGTATCGCCCAAAAATCAAAGGAGAACATCCTGGCCTGTCCATTGGTGATGTTG
CGAAGAAACTGGGAGGGATGTGGAATAACACAGCTGCAGATGACAAGCAGCCTTATGAA
AAGAAGGCTGCGAAGCTGAAGGAAAAATCGAAAAGGATATTGCTGCATATCGAGCTAAA
GGAAAGCCTGATGCAGCAAAAAAGGGAGTTGTCAAGGCTGAAAAAAGCAAGAAAAAGA
AGGAAGAGGAGGAAGATGAGGAAGATGAGAGGATNAGGGG

Clone 174 (ad2)
TTTTTTCGNCTTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCG
ATT**AGCGTGGTCGCGGCCGAGGT**ACTGTAGATATACAGAAGAATGGTGTTAAATTTACC
AACAGCAAATTGACTTCTTTAAATGAAGAGTATACCAAAAATAAAACAGAATATGAAGAA
GCCCAGGATGCCATTGTTAAAGAAATTGTCAATATTTCTTCAGGCTATGTAGAACCAATGC
AGACACTCAATGATGTGTTAGCTCAGCTAGATGCTGTTGTCAGCTTTGCTCACGTGTCAAA
TGGAGCACATGTTCCATATGTACGACCAGCCATTTTGGAGAAAGGACAAGGAAGAATTAT
ATTAAAAGCATCCAGGCGTGCTTGTNTGAAAGTTCAAGATGAAATTGCATTTATTCCTAAT
GACG

Clone 177 (ad2)
NTTACGNCNTGGGCCCGTACGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCGGGAATTC
GATT**AGCGTGGTCGCGGCCGAGGT**ACGGTTCTTCCTGTGTCAGCTCAATAGCTTGCTGCT
TTTTAAGAACCAAGAAGCTGTAGAACTTTGCGGCAGCTTGTTTTCTGTTCGTATTTCGACA
TAACTCAAGCAAACTGATAGATTCAGCTCCAGTTTAAGCAAGAGCACGCTGAAGACCATG
AAGCATCTGCTGAGTCCTTTTGTTCCATCTTCTTTCTTCCTGATCTTGATCGCCCCCTGATG
CATCTTCATCCTCTTCCTCTTCATCATCTTCTTTTTCCTTCTCTTTCTCCTTCTCTTTTTCTGG
AGAAGTTCTAACTCTGGTATTAGCTGACAGATATTGNAGGTTCTTCTGGGGGAAG

Clone 190 (ad2)
TTTNTTACGNCTTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCGGGAATT
CGATT**AGCGTGGTCGCGGCCGAGGT**ACCACTAATCGGGCCAATATGGCTTGTGAGCAAA
ATGGGTGAAAATAGAGGGAACTGACTGGGACCTGTTGTCTAACATCATTTTAAGGTTGAA
CTTGAGCAAAGACTTAGAGAAAGCTGATGGAATGCCAAGCATCAACGTGAAGAGACCCTC
TATGCACACAGTGGATATGTTATGCCTGGCTGTAGCCACCTTCTCTTCTGAAAACAAGATG
GGAATGACGGAACTGTATTAAAAGATTAAGTTATCCACTAGGGAGCATACTAGCACACTT
CCAGATCAGCCTTGAGATGCTAGACTTCAGATACAGGGACTTGTGTCAGGTGGCCTCTAG
AAATGT

Clone 192 (ad2)
NNGGGGGACCNCNNANCNTNCGTANCCCGCATGCCTNCCGNCCGCCATGGCCGGCCGCG
GGTAATTCGATT**AGCGTGGTCGCGGCCGAGGT**CCAGAATGTTGCAGATTACATGCGCTA

CTTAAAGGAAGAAGATGAAGATGCTTACAAGAAACAGTTCTCTCTATACATAAAGAACAG
CGTAACTCCAGACATGATGGAGGAGATGTATAAGAAAGCTCATGCTGCTATACGAGAGAA
TCCAGTCTATGAAAAGAAGCCCAAGAAAGAAGTTAAAAAGAAGAGGTGGAACCGTCCCG
AAATGTCCCTTGCTCAGAAGAAGGATCGGGTAGCTCAAAAGAAGGCAAGCTTCCTCAGAG
CTCAGGAGCGGGCTGCTGAGAGCTAAACCCAGCAATTTTCTATGATTTTTTCAGATATAGA
TAATAAACTTATGAACAGCAAAAAAAAAACCTGCCCGNNGNNCNNTNNAAATCCTANGA
ATTCC

Clone 218 (ad2)

ATTTTTACGNCTTGGGCCCGTNGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCGGGAATT
CGATT**AGCGTGGTCGCGGCCGAGGT**GGCTTGGCACCCAGAGAAAGCCTCTGCCCCCGA
AAGAGACTTTCTACATAGGGTTAAGCTTCATTAAATGAGGTGCAACCTTTCATTTTCAGGG
CATCTCTTTTCTTGCAAGGTCTTTAGAGGCAGAAGTTCCACTTGGATTCTAATACACATCTC
TGTGAGCTCAGTTTCCTGAAAATATACACCTACTGGGTTCTAGGTTCTCCCTTACCAGTGA
CTGTATTCATTATTTCACAGCCACCAGAATCGGACATACNCTATTAACATGATGAAAAATA
CAGCTACTGCTGCAAGTTTGGCATAAGTGGAACGCATGTTCAAGTACTTCNCATCCTGGCG
GA

Clone 234 (ad1)

NNTTNCCNCAACCNCTTNTTTACCGTACCTCNGCATGNCTNCCGGCCGCCATGGNCGGCCG
CGGGAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**GGCGTGATGTCTCACAGAAAGTT
CTCCGCTCCCAGACATGGGTCCCTCGGCTTCCTGCCTCGGAAGCGCAGCAGCAGGCATCGT
GGGAAGGTGAAGAGCTTCCCTAAGGATGACCCGTCCAAGCCGGTCCACCTCACAGCCTTC
CTGGGGTACAAGGCTGGCATGACTCACATCGTACGGGAAGTCGACAGGCCGGGATCCAAG
GTGAACAAGAAGGAGGTGGTGGAGGCTGTGACCATTGTAGAGACACCACCCATGGTGGTT
GTGGGCATTGTGGGCTGCGTGGAAACCCCTCGAGGCCTCCGGACTTCAAGACTGTCTTTGC
TGAGCACATCAGTGATGAATGCAAGAGGCGTTTCTATAAGAATTGGCATAAATCTAAGAA
GAAGCCTTTACAAGTACCTCGGNCGCGACACGCTAATCCTATGAATTCGCGGCCGCTGAG
TCGACATATG

Clone 259 (ad1)

TTTNTTNCCTTGAACCCNGTTCCTACGTCNCATGCTCCCGGCCGCCATGGNCGGCCGCGGG
AATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACCAAATTCAGGAGCATCAGCCAAAAG
TAGCACTGAGCACGTTGAGAGATCTCCAGTGCCCAGTGCTGCAGAGCAGCGAGCTGGAGG
GAACGCCAGAGGTGTCCTGCCGGGCTCTGGAGCTCTTCGACTGGCTCGGCGCCGTCTTCAG
TAATGTCGACCTTAATAATGAGCCTAATAATTTCATATCAACCTATTGCTGTCCTGAGCCA
AGCACAGTGGCGGCAAAAGCTTATTTTGTGTACCTCGGCCGCGACCACGCTAATCACTAG
TGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCAT
ACTTGAGTATTTAT

Clone 268 (ad2)

NGNCNNCTTCTGATTCCTATCGTGNTGCNTNCNNCCGCCATGGNCGGCCGCGGGAATTCG
ATT**AGCGTGGTCGCGGCCGAGGT**ACATTAAGTAGAAAGTTTCTAACTACCCTAAAGTCA
CAAACCAAGATTCAAAATGAACCTCTAGGCCTTGCCCTAACATTTCCCCAACACCACAAA
ATTGTCCAACTTGCTCTATTGTAAGTAACCTTTAAACTATTAGCGAGTTTAGTAACATCCC
GCGAGGCAGATTTTAAACTACGATACAGTTTCTATCTAGGCCACTGTGTTTAGTGTGTTTA
CAAACAGCTTGGAGTTCTAGGTTTCTTCATCATCAGTGAACACAGAGCGTCCTANAGCGCC
AGCCTGTTCATGAGGGGTGTCCACAAAAGCACGCAAACTATAGCAGAGTGNGNGGGAGT
G

Clone 277 (ad2)

CTCCNNCACCCNCTTGTNCCCGACCNCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAA
TTCGATT**AGCGTGGTCGCGGCCGAGGT**ACCTCCACTGGCTCTGTGGAACCTGTCTGGTA
CAGCTCTAAAATGAAGTCATGGAGTGGGTGATGTTTCCCCACAAATAAGACATCACCTCC
AAGGCTGTTTCTTCTGGTCTCTTCTGGAGTGAGGTCTGGGTATACCTCTTCTAGGGCAGCT
CGTAGCCTTCGCTCATCCACGAATGGCAAGAGAGCGACACCTTGCCATGCATATTTCTTCC
CATTCAAATCAATAGCAAAATCTTCAGGATAGAGGTCAACTATACTAGAATCAGGATCAC
TCATGAGCTTCCGCCATGATGGAGGTAGAAAATTCCACTTGCAGCTGGAAATCCCCCATA

AGTTGTTCTAGTGGTTAAACGGTTCGACCTNCCCGGGGNCGTCAAATCCTANNGAATTCNC
GGNCGCTGCANTCNACTATGGGANACTCCAACNCNTGGANCTACTTGAGATTCTATA

Clone 282 (ad1)

TNAAGGCGACNTCTTGTTCCCGTCNCNGCNGATGCCTTCCGNCCGCCATGGNCGGCCGCG
GGAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACCTATCGAGGACAAGAATTTATTA
GAGTTGGCTATTATGTAAATAATGAATATACTGAGACAGAATTAAGGGAAAATCCACCAG
TAAAACCAGACTTTTCTAAGATTCAAAGGAATATTTTGGCATCTAATCCCAGGGTCACAAG
ATTCCACATTAATTGGGAAGATAACACAGAAAAACTGGAAGATGCAGAGAGCAGTAATC
CAAATCTACAGTCACTTCTTTCAACAGATGCATTACCTTCAGCATCAAAGGGATGGTCCAC
ATCAGAAAACTCACTAAATGTCATGTTAGAATCCCACATGGACTGCATGTGACCACCTAC
CATCCCTTTAGTACCTCGGCGCGACACGCTAATCCTAGTGAATTCGCGGCGCTGCAGGTCG
ACTATGGGAGAGCTCCACGCGTGGATGCATAGCTTGAGATTCTATAGTGCNCCCNAAA

Clone 293 (ad1)

AGNGTCCNGGGACNNNCTTNNGTCCCGCACNCTTCGCATGCTCCCGGCCGCCATGGACGG
CCGCGGGAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACACCAAGCATGTGGTAAGT
TAGAAATGACAGGAAAGTGCTCCCCAAAGCAGTGGGAGAGGCAATGTGGTCTCTCACATC
CATTCAATGTGCATTCTTTTTCCTGGAATATCTGTTACCCACCCTTCTCAGGAACAAGAAC
CAGGACGACGGGGAGTATGTGTCAAATAAGTACCTCGGCCGCGACCACGCTAATCACTAG
TGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCAT
AGCTTGAGTATTCTATAGTGTACCTAATCA

Clone 295 (ad1)

TTTNTTNNTNAACCANTCCTATAGCGNTGCNTACCNNCCGTCATGGGCGGCCGCGGGTAA
TTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACCAAATTCAGGAGCATCAGCCAAAAGTA
GCACTGAGCACGTTGAGAGATCTCCAGTGCCCAGTGCTGCAGAGCAGCGAGCTGGAGGGA
ACGCCAGAGGTGTCCTGCCGGGCTCTGGAGCTCTTCGACTGGCTCGGCGCCGTCTTCAGTA
ATGTCGACCTTAATAATGAGCCTAATAATTTCATATCAACCTATTGCTGTCCTGAGCCAAG
CACAGTGGCGGCAAAAGCTTATTTGTGTACCTCGGCCGCGACCACGCTAATCACTAGTGA
ATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGC
TTGAGTATTCTATAC

Clone 305 (ad1)

TGACNNCNCCTACCTCCTATCCCGNNNCCTACNNTCCGCCATGGCNGCCGCGGGAATTCG
ATT**TCGAGCGGCCGCCCGGGCAGGT**ACTACTTTTCTCACTTTTTCTGGTTAGCCAGAATG
TTCCATTAAGAAACAATAAAAGTTGTATAGTTCTCTAAGATGAAAGATTAGTATATTCAAT
GGCTATTATATTAACCATTTAGTGAACACACAACAAAAACTATCCTTATATTAATTGACTG
AAGTTATAACATAAGAAATAAGTTACACTACTACTTTGTCATTCACTTAATGCTTACAAGA
TTACTCAAGAAATCAAAATGGCTTCCCATTGCTTGACGTTTGTTGTCCAAATACTTCACAT
TTCAATCTCATATACCTACAAAGAGGAAAAAATCCAAACATACTTTCCTTACCTAAAAATA
TTAAAGAAGGCTAAAAGGCATTAGGAATTTTTTTAAACCTTGAAAAC

Clone 314 (ad1)

TTTNTTTTTTGGAAACCGGNCCGTCGTCNCATGCTCCCGGCCGCCATGGCCGGCCGCGGGA
ATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACAGTGTGTTAATGATTTTCGCAGTGTTT
CCTTGAACTTGCTACAGACACATTTTAAGAAAGCCCAAGAAAATCAGCAGATTGGGAGGG
TAGAATTTCTTCCAGTCAACTGGCACAGTCCTTTGCATTCTACTGGTGTGGATGTAGATCT
GCAGCGAATAACCCTGCCCAGCATTAACCGCCTCAGGCACTTCACCAATGACACAATTCT
GGATGTCTTCTTCTACAATAGTCCCACCTACTGTCAGACTATTGTGGACACAGTTGCTTCT
GAAATGAACCGAATATACACACTTTTTCTACAGAGGAACCCTGATTTCAAAGGGGGGTGT
ATCCATTGCTGGACAT

Clone 321 (ad2)

GNNNNTTTTTCGNCTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAA
TTCGATT**AGCGTGGTCGCGGCCGAGGT**ACAGTGCCAGACCATGACTGTCAATCGTCAGA
TGAAGCGCTACAACGTTCCGTTTCTAACTTTTATTAACAAATTGGACCGAATGGGCTCCAA

CCCAGCCAGGGCCCTGCAGCAAATGAGGTCTAAACTAAATCATAATGCAGCGTTTATGCA
GATACCCATGGGTTTGGAGGGTAATTTTAAAGGTATTGTAGATCTTATTGAGGAACGAGC
CATCTATTTTGATGGAGACTTTGGTCAGATTGTTCGATATGGTGAGATTCCAGCTGAATTA
AGGGCGGCGGCCACTGACCACCGGCAGGAGCTAATTGAATGTGTTGCAATTCAGATGAAC
AGCTTGGTGAGATGTTTCTGGAAGAAAAAATCCCCTCGATTTCTGATTAAAGCTAGCAATT
CGAAGAGCTCTCTGAAAAGACATTTACTCCTGATTTTT

Clone 326 (ad1)

CGNCCNGGACCCCTTGNGCCCGACCNCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAA
TTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACAACTACCCCTATCAGTCACTCACAACTG
ACCTTCACTACTATTACATCCTGGAGCTGTCGTTTTATTGGTCTTTGATGTTTTCTCAGTTC
ACTGATATCAAAAGAAAGGACTTTGGCATTATGTTCCTGCACCACCCTGTATCTATTTTCT
TGATTACCTTTTCATATGTCAACAATATGGCCCGAGTAGGAACGCTGGTCCTTTGTCTTCA
TGATTCAGCTGATGCTCTTCTGGAGGCTGCAAAATGGCAAATTATGCCAAGTTTCAGAAA
ATGTGTGATCTCCTGTTTGTATGTTTGCCGTGGTTTTTATCACCACACGACTGGGTATATTC
CCTCTCTGGGTGTTAAATACCACATTATTTGAAAGCTGGGAGACGTTGGACCTTACCCTTC
TGGGGGTTTTAACCTACTGTATTGTAGTCCTCGGCGCGACACCTAATNCTANGAATCGCGG
CT

Clone 332

GGGAAATTCGTTACCTCACNTAATCNNCTGCTANGANCNTGATATATGCACGTCATANTG
GCGCNCAATCAGNGGNANATACACTTCATAAGTAGTCAGATGNCTGCANNNNGACCTGAT
NGGTGACTAGAATACTCGGGAACAGNTATTTCANAGGNGNCAANACGTGNGNAGNATGT
GNNCAGNACCTGCCTTGGTGACTGTGCTGAGCATAAAGNAATGTGTTCAGTGCAGNAGCC
TTCAATAAAGGNAGNAAAAGNAAAGNACACATGCACACAGGGNATGGNNTCCTAATTTT
AACATTACCAAGGGTAGNAAAGTNGGGNACAANATTACCCCAAGNCGGTCCAACCTGNA
TCCTGGTGGTNCCATTGNAAGGG

Clone 339 (ad2)

TCCCCNTACCCCTTGTGCCCGACCTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATT
CGATT**AGCGTGGTCGCGGCCGAGGT**ACTTTCAACAAAGATTGAAATTAAACTGAAAAA
GCCAGAGGCTGTGAGATGGGAAAAGCTAGAGGGGCAAGGAGATGTGCCTACGCCAAAAC
AATTCGTAGCAGATGTAAAGAACCTATATCCATCATCATCTCCTTATACAAGAAATTGGGA
TAAATTGGTTGGTGAGATCAAAGAAGAAGAAAAGAATGAAAAGTTGGAGGGAGATGCAG
CTTTAAACAGATTATTTCAGCAGATCTATTCAGATGGTTCTGATGAAGTGAAACGTGCCAT
GAACAAATCCTTATGGAGTCGGGTGGACCTGCCGNNGNNCNNTNNNAATCCTATGAATTC
NNGGCCGCTGCNNNCNCCTATGGGAGACTCCACGCNNGATGCATACTNGATATTCTATAN
GCNCCANT

Clone 358 (ad2)

GNNNNTTTTTTCGACCTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGG
GAATTCGATT**AGCGTGGTCGCGGCCGAGGT**ACACATTCGATGTTGGGAACTAACACACA
GCGATGGGTGGGAAGGAAGGATGTTCAGGCAAGGTTCTTACTCCTTTACTCATCTGGTTCT
GGCTTTGGGAAAAAATAAGGTTTCATGTGCTGGGAAATACTTAGCAGTAATAAGTACCAA
AAAGGAAACATTGCCCTCTCATTTTGCCTAGTAGGAACTTACTGTGGTGATAAGAAATATG
AAACCCATTACTCTCTTGAACCCCATACTTGGGAGTAGATGCAGAGAGCTTGGTTGTAGTT
GGTTGCAGGGAGGTAGGAATTCAGGAGTAGTATTTCCCATTGATTCATCTTCAGCAGCACT
AGAATTCTAGTCTGCAAATCCACTCTCCCTCAGGCAGGAAGTGTCTTCAGACTGGCTGCTT
GCATTAAACACTCTTAGGGGACAAGCACTCCTCTTTTTT

Clone 362 (ad1)

TTTTTTACGACNTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTC
GATT**TCGAGCGGCCGCCCGGGCAGGT**TCAATCTCCAACAACATGCCAGTGATTTTACCA
GCAAGAGTAGGGTGCATGGCTTGAATAAGAGGAAACAGCCGTTCACCCAACATTTGCTTT
TGCTCTTGAGGAGGGGCAGATGCCAACATGGAAGCAGTCAAAGGTTCCTGACCTTGTACA
TGAACAGCAGGCTGTTGCATTGTAACTTGTGGCTGTGCATTAAGATGTTGCTGAGGATTGC
GAACTCCTGCAGCATATTTATACTGTGGAACGGTGCGGACAGCAGGAGTAGCTGCAGCGG
CTGCAGCTGCAGGACGTGGACCCATTGTCTGTGTTGATGTGTTAGCAACACGCTGTGTTGA

CATGACTCGTGGACCTGTGAAGAACTGGCTATAGTACCTCGGCGCGACACGCTATCCTAT
GAATTCNGGCGCCTGAGTCGACATATGGA

Clone 370 (ad2)

NNNNNTTTTTTACGCCTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCGG
GAATTCGATT**AGCGTGGTCGCGGCCGAGGT**GCAGAGAGAGAAGGTGCTGGGGCACAAG
AGGCAAGGGTATGAAGTCCCTCCAAATAGGAGTGGAGTGCCAACTGCCCTGCCTCGCTCC
AAACACCTGACTCCTGGGCCATGGCAAGAGTCCAGTCCATTAAGTGCAGCGTGCAATACT
AGCGCTTGGAGTCTCCTGTCCTCATCAATGAAGCGGTGTGGACGGGATAGCAGTCACCTG
GCAGGAGGCCTTGACCCCTGGCAGGCTAGTTGTATCGGCCCTTGATCATTGTGTTCAACAA
GGGCCCCACCTCTTGTGGGTTCCCCAAGGCCTCTCCAAGCATCTTCTCAATGTTTCTCATTA
TGGCACTTTCTGATGAGCTTTGAGAGGATATTCAATTCTCTTAGGGGTGGGAGCCCCTTAT
ACCNGAAGTTACAGTNTGGAATCCCCCATTAGTAATGACTTN

Clone 371 (ad2)

NNNNNTTTNTTACGNCNTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGG
GAATTCGATT**AGCGTGGTCGCGGCCGAGGT**ACCAAGGCTTTAACGTGTCTGTGCAGGGT
ATTATCATCTACCGAGCCGCCTACTTCGGTATCTATGGCACTGCAAAGGGAACGCTTCCGG
ATCCCAAGAACACTCACATCGTCATCAGCTGGATGATCGCACAGACTGTCACTGCTGTTGC
CGGGTTGACTTCCTATCCATTTGACACTGTTCGCCGCCGCATGATGATGCAGTCAGGGCGC
AAAGGAACTGACATCATGTACACAGGCACGCTTGACTGCTGGCGGTAGATTGCTCGTGAT
GAAGGAGGCAAAGCTTTTTTAAGGGTGCATGGTCCAATGTTCTCAGAGGCATGGGTGGTG
CTTTTGTGTTGCTTGTATGATGAAATCAAGAAGCACCTGGCCCNGGCGCTCGAAATCCTAT
GAATCGCGGCGCTGAGTCGACATATGGAGACTT

Clone 384 (ad2)

GNNNNTTTTTTGCGNCCTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGG
GAATTCGATT**AGCGTGGTCGCGGCCGAGGT**ACAAATTCAAGTGCAGAATTATTACGCCA
TTTTAAGTATTATCTACTTTTTTAAAAAATCACAGTGGTCTTCATCACATGGCGAAAAAAC
AAACTTCACTCTTCTCCAAAGGGATAAGTCATCCTAAATGTAACTACAAACAATTGTATAA
ACCTTTATAGTTTTTCCCCCTAATCTACTAAACCTATTCCAGTGTCATTTAGTAGTTTTACA
TAGAATCTGCACTATACTTGGATACTCAGGGAAAATAAGATGACATATATTGTAGTTTTAG
AACGCAAAAGAGCTTTCATTTTAGACAAATATACAACATAGAAGCCCTAAAAAATATGGG
TCTATTAATCAAGAGCCCATTTCAAAATGATAAGAGCATTTAGATGTAGATCTGGAATGCC
AGTTCTGAGTATTAGAACTAGGGTTACAGTGCCTTATCN

Clone 406 (ad1)

NGNACGCGGACCCCTTGNGTNCGTCCCGCGGATGCNTTCCGGCCGCCATGGCGGCCGCGG
GAAATTCGAT**TCGAGCGGCCGCCCGGGCAGGT**ACCACACACCTTTCTCCAAGGTGGTCT
CATAATTCTAGAATGGAAGGTCCAGCTGTTACAAAATGAAGACAGACAACACAACATTTA
CTCTGTGGAGATATCCTACTCATACTACGCACGTGCTGNGATTTTGAACATAACTCGTCCC
AAAAACTTGTCACGATCATCCTGACTTTTTAGGNTGGCTGATCCNTCAATCTTGCACTCAA
CTGNTACTTNTTTCCCAGNGNTGNTAGGAGCNAAGCTGACCTGAACAGNAACCAATGGCT
GGAGATCCCNACATGCNGGTTTTTCCCATAATATGGGAAAATTTAAGNCTATCATTCCTTT
GAGGAAAACTGNACNTTTGGGATATCTTCATTTTTGAAACACAATCTATNCTTGGNCTCCT
TAGGCTTAATCCANTATTTTGNGATTTTNCANNAAACANGGGTTCTTGAGANACCCAATA
GATATT

Clone 410 (ad1)

GNNNNNTTTTTNCGACNTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGNCGGCCGCG
GGAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**GGACAGACAAGAGAACATGCAATGT
TGGCAAAGACAGCAGGTGTAAAACACCTAATTGTGCTAATTAATAAGATGGATGATCCAA
CAGTAAATTGGAGCAATGAGAGATATGAAGAATGTAAGGAGAAACTAGTGCCATTTTTGA
AAAAAGTTGGCTTCAATCCCAAAAAGGACATTCACTTTATGCCCTGCTCAGGACTTACTGG
AGCAAATCTCAAAGAGCAGTCGGATTTCTGTCCTTGGTACATTGGATTACCGTTTATTCCA
TATCTGGATAATTTGCCGAACTTCAATAGATCAGTTGATGGACCAATCAGATTTCAGCAAC
AACTGGAACAACTCAGTGCAATGGGATTTTTGAACCGTGAAGCAAACTTGCAAGCTCTAA
TAGCAACAGAGGTGATATCAATGCAGCTATTGAAGGTACTGGGCTCCCACA

Clone 415 (ad1)

GANCGGNGACCNCTTNNTCCCGTCCNCGCATGCTNCCGGCCGCCATGGNCGGCCGCGGGA
ATTCGATT**TCGTGCGGCCGCCCGGGCAGGT**ACATCCAAAAGTCTCAGTGTAATAGCAGG
ACCAAAATATTCTGTCAATCAGCTGACCATATACTTAATGACTCCTAAAATCTCGTGGACT
TCTAAGAAAGCGCCATGGCCTGTGCTGCTGTTATGATTCCTGGGTTGTTGCGGTGCTCTGT
TGGAGCCATCCGTATTGAGGCTGCGTCACTGAGATTGACACTCAGCACTTTGCGCCATCTT
ACTCTAACCAGCATAATGAAATCCAAAAGGAAAACTGATCACATGGAGAGAACTGCAAG
TGTCCTTCGACGGGAGAGCAACTTATTTCATCAAGGGAGAAAGCACTAACATCTGAGAAA
GGAAAGTGGACGAGCTCTTGTACCCATCTAATGTACCTCGGCGCGACACGCTAATACTAG
TGAATTCCGGCCGCTGAGGTCGACTATGGGAGAGCTCCCACGCGTGGATGCATAGCTTGA
TATTTATAGTGCACA

Clone 420 (ad1)

TTTNNTTTNCTNNAAACCCAGTCCGTATAGCCGCATGCNTACCGNCCGCCATGGGCGGCC
GCGGGAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACAACTACCCCTATCAGCCACTC
ACAACTGACCTTCACTACTATTACATCCTGGAGCTGTCGTTTTATTGGTCTTTGATGTTTTC
TCAGTTCACTGATATCAAAAGAAAGGACTTTGGCATTATGTTCCTGCACCACCTTGNATCT
ATTTTTCTTGATTACCTTTTCATATGTCAACAATATGGCCCGAGTAGGAACGCTGGTCCTTT
GTCTTCATGTTTCAGCTGATGCTCTTCTGGAGGCTGCCAAAATGGCAAATTATGCCNAGTT
TCAGAAAATGNGTGANCTCCTGNTTGGTATGTTTGCCGCGGGTTTTATCACCCACNACTGG
GNATATTTCCTCTCTA

Clone 421 (ad2)

NNNTNNTTTTTTTGAGACCCTGGNTTCCGTACNTCNGCATGCCTCCCGGCCGCCATGGNCG
GCCGCGGGAATTCGATT**AGCGTGGTCGCGGCCGAGGT**GTAGGATTAATGCATGGTTATA
TGGACCAGAAAAAAGTGCCATAGAAGACCAATAACTGTTTAGTTGAGGCTAGTCTGGAAC
CTTTCATTAGAGCAATATTTGGTTATTGCACTTCATTTTTATTTACTAAGAAATGCAATTTG
GGAATTTTTAATCTGTTATGCTTTGTTTATCAACCTTGATTTTAATTAAGACTTTTATAAGA
CTAGCATAAAACACCAACCAACATTATTTTTGCAAAAGTGAGTTGGTCTCACTTTCCATTC
TTGCTAGTCAGAGTAAGTAGGCAGCACTTTTAAAAATATGTGAACTCAAATATTGCACTTC
TTTCAAGATGTATCAATTGCTATTGACCTGCCGCGGGNCGTCGAAATCCTAGTGAATCGCG
GCGCTGAGGTCGACTATGGGAGACTC

Clone 423 (ad2)

TTTNTTNCTTNAACCCACTCCTATAGNGNNGCNTACCNNCCGCCATGGGCGGCCGCGGGA
ATTCGATT**AGCGTGGTCGCGGCCGAGGT**ACGCTGAACGAGATCTACTTTGGAAAAACAA
AGGATATCGTCAATGGGCTGAGGTCTGTGCAGACTTTTGCAGACAAATCAAAACAAGAAG
CTCTGAAGAATGACCTGGTGGAGGCTCTGAAGAGAAAGCAGCAATGCTAAACCTCTGTTT
CATGCTAACCAGACACGCCGTGCACTCGTTAGATTCCTCTCTTAGAAAACTCGTTTACTGC
TCCCTTCCCTCGTCCCTTCCCTCCCCGACAGGTCACATAACAGCTGCATCATTGACCGCAC
AGCGCCATCTCTCCCTGAGAATAAAGCCGATAGCCACCCTCCTCCGGCTCCGAGCCTGCTT
CTGCCACACCTCC

Clone 424 (ad2)

TGATANNNCCNTNNCTNNANTNAACCGCCCGATGNTTTTCGGCANGAACANCCCCGGCCG
CGGGAATTCGATT**AGCGTGGTCGCGGCTCGAGGT**NNTATGCCAATCAGAGTANACTGN
CCCTCAGTTTNAAAAGAAGATGGATTAAACCAAAACAATTTTGTAGATTCATCGTATGTG
AACATACCAATATCTGGATTGAAGATTTCCTCCACAACCAGATGAAAAAATTNTNTGNAA
ACANCTCNCTNATCAACTCCTTGNTNTCCCACAAATTCCACATACAAGNGCTTCTTNAAGN
CTGCAGGATTTTCCATAGGGATCATCTNTAGCCGGACAAGTGCATCATCTATGATATGGNC
ACGTCTAACTTTGAGNCTCAAATATGGNTTCAACTGCTGNNCTTGAACTAANCTGGNAGA
ACANAGANNNTTNNTTCACTGTACCTGCCCGGGNCGGNCACTCCAANTCGAAATAATCNA
GACNNNCGCCCTNNNTNGGACNCCCCNCCCGACANGNTNCNGNACNCTNCAGCCNCCCG
NCNCNTCCGGCCCCCTANC

Clone 426 (ad1)

TTTNTTCACTAAACCCATTNCTATAGCGCATGCNTACCNNNCGCCATGGGCGGCCGCGGG
AATTCGATT**TCGAGCGGNCGNCCGGGCAGGT**ACCATGCTGGGTGCCTCTGGCGACTACG
CTGATTTCCAGTATTTGAAGCAAGTTCTCGGCCAGATGGTGATTGATGAGGAGCTTCTGGG
AGATGGACACAGCTATAGTCCTAGAGCTATTCATTCATGGCTGACCAGGGCCATGTACAG
CCGGCGCTCGAAGATGAACCCTTTGTGGAACACCATGGTCATCGGAGGCTATGCTGATGG
AGAGAGCCTCCTCGGTTATGTGGACATGCTTGGTGTAGCCTATGAAGCCCCTTCGCTGGCC
ACTGGTTATGGAGCATACTTGGCTCANCCTCTGCTGCGAGAANTTCTGGNAGAANCAACC
AGTGCTNAGCCAGACCGA

Clone 431 (ad2)

TTNTANANNCTACCTTCTNTTCCCGTCCCCCGCATGCTCCCGGCCGCCATGGNCGGCCGCG
GGAATTCGATT**AGCGTGGTCGCGGCCGAGGT**ACTGGATTTTAAATCTGTATGTATCTGTT
GATTTTTTTTTCTAATATTTCAGTTGAGCTGCTGTTTTCTTCCATGCAATATTGTATACTCAA
TTGTGTATAGAAGAAGCTGGTGAGAGTGCCCTCCTACATAAATAAGCAATTGCAGTGTTTT
GCATGCAAAATATAAAAAATTTAAATTGTCCTGATTCTATTTTGTAAATGGAGAAACAATC
ATATCTTTCTAAGCGGTAATGGAGGAAGACTAGTGCTTTGTGCATTTTGATATATTTGAGT
CCATTTTTTCCCAATGTCATACTTTTNCCGCAGTTGGGTTTCTCATAAGTATCCTAGTCATG
TCCTGCCGGGC

Clone 434 (ad1)

NTNTNNTTTNCCTTAGACCCTGNTTCCGTATNCGCATGCCTNCCGGCCGCCATGGNCGGCC
GCGGGAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACATCCAAAAGTCTCAGTGTAA
TAGCAGGACCAAAATATTCTGTCAATCAGCTGACCATATACTTAATGACTCCTAAAATCTC
GTGGACTTCTAAGAAAGCGCCATGGCCTGTGCTGCTGTTATGATTCCTGGGTTGTTGCGGT
GCTCTGTTGGAGCCATCCGTATTGAGGCTGCGTCACTGAGATTGACACTCAGCACTTTGCG
CCATCTTACTCTAACCAGCATAATGAAATCCAAAAGGAAAACTGATCACATGGAGAGAAC
TGCAAGTGTCCTTCGACGGGAGAGCAACTTATTTCATCAAGGGAGAAAGCACTACATCTG
AGAAAGGAAAGTGGACGAGCTCTTGTACCCATCTAATGTCCTCGGCGCGACACGCTAATA
CTATGAATTCGCGGCCGCTGAGTCGACTAT

Clone 438

TTTTTNGNGNGAACCCCGTTACCTTCACNTAATCCCCCGGCTATGCCTTCNCNANCACGCN
CATTGGCCCGCGNCNCGCGGGNAATTCGNATTTCGNAGNCGGCNCGCCCGGGNCAGGTAC
NCCATTCACGGGNTGTTTGTGGGNAGNATACCTTCTGTTACNTTTGCTGGCATGNACNCTT
TGCNCGTGGTGGGCATCTTGGGNACACTTCAGCAAGNACCATGCTACNTATTCTTCATGCC
CCAGGNTGTTCAAACNTTCCTCTACTCACNTGCCTCAAGCTCCTGCAATATCATCCCCTGC
CCTCGCCACCGCATACCCAAGNACTCAAATTATTCAAGNACAGGGCAAACTGGGNAGNAT
GGAAGCTTATTCCAAGGTTCAAAGGAACCAAGGAAGCCTCTTCTTTCTTGGGGGCCACCTT
TTATTTTTAAAAAGGGTGGGGCAAGAAGAAAGCCCTNCAAGCTTGGGTGGACAAGGTAAC
CCTTCGGGNNCNGNGGAANCAACGGCTTAAATCAACTTAAGNGGAAATTTCNNGGGNCG
GNCTGGCANGGNNCGGAACCATTAATGGGGAAANAA

Clone 446 (ad2)

NNTTNCCNNANCCCTTNTGCCCGACCTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGA
ATTCGATT**AGCGTGGTCGCGGCCGAGGT**ACCCCATTATCTATGACATTCGGGCCAGACC
TCGAAAAATCTCCTCCCCTACAGGCTCCAAAGACCTACAGATGGTGAATATCTCCCTGCGA
GTGTTGTCTCGACCCAATGCTCAGGAGCTTCCTAGCATGTACCAGCGCCTAGGGCTGGACT
ACGAGGAACGAGTGTTGCCGTCCATTGTCAACGAGGTGCTCAAGAGTGTGGTGGCCAAGT
TCAATGCCTCACAGCTGATCACCCAGCGGGCCCAGGTATCCCTGTTGATCCGCGGGAGCT
GACAGGGAGGGCCAAGGACTTCAGCCTCATCCTGGATGATGTGGCATCACAGAGCTGAGC
TTTACCGAGAGACCTGCCCGCGGNCGTCNAAATCCTANNGAATTCCGGNCGCTGCAGTCG
ACCTATGGGAAGCTCCAAGCGTTGGATCTANTTGATATTTATANTGNNNCCT

Clone 447 (ad1)

GGNNNNNNAGTTTGNNNNCCNNNNNTTTNNNTTTCCTTACGCCCTGNNTCCCGTACGTCG
CATGCCTCCCGGCCGCCATGGCCGGCCGCGGGNAATTCGATT**TCGAGCGGCCGCCCGGG**

CAGGTACCTATCGAGGACAAGAATTTATTAGAGTTGGCTATTATGTAAATAATGAATATA
CTGAGACAGAATTAAGGGAAAATCCACCAGTAAAACCAGACTTTTCTAAGATTCAAAGGA
ATATTTTGGCATCTAATCCCAGGGTCACAAGATTCCACATTAATTGGGAAGATAACACAG
AAAAACTGGAAGATGCAGAGAGCAGTAATCCAAATCTACAGTCACTTCTTTCAACAGATG
CATTACCTTCAGCATCAAAGGGATGGTCCACATCAGAAAACTCACTAAATGTCATGTTAG
AATCCCACATGGACTGCATGTGACCACCTACCATCCCTTTAGTACCTCGGCGCGACACGCT
AATCACTAGTGAATTCGCGGCCGCTGCAGTCGACNTATGGGAGAGCTCCC

Clone 452 (ad2)

ATTTTTACGNCNTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATT
CGATT**AGCGTGGTCGCGGCCGAGGT**GCGTTGTTCTCTTCAGGTTTAGGCATGTAAGCCA
ATGACATTCAAAAATGTATCAATAAGTTTTGGTATGGAGAGTCTCAAATCACAGGGTGGC
AGGTTTATCCCCAAATCACAGCCAGCCTCAATTTCTTATCAGGCAAAGTAAGTCACACTAT
TCACAACAGCTCTGTGTTGATTCAATTAAACAATACGTAAACATTCACTGAACATTGCTGG
TTGGCCCTTANACCTTTCGTTGTGCCCTTTCTGGCTTCTTCTCATCTCTCCATTCATACATAT
CCATATCTATATCTATATATATATAATCCCTATAAGGCAAATGTTACTTTCTAATCAATAC
ATTTTTCCTTG

Clone 458 (ad1)

NTATCTNAANACCTCTTNTTCCTATCCTGANNCNTACNTCCGCCATGGCGGCCGCGGGAAT
TCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACCATCTTATTTCTTTTGGGGGATTATACAC
CTTAACCNGCCTGAGATGGGGGTCAGCTCTTTATATAAAGGGAAACCAGACCAGGCCTAA
AGCCCACCCCTACCCTCACCCCCCCAATCCTCTCCTGAAACTTAAAANCAGNGGGAATAT
AGGAANGGGAACCAAATCTCATTAATTAATGGTTCTCCCCCATTACCCCACTGAATGAAN
GGCCATACAGGCTAAGCTGAATAGTGNCGAAGTTGAAAGGACCAATACAGCCCCTTTTAT
AAGGATTTTGAATGTTTTGCAAATGTATTGCCCCTGTGTGTATTTTGTACCCTTTTNTGGGC
TTCANCTCNCTATTTTGNNTGATNTGCATACTGCTCTNACCTTANGTNATGACCAAAAAAA
AAAAAAAT

Clone 460 (ad1)

GAGGATTNNTTTCCGNNATANTTACNCATGACCNCTNNNTGCANTATCCNTGTATNCCNTA
CCNTCACGCCATGGNCGGCCGCGGGCAATTCGATT**TCGAGTCGGCCGCCCGGGCAGGT**
ACACTCAGGTATTCAGAGCACCAGAGGTCTTGACAAAGTGCCCCATNAAACTACAGCAAT
TGACATGTGGTTTGCAGGTGTCATATTTCTTTCTTTGCTTAGTGGACGATATCCATTTTATA
AAGCAAGTGATGATTTAACTGCTTTGGCCCAAATTATGACAATTAGGGGATCCAGAGAAA
CTATCCAAGCTGCTAAAACTTTTGGGAAATCAATATTATGTAGCGAAGAAGTTCCAGCAC
AANACTTGNGAAAACTCTGTGAGAGACTCAGGGGTATGGATTTAGCACTCCCAAGNTAAC
AAGTGATATACAANGGCATGCTTCTCTCAACAGCTATTTANAGAANACTGATATNNAGTT
NTGCTNGTTATACCCTNAGGACATACTCAGA

Clone 470 (ad1)

TNTNTANGNNCNNCATNCNNAGNTNACTATNCTGTNNCTTTAATCACGNCATGNCGGCCG
CGGGTAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACGAATGCAACCCCATGGCCCA
CGTCATGGAGAAGGCTGGGGGAATGGCCACCACTGGGAAGGAGGCCGTGTTAGACGTCA
TTCCCACAGACATTCACCAGAGGGCGCCGGTGATCTTGGGGTCCCCCGACGACGTGCTCG
AGTTCCTGAAGGTGTATGAGAAGCACTCTGCCCAGTGAGCACCTGCCCTGCCTGCATCCG
GAGAATTGCCTCTACCTGGACCTTTTGTCTCACACAGCAGTACCTCGGCCGNGACCCGCTA
ATCACTAGTGAATTCGCGGCGCTGCAGGTNGACATATGGGAGAGCTCCAACGCGTTGGAT
GATAGTTGAGTATTCTTANTGCCCTAAATCANCCATTCTNCCCTCTCCCATCNTTCCTCNNC
TTCC

Clone 474 (ad2)

TTNTGNCCGATNCNCNNCNTACGTATCCCCGATGNCTNCTTTCCGNACATGGCCNGCTCGC
GGGTAATTCGATT**AGCGTGGTCGCGGCCGAGGT**ACTGGGGGTTCCCGTACACAGTGGCT
GAGCTGCTGAACACCAGGTTCTTCACCCCGTGGGCCTTCATGATCTCCAGAAGCTGGATGG
TCCCGGTCAGGTTAACTCTGTAATAATCCAGAGGCTTCTGCACCGACTCGCCCACGGCCTT
GAGCCCCGCAAAGTGGATGACCGCCATAAAGCTGTACCTGCCCGGGCG

Clone 475 (ad1)

NTNTNNGNNCGNNTTTTNTTCCTATNCTGTNNCNTAAATNCGCCATGGCGGCCGCGGGAA
TTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACCGTCAGCATCCACAGCCATACATCTTCC
CTGACTCTCCTGGGGGCACCTCCTATGAGAGATACGATTGCTACAAGGTCCCAGAATGGT
GCTTAGATGACTGGCATCCTTCTGAGAAGGCAATGTATCCTGATTACTTTGCCAAGAGAGA
ACAGTGGAGGAAACTGCGGAGGGAAAGCTGGGAACGAGAGGTTAAGCAGCTGCAGGAGG
AAACGCCACCTGGTGGTCCTTTAACTGAAGCTTTGCCCCCTGCCCGAAAGGAAGGTGATTT
GCCCCATGTGGTGGATATTGTGACCAGACCCGGGAGCGGCCATGTAGAAAGAGAGAGAC
CTCATCTTTCTGCTTGCAAGTGAAATATGTCAGACATGCACTTGCCTATAAAAAAAAAAAA
AAAAAAAN

Clone 1026 (ad2)

NTCCTACGCCCTGNNCCCGTCNNCGGATGCTCCCGGCCGCCATGGNCGGCCGCGGGAATT
CGATT**AGCGTGGTCGCGGCCGAGGT**ACAAGCCTGGCGTGTTGCTGGGGTTTAACATGAA
AGAATTAAATAAAGTCAAGCATGAGATGGATTTTGATGCTGAGTGGCTATTTGAAAATCA
TGATGAGAAGGATTCCAGAGTCAATGCTAGTAAGACTGCTGACAAAAAGCTCAACCTTGG
GAAGGAAGATGACAGTTCCTCAGACAGTTTTGAAAATTTGGAAGAAGAATCCAATGAAAG
TGGTAGCCCTTTTGACCCTGTTTTTGAAGTTGAACCTAAAATCTCTAACGATAACCCAGAG
GAACATGTACCTGCCGGGGCCGGNCGCTCGAAATCCTAGTGAATTCCGGNCGCTGCAGGT
CGAC

Clone 1029 (ad1)

AGTGTNGGGNNTCTCTTTCNNTTNGAATNTANTGTCANTTATNTGACGTATTCCGGGAGCG
TGNGTNATTCGATT**TCGAGCGGNCGCCCGGGCAGGT**NCCCACAGCAAGGTTCTTTATGT
CTATGANATGCTGGCTGGAAAGTTAATTCCTGTGCATCANGTGAGAGGTTTGAANGAGAA
GATAGNGAGGAGCTTTGAAGTCTNCCCANATGGGTCCTTCTTGCTCATANATGGCATTGCT
GGATATTTGCATTTGCTAGCAATGAAGACCAAAGAACTGATTGGAAGCATGAAAATTAAT
GGAAGGGTTGCAGCATCCNCACTCTCTTCAGATAGNAAGAAAGTNTACGCCTATTNGGGG
ANGGANAANTANNGTTACANGNTCACTNTTTNNNANNNNANANCANTNNTNAANNNANT
NNCNGTCAATCNTCNNCNTTNNATGNNANTCNTCNCTANNNNTTNCNTNCTNGAA

Clone 1034 (ad2)

GNATTTANTACNANCCNTGCATTTNNNGTAANTACCCCTGANGCATTNCCGNCACGTACA
TGNCGGCTCGNGGGNAATTCGATT**AGACGTGGTCGCGGCCGAGGT**ACTCCAATCCCTCT
CCAACTATCCCAATGAAGGAGATCCAAGCAATTAGCACCTTCAGTTGGCGCTGCTCCTAA
GTTGTAAGCCTCTAACTACTATCAGCTGGACAAAAGCCACATCCTATATGCCCTGTTAGCA
CACCAATGTATTAATACTAACCAGCCACCCTAGAGCTCAATTAAGCATCTGGCCTTAATGC
AGTAAACCACACTCAAAGGAGACATGAAATATTGGCTGNTGTACCTGCCCGGGCGNCNCC
NTCNANCACTCTCNNNNNNANTCCCTNTCANNNNNNNTATNTNCNATATCNNCCNANNNA
ACGAGCNTNACTNCTTNCNTANTNNATNNNCCCCCCTCACCCTCNCCCCNNTANNNCACC
CANCNNCNTACCCCA

Clone 1040

GTTNTANGNCTNNTTCTACTCCTACNCGGTANNANGACNCCTATTGGGNAATACGACTCA
CTATAGGGTANTGCGAGTCCGTATAGGGNCTTCTATGNNCTGTGNGNGGNCACNGAAGNT
GCTGCTGACGCTCTGGGTGAAGAATGGAAGGGTTATGTGGTCCGAATCAGTGGTGAGAAC
GACNAACNNGGTTTCCCCATGAAGCAGGGTGTCNTGACCCATGGCCGTGTCCGCCTGCTA
CTGANTAAGGGGCATTGCTGTTACAGACCNAGGAGAACTGGAGAACGAANGAGAAAATC
TNTCCGTGGNGCATNGAGGNNGCCNACNNGCCGTNCTCACNNTGGACTGAGCNTANNCCT
AGCNTANAAGGACNGACCCCNTCTGCCATGCNNCTCCTTCNCTGNTACCCTNGCCTCTGCT
TCATCGNNNCNGCCTNGNNCCNNCCTNTNCNGGNAANNCCCNNCA

Clone 1049 (ad1)

CTTATNNNNNCNTCCTCNGANTCCTACANTGTNNCTTAAANCCGTACATGGNTGGCCGNG
GGTAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACTTTCTATGAGAAGCGTATGGCCA
CAGAAGTTGCTGCTGACGCTCTGGGTGAAGAATGGAAGGGTTATGTGGTCCGAATCAGTG
GTGGGAACGACAAACAAGGTTTCCCCATGAAGCAGGGTGTCTTGACCCATGGCCGTGTCC
GCCTGCTACTGAGTAAGGGGCATTCCTGTTACAGACCAAGGAGAACTGGAGAAAGAAAG

AGAAAATCANTTCGTGGTTGCATTGTGGATGCNAATCTGAGCGTTCTCAACTTGGTTTTGN
NNNNAGGAGAGAAGGATATTCTGGACTGACTGATACTACANTGCTCGCCGCTGGCCCCNA
AAGACTANCATAATCGCAACTTTAATCTCTTAAGAAGAGATGTCCCCATATGNCTNNAAA
GCCN

Clone 1060 (ad2)

ATNTTTGCGCCNTGGGCCCGTNGTGCGGCATGCCTCCCGGCCGCCATGGTCGGCCGCGGG
TAATTCGATT**AGCGTGGTCGCGGCCGAGGT**GGCTTGGCACCCAGAGAAAGCCTCTGCCC
CCAAAAGAGACTTTCTACATAGGGTTAAGCTTCATTAAATGAGGTGCAACCTTTCATTTTC
AGGGCATCTCTTTTCTTGCAAGGTCTTTAGAGGCAGAAGTTCCACTTGGATTCTAATACAC
ATCTCTGTGAGCTCAGTTTCCTGAAAATATACACCTACTGGGTTCTAGGTTCTCCCTTACCA
GTGACTGTATTCATTATTTCACAGCCACCAGAATCGGACATACACTATTAACATGATGAAA
AATACAGCTACTGCTGCAAGTTTGGCATAAGTGGAACGCATGTTCAAGTACTTCGCATCCT
GGCA

Clone 1064 (ad1)

TTTATNNNAACNANCNTCTGTTTACCGTATCCCGGCATGTNTCCCGNCCGTACNTGGNGGG
CCGCGNGTAATTTAGATT**TCGAGTCGGCACGNCCGNNCAGGT**ACTTCATTCGAGCATGA
GTTTCTGAGGATCAGCACTGCCAGTGGAGATGGGCGTCACTACTGCTACCCTCACTTCACC
TGCGCTGTGGACACTGAGAACATCCGCCGTGTGTTCAACGACTGCCGTGACATCATTCAGC
GCATGCACCTTCGTCAGTACGAGCTGCTCTAAGAAGGGAACCCCCAAATTTAATTAAAGC
CTTAAGCACAATTAATTAAAAGTGAAACGTAATTGTACAAGCAGTTAATCACCCACCATA
GGGCATGATTAACAAAGCAACCTCCCCCTTCCCCCGAGTGATTTTGCGAAACCCCCTTTTC
CCTTCAGCTTGTTAGATGTCCAAATTTAGAAAGCTTAAGGCGGCTACAGAAAAGGAAAAA
AGCCAAAAGTTCCTCCCN

Clone 1065 (ad2)

TTTTCTCAAGTACCCGNTTCCTATANNGATGCTTCCGNCCGCCATGGCGGCCGCGGGAATT
CGATT**AGCGTGGTCGCGGCCGAGGT**ACTGATGCAACAGTTGGGTAGCCAATCTGCAGAC
AGACACTGGCAACATTGCGGACACCCTCCAGGAAGCGAGAATGCAGAGTTTCCTCTGTGA
TATCAAGCACTTCAGGGTTGTAGATGCTGCCATTGTCGAACACCTGCTGGATGACCAGCCC
AAAGGAGAAGGGGGAGATGTTGAGCATGTTCAGCAGCGTGGCTTCGCTGGCTCCCACTTT
GTCTCCAGTCTTGATCGGCTGCACATCACTCAGGATTTCAATGGTGCCCCTGGAGATTTTA
GTGGTGATACCTAAAGCCTGGAAAAAGGAGGTCATCTCGGGCCCGAGNCAGTGTTCTGGA
CTGCCGGCGGCGTCGAAATCCTATGAATTCCGGCGCTGCAGTCGACTATGGGAACTCCAC
GCGTGATGCTAN

Clone 1070

TTTTTNGGGCGGGAANCTTCNTTAACCTCACNTAATCCCCCTGCTATGCCATTTNCTATTCA
CGTACTATGGCCCGGNCTCCGTCGGGATACATTCGTATNTAAGNCGTGGTCGCGGCNCGN
AGGNTACNCGCAAGGNGTGGTTTGGNATGNAAGNTCTGNACTACTTTCTTTCAAACAANA
CTATTATATTAAAACTGTCATATTTTGGCTAAAGTTTGGGNACCTATAACTACACTTTCAT
NTGTTTGGCAATCTCTCTATGNAAGNATACGTCTGTCCAAACTTTTAAAAGGGCAATAAAC
TGGTAATTTTTATGGTGGTTTAATNTCTTTATAATAAGGAATANGTAATNTTTAATAATTTT
AATTTCTTCAACCCGAAAAGTATTTCAACAACAAATCTTTTTTTAAAAAAAAAAATTTGGG
GAAAATGGGGCAATTTTTGGTAATTGGCCCACAAGAAGGGTAANGGAATGGAAGCCATTA
ATAATTTAANTGGAAAATGGTNTNTAATTTTTGGNNAAAAATATAAANNNGGGGAATTAA
TTTNNGNCCATTNNNTTTAANTAACCTGGNCCCGGG

Clone 1071 (ad2)

NNTGTNCCGNCNNACNTCTTNTTTNCTCCCCNGCATGNNTTCCGNCCGTCATGGNGGGCCG
CGGGTAATTCGATT**AGCGTGGTCGCGGCCGAGGT**ATAATCTTGAAATTAAGAAGGCAA
AAATGTAAAGGAGTCAAAACTATAAATCAAGTATTTGGGAAGTGAAGACTGGAAGCTAAT
TTGCATTAAATTCACAAACTTTTATACTCTTTCTGTATATACATTTTTTTTCTTTAAAAAAC
AACTACGGATCAGAATAGCCACATTTAGAACACTTTTTGTTATCAGTCAATATTTTTAGAT
AGTTAGAACCTGGTCCTAAGCCTAAAAGTGGGCTTGATTCTGCAGTAAATCTTTTACAACT
GCCTCGACACACATAAACCTTTTTAAAAATAGACACTCCCCGAAGTCTTTTGTCGCATGGT

CACACACTGATGCTAGATGTCCAGTAATCTAATATGGCCCAGTAGCTTGATGACCAAAGC
CTTTTTTCTCTTTAGAAACT

Clone 1072 (ad2)

NTTNCCGGNGAACCNCTTTTTCCCGTACGCCCGGCATGCCTNCCGGCACGCCATGGNCGG
CCGCGGGAATTCGATT**AGCGTGGTCGCGGCCGAGGT**ACAGCTACAGCTTCATCAACCTT
AGAACGGAGTGACTCTGGAGACTCGAGCATATGAAGAAGTTCTGAATTATCAATCTCCAA
CAACATGCCAGTGATTTTACCAGCAAGAGTAGGGTGCATGGCTTGAATAAGAGGAAACAG
CCGTTCACCCAACATTTACTTTTGCTCTTGAGGAGGGGCAGATGCCAACATGGAAGCAGTC
AAAGGTTCCTGACCTTGTACCGCCGGGGCGGGCGCTCGAAATCCTAGTGAATTCGCGGCC
GCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCT
ATAGTGCACCCTATA

Clone 1073 (ad1)

AGTTTNNGNAGCNCATNCGNACNTCACTACNCGGTACACNTAAATCCGTATTGGGCNGAN
CGTNANNCTTTATATT**TGCGAGCCGGAACGTCCGGGCAGGT**ACAGGGCAGGGCCCTTA
TAATTTGGGCCTCAAGAATTGATAGTTGTCTCTTAACTGGTTCTGCTGAGGCGCACTCACA
GCAGTGAGTGGAGGGTCCTGGGAGGGCATGGATGACGGAGGCTGCCCCTAGAGGAGCTG
GATGTAGGAGCAGAGACATTTCTTGATCTTTCACTCTGCAAACTGGTCCAAGAACTTGAGG
TAGGCCTGACGCTGGGGTGCAGCTGCTGGAATGAAGTGGCCACCAGAGTGGGTGAGGGTG
ATGGCTCCGGGAAATTGCTGACCAGTTGCACACTCTCTGAGAGGGGATGACTTTGTCAGT
GCCCCAAAACATGGAGCGAAGCAATGACAGGCCTTTCAGATGGATTCTTGACCCATGCCC
GGGGACAGAACCAGCCCAGA

Clone 1073 (sequenced with sp6 primer)

GAGTTTTTTTNNTTCAAANGATGAAACCNCGTTNTTTTANTNNGCTATCCGCNGCNGCGNG
TGNTGTAGACTCTACCCATGATGGGGCTGCGAACTCTGCAGGTCGGCCGCGNAATTCACN
TAGNTGNATNTAGCGTGGTCGCGGCCGNAGGTCGNACGTTTTCTCCGCATTGGNAAGNAG
CCCGCCGTCTGCAGGGGCCTGGNAGGGAATCACTGGGGATGGTGGCACCAAGGCACTGG
AACAGGCTTGGGGCCTTTTGACGGCCTTCTTGGGTTTCAAGCCAAGGGGCTGCGCTAGCA
GCCCTTGTGTGTGCCCTGGGGCCAAGGCAGGCGATCCCCGCTTCTCCTTGCCACGGTTTAT
CCTCTTGGTGGTCTGGGTTTCTGTCCCCGGGGCCATTGGGGTTCAAGGGAATCCATCCTTG
CAAAGGCCCTTGTCATTGCCTTCGCTCCATGTTTTTGGGGGGACACTTGGACAAAAGTCAT
CCCCTCTCAGGGAGAGTGTGCAAACTGGGTCAAGCCAATTTCCCGGGAGCCATCACCCTN
ACCCACTCTGGGTGGGCCACTTCATTCCA

Clone 1075 (ad1)

TCTNCTTCNCNNNAANCACGANTCCTATNCTGGTCACNTAAATCCGNCATGCCGGCCGCG
GGNAATTCGATT**TCGAGCGGCCGCCCGGGCAGGT**ACGCAAATGTTTTTCAACAGTTATT
GAAAATAGGAGACCAAGTTGAACGTGCCAAAGAGATACCATTCAGTTGGATTTTCTAGGG
TCAACATGAAGAAAATTAGAGAAGGGATTTACTTGGTTGGTTTCCATAGCTTGATAGACC
AAAAACAAAAAAACTGCCACAACAACAAACAGCAAAATTTTTATCCATACGGGAATGGA
GCGTCCCTTTTTTGTCTTTTCTGACTTGACATCTGCCAAGGGAACATACTTAGGAACATATT
TAGATGAAAAAGACTCCTCCGTCCTGAAATCCTGAGTTCTGATGGCGGCTGAGCCTTTGAT
GGCTGCGGCACTAGCCTAATTCTGNGGGTAATGTTATANGGGACATTTCTTAAGATATCCC
TTTTCTCC

Clone 1078 (ad2)

GNNNNGAGGNNNNNNTTTANTTTCAAACCGCNTCTNNTTCCTATCNNNGNNNCNTANNTC
CGTATTGGGNGGACGTNNNTAATTCGATT**AGTCGTGGTCCGCGGCCGAGGT**ACGAAAA
TGGGACCAGACACATGAACAGCCTGGCCTAAATGAACTCCATCAATCTTAACTGTGACAG
AAGTAATGGAGGATAAGGAAAAGGCCAAGACTCTGATGTGTGTTGAGTGAAGAAGTCTTT
CTAGTGGTTCATGTTCACCACAACTATAAAGGAGTGATTTAGGATTGGTGATAAGAACCA
CAGGCCACTTCCCAAAGATCAAATCTGCAAAGCTAAAGAGGTCGTGATCAAAAGCAAAA
ATCCGGTACCTCCCGGGCG

Clone 1083 (ad1)

NTNTNCNNGNNACCNCGTTTCCTATCCGATGCTTCCGNCCGTACATGGCGGCCGCGGGAA
TTCGATT**TCGAGCGGCCGCCCGGGCAGGT**CTGTAGGGATACTGCTTCATAAGCACCAGC
AGAACACCAAAGGAGACCATATGGGTGAAAGCAACCAGCACTGCCCTGGCGCTTCATAG
GTTCTTAGAGTTTTTATCTTTTACTTTCAGTCTAACACAGCACTGCCTGCTTTTTGTTTTTGT
TGCTTGGTTTGTTTTTTTCTTACCGTGTTCACCAAACTTGTGTCCAAATAGCTTTGGGCTGA
TGCAAAAATATCTATGTGGAAGAGAAGAAGTTGTTCTCATGGAGGGCCTTCAGATGAGTG
CTATAGACTCTCTAGGCAACTCCAAGAGGCTTTAAGCAGGGTGGGCAGTGAGAGCTGTAT
GGAATCAATGGACAAACTGACAGGGACTGTTTGAAAGACAGACCTCGGCGGACACGCTA
ATCCTATGAATA

Clone 1085

GTTNTAGNNCCATATACGGANCGTAACTAATCGCGGGTANNACAACNCACTATAGGGNAA
TACGACTCACTATAGGGTNATNCGAGTCACTATAGGGTCTNCCGAATCACNATNGGGNGA
TAGCTGNGAGAANAAAGCNTGNCCTTACANAGAGCCACNGAGCNNGCNNGGNCACATTT
CTCTGCAGCANTNTCATTCATTCTGANAGTTNACNANCANGAACTGCCGANGCCTTNAAC
CTGGATGCGGCGCCANTNGAACTAATCAAGCGACGNCAGCAGANGTGNTCGNNTGCATC
ACAGCTTAACAGCTCANNGTCTGAGTNAGAGCTNCNCTNATTNCAGANAGTGGACACTGN
NCTTGNACATCCTAANGGCACCTCTGNATGGCTCNTGCAGCNCACAAACAGCNGNCCCAG
GANATGNCTACTCANAGGGCGNTAGACAGAGACGAGCANANATCCANCGGCTGCAATAN
TCNGNTCNGCTGNACTNCACN

Appendix F: BLAST search results of the sequenced clones

Clone and accession numbers have been indicated.

Blast bp: Number of base pairs subjected to BLAST search.

Identities: Percentage of homology of the sequence identified by BLAST search.

Appendix F: BLAST search results of the sequenced clones

Clone #	accession #, gene	BLAST bp	Identities
33	gi|16551409|dbj|AK056096.1|AK056096 Homo sapiens cDNA FLJ31534 fis, clone NT2RI2000671	338	168/169 (99%)
41	gi|13642967|ref|XM_018280.1| Homo sapiens poly(A)-binding protein, cytoplasmic 1 (PABPC1), mRNA	349	345/349 (98%)
362	gi|13642967|ref|XM_018280.1| Homo sapiens poly(A)-binding protein, cytoplasmic 1 (PABPC1), mRNA	424	359/360 (99%)
1072	gi|13642967|ref|XM_018280.1| Homo sapiens poly(A)-binding protein, cytoplasmic 1 (PABPC1), mRNA	339	223/224 (99%)
48	gi|13640156|ref|XM_015349.1| Homo sapiens ribosomal protein S6 (RPS6), mRNA	354	345/353 (97%
1040	gi|13640156|ref|XM_015349.1| Homo sapiens ribosomal protein S6 (RPS6), mRNA	280	177/189 (93%)
1049	gi|13640156|ref|XM_015349.1| Homo sapiens ribosomal protein S6 (RPS6), mRNA	388	301/313 (96%)
55	gi|13376244|ref|NM_024835.1| Homo sapiens C3HC4-type zinc finger protein (LZK1), mRNA	355	353/355 (99%)
59	gi|21361889|ref|NM_021633.2| Homo sapiens kelch-like protein C3IP1 (C3IP1), mRNA	286	170/172 (98%)
293	gi|21361889|ref|NM_021633.2| Homo sapiens kelch-like protein C3IP1 (C3IP1), mRNA	291	174/174 (100%)
66	gi|18375675|ref|NM_080599.1| Homo sapiens regulator of nonsense transcripts 2 (RENT2), transcript variant 1, mRNA	352	350/352 (99%)
67	gi|13650772|ref|XM_016712.1| Homo sapiens hypothetical protein FLJ23375 (FLJ23375), mRNA	352	161/167 (96%)
1078	gi|13650772|ref|XM_016712.1| Homo sapiens hypothetical protein FLJ23375 (FLJ23375), mRNA	265	259/259 (100%)
82	gi|14150014|ref|NM_032299.1| Homo sapiens hypothetical protein MGC2714 (MGC2714), mRNA	263	203/236 (86%)
89	gi|1552325|emb|Y07969.1|HSAPRIL H.sapiens mRNA for APRIL protein	337	253/258 (98%) 51/60 (85%)

93	gi|11425596|ref|XM_001901.1| Homo sapiens Sjogren syndrome antigen A2 (60kD, ribonucleoprotein autoantigen SS-A/Ro) (SSA2), mRNA	353	324/326 (99%)
107	gi|19923906|ref|NM_138381.1| Homo sapiens hypothetical protein BC008322 (LOC92106), mRNA	395	289/290 (99%)
434	gi|19923906|ref|NM_138381.1| Homo sapiens hypothetical protein BC008322 (LOC92106), mRNA	415	287/288 (99%)
415	gi|19923906|ref|NM_138381.1| Homo sapiens hypothetical protein BC008322 (LOC92106), mRNA	466	287/288 (99%)
113	gi|13751653|gb|XM_028311| Homo sapiens solute carrier family 38,member 2 (SLC38A2), mRNA (ATA2)	348	260/261 (99%)
114	gi|20561660|ref|XM_168026.1| Homo sapiens KIAA1007 protein (KIAA1007), mRNA	349	340/349 (97%)
458	gi|20561660|ref|XM_168026.1| Homo sapiens KIAA1007 protein (KIAA1007), mRNA	402	318/345 (92%)
118	gi|20551719|ref|XM_166297.1| Homo sapiens CG2277 gene product (LOC221294), mRNA	351	342/353 (96%)
121	>gi|14150079|ref|NM_032314.1| Homo sapiens hypothetical protein MGC4767 (MGC4767), mRNA	345	339/345 (98%)
133	gi|13160958|gb|AF321876.1|AF321876 Homo sapiens candidate tumor suppressor (DPH2L1) mRNA, complete cds	365	357/360 (99%)
1073	gi|13160958|gb|AF321876.1|AF321876 Homo sapiens candidate tumor suppressor (DPH2L1) mRNA, complete cds	398	343/352 (97%)
153	gi|13646652|ref|XM_008741.3| Homo sapiens suppressor of K+ transport defect 1 (SKD1), mRNA	351	348/352 (98%)
158	gi|11429541|ref|XM_005622.1| Homo sapiens dystonia 1, torsion (autosomal dominant; torsin A)(DYT1), mRNA	351	348/351 (99%)
162	gi|13097233|gb|BC003378.1|BC003378 Homo sapiens, high-mobility group (nonhistone chromosomal) protein 1,	369	286/289 (98%)
174	gi|13637791|ref|XM_002696.3| Homo sapiens mutS (E. coli) homolog 2 (colon cancer, nonpolyposis type 1) (MSH2)	343	337/343 (98%)
177	gi|13641527|ref|XM_005078.3| Homo sapiens RAD21 (S. pombe) homolog (RAD21), mRNA	338	223/224 (99%)
190	gi|307154|gb|L19183.1|HUMMAC30X Human MAC30 mRNA, 3' end	341	337/341 (98%)
268	gi|307154|gb|L19183.1|HUMMAC30X Human MAC30 mRNA, 3' end	443	379/392 (96%)

192	gi|13636890|ref|XM_018498.1| Homo sapiens ribosomal protein L5 (RPL5)	391	343/346 (99%)
218	gi|12655032|gb|BC001364.1|BC001364 Homo sapiens, SEC22, vesicle trafficking protein (S. cerevisiae)-like 1	341	338/340 (99%)
1060	gi|12655032|gb|BC001364.1|BC001364 Homo sapiens, SEC22, vesicle trafficking protein (S. cerevisiae)-like1,mRNA	335	333/334 (99%)
234	gi|14250147|gb|BC008492.1|BC008492 Homo sapiens, ribosomal protein L3, clone MGC:14821, mRNA	455	386/390 (98%)
259	gi|12731938|ref|XM_004104.2| Homo sapiens ribonuclease P, 40kD subunit (RPP40), mRNA	343	231/235 (98%)
295	gi|12731938|ref|XM_004104.2| Homo sapiens ribonuclease P, 40kD subunit (RPP40), mRNA	346	237/241 (98%)
277	gi|13630833|ref|XM_009601.3| Homo sapiens 5'-3' exoribonuclease 2 (XRN2), mRNA	452	355/361 (98%)
282	gi|15076870|gb|AF279306.1|AF279306 Homo sapiens anti-silencing function 1A (ASF1A) mRNA	445	339/340 (99%)
447	gi|15076870|gb|AF279306.1|AF279306 Homo sapiens anti-silencing function 1A (ASF1A) mRNA, complete cds	406	339/340 (99%)
305	gi|13640144|ref|XM_005467.4| Homo sapiens hypothetical protein FLJ20060 (FLJ20060), mRNA	387	366/367 (99%)
314	gi|3882170|dbj|AB018268.1|AB018268 Homo sapiens mRNA for KIAA0725 protein, partial cds	347	342/343 (99%)
321	gi|14039833|gb|AF367998.1|AF367998 Homo sapiens elongation factor G1 (EFG1) mRNA, complete cds	433	427/431 (99%)
326	gi|217461916|ref|XM_065847| Homo sapiens similar to tumor metastasis suppressor; LAG1 homolog	460	326/331 (98%)
420	gi|217461916|ref|XM_065847| Homo sapiens similar to tumor metastasis suppressor; LAG1 homolog	346	264/277 (95%)
332	gi|13629831|ref|XM_005799.3| Homo sapiens integrin, beta 1 (fibronectin receptor, beta) (ITGB1)	335	61/64 (95%)
339	gi|12654186|gb|BC000911.1|BC000911 Homo sapiens, suppressor of G2 allele of SKP1, mRNA, complete cds	399	299/300 (99%)
358	gi|13645714|ref|XM_011883.2| Homo sapiens vinculin (VCL), mRNA	433	427/436 (97%)
370	gi|14741441|ref|XM_030426.1| Homo sapiens hypoxia-inducible factor 1, alpha subunit inhibitor (HIF1AN), mRNA	433	401/404 (99%)

371	gi|11421392|ref|XM_010362.1| Homo sapiens solute carrier family 25,member 5 (SLC25A5),nuclear gene	426	362/368 (98%)
384	gi|21700561|gb|AC092140.3| Homo sapiens chromosome 16 clone RP11-413H22, complete sequence	433	393/397 (98%)
406	gi|4502280|ref|NM_001679.1| Homo sapiens ATPase, Na+/K+ transporting, beta 3 polypeptide (ATP1B3), mRNA	457	363/393 (92%)
410	gi|13627221|ref|XM_007816.3| Homo sapiens G1 to S phase transition 1 (GSPT1), mRNA	439	317/317 (100%)
421	gi|13643398|ref|XM_003719.3| Homo sapiens erbb2-interacting protein ERBIN (LOC55914), mRNA	415	342/346 (98%)
423	gi|13627242|ref|XM_001436.2| Homo sapiens capping protein (actin filament) muscle Z-line, beta (CAPZB), mRNA	347	343/346 (99%)
424	gi|12803510|gb|BC002582.1|BC002582 Homo sapiens, ubiquitin protein ligase E3A (human papilloma virus E6-associated protein)	436	334/336 (99%)
426	gi|12653128|gb|BC000331.1|BC000331 Homo sapiens, proteasome (prosome, macropain) subunit, beta type, 4, PSMB4, clone MGC:8522, mRNA, complete cds	348	340/348 (97%)
431	gi|11493480|gb|AF130088.1|AF130088 Homo sapiens clone FLB9213 PRO2474 mRNA, complete cds	348	321/335 (95%)
438	gi|4557530|ref|NM_001382.1| Homo sapiens dolichyl-phosphate (UDP-N-acetylglucosamine) N-acetylglucosaminephosphotransferase 1 (GlcNAc-1-P transferase) (DPAGT1), mRNA	525	161/175 (92%)
446	gi|7271466|gb|AF178980.1|AF178980 Homo sapiens D-prohibitin mRNA, complete cds	445	336/339 (99%)
452	gi|11968293|gb|AC010368.4|AC010368 Homo sapiens chromosome 5 clone CTD-2046J7, complete sequence	351	265/268 (98%)
460	gi|11038647|ref|NM_003503.2| Homo sapiens CDC7 (cell division cycle 7, S. cerevisiae,homolog)-like 1 (CDC7L1)	393	311/320 (97%)
470	gi|13641498|ref|XM_005572.3| Homo sapiens fructose-1,6-bisphosphatase 1 (FBP1), mRNA	388	243/245 (99%)
474	gi|9945333|ref|NM_000403.2| Homo sapiens galactose-4-epimerase, UDP- (GALE)	196	185/185 (100%)

475	gi|14043355|gb|BC007672.1|BC007672 Homo sapiens, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9 (22kD, B22), clone MGC:1186, mRNA, complete cds	398	358/366 (97%)
1026	gi|13653412|ref|XM_016771.1| Homo sapiens activity-dependent neuroprotective protein (ADNP), mRNA	339	285/285 (100%)
1029	gi|13653050|ref|XM_008502.3| Homo sapiens CGI-48 protein (LOC51096), mRNA	258	244/255 (95%)
1034	gi|13652652|ref|XM_006998.3| Homo sapiens retinoblastoma-binding protein 2 (RBBP2), mRNA	269	239/241 (99%)
1064	gi|12803766|gb|BC002722.1|BC002722 Homo sapiens, guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1, clone MGC:3330, mRNA	397	376/384 (97%)
1065	gi|13543411|gb|BC005863.1|BC005863 Homo sapiens, ribosomal protein, large, P0, clone MGC:3679, mRNA	407	332/336 (98%)
1070	gi|11419784|ref|XM_010122.1| Homo sapiens sex comb on midleg (Drosophila)-like 1 (SCML1)	485	123/134 (91%)
1071	gi|14043361|gb|BC007674.1|BC007674 Homo sapiens, CD24 antigen (small cell lung carcinoma cluster 4 antigen)	409	385/400 (96%)
1075	gi|885681|gb|U18271.1|HSTMPO6 Human thymopoietin (TMPO) gene, partial exon 6, complete exon 7, partial exon 8, and partial cds for thymopoietin beta	396	142/142 (100%)
1083	gi|18181683|emb|AL583839.12|AL583839 Human DNA sequence from clone RP11-165J3 on chromosome 9	405	203/209 (97%)
1085	gi|14132743|gb|AY014957.1| Homo sapiens PI-3-kinase-related kinase SMG-1 (SMG1) mRNA	371	256/321 (79%)

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Identification of genes induced by BRCA1 in breast cancer cells

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Abstract

Inherited mutations of the BRCA1 gene predispose to breast, ovarian, and other cancers. The role of the BRCA1 gene in the maintenance of chromosomal integrity is linked to a number of biological properties of its protein product, including transcriptional regulation. In the present study, we have used suppression subtractive hybridisation (SSH) to identify genes induced by BRCA1 by comparing control MCF7 breast carcinoma cells (driver) with MCF7 cells ectopically expressing BRCA1 (tester) and generated a forward subtracted cDNA library. We screened 500 putative positive clones from this library. Two hundred and ten of these clones were positive by differential screening with forward and reverse subtracted probes and the 65 cDNA clones which showed more than fivefold increase were selected for sequencing analysis. We clustered 46 different genes that share high homology with sequences in the GenBank/EMBL databases. Among these, 30 were genes whose function had been previously identified while the remaining 16 clones were genes with unknown functions. Of particular interest, BRCA1 gene induces the expression of genes encoding DNA repair proteins RAD21 and MSH2, ERBB2/HER2 interacting protein ERBIN, meningioma-associated protein MAC30, and a candidate ovarian tumour-suppressor OVCA1. Northern and Western blot analyses confirmed that the expression of these five genes are up-regulated following BRCA1 overexpression in MCF7 and UBR60-bcl2 cells. This is the first study reporting a set of BRCA1-induced genes in breast carcinoma cells by the SSH technique. We suggest that some known genes identified in this study may provide new insights into the tumour-suppressor function of BRCA1.

Keywords: BRCA1; Breast cancer; SSH; MCF7; Gene expression; Target gene

Germ-line mutations in the BRCA1 tumour-suppressor gene predispose carriers to breast and ovarian cancers and account for approximately 50% of inherited breast cancers [1]. BRCA1 gene encodes a 220 kDa multifunctional nuclear phosphoprotein involved in different cellular pathways including cellular response to DNA damage, cell cycle, growth suppression, apoptosis induction, ubiquitin ligation, and transcription regulation [2]. Several functional regions have been described in BRCA1: an N-terminal RING finger domain, two nuclear localisation signals, and two BRCA1 carboxyl terminal (BRCT) motifs at the carboxyl terminus. BRCA1 is the main component of the BRCA1-associated genome surveillance complex (BASC) which serves as a sensor for DNA damage. BASC comprises tumour suppressors and DNA damage repair proteins MSH2, MSH6, MLH1, ATM, BLM, the RAD50–MRE11–NBS1 protein complex, and DNA replication factor C [3,4]. In addition, the carboxyl terminal of BRCA1 acts as a strong transcriptional activator when fused to a heterologous DNA binding domain [5]. BRCA1 co-purifies with RNA polymerase II holoenzyme complex, suggesting that it is a component of core transcription machinery [6]. BRCA1 also interacts with several transcription factors such as p53, CtIP, c-myc, ZBRK1, ATF, E2F, and signal transducer STAT1 [7] and modulates their activity. These findings, together with the interaction of BRCA1 with histone deacetylases (HDACs) and the SWI/SNF-related chromatin remodelling complex, imply that transcriptional regulation is one of the main functions of BRCA1 [8]. In addition, nearly all germ-line BRCA1 mutations involve truncation or loss of the C-terminal BRCT transcriptional activation domain, suggesting that transcriptional regulation is a critical function of the BRCA1 gene. Our

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purpose in this study was to identify the genes whose expression is regulated by BRCA1 and therefore contribute to a better understanding of its cellular functions. We used MCF7 breast carcinoma cell line which has low endogenous BRCA1 expression level and maintains wild-type p53 [9]. We compared the expression profile of control MCF7 cells with MCF7 cells ectopically expressing BRCA1 by performing SSH. We generated a subtracted cDNA library and identified novel BRCA1-induced genes as candidate mediators of tumour suppression by BRCA1.

Materials and methods

Cell lines and cell culture. MCF7 breast carcinoma cells (American Type Culture Collection) and UBR60-bcl2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1 mM glutamine, 10 U/ml penicillin G, and 10 μg/ml streptomycin, at 37 °C in 5% CO_2-containing atmosphere. The UBR60-bcl2 cell line (a gift from Dr. Paul Harkin) which expresses BRCA1 under the control of tetracycline-regulated promoter has been previously described [10].

Plasmids and transfections. pCMVmycBRCA1 was constructed by subcloning the full-length BRCA1 insert from pCR3.BRCA1 (provided by Dr. Barbara Weber) into pCMVmyc vector (Clontech laboratories, Palo Alto, CA). pCR3.BRCA1 was cut with *Hin*dIII, the ends were filled with Klenow, and *Sal*I linkers were ligated into the ends. The plasmid was digested with *Sal*I/*Not*I and the BRCA1 cDNA was cloned into pCMVmyc vector (Clontech). MCF7 cells were grown to 80% confluency 24 h prior to electroporation. Briefly, cells were harvested with trypsin, washed twice with ice-cold calcium and magnesium-free PBS, and re-suspended in ice-cold PBS. Plasmid DNA was added ($30\,\mu g/15 \times 10^6$ cells/cuvette) and electroporation was carried out at 950 μF, 0.22 kV/cm ($t = 19$–22 ms) (BioRad Gene Pulser). pEGFP-N2 (Clontech) was used as a control plasmid to calculate the transfection efficiency using the same experimental conditions. Total RNA and protein extractions were performed 24 h after transfection.

Annexin V staining. Binding of Annexin V to the cell surface, which is an early indication of apoptosis, was determined with the Annexin V-PE stain (PharMingen, BD Biosciences). Cells were washed twice with cold PBS and then incubated in the dark in 100 μl binding buffer (0.01 M Hepes, pH 7.4, 0.14 M NaCl, and 0.25 mM $CaCl_2$) containing 5 μl Annexin V-PE stain per coverslide. The coverslides were washed with binding buffer and fixed in 1 ml of 90% cold ethanol for 30 min under dim light. After washing with PBS, Hoechst staining (300 μg/ml final concentration) was performed for 5 min in the dark. The coverslides were extensively washed with H_2O and mounted onto 80% glycerol droplet. As a positive control for apoptosis, MCF7 cells were treated under 50 J/m^2 ultraviolet light and the same experimental conditions were applied.

Western blot analysis. Cells were harvested using RIPA lysis buffer (10 mM Tris–Cl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1% NaDOC, and protease inhibitors). Equal amounts of protein extracts were loaded in 10% (for MSH2 and cytokeratin 18) or 5% (for BRCA1, Ab-1) SDS–polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After overnight incubation at 4 °C in blocking solution (TBS-T: 20 mM Tris–HCl, pH 7.6, 0.137 M NaCl, 0.5% Tween 20, and 3% milk) membranes were incubated for 2 h at room temperature with mouse anti-human BRCA1 monoclonal (Ab-1, Oncogene Research Products), mouse anti-human MSH2 monoclonal (Ab-1, Oncogene Research Products), or cytokeratin 18 (JAR13 clone, a gift from Dr. D. Bellet, IGR, France) antibodies. Blots were washed three times for 10 min in TBS-T and incubated with the secondary antibody (Horseradish peroxidase-conjugated anti-mouse IgG, 1:2000, Santa Cruz Biotechnology) for 1 h at room temperature with constant shaking. After extensive washing, chemiluminescence detection was carried out with the Amersham ECL detection kit according to manufacturer's instructions.

RNA isolation for cDNA subtraction. Total RNA was prepared with TRI Reagent (Sigma). mRNA was isolated from total RNA with poly(A) Spin mRNA Isolation Kit (New England Biolabs) according to manufacturer's specifications. For removal of DNA contamination from RNA samples, the MessageClean Kit (GenHunter) was used according to manufacturer's instructions.

Suppression subtractive hybridisation (SSH). SSH was performed with PCR-Select cDNA Subtraction Kit (Clontech) as described by the manufacturer with minor modifications. In brief, 3.5 μg poly(A)$^+$ RNA from MCF7 cells electroporated with pCMVmyc used as the driver and 3.5 μg from pCMVmycBRCA1-transfected cells was used as the tester to construct a forward subtracted library. Reverse subtraction was also performed where tester cDNA was derived from MCF7 electroporated with pCMVmyc and driver cDNA derived from MCF7 electroporated with pCMVmycBRCA1 using the same experimental conditions. SSH was performed with double-strand tester and driver cDNAs. Primary PCR condition was 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 90 s for 30 cycles in 25 μl reaction volume. One microliter of 1/10th diluted primary PCR product was added into a new PCR tube for a second round of PCR. The secondary PCR condition was 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 90 s for 15 cycles. All PCR and hybridisation steps were performed on a Perkin–Elmer 9600 thermal cycler.

Cloning of cDNA mixture. The final PCR-generated forward subtracted cDNA mixture, enriched for BRCA1 up-regulated sequences, was cloned into the cloning vector pGEM-T Easy (Promega) and transformed into supercompetent *Escherichia coli* strain JM109. The transformed bacteria were plated on 150 mm ampicillin agar plates containing 100 mM IPTG and 50 mg/L X-Gal and bacteria were grown overnight at 37 °C. Plates were then incubated further at 4 °C until blue/white staining could be clearly distinguished.

Differential screening of clones. The PCR-Select Differential Screening Kit (Clontech) was used to screen the subtracted cDNA library to analyse the differentially expressed sequences according to manufacturer's recommendations. Five hundred white clones were picked randomly, inoculated into sterile 96-well plates containing LB medium with ampicillin, and grown overnight shaking at 37 °C. Then 1 μl of each bacteria culture was used to amplify the cDNA inserts with SP6 and T7 universal primers. The PCR products were then transferred into 96-well plates and denatured in equal amount of NaOH and 1.5 μl of each sample was blotted onto two nylon membranes (Hybond N$^+$) in an identical order. Negative hybridisation controls cDNA1 and cDNA2 (provided by the manufacturer), and BRCA1 cDNA were included as control in the blots. The forward and reverse subtracted cDNA pools in SSH steps were used as probes which were labelled with [α-^{32}P]dCTP using Random Primers DNA Labeling System (Clontech). Two identical blots were hybridised with either forward or reverse probes with the same number of cpm for each pair and signal intensity was measured by phosphorimaging (Bio-Rad Molecular Imaging System). Two hundred and ten clones which showed more than fivefold increase in signal intensity were again dotted onto membranes and differential screening was repeated two further times.

Sequencing and homology search. All sequencing reactions were performed on double-stranded plasmid templates with T7 and SP6 primers. Sequencing reactions were carried out with the Big Dye terminator cycle sequencing kit and analysed with the ABI 377 DNA sequencer. Partial cDNA sequences were compared with entries in the GenBank/EMBL database using the BLAST homology search program at http://www.ncbi.nlm.nih.gov/blast/.

Northern blot analysis. Total RNA was isolated with TRI Reagent (Sigma). Thirty μg of total RNA samples was resolved by formaldehyde gel electrophoresis, run overnight at 50 V, and then transferred to

nylon membrane (Hybond N^+). Purified cDNA inserts were labelled with HexaLabel Plus Kit (MBI Fermentas) and separated by Quick Spin columns (Boehringer–Mannheim). Membrane hybridisation was carried out overnight at 65 °C in hybridisation buffer (0.5 M phosphate buffer, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA, and 10 μg/ml salmon sperm DNA, and yeast total RNA) containing at least 10^7 cpm [α-^{32}P]dCTP-labelled cDNA PCR products as probes per membrane. Membranes were sequentially washed in 2× SSC and 0.5% SDS, 1× SSC and 0.5% SDS, and 0.2× SSC and 0.5% SDS at 68 °C and the filters were exposed to X-ray film with an intensifying screen at −70 °C.

Results

Ectopic expression of BRCA1 in MCF7 breast carcinoma cells

MCF7 cells were transiently transfected with either pCMVmycBRCA1 or a control plasmid pCMVmyc lacking BRCA1 insert. The transfection efficiency, verified by pEGFP-N2 reporter plasmid, was approximately 40%. Under these conditions, less than 1–2% of cells were apoptotic, as tested by Annexin V staining at 24 h, following transfection with either BRCA1 or control plasmid (data not shown). Thus, at a transfection efficiency of 40%, no more than 5% of BRCA-1 expressing cells were apoptotic. This indicated that ectopically expressed BRCA1 was not significantly cytotoxic to MCF7 cells under test conditions. BRCA1 or control plasmid-transfected cells were examined for the BRCA1 protein level by Western blot analysis. The results of two representative experiments are shown in Fig. 1. Both control MCF7 cells (lane 1) and the cells transfected with control plasmid without BRCA1 insert (lane 2) demonstrated endogenous BRCA1 protein. As shown in Fig. 1 (experiment 1) ectopically expressed BRCA1 protein level was similar to endogenous BRCA1 level, suggesting that transient transfection resulted in a significant increase in total BRCA1 levels in pCMVmycBRCA1-transfected MCF7 cells (lane 3). Taken together, these results indicated that it was possible to increase BRCA1 levels in MCF7 by transient transfection, without compromising cellular integrity.

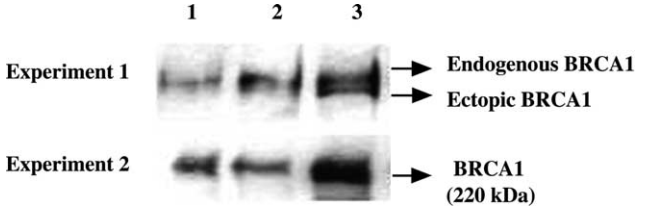

Fig. 1. Ectopic expression of BRCA1 in MCF7 breast carcinoma cells. MCF7 cells electroporated without any plasmid (lane 1), cells transfected with control plasmid pCMVmyc (lane 2), and pCMVmycBRCA1 plasmid (lane 3) are shown. Total protein extracts were prepared and analysed for BRCA1 by Western blot using Ab-1 antibody specific to BRCA1. Blots shown are representative of Western blot analysis from six separate experiments.

Generation of a subtracted library and screening of differentially expressed clones

We used suppression subtractive hybridisation (SSH) technology to identify genes that are differentially expressed in BRCA1-transfected MCF7 cells. SSH was performed with the double-strand tester (MCF7/pCMVmycBRCA1) and driver (MCF7/pCMVmyc) cDNAs (forward subtraction). Reverse subtraction was also performed where tester cDNA was derived from pCMVmyc-transfected MCF7 cells and driver cDNA derived from pCMVmycBRCA1-transfected cells. Following SSH, differential screening was performed with these forward and reverse subtracted probes in order to identify differentially expressed genes. Five hundred clones were selected at random from the forward subtracted library enriched for BRCA1 up-regulated sequences and the cDNA inserts were amplified. The PCR products were then blotted onto membranes together with control cDNAs and probed with the forward and reverse subtracted cDNA pools. Two hundred and ten of them were screened positive by differential screening with forward and reverse subtracted probes and 65 cDNA clones which showed more than fivefold increase were selected for sequencing analysis (Table 1). Fig. 2 shows an example of duplicate dot blots hybridised with forward (Fig. 2A) and reverse subtracted cDNA probes (Fig. 2B), respectively. Control experiments indicated the subtraction allowed the enrichment of BRCA1 cDNA. As expected, the negative hybridisation controls cDNA1 and cDNA2 were not hybridised.

BRCA1-induced genes in breast cancer cells form several distinct functional classes

To characterise the 65 differentially expressed clones, the inserts were partially sequenced and the output sequences, compared to the GenBank/EMBL database in order to find homologies with already known genes. Eight clones yielded poor sequence data and eleven genes were represented more than once in the library (Table 1). Overall, 57 clones showed more than 90%

Table 1
Summary of the analysed clones

	Number
Analysed putative positive clones after SSH	500
Confirmed by differential screening[a]	210
Number of clones sequenced[b]	65
>90% homology	57
Known function	30
Unknown function	16

[a] The cDNA clones which showed more than 5-fold induction were selected for sequencing.

[b] Eight clones yielded poor sequence data and eleven genes were represented more than once in the library.

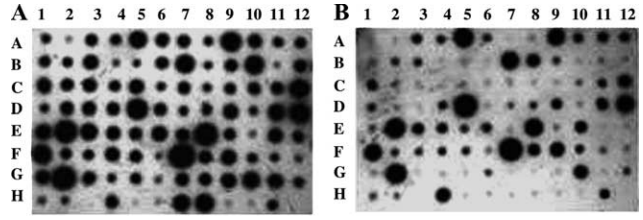

Fig. 2. Differential screening of SSH-selected cDNA clones with forward (A) and reverse (B) subtracted probes. Selected cDNA inserts were PCR-amplified from forward subtracted cDNA library, enriched for BRCA1 up-regulated sequences, spotted in two identical membranes, and hybridised with [α-^{32}P]dCTP-labelled forward or reverse subtracted cDNA probes. Rows A–H; test cDNA samples, Rows H3: negative PCR control, H5–H6: cDNA1, H9–H10: cDNA2 as negative control cDNAs, H7–H8: BRCA1, and H12: NaOH + water). For example E2 cDNA showed no significant increase (1.76-fold), but C3 cDNA (zinc finger protein, LZK1) displayed 7.0-fold increase. The signal intensities were measured by phosphorimager.

homology to known genes. As shown in Table 2, we associated 30 of the genes to a known or putative function: 5 in transcriptional regulation, 3 in intermediate metabolism, 3 in ubiquitin-mediated proteolysis, 2 in chromatin organisation, 3 in DNA repair, 2 in receptor-mediated signalling, and 2 in cytoskeletal organisation. The others were involved in amino acid or ion transport, cell–cell and cell–ECM interaction, endosome or vesicle-trafficking, and *N*-glycan biosynthesis. The Cdc7 protein kinase which is involved in the initiation of DNA synthesis was also induced by BRCA1. The function of the remaining 16 genes was unknown.

Confirmation of BRCA1-induced genes by Northern and Western blot analysis

Several BRCA1-induced genes reported here are known to be involved in DNA repair (RAD21, MSH2); [11,4] and chromosomal structure maintenance (RAD21, CDC7, SGT1, and HMG1); [12–15]. This observation strongly suggests that at least some of the effects attributed to BRCA1 for chromosomal stability are mediated by these genes. To further analyse their BRCA1-regulated expression, we analysed RAD21 and MSH2 expression in two different cell lines. Both RAD21 and MSH2 were up-regulated following overexpression of BRCA1 in MCF7 cells (Figs. 3A and B) as well as in the UBR60-bcl2 cell line following BRCA1 induction (Fig. 3C).

Northern blot assays, using both MCF7 and UBR60-bcl2 cells, demonstrate that BRCA1 induces ERBIN expression (Fig. 4). OVCA1 that was identified as a candidate tumour-suppressor gene was also studied. The levels of OVCA1 mRNA transcripts (1.1 and 2.3-kb species) were low in MCF7 cells and BRCA1 overexpression caused induction of both transcript forms (Fig. 4A). Finally, MAC30 transcript, whose expression has been reported to be decreased in meningiomas, schwannomas, and neurofibromas [16] was also induced in MCF7 as well as UBR60-bcl2 by BRCA1 (Fig. 4B). These studies performed with UBR60-bcl2 cells that stably express BRCA1 at modest levels in an inducible manner [10] confirm and validate our results obtained with a transient expression approach to identify potential BRCA1 target genes.

Discussion

In the present study, we have used SSH technology to generate a library of partial-length cDNAs representing differentially expressed mRNAs in BRCA1-overexpressing MCF7 cells. SSH combines subtractive hybridisation with PCR to generate a population of PCR fragments enriched for sequences from genes expressed differentially. SSH-mediated cDNA enrichment allows the equalisation of wide differences in abundance of different transcript species. Consequently, differentially expressed transcripts of low abundance can be cloned [17]. Using this approach we were able to identify 46 genes that are up-regulated as a result of BRCA1 overexpression in breast cancer cells.

Several studies have previously reported the identification of BRCA1 target genes in osteosarcoma [10], colorectal cancer [18], mouse BRCA−/− embryonic stem [19], and human embryonal kidney epithelial [20] cells. None of the genes that we report here have been identified in these previous studies. This could be due to the fact that we used a breast cancer cell line to identify potential BRCA1 target genes by the highly sensitive SSH technique.

We report that several DNA damage response (RAD21, MSH2, ASF1A, and CDC7) and chromosomal structure maintenance (RAD21, ASF1A, CDC7, SGT1, and HMG1) genes are positively regulated targets of BRCA1. As BRCA1-deficient cells suffer from both DSB repair deficiency and chromosomal instability [21], at least some of these defects may be attributed to inefficient expression of these target genes in the absence of functional BRCA1.

Several other genes (such as SGT1, UbE3A, and PSMB4) are involved in ubiquitin-mediated protein degradation. This may be expected since BRCA1 itself (together with BARD1) functions as an E3 ubiquitin ligase [2,22,23]. The involvement of BRCA1 in ubiquitin-mediated protein degradation could help explain the multiplicity of biological roles ascribed to this protein, including coordination of DNA repair-related events. Other genes involved in distant cellular processes such as intermediate metabolism, cell–cell interaction, receptor-mediated signalling, endosome-trafficking, or cytoskeletal organisation also appear to be up-regulated by BRCA1 (Table 2). In this context, BRCA1 seems to act like another tumour-suppressor gene, namely p53 which

Table 2
List of BRCA1 up-regulated genes in MCF7 breast carcinoma cells

Putative function	Gene name	Accession No.	Fold induction	Redundancy	cDNA insert size
Amino acid transport	Solute carrier family 38, member 2 (SLC38A2)	XM_028311	9	1	800 bp
Cell–cell interaction	CD24 antigen (small cell lung carcinoma cluster 4 antigen) (CD24)	XM_087865	29	1	800 bp
Cell–ECM interaction	Integrin, β1 (fibronectin receptor, β polypeptide) (ITGB1), transcript variant 1E	NM_133376	6	1	800 bp
Chaperon	Dystonia 1, torsion (autosomal dominant; torsin A) (DYT1)	NM_000113	11	1	1.1 kb
Chromatin assembly/DNA repair and response	Anti-silencing function 1A (ASF1A)	AF279306	13	2	500 bp
Chromatin structure	High-mobility group (nonhistone chromosomal) protein 1 (HMG1)	BC003378	9	1	650 bp
Cytoskeleton organisation	Vinculin (VCL), transcript variant meta-VCL	NM_014000	7	1	800 bp
Cytoskeleton organisation	Capping protein (actin filament) muscle Z-line, β	BC008095	14	1	800 bp
DNA repair	MutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*) (MSH2)	XM_034901	9	1	600 bp
DNA repair	RAD21 homolog (*S. pombe*) (RAD21)	NM_006265	11	1	500 bp
Vesicle-trafficking	SEC22, vesicle-trafficking protein (*Saccharomyces cerevisiae*)-like 1	BC001364	11	2	900 bp
Endosome-trafficking	Suppressor of K^+ transport defect 1 (SKD1)	NM_004869	9	1	700 bp
Intermediate metabolism	Fructose-1,6-bisphosphatase 1 (FBP1)	NM_000507	9	1	500 bp
Intermediate metabolism	Galactose-4-epimerase, UDP- (GALE)	XM_032314	9	1	400 bp
Intermediate metabolism	NADH dehydrogenase (ubiquinone) 1β subcomplex 9	BC007672	9	1	750 bp
Ion transport	ATPase, Na^+/K^+ transporting, β3 polypeptide (ATP1B3)	NM_001679	10	1	800 bp
N-Glycan biosynthesis	Dolichyl-phosphate *N*-acetylglucosaminephospho-transferase 1 (GlcNAc-1-P transferase)	BC008817	6	1	550 bp
Nuclear envelope protein	Thymopoietin (TMPO)	U18271	11	1	900 bp
Protein kinase/DNA synthesis/Meiosis	Cdc7 (CDC7)	AF015592	11	1	800 bp
Receptor-mediated signalling	Erbb2 interacting protein (ERBB2IP) (ERBIN)	NM_018695	6	1	700 bp
Receptor-mediated signalling	G protein α stimulating activity polypeptide 1 (GNAS)	BC002722	13	1	700 bp
Transcription regulation	C3HC4-type zinc finger protein (LZK1) (Dif3 homolog)	NM_024835	7	1	700 bp
Transcription regulation	Activity-dependent neuroprotector (ADNP)	NM_015339	9	1	500 bp
Transcription regulation	Hypoxia-inducible factor 1, α subunit inhibitor HIF1AN (FIH-1)	XM_030426	7	1	700 bp
Transcription regulation	Sex comb on midleg-like 1 (*Drosophila*) (SCML1)	NM_006746	9	1	600 bp
Transcription regulation	Kelch-like protein C3IP1 (C3IP1)	XM_086284	7	2	400 bp
Mitochondrial stress response	D-Prohibitin (Bap37)	AF178980	8	1	500 bp
Ubiquitin-mediated proteolysis	Suppressor of G2 allele of SKP1, *S. cerevisiae*, homolog of (SGT1)	NM_006704	8	1	450 bp
Ubiquitin-mediated proteolysis	Ubiquitin protein ligase E3A, transcript variant 2 (UbE3A)	NM_000462	9	1	600 bp
Ubiquitin-mediated proteolysis	Proteasome subunit, β type, 4 (PSMB4)	XM_047881	9	1	800 bp
Unknown	KIAA0725 protein (KIAA0725)	XM_049445	12	1	1.0 kb
Unknown	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B (ANP32B)	NM_006401	9	1	800 bp
Unknown	Candidate tumour-suppressor OVCA1 (OVCA1)	NM_080822	12	2	1.0 kb
Unknown	Hypothetical protein (MAC30), mRNA	XM_031536	17	2	900 bp
Unknown	Similar to tumour metastasis-suppressor; longevity assurance (LAG1, *S. cerevisiae*) homolog 2	XM_065847	12	2	600 bp
Unknown	DNA sequence from clone RP11-165J3 on chromosome 9	AL583839.1	16	1	800 bp
Unknown	Hypothetical protein FLJ23375 (FLJ23375)	NM_024956	16	2	500 bp
Unknown	Hypothetical protein MGC2714 (MGC2714)	NM_032299	8	1	500 bp
Unknown	Hypothetical protein BC008322 (LOC92106)	NM_138381.1	8	3	700 bp
Unknown	Hypothetical protein FLJ20060	XM_005467.4	12	2	700 bp
Unknown	CG2277 gene product (LOC221294)	XM_166297.1	7	1	500 bp
Unknown	Hypothetical protein MGC4767 (MGC4767)	XM_045844	10	1	700 bp
Unknown	KIAA1007	XM_168026.1	11	2	700 bp

Table 2 (*continued*)

Putative function	Gene name	Accession No.	Fold induction	Redundancy	cDNA insert size
Unknown	Clone FLB9213 PRO2474	AF130088	11	1	700 bp
Unknown	Chromosome 5 clone CTD-2085H24	AC025447	10	1	800 bp
Unknown	CGI-48 protein (LOC51096)	NM_016001	13	1	750 bp

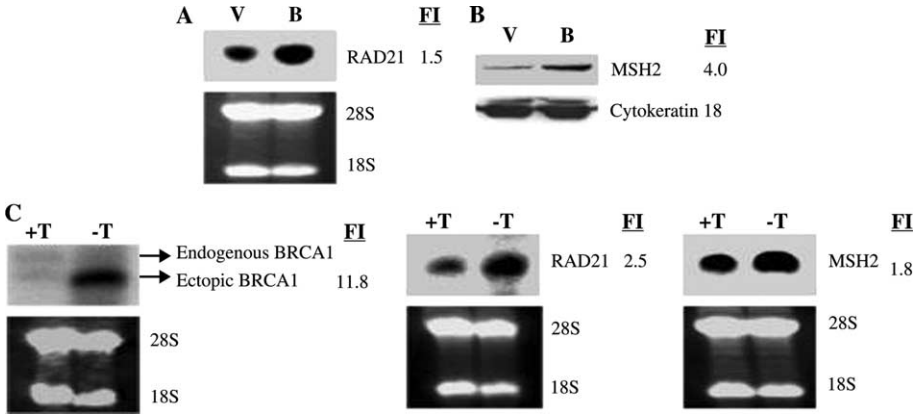

Fig. 3. BRCA1 induces up-regulation of RAD21 and MSH2 in MCF-7 (A, B) and UBR60-bcl2 (C) cells following BRCA1 induction. (A) Northern blot with total RNA from MCF7 cells transfected with control plasmid pCMVmyc (lane V) and pCMVmycBRCA1 plasmid (lane B) confirms BRCA1-mediated induction of RAD21 expression. (B) Western blot analysis of MSH2 protein levels confirms induced expression of MSH2. Cytokeratin 18 was used for equal protein loading control. (C) Northern blot of total RNA from UBR60-bcl2 cells with inducible BRCA1 expression shows over-expression of ectopic BRCA1, RAD21, and MSH2 following tetracycline withdrawal (−T) at 24 h. Ethidium bromide staining shows equal RNA loading in each lane. "FI" indicates fold induction.

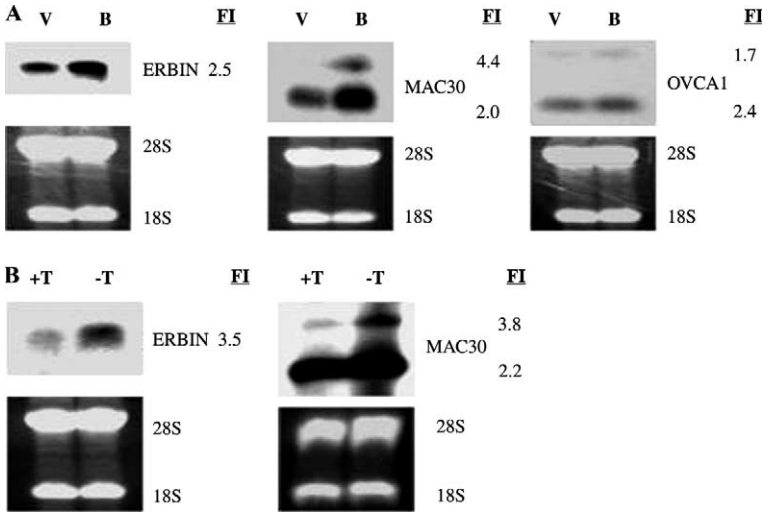

Fig. 4. BRCA1 mediated up-regulation of genes involved in cell signalling or tumour suppression in MCF7 cells and UBR60-bcl2 cells. (A) ERBIN, MAC30, and OVCA1 transcript induction in MCF7 cells (B) ERBIN and MAC30 induction in UBR60-bcl2 cells. Cells were treated and Northern blotting was performed as described in Fig. 3.

also up-regulates a high number of genes involved in many different cellular processes [24]. Although we have not investigated these target genes in tumours yet, we notice that 10 of the 46 genes (22%) that we report here (RAD21, ERBB2IP, UbE3A, FBP1, KIAA0725, TMPO, PSMB4, CD24, MSH2, and ATP1B3) are among markers of a 'poor prognosis' gene expression signature identified by DNA microarray analysis of early primary breast tumours [25]. Therefore, we believe that we identified a large set of BRCA1 target genes that may be involved in BRCA1-mediated cellular processes as well as breast carcinogenesis.

In relation to BRCA1-related breast/ovarian carcinogenesis, we further analysed the expression of ERBIN

and OVCA1. ERBIN, an ERBB2/HER2-binding protein which locates this receptor to the basolateral membrane [26], was also up-regulated by BRCA1. Overexpression of ERBB2/HER2 is frequently observed in breast cancers and its overexpression is associated with poor tumour prognosis [27]. Although it is not known whether ERBIN is able to inhibit ERBB2/HER2 activity, its deregulation results in the mislocalisation of the receptor [26]. This raises the possibility that ERBIN deficit resulting from inactivation of BRCA1 could lead to a loss of epithelial homeostasis, and in consequence, pathological disorganisation in breast carcinoma. Another gene, namely OVCA1 which we show to be up-regulated by BRCA1, was initially identified as a candidate ovarian tumour-suppressor gene located on chromosome 17p13.3 [28]. This locus displays frequent LOH in both ovarian and breast cancers [29] and OVCA1 was reported to display reduced expression in breast and ovarian cancers [30]. In addition, ectopic expression of OVCA1 causes a dramatic reduction in cell proliferation in association with accelerated cyclin D1 degradation [30]. Even if additional studies are needed, these observations strongly suggest that BRCA1 mediates its tumour-suppressor functions through a large set of downstream genes involved in DNA repair, receptor-mediated signalling, and ovarian cancer suppression.

In conclusion, we have identified 46 genes whose expression levels are up-regulated as a result of BRCA1 overexpression in breast cancer cells. The properties of several of these genes are consistent with putative tumour-suppressor functions in breast neoplasia. To our knowledge, our study is the first to report BRCA1-induced genes in breast carcinoma cells with the SSH technique. It will now be important to construct a complete profile of BRCA1-regulated genes in order to achieve an integrated view of all the functional events regulated by BRCA1, and to assess how expression of these genes is affected by the BRCA1 status of cells.

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