

**DEVELOPMENT OF A FUNCTIONAL ASSAY FOR
DETERMINATION OF *BRCA1* CARRIER STATUS**

by

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Abstract

Given the high risk of cancer development associated with *BRCA1* mutation carriers, it is important that they be identified early and accurately. Identification is impeded however, by the large number of variants of unknown significance (VUS) and the pleiotropic nature of *BRCA1* function. Our lab has developed a novel functional assay that identifies *BRCA1* mutation carriers based on gene signatures in Epstein-Barr virus (EBV)-transformed lymphoblastoid cells (LCLs). Given that this assay was developed using blood-derived cells, we hypothesized that the same abnormally regulated genes would be detected in fresh blood samples and that this could be used to develop a novel blood-based functional assay for the determination of *BRCA1* status in a patient population. The first part of the study used selected reaction monitoring-mass spectrometry (SRM-MS) as well as flow cytometry (FC) to determine protein expression in LCLs and identify potential targets for the development of a protein-based functional assay. Interestingly, many of the strong predictors of *BRCA1* carriers were proteins involved in two signalling pathways: Interferon (IFN)-regulated signalling and B cell development. If validated in fresh blood samples, these observations represent potentially novel findings and suggest yet unexplored functions for *BRCA1*. Next, this study established the methodology to identify the desired cell population within a heterogeneous whole-blood sample by determining that LCLs express the pan B cell surface marker CD20. To determine which targets were detectable in CD20⁺ B cells isolated from fresh blood samples, mRNA and protein expression of the selected targets was examined using a sample cohort of *BRCA1* mutation carrier and non-carrier control patients. The results showed that four mRNA targets, *CXCR3*, *GLDC*, *IFIT1* and *TBX21* were detectable in these fresh blood samples

identifying them as appropriate targets for further development of a qRT-PCR-based assay. However, given poor RNA quality and changes in transcript expression levels as the age of the blood samples increased, the development of a protein-based assay is the best approach moving forward. Moreover, four proteins, IGHM, IGHD, CD24 and CXCR3, had high expression on CD20⁺ B cells, identifying them as the best targets for further development of a FC-based functional assay.

Co-Authorship

All work was performed by the author, under the supervision of Dr. Scott Davey. LCL protein lysates for mass spectrometry analysis were prepared by Nicole Archer. Selected reaction monitoring-mass spectrometry was performed by Jian Chen and Moyez Dharsee from the Ontario Cancer Biomarker Network. RNA extraction for microarray analysis was performed by Claire Michel and prediction analysis for microarrays (PAM) was performed by Dr. Scott Davey. Matthew Gordon and Jeff Mewburn provided technical support with flow cytometry.

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List of Abbreviations

| | |
|----------|--------------------------------------|
| alt-NHEJ | alternative NHEJ |
| ATP | adenosine triphosphate |
| AUC | area under the curve |
| BRCT | BRCA1 C-terminal |
| cDNA | complementary DNA |
| CSR | class switch recombination |
| CV | coefficient of variation |
| DDR | DNA damage response |
| DNA | deoxyribonucleic acid |
| DSB | double stranded DNA break |
| EBV | Epstein-Barr virus |
| ER | estrogen receptor |
| ES | embryonic stem |
| FC | flow cytometry |
| HR | homologous recombination |
| IFN | interferon |
| Ig | immunoglobulin |
| IgH | Ig heavy chain |
| IR | ionizing radiation |
| IRIF | ionizing radiation induced foci |
| LCL | EBV-transformed lymphoblastoid cells |
| LOH | loss of heterozygosity |
| MFI | mean fluorescent intensity |
| MMEJ | microhomology-mediated end joining |
| mRNA | messenger RNA |
| NHEJ | non-homologous end joining |
| NYGH | North York General Hospital |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PFA | paraformaldehyde |
| PR | progesterone receptor |
| qRT-PCR | quantitative real-time PCR |
| RIN | RNA integrity number |
| RING | really interesting new gene |
| RNA | ribonucleic acid |
| ROC | receiver operator characteristics |
| S | switch |

| | |
|--------|--|
| SEM | standard error of mean |
| SRM-MS | selected reaction monitoring-mass spectrometry |
| ssDNA | single-stranded DNA |
| Th1 | T-helper cell type 1 |
| TN | triple negative |
| Ub | ubiquitin |
| VUS | variants of unknown significance |
| WT | wild-type |

Chapter 1

Introduction

1.1 Breast cancer overview

Breast cancer is the most common form of cancer in Canadian women; one in every nine women is expected to develop breast cancer during her lifetime (1). The Canadian Cancer Society estimates that breast cancer will represent 26% of all newly diagnosed cancers in women in 2013, with 23,800 new cases expected (1). Breast cancer will cause the deaths of 5,000 Canadian women in 2013 and be second only to lung cancer as the most common cause of cancer death (1). Given the vast devastation of this disease, it is important that the underlying molecular mechanisms and etiology be better understood to facilitate the improvement of preventative strategies and disease treatments.

The majority of breast cancers are sporadic cases. Risk factors for development of the disease are both environmental and physiological, including, early age at menarche, late onset menopause, lifetime duration of breastfeeding (2, 3), alcohol consumption (4), obesity (3, 5), the use of oral contraceptives (6) and the use of hormone replacement therapy (7).

Conversely, up to 10% of breast cancer cases are hereditary in origin and may be caused by the inheritance of germline mutations in several breast cancer susceptibility genes including, *ATM*, *BRCA1*, *BRCA2*, *BRIP1* (*BACH1*), *CHEK2*, *NBN*, *PTEN*, *RAD51C* and *TP53* (8). A large portion of these hereditary breast cancer cases however,

can be attributed to germline mutations in *breast cancer 1 or 2, early onset (BRCA1 or BRCA2)*, accounting for up to 50% of all hereditary breast cancer cases (9). The identification of women carrying these mutations is essential for effective disease screening, prevention and treatment strategies.

1.2 BRCA1 tumour suppressor

BRCA1 is a tumour suppressor gene associated with hereditary breast and ovarian cancer syndrome. The link between *BRCA1* and breast cancer was uncovered in the early 1990s through linkage analysis of families with multiple cases of early onset breast and ovarian cancer (10–12). *BRCA1* mutation carriers possess one normal and one mutated copy of the gene (*BRCA1*^{+/-}) and carry a lifetime risk of up to 80% for developing breast cancer and up to 50% for ovarian cancer (9). Manifestation of *BRCA1*-related breast cancer is believed to require the loss of heterozygosity (LOH) of the wild-type allele according to the ‘two-hit’ hypothesis of classic tumour suppression as is reflected in early studies of *BRCA1*-related breast cancer (13–15). However, many of these studies had a low number of samples and provided contradicting evidence, with some breast cancer tissue showing retention of the wild-type allele or loss of the mutated allele. A later study of breast cancer tissue samples from *BRCA1* mutation carriers showed that out of 18 cases in which LOH was observed, 11 patients showed loss of the mutant allele, also noting that LOH often occurred as a late-stage event in tumourigenesis (16). These results suggested *BRCA1* may not necessarily follow the classic ‘two hit’ paradigm of tumour suppression and that loss of the wild-type *BRCA1* allele may not be required for *BRCA1*-associated tumourigenesis. Interestingly, several more recent studies have described

defects in cellular differentiation and proliferation in breast epithelial cells from haploinsufficient *BRCA1* mutation carriers compared to non-carriers, providing some support for the theory that inactivation of a single allele may have functional consequences (17–19).

1.3 *BRCA1*-related breast cancer

More than 75% of tumours arising in *BRCA1* mutation carriers have a triple negative (TN), a basal-like phenotype, or both (20). Basal-like breast cancers are one of the five defined subgroups of breast cancer identified through microarray-based gene expression profiling (21–23) and are characterized by the expression of genes found in normal basal or myoepithelial breast cells, such as cytokeratins 5/6, 14 and 17, p-cadherin and caveolin 1 (24). Triple negative tumours are defined as tumours that lack Estrogen Receptor (ER), Progesterone Receptor (PR) expression and HER2/Neu (ErbB-2) overexpression. Morphologically, *BRCA1*-related breast cancers are often high grade with high mitotic indices, necrosis and lymphocyte infiltration (20). They are typically aggressive and associated with poor outcome (20). In addition, *BRCA1*-related breast cancers often possess distinct somatic mutations of the p53 tumour suppressor at a higher frequency than sporadic breast cancers (25–29). The aggressiveness of *BRCA1*-related breast cancers and the lack of hormone receptor expression, which renders hormone therapy ineffective to treat the disease, underscore the importance of preventative screening for *BRCA1* mutation carriers.

1.3.1 *BRCA1* and sporadic breast cancer

Although *BRCA1* is rarely inactivated by somatic mutation in sporadic breast cancer (30, 31), there is increasing evidence that a proportion of sporadic breast tumours with a basal-like and/or TN phenotype have decreased expression of *BRCA1*. This has been attributed to epigenetic silencing through *BRCA1* promoter hypermethylation (32–34) as well as transcriptional silencing involving the *BRCA1*-silencing proteins, high mobility group protein A1 (HMGA1) and DNA-binding protein inhibitor ID-4 (ID4) (35, 36). Tumours expressing low levels of *BRCA1* through promoter methylation are aggressive with high histological grade and are also associated with poor outcome (37). Together these studies imply that the downregulation of *BRCA1* expression may be a driving event in sporadic basal-like/TN breast cancer carcinogenesis.

1.4 *BRCA1* structure and function

The human *BRCA1* gene is located on chromosome 17q21 and encodes an 1,863 amino acid protein, breast cancer type 1 susceptibility protein 1 (BRCA1) (11) (Figure 1). Controlled by its nuclear localization and nuclear export signals, BRCA1 shuttles between the nucleus and cytoplasm (38, 39). The C-terminal Coiled-coil domain binds the partner and localizer of BRCA2 (PALB2), physically linking the BRCA1 and BRCA2-RAD51 proteins and connecting them within the homologous recombination (HR) repair pathway (40). In addition, the C-terminal SQ cluster domain (SQCD) contains several threonine and serine residues that can become phosphorylated by ataxia telangiectasia mutated (ATM) (41). ATM-mediated phosphorylation within the BRCA1

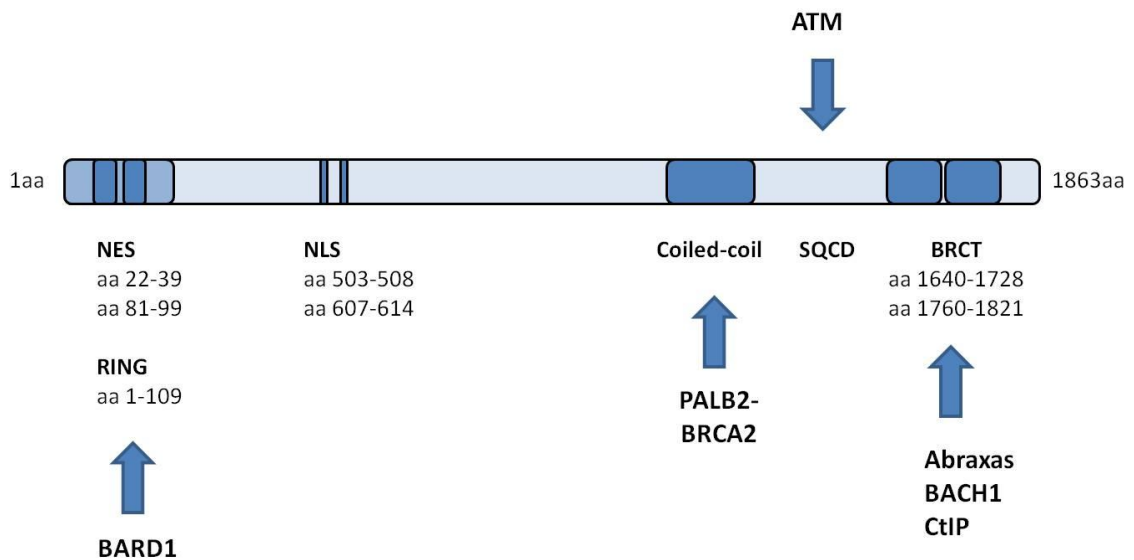


Figure 1: BRCA1 domain organization and interaction partners.

The *BRCA1* gene encodes an 1,863 aa protein. The two most highly conserved domains are the RING finger domain at the N-terminus and the tandem BRCT repeats at the C-terminus. The RING domain is important for the interaction with the BARD1 tumour suppressor. Through the BRCT domains, BRCA1 associates with several different proteins including Abraxas, BACH1 and CtIP, in a phosphorylation-dependent and mutually exclusive manner. The Coiled-coil domain mediates the interaction of BRCA1 with PALB2 and BRCA2 to facilitate homologous recombination. The SQ cluster domain (SQCD) contains multiple ATM phosphorylation sites, that are essential for efficient cell cycle checkpoint activation following DNA damage. BRCA1 also contains two nuclear localization signals (NLS) and two nuclear export signals (NES).

SQCD domain is essential for efficient cell cycle checkpoint activation following DNA damage (42–44).

The two most highly conserved domains however, are the really interesting new gene (RING) finger domain located at the N-terminus and the tandem BRCA1 C-terminal (BRCT) repeats located at the C-terminus (Figure 1). Through these domains, BRCA1 interacts with several tumour suppressor and DNA repair proteins, forming different complexes with distinct functions.

1.4.1 N-terminal RING finger domain

RING fingers are cysteine rich zinc-binding motifs that function as E3 ligase enzymes involved in ubiquitination (45). Through the RING finger domain, BRCA1 forms a constitutive heterodimer with BRCA1-associated RING domain 1 (BARD1) that is enzymatically active as an E3 ubiquitin (Ub) ligase. Cancer-associated mutations within the RING motif, such as C61G and C64G, abrogate the association of BRCA1 with BARD1 and do not exhibit Ub ligase activity (46). The BRCA1-BARD1 heterodimer catalyzes unconventional Lys-6 linked Ub chains (47). Targets include the autoubiquitination of the BRCA1-BARD1 proteins themselves, enhancing their E3 ligase activity (47–49), γ H2AX (48, 50), CTBP-interacting protein (CtIP), facilitating double stranded break (DSB) repair mechanism choice and G2/M checkpoint activation (51), as well as γ -tubulin, which regulates centrosome duplication (52).

The function of E3 ligase enzymatic activity in *BRCA1* tumour suppression is controversial, however. Mouse embryonic stem (ES) cells expressing an enzymatically inactive I26A BRCA1 mutation that maintains the ability to heterodimerize with BARD1

had no observable defects in HR (53). Conversely, another study demonstrated that the E3 ligase activity of BRCA1-BARD1 is essential for tumour suppression through its ubiquitination of the histone variant H2AX at sites of heterochromatin. Loss of BRCA1-BARD1-mediated H2AX ubiquitination led to an increase in transcriptional activation of heterochromatic regions and defects in HR (50). Additional data from another mouse model demonstrated that BRCA1 E3 ligase activity is essential for tumour suppression, but that mice expressing the C61G BRCA1 mutation rapidly developed resistance to treatment with cisplatin, suggesting that the mutation maintains some function (54).

1.4.2 C-terminal BRCT domains

Through the C-terminal BRCT repeats, BRCA1 functions as a transcriptional regulator. Wild-type BRCA1 BRCT domains co-activate transcription when fused to a GAL4-DNA binding domain, whereas pathogenic mutations in the BRCT domains are unable to activate transcription, indicating that transcriptional regulation may be important for *BRCA1* tumour suppression (55, 56). Furthermore, wild-type BRCA1 has been shown to interact with RNA polymerase II by association with the RNA helicase A component, while a cancer associated point mutation was found to abrogate this association (57, 58). BRCA1 also co-activates transcription through association with several well-known transcription factors, including p53, leading to the induction of genes involved in cell cycle arrest and the DNA damage response (DDR) (59, 60), as well as signal transducer and activator of transcription 1 (STAT1) resulting in the induction of p21 transcription following stimulation with Interferon (IFN)- γ (61).

BRCT domains are also commonly found in proteins associated with DNA damage repair and cell cycle checkpoint control (62) and are known to interact with proteins phosphorylated on the pSer-X-X-Phe motif (63, 64). Through the BRCT domains, BRCA1 interacts with several proteins in a phosphorylation-dependent and mutually exclusive manner forming several distinct complexes involved in DNA damage repair (Figure 1). The BRCT-mediated association with the Abraxas-RAP80 complex localizes BRCA1 to γ H2AX foci at sites of DSBs (65, 66), whereas the BRCT-mediated association of BRCA1 with the BRCA1-associated C-terminal helicase (BACH1) is linked to S-phase checkpoint activation and HR-mediated repair (64, 67, 68). Lastly, the phosphorylation-dependent interaction of CtIP with BRCA1 determines in part the repair mechanism implemented in response to DSBs through DNA end-resection (69, 70). Indeed BRCA1 tumour suppression may be dependent on BRCA1 association with one or more of these BRCT-binding phosphoproteins; ablation of phosphoprotein recognition by the BRCT domains generated tumours in mice, whereas ablation of the E3 ligase activity of BRCA1 did not (71). In conclusion, although multiple functions have been ascribed to the BRCA1 protein, there is presently no known unifying mechanism linking all the biochemical functions of BRCA1 to its tumour suppressor function.

1.5 Additional cellular functions of BRCA1

The BRCA1 protein plays a role in a number of cellular processes involved in the maintenance of genomic integrity. The protein is a key component of the DDR signalling cascade and plays an essential role during HR repair of DNA DSBs. It is also involved in the activation of cell cycle checkpoints in response to DNA damage as well as chromatin

remodeling. More recent studies have also described the role of BRCA1 in breast epithelial cell differentiation and proliferation. However, it remains to be established which, if any, of these processes represent the biologically relevant tumour suppression function of BRCA1.

1.5.1 DNA damage response (DDR) signalling

DNA damage can arise from external sources such as exposure to ionizing radiation (IR), ultraviolet radiation (UV), environmental toxins or some classes of chemotherapeutic drugs. Damage may also result from endogenous sources such as reactive oxygen species or errors occurring during DNA replication. Exposure to these sources generates different types of DNA lesions including modified DNA base or sugar moieties, the formation of DNA adducts, the crosslinking of the DNA strands as well as the generation of single stranded breaks or DSBs. DSBs are especially cytotoxic lesions - both strands of DNA are broken at the same time and inaccurate repair can lead to chromosomal rearrangements and tumourigenesis. DSBs can be caused by exposure to IR, replication fork collapse during DNA replication, or during the processing of intrastrand crosslinks, but are also essential intermediates during normal biological processes, including lymphocyte development in V(D)J and immunoglobulin (Ig) class switch recombination (CSR) (72).

IR exposure generates DSB lesions and results in the formation of ionizing radiation-induced foci (IRIF), in which multiple proteins form nuclear foci flanking the DSB (Figure 2). These proteins are part of a signalling cascade, comprising sensors that detect damage, effectors that execute repair and mediators that facilitate interactions

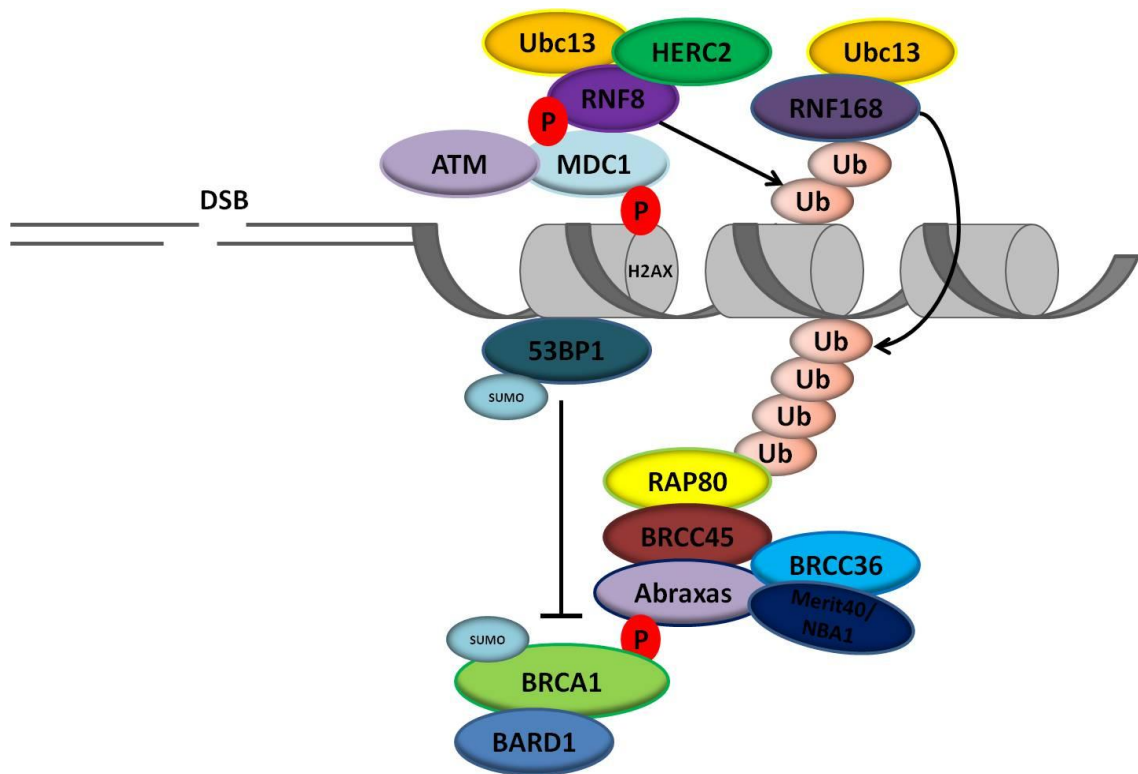


Figure 2: BRCA1 participates in DDR signalling at sites of DSBs.

DSB detection is initiated by the MRN sensor complex that binds the ends of the DSB site. MRN recruits the ATM protein kinase that quickly initiates a phosphorylation-dependent signalling cascade to recruit other DDR factors. A key event in this process is the ATM-mediated phosphorylation of histone H2AX. Phosphorylated H2AX creates a docking site for DNA repair protein assembly. A complex series of modifications is involved in recruiting BRCA1 and many other factors - including phosphorylation, ubiquitination and sumoylation - to sites of DNA damage. The end result is the localization of BRCA1 at sites of DNA damage through association with the Abraxas protein, where it helps facilitate DNA damage repair and cell-cycle checkpoint activation. 53BP1 inhibits BRCA1 accumulation at DSB sites, preventing DNA end resection and HR-mediated repair. P, ATM-mediated phosphorylation. Ub, Ubiquitin. SUMO, Sumoylation.

between sensors and effectors (9). BRCA1 has been identified as a key player in this molecular cascade that promotes DNA damage repair and cell cycle checkpoint activation. A complex series of modifications is involved in recruiting BRCA1 and many other factors - including phosphorylation (73–75), ubiquitination (76–79) and sumoylation (80, 81) - to sites of DNA damage (Figure 2).

DSB detection is initiated by the Mre11-RAD50-Nbs1 (MRN) sensor complex that binds the ends of the DSB site (73). MRN recruits the ATM protein kinase that quickly initiates a phosphorylation-dependent signalling cascade to recruit other DDR factors (73) (Figure 2). A key event in this process is the ATM-mediated phosphorylation of Ser-139 on histone H2AX, a member of the H2A histone family (74). Phosphorylated H2AX is designated as γ H2AX and occurs within minutes of DNA damage, spreading over large distances around the DSB and creating a docking site for DNA repair protein assembly (75). Mediator of DNA damage checkpoint-1 (MDC1) is localized to γ H2AX foci through its C-terminal BRCT domains (82). It is also directly associated with the forkhead-associated (FHA) domain of ATM, which further propagates γ H2AX spreading and is important for sustaining the DDR (83). MDC1 is subsequently phosphorylated in an ATM-dependent manner and recruits RING finger protein 8 (RNF8), an E3 ligase that initiates an ubiquitination signalling cascade (77, 84, 85). In association with ubiquitin-conjugating enzyme E2 N (Ubc13), an E2-conjugating enzyme, as well as the E3 ligase, hect domain and RCC1-like domain-containing protein 2 (HERC2), RNF8 initiates the formation of Lys-63 linked polyubiquitin chains on γ H2AX (76, 77, 86–88). The E3 ligase, RING finger protein 168 (RNF168), directly binds the polyubiquitin chains on γ H2AX and, in association with Ubc13, further propagates the Lys-63 ubiquitination to

amplify and spread the ubiquitination signalling initiated by RNF8 (78, 79). The Lys-63 chains generated by RNF8 and RNF168 are recognized by the ubiquitin interacting motif of receptor-associated protein 80 (RAP80) mediator, which recruits the adaptor protein, BRCA1-A complex subunit Abraxas (Abraxas) (65, 66, 76, 77, 86, 89, 90). ATM-mediated phosphorylation of Abraxas allows for the association of Abraxas and BRCA1 through the BRCA1 BRCT domains (65). The end result is the localization of BRCA1 at sites of DNA damage, where it helps facilitate DNA damage repair and cell-cycle checkpoint activation. The BRCA1-Abraxas complex also includes brain and reproductive organ-expressed protein (BRE), Lys-63-specific deubiquitinase BRCC36 and the BRISC and BRCA1-A complex member 1 (NBA1) adaptor protein (91–93) (Figure 2).

The tumor suppressor p53-binding protein 1 (53BP1) is another repair protein that accumulates at DSBs through its association with the dimethylated Lys-20 residue on histone H4 (H4K20me2) (94). Similar to BRCA1, RNF8/RNF168-mediated ubiquitination is also required for the recruitment of 53BP1 to damaged chromatin (76–79, 87), however these two proteins have opposing roles at sites of DSBs, promoting different repair mechanisms depending on the phase of the cell cycle (70). 53BP1 inhibits BRCA1 accumulation at DSBs during the G1-phase of the cell cycle (70, 95).

1.5.2 Homologous recombination (HR) repair

The inaccurate repair of DSBs can result in chromosomal translocations, genomic instability and cell death. Consequently, several different independent mechanisms have

evolved to repair DSBs including: homologous recombination (HR), non-homologous end joining (NHEJ) and alternative-NHEJ (alt-NHEJ), also known as microhomology-mediated end joining (MMEJ). The repair pathway implemented depends on the phase of the cell cycle as well as the degree of end-resection of the DSB. HR is the most accurate form of repair, and involves extensive 5' end-resection of the DSB followed by the use of an homologous stretch on a sister chromatid as a template to guide repair of the broken strand (96). HR functions during the S- and G2-phases of the cell cycle during which time sister chromatids are present. NHEJ is a more error-prone process that repairs DSBs by Ku70/80-mediated direct ligation of the broken ends. This can result in small insertions or deletions at the break site as well as translocations if DSBs from different parts of the genome are joined (97). NHEJ functions throughout the cell cycle but is particularly important during the G1-phase and during certain biological processes such as V(D)J rearrangement and CSR in B cells. Lastly, alt-NHEJ utilizes short sequences of microhomologies (4-6 nucleotides) created by limited end-resection to align broken ends prior to ligation (97). BRCA1 is involved in HR-mediated repair of DSBs (98, 99) and the mechanism of repair pathway choice (70).

As described above, DSBs are recognized through the Mre11-RAD50-Nbs1 (MRN) sensor complex. The DNA repair protein RAD50 (RAD50) component bridges both ends of the DSB and facilitates initial DNA end-resection by double-strand break repair protein MRE11A (Mre11) exonuclease activity (100). The third component of the complex, nibrin (Nbs1), associates with Mre11 as well as ATM, thereby recruiting ATM to DSBs (100) and initiating the phosphorylation cascade described above. CtIP is recruited by its association with the MRN complex to facilitate further end-resection

(101) (102). During the S- and G2-phases of the cell cycle, CtIP phosphorylation on Ser-327 allows CtIP to associate with the BRCA1 BRCT domains (102, 103). BRCA1 subsequently ubiquitinates CtIP, increasing its end-resection activity required to initiate HR (51). In contrast, the NHEJ pathway predominates during the G1-phase through 53BP1-mediated inhibition of BRCA1 accumulation at DSB sites and thereby CtIP-BRCA1-mediated end-resection (70, 95). Indeed, loss of 53BP1 promotes end-resection and HR-mediated repair and also restores the lethality and hypersensitivity to DNA-damaging agents associated with BRCA1 deficiency (95, 104). A recent study has suggested that a complex cell cycle-regulated circuit governed by the opposing activities of 53BP1 and BRCA1 determines DSB repair pathway choice to ensure that NHEJ dominates in G1- and that HR is favoured in S/G2-phase (70).

The initial end-resection facilitated by MRN and CtIP during HR is followed by recruitment of exonuclease 1 (EXO1) in association with the Bloom syndrome protein (BLM) DNA helicase in order to generate long-range resection (105). The single-stranded DNA (ssDNA) tails generated through processive DNA resection are rapidly bound by replication protein A (RPA), which is subsequently replaced by DNA repair protein RAD51 (RAD51) (106), forming a RAD51-nucleofilament that initiates strand invasion and HR repair. BRCA1 also mediates HR through its recruitment of PALB2, which is required for BRCA2-RAD51 colocalization into nuclear foci, culminating in HR (40).

1.5.3 Cell cycle checkpoint activation

In addition to the elaborate repair mechanisms implemented in response to DNA damage, cells have evolved several cell cycle checkpoints that delay the cell cycle to ensure the repair of DNA lesions before cell cycle progression resumes. BRCA1 regulates the activation of the G1/S-, S- and G2/M-phase cell cycle checkpoints in response to DNA damage. Phosphorylated BRCA1 regulates the ATM and ataxia telangiectasia and Rad3 related (ATR)-mediated phosphorylation of p53, leading to p21 induction and cell cycle arrest at the G1/S-phase checkpoint following exposure to IR (107). BRCA1 phosphorylation at serines 1387 and 1423 by ATM and ATR are critical for activation of the intra-S-phase and G2/M-phase checkpoints, respectively (42–44). Moreover, BRCA1-mediated phosphorylation of serine/threonine protein-kinase Chk1 (Chk1) in response to IR (108) as well as BRCA1-mediated degradation of cyclin B and Cdc25 also regulate the G2-M checkpoint (109). Lastly, BRCA1-mediated phosphorylation of Chk1 regulates the intra-S-phase checkpoint in response to replication inhibition (110). Therefore, BRCA1 plays an important role in linking DNA damage signalling to cell cycle checkpoint activation.

1.5.4 Chromatin remodelling

The repair of DSB lesions also involves posttranslational histone modification and remodelling of the chromatin surrounding the DSB. This facilitates an open chromatin structure to allow DNA repair proteins access to damaged DNA. BRCA1 has been associated with several different proteins involved in chromatin remodelling. SWI/SNF is a chromatin remodelling complex that uses ATP hydrolysis to alter histone-DNA

contacts to both positively and negatively regulate transcription. BRCA1 has been shown to interact directly with transcription activator BRG1 (BRG), an ATPase subunit of the SWI/SNF complex, suggesting that BRCA1 may regulate transcription through modulation of chromatin structure (111). Moreover, Harte et al. 2010 demonstrated a direct interaction between BRCA1 and the bromodomain-containing protein 7 (BRD7) subunit of the SWI/SNF complex and suggested that BRD7 regulates up to 30% of BRCA1-regulated genes (112). Other chromatin remodelling complexes shown to interact with BRCA1 include the catalytic subunits of the histone deacetylase complex, HDAC1 and HDAC2 (113), as well as the histone acetyltransferases p300/CBP (114). A previous study found that BRCA1 induced large-scale chromatin decondensation. Interestingly, this process was impaired or enhanced by cancer-predisposing mutations depending on their location within the gene (115). In contrast, a more recent study demonstrated that BRCA1 deficiency led to transcriptional de-repression of satellite DNA in heterochromatic regions. The silencing of constitutive heterochromatin by BRCA1 was dependent on its E3 ligase activity and the mono-ubiquitination of histone H2AX within heterochromatic regions, thus demonstrating a role for BRCA1 in the maintenance of heterochromatic structure (50). For these reasons, it is possible that the transcriptional regulation function associated with BRCA1 may actually be attributed to its regulation of chromatin structure.

1.5.5 Breast epithelial cell differentiation and proliferation

The first evidence of BRCA1 function in breast epithelial cell differentiation came from a study demonstrating that knockdown of BRCA1 in MCF10A mammary

epithelial cells abrogated the ability of the cells to form acini in 3D culture (116). Studies using primary mammary epithelial cells subsequently showed that BRCA1 knockdown reduced the number of luminal ER⁺ and epithelial specific antigen (ESA/EPCAM) expressing cells and increased the number of ER⁻ undifferentiated stem/progenitor and myoepithelial cells, demonstrating that loss of BRCA1 function results in blocked epithelial differentiation (117). Primary mammary epithelial cells from *BRCA1* mutation carriers grown in 3D culture were also found to have increased clonality and proliferation as well as different morphology compared to non-carriers (17).

The emergence of distinct biological markers has led to the ability to isolate different subpopulations of breast epithelial cells. It was found that normal breast tissue from *BRCA1* mutation carriers had significant expansion of the luminal progenitor population compared to other cell types as well as a reduction in basal cells. This expanded population demonstrated increased clonality *in-vitro* and unusual growth factor dependence and had a molecular signature similar to basal-like tumours (18). Furthermore, deletion of *BRCA1* in mammary ER⁻ luminal progenitor cells generated basal-like mammary tumours in mice that phenocopy human BRCA1-related breast tumours (118). These studies suggest that luminal progenitors are the cells of origin for the basal-like tumours arising in *BRCA1* mutation carriers. In contrast, a different study demonstrated that normal breast tissue from *BRCA1* mutation carriers had a statistically significant increase in basal progenitor cells and a slight decrease in the number of luminal progenitor cells (19). Together, these studies highlight the role of BRCA1 in regulating breast epithelial cell differentiation and proliferation.

In conclusion, BRCA1 has been ascribed numerous roles in the maintenance of genomic integrity and breast epithelial cell development. Moreover, non-functional mutations that are associated with cancer initiation are known to impair the protein's biological activities. However, it still remains unknown which biological activity is crucial for tumour suppression. As described below, this lack of knowledge has significant implications for the identification of *BRCA1* mutation carriers.

1.6 *BRCA1* carrier identification and variants of unknown significance (VUS)

Mutations can occur throughout the coding region of the *BRCA1* gene and are identified by direct sequencing. Nonsense and frameshift mutations occurring through nucleotide deletions or insertions lead to the translation of a non-functional truncated protein and pose few problems with risk assessment. Truncations as small as the last 8 residues of the C-terminal BRCT domains, for example, completely abolish the transcriptional activity of BRCA1 and are associated with cancer predisposition (119). The prevalence of pathogenic *BRCA1* mutations in Ontario, Canada is estimated to be 1:140 to 1:800 (120). While mutations resulting in truncated forms of BRCA1 are unmistakably disease causing, there are many others where the clinical significance of the mutation is unknown. These are known as variants of unknown significance (VUS) and are often missense mutations resulting in single amino acid substitutions, but may also include in-frame insertions or deletions, changes in splicing sites and intronic and regulatory regions, all with unknown effects on BRCA1 function. Approximately 13% of *BRCA1* and *BRCA2* genetic tests reveal mutations identified as VUS (121). These kinds of mutations are problematic in that they are difficult to interpret clinically and hence

decisions regarding prophylactic mastectomies are made in the absence of critical information.

Tracking of how these *BRCA1* mutations segregate with disease within families as well as case-control studies provide the most reliable information for classifying variants as pathogenic or neutral. This is made difficult, however, by the rarity of specific mutations in the population, and in the case of co-segregation studies, the lack of availability of family members for genetic testing. Given these challenges, several studies have developed models that integrate various sources of genetic evidence, including the frequency of the VUS in cases and controls (case-control study), co-segregation of the VUS with disease in families, co-occurrence in *trans* with a deleterious mutations (given that homozygosity for deleterious mutations is embryonic lethal), phylogenetic conservation and severity of amino acid substitution as well as pathological classification using tumour samples with the distinct basal-like phenotype of *BRCA1*-related breast cancers (122–125). These models integrate these pieces of evidence, generating a ratio of likelihood that the VUS is pathogenic. These are known as multi-factorial probability-based models (122–125).

As an alternative to genetic analyses, functional assays have been developed to assess the effects of specific mutations on BRCA1 protein function and provide additional sources of information in the classification of VUS (125). Currently, there are several different functional assays to assess the impact of VUS. Tests are limited however by the multi-functionality of the BRCA1 protein and the fact that it has not been established which functions are important for tumour suppression, as well as other practical limitations.

1.6.1 Transcriptional activation assay

The Transcriptional Activation assay is the most extensively validated assay to date and is the only functional assay to be incorporated into a multi-factorial probability-based model for the assessment of VUS (125). The assay utilizes the ability of the BRCT domains to activate transcription of a reporter gene when fused with a GAL4-DNA binding domain (55, 126–130). However, this assay is only feasible for the assessment of mutations in the BRCT domains. Moreover, because the assay focuses on the transcriptional functions of the BRCT domains, variants that impact other functions can be classified as neutral (131).

1.6.2 Phosphoprotein-binding assay

The Phosphoprotein-Binding assay also examines the integrity of the BRCA1 BRCT domains and has been extensively tested with a number of VUS (132). Given that the BRCT domains are known to bind to phosphorylated proteins with the pSer-X-X-Phe motif, examination of the ability of the BRCT domains containing different VUS to bind phosphorylated peptides was conducted using a pull-down assay in which a biotinylated phosphorylated-peptide derived from BACH1 was bound to streptavidin beads (132). Like the previous assay however, this assay is restricted to the analysis of a specific domain and function.

1.6.3 Rescue of radiation resistance assay

Experiments using colony formation assays have shown that *BRCA1*-deficient HCC1937 cells are hypersensitive to IR and that radiation resistance was restored by exogenous *BRCA1* expression (133, 134), whereas *BRCA1* constructs of four different known pathogenic mutations were unable to restore radiation resistance (134). The authors therefore suggest that this rescue of radiation sensitivity assay may be applied to identify non-functional *BRCA1* mutations. Only four variants were assessed however, and much additional validation would be required to determine its utility. Moreover, the assay requires a large amount of time to complete as it involves the generation of individual *BRCA1*-mutant constructs and the monitoring of cells over several weeks following IR exposure.

1.6.4 Embryonic stem cell-based assays

Homozygous-null *BRCA1* mutations in mice are known to be embryonic lethal (135, 136). Embryonic stem (ES) cell-based functional assays have been developed that introduce bacterial artificial chromosomes (BAC) or cDNA constructs expressing *BRCA1* variants into *BRCA1*-null murine ES cells and look for the rescue of these cells from lethality or the restoration of cisplatin response (137, 138). These assays are promising since they are not domain specific. However, they are extremely complex and timely. Both assays require the generation of individual BAC clones or cDNA expression vectors expressing the variant transgene, subsequent complementation of ES cells and are assayed over several days or weeks.

In summary, many of the functional assays developed to date are complex and time and labor intensive, preventing their implementation within a clinical setting. Moreover, many of the functional assays are also limited to the evaluation of mutations within a specific domain of the protein. Given that mutations occur throughout the protein, the effects of many mutations cannot be determined by these tests. Regardless of any functional assay result, it remains uncertain whether this is in fact the function associated with *BRCA1*-related pathogenesis. There would therefore be several advantages to a novel functional assay, such as a simple peripheral blood-based assay, as it would identify non-functional *BRCA1* mutations irrespective of their location in the *BRCA1* sequence, and provide a quick and accurate alternative to the methods described above that could be easily standardized and adapted for use in a clinical laboratory.

1.7 Previous work in our lab

Previous work in our lab has focused on the development of a novel functional assay to predict *BRCA1* status based on gene expression profiles in Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell lines (LCLs). This study analyzed the transcriptome microarray data of 69 LCLs, including 31 *BRCA1* mutation carriers and 38 non-carrier controls. The results showed that lymphocytes from *BRCA1* carriers could be distinguished with high fidelity (~90% accuracy) from individuals with two wild-type copies of the gene (139). This modeling was performed using prediction analysis for microarrays (PAM), which implements a nearest shrunken centroid (NSC) analytical approach (140). Interestingly, carriers were recognized through their basal gene expression profiles. Global gene expression level changes following IR to induce the

DDR did not support the development of an accurate class predictor (139). The final predictor included a set of 43 genes and it was found that many of these genes were also markers of differentiation in blood cells (139) (Table 1). A top predictor of *BRCA1* carriers was the cytokine-regulating transcription factor *TBX21* known as a master regulator of lymphocyte differentiation, directing CD4⁺ Th1 lineage development (141) as well as differentiation of mature B cells in mice (143–145). Another strong predictor of carrier status was the chemokine receptor, *CXCR3*, known to be induced by *TBX21* (146). Both genes were downregulated in *BRCA1* mutation carriers. Five genes selected as top predictors of *BRCA1* carriers with high expression levels were validated using qRT-PCR: *CXCR3*, *GLDC*, *IFIT1*, *MX2* and *TBX21* (142). Three of these genes: *CXCR3*, *IFIT1* and *TBX21* confirmed initial microarray findings of altered expression levels in *BRCA1* carriers, identifying them as appropriate targets in the development of a qRT-PCR-based functional assay (142) (Table 1).

1.8 Rationale, hypothesis and objectives

1.8.1 Rationale and hypothesis

Given the high risk of breast cancer development associated with *BRCA1* mutation carriers, it is important that they be identified early and accurately. However, identification is impeded by the number of VUS and the pleiotropic nature of *BRCA1* function. This provides a compelling rationale for a functional assay that will identify pathogenic mutations and distinguish them from neutral polymorphisms. Our lab has developed a novel functional assay that identifies *BRCA1* mutation carriers based on gene

Table 1: Summary of genes identified as predictors of *BRCA1* mutation carriers.

Microarray analysis of LCLs was used to develop an accurate class predictor comprised of 43 genes to distinguish *BRCA1* carriers from non-carriers (139). Genes included in the 43-gene classifier and their predicative values are listed. Predictive values <0 or >0 identify strongly predictive genes of carrier status and indicate downregulation or upregulation in carrier LCLs relative to non-carriers, respectively. Initial microarray findings were validated using qRT-PCR for 5 genes (142). Three genes, *CXCR3*, *IFIT1* and *TBX21*, confirmed initial microarray results (Yes) (142). qRT-PCR, quantitative real-time PCR.

| Gene | Full Gene Name | Predictive Value | qRT-PCR Validated |
|-----------------|--|-------------------------|--------------------------|
| <i>PXDN</i> | Peroxidasin homolog | 4.247 | |
| <i>JAKMIP2</i> | Janus kinase and microtubule interacting protein 2 | 2.147 | |
| <i>MMP7</i> | Matrix metalloproteinase 7 | 2.063 | |
| <i>CSRP2</i> | Cysteine and glycine-rich protein 2 | 1.797 | |
| <i>CD24</i> | CD24 molecule | 1.757 | |
| <i>LFNG</i> | O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase | 1.707 | |
| <i>ENPP2</i> | Ectonucleotide pyrophosphatase/phosphodiesterase 2 | 1.706 | |
| <i>FOXP1</i> | Foxhead box P1 | 1.503 | |
| <i>PWWP2</i> | PWWP domain containing 2B | 1.479 | |
| <i>PRLR</i> | Prolactin receptor | 1.383 | |
| <i>IFNA5</i> | Interferon alpha 5 | 1.228 | |
| <i>FCGRT</i> | Fc fragment of IgG, receptor, transporter, alpha | 0.998 | |
| <i>IFNA4</i> | Interferon alpha 4 | 0.992 | |
| <i>IFIT3</i> | Interferon-induced protein with tetratricopeptide repeats 3 | 0.894 | |
| <i>SERPINF1</i> | Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 | 0.887 | |
| <i>IGHD</i> | Immunoglobulin heavy constant delta | 0.849 | |
| <i>IFIT1</i> | Interferon-induced protein with tetratricopeptide repeats 1 | 0.788 | Yes |
| <i>ZBED3</i> | Zinc finger BED-type containing 3 | 0.786 | |
| <i>IFIT2</i> | Interferon-induced protein with tetratricopeptide repeats 2 | 0.736 | |
| <i>USP18</i> | Ubiquitin specific peptidase 18 | 0.728 | |
| <i>IFI44L</i> | Interferon-induced protein 44-like | 0.689 | |
| <i>SOX4</i> | SRY (sex determining region Y)-box 4 | 0.471 | |
| <i>MX2</i> | Myxovirus (influenza) resistance 2 | 0.468 | No |
| <i>MX1</i> | Myxovirus (influenza) resistance 1 | 0.462 | |
| <i>ETV7</i> | Ets variant 7 | -2.721 | |
| <i>TBX21</i> | T-box expressed in T cells | -2.668 | Yes |
| <i>CXCR3</i> | Chemokine (C-X-C motif) receptor 3 | -2.203 | Yes |
| <i>FYN</i> | Oncogene related to SRC, FGR, YES | -2.114 | |
| <i>IGHG1</i> | Immunoglobulin heavy constant gamma 1 | -2.111 | |
| <i>IFNG</i> | Interferon, gamma | -1.976 | |
| <i>FAM79B</i> | Tumor protein p63-regulated 1 | -1.900 | |
| <i>CYP11B1</i> | Cytochrome P450, family 1, subfamily B | -1.779 | |
| <i>PLA2G4A</i> | Phospholipase A2, group IVA (cytosolic, calcium dependent) | -1.621 | |
| <i>SLC16A10</i> | Solute carrier family 16 (aromatic amino acid transporter), member 10 | -1.558 | |
| <i>TNS4</i> | Tensin 4 | -1.475 | |
| <i>UBD</i> | Ubiquitin D | -1.174 | |
| <i>IL18BP</i> | Interleukin 18 binding protein | -1.173 | |
| <i>LAG3</i> | Lymphocyte activation gene 3 | -1.068 | |
| <i>BCR</i> | Breakpoint cluster region | -1.005 | |
| <i>ZBTB38</i> | Zinc finger and BTB domain containing 38 | -0.895 | |
| <i>GLDC</i> | Glycine dehydrogenase (decarboxylating) | -0.879 | No |
| <i>DUSP23</i> | Dual specificity phosphatase 23 | -0.861 | |
| <i>HLA-DMB</i> | Major histocompatibility complex, class II, DM beta | -0.378 | |

signatures in LCLs (139). Given that this assay was developed using blood-derived cells, we hypothesized that the same abnormally regulated genes would be detected in fresh blood samples and that this could be used to develop a novel blood-based functional assay for the determination of *BRCA1* status in a patient population. The expectation is that this study will work towards building a new and powerful tool for the identification of *BRCA1* carriers and ultimately lead to improved outcomes through easier and earlier diagnosis.

1.8.2 Objectives of this study

- Verify microarray data using protein-based methods and cultured LCLs to select appropriate targets for the development of a protein-based functional assay.
- Develop the methodology to identify the desired cell population from whole-blood samples using cultured LCLs.
- Determine whether mRNA and protein targets are detectable in lymphocytes isolated from fresh blood samples using a cohort of *BRCA1* mutation carrier and non-carrier patient blood samples. This will determine their feasibility in the development of a blood-based functional assay.

Chapter 2

Materials and Methods

2.1 Blood samples and patients

Fresh blood samples were obtained from proven *BRCA1* mutation carriers and mutation negative women recruited through the North York General Hospital (NYGH) Genetics Program. Informed written consent was obtained from each individual before participation, and the study protocol was approved by the Queen's University and NYGH Research Ethics Boards. Subjects currently undergoing chemotherapy or radiation treatment were ineligible for the study. Blood samples were collected in K₂EDTA tubes (BD Biosciences, Mississauga, ON) approximately 24 h prior to processing. Blood samples for the time-course experiments were provided by a lab volunteer, stored at 4°C and processed at the indicated time points.

2.2 Isolation of primary B cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (GE Healthcare, Baie d'Urfe, QC) density centrifugation according to the manufacturer's instructions. Briefly, 10 mL of blood diluted 1:1 using 1x phosphate buffered saline (PBS; Life Technologies, Burlington, ON) solution was layered over 15 mL Ficoll-Hypaque and centrifuged for 30 min at 400 g. The buffy coat was extracted and PBMCs were washed twice in 1x PBS with 2 mM EDTA (Life Technologies) for 5 min at 800 g. For qRT-PCR studies, peripheral B cells were enriched from PBMCs with CD19

microbeads (Miltenyi Biotec, Cambridge, MA) according to the manufacturer's instructions.

2.3 Cell culture

LCLs were obtained through the NIH Breast Cancer Family Registries. The 69 cell lines included in this study consisted of 31 *BRCA1* mutation carrier, and 38 non-carrier control cell lines. The mutations were primarily frameshift and nonsense mutations, but also included some missense and splicing mutations. See Table 2 for an exact list of the *BRCA1* mutations used in this study. The medium used for LCL cell culture was RPMI-1640 (Sigma Aldrich, Oakville, ON) supplemented with non-heat inactivated 15% fetal bovine serum (FBS; Life Technologies). All cell culture was carried out in 25 cm² flasks (Corning, Nepean, ON) at 37°C in 5% CO₂ atmosphere. Cell number was normalised to 6.5x10⁵ cells/mL and fresh medium was added to cells 24 h prior to flow cytometry (FC) experiments to ensure cells were actively cycling. Primary B cells were short-term cultured for 24 h in 24-well flat-bottom plates (Sarstedt, Montreal, QC) in 1 mL of complete media consisting of RPMI 1640 (Sigma Aldrich) supplemented with non-heat inactivated 15% FBS (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies) and 50 µM β-mercaptoethanol (Life Technologies), at 37°C in 5% CO₂ atmosphere.

Table 2: List of LCL cell line *BRCA1* mutations.

| Cell Line | Mutation | Class |
|------------------|--------------------|--------------|
| B12928 | c.4689C>G | N |
| B13135 | c.66_67delAG | F |
| B13416 | c.5263insC | F |
| B13537 | c.3607C>T | N |
| B14023 | c.2071delA | F |
| B14643 | c.1175_1214del | F |
| B14663 | c.4327C>T | N |
| B14703 | c.2834_2836delGT | F |
| B14832 | c.2475_2476delC | F |
| B14834 | c.1016insA | F |
| B15268 | c.5263insC | F |
| B15285 | exon13ins6kb | O |
| B15736 | c.191G>A | M |
| B15737 | IVS1-22A>G | O |
| B16236 | c.2561insGC | F |
| B17082 | c.66_67delAG | F |
| B17653 | c.5263insC | F |
| B18318 | c.3756_3759delGTCT | F |
| B18700 | IVS9-2A>C | S |
| B19018 | c.4327C>T | N |
| B21303 | c.66_67delAG | F |
| B22893 | c.1175_1214del | F |
| B24262 | c.851ins7 | F |
| B25453 | c.2934T>G | N |
| B26842 | c.4327C>T | N |
| B26950 | c.3695_3699del5 | O |
| B27129 | exon13 dup | F |
| B27131 | c.4484G>T | M |
| B27348 | c.66_67delAG | F |
| B27636 | c.3607C>T | N |
| B33139 | c.66_67delAG | F |

F = Frameshift, N = Nonsense, M = Missense, S = Splicing, O = Other

2.4 RNA extraction and qRT-PCR analysis

Total RNA was extracted from CD19⁺ B cells using RNeasy Mini kits (Qiagen, Toronto, ON) according to the manufacturer's instructions. RNA quality was determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA was generated by reverse transcribing 26 ng of RNA using the High Capacity cDNA Reverse Transcription kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The PCR protocol consisted of 10 min at 25°C, 120 min at 37°C and 5 min at 85°C and was performed in an Eppendorf Thermocycler (Eppendorf, Mississauga, ON). qRT-PCR was performed using Taqman Gene Expression Master Mix (Applied Biosystems) with 12 ng of cDNA per reaction according to the manufacturer's instructions. The following gene expression assays were obtained from Applied Biosystems: *CXCR3* Hs00171041_m1, *TBX21* Hs00203436_m1, *IFIT1* Hs00356631_g1, *GLDC* Hs01580591_m1 and *GUSB* Hs99999908_m1. The qRT-PCR protocol consisted of 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C, performed in an Eppendorf Realplex MasterCycler (Eppendorf). Targets were normalized by the corresponding *GUSB* expression in each sample. Target expression for each sample was then calculated relative to the results for sample B-1 using the $\Delta\Delta C_t$ method presented by Applied Biosystems.

2.5 Selected reaction monitoring-mass spectrometry (SRM-MS)

Proteomic SRM-MS was conducted by the Ontario Cancer Biomarker Network using LCL lysates that had been previously prepared from non-cycling cells. Seven *BRCA1* carrier and 6 non-carrier control LCL cell lines were used in this analysis. Peak

area for each protein fragment was averaged over two replicate experiments per *BRCA1* mutation carrier and control LCL cell line. Fold-changes for each fragment were calculated from the ratio of the average peak area in *BRCA1* mutation carrier LCLs to that in control LCLs. A fragment was discarded if peak area coefficient of variation (CV) across replicates was greater than 0.4 as cut-offs lower than 0.4 were considered too stringent. Peptides with at least two fragments satisfying this constraint were conserved. Fragment peak areas were averaged to determine protein peak area. Fold changes for each protein were calculated from the ratio of the average protein peak area in *BRCA1* mutation carrier LCLs to that in control LCLs.

2.6 Flow cytometry (FC)

For flow cytometry analysis, 1×10^6 LCLs or 0.5×10^6 primary B cells were re-suspended in 100 μ l PBS containing 10% FBS. All steps were performed in the dark from this point onwards. Cells were incubated with primary antibody or primary-conjugated antibody for 30 min on ice and washed twice. All washes were performed by centrifugation for 5 min at 800 rpm. Primary B cells were double stained for CD20 and the protein target of interest; all antibodies and conditions used are listed in Appendix C. Where required, cells were incubated with secondary antibody for 20 min on ice and washed twice. Following cell surface staining, cells were fixed for 10 min at room temperature using 4% paraformaldehyde (PFA). Cells were then washed and analyzed on a FC500 flow cytometer (Beckman Coulter, Mississauga, ON).

For analysis of intracellular proteins, cells were permeabilized for 15 min on ice after fixation using 2 mL PBS containing 1% saponin (Sigma). All wash steps were performed using saponin solution from this point onwards and staining procedures as described above. At least 30,000 events were analyzed on a FC500 flow cytometer (Beckman Coulter). Analysis was performed using Flowjo 7.6.5 software (TreeStar, Ashland, OR). Mean fluorescent intensity (MFI) values were determined relative to an unstained control.

2.7 Statistical analysis

Unpaired Student's t-test was used for statistical comparisons between *BRCA1* carriers and controls, and p values ≤ 0.05 were considered significant. The construction of receiver operating characteristic (ROC) curves was conducted using GraphPad PRISM software (GraphPad Software Inc., San Diego, CA). The area under the curve (AUC) was used to evaluate the diagnostic performance of targets identified by microarray or FC analysis as being differentially expressed in *BRCA1* mutation carriers compared to non-carrier controls. An AUC close to 1 indicates good sensitivity and specificity of the method at carrier/non-carrier discrimination. Sensitivity is defined as the probability that the test identifies a mutation carrier when the patient is truly a mutation carrier and is determined by the equation: $\text{sensitivity} = \text{number of true positives} / (\text{number of true positives} + \text{number of false negatives})$. Specificity is defined as the probability that the test identifies a non-carrier when the patient is truly a non-carrier and is determined by the equation: $\text{specificity} = \text{number of true negatives} / (\text{number of true negatives} + \text{number of false positives})$. Youden index was used to define cut-off points on the ROC curves

with maximal sensitivity and specificity, and is determined by the equation: Youden index = (sensitivity + specificity) - 1 (147).

Chapter 3

Results

3.1 Development of the *BRCA1* functional assay using LCLs

3.1.1 *In-silico* assay modelling using LCL gene expression

The first initiative in the development of a functional assay was to design a potential assay *in-silico* using the microarray data previously collected in our lab from 69 LCLs, including 31 *BRCA1* mutation carriers and 38 non-carrier controls. This was done to facilitate the selection of targets for the final assay as well as to provide a potential model that could be extended to the development of the blood-based assay. Top predictors of *BRCA1* carriers were selected from the 43-gene classifier and made into gene pairs by pairing genes of opposite direction (one gene is upregulated and the other downregulated in carriers relative to controls). In each LCL cell line, final values were obtained by taking the ratio of gene expression values. This approach was designed to amplify differences in gene expression levels (using high:low ratios). The gene pairs were tested by ROC curve analyses as individual predictive gene pairs, which showed that *TBX21/IFIT3* had the highest value for discriminating *BRCA1* carrier from control LCLs in this sample set (AUC=0.9448; $p < 0.0001$; Table 3). Next, combined sensitivity and specificity was calculated for the minimal number of gene pairs which could improve the diagnostic performance over a single gene pair. The resulting model comprised of *TBX21/IFIT3* and *PLA2G4A/FCGRT* (Table 4). The model had sensitivity 93.55% and specificity 94.74% in the process of distinguishing *BRCA1* carriers from controls and

Table 3: ROC curve AUC data of the three best gene pairs.

ROC curves were constructed for the three best predictive gene pairs of *BRCA1* status identified by microarray analysis. AUC values were determined from the ROC curves. An AUC value close to 1 indicates good sensitivity and specificity of the gene pair at carrier/non-carrier discrimination.

| Gene pair | AUC | | | 95% confidence interval | |
|-----------------------|--------|---------|----------|-------------------------|-------------|
| | Area | SE | p-value | Lower bound | Upper bound |
| <i>TBX21 /IFIT3</i> | 0.9448 | 0.02911 | < 0.0001 | 0.8878 | 1.002 |
| <i>PLA2G4A /FCGRT</i> | 0.8998 | 0.03589 | < 0.0001 | 0.8295 | 0.9702 |
| <i>CXCR3 /MX2</i> | 0.8964 | 0.03853 | < 0.0001 | 0.8209 | 0.972 |

Table 4: Sensitivity and specificity of predictive gene pairs.

For each gene pair, Youden index was used to define cut-off points on the ROC curves with maximal sensitivity and specificity. Sensitivity and specificity was then calculated for different combinations of gene pairs, such that each sample must meet the gene expression ratio cut-off of multiple gene pairs to be classified as a carrier. This determined the minimal number of gene pairs that could improve the diagnostic performance over a single gene pair.

| Gene pair | Sensitivity(%) | Specificity(%) |
|---|-----------------------|-----------------------|
| <i>TBX21 /IFIT3</i> | 96.77 | 89.47 |
| <i>PLA2G4A /FCGRT</i> | 93.55 | 76.32 |
| <i>CXCR3 /MX2</i> | 90.32 | 84.21 |
| <i>TBX21 /IFIT3 + PLA2G4A /FCGRT</i> | 93.55 | 94.74 |
| <i>TBX21 /IFIT3 + CXCR3 /MX2</i> | 90.32 | 92.1 |
| <i>CXCR3 /MX2 + PLA2G4A /FCGRT</i> | 87 | 94.74 |
| <i>TBX21 /IFIT3 + PLA2G4A /FCGRT + CXCR3 /MX2</i> | 87 | 100 |

correctly predicted 66/69 LCLs. The positive and negative predictive values were 93.55% and 94.6%, respectively. The diagnostic performance was not improved by the addition of a third gene pair *CXCR3/MX2* (Table 4). These results represent a potential model that could be applied to the development of a blood-based assay. Many of the genes used in this model were selected for analysis using qRT-PCR and protein-based methods.

3.1.2 LCLs express surface marker CD20

Initial assay development was performed using LCLs of both *BRCA1* carriers and controls. The first step in extending the assay to fresh blood samples was to develop the methodology to identify the relevant cells within heterogeneous whole-blood samples. LCLs were stained with surface markers of both B cells (CD20 and CD38) as well as T-cells (CD4 and CD8). The LCLs are EBV-transformed, which is known to infect B cells through binding of CD21, a component of the B cell co-receptor. As expected, LCLs did not express T-cell markers CD4 and CD8 (Figure 3). LCLs had high expression of the pan B cell marker, CD20, identifying the LCLs as a B cell population (Figure 3). LCLs also expressed the plasma B cell marker, CD38 (Figure 3). This result will become important once the assay is extended to lymphocytes isolated from fresh blood samples and will allow for the identification of relevant cells through the selection of CD20⁺ cells.

3.1.3 Protein expression in LCLs

Three genes identified as top predictors of *BRCA1* carriers based on initial microarray analysis were validated by qRT-PCR and selected as targets for a qRT-PCR-

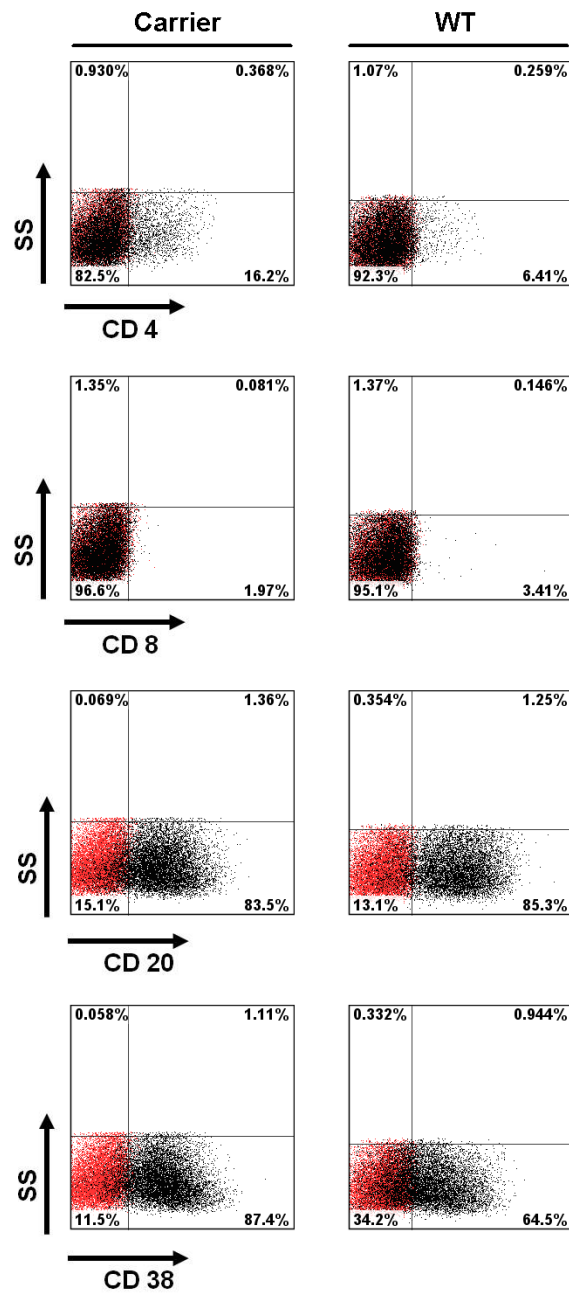


Figure 3: *BRCA1* carrier LCLs express B cell surface markers.

Flow cytometry for CD4, CD8, CD20 and CD38 from a representative *BRCA1* carrier and control (WT) LCL cell line. Red: unstained control. Black: target protein. SS, side scatter.

based assay (142) (Table 1). However, protein expression unique to *BRCA1* mutation carriers, remained to be validated in order to identify appropriate proteins for the development of a FC-based functional assay. To accomplish this aim, SRM-MS was used to analyze the corresponding protein expression of the 43-gene classifier. Seven *BRCA1* carrier and 6 non-carrier control LCL cell lines were used in this analysis. In contrast to initial microarray studies, cell lysates were prepared from non-cycling cells. SRM-MS results identified 8 aberrantly expressed proteins in carrier LCLs relative to controls (Table 5; Appendix B). FC was then used to analyze individual proteins in order to further validate possible targets for the development of the FC-based functional assay. LCLs used for FC analysis were actively cycling and successfully identified 6 proteins with altered expression in *BRCA1* carriers (Figure 4). Interestingly, many of these proteins identified by SRM-MS and FC analyses were associated with two biological pathways: IFN-regulated signalling and B cell development.

Interferon-regulated proteins: MX1 is a key mediator of the IFN-induced antiviral response and its expression is tightly regulated by the type I (α/β) IFNs and the JAK/STAT signalling pathway. FC found no significant difference in MX1 expression between the two groups ($p=0.8379$; Figure 4), whereas SRM-MS identified MX1 as being upregulated in carriers relative to controls (21/21 fragments (+); fold change=1.39; $p=0.0126$; Table 5; Appendix B), thereby corroborating initial microarray findings.

Expression levels of five other type I (α/β) IFN-induced proteins included in the 43-gene classifier, IFIT1, IFIT2, IFIT3, IFI44L and USP18, were not found to be significantly different between carriers and controls as determined by SRM-MS and FC (Table 5; Appendix B; Figure 4). While the overall differences between carriers and

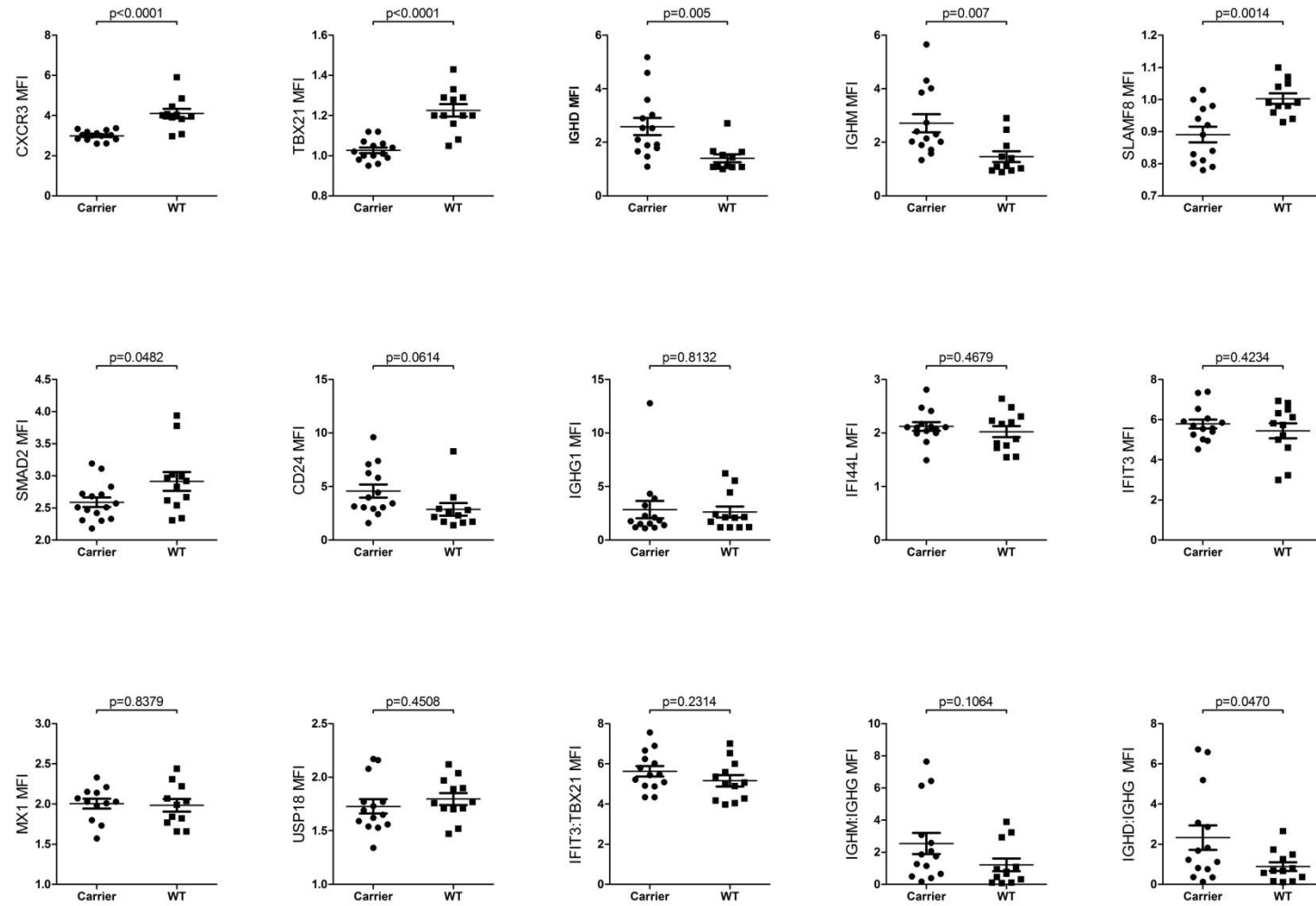
Table 5: Summary of SRM-MS analysis of target proteins.

The corresponding protein expression of the 43-gene classifier was determined using SRM-MS. Fragment peak areas were averaged to determine protein peak area. Fold changes for each protein were calculated from the ratio of the average protein peak area in *BRCA1* mutation carrier LCLs to that in control LCLs. Significance was determined by Student's unpaired t-test. (+) upregulated in carriers, (-) downregulated in carriers relative to controls.

| Protein | Number of fragments | | Mean peak area | | Fold change | p-value |
|----------|---------------------|---------------|----------------|------------|-------------|---------|
| | Upregulated | Downregulated | Control | Carrier | | |
| | (+) | (-) | | | | |
| SMAD2 | 0 | 9 | 65739.42 | 20469.61 | -3.21 | <0.0001 |
| IGHG1 | 0 | 6 | 1304318.95 | 292703.46 | -4.46 | 0.0008 |
| IGHD | 4 | 2 | 188404.74 | 330353.64 | 1.75 | 0.0009 |
| BCR | 0 | 6 | 67038.11 | 37925.93 | -1.77 | 0.0013 |
| SMARCAD1 | 2 | 7 | 116394.98 | 68274.53 | -1.70 | 0.0035 |
| SERPINF1 | 2 | 3 | 29310.93 | 20700.00 | -1.42 | 0.0092 |
| MX1 | 21 | 0 | 306155.29 | 425122.75 | 1.39 | 0.0126 |
| SOX4 | 3 | 5 | 237303.38 | 128047.91 | -1.85 | 0.0256 |
| IFNA4 | 4 | 2 | 124200.98 | 77955.16 | -1.59 | 0.092 |
| LAG3 | 0 | 5 | 822087.92 | 586107.90 | -1.40 | 0.2065 |
| SP1 | 5 | 0 | 183350.75 | 220105.58 | 1.20 | 0.2221 |
| PLA2G4A | 5 | 0 | 109931.84 | 128503.12 | 1.17 | 0.38 |
| RHOA | 2 | 7 | 167573.56 | 132932.48 | -1.26 | 0.4034 |
| ENPP2 | 2 | 4 | 273674.43 | 209148.29 | -1.31 | 0.41 |
| UBD | 0 | 11 | 167871.24 | 144157.26 | -1.16 | 0.4199 |
| JAKMIP2 | 2 | 8 | 87716.05 | 79898.00 | -1.10 | 0.4236 |
| USP18 | 0 | 8 | 1084509.10 | 815565.69 | -1.33 | 0.4329 |
| IFIT2 | 1 | 8 | 132056.29 | 115916.47 | -1.14 | 0.4872 |
| DUSP23 | 2 | 0 | 99298.00 | 128652.21 | 1.30 | 0.4976 |
| IL18BP | 0 | 6 | 60512.55 | 54999.16 | -1.10 | 0.5344 |
| CYP11B1 | 4 | 4 | 800180.15 | 641575.91 | -1.25 | 0.5415 |
| BRCA1 | 2 | 7 | 137722.94 | 119947.53 | -1.15 | 0.5501 |
| GRB2 | 0 | 10 | 413932.50 | 360275.48 | -1.15 | 0.5833 |
| CXCR3 | 0 | 3 | 181059.39 | 167872.35 | -1.08 | 0.6327 |
| FAM79B | 7 | 6 | 426039.13 | 382440.23 | -1.11 | 0.6464 |
| FCGRT | 0 | 2 | 232177.25 | 203206.85 | -1.14 | 0.6631 |
| PXDN | 3 | 0 | 59806.11 | 67218.93 | 1.12 | 0.6664 |
| SLC16A10 | 3 | 3 | 502775.65 | 457915.92 | -1.10 | 0.7354 |
| FOXP1 | 1 | 7 | 232580.60 | 202897.01 | -1.15 | 0.7402 |
| ZBTB38 | 0 | 4 | 100645.25 | 93783.55 | -1.07 | 0.7599 |
| TBX21 | 7 | 0 | 224366.53 | 244413.37 | 1.09 | 0.7657 |
| GLDC | 2 | 4 | 40521.96 | 38052.72 | -1.06 | 0.7748 |
| IFIT1 | 4 | 9 | 717225.80 | 655668.60 | -1.09 | 0.7838 |
| ZZZ3 | 4 | 4 | 76460.42 | 72815.13 | -1.05 | 0.7909 |
| FYN | 4 | 0 | 408763.93 | 437931.43 | 1.07 | 0.7944 |
| IFNG | 3 | 6 | 1555460.37 | 1771871.69 | 1.14 | 0.8011 |
| HLA-DMB | 2 | 4 | 185231.37 | 171392.02 | -1.08 | 0.802 |
| ETV7 | 5 | 2 | 349058.35 | 380408.31 | 1.09 | 0.8055 |
| PRLR | 3 | 3 | 557297.84 | 575227.94 | 1.03 | 0.8518 |
| IF44L | 4 | 4 | 121839.14 | 125574.45 | 1.03 | 0.8812 |
| NFKB1 | 4 | 4 | 38419.54 | 37767.64 | -1.02 | 0.9118 |
| MX2 | 3 | 7 | 536644.12 | 511788.19 | -1.05 | 0.9157 |
| MMP7 | 4 | 5 | 188160.20 | 186705.63 | -1.01 | 0.9528 |
| LFNG | 7 | 0 | 1126928.92 | 1153657.21 | 1.02 | 0.9596 |
| ZBED3 | 1 | 2 | 147664.75 | 149075.42 | 1.01 | 0.965 |
| IFNA5 | 2 | 5 | 167592.36 | 166118.44 | -1.01 | 0.9664 |
| TNS4 | 6 | 3 | 617211.81 | 611426.65 | -1.01 | 0.9669 |
| CSRP2 | 5 | 3 | 153585.91 | 154401.85 | 1.01 | 0.9803 |

Figure 4: LCL protein expression determined by flow cytometry.

Mean fluorescent intensity (MFI) of target protein expression in *BRCA1* carrier (n=12) and control (WT) (n=13) LCL cell lines. MFI was determined relative to an unstained sample. Means are indicated by a solid line. Error bars represent standard error of the mean (SEM). Significance was determined by Student's unpaired t-test.



controls were not statistically significant, SRM-MS analysis demonstrated that a large number of fragments were consistently downregulated in *BRCA1* carriers for IFIT1 (9/13 fragments (-); fold change=-1.09; p=0.7838; Table 5; Appendix B), IFIT2 (8/9 fragments (-); fold change=-1.14; p=0.4872; Table 5; Appendix B) and USP18 (8/8 fragments (-); fold change=-1.33; p=0.4329; Table 5; Appendix B), suggesting that these proteins may be appropriate targets for further assay development.

SRM-MS found no difference in TBX21 or CXCR3 expression in carriers relative to controls using non-cycling cells (Table 5; Appendix B). In accordance with initial microarray results however, FC analysis found both proteins to be significantly downregulated in *BRCA1* carriers (p<0.0001; Figure 4). Similar to initial microarray results, TBX21 and CXCR3 were the two top predictive proteins of *BRCA1* carriers in actively cycling cells. These results successfully identified several proteins as appropriate targets for the next stages of assay development. Moreover, these results implicate BRCA1 in mediating IFN-regulated signalling pathways and suggest that other IFN-regulated proteins may be potential targets for assay development.

B cell development: SRM-MS analysis confirmed upregulation of IGHD (4/6 fragments (+); fold change=1.75; p=0.0009; Table 5; Appendix B), and downregulation of IGHG1 (6/6 fragments (-); fold change=-4.46; p=0.0008; Table 5; Appendix B) in *BRCA1* carriers relative to controls, suggesting that BRCA1 may influence B cell CSR. Therefore, different Ig isotypes were analyzed by FC, including IGHM, IGHD and IGHG1, and demonstrated that both IGHM (p=0.007; Figure 4) and IGHD (p=0.005; Figure 4) expression was significantly higher on carrier LCLs relative to controls. IGHG1 expression, however, was not significantly different between groups (p=0.8132; Figure

4). Moreover, the ratio of IGHM:IGHG1 or IGHD:IGHG1 expression as determined by MFI (Figure 4) as well as the percentage of positively expressing cells did not improve the ability of IGHM or IGHD to distinguish *BRCA1* carriers from non-carriers. The differences in IGHG1 expression between protein analyses may be partially attributed to the differences in cell cycling as well as the possibility that IGHG1 is less abundant on the cell surface relative to intracellular secretory vesicles. Therefore intracellular expression of IGHG1 remains to be examined. Nonetheless, the increased expression of IGHM and IGHD immunoglobulins on carrier LCLs suggests that these proteins are appropriate for the next phase of assay development. Moreover these results suggest that loss of a single *BRCA1* allele may influence CSR and B cell development.

The reduced expression of additional proteins involved in B cell differentiation provides further support to this theory. FOXP1 is a transcription factor that regulates both early as well as mature B cell development. While the overall differences between carriers and controls were not statistically significant, SRM-MS analysis demonstrated that a large number of FOXP1 fragments were consistently downregulated in *BRCA1* carrier LCLs (7/8 fragments (-); fold change=-1.15; p=0.7402; Table 5; Appendix B), suggesting that FOXP1 may be appropriate for further assay development. SOX4 is a transcription factor with well described roles in B cell development that was found to be significantly downregulated in *BRCA1* carriers (5/8 fragments (-); fold change=-1.85; p=0.0256; Table 5; Appendix B). High expression of CD24 is typically found on mature B cells prior to terminal differentiation. Overall, CD24 expression was not significantly different between carriers and controls (p=0.0614; Figure 4); however, these experiments must be repeated a third time as one replicate found CD24 to be significantly increased

on *BRCA1* carrier LCLs. Lastly, SLAMF8 is a novel B cell antigen with unknown functions, but is believed to play a role in B cell development and was found to be significantly downregulated on carrier LCLs relative to controls ($p=0.0014$; Figure 4).

Other proteins: Interestingly, SRM-MS and FC screening led to the identification of downregulated transcription factor, SMAD2, that was not previously identified as a predictor of *BRCA1* carriers by microarray analysis (9/9 fragments (-); fold change=-3.21; $p<0.0001$; Table 5; Appendix B) ($p=0.0482$; Figure 4). SRM-MS also identified two other proteins as downregulated in *BRCA1* carriers: BCR (6/6 fragments (-); fold change=-1.77; $p=0.0013$; Table 5; Appendix B) and SMARCAD1 (7/9 fragments (-); fold change=-1.7; $p=0.0035$; Table 5; Appendix B). BCR plays a role in the cellular trafficking of growth factor receptors, while SMARCAD1 is a component of the SWI/SNF chromatin remodelling complex.

Together these results successfully identified several proteins, aberrantly expressed in *BRCA1* mutation carrier LCLs, that could be used in the development of a FC-based functional assay. These results are summarized in Table 6. Interestingly, many of the proteins were also involved in two biological pathways: IFN-regulated signalling and B cell development. This suggests that other proteins involved in these pathways may also serve as additional targets for the development of a FC-based functional assay.

3.1.4 Assay modelling using LCL protein expression levels determined by FC

Following the identification of proteins preferentially expressed in *BRCA1* carrier LCLs, the next step was to use these proteins to model a potential functional assay which

Table 6: Summary of genes and proteins identified as predictors of *BRCA1* mutation carriers.

Microarray analysis of LCLs was used to develop an accurate class predictor comprised of 43 genes to distinguish *BRCA1* carriers from non-carriers (139). Genes included in the 43-gene classifier and their predicative values are listed. Predictive values <0 or >0 identify strongly predictive genes of carrier status and indicate downregulation or upregulation in carrier LCLs relative to non-carriers, respectively. Initial microarray findings were validated using qRT-PCR for 5 genes (142). Protein expression was determined by SRM-MS and FC. Yes, indicates a significant difference in target expression between *BRCA1* carrier and non-carrier LCLs as determined by that method. No, indicates no significant difference.

qRT-PCR, quantitative real-time PCR. SRM-MS, selected reaction monitoring-mass spectrometry. FC, flow cytometry. ** = genes not included in the 43-gene classifier.

| Gene | Predictive Value | qRT-PCR | SRM-MS | FC |
|-----------------|-------------------------|----------------|---------------|-----------|
| <i>PXDN</i> | 4.247 | | No | |
| <i>JAKMIP2</i> | 2.147 | | No | |
| <i>MMP7</i> | 2.063 | | No | |
| <i>CSRP2</i> | 1.797 | | No | |
| <i>CD24</i> | 1.757 | | | No |
| <i>LFNG</i> | 1.707 | | No | |
| <i>ENPP2</i> | 1.706 | | No | |
| <i>FOXP1</i> | 1.503 | | No | |
| <i>PWWP2</i> | 1.479 | | | |
| <i>PRLR</i> | 1.383 | | No | |
| <i>IFNA5</i> | 1.228 | | No | |
| <i>FCGRT</i> | 0.998 | | No | |
| <i>IFNA4</i> | 0.992 | | No | |
| <i>IFIT3</i> | 0.894 | | | No |
| <i>SERPINF1</i> | 0.887 | | Yes | |
| <i>IGHD</i> | 0.849 | | Yes | Yes |
| <i>IFIT1</i> | 0.788 | Yes | No | No |
| <i>ZBED3</i> | 0.786 | | No | |
| <i>IFIT2</i> | 0.736 | | No | |
| <i>USP18</i> | 0.728 | | No | No |
| <i>IFI44L</i> | 0.689 | | No | No |
| <i>SOX4</i> | 0.471 | | Yes | |
| <i>MX2</i> | 0.468 | No | No | |
| <i>MX1</i> | 0.462 | | Yes | No |
| <i>ETV7</i> | -2.721 | | No | |
| <i>TBX21</i> | -2.668 | Yes | No | Yes |
| <i>CXCR3</i> | -2.203 | Yes | No | Yes |
| <i>FYN</i> | -2.114 | | No | |
| <i>IGHG1</i> | -2.111 | | Yes | No |
| <i>IFNG</i> | -1.976 | | No | |
| <i>FAM79B</i> | -1.900 | | No | |
| <i>CYP1B1</i> | -1.779 | | No | |
| <i>PLA2G4A</i> | -1.621 | | No | |
| <i>SLC16A10</i> | -1.558 | | No | |
| <i>TNS4</i> | -1.475 | | No | |
| <i>UBD</i> | -1.174 | | No | |
| <i>IL18BP</i> | -1.173 | | No | |
| <i>LAG3</i> | -1.068 | | No | |
| <i>BCR</i> | -1.005 | | Yes | |
| <i>ZBTB38</i> | -0.895 | | No | |
| <i>GLDC</i> | -0.879 | No | No | |
| <i>DUSP23</i> | -0.861 | | No | |
| <i>HLA-DMB</i> | -0.378 | | No | |
| **IGHM | | | | Yes |
| **SLAMF8 | | | | Yes |
| **SMAD2 | | | Yes | Yes |
| **SMARCD1 | | | Yes | |

could then be applied to the fresh blood-based assay. Similar to the assay modelling based on initial transcriptome data, the selected proteins were tested by ROC curve analysis as individual predictive proteins. This showed that TBX21 had the highest value for discriminating *BRCA1* carrier from non-carrier LCLs in this sample set (AUC=0.9685; $p < 0.03073$; Table 7). Next, combined sensitivity and specificity was calculated for the minimal number of proteins which could improve the diagnostic performance over a single protein. No combination of proteins was found to improve the diagnostic performance over TBX21, which had a sensitivity of 92.31% and specificity of 90.91% in the process of distinguishing *BRCA1* carriers from controls, and correctly predicted 22/24 LCLs (Table 8). The positive and negative predictive values were 92.31% and 90.91%, respectively. This represents an example of the potential modelling that could be done using protein expression levels in fresh blood samples.

3.2 Development of the *BRCA1* functional assay using fresh blood samples

3.2.1 Sample characteristics

Following selection of appropriate mRNA and protein targets, design of a potential assay, and establishment of a methodology to identify the desired cell population, the next step was to use a sample cohort of patients to determine whether detection of these targets is feasible in lymphocytes isolated from fresh blood samples. To accomplish this, 13 *BRCA1* mutation carriers and 4 non-carrier controls all confirmed by direct sequencing, were recruited to participate in this study. Sample characteristics are listed in Table 9. At the time of blood sample collection, all patients were healthy and

Table 7: ROC curve AUC data of predictive proteins.

ROC curves were constructed for proteins identified by flow cytometry as significantly different between carrier and non-carrier LCLs. AUC values were determined from the ROC curves. An AUC value close to 1 indicates good sensitivity and specificity of the protein at carrier/non-carrier discrimination.

| Protein | AUC | | | 95% confidence interval | |
|----------------|-------------|-----------|----------------|--------------------------------|--------------------|
| | Area | SE | p-value | Lower bound | Upper bound |
| TBX21 | 0.9685 | 0.03073 | 0.000105 | 0.9083 | 1.029 |
| CXCR3 | 0.9021 | 0.07067 | 0.0008705 | 0.7635 | 1.041 |
| IGHD | 0.8776 | 0.07213 | 0.001767 | 0.7362 | 1.019 |
| IGHM | 0.8462 | 0.08459 | 0.00415 | 0.6803 | 1.012 |
| SLAMF8 | 0.8427 | 0.08004 | 0.004545 | 0.6857 | 0.9996 |
| SMAD2 | 0.7448 | 0.1039 | 0.04264 | 0.541 | 0.9485 |

Table 8: Sensitivity and specificity of predictive proteins.

For each protein, Youden index was used to define MFI cut-off points on the ROC curves with maximal sensitivity and specificity. Sensitivity and specificity was then calculated for different combinations of proteins, such that each sample must meet the MFI cut-off of multiple proteins to be classified as a carrier. This determined the minimal number of proteins that could improve the diagnostic performance over a single protein.

| Proteins | Sensitivity(%) | Specificity(%) |
|--|-----------------------|-----------------------|
| TBX21 | 92.31 | 90.91 |
| CXCR3 | 100 | 81.82 |
| IGHD | 76.92 | 90.91 |
| IGHM | 92.31 | 72.73 |
| SLAMF8 | 61.54 | 100 |
| SMAD2 | 92.31 | 54.55 |
| TBX21 + CXCR3 | 92.31 | 90.91 |
| TBX21 + CXCR3 + IGHD | 76.92 | 100 |
| TBX21 + CXCR3 + IGHD + IGHM | 76.92 | 100 |
| TBX21 + CXCR3 + IGHD + IGHM + SLAMF8 | 69.23 | 100 |
| TBX21 + CXCR3 + IGHD + IGHM + SLAMF8 + SMAD2 | 53.85 | 100 |

Table 9: Sample characteristics for *BRCA1* mutation carriers and non-carrier controls.

| Sample | Carrier yes/no | <i>BRCA1</i> Mutation | Mutation Type | Nuc. | Exon | Codon | AA Change | Age | Sex | Age at Breast Cancer Diagnosis |
|--------|-------------------|-----------------------|---------------|--------|------|-------|-------------|-----|-----|-----------------------------------|
| A-1 | yes | c.68_69delAG | F | 185 | 2 | 23 | Stop 39 | 54 | F | N/A |
| A-2 | yes | c.1961delA | F | 2080 | 11b | 654 | Stop 700 | 34 | F | N/A |
| A-3 | yes | c.68_69delAG | F | 185 | 2 | 23 | Stop 39 | 36 | F | 36 |
| A-4 | yes | c.5319dupC | F | 5438 | 21 | 1773 | Stop 1829 | 42 | F | N/A |
| A-5 | yes | c.4327C>T | N | 4446 | 13 | 1443 | Arg to Stop | 56 | F | 47 |
| A-6 | yes | c.4524G>A | N | 4643 | 15 | 1508 | Trp to Stop | 54 | F | 52 |
| A-7 | yes | c>5152+1G>T | IVS | 5271+1 | 18 | | | 70 | F | 57 |
| A-8 | yes | c.68_69delAG | F | 185 | 2 | 23 | Stop 39 | 34 | F | N/A |
| A-9 | yes | c.3764dupA | F | 3883 | 11d | 1255 | Stop 1266 | 54 | F | 28 |
| A-10 | yes | c.895_896delGT | F | 1014 | 11a | 299 | Stop303 | 35 | F | N/A |
| A-12 | yes | c.2216_2217delAA | | | | 741 | | 39 | F | N/A |
| A-13 | yes | c.4524G>A | N | 4643 | 15 | 1508 | Trp to Stop | 29 | F | N/A |
| A-14 | yes | c.4524G>A | N | 4643 | 15 | 1508 | Trp to Stop | 24 | F | N/A |
| B-1 | no | | | | | | | 37 | F | N/A |
| B-2 | no | | | | | | | 69 | F | 47 |
| B-3 | no | | | | | | | 54 | F | 50 |
| B-4 | no | | | | | | | 69 | F | 60 |

F=Frameshift, N=Nonsense, IVS=Intervening Sequence

not undergoing treatment, although some had been previously diagnosed with breast cancer between 2 to 26 years prior. All mutations had been previously reported as pathogenic and included deleterious nonsense or frameshift mutations, and one intervening sequence (IVS) mutation, scattered throughout the entire gene. All patients were female and age distribution was similar between the two groups ranging from 24-69. Patient mutations also corresponded to mutations used in the LCL cell lines.

3.2.2 Target detection in fresh blood samples

Blood samples were received and processed approximately 24 h after blood draw. The B cell population of interest represents between 5-15% of the total lymphocyte population depending on the sample. The low abundance of the B cells precluded analysis by both qRT-PCR and FC techniques for many patient samples. For qRT-PCR analysis, CD19⁺ B cells were enriched using magnetic separation from whole lymphocytes. This resulted in very low cell numbers, ranging from 5×10^5 - 5×10^6 B cells depending on the sample. RNA quality was determined using an Agilent 2100 bioanalyzer and demonstrated that RNA extracted from the majority of samples had an RNA integrity number (RIN) of 7 or lower. Despite low B cell numbers and poor RNA quality, expression of all four target genes tested, *CXCR3*, *GLDC*, *IFIT1* and *TBX21*, was detected in all patients in this sample cohort (Figure 5). This suggested that a qRT-PCR-based assay may be feasible using fresh blood samples.

To establish the optimum time for processing samples after blood draw, a time course experiment was conducted using blood samples from a lab volunteer. Transcript

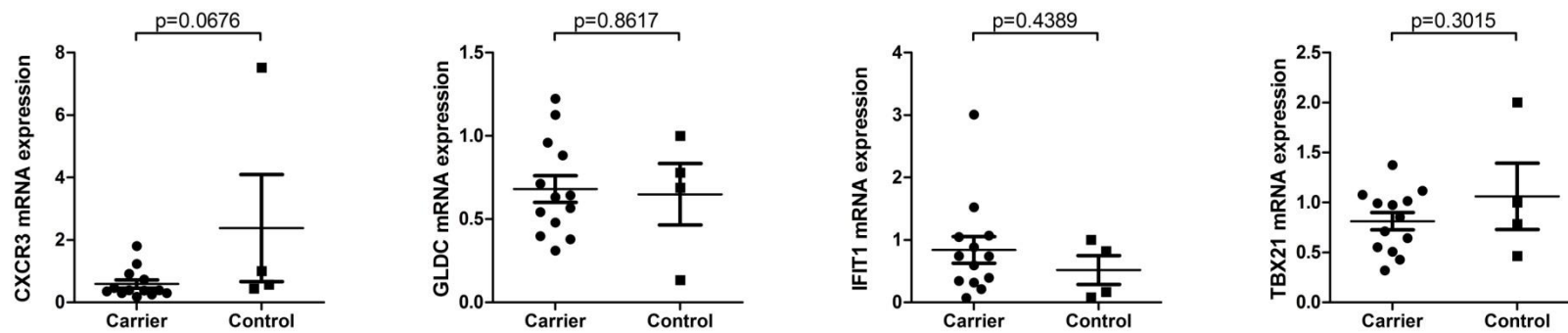


Figure 5: mRNA expression in CD19⁺ B cells isolated from fresh blood samples.

mRNA expression of four genes *CXCR3*, *GLDC*, *IFIT1* and *TBX21* was determined by qRT-PCR, using CD19⁺ B cells isolated from the fresh blood samples of a sample cohort of *BRCA1* mutation carriers (n=13) and non-carrier controls (n=4). Means are indicated by a solid line. Error bars represent SEM. Significance was determined by Student's unpaired t-test.

expression levels were found to be different in blood samples processed immediately after blood draw compared to 24 or 48 h later (Figure 6A). Unknown is whether this would affect the ability to detect a difference in target expression between carriers and controls. Previous studies have demonstrated that B cells isolated from fresh blood samples may be cultured for short durations of time. To determine whether cell culture would restore RNA quality, primary B cells from 2 patient samples were cultured without stimulation for 24 h following isolation. This, however, resulted in poor RNA quality and changes in target gene expression compared with the blood samples processed immediately (Figure 6B,C).

The changes in transcript expression levels between samples processed immediately and samples processed 24 h later, suggested that a qRT-PCR-based assay may not be feasible and that the development of a FC-based assay would be the best approach for further development of a blood-based assay. FC was then used to determine which target proteins identified in LCLs were also detectable in B cells isolated from fresh blood samples. Blood samples from 8 patients had enough lymphocytes for FC analysis in addition to qRT-PCR (Figure 7; Figure 8). The desired B cell population was selected for by gating CD20⁺ cells. SLAMF8 was virtually undetectable on primary CD20⁺ B cells, suggesting it would not be appropriate for assay development and the expression of IGHG1 varied between samples (Figure 7; Figure 8). While expression of TBX21 was significantly different between samples from *BRCA1* carriers and non-carriers, the power of experiment was not strong enough to be conclusive based on low sample size (Figure 8). Moreover, the low expression of TBX21 on CD20⁺ B cells

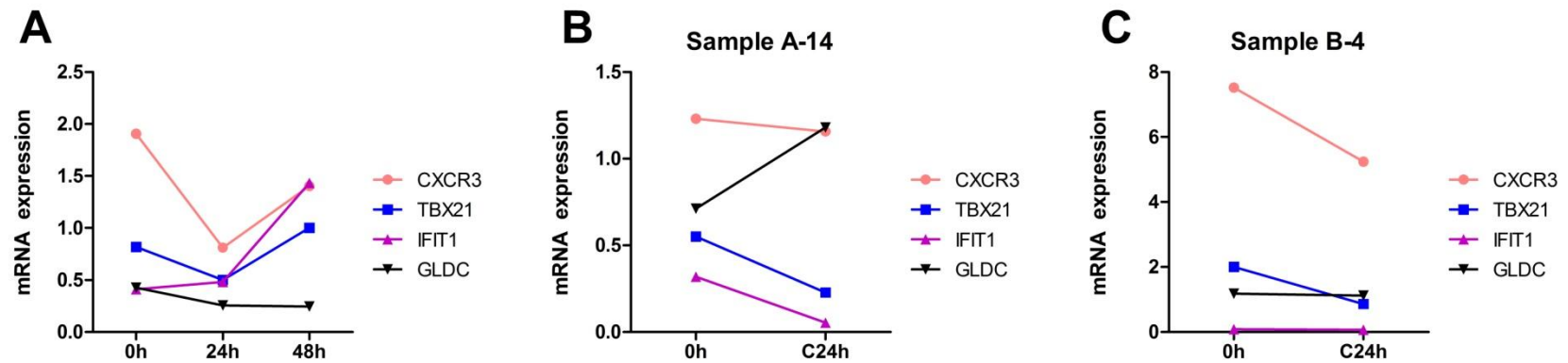


Figure 6: mRNA expression levels change as the age of the blood sample increases.

A. mRNA was extracted from CD19⁺ B cells isolated from the fresh blood of a volunteer at 3 different time points: immediately following blood draw (0 h), 24 h or 48 h following blood draw. mRNA expression of four genes *CXCR3*, *TBX21*, *IFIT1* and *GLDC* was determined by qRT-PCR and varied between time points. **B/C.** mRNA was extracted from CD19⁺ B cells from two patients immediately following sample delivery (0 h) or after 24 h primary B cell culture (C24 h). mRNA expression of four genes *CXCR3*, *TBX21*, *IFIT1* and *GLDC* was determined by qRT-PCR and varied between B cell conditions. **B.** *BRCA1* mutation carrier A-14. **C.** Non-carrier B-4.



Figure 7: Target protein expression in B cells isolated from fresh blood samples.

Flow cytometry for IGHM, IGHD, CD24, IGHG1, SLAMF8, CXCR3 and TBX21 in electronically gated CD20⁺ B cells from *BRCA1* carrier (A-1-5, A-9) and non-carrier (B-1-2) patient samples. Red line: unstained control. Black line: target protein.

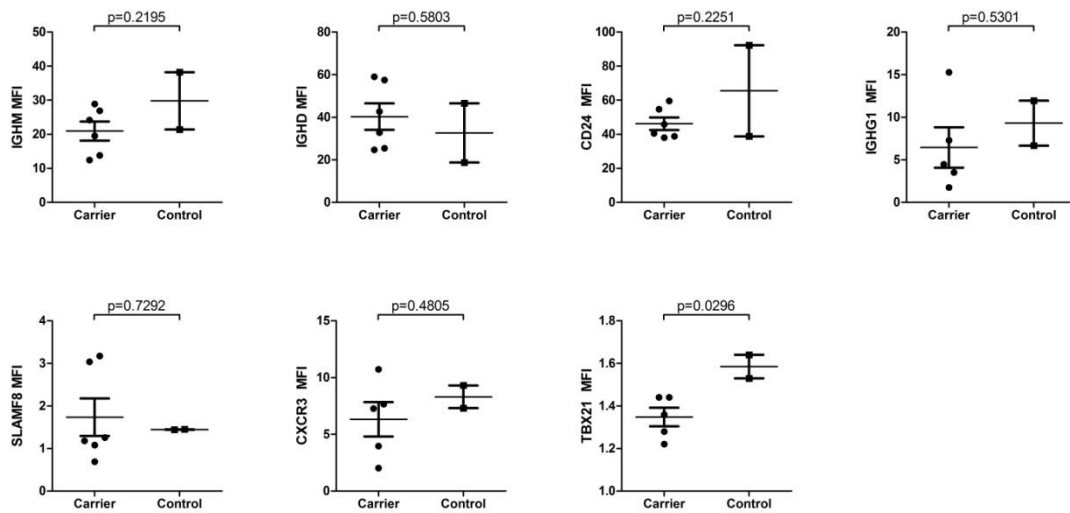


Figure 8: MFI values of target proteins in B cells isolated from fresh blood samples.

MFI values of target protein expression in a sample cohort of *BRCA1* mutation carrier (n=6) and non-carrier (n=2) patient samples. MFI was determined relative to an unstained sample. Means are indicated by a solid line. Error bars represent standard SEM. Significance was determined by Student's unpaired t-test.

suggests that it is not an ideal target for assay development (Figure 7). In contrast, four target proteins, IGHM, IGHD, CD24 and CXCR3 had high expression on B cells from this sample cohort, successfully identifying these proteins for further development of a FC-based functional assay (Figure 7). In summary, the results of this study have identified four proteins for further development of a FC-based functional assay to identify *BRCA1* mutation carriers.

Chapter 4

Discussion

The high risk of breast cancer development associated with *BRCA1* mutation carriers makes it essential that these women be identified early and accurately. However, the development of a functional assay to predict the effects of *BRCA1* mutation is impeded by the pleiotropic nature of BRCA1 function. As described above, our lab developed a novel functional assay using gene expression profiles in LCLs to accurately distinguish *BRCA1* mutation carriers from non-carriers. This study sought to validate this further by extending the functional assay from immortalized cell lines to fresh blood samples.

As a first step, initial LCL transcriptome data was used to develop a prototype functional assay. This involved making different combinations of predictive gene pairs to maximize diagnostic performance of the assay, creating a potential model that could be extended to the development of the blood-based assay and facilitating the selection of targets for protein analysis. Corresponding protein expression was determined using SRM-MS as well as FC, which successfully identified several proteins as strong predictors of *BRCA1* carriers. Interestingly, many of these proteins were involved in two signalling pathways: IFN-regulated signalling and B cell development. If validated in fresh blood samples, these represent potentially novel findings and suggest yet unexplored functions for BRCA1 in the innate immune response to viral infection, and in CSR and B cell development. Moreover, these results suggested that other proteins involved in these pathways may also serve as potential targets for the development of a protein-based functional assay. It was in fact further demonstrated that protein expression

in LCLs could be used to model a functional assay that predicts carrier status. Additionally, this work provided a potential model that could be extended to the development of the blood-based assay and facilitated the selection of targets for protein analysis using fresh blood samples.

Next, we established the methodology to identify the desired cell population within a heterogeneous whole-blood sample by determining that LCLs express the pan B cell surface marker CD20. This allowed for B cells to be isolated from heterogeneous whole-blood samples either by using magnetic beads or electronic gating, depending on the type of analysis. Lastly, to determine which targets were detectable in B cells isolated from fresh blood samples, mRNA and protein expression of the selected targets were examined using a sample cohort of *BRCA1* mutation carrier and non-carrier control patients. The results showed that four mRNA targets, *CXCR3*, *GLDC*, *IFIT1* and *TBX21*, were detectable in these fresh blood samples identifying them as appropriate targets for further development of a qRT-PCR-based assay. However, given poor RNA quality and changes in transcript expression levels as the age of the blood samples increased, the findings suggest that development of a protein-based assay is the best approach moving forward. These results also identified four proteins, IGHM, IGHD, CD24 and CXCR3, with high expression on CD20⁺ B cells, highlighting their potential for further development of a FC-based functional assay.

4.1 BRCA1 and IFN-regulated signalling

To identify proteins appropriate for the development of a protein-based functional assay, SRM-MS and FC were used to identify those proteins with abrogated expression in

carrier LCLs relative to controls. Interestingly, the results demonstrated altered expression of several IFN-regulated proteins in carrier LCLs. IFNs are pleiotropic molecules that are crucial components of the innate and adaptive immune response and possess antiviral activity. There are 3 types of IFNs: Type I (α/β), Type II (γ) and Type III (λ). Binding of the IFNs to their cognate receptors results in activation of the Janus kinase (JAK) that phosphorylates STAT transcription factors. The STAT transcription factors then dimerize and translocate to the nucleus where they bind specific motifs called *interferon-stimulated regulatory elements* (ISRE) or *gamma activating sequences* (GAS) that are found in the promoters of genes induced by IFNs.

The strongest predictors of *BRCA1* carrier status in LCLs as determined by FC, were the IFN- γ regulated proteins, TBX21 and CXCR3. FC analysis demonstrated downregulation of these two proteins in *BRCA1* carriers relative to controls. The transcription factor, 'T-box expressed in T cells' (TBX21), was initially identified as a master regulator of Th1 cell differentiation through induction of IFN- γ (141, 148). It is now known to be expressed and have important roles in several different cell types, including B cells, where it is required to produce IGHG2a (IgG2a) (143, 144, 149–151). TBX21 expression in both Th1 and B cells is initially driven by IFN- γ and STAT1-mediated signalling (151, 152). TBX21 then induces expression of IFN- γ , creating a positive feedback loop. In addition, TBX21 directly controls the expression of the chemokine receptor CXCR3 in both Th1 and B cells (146, 153) thereby orchestrating their migration to sites of inflammation. Consistent with these findings, decreased expression of TBX21 in *BRCA1* carrier LCLs coincides with decreased expression of CXCR3.

SRM-MS analysis demonstrated altered expression of MX1 and the IFIT family of proteins in *BRCA1* carrier LCLs. Expression of these proteins is tightly regulated by Type I (α/β) and Type III (λ) IFNs and is dependent on STAT1-mediated signalling. The MX1 GTPase is an important component of the early innate immune response and has broad antiviral activity against a wide range of RNA and some DNA viruses by blocking viral replication. The IFIT family of proteins also possesses antiviral activity and functions to restrict viral replication by blocking protein synthesis.

The altered expression of IFN-regulated proteins in carrier LCLs relative to controls suggested that *BRCA1* may mediate IFN-regulated signalling pathways. These observations are supported by previous findings that *BRCA1* physically associates with the transcription factor STAT1 and differentially activates a subset of IFN- γ target genes, such as p21, in both embryonic kidney and fibrosarcoma cell lines (61). Moreover, a study using breast epithelial cell lines demonstrated that several IFN- γ regulated genes are synergistically upregulated by *BRCA1*, including the MX1 protein identified in our analysis (154). The opposing MX1 expression patterns between the observations presented here and the study by Andrews et al. (154) may reflect cell type specific differences in transcript regulation. Lastly, in breast epithelial cells, *BRCA1* was also found to indirectly increase IFN-induced gene expression through upregulation of STAT1 as well as Type I (α/β) IFNs in response to IFN- γ (155). Together these studies support the theory that *BRCA1* mediates IFN-regulated signalling, possibly through the regulation of STAT1 transcriptional activation.

Although the *IFNA5*, *IFNB4* and *IFNG* genes were included in the initial 43-gene classifier, SRM-MS analysis suggested that these IFNs were not differentially expressed

between carriers and controls. In addition there were several differences in protein expression observed between SRM-MS and FC analyses, including MX1, CXCR3 and TBX21. Differences in target expression levels between microarray/FC and SRM-MS analyses may in part be attributed to differences in cell cycling. LCLs used for SRM-MS were non-cycling whereas LCLs used in initial microarray and FC experiments were actively cycling. Given that expression of many genes is dependent on cell growth, including BRCA1 which is actively downregulated in non-cycling cells (156), changes in cell cycling may have caused differences in target expression between analyses and represents a clear disadvantage of the SRM-MS data. The expression of the IFN proteins remains to be examined in cycling cells.

An additional observation regarding SRM-MS analysis was that fragments of the same protein frequently have opposing expression patterns. There are several reasons why this might be observed, including the chemical composition of the peptide, which may allow it to ionize more readily and therefore be detected more easily than other peptides of the same protein. In addition, protein degradation prior to digestion may have created an uneven distribution of input peptides. It is also possible that certain peptide sequences may not be unique to the parent protein. Instead they may be measuring signals from other proteins in addition to the parent. Proteins with an equal number of fragments in both directions are difficult to interpret. Proteins with the majority of fragments expressed in the same direction were considered more reliable.

Despite the limitations described above, the findings presented here combined with data from previous studies suggest that IFN-regulated proteins represent potential targets for the development of a blood-based assay. One of two IFN-regulated proteins

tested in fresh blood thus far, CXCR3, was highly expressed on primary B cells, identifying CXCR3 for the next phase of assay development. Moreover, if initial observations of IFN signalling deregulation in carrier LCLs are validated in haploinsufficient B cells from fresh blood, then this would represent a novel finding and suggest another role for BRCA1 in the innate immune response to viral infection.

4.2 BRCA1 and B cell development

Interestingly, initial microarray analysis as well as subsequent SRM-MS and FC analysis demonstrated that LCLs of *BRCA1* mutation carriers have altered expression of the Igs IGHM, IGHD and IGHG1. Igs are responsible for clearing pathogens during the effector phase of the adaptive immune response. There are five different Ig isotypes: IGHM (IgM), IGHD (IgD), IGHG (IgG), IGHE (IgE) and IGHA (IgA), that are distinguished by the constant region in their heavy chains, allowing for differences in structural features including size, solubility and tissue distribution of the Igs. Initially mature B cells express surface IGHM and IGHD. During the course of an immune response, proliferating B cells undergo a programmed cell change, CSR, which involves a series of cutting and rejoining events at the DNA level. This gives rise to class-switched B cells that express Igs of isotypes other than IGHM and IGHD and alters the effector function of the antibody molecules. The constant regions of different Ig isotypes are encoded by distinct C_H exons (C_μ , C_δ , C_γ , C_ϵ , C_α) ordered sequentially within the Ig heavy chain (IgH) locus. The 5' intron immediately upstream of each C_H exon possesses a switch (S) region (except C_δ). The S region preceding each C_H is denoted as S_H : S_μ , S_γ , S_ϵ or S_α . The CSR events are initiated by active transcription within S regions, and this

recruits the activation-induced cytidine deaminase (AID) enzyme. Transcription provides an accessible ssDNA substrate for AID activity. The AID enzyme then deaminates deoxycytosines in S_{μ} and another downstream S_H region, yielding deoxyuracils. DNA deamination by AID is processed by the combined activities of base-excision and mismatch repair proteins to generate staggered DNA breaks in the upstream S_{μ} (donor) and downstream S_H (acceptor) regions. The DSB intermediates trigger the same set of DDR repair factors that repair DSBs generated by exogenous sources, such as IR. DSB resolution involves the looping out and deletion of the intervening DNA and the formation of the S_{μ} - S_H junction that brings the downstream C_H region DNA closer to the $V_H D_H J_H$ variable region DNA. CSR occurs primarily during the G1-phase of the cell cycle and utilizes NHEJ as the main mechanism of DSB repair (157); however, a subset of CSR also occurs by alt-NHEJ that results in S-S junctions with an increase in microhomology (158–160).

CSR is greatly impaired by defects in several different proteins that form IRIF at sites of DSBs following exposure to IR. ATM is required for the efficient joining of distal switch regions (161). Moreover, ATM mutations are associated with an immunodeficiency characterized by low serum levels of switched antibodies in both mice and humans (162, 163). H2AX regulates chromatin remodelling that facilitates switch region synapsis and recruits other DNA repair factors such as 53BP1 (157, 161, 164). Deletion of H2AX in mice resulted in decreased IGHG1 expression (157). 53BP1 is required for effective CSR in mice and was found to regulate the choice between NHEJ and alt-NHEJ during CSR by protecting DNA ends from resection (165–168). Moreover, CtIP was found to promote alt-NHEJ during CSR through end resection (169).

Deficiency in other proteins involved in the DSB signaling cascade, including the MRN complex (170), as well as the RNF8 and RNF168 Ub ligases (171–173), also resulted in impaired CSR.

The results of this study demonstrate that *BRCA1* haploinsufficient LCLs display reduced CSR from IGHM/IGHD to IGHG1. Since *BRCA1* is activated and recruited to IRIF in the context of the DSB signalling cascade, it is reasonable to speculate that *BRCA1* may act in a similar way for DSBs generated during CSR. Moreover, the well established role of *BRCA1* in facilitating CtIP-mediated DNA end resection (69) suggests that *BRCA1* may facilitate this process during the alt-NHEJ process of CSR. This theory is further supported by another study demonstrating that AID-dependent DNA lesion repair during the process of gene conversion in chicken B cells, was regulated by *BRCA1* (174).

Interestingly however, *BRCA1* did not co-localize with H2AX at the IgH locus in murine lymphocytes stimulated to differentiate (157). It is also known that CtIP is not phosphorylated during the G1-phase of the cell cycle. CtIP mediates DNA-end resection during the G1-phase without *BRCA1* association (69). Therefore, if as proposed, CSR occurs during the G1-phase of the cell cycle, then other possibilities must be considered. As described above, *BRCA1* has been implicated in regulating chromatin structure and physically associates with several chromatin remodelling proteins (111, 113, 114). *BRCA1* was found to induce large-scale chromatin de-condensation (115), and conversely, to maintain heterochromatin silencing through ubiquitination of histone H2AX (50). The IgH locus must be actively transcribed to undergo CSR, therefore it is possible that *BRCA1* may regulate chromatin structure in the IgH locus, generating an

open chromatin structure for active transcription as required for targeting AID and other CSR machinery to S regions. To determine whether BRCA1 mediates CSR prior to AID-induced generation of DSBs, it would be interesting to measure germline and productive transcript levels using haploinsufficient LCLs or a *BRCA1* knockdown model. A decrease in germline transcript levels in *BRCA1* deficient cells would indicate that BRCA1 is required for transcription through the IgH locus. Development of the blood-based assay will also provide further insight into the role of BRCA1 in CSR. The Ig proteins were easily detected on primary B cells using FC and will therefore be used in the next stages of assay development. If initial observations of increased expression of IGHM/IGHD and decreased expression of IGHG1 on *BRCA1* carrier LCLs are validated in B cells from fresh blood, this would represent a novel finding and suggest yet another role for BRCA1 in mediating CSR in human B cells.

Several other protein targets identified by SRM-MS and FC analysis of LCLs also function in B cell development and were downregulated in *BRCA1* carriers. Forkhead box protein P1 (FOXP1) is an essential transcription factor during early B cell development, controlling the pro-B to pre-B cell transition in the bone marrow. FOXP1 regulates the expression of the Rag1 and Rag2 enzymes that initiate V(D)J recombination in the IgH locus (175, 176). Recent studies have also implicated FOXP1 in mature B cell development. Ectopic expression of hFOXP1 in mice led to a decrease in germinal centre B cells as well as an increase in naive and marginal zone B cells. This suggests that FOXP1 serves a transcriptional regulatory function in naive B cells. Moreover, the aberrant expression of FOXP1 in mouse B cells led to a significant decrease in transcription through the C_{γ1} exon and switching to IGHG1 isotype (177). The SRY-

related HMG-box (SOX4) is another essential transcription factor required for pro-B cell survival and the transition of pro-B to pre-B cells (178, 179), however the role of SOX4 in mature B cell development is currently unknown. Lastly, much less is known about the SLAMF8 protein, although it expressed on a small subset of mature B cells and may play a role in B cell development (180, 181). Taken together, these results demonstrate that *BRCA1* haploinsufficiency in LCL cell lines results in altered expression of several proteins involved in B cell development. It is therefore possible to speculate that loss of one *BRCA1* allele may result in impaired B cell lineage differentiation. Support for this theory also comes from several studies implicating BRCA1 in cellular differentiation. As previously noted above, numerous studies have demonstrated the effects of *BRCA1* haploinsufficiency in breast epithelial cell development, leading to impaired differentiation and expanded populations of certain epithelial lineage subsets in *BRCA1* carriers (17–19). Interestingly, *BRCA1* deficiency also impaired differentiation of lymphocyte lineage cells. *BRCA1* knockout in murine T cells was associated with a severe decrease in the number of mature thymocytes compared to wild-type mice (182). The defects in T cell lineage development associated with loss of *BRCA1* expression makes it reasonable to speculate that *BRCA1* may function in the development of other lymphocytes as well.

4.3 Fresh blood assay development and future directions

Following the selection of mRNA and protein targets using LCLs, the first step in the development of a blood-based assay was to develop the methodology to identify the desired cell population within a heterogeneous blood sample. Immunophenotyping

successfully determined that LCLs express the pan B cell marker CD20. This allowed for the selection of appropriate protocols to isolate the CD20⁺ population from fresh blood samples either by using magnetic beads or electronic gating, depending on the type of analysis.

Once this methodology was established, the next step in assay development was to determine whether the mRNA and protein targets identified using LCLs were detectable in CD20⁺ B cells isolated from fresh blood samples. The blood samples were received and processed approximately 24 h after blood draw and sample degradation was evident. Cell yield is known to decrease by up to 40% even 8 h after blood draw. Given that B cells comprise only 5-15% of the lymphocyte population, this is particularly problematic for qRT-PCR and may result in insufficient amounts RNA to facilitate qRT-PCR analysis. In addition, bioanalyzer results indicated that many sample RIN values were below 7. Poor RNA quality may be attributed to low cell numbers as well as sample degradation. It was determined that transcript expression levels are different in blood samples processed immediately after blood draw compared to 24 or 48 h later. Unknown is whether or not this might impact on the ability to detect a difference in target expression between carriers and controls. An additional concern is that sample degradation may upregulate cell death signalling pathways that mask the detection of the targets of interest and whether target expression is in fact attributed to *BRCA1* haploinsufficiency and not the cell death signalling response. Blood collection tubes with RNA preservative reagents are not a feasible option as the preservatives degrade surface proteins, precluding the ability to isolate the CD20⁺ cell fraction. As an alternative, the primary B cells were short-term cultured in attempt to restore RNA quality, though

without stimulation to differentiate and proliferate, short-term culture did not restore RNA quality as determined by bioanalyzer analysis. Nonetheless, four mRNA targets, *CXCR3*, *GLDC*, *IFIT1*, and *TBX21*, were detectable in the fresh blood samples identifying them for further development of a qRT-PCR-based assay. The development of a FC-based assay, however, is the best approach moving forward. Moreover, FC analysis does not require magnetic enhancement of the B cell population, making the procedure more easily implementable in a clinical setting. Four proteins, IGHM, IGHD, CD24 and CXCR3, all had high expression on CD20⁺ B cells, identifying them as having potential for further development of a FC-based functional assay. The next step in assay development will be to test these protein targets in a larger patient cohort.

4.4 Significance and conclusions

Given the pleiotropic nature of BRCA1 function, it is a significant challenge to develop a comprehensive functional assay to identify pathogenic mutations. Many assays are limited to specific domains of BRCA1 and assess only certain functions ascribed to the protein. Given that the precise tumour suppressor function of BRCA1 remains unknown, it is difficult to extrapolate these assay results to cancer risk for the patient. Other assays that are not domain specific are extremely timely and technically complex, making them unsuitable for implementation in a clinical setting. This study has worked towards the development of a novel functional assay by testing mRNA and protein expression known to be abrogated in LCLs derived from *BRCA1* mutation carriers in B cells isolated from fresh blood samples. The results identified four proteins for further development of a FC-based functional assay.

There would be several advantages to this simple peripheral blood-based assay as it would assess the biological effects of *BRCA1* mutations irrespective of their location within the protein. The assay could also be used to classify other previously reported VUS as either pathogenic or neutral. Moreover, the assay would not require pre-treatment of the blood cells with IR to induce the DDR and could be easily standardized and reproduced within a clinical setting. Apart from the clinical significance of a quick and accurate functional tool to identify *BRCA1* mutation carriers, the targets themselves would also provide additional information about the biological effects of haploinsufficiency of this protein.

In conclusion, this study has worked towards the development of a novel functional assay to identify haploinsufficient *BRCA1* mutation carriers that would help clinicians in prediction and risk assessment. The hope is that this assay can be further developed and eventually implemented in a clinical setting to facilitate easier and earlier identification of carriers. This would help target these patients for increased surveillance or preventative treatments such as prophylactic mastectomies, ultimately saving health care costs and personal anguish of unnecessary procedures and potentially lives.

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Appendix A

Research Ethics Board Approval



QUEEN'S UNIVERSITY HEALTH SCIENCES AND AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD ANNUAL RENEWAL

Queen's University, in accordance with the "Tri-Council Policy Statement 2, 2010" prepared by the Interagency Advisory Panel on Research Ethics for the Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada and Social Sciences and Humanities Research Council of Canada requires that research projects involving human participants be reviewed annually to determine their acceptability on ethical grounds.

A Research Ethics Board composed of:

- Dr. A.F. Clark**, Emeritus Professor, Department of Biomedical and Molecular Sciences, Queen's University (Chair)
- Dr. H. Abdollah**, Professor, Department of Medicine, Queen's University
- Dr. C. Cline**, Assistant Professor, Department of Medicine, Director, Office of Bioethics, Queen's University, Clinical Ethicist, Kingston General Hospital
- Dr. R. Brisson**, Professor, Department of Emergency Medicine, Queen's University
- Dr. M. Evans**, Community Member
- Ms. J. Hudacin**, Community Member
- Dr. B. Kisilevsky**, Professor, School of Nursing, Departments of Psychology and Obstetrics and Gynaecology, Queen's University
- Dr. J. MacKenzie**, Pediatric Geneticist, Department of Paediatrics, Queen's University
- Mr. D. McNaughton**, Community Member
- Ms. P. Newman**, Pharmacist, Clinical Care Specialist and Clinical Lead, Quality and Safety, Pharmacy Services, Kingston General Hospital
- Ms. S. Rohland**, Privacy Officer, ICES-Queen's Health Services Research Facility, Research Associate, Division of Cancer Care and Epidemiology, Queen's Cancer Research Institute
- Dr. A. Singh**, Professor, Department of Psychiatry, Queen's University
- Ms. K. Weisbaum**, LL.B. and Adjunct Instructor, Department of Family Medicine (Bioethics)

has reviewed the request for renewal of Research Ethics Board approval for the project **Development of a Functional Assay for Detection of BRCA1 Carriers** as proposed by **Dr. Scott Davey** of the **Cancer Research Institute**, at **Queen's University**. The approval is renewed for one year, effective **September 16, 2013**. If there are any further amendments or changes to the protocol affecting the participants in this study, it is the responsibility of the principal investigator to notify the Research Ethics Board. Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other adverse events must be reported within 15 days after becoming aware of the information.

Albert F. Clark.

Date: September 17, 2013

Chair, Health Sciences Research Ethics Board

Renewal 1[] Renewal 2 [] Extension [X] Code# PATH-115-10 Romeo file# 6005745

Appendix B

SRM-MS Data

| Protein | Peptide | Fragment change | Fold change | Mean peak area | | CV | |
|---------|---------------------------|-----------------|-------------|----------------|------------|---------|---------|
| | | | | Control | Carrier | Control | Carrier |
| BCR | SVGDIQEELER | 2y8 | -3.11 | 40375.37 | 12984.09 | 0.14 | 0.31 |
| | | 2y9 | -2.79 | 112741.40 | 40351.18 | 0.06 | 0.21 |
| | | 2b10 | -3.24 | 31274.73 | 9640.32 | 0.21 | 0.36 |
| | VQQWSHQQR | 2y7 | -1.29 | 123582.43 | 95456.76 | 0.06 | 0.13 |
| | | 2b7 | -1.37 | 49806.54 | 36300.92 | 0.05 | 0.15 |
| | | 2y5 | -1.35 | 44448.17 | 32822.32 | 0.06 | 0.13 |
| BRCA1 | FSQLV EELLK | 2y9 | -1.08 | 18563.04 | 17263.75 | 0.21 | 0.27 |
| | | 2b8 | 1.26 | 9372.46 | 11812.40 | 0.34 | 0.38 |
| | | 2b7 | 1.18 | 31321.74 | 37086.06 | 0.31 | 0.22 |
| | LGV LQPEVYK | 2y8 | -1.21 | 201008.26 | 165959.46 | 0.08 | 0.08 |
| | | 2y9 | -1.23 | 576997.27 | 468144.08 | 0.07 | 0.08 |
| | | 3b7 | -1.20 | 21969.98 | 18379.52 | 0.13 | 0.15 |
| | DLVYV LAEENK | 2y7 | -1.12 | 63505.63 | 56836.95 | 0.07 | 0.12 |
| | | 2y8 | -1.09 | 149963.44 | 137406.00 | 0.06 | 0.09 |
| | | 2y9 | -1.00 | 166804.65 | 166639.51 | 0.09 | 0.17 |
| CSR P2 | LGIKPESVQPHRPTTNPNTSK | 3y9 | -1.01 | 491710.06 | 485140.58 | 0.02 | 0.10 |
| | | 3y10 | -1.08 | 31759.58 | 29516.32 | 0.15 | 0.18 |
| | | 3b9 | 1.06 | 57428.04 | 61022.98 | 0.08 | 0.20 |
| | FAQKYGGA EK | 2y8 | 1.05 | 89251.30 | 93417.62 | 0.11 | 0.13 |
| | | 2b6 | 1.02 | 351268.36 | 359967.97 | 0.06 | 0.11 |
| | | 2y6 | 1.05 | 25689.72 | 26918.97 | 0.12 | 0.24 |
| | SLESTTLTEK | 2b9 | 1.01 | 96309.96 | 97414.12 | 0.11 | 0.10 |
| | | 2b8 | -1.04 | 85270.23 | 81816.24 | 0.10 | 0.16 |
| DUSP23 | FVQIVDEANAR | 2y7 | 1.28 | 182800.10 | 233233.84 | 0.07 | 0.11 |
| | | 2b8 | 1.52 | 15795.90 | 24070.58 | 0.22 | 0.21 |
| ENPP2 | TEFLSNYL TNVDDITLVP GTLGR | 3y10 | -1.35 | 49852.24 | 36872.99 | 0.17 | 0.20 |
| | | 3y11 | -1.22 | 133404.31 | 109000.26 | 0.06 | 0.14 |
| | LHYANNRR | 2y7 | -1.28 | 161597.48 | 126123.83 | 0.10 | 0.16 |
| | | 2b6 | -1.41 | 1102715.20 | 782966.02 | 0.09 | 0.12 |
| | VRDIEHLTSLDFFR | 2y8 | 1.01 | 149923.54 | 151179.46 | 0.13 | 0.17 |
| | | 2b10 | 1.09 | 44553.80 | 48747.17 | 0.11 | 0.19 |
| FCGRT | LFLEAFKALGGK | 2b9 | -1.12 | 69492.52 | 61970.28 | 0.09 | 0.16 |
| | | 2y11 | -1.15 | 394861.98 | 344443.41 | 0.05 | 0.06 |
| FYN | LIEDNEY TAR | 2y6 | 1.06 | 551809.17 | 585104.68 | 0.05 | 0.11 |
| | | 2y7 | 1.07 | 948998.71 | 1018936.78 | 0.04 | 0.09 |
| | | 2y8 | 1.12 | 59206.95 | 66412.38 | 0.19 | 0.22 |
| | | 2b8 | 1.08 | 75040.89 | 81271.87 | 0.06 | 0.15 |
| GLDC | AGHQLQHDLFFDTLK | 2y9 | -1.14 | 9618.34 | 8411.66 | 0.35 | 0.27 |
| | | 2b10 | -1.43 | 14961.78 | 10491.43 | 0.13 | 0.37 |
| | | 2b9 | -1.53 | 32728.76 | 21414.63 | 0.23 | 0.12 |

| | | | | | | | |
|---------|-------------------------|------|-------|------------|------------|------|------|
| | | 2y7 | 1.05 | 114190.15 | 119599.60 | 0.09 | 0.15 |
| | QEIADIEEGR | 2y8 | 1.01 | 39562.75 | 39806.78 | 0.14 | 0.12 |
| | | 2b9 | -1.12 | 32070.00 | 28592.23 | 0.12 | 0.09 |
| | | 2y6 | -1.13 | 206432.38 | 182210.16 | 0.11 | 0.13 |
| | ATADDELSFK | 2y7 | -1.18 | 471084.19 | 399706.49 | 0.08 | 0.09 |
| | | 2y8 | -1.17 | 701177.68 | 599922.71 | 0.09 | 0.10 |
| | | 2y8 | -1.17 | 141038.42 | 120227.61 | 0.10 | 0.14 |
| GRB2 | ESESAPGDFSLSVK | 2y9 | -1.14 | 2033755.72 | 1776994.13 | 0.06 | 0.10 |
| | | 2y10 | -1.17 | 179022.75 | 153463.12 | 0.08 | 0.12 |
| | | 2y11 | -1.11 | 190120.93 | 170864.82 | 0.11 | 0.13 |
| | FNSLNELVDYHR | 2y7 | -1.04 | 51707.88 | 49886.97 | 0.19 | 0.19 |
| | | 2y8 | -1.09 | 118065.58 | 108680.09 | 0.11 | 0.19 |
| | | 3y7 | -1.15 | 46919.51 | 40798.75 | 0.11 | 0.18 |
| | | 2y7 | -1.23 | 21163.64 | 17231.86 | 0.13 | 0.21 |
| | TRPPSVQVAK | 2y9 | -1.52 | 226619.76 | 148996.47 | 0.06 | 0.09 |
| HLA-DMB | | 2y6 | -1.57 | 51979.04 | 33184.19 | 0.10 | 0.18 |
| | | 3y8 | -1.06 | 25768.04 | 24271.82 | 0.19 | 0.19 |
| | VSVSAVTLGLGLIIFSLGVISWR | 3y9 | 1.02 | 660385.04 | 674183.93 | 0.07 | 0.22 |
| | | 3b12 | 1.04 | 125472.68 | 130483.85 | 0.10 | 0.21 |
| | | 2y7 | 1.63 | 125032.14 | 203834.08 | 0.06 | 0.13 |
| | VLDQIEFLDTK | 2y8 | 1.67 | 50067.07 | 83719.01 | 0.13 | 0.12 |
| | | 2y9 | 1.83 | 154484.79 | 283349.52 | 0.10 | 0.08 |
| | | 2y10 | 1.86 | 13668.81 | 25393.89 | 0.17 | 0.11 |
| | | 2y8 | -1.10 | 104408.66 | 95226.77 | 0.06 | 0.15 |
| | YSVGIHNLAYVK | 2y9 | -1.10 | 76888.95 | 69632.12 | 0.05 | 0.14 |
| IFIT1 | | 2b11 | -1.05 | 115966.88 | 110206.05 | 0.04 | 0.20 |
| | | 2b9 | -1.13 | 85223.85 | 75328.69 | 0.08 | 0.13 |
| | LQDEGQEAEGEK | 2y8 | -1.42 | 32361.98 | 22776.44 | 0.13 | 0.25 |
| | | 2y10 | -1.54 | 219038.76 | 142649.45 | 0.10 | 0.16 |
| | LAADFENSVR | 2y7 | -1.13 | 3295493.32 | 2923638.26 | 0.06 | 0.07 |
| | | 2y8 | -1.12 | 5025045.47 | 4467057.91 | 0.06 | 0.07 |
| | | 2b9 | -1.26 | 26254.69 | 20879.61 | 0.16 | 0.15 |
| | NTGRGGEEK | 2y7 | -1.06 | 561618.98 | 529795.49 | 0.07 | 0.09 |
| | | 2y8 | -1.10 | 289122.82 | 263802.49 | 0.08 | 0.16 |
| IGHD | | 2y7 | 4.23 | 61162.72 | 258843.65 | 0.12 | 0.11 |
| | VPTGGVEEGLLER | 2y9 | 4.25 | 28790.13 | 122478.74 | 0.12 | 0.12 |
| | | 2y10 | 4.22 | 94584.25 | 399062.47 | 0.19 | 0.11 |
| | | 2y11 | 4.29 | 95149.56 | 408139.02 | 0.03 | 0.09 |
| | GPSVFPLAPSSK | 2y7 | -4.02 | 1758992.63 | 437755.20 | 0.07 | 0.19 |
| | | 2y8 | -4.04 | 1056803.63 | 261320.72 | 0.10 | 0.27 |
| | | 2y10 | -3.36 | 165023.71 | 49111.01 | 0.13 | 0.19 |
| IGHG1 | | 2y5 | -4.69 | 390544.78 | 83248.40 | 0.11 | 0.35 |
| | ALPAPIEK | 2y6 | -4.57 | 2078303.30 | 455002.03 | 0.06 | 0.23 |
| | | 2y4 | -5.06 | 2376245.64 | 469783.40 | 0.07 | 0.34 |
| | LWEGSTSRER | 2y6 | -1.03 | 48613.53 | 47408.65 | 0.07 | 0.13 |
| IL18BP | | 2y8 | -1.11 | 33135.38 | 29857.44 | 0.18 | 0.22 |
| | | 3y6 | -1.04 | 79560.58 | 76730.37 | 0.08 | 0.11 |
| | ATLPPTQEALPSSHSPQQQG | 3y8 | -1.13 | 58068.80 | 51233.19 | 0.11 | 0.16 |

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|------------|-------------------------|------|-----------|------------|------------|------|------|
| | | 3y9 | -1.24 | 13850.11 | 11171.71 | 0.12 | 0.31 |
| | | 3y11 | -1.14 | 129846.87 | 113593.58 | 0.06 | 0.10 |
| LFNG | ARLDLLETWISR | 2b9 | 1.15 | 84558.52 | 97616.08 | 0.09 | 0.14 |
| | | 2b8 | 1.17 | 141784.79 | 166553.92 | 0.09 | 0.15 |
| | LDLLETWISR | 2y10 | 1.21 | 276047.62 | 334677.04 | 0.06 | 0.07 |
| | | 2b8 | 1.19 | 24848.34 | 29456.45 | 0.14 | 0.19 |
| | LDLLETWISRHK | 2y7 | 1.05 | 247024.77 | 259781.04 | 0.12 | 0.25 |
| | | 2y9 | 1.01 | 7063741.94 | 7136934.58 | 0.04 | 0.09 |
| | | 2b9 | 1.00 | 50496.43 | 50581.35 | 0.06 | 0.17 |
| MMP7 | WTSKVVTYR | 2y6 | -1.09 | 351395.92 | 323681.83 | 0.05 | 0.14 |
| | | 2y8 | -1.03 | 181540.48 | 176139.58 | 0.07 | 0.07 |
| | | 2y5 | -1.05 | 227787.54 | 216911.86 | 0.05 | 0.12 |
| | VVTYRIVSYTR | 2y7 | -1.15 | 117106.65 | 102070.20 | 0.07 | 0.20 |
| | | 2y8 | 1.02 | 360459.23 | 367232.39 | 0.07 | 0.12 |
| | | 2b7 | 1.12 | 18223.00 | 20416.03 | 0.23 | 0.19 |
| | IVSYTRDLPHTVDR | 2y10 | 1.07 | 14044.85 | 15087.85 | 0.32 | 0.15 |
| | | 2b9 | 1.16 | 255731.49 | 297382.98 | 0.17 | 0.29 |
| | | 2y8 | -1.04 | 167152.70 | 161427.94 | 0.15 | 0.22 |
| MX1 | ALGVEQDLALPAIAVIGDQSSGK | 3y9 | 1.40 | 200599.26 | 281103.53 | 0.06 | 0.15 |
| | | 3y10 | 1.40 | 180576.67 | 251938.89 | 0.05 | 0.15 |
| | | 3y11 | 1.45 | 38297.06 | 55601.10 | 0.11 | 0.21 |
| | | 3y13 | 1.39 | 43518.19 | 60646.79 | 0.08 | 0.14 |
| | | 2y9 | 1.50 | 774351.06 | 1161673.90 | 0.05 | 0.13 |
| | SSVLEALSGVALPR | 2y10 | 1.48 | 699924.39 | 1036232.22 | 0.03 | 0.13 |
| | | 2y11 | 1.49 | 298375.27 | 445053.72 | 0.05 | 0.15 |
| | | 2b11 | 1.58 | 19417.04 | 30664.14 | 0.12 | 0.12 |
| | DVPDLTLIDLPGITR | 2y9 | 1.34 | 734023.35 | 986994.83 | 0.06 | 0.13 |
| | | 2y10 | 1.38 | 753350.77 | 1037139.30 | 0.05 | 0.14 |
| | | 2y11 | 1.37 | 164966.49 | 226316.00 | 0.04 | 0.15 |
| | | 2b9 | 1.11 | 27675.02 | 30674.05 | 0.15 | 0.15 |
| | VAVGNQPADIGYK | 2y8 | 1.38 | 161861.29 | 223648.75 | 0.09 | 0.11 |
| | | 2y9 | 1.30 | 334387.66 | 434683.59 | 0.05 | 0.09 |
| | | 2y10 | 1.38 | 1077186.09 | 1484877.74 | 0.06 | 0.08 |
| 2y11 | | 1.38 | 224289.95 | 308717.96 | 0.06 | 0.10 | |
| 2y12 | | 1.30 | 36875.90 | 47974.70 | 0.16 | 0.11 | |
| NFEEFFNLHR | 2y6 | 1.26 | 177561.11 | 222981.77 | 0.04 | 0.12 | |
| | 2y7 | 1.27 | 195031.37 | 246820.88 | 0.09 | 0.14 | |
| | 2y8 | 1.23 | 226266.14 | 277918.72 | 0.07 | 0.13 | |
| | 3y6 | 1.25 | 60726.96 | 75915.23 | 0.09 | 0.13 | |
| NFKB1 | LEPVVSDAIYDSK | 2y8 | 1.19 | 31304.28 | 37240.00 | 0.31 | 0.21 |
| | | 2y9 | 1.07 | 38825.97 | 41500.56 | 0.12 | 0.18 |
| | | 2y10 | -1.00 | 5574.19 | 5567.83 | 0.28 | 0.18 |
| | EESAGVQDNLFLEK | 2y11 | 1.33 | 34650.27 | 46012.16 | 0.33 | 0.32 |
| | | 2y8 | 1.08 | 59981.78 | 64937.49 | 0.16 | 0.17 |
| | | 2b11 | -1.21 | 30828.86 | 25508.28 | 0.20 | 0.33 |
| | | 2b10 | -1.31 | 102964.06 | 78518.36 | 0.06 | 0.14 |
| | | 2b9 | -1.13 | 3226.88 | 2856.42 | 0.39 | 0.30 |
| PLA2G4A | DVPVVAILGSGGGFR | 2y9 | 1.24 | 123180.20 | 153119.22 | 0.05 | 0.11 |

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|----------|----------------------|------|-------|------------|------------|------|------|
| | | 2y10 | 1.13 | 237171.33 | 268097.24 | 0.02 | 0.11 |
| | | 2y13 | 1.22 | 19040.16 | 23181.00 | 0.10 | 0.12 |
| | NVSHNPLLLLTPQK | 2y9 | 1.17 | 59305.08 | 69646.83 | 0.06 | 0.13 |
| | | 2y10 | 1.16 | 110962.44 | 128471.32 | 0.06 | 0.19 |
| PXDN | SPNDLLALFR | 2y7 | 1.18 | 51901.00 | 61383.55 | 0.13 | 0.23 |
| | | 2y8 | 1.10 | 119823.62 | 131834.55 | 0.16 | 0.12 |
| | | 2y9 | 1.10 | 7693.70 | 8438.68 | 0.23 | 0.27 |
| | | 3y7 | -1.33 | 127804.31 | 96023.71 | 0.07 | 0.15 |
| | QVELALWDTAGQEDYDR | 3y8 | -1.44 | 40657.99 | 28300.54 | 0.21 | 0.21 |
| | | 3y9 | -1.57 | 23858.07 | 15170.52 | 0.14 | 0.25 |
| RHOA | QEPVKPEEGR | 2b9 | 1.14 | 107090.90 | 121764.18 | 0.08 | 0.12 |
| | | 2b7 | 1.07 | 53110.93 | 57051.46 | 0.16 | 0.12 |
| | | 2b6 | -1.07 | 38401.71 | 35980.07 | 0.17 | 0.15 |
| | | 2y4 | -1.33 | 804532.23 | 605312.39 | 0.04 | 0.12 |
| | AALQARR | 2y5 | -1.32 | 283164.93 | 214792.61 | 0.06 | 0.14 |
| | | 2y6 | -1.34 | 29540.96 | 21996.85 | 0.15 | 0.22 |
| | | 2y8 | 1.14 | 16724.93 | 18984.78 | 0.31 | 0.22 |
| | LAAAVSNFGYDLYR | 2b12 | 1.16 | 34020.74 | 39344.71 | 0.11 | 0.30 |
| SERPINF1 | | 2y7 | -2.70 | 29467.16 | 10906.32 | 0.12 | 0.28 |
| | DTDTGALLFIGK | 2y9 | -1.41 | 35463.42 | 25145.83 | 0.12 | 0.27 |
| | | 2y11 | -3.39 | 30878.38 | 9118.37 | 0.16 | 0.36 |
| | | 2y9 | -1.36 | 23469.14 | 17274.97 | 0.18 | 0.17 |
| | KSAGGSGGAGGGEQNGQEEK | 2y10 | -1.96 | 97159.55 | 49553.01 | 0.09 | 0.19 |
| | | 2y12 | -1.98 | 39112.23 | 19790.23 | 0.06 | 0.22 |
| | | 2y11 | -2.02 | 23761.06 | 11765.76 | 0.26 | 0.33 |
| SMAD2 | SAGGSGGAGGGEQNGQEEK | 2b15 | -2.57 | 27733.58 | 10774.56 | 0.06 | 0.20 |
| | | 2b14 | -1.64 | 12411.82 | 7571.94 | 0.13 | 0.36 |
| | | 2y9 | -5.58 | 173368.59 | 31071.46 | 0.19 | 0.34 |
| | LWRWPDLSHSHHELK | 2y10 | -5.88 | 134150.79 | 22833.77 | 0.17 | 0.39 |
| | | 2b9 | -4.45 | 60488.05 | 13590.80 | 0.12 | 0.22 |
| | | 2y8 | -1.11 | 210812.86 | 190058.79 | 0.05 | 0.13 |
| | TEDSSVPETPDNER | 2y9 | -1.11 | 52438.49 | 47229.65 | 0.07 | 0.11 |
| | | 2b10 | 1.02 | 18484.33 | 18795.17 | 0.11 | 0.14 |
| | | 3y9 | 1.02 | 10873.82 | 11064.70 | 0.05 | 0.15 |
| SMARCAD1 | RNDDISELEDLSELEDLK | 3y10 | -1.05 | 6201.68 | 5893.57 | 0.13 | 0.18 |
| | | 3b9 | -1.03 | 162844.51 | 158415.17 | 0.03 | 0.15 |
| | | 2y7 | -3.82 | 244333.54 | 63907.86 | 0.04 | 0.14 |
| | VGLNWLALVHK | 2b8 | -2.77 | 147611.74 | 53336.50 | 0.11 | 0.20 |
| | | 2b7 | -2.95 | 193953.86 | 65769.34 | 0.07 | 0.16 |
| CXCR3 | ESRVDVAK | 2y6 | -1.10 | 245254.38 | 223786.91 | 0.04 | 0.09 |
| | | 2b6 | -1.04 | 116864.39 | 111957.79 | 0.05 | 0.08 |
| | | 2y5 | -1.29 | 716732.49 | 554491.68 | 0.04 | 0.13 |
| | ELVALLVR | 2y6 | -1.26 | 4464025.55 | 3546066.40 | 0.03 | 0.10 |
| | | 2b7 | -1.28 | 890392.05 | 697966.70 | 0.03 | 0.11 |
| CYP1B1 | | 2b5 | -1.30 | 180431.50 | 138358.64 | 0.13 | 0.16 |
| | YSHDDPEFR | 2y7 | 1.36 | 59202.73 | 80330.84 | 0.10 | 0.14 |
| | | 2y8 | 1.37 | 52331.48 | 71680.83 | 0.15 | 0.18 |
| | TVFREFEQLNR | 2y8 | 1.17 | 11333.21 | 13225.19 | 0.10 | 0.10 |

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|--------|---------------------|-----------|-----------|------------|------------|------|------|
| | | 3y7 | 1.13 | 26992.15 | 30487.02 | 0.07 | 0.23 |
| ETV7 | LLWDYVYQLLDTRYEPYIK | 4y8 | 1.09 | 704039.27 | 770637.74 | 0.11 | 0.14 |
| | | 4y9 | 1.12 | 37322.08 | 41653.36 | 0.16 | 0.16 |
| | | 4b9 | 1.09 | 1566105.68 | 1708688.06 | 0.11 | 0.14 |
| | | 4b8 | 1.14 | 49528.58 | 56287.81 | 0.15 | 0.20 |
| | YEPYIKWEDK | 2y7 | 1.05 | 25794.57 | 26955.50 | 0.16 | 0.15 |
| | | 2b8 | -1.07 | 28766.80 | 26810.66 | 0.18 | 0.32 |
| | | 2b7 | -1.00 | 31851.45 | 31825.03 | 0.19 | 0.13 |
| FAM79B | IDHWNNEKER | 2y8 | 1.08 | 171644.37 | 185866.50 | 0.07 | 0.16 |
| | | 2y9 | 1.03 | 248196.88 | 255158.19 | 0.09 | 0.13 |
| | | 2b7 | -1.05 | 114168.60 | 108393.09 | 0.06 | 0.07 |
| | | 2b6 | -1.01 | 117526.14 | 116710.81 | 0.10 | 0.13 |
| | ILLVTDK | 2b6 | -1.71 | 474570.31 | 276892.83 | 0.14 | 0.38 |
| | | 2b5 | -1.37 | 1400841.84 | 1020159.56 | 0.08 | 0.16 |
| | | 2y4 | -1.30 | 121817.52 | 93635.17 | 0.13 | 0.19 |
| | IPLSAVYR | 2b4 | -1.35 | 508885.78 | 377098.25 | 0.08 | 0.15 |
| | | 2y6 | 1.13 | 85219.55 | 96496.27 | 0.15 | 0.19 |
| | | 2y7 | 1.34 | 46989.07 | 62770.25 | 0.20 | 0.15 |
| | RQGEGLR | 2y5 | 1.06 | 2100085.35 | 2218127.57 | 0.04 | 0.16 |
| | | 2b5 | 1.09 | 86194.29 | 93580.26 | 0.07 | 0.15 |
| | | 2b4 | 1.07 | 62368.94 | 66834.20 | 0.09 | 0.18 |
| FOXP1 | AAPQPLNLVSSVTLK | 2y9 | -1.08 | 1428338.19 | 1319408.62 | 0.07 | 0.12 |
| | | 2b11 | 1.03 | 20702.95 | 21326.84 | 0.16 | 0.19 |
| | | 2b10 | -1.01 | 37840.96 | 37491.60 | 0.06 | 0.21 |
| | YNVPISSADIAQNQEFYK | 3y6 | -1.22 | 72112.90 | 58988.25 | 0.06 | 0.10 |
| | | 4y6 | -1.23 | 135782.00 | 110127.24 | 0.10 | 0.10 |
| | NAEVRPPFTYASLIR | 2y10 | -1.88 | 10109.01 | 5385.83 | 0.09 | 0.24 |
| | | 2b9 | -1.87 | 16421.65 | 8789.88 | 0.06 | 0.30 |
| 2y8 | -2.26 | 139337.12 | 61657.82 | 0.10 | 0.20 | | |
| IFI44L | FYGHR | 2y3 | -1.08 | 145489.91 | 134558.18 | 0.14 | 0.26 |
| | | 2b3 | -2.03 | 39909.40 | 19679.16 | 0.29 | 0.16 |
| | DNLDDIK | 2y4 | 1.15 | 353809.55 | 408185.84 | 0.09 | 0.18 |
| | | 2b4 | 1.06 | 41583.13 | 43951.20 | 0.21 | 0.39 |
| | DNLDDIKR | 2y5 | -1.01 | 133019.13 | 131808.63 | 0.07 | 0.12 |
| | | 2y6 | 1.08 | 35269.78 | 38133.49 | 0.14 | 0.14 |
| | | 2b5 | -1.05 | 165138.19 | 157510.99 | 0.07 | 0.14 |
| | | 2y4 | 1.17 | 60494.03 | 70768.07 | 0.09 | 0.15 |
| | | 2y9 | -1.88 | 10609.60 | 5641.58 | 0.16 | 0.20 |
| IFIT2 | LDNWPPSQNAIDPLR | 2y10 | -1.94 | 5729.74 | 2950.03 | 0.15 | 0.22 |
| | | 2y11 | -1.79 | 48345.54 | 27022.40 | 0.05 | 0.20 |
| | | 2y7 | -1.02 | 399692.26 | 392980.43 | 0.06 | 0.13 |
| | LNPDNQYLK | 2y8 | 1.03 | 212390.56 | 218873.45 | 0.07 | 0.12 |
| | | 2y5 | -1.33 | 52302.94 | 39286.11 | 0.19 | 0.30 |
| | VLLALKLHK | 2y5 | -1.30 | 106139.96 | 81902.20 | 0.08 | 0.16 |
| | | 2y6 | -1.28 | 167099.44 | 130822.95 | 0.08 | 0.14 |
| 2y7 | | -1.30 | 186196.53 | 143769.05 | 0.07 | 0.06 | |
| IFNA4 | HDFGFPEEEFDGHQFQK | 3y8 | 1.21 | 34406.16 | 41514.21 | 0.08 | 0.29 |
| | | 3b8 | 1.23 | 25412.72 | 31240.86 | 0.14 | 0.21 |

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|---------|------------------|------|--------|------------|------------|------|------|
| | KYFQR | 2y4 | -5.80 | 36688.18 | 6323.51 | 0.27 | 0.28 |
| | | 2b3 | -16.79 | 300849.85 | 17916.27 | 0.29 | 0.30 |
| | SLSFSTNLQK | 2y6 | 1.07 | 242468.20 | 259124.66 | 0.07 | 0.14 |
| | | 2b9 | 1.06 | 105380.80 | 111611.43 | 0.12 | 0.15 |
| IFNA5 | HDFGFPEEFDGNQFQK | 3y7 | -1.07 | 32161.34 | 30081.68 | 0.25 | 0.19 |
| | | 3y8 | -1.10 | 23390.74 | 21282.60 | 0.16 | 0.29 |
| | SFSLSANLQER | 2y6 | -1.03 | 396390.90 | 384172.17 | 0.05 | 0.07 |
| | | 2y8 | 1.03 | 49778.39 | 51501.75 | 0.14 | 0.22 |
| | | 2b11 | -1.06 | 243562.79 | 229212.99 | 0.08 | 0.10 |
| | SFSLSANLQERLR | 2b10 | 1.41 | 50776.45 | 71755.42 | 0.27 | 0.36 |
| | | 2b9 | -1.01 | 377085.92 | 374822.42 | 0.16 | 0.17 |
| IFNG | EESDRK | 2b5 | -1.33 | 32103.48 | 24173.44 | 0.19 | 0.15 |
| | | 2b4 | -1.38 | 48627.18 | 35279.17 | 0.32 | 0.26 |
| | | 2y3 | -1.37 | 109355.42 | 79859.07 | 0.09 | 0.25 |
| | LFKNFK | 2y4 | 1.13 | 290525.40 | 327023.34 | 0.04 | 0.13 |
| | | 2y5 | 1.15 | ##### | ##### | 0.03 | 0.09 |
| | | 2y7 | -1.14 | 37004.16 | 32446.62 | 0.16 | 0.18 |
| | NFKDDQSIQK | 2b8 | -1.05 | 23612.72 | 22401.18 | 0.23 | 0.10 |
| | | 2b7 | -1.09 | 73276.93 | 67311.18 | 0.08 | 0.15 |
| | | 2b6 | 1.01 | 94029.44 | 94541.71 | 0.10 | 0.15 |
| | | 2y7 | 1.15 | 102062.45 | 117268.85 | 0.14 | 0.18 |
| JAKMIP2 | LKLLQEIADLK | 2y9 | -1.19 | 185361.03 | 155359.60 | 0.06 | 0.08 |
| | | 2b9 | -1.03 | 54528.70 | 52707.23 | 0.05 | 0.18 |
| | | 2y8 | -1.16 | 35644.30 | 30677.64 | 0.16 | 0.15 |
| | SEHQSHQEAIK | 2y9 | -1.20 | 57033.81 | 47495.81 | 0.08 | 0.12 |
| | | 2b9 | -1.23 | 64010.55 | 51995.13 | 0.10 | 0.18 |
| | | 2b8 | -1.21 | 189195.53 | 156225.88 | 0.08 | 0.13 |
| | LEDRNTLLGDER | 2y10 | 1.08 | 92128.37 | 99744.40 | 0.10 | 0.13 |
| | | 2b10 | -1.07 | 36271.16 | 33836.94 | 0.17 | 0.27 |
| | | 2b9 | -1.14 | 60924.54 | 53668.57 | 0.13 | 0.13 |
| LAG3 | RADAGEYR | 2y5 | -1.35 | 557786.20 | 413387.01 | 0.03 | 0.09 |
| | | 2b6 | -1.06 | 35129.82 | 33015.24 | 0.17 | 0.22 |
| | | 2y4 | -1.34 | 677901.44 | 506700.32 | 0.05 | 0.09 |
| | ADAGEYRAAVHLR | 2y10 | -1.44 | 2323382.77 | 1612506.85 | 0.04 | 0.09 |
| | | 2y11 | -1.41 | 516239.36 | 364930.10 | 0.06 | 0.08 |
| MX2 | VAVDNQPRDIGLQIK | 2y10 | 1.01 | 40970.62 | 41181.64 | 0.07 | 0.15 |
| | | 2b11 | 1.04 | 97339.84 | 101395.56 | 0.07 | 0.15 |
| | | 2b9 | 1.06 | 10400.15 | 10982.45 | 0.27 | 0.31 |
| | | 2y6 | -1.10 | 175658.21 | 160186.70 | 0.09 | 0.11 |
| | NLTYPLKK | 2y7 | -1.04 | 4460386.39 | 4288502.26 | 0.04 | 0.08 |
| | | 2b7 | -1.15 | 128175.93 | 111512.40 | 0.14 | 0.12 |
| | | 2y5 | -1.18 | 64130.73 | 54390.47 | 0.12 | 0.23 |
| | EITFFQTHPYFR | 2y7 | -1.21 | 21589.35 | 17855.19 | 0.12 | 0.17 |
| | | 2y8 | -1.31 | 21246.11 | 16165.66 | 0.16 | 0.19 |
| | | 2b7 | -1.10 | 346543.87 | 315709.57 | 0.03 | 0.13 |
| PRLR | ILSLHPGQK | 2y6 | 1.21 | 601548.39 | 725344.69 | 0.06 | 0.10 |
| | | 2y8 | 1.20 | 532759.47 | 640491.53 | 0.07 | 0.09 |
| | | 2y5 | 1.21 | 763557.16 | 926191.17 | 0.06 | 0.09 |

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|----------|-----------------------|------|-------|------------|------------|------|------|
| | | 2y6 | -1.24 | 1274157.54 | 1025168.50 | 0.05 | 0.12 |
| | DGALSLLPK | 2y7 | -1.33 | 67080.01 | 50321.52 | 0.10 | 0.09 |
| | | 2y4 | -1.25 | 104684.47 | 83850.24 | 0.14 | 0.18 |
| SLC16A10 | FSPPKK | 2y4 | -1.34 | 88806.12 | 66428.07 | 0.11 | 0.13 |
| | | 2b4 | -1.42 | 193859.44 | 136326.94 | 0.07 | 0.04 |
| | | 2y3 | -1.40 | 1163700.21 | 830059.10 | 0.07 | 0.07 |
| | IFNFAIFK | 2b7 | 1.27 | 30876.50 | 39353.05 | 0.25 | 0.15 |
| | | 2y5 | 1.09 | 1396094.38 | 1522180.83 | 0.04 | 0.11 |
| | | 2b4 | 1.07 | 143317.28 | 153147.54 | 0.20 | 0.12 |
| SOX4 | YRPRK | 2b4 | 1.16 | 28465.98 | 32985.42 | 0.13 | 0.18 |
| | | 2b3 | 1.06 | 95345.08 | 100762.41 | 0.13 | 0.27 |
| | | 2y3 | 1.25 | 116339.67 | 145701.42 | 0.17 | 0.23 |
| | VKSGNANSSSSAAASSKPGEK | 2y12 | -1.13 | 67077.15 | 59341.57 | 0.06 | 0.21 |
| | | 2b13 | -1.08 | 502295.28 | 464478.30 | 0.03 | 0.14 |
| | | 3b13 | -1.02 | 43480.09 | 42451.54 | 0.09 | 0.12 |
| | VAGGAGGGVSKPHAK | 2y7 | -5.98 | 851984.41 | 142437.75 | 0.04 | 0.13 |
| | | 2y9 | -5.34 | 193439.40 | 36224.86 | 0.07 | 0.07 |
| SPI | DSEGRGSGDPGK | 2y10 | 1.04 | 305923.12 | 317720.49 | 0.07 | 0.10 |
| | | 2b10 | 1.08 | 257413.75 | 278742.66 | 0.05 | 0.21 |
| | | 2b9 | 1.24 | 159958.50 | 198902.25 | 0.09 | 0.20 |
| | VYGKTSHLR | 2b8 | 1.52 | 112471.71 | 171016.23 | 0.06 | 0.13 |
| | | 2b7 | 1.66 | 80986.66 | 134146.27 | 0.17 | 0.14 |
| TBX21 | EDYALPAGLEVSGK | 2y9 | 1.00 | 24771.50 | 24887.07 | 0.20 | 0.22 |
| | | 2y11 | 1.20 | 95211.51 | 114210.34 | 0.06 | 0.13 |
| | | 2b10 | 1.15 | 64652.01 | 74636.76 | 0.05 | 0.14 |
| | LRVALNNHLLWSK | 2y9 | 1.07 | 943616.33 | 1011030.61 | 0.06 | 0.15 |
| | | 2b10 | 1.11 | 237104.41 | 263292.17 | 0.06 | 0.15 |
| | | 2b9 | 1.10 | 107485.71 | 118052.28 | 0.08 | 0.12 |
| | | 2b8 | 1.07 | 97724.25 | 104784.39 | 0.08 | 0.14 |
| UBD | ILKPRR | 2y4 | -1.28 | 146607.12 | 114917.62 | 0.12 | 0.14 |
| | | 2b5 | -1.02 | 153457.83 | 149919.61 | 0.11 | 0.14 |
| | | 2b4 | -1.24 | 121385.91 | 97818.12 | 0.13 | 0.30 |
| | | 2y3 | -1.05 | 651551.11 | 623036.60 | 0.10 | 0.16 |
| | | 2y7 | -1.23 | 142677.98 | 116390.66 | 0.10 | 0.12 |
| | RSLSSYGIDK | 2y8 | -1.23 | 156044.67 | 126498.13 | 0.10 | 0.15 |
| | | 2b8 | -1.24 | 144554.88 | 116854.14 | 0.10 | 0.21 |
| | | 2b7 | -1.30 | 99923.15 | 77070.07 | 0.07 | 0.13 |
| | EKTIHLTLK | 2b7 | -1.52 | 29159.69 | 19139.42 | 0.16 | 0.18 |
| | | 2b6 | -1.40 | 166603.51 | 119309.10 | 0.09 | 0.09 |
| | | 2y5 | -1.40 | 34617.77 | 24776.37 | 0.14 | 0.15 |
| USP18 | GADEQR | 2y4 | -1.19 | 106474.13 | 89560.77 | 0.07 | 0.13 |
| | | 2y5 | -1.34 | 21665.87 | 16186.01 | 0.11 | 0.17 |
| | | 2y3 | -1.21 | 90139.49 | 74283.54 | 0.21 | 0.28 |
| | GADEQRR | 2y6 | -1.00 | 123660.56 | 123437.13 | 0.09 | 0.11 |
| | | 2b5 | -1.09 | 240010.32 | 219427.24 | 0.07 | 0.14 |
| | | 2y5 | -1.34 | 5894511.06 | 4396815.25 | 0.03 | 0.12 |
| | LWNLIK | 2b4 | -1.38 | 473128.88 | 343335.09 | 0.04 | 0.11 |
| | | 2b3 | -1.37 | 1726482.46 | 1261480.53 | 0.06 | 0.13 |

| | | | | | | | |
|--------|-------------------|------|-------|------------|------------|------|------|
| ZBED3 | ELAVEQGER | 2y7 | 1.04 | 198463.70 | 205576.76 | 0.06 | 0.14 |
| | | 2y8 | -1.06 | 17485.39 | 16550.99 | 0.18 | 0.13 |
| | | 2y5 | -1.01 | 227045.16 | 225098.50 | 0.06 | 0.12 |
| ZBTB38 | VFALAEYRTR | 2b8 | -1.17 | 69197.27 | 59168.32 | 0.11 | 0.19 |
| | | 2b7 | -1.21 | 55791.98 | 46243.07 | 0.06 | 0.10 |
| | KTANGGLKPSVYPYK | 2y9 | -1.17 | 50319.34 | 43124.65 | 0.19 | 0.21 |
| | | 2y10 | -1.00 | 227272.43 | 226598.15 | 0.10 | 0.12 |
| ZZZ3 | SEAPNSSEEDSPIKSDK | 2b11 | 1.16 | 48292.11 | 56133.89 | 0.15 | 0.25 |
| | | 2b10 | -1.03 | 43355.86 | 42261.51 | 0.19 | 0.32 |
| | | 2y9 | 1.17 | 22624.53 | 26489.91 | 0.11 | 0.32 |
| | STVVDNDADFGTKR | 3y8 | 1.30 | 8206.66 | 10679.54 | 0.11 | 0.26 |
| | | 3y9 | 1.41 | 101584.22 | 142847.73 | 0.05 | 0.12 |
| | | 2y8 | -1.29 | 246877.46 | 190678.72 | 0.07 | 0.14 |
| | KEDSYIDHK | 2b8 | -1.18 | 35897.74 | 30549.56 | 0.08 | 0.16 |
| | | 2y5 | -1.27 | 104844.81 | 82880.14 | 0.04 | 0.13 |
| TNS4 | ETRSSSESLIFSGNQGR | 2y10 | 1.01 | 49747.93 | 50046.28 | 0.10 | 0.28 |
| | | 2y11 | 1.00 | 2365360.95 | 2365708.39 | 0.03 | 0.18 |
| | | 2b10 | -1.06 | 917069.40 | 861584.17 | 0.04 | 0.19 |
| | | 2y9 | -1.08 | 40830.75 | 37759.17 | 0.11 | 0.17 |
| | ASSPHGLGSPLVASPR | 2y9 | -1.02 | 811811.96 | 792132.54 | 0.05 | 0.15 |
| | | 2y10 | 1.01 | 870106.87 | 877069.80 | 0.05 | 0.16 |
| | | 2y11 | 1.07 | 137127.16 | 146599.35 | 0.11 | 0.15 |
| | | 2y12 | 1.01 | 213457.10 | 215929.07 | 0.07 | 0.21 |
| | | 2b13 | 1.04 | 149394.18 | 156011.05 | 0.04 | 0.18 |

b = N-terminal ion; y = C-terminal ion

Appendix C

Conditions Used for Flow Cytometry Experiments

| Antibody | Conditions | Company/catalogue number |
|------------------------------|-------------------|--|
| CD4-PE-Cy5 | 10 μ l | Abcam ab95512 |
| CD8-PE-Cy5 | 10 μ l | Abcam ab25444 |
| CD38-PE-Cy5 | 10 μ l | Abcam ab95569 |
| CD20-PE-Cy5 | 10 μ l | Abcam ab25443 |
| CXCR3-PE | 5 μ l | BD Biosciences 557185 |
| T-bet-PE | 10 μ l | BD Biosciences 561268 |
| IgD-FITC | 15 μ l | BD Biosciences 555778 |
| IgM-FITC | 15 μ l | BD Biosciences 555782 |
| SLAMF8-PE | 10 μ l | R&D Systems FAB19072P |
| SMAD2 | 1:100 in PBS | Abcam ab33875 |
| CD24-FITC | 15 μ l | BD Biosciences 555427 |
| IgG1-PE | 10 μ l | Miltenyi Biotec 130-093-188 |
| IFI44L | 5 μ l | Santa Cruz sc-101981 |
| IFIT3 | 2.5 | Santa Cruz sc-133687 |
| MxA | 1:200 in PBS | Gift from Georg Kochs, University of Freiberg, Germany |
| USP18 | 5 μ l | Santa Cruz sc-374064 |
| IgG-PE | 10 μ l | BD Biosciences 555787 |
| FITC goat anti-mouse IgG/IgM | 1:250 in PBS | BD Biosciences 555988 |
| FITC goat anti-rabbit Ig | 1.5 μ l | BD Biosciences 554020 |