

# **The role and mechanism of action of BRK in breast cancer progression**

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## ABSTRACT

Breast cancer is unanimously considered a highly heterogeneous disease due to its diverse molecular features. Breast tumor kinase (BRK), also known as protein tyrosine kinase 6 (PTK6), is a non-receptor tyrosine kinase that is highly expressed in over 80% of breast carcinomas. The role and mechanism of action of enzymatically activated BRK in breast pathology are unclear. The objectives of this project were to reveal the effect of BRK activation on cell migration, proliferation and tumorigenesis. We also aimed to determine the mechanism of action of BRK in the promotion of cell proliferation. We used BRK-negative cells (MCF10A, MDA-MB-231 and HEK293) to generate three sets of stable cell lines that stably expressed GFP alone, GFP-BRK-WT or GFP-BRK-Y447F (constitutively active) by retroviral infections. We also stably knocked down BRK from BRK-positive cells BT20 and SKBR3 by RNA interference using shRNAs against BRK. Western blotting, immunoprecipitation and qPCR studies were conducted to evaluate protein expression, protein-protein interaction and mRNA expression, respectively. Both sets of cell lines were used to determine the effect of BRK on cell proliferation (automated cell counter), cell migration (transwell and wound healing assay), transformation (colony formation assay) and tumor formation (mouse Xenograft assay). To investigate the mechanism of action of BRK, we validated downstream of tyrosine kinases 1 (Dok1), a tumor suppressor, as a BRK substrate. Deletion or site-directed mutagenesis was performed to map BRK-targeted tyrosines in Dok1 protein. Results obtained from this research project showed that stable expression of the constitutively active mutant of BRK (BRK-Y447F) in MDA-MB-231 cells led to a significant increase in the cell proliferation, migration rate and promoted colony formation and drastically enhanced tumor formation in athymic nude mice in comparison to control cells. Additionally, depletion of BRK abrogated the migration of BT20 and SKBR3 cells. Furthermore, we showed that BRK interacts with and phosphorylates Dok1, inducing Dok1 downregulation via a ubiquitin-proteasome-mediated mechanism. Together, our results show that the activation of BRK is essential for mammary gland tumorigenesis and suggest that targeting of Dok1 for degradation is a novel mechanism of action of BRK in the promotion of cell proliferation, migration and tumor formation.

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## **DEDICATION**

I dedicate this thesis to:

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## LIST OF ABBREVIATIONS

|       |  |
|-------|--|
| ABL   | abelson tyrosine-protein kinase                          |
| ACK   | activated Cdc42-associated kinase 1                      |
| ALK   | anaplastic lymphoma kinase                               |
| ANOVA | analysis of variance                                     |
| AP-1  | activator protein-1                                      |
| APS   | ammonium persulfate                                      |
| ARG   | abelson-related gene                                     |
| ATCC  | american type culture collection                         |
| ATF2  | activating transcription factor 2                        |
| ATP   | adenosine triphosphate                                   |
| AXL   | receptor tyrosine kinase AXL                             |
| BLK   | B lymphoid tyrosine kinase                               |
| BMX   | bone marrow tyrosine kinase gene in chromosome X protein |
| BRK   | breast tumor kinase                                      |
| BSK   | B-cell Src-homology tyrosine kinase                      |
| BTK   | B-cell progenitor kinase                                 |
| cDNA  | complementary DNA  |
| CSF1R | colony stimulating factor 1 receptor                     |
| CSK   | C-terminal src kinase                                    |
| DDR   | discoidin domain receptor family                         |
| DLK   | dual leucine zipper bearing kinase                       |
| DMEM  | Dulbecco's modified eagle medium                         |
| ECL   | enhanced chemiluminescence                               |
| EGF   | epidermal growth factor                                  |

|        |   |
|--------|---|
| EGFR   | epidermal growth factor receptor                      |
| EPH    | ephrin  |
| ER     | estrogen receptor                                     |
| ERK    | extracellular signal-regulated kinase                 |
| FAK    | focal adhesion kinase                                 |
| FBS    | fetal bovine serum                                    |
| FER    | fer (fps/fes related) tyrosine kinase (p              |
| FES    | feline sarcoma oncogene                               |
| FGFR   | fibroblast growth factor receptor                     |
| FGFR-1 | fibroblast growth factor receptor-1                   |
| FGR    | feline Gardner-Rasheed sarcoma viral oncogene homolog |
| FITS   | 4', 6-diamidino-2-phenylindole                        |
| FRK    | fyn-related tyrosine kinase                           |
| FYN    | fibroblast endothelial kinase                         |
| GAP    | GTPase-activating protein                             |
| GDP    | guanosine diphosphate                                 |
| Grb2   | growth factor receptor-bound protein 2                |
| Grb7   | growth factor receptor-bound protein 7                |
| GTK    | gastrointestinal tyrosine kinase                      |
| GTP    | guanosine-5'-triphosphate                             |
| HCK    | hemopoietic cell kinase                               |
| HER2   | human epidermal growth factor receptor 2              |
| IGF-1R | insulin-like growth factor-1 receptor                 |
| IGFR   | insulin-like growth factor receptor tyrosine kinase   |
| INSR   | insulin receptor                                      |
| IRS-2  | insulin receptor substrate-2                          |

|       |  |
|-------|--|
| ITK   | interleukin-2-inducible T-cell kinase                    |
| IYK   | intestine tyrosine kinase                                |
| JAK   | janus kinase   |
| LB    | lysogeny broth   |
| LMR1  | serine/threonine-protein kinase LMTK1                    |
| LOH   | loss of heterozygosity                                   |
| LTK   | leukocyte tyrosine kinase                                |
| LYN   | v-src-1 yamaguchi sarcoma viral related oncogene homolog |
| MAP   | mitogen-activated protein                                |
| MAPK  | mitogen-activated protein kinases                        |
| MATK  | megakaryocyte-associated tyrosine kinase                 |
| MEK   | MAP/ERK kinase   |
| MEKK  | MEK kinase   |
| MER   | c-mer proto-oncogene tyrosine kinase                     |
| MET   | met proto-oncogene (hepatocyte growth factor receptor)   |
| MUSK  | muscle, skeletal, receptor tyrosine kinase               |
| NLS   | nuclear localization signal                              |
| NRTK  | non-receptor tyrosine kinase                             |
| PCR   | polymerase chain reaction                                |
| PDGF  | platelet-derived growth factor                           |
| PDGFR | platelet-derived growth factor receptor                  |
| PDK1  | phosphoinositide-dependent kinase 1                      |
| PH    | pleckstrin homology                                      |
| PI3K  | phosphoinositide 3-kinase                                |
| PKB   | protein kinase B   |
| PMSF  | phenylmethylsulfonyl fluoride                            |

|          |  |
|----------|--|
| PR       | progesterone receptor  |
| PTB      | phosphotyrosine binding  |
| PTK      | protein tyrosine kinase  |
| PTK7     | inactive tyrosine-protein kinase 7                                       |
| PTPase   | protein tyrosine phosphatase   |
| PYK2     | protein tyrosine kinase 2  |
| RAS      | rat Sarcoma  |
| RET      | rearranged during transfection; ret proto-oncogene                       |
| RON      | recepteur d'origine nantais  |
| ROR      | receptor tyrosine kinase-like orphan receptor                            |
| ROS      | c-Ros receptor tyrosine kinase   |
| RTK      | receptor tyrosine kinase   |
| RT-PCR   | real-time polymerase chain reaction                                      |
| RYK      | RYK receptor-like tyrosine kinase  |
| S. D.    | standard deviation   |
| Sam68    | a Src-associated substrate in mitosis of 68 kDa                          |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis                |
| SFK      | Src family kinase  |
| SH       | Src homology   |
| SHB      | Src homology 2 domain adapter protein                                    |
| SKY      | tyrosine-protein kinase receptor TYRO3                                   |
| Src      | sarcoma  |
| SRMS     | Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal |
| STAT     | signal transducer and activator of transcription                         |
| SuRTK106 | protein PK-unique  |
| SYK      | spleen tyrosine kinase   |

|         |   |
|---------|---|
| TEC     | tec protein tyrosine kinase                                   |
| TEK     | tunica interna endothelial cell kinase                        |
| TEMED   | N,N,N',N'-tetramethylethylenediamine                          |
| TIE     | tyrosine kinase with immunoglobulin-like and EGF-like domains |
| TNK1    | tyrosine kinase, nonreceptor, 1                               |
| TRK     | tyrosine receptor kinase                                      |
| TXK     | PTK4 protein tyrosine kinase                                  |
| TYK2    | tyrosine kinase 2   |
| Tyr     | tyrosine  |
| VEGFR-2 | vascular endothelial growth factor receptor-2                 |
| YES     | yamaguchi sarcoma viral oncogene homolog 1                    |
| YRK     | yes-related kinase  |
| ZAP70   | zeta-activating protein                                       |

# **1. Introduction**

## **1.1 Impact of breast cancer**

In both the developed and the developing world, breast cancer is the most commonly diagnosed female malignancy and one of the leading causes of cancer related death in women. It was predicted that every year over 1.5 million women will be diagnosed with breast cancer, accounting for approximately 23 % of all cancers and ultimately taking over half a million lives per year worldwide (Ginsburg and Love, 2011). The global economic impact of breast cancer is staggering, which is estimated at \$88 billion in 2008 (American Cancer Society-2008). Statistics Canada projects more than 24,400 new cases of breast cancer diagnoses and over 5000 deaths of Canadian women each year. One in nine women will be diagnosed with breast cancer and 1 in 30 will die of this terrible disease in their lifetime (Canadian Cancer Statistics 2014).

### **1.1.1 Breast cancer and molecular classifications**

Breast cancer is a highly heterogeneous group of cancers, with diversity in its morphology, molecular genetics, biology, and clinical outcome. Adenocarcinoma is a cancer of the epithelium that originates in glandular tissue and most breast cancers start as adenocarcinoma. Adenocarcinoma progresses into ductal carcinoma in situ (DCIS), also arising from the ductal epithelium, or lobular carcinoma in situ (LCIS), which originates from the epithelium of the lobules. The most common kind of breast cancer, invasive ductal carcinoma (IDC) also called infiltrating ductal carcinoma and infiltrating lobular carcinoma (ILC), grows from DCIS and LCIS respectively (Sharma et al., 2010). The invasive tumor cells metastasize preferentially to the bone and the lungs (Figure 1.1).

The biology of breast tumors remains poorly understood, although, several measures have been undertaken to distinguish tumor features according to tumor grade, stage and gene profile. Grading is based on the microscopic structure of the tumor and scaled from 1-3, from the least aggressive in appearance to the most aggressive. The stages are classified from noninvasive Stage 0 to the highly metastatic Stage IV, based on measurement of the tumor, lymph node involvement, and the metastatic spread (Perou et al., 2000; Sorlie et al., 2001; Sotiriou et al., 2003).



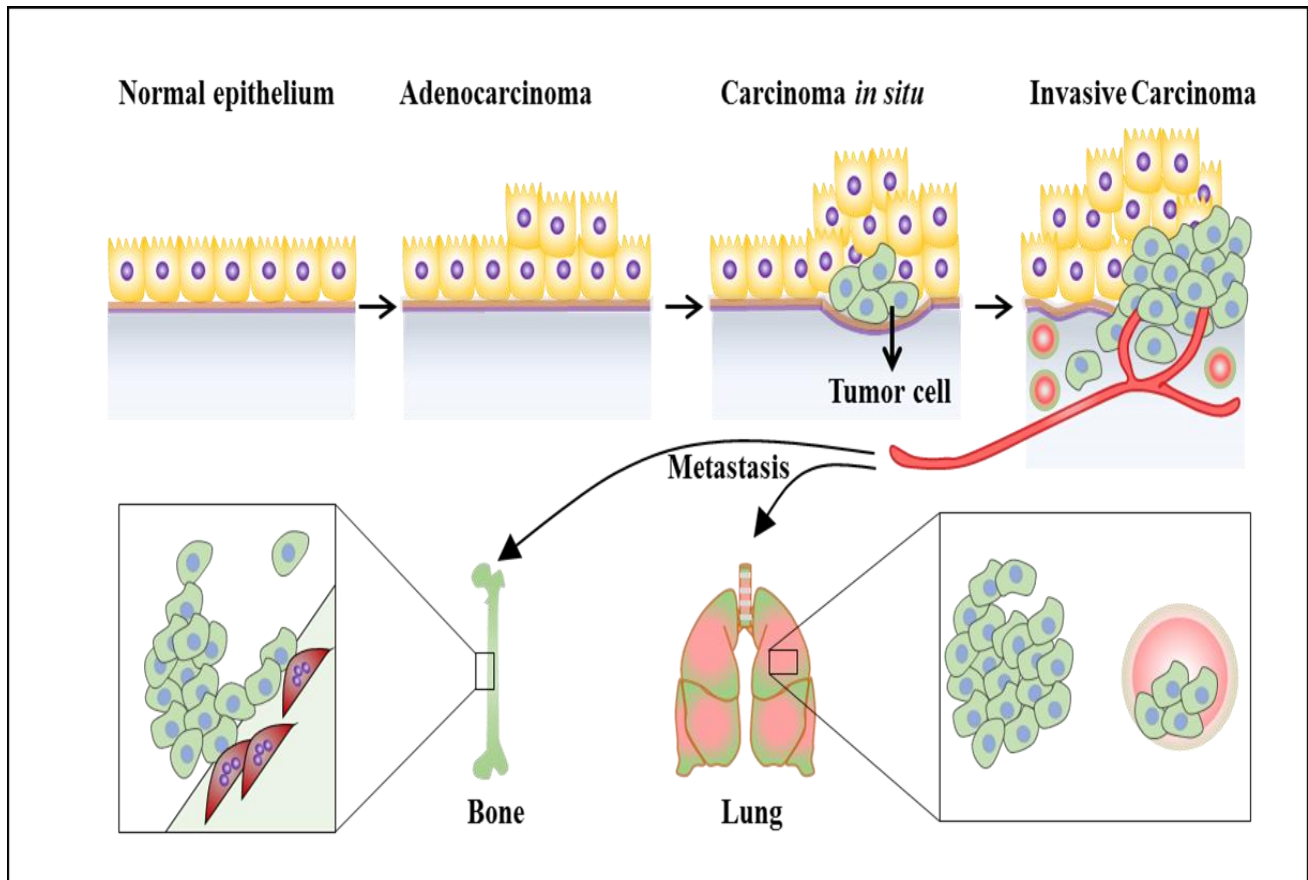


Figure 1.1: Progression of human breast cancer. Normal epithelium of glandular tissues alters into malignant adenocarcinomas, then becomes tumorigenic (carcinoma *in situ*) and subsequently develops into invasive carcinomas. Malignant tumor cells metastasize to the bone and the lungs. (Adapted from Siegel and Massague, 2003).

Six molecular classes of breast cancer have also been defined through gene expression profiling (Andre and Pusztai, 2006; Sorlie *et al.*, 2001) (Table 1). They include: 1. Normal breast-like, 2. Basal-like (Triple-negative), 3. Luminal A, 4. Luminal B, 5. Human epidermal growth factor receptor 2 (HER2)-positive, and 6. Claudin-low.

The luminal subtypes that make up hormone receptor expressing breast cancer represent 67% of the tumors and express luminal cytokeratins 8/18, estrogen receptors (ER) and genes associated with ER activation such as LIV1 and Cyclin D1. The basal-like tumors are usually ER-, PR- and HER2-negative and are also associated with germ-line BRCA1 mutations, one of the most important forms of the hereditary breast cancer (Andre and Pusztai, 2006; Foulkes *et al.*, 2003).

Table 1: Molecular subtypes of breast cancer. This table shows the gene expression pattern of each molecular class of breast cancers including histological grade and metastatic potential (Munirah *et al.*, 2011; Sabatier *et al.*, 2014; Taube *et al.*, 2010). ER (estrogen receptor); PR (progesterone receptor); GRB7 (Growth factor receptor- bound protein7) and BRCA1 (Breast Cancer 1)

|                    | Normal Breast-like             | Basal-like (Triple-negative)                                    | Luminal A                                  | Luminal B                                  | HER2-Positive                               | Claudin-low  |
|--------------------|--------------------------------|---|--|--|---|--|
| Gene expression    | Cytokeratin 5-, 6- and 8+, 18+ | ER-, PR-, HER2-, and BRCA1 mutations. Cytokeratin 5, 6, 14 & 17 | High ER+ and PR+<br><br>HER-               | Low ER+ and PR+<br><br>HER+                | HER+<br><br>GRB7+                           | Low expression of claudin and E-cadherin and high expression of Vimentin, Snail, TWIST and ZEB |
| Histological grade |                                | Histological high grade and highly metastatic                   | Histological low grade and less metastatic | Histological low grade and less metastatic | Histological high grade and very aggressive | High metastatic potential  |

HER2-positive breast cancers are characterized by high expression of HER2 usually as a result of amplification of 17q12q21 locus that contains the HER2 gene. Unlike other breast tumors, basal-like and HER2 tumors have a higher proportion (40-80%) of TP53 mutations (Andre and Pusztai, 2006). The normal breast-like subtype has expression patterns similar to nonmalignant tissue. Luminal A tumors have the longest survival times, luminal B tumors have intermediate survival times, whereas the basal-like and HER2 positive subtypes display the shortest survival times. Luminal A and B subtypes possess low and intermediate metastatic properties respectively, while the basal-like and HER2 positive subtypes are highly aggressive (Sorlie *et al.*, 2003) tumors that express low levels of the tight junction claudin 3, as well as E-cadherin. They are equally characterized by a low expression of luminal markers and a high expression of mesenchymal markers and are associated with poor prognosis. Claudin-low tumors are more enriched in epithelial-to-mesenchymal transition (EMT) and tumor initiating cell features, immune system responses and stem cell-associated biological processes (Prat *et al.*, 2012).

Despite tremendous progress in cancer research, the heterogeneous nature of breast cancer is limiting the design of effective therapies. Breast cancer treatment typically includes surgical excision of the tumor mass in combination with chemotherapy and/or radiation therapy (Horgan *et al.*, 2012). However, these conventional therapies are often ineffective due to several reasons such as treatment target inaccuracy, recurrence of the tumor and in some cases primary or acquired resistance to conventional therapies (Bonavida and Kaufhold, 2015). In order to overcome these common challenges in breast cancer treatment, the major focus of research today is to identify and characterize potential molecules that play a significant role in pathogenesis, progression and resurgence of cancer. Targeting these potential markers will potentially pave a new window in breast cancer treatment. Examples of successful targeted therapies include Tamoxifen, an antagonist of the ER that blocks the effects of estrogen (Jordan, 2014), and Trastuzumab (Herceptin), a monoclonal antibody that inhibits the activity of HER2 (Roskoski, 2014). Overexpression, gene amplification, or mutations of other tyrosine kinases have a strong association with carcinogenesis (Foth *et al.*, 2014; Ko *et al.*, 2014; Morel *et al.*, 2014; Peng *et al.*, 2014b), thus making them promising therapeutic targets. The goal of my Ph.D. project was to investigate the role of a non-receptor tyrosine kinase, breast tumor kinase (BRK), which is overexpressed in approximately 80% of breast tumors.

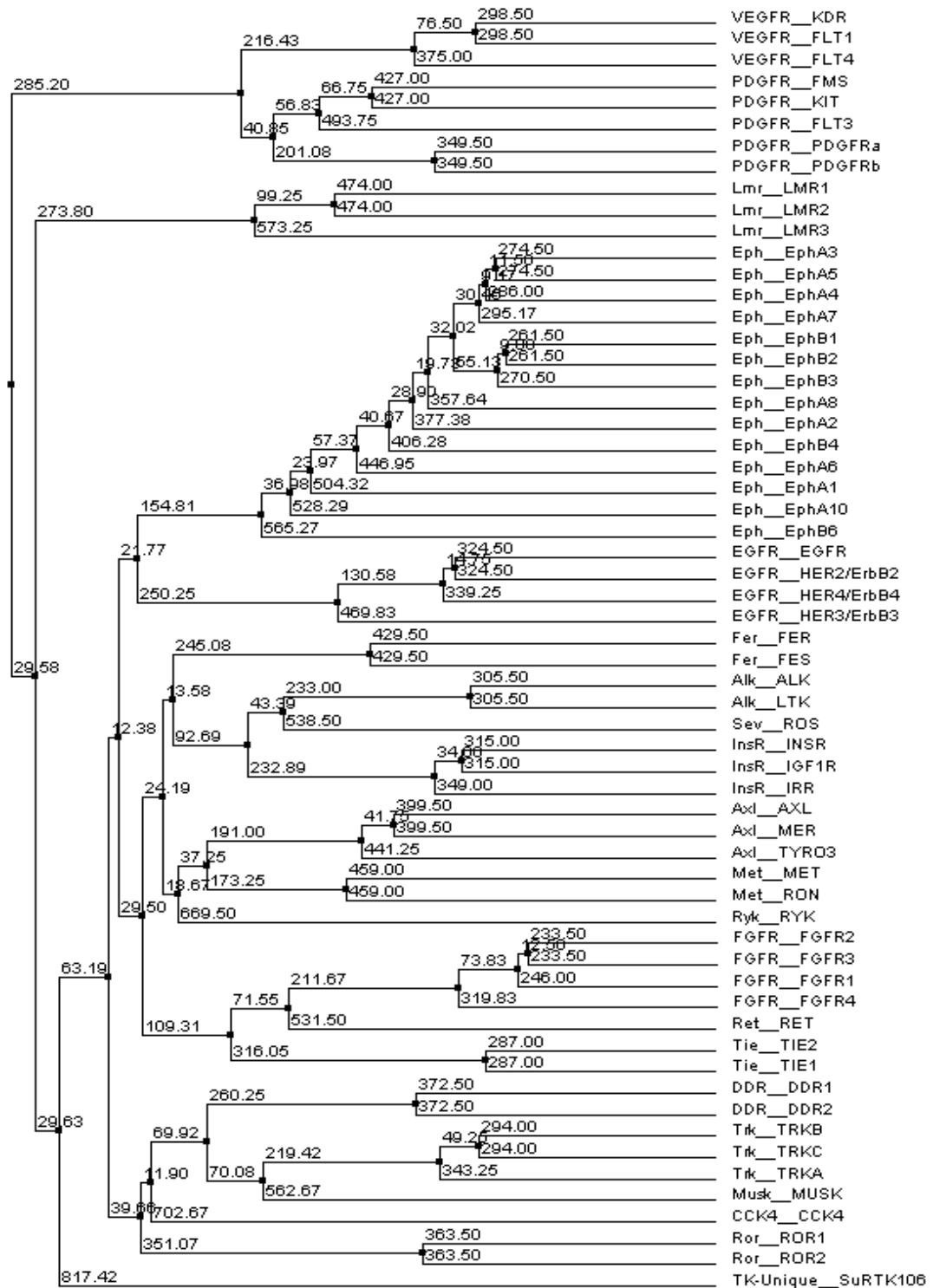
## 1.2 Protein kinases

A large family of enzymes known as kinases catalyzes protein phosphorylation. Kinases transfer the  $\gamma$ -phosphate group of ATP onto a selective residue (such as Ser, Thr or Tyr) of a target protein substrate (Hunter, 2014). Phosphorylation plays a major role in protein functioning and can turn enzymes on and off. For example, phosphorylation of Tyrosine 416 and 527 turns Src kinase on and off, respectively (Guarino, 2010). Phosphorylation thus is an indispensable part of cellular processes.

Approximately 2% of the eukaryotic genome encode protein kinases, which make them one of the largest and most influential gene families (Manning, 2005). There are 518 protein kinases in the human genome (Manning *et al.*, 2002) of which 90 are classified as protein tyrosine kinases (Figure 1.2). A protein tyrosine kinase transfers the phosphate group specific to the tyrosine on the target protein. Based on sequence similarity and divergent genetic structural organization similarities, tyrosine kinases have been further separated into either receptor or non-receptor tyrosine kinase families. The receptor tyrosine kinase family has 58 members which are divided into 20 subfamilies (Figure 1.3), whereas non-receptor tyrosine kinase family is comprised of 32 members which are divided into 10 subfamilies (Figure 1.4) (Robinson *et al.*, 2000).

Tyrosine kinases play a major role in various biochemical pathways and it has been reported that up to 30% of the proteome can potentially be phosphorylated by protein kinases (Manning, 2005). In fact, they participate in multiple cellular functions including cell growth, differentiation, cell motility, metabolism, survival as well as apoptosis. Hence, any deregulation of the phosphorylation process may alter cell function and result in a disease state (Rauch *et al.*, 2011). Therefore, protein kinases have emerged as major targets of drug discovery. In fact, approximately one-third of the contemporary validated drugs used to treat cancer are based on kinase inhibition in the pharmaceutical armoury (Pearl and Barford, 2002; Sun *et al.*, 2015).

A



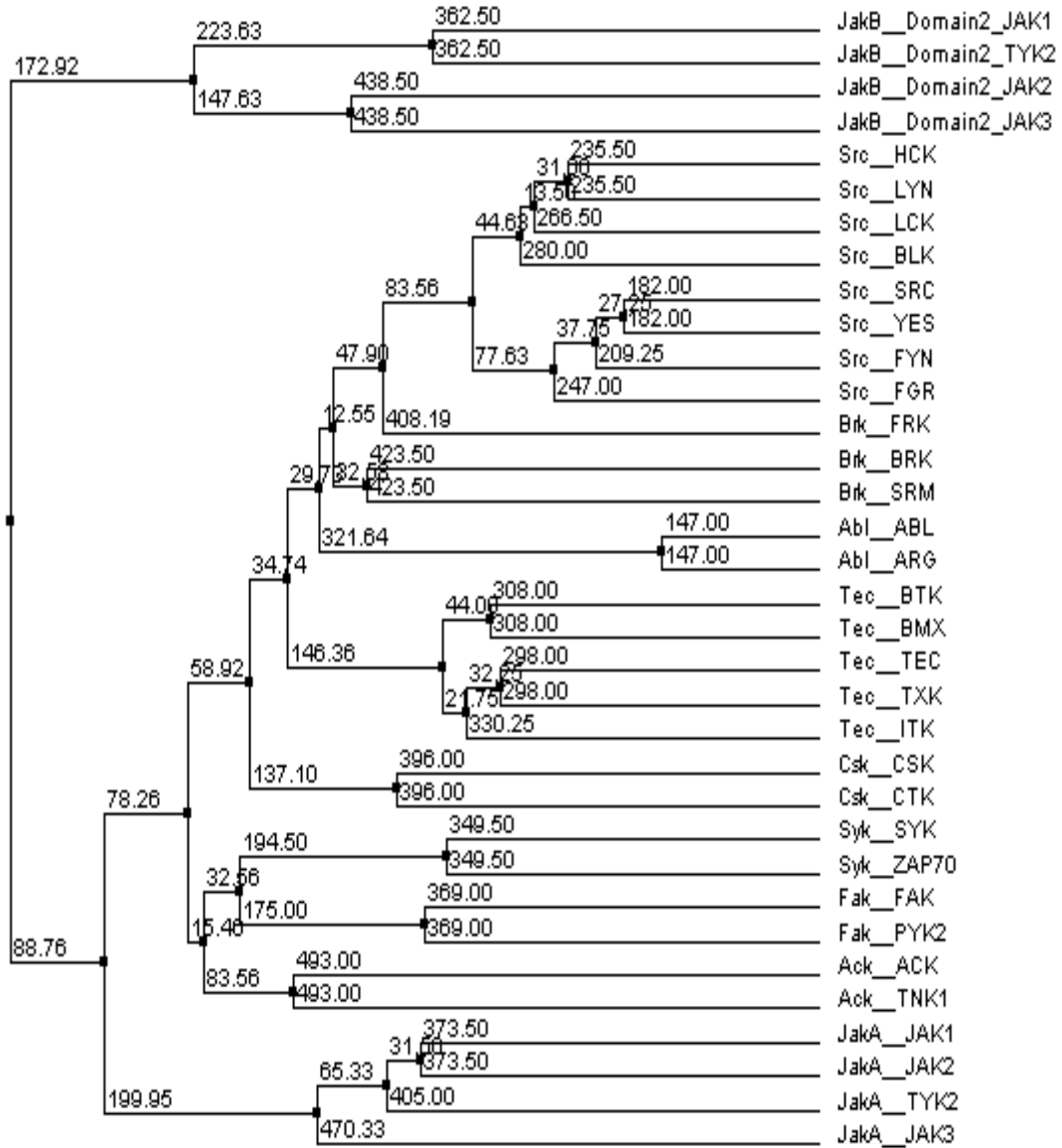
**B**

Figure 1.2: Phylogram of the human protein tyrosine kinase family A. Receptor tyrosine kinases. B. Non-receptor tyrosine kinase family. These kinase family trees are generated based on amino acid sequence identity and constructed by the EBI clustalw and numbers on each node dictate the evolutionary distance of most recent common ancestor in years (million).

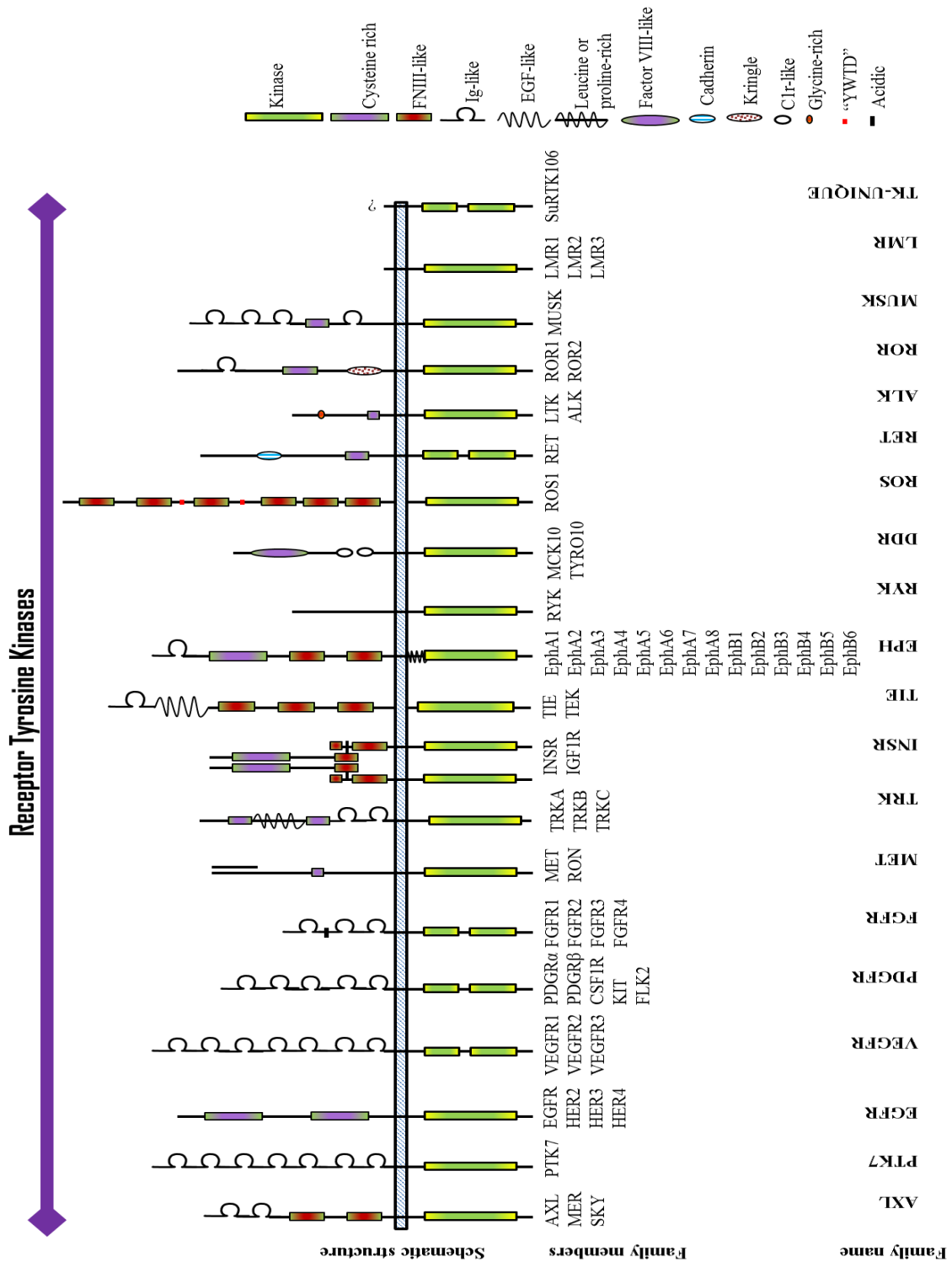


Figure 1.3: Class and domain organization of human receptor RTKs are shown schematically. The cytoplasmic part of the receptor is on the bottom and top is the extracellular portion of the receptors (Hubbard and Till, 2000; Robinson *et al.*, 2000).

# Non-Receptor Tyrosine Kinases

In context of Breast cancer











| Family name | Family members                              | Schematic structure  | Tumor suppressor | Oncogene                                    |
|-------------|---|--|------------------|---|
| BRK         | BRK, FRK, SRMS                              |    | FRK              | BRK   |
| SRC         | SRC, YES, FYN, LYN, LCK, BLK, HCK, FGR, YRK |    |                  | SRC, YES, LYN, FYN, LCK, BLK, HCK, FGR, YRK |
| TEC         | BTK, ITK, TEC, BMX, TXK                     |    |                  | BTK, ITK, TEC, BMX                          |
| CSK         | CSK, MATK                                   |    | CSK, MATK        | BTK, ITK, TEC, BMX                          |
| ABL         | ABL, ARG                                    |    |                  | ABL, ARG                                    |
| SYK         | ZAP70, SYK                                  |   |                  |   |
| FES         | FES, FER                                    |  |                  | FES, FER                                    |
| FAK         | FAK, PYK2                                   |  |                  | FAK, PYK2                                   |
| JAK         | JAK1, JAK2, TYK2, JAK3                      |  | TYK2             | JAK1, JAK2, JAK3                            |
| ACK         | ACK, ACK2                                   |  | ACK2 (TNK1)      | ACK   |



Figure 1.4. Class and domain topology of non-receptor RTKs are shown schematically. The boxes indicating SH3, SH2 and Kinase domain are defined by different colors as indicated in the figure. Family members are involved in tumor progression or suppression is also shown in figure. (Goel and Lukong, 2015; Chakraborty *et al.*, 2006; Cohen *et al.*, 2010; Coopman and Mueller, 2006; Di Stefano *et al.*, 2007; Gil-Henn *et al.*, 2013; Golubovskaya *et al.*, 2009; Hoare *et al.*, 2008; Hornakova *et al.*, 2011; Hussain *et al.*, 2011; Ivanova *et al.*, 2013; Mahajan and Mahajan, 2010; Marotta *et al.*, 2011; Miah *et al.*, 2012; Montero *et al.*, 2011; Sang *et al.*, 2012; Tabaries *et al.*, 2015; Wendt *et al.*, 2013; Yadav and Denning, 2011; Ye *et al.*, 2013a; Ye *et al.*, 2013b; Zhao *et al.*, 2010; Zhu *et al.*, 2008).

### 1.3 BRK family kinases (BFKs)

Based on exon-intron boundary organization, BRK family kinases (BFKs) are comprised of three members namely BRK or protein tyrosine kinase 6 (PTK6), Fyn related kinase (FRK) or protein tyrosine kinase 5 (PTK5), and Src-Related tyrosine kinases lacking C-terminal Regulatory tyrosine and N-terminal Myristoylation Sites (SRMS) or protein tyrosine kinase 70 (PTK70) (Goel and Lukong, 2015). The BFKs have 8 exons and this unique exon-intron pattern of BFKs is evolutionary distinct from Src family kinases (SFKs), which have 12 exons (Serfas and Tyner, 2003).

BRK is a non-receptor tyrosine kinase also known as PTK6, murine ortholog, Sik (Src-related intestinal kinase) was cloned in three separate studies in the early 1990s. BRK was first identified in a screen for protein tyrosine kinases in human melanocytes (Lee *et al.*, 1993), followed by a study screening for novel kinases in metastatic breast cancers (Mitchell *et al.*, 1994), and lastly in an experiment assessing epithelial cell differentiation in the mouse small intestine (Siyanova *et al.*, 1994). The human *PTK6* gene was mapped to chromosome 20q13.3. The gene is 10 kb long and is comprised of 8 exons that code for the BRK protein, consisting of 451 amino acid residues. In addition, a BRK isoform produced by alternative splicing has been identified, which encodes a 15 kDa protein called ALT-PTK6 (Brauer *et al.*, 2011), initially termed  $\lambda m5$ , and is composed of 134 amino acids (Mitchell *et al.*, 1997).

FRK also known as PTK5, Rak, Bsk, Iyk and Gtk, was cloned by several research laboratories in the early 1990s. FRK was first cloned from a human hepatoma cell line (Lee *et al.*, 1994) as well as from human breast cancer cells (Cance *et al.*, 1994). The murine homolog of FRK, Bsk, was cloned from the kidney and islets of Langerhans of the mouse (Obergh-Welsh and

Welsh, 1995). The human *FRK* localizes to chromosome 6q22.1, and the gene encodes a protein of 58 kDa, composed of 505 amino acids (Lee *et al.*, 1994). An alternative transcript variant of *FRK* has also been identified in transcriptome studies of the entire human cDNAs library (Ota *et al.*, 2004).

*SRMS* is the 3<sup>rd</sup> member of the *BRK* family. It was cloned from mouse embryonic neuroepithelial cells (Kohmura *et al.*, 1994). The human orthologue of *SRMS* maps on chromosome 20q13.33 and is composed of 8 exons which encode a protein of 54 kDa, composed of 488 amino acids (Deloukas *et al.*, 2001).

The *BFKs* are structurally homologous with *Src* tyrosine kinase and composed of *Src* homology domains 3 and 2 (SH3 and SH2), and a kinase domain. Both *BRK* and *FRK* have a putative C-terminal regulatory tyrosine and display a similar architecture to *Src* kinases, however, *SRMS* is lacking a C-terminal tail (Serfas and Tyner, 2003) (Figure 1.5). Unlike *Src* family kinases, the human *BFKs* lack the myristoylated N-terminal consensus sequence required for membrane anchorage. Conversely, rodent *FRK* has a glycine residue at position 2, which allows it to myristoylate and localizes to the cell membrane (Sunitha and Avigan, 1996). *BRK* preferentially localizes to the cytoplasm but some is found in the nucleus (Miah *et al.*, 2014; Serfas and Tyner, 2003). *SRMS* was shown to have a punctate cytoplasmic localization (Goel *et al.*, 2013). However, *FRK* has a putative nuclear localization signal (NLS) motif (KRxxxxxFFxxRRR) in the SH2 domain that contains two groups of basic amino acids separated by spacer amino acids that dictates its nuclear localization in COS7 Monkey kidney cells (Serfas and Tyner, 2003), although other studies have indicated that *FRK* localizes to specific structures in the cytoplasm (Sunitha and Avigan, 1996).

Functional redundancy of the *PTKs* is common, since these enzymes tend to share their substrates (Carreno *et al.*, 2002; Xian and Zhou, 2004). For example, Docking protein 1 is a substrate of both *BRK* (Miah *et al.*, 2014) and *Src* (Niu *et al.*, 2006). Homozygous deletion of *BRK*, *FRK* and *SRMS* in mice did not show any detectable phenotypic effect, perhaps due to functional redundancy. More specifically, *BRK* or *SRMS* deficient mice were viable and fertile, and depletion of *FRK* did not demonstrate any developmental abnormalities (Kohmura *et al.*, 1994); (Chandrasekharan *et al.*, 2002; Haegebarth *et al.*, 2006). However, overexpression of *PTKs* is associated with different human malignancies including cancer (Shchemelinin *et al.*, 2006). *FRK* has been shown to be a potential inhibitor of tumor progression. Overexpression of

FRK in breast ductal carcinoma cells arrests the cells in G1 phase of the cell cycle, hindering their proliferation (Meyer *et al.*, 2003), while the depletion of FRK promotes epithelial cell transformation to mesenchymal cells (Yim *et al.*, 2009). Likewise, in glioma cells, both mRNA and protein levels of FRK are significantly repressed suggesting a tumor suppressive function of FRK (Shi *et al.*, 2015; Zhou *et al.*, 2012). Conversely, it has recently been reported that SRMS levels increase with breast tumor grades (Goel *et al.*, 2013).

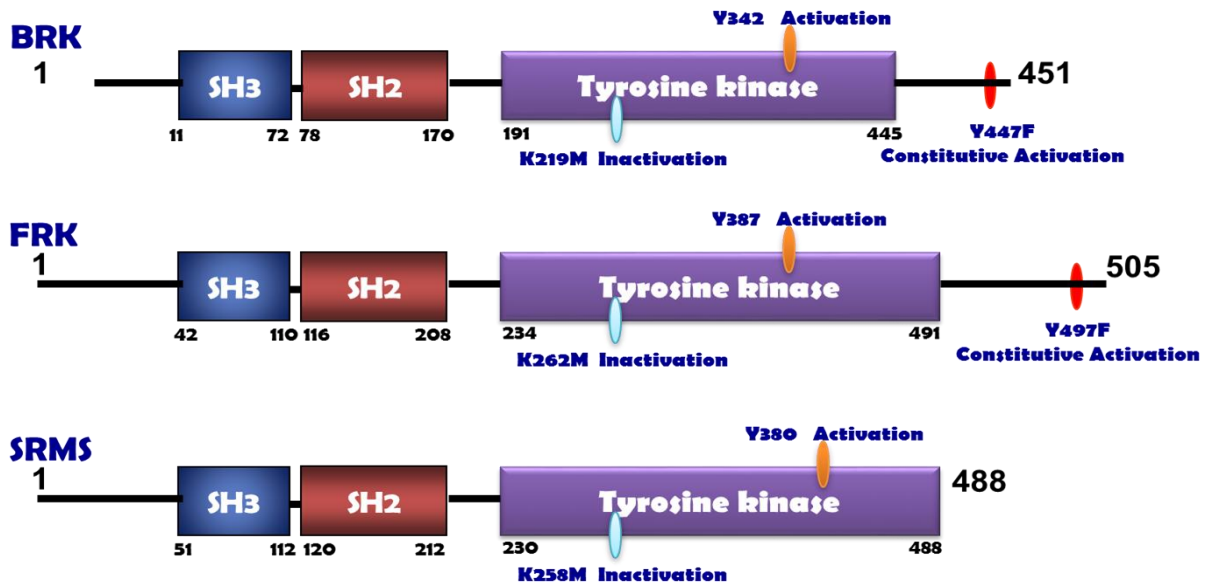


Figure 1.5. Schematic structure of BRK, FRK and SRMS tyrosine kinases. They share structural similarity, including the SH3 and SH2 that regulate protein-protein interaction and a conserved catalytic domain. BRK, FRK and SRMS are activated by phosphorylation of Y342, Y387 and Y380, and inactivated by K219M, K262M and K258M mutations, respectively. The tyrosine at 447 in BRK and at 497 in FRK regulates kinase activity. In contrast to BRK and FRK, SRMS lacks a C-terminal regulatory tail.

Although BRK deficient mice are viable and fertile, overexpression of BRK has been detected in many breast cancer cell lines, primary mammary gland tumors, and is typically undetected in normal mammary glands or benign tumors (Barker *et al.*, 1997; Ludyga *et al.*, 2011). However, it has been recently reported that BRK is also expressed in the mammary glands of healthy individuals, but it becomes enzymatically active only in the malignant form of mammary tumors (Peng *et al.*, 2014a).

## 1.4 BRK structure, activity and regulation

BRK has the Src homology (SH) domains SH3, SH2, a kinase domain and a putative C-terminal regulatory tyrosine. It also displays a similar architecture and has 30–45% sequence identity with Src kinases (Serfas and Tyner, 2003). However, it lacks the amino-terminal myristoylation signal that localizes Src to the cell membrane, and therefore, it is not specifically targeted to the membrane (Figure 1.6) (Vasioukhin *et al.*, 1995). Hence, its intracellular localization is flexible and it therefore localizes in the nucleus and cytoplasm, as well as at the cell membrane (Haegbarth *et al.*, 2004). The SH3 domain binds to proline-rich regions of the consensus PXXP in substrate proteins. This domain is involved in intramolecular interactions that regulate kinase activity, interactions with substrates, cellular localization, and associated with other protein targets (Pawson, 1995). The SH2 domain recognizes and binds to phosphorylated tyrosine residues, with the specificity being determined by the 3-5 amino acids following the tyrosine residue (the peptide motifs for Src-SH2 was (H)(Y/M/F/H)pY(T/A/N)(M/Q/V/I)(I/M) and for BRK was (H/E/N/D)(M/Y/H/F)pY(D/E)(I/N/V/M)(C/V/Y)) (Songyang *et al.*, 1993; Zhao *et al.*, 2013a).

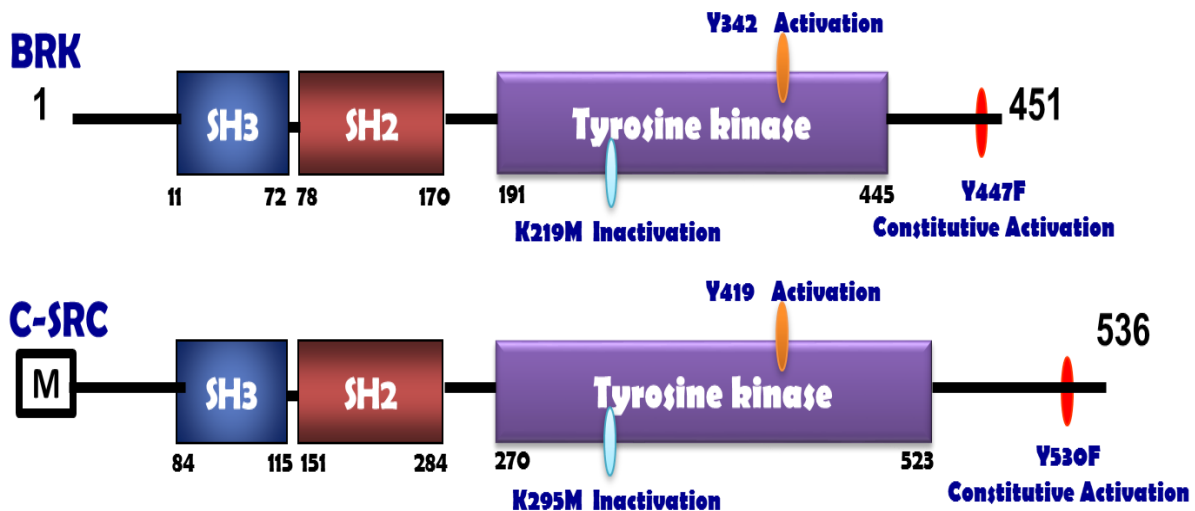


Figure 1.6. Schematic structure of BRK and Src tyrosine kinases. BRK and Src share 44% amino acid identity. These include the SH3 and SH2 that regulate protein-protein interaction and a conserved catalytic domain. BRK and Src are activated by phosphorylation of Y342 and Y419, and inactivated by K219M and K295M mutations, respectively. The tyrosine at 447 in BRK and at 527 in Src regulates kinase activity. In contrast to Src, BRK lacks an N-terminal consensus myristoylation site.

The SH3 and SH2 domains of BRK like Src family members are involved in intramolecular interactions with the kinase domain to form an autoinhibited conformation (Qiu and Miller, 2002). Similar to Src kinases, BRK is regulated negatively by phosphorylation of C-terminal tyrosine 447 (which is analogous to the regulatory Y530 of Src) (Figure 5). However, it remains to be ascertained how this tyrosine becomes phosphorylated in BRK. It is known that it is neither phosphorylated by itself, nor by Csk (Qiu and Miller, 2002) which plays this role in Src-family PTKs (Liu *et al.*, 1993). It is deduced that phosphorylation of BRK on Tyr-447 induces the intramolecular interaction of this residue with the SH2 domain of the protein. This subsequently induces the binding of the SH3 domain to the linker region connecting the SH2 domain and the kinase domain, which comprises the series of events that constitutively prevent the binding of ATP to the critical catalytic residues rendering BRK inactive. Mutation of tyrosine 447 to phenylalanine significantly enhances the kinase activity of BRK (Derry *et al.*, 2000; Lukong and Richard, 2003; Qiu and Miller, 2002; Miah *et al.*, 2012), suggesting an inhibitory role for this residue.

The biochemical and molecular analysis has revealed that BRK is capable of autophosphorylation at tyrosine 342 which is located in the kinase domain (Qiu and Miller, 2002). It is also observed that this tyrosine residue is located in the activation loop of the active site of the catalytic domain and is conserved through evolution in different members of PTKs. Autophosphorylation of this conserved tyrosine results in conformational alterations within the activation loop, which results in full activation of the enzyme (Lin *et al.*, 2003).

### **1.5 BRK interacting partners, substrates and biology**

Although the biological role of BRK remains largely unknown, progress has been made in identifying the endogenous physiological substrates of BRK in the normal cells as well as in various cancers. Xian *et al.* reported that BRK is co-amplified and co-overexpressed with HER2 in human breast cancer (Xiang *et al.*, 2008). Consistent with its potential role in tumorigenesis, BRK has been found in complexes with proteins such as epidermal growth factor receptor (EGFR) (Kamalati *et al.*, 1996), the putative adaptor protein BKs (Mitchell *et al.*, 2000), GTPase activating protein-associated p65 (Vasioukhin and Tyner, 1997), RNA binding proteins (e.g. Sam68, SLM-1 and SLM2) (Derry *et al.*, 2000; Haegerbarth *et al.*, 2004), the EGFR family

member HER3 (Kamalati *et al.*, 2000), and the focal adhesion protein paxillin (Chen *et al.*, 2004), the serine-threonine kinase PKB/Akt (Zhang *et al.*, 2005). It has also been shown to enhance the mitogenic signals of EGF (Brauer and Tyner, 2010; Hussain and Harvey, 2014; Kamalati *et al.*, 2000). Thus, BRK may also directly or indirectly regulate the downstream signaling molecules in the EGFR pathway.

Knockdown of BRK in breast carcinoma cells can significantly suppress proliferation (Harvey and Crompton, 2003). It is also possible that BRK can mediate cell proliferation by potentially functioning as an adaptor protein via a kinase-independent mechanism (Harvey and Crompton, 2003). It has been reported that BRK directly interacts with Akt, which results in the inhibition of Akt kinase activity and downstream signaling in unstimulated cells (Zhang *et al.*, 2005). However, upon EGF stimulation the BRK-Akt complex dissociates, resulting in activation of Akt signaling (Serfas and Tyner, 2003). Taking all the aforementioned information into account, it can be assumed that the biological function of BRK mostly depends on growth factor receptor inputs that mediate multiple intracellular signaling pathways.

The first identified BRK substrate was RNA-binding protein Sam68 (Src associated during mitosis, 68 kDa) (Derry *et al.*, 2000). Lukong *et al.* showed that Sam68 is phosphorylated upon EGF stimulation in a BRK-dependent manner in breast cancer cells (Lukong *et al.*, 2005; Lukong and Richard, 2003). Although Sam68 can be phosphorylated by several intracellular tyrosine kinases, BRK phosphorylates and sequesters Sam68 in the nucleus, resulting in negative regulation of the RNA binding function of Sam68 (Derry *et al.*, 2000). BRK substrates also link BRK to several signal transduction pathways as shown in Figure 1.7. They include the serine-threonine kinase Akt (Zhang *et al.*, 2005), insulin receptor substrate-4 (IRS-4) (Qiu *et al.*, 2005), signal transducer and activator of transcription 3 (STAT3) (Liu *et al.*, 2006), STAT5b (Weaver and Silva, 2007), p190 (Shen *et al.*, 2008), kinesin-associated protein 3A (Lukong *et al.*, 2008), polypyrimidine tract-binding (PTB) protein-associated splicing factor (PSF) (Lukong *et al.*, 2009), and  $\beta$ -catenin (Palka-Hamblin *et al.*, 2010). STAT3, for instance, is phosphorylated and specifically activated by BRK, resulting in increased cell proliferation (Liu *et al.*, 2006), while phosphorylation of Tyr 1105 in p190 enhanced its association with p120RasGAP, leading to Rho inhibition and Ras activation, which results in turn in cell migration (Bradley *et al.*, 2006; Ostrander *et al.*, 2010). Overall, the identified and validated substrates of BRK are associated with the mitogenic and cell migration pathways.

Tyrosine phosphorylation sites have been identified for a number of BRK substrates. Paxillin has been identified as a binding partner and a substrate of BRK, being phosphorylated at Y31 and Y118 upon EGF stimulation. Phosphorylation at these two positions creates a binding site for Crk11, leading to small GTPase Rac1 activation (Chen *et al.*, 2004). Through this action, BRK promotes cell motility and invasion and acts as a mediator of EGF-induced migration and invasion (Chen *et al.*, 2004).

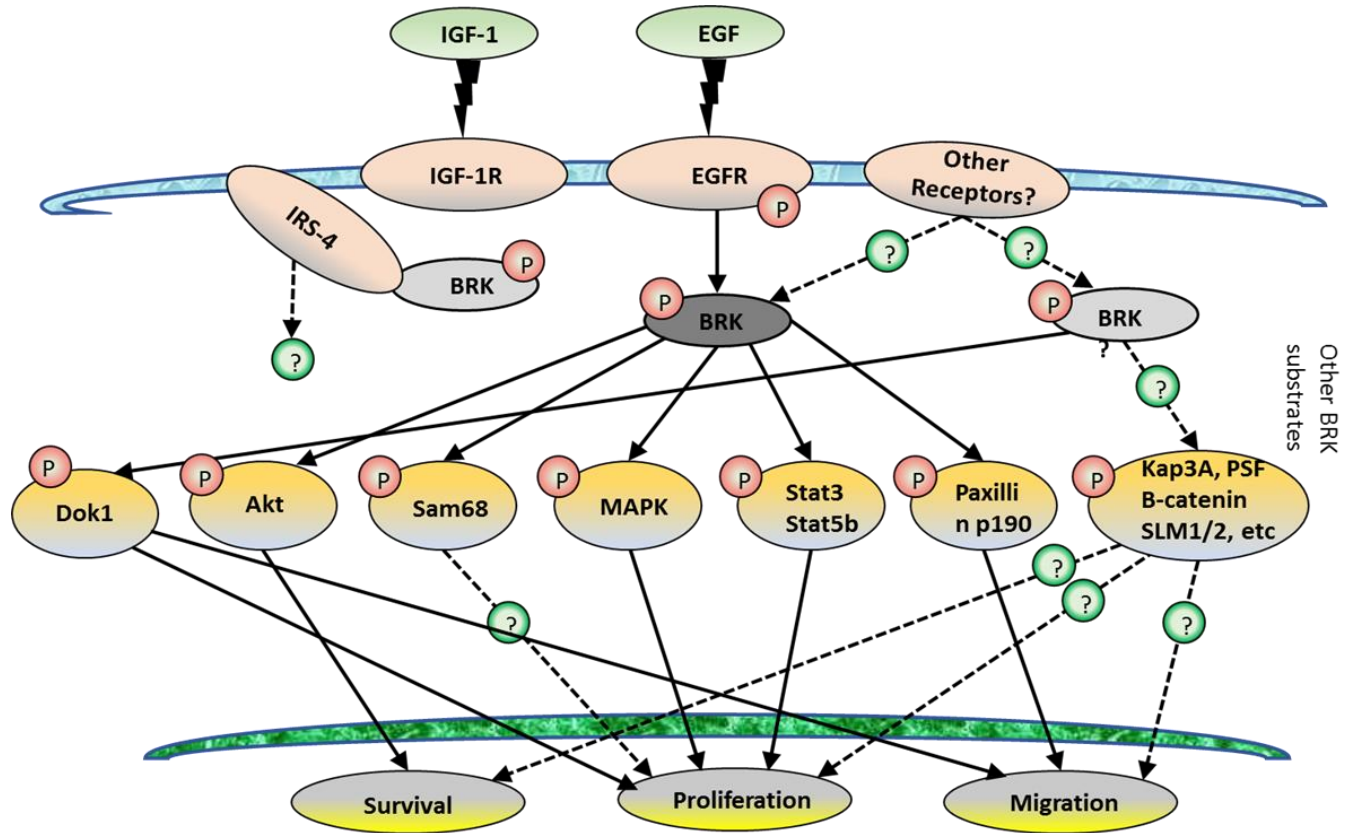


Figure 1.7. BRK acts as mediator of multiple signaling pathways. BRK signaling is downstream of EGF receptors and stimulates multiple signaling pathways which control several cellular processes, including cell migration, proliferation and survival. Other receptors like IGF-1R can activate BRK, resulting in the phosphorylation of substrates such as Dok1, IRS-4, KAP3A, PSF,  $\beta$ -catenin, SLM1 and SLM2 or other unidentified substrates.

BRK-induced phosphorylation of STAT5b was mapped to Y699 that is essential for transcriptional activation and suggesting that BRK signals downstream to STAT5b to mediate proliferation of breast cancer cells (Weaver and Silva, 2007). In addition, Lukong *et al.* showed

that KAP3A is required by BRK to promote cell migration, and BRK-induced phosphorylation of PSF leads to cell cycle arrest (Lukong *et al.*, 2008; Lukong *et al.*, 2009). Moreover, in human breast cancer, BRK becomes activated by the stimulation of insulin-like growth factor-1 receptor (IGF-1R) (Qiu *et al.*, 2005) (Figure 1.7). However, BRK is associated with nuclear and cytoplasmic  $\beta$ -catenin and inhibits  $\beta$ -catenin-regulated transcription in colorectal cancer (Palka-Hamblin *et al.*, 2010). In a recent proteomic study, a tumor suppressor, downstream of tyrosine kinase 1 (Dok1), was identified as a potential substrate of BRK (Takeda *et al.*, 2010). In addition, my studies in this thesis have demonstrated that BRK interacts with and phosphorylates Dok1 and to promote cell proliferation and migration

## **1.6 Downstream of tyrosine kinase 1 (DOK1)**

Dok1, also known as p62dok, is the prototypical member of a family of 7 adaptor proteins comprising Dok1 to Dok7. The cytoplasmic protein Dok1 is functionally characterized by an N-terminal pleckstrin homology (PH) domain that allows anchorage to the membrane, followed by a phosphotyrosine-binding (PTB) domain that is involved in protein-protein interactions, and a C-terminal region rich in tyrosine, proline and serine residues (Mashima *et al.*, 2009). Since p62Dok was first identified as a substrate of p210bcr-abl, v-Abl (Carpino *et al.*, 1997); (Yamanashi *et al.*, 1997) and many other protein tyrosine kinases, it was therefore termed Dok1, for downstream of tyrosine kinase 1 (Bose *et al.*, 2006; DeClue *et al.*, 1993; Mashima *et al.*, 2009; Niu *et al.*, 2006; Woodring *et al.*, 2004). Dok1 was functionally identified as a tumor suppressor based on several studies that demonstrated an antagonizing role of the adaptor protein towards p210bcr-abl-mediated cell transformation *in vivo* (Di Cristofano *et al.*, 2001; Niki *et al.*, 2004). An understanding of the physiological tumor suppressor role of Dok1 emerged from mice studies, which revealed a significantly accelerated onset of the p210bcr-abl-induced chronic myelogenous leukemia (CML), a myeloproliferative disorder of the hematopoietic stem cell, upon Dok1 inactivation (Di Cristofano *et al.*, 2001; Niki *et al.*, 2004). In addition, mice with combined knockouts of Dok1, Dok2, and Dok3 developed aggressive histiocytic sarcoma (Mashima *et al.*, 2010) or lung adenocarcinoma (Berger *et al.*, 2010).



The *Dok1* gene localizes to human chromosome 2p13, a locus that is prone to genetic alterations in various human tumors (Inaba *et al.*, 1991; Nelms *et al.*, 1998; Yoffe *et al.*, 1990). Dok-1, Dok-2, and Dok-3 proteins are highly expressed in hematopoietic cells (Di Cristofano *et al.*, 2001; Kawamata *et al.*, 2011). In addition, higher expression levels of Dok1 were detected in serous epithelial ovarian cancer as compared to normal tissues and this overexpression significantly correlated with disease-free survival of serous epithelial ovarian cancer patients (Siouda *et al.*, 2012). *Dok1* was also shown to be repressed in other forms of cancer including head and neck cancer (HNC), lung, liver, and gastric cancers, likewise in Burkitt's lymphoma (Balassiano *et al.*, 2011; Lambert *et al.*, 2011; Saulnier *et al.*, 2012). The function of Dok1 is regulated upon phosphorylation by a variety of receptor and non-receptor tyrosine kinases including the Src tyrosine kinase family members Lck and Fyn (Nemorin and Duplay, 2000), as well as tyrosine kinases such as Tec and Bcr-Abl (Gerard *et al.*, 2004; Lee *et al.*, 2004; Liang *et al.*, 2002; Noguchi *et al.*, 1999; Woodring *et al.*, 2004). It has also been demonstrated that Src phosphorylates Dok1 and prevents its entry into the nucleus (Niu *et al.*, 2006). Recently, Takeda *et al.* identified Dok1 as a substrate of several tyrosine kinases including BRK (Takeda *et al.*, 2010).

### **1.7 BRK expression profile in normal and cancer tissues**

Physiological expression of BRK has been reported in a number of epithelial cells, including oral (Petro *et al.*, 2004), intestinal (Vasioukhin *et al.*, 1995), prostate (Lee *et al.*, 1998), and mammary epithelial cells (Peng *et al.*, 2014a). In addition, BRK is expressed in vascular endothelial cells (Haines *et al.*, 2015), melanocytes (Lee *et al.*, 1993), lymphocytes (Kasprzycka *et al.*, 2006), normal ovary (Schmandt *et al.*, 2006), and esophageal tissues (Chen *et al.*, 2014). It has been reported that BRK expression is initiated as cells migrate away from the proliferative zone and begin the process of terminal differentiation (Vasioukhin and Tyner, 1997). Overexpression of BRK in mouse keratinocytes resulted in elevated expression of the differentiation marker filaggrin during calcium-induced differentiation (Vasioukhin and Tyner, 1997). Further, a report suggests that BRK is highly expressed in non-dividing villus epithelium of small intestine and also detectable in post irradiated crypt cells (Haegebarth *et al.*, 2009; Llor *et al.*, 1999).

BRK overexpression has also been observed in many cancers, including breast cancers (Mitchell *et al.*, 1994), some metastatic melanomas (Easty *et al.*, 1997), colon cancers (Llor *et al.*, 1999), squamous cell carcinomas (Petro *et al.*, 2004), prostate cancers (Derry *et al.*, 2003), malignant lymphocytes (Kasprzycka *et al.*, 2006), as well as in high-grade serous carcinomas and ovarian cancer cell lines (Schmandt *et al.*, 2006). It has also been reported that a detectable level of BRK is present in transforming B and T cells as well as cutaneous T-cell lymphomas (Kasprzycka *et al.*, 2006). BRK is also expressed in pancreatic cancer and promotes cell migration and invasion through ERK signaling cascade (Ono *et al.*, 2014). Additionally, it has been reported that BRK is significantly downregulated in esophageal squamous cell carcinoma and is associated with a poor prognosis (Chen *et al.*, 2014). However, overexpression of BRK in a subgroup of non-small cell lung cancers is associated with a poor prognosis (Zhao *et al.*, 2013b), suggesting that the role of BRK may be tissue specific.

### **1.8 Implication of BRK in breast cancer**

BRK is expressed in many breast cancer cell lines and primary breast tumors (Barker *et al.*, 1997; Mitchell *et al.*, 1994). However, the expression of BRK is low or absent in the normal human breast tissue or at any stage of mammary gland differentiation in the mouse (Llor *et al.*, 1999). Recently, it has been reported that the activated form of BRK occurs only in mammary tumors, and not in normal tissue (Peng *et al.*, 2014a). This suggests that the enzymatic activity of BRK is associated with mammary tumor progression.

It was recently determined that approximately 85% of surgical samples of breast carcinomas show BRK mRNA expression (Harvey *et al.*, 2009). The dramatic induction of BRK in a significant percentage of human breast tumors, therefore, suggests a role for BRK in the etiology of breast cancer. In fact, *BRK* localizes to chromosome 20q13.3, a region of the genome that is frequently amplified in breast cancer (Chin *et al.*, 2006; Harvey and Crompton, 2004). BRK overexpression has also been associated with ER-positive status (Zhao *et al.*, 2003) and with long-term survival in breast cancer patients (Aubele *et al.*, 2007; Aubele *et al.*, 2009; Aubele *et al.*, 2008), suggesting that BRK may play different roles in breast cancer depending on the cellular and/or molecular context. These studies, as a whole, propose that although cell proliferation and migration are potential molecular processes modulated by BRK in breast

cancer, the cellular role of BRK may depend on its activation status and may also be cell type specific.

### 1.9 Physiological role of BRK

BRK (PTK6)-null mice and mouse xenografts as well as recent transgenic mouse models have helped in the understanding of the potential physiological role of BRK in tumorigenesis. BRK-deficient mice showed delayed and decreased expression of I-FABP, a differentiation marker, and enhance proliferation and growth of enterocytes of the small intestine (Haegebarth *et al.*, 2006). However, transplantation of BRK alone, ErbB2 alone or both BRK and ErbB2 stably overexpressing immortalized pluripotent mammary epithelial cell line (Comma-1D) into mouse mammary fat pad induce tumor mass in BALB/c mice, suggesting a role of BRK in tumorigenesis (Xing *et al.*, 2008).

Muller *et al.* for the first time showed the link of ErbB2 in mammary tumorigenesis (Muller *et al.*, 1988). In this study, the rat homolog of HER2/ErbB2 (*Neu*) was targeted under mouse mammary tumor virus (MMTV) promoter to the mouse mammary gland. This MMTV-*Neu mouse* spontaneously developed multifocal invasive tumors as early as 12 weeks with 100% penetrance (Muller *et al.*, 1988). Additionally, it was shown that polyoma middle T antigen induces mammary gland tumorigenesis with 100% penetrance with a latency 1-6 months and these tumors showed high lung metastatic potential (Fantozzi and Christofori *et al.*, 2006). However, c-Src-induced mammary gland tumorigenesis was not observed when it was targeted to the mouse mammary gland under the MMTV promoter (Webster *et al.*, 1995).

In the first transgenic mouse model, BRK (PTK6) was targeted under the whey acidic protein (WAP) promoter for mammary gland specific expression. Only 30% of these BRK transgenic mice developed tumors, with a latency average of 10 months and showed delayed involution (Lofgren *et al.*, 2011). Additionally, in another study in which BRK was guided to mouse mammary gland under the MMTV promoter, only 11% penetrance was observed with a long latency time of 21 months (Peng *et al.*, 2013). Additionally, it was also reported that Ptk6<sup>-/-</sup> mice developed skin tumor with a latency 11 months (Chastkofsky *et al.*, 2015). Further, when this MMTV-BRK transgenic strain was crossed with MMTV-*Neu* (HER2), no significant incident of tumorigenesis was observed (whereas 80% MMTV-*Neu* strain developed tumors

within 8 months) (Peng *et al.*, 2013). This was surprising since BRK and HER2 coexpression has been shown to have synergistic effect in the induction of cell proliferation and the promotion of tumor development (Peng *et al.*, 2013; Xing *et al.*, 2008). In fact, it was observed that the latency time was increased for tumor formation in these composite mice (Peng *et al.*, 2013), suggesting undetectable involvement of wild type BRK in mammary gland tumorigenesis.

## **2.0 Hypothesis and Objectives**

### **2.1 Hypothesis**

Despite significant progress, the mechanism of BRK overexpression, as well as the role of BRK in tumorigenesis, prognosis and in signaling is poorly understood and the physiological role of BRK is unknown. Since wild-type BRK does not seem to play a significant role in mammary tumorigenesis and the activated form of BRK is observed in breast tumor tissues and not the normal mammary gland, it is possible the activated form of BRK plays a major role in mammary tumorigenesis. Therefore, we hypothesized that BRK contributes to the mammary tumor formation and that the activity of BRK is required for the morphogenesis of mammary gland tumors and metastasis of breast cancer *in vivo*. The overall objective of this project is to determine how the activation of BRK affects the cellular and physiological role of this kinase in breast cancers.

### **2.2 Objectives of the project:**

1. To determine the effect of BRK activation on cell migration, proliferation and anchorage-independent cell growth.
2. To examine the role of BRK activation in tumor formation in the mammary glands of mice.
3. To determine the mechanism of action of BRK in the promotion of cell growth and proliferation.

### 3. Materials and Methods

#### 3.1. Antibodies and Reagents.

The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): anti-BRK (N19, sc-916), anti-ERK1/2 (sc-1647), anti-pERK1/2 (sc-16982), anti-GFP (sc-8334), anti-pTyr pY20 (sc-508), anti-p-p38 (sc-17852-R), anti-p38 (sc-535), anti- $\beta$ -actin (sc-130300). Both anti-phosphotyrosine (anti-pTyr) clone 4G10 and anti-pBRK (Y342) were from Upstate (Lake Placid, NY). Anti-PRMT1 antibody was obtained from Millipore (Billerica, MA, USA). ). Anti-Dok1 was a gift from Dr. Ryuji Kobayashi (University of Texas, Austin, USA). The anti-Sam68 (AD1) polyclonal antibody was generously given by Dr. Stephane Richard (McGill University, Canada). Proteasome inhibitors MG132 or Lactacystin were purchased from Calbiochem (MA, USA), cycloheximide from Sigma-Aldrich Corporation (St. Louis, MO) and EGF from Upstate (Lake Placid, NY). The reagents that were used in the experiments are listed in **Table 3.2**, and names and addresses of suppliers are listed in **Table 3.3**.

**Table 3.2. List of reagents and suppliers**

| Reagents                                     | Suppliers      |
|--|----------------|
| 1 kb DNA ladder, N3232S                      | NEB            |
| 4', 6-diamidino-2-phenylindole (FITS), D9542 | Sigma- Aldrich |
| 100 bp DNA ladder, N3231S                    | NEB            |
| Acrylamide, 0341                             | AMRESCO        |
| Adenosine Triphosphate (ATP), A1205          | Teknova        |
| Agarose I <sup>TM</sup> , 0710               | AMRESCO        |
| Ampicillin, 0339                             | AMRESCO        |
| Ammonium persulfate (APS), A3678             | Sigma- Aldrich |
| Alkaline phosphatase, M0290S                 | NEB            |
| Aprotinin, A6279                             | Sigma- Aldrich |

|  |                          |
|--|--------------------------|
| Bisacrylamide, 0172                                  | AMRESKO                  |
| Crystal violet, 65092A-95                            | EMD                      |
| Difco™ skim milk, 232100                             | BD                       |
| dNTP, D0056  | GeneScript               |
| Dulbecco's Modified Eagle Medium (DMEM), SH30022.01  | Thermo Scientific Fisher |
| Enhanced Chemiluminescence Substrate (ECL), 104001EA | PerkinElmer              |
| Fermtech® Yeast Extract, 1.11926.1000                | EMD                      |
| Fetal Bovine Serum (FBS), SH30397.03                 | Thermo Scientific Fisher |
| Gelatin, G1890                                       | Sigma- Aldrich           |
| Glycine, 0167  | AMRESKO                  |
| Kanamycin sulfate, 0408                              | AMRESKO                  |
| Laemmli sample buffer, S3401                         | Sigma- Aldrich           |
| Lysogeny Broth (LB) agar, L2897                      | Sigma- Aldrich           |
| Methanol, MX0485                                     | EMD                      |
| N,N,N',N'-Tetramethylethylenediamine (TEMED), 87689  | Sigma- Aldrich           |
| Paraformaldehyde, PX0055-3                           | EMD                      |
| Phenylmethylsulfonyl fluoride (PMSF), P7626          | Sigma- Aldrich           |
| Polyethylenimine, 25987-06-8                         | Sigma- Aldrich           |
| SDS, 151-21-3  | Sigma- Aldrich           |
| Sodium azide, S8032                                  | Sigma- Aldrich           |
| Sodium chloride (NaCl), 0241                         | AMRESKO                  |
| T4 DNA ligase, M0202S                                | NEB                      |
| Taq Polymerase, M0273S                               | NEB                      |

|                       |                |
|-----------------------|----------------|
| Tris, 0826            | AMRESCO        |
| Trptone, 1.07213.1000 | EMD            |
| Triton™ X-100, X-100  | Sigma- Aldrich |
| Trypsin-EDTA, T4049   | Sigma- Aldrich |
| TWEEN® 20, 0777       | AMRESCO        |

**Table 3.3. List of names and Addresses of the Suppliers.**

| Company Name             | Address                        |
|--------------------------|--------------------------------|
| AMRESCO                  | North York, Ontario, Canada    |
| Bio-Rad                  | Hercules, California, USA      |
| BD                       | Mississauga, Ontario, Canada   |
| Cell signaling           | Whitby, Ontario, Canada        |
| Corning                  | NY, USA                        |
| Dojindo                  | Rockville, Sunnyvale, USA      |
| EMD                      | Madison, Wisconsin, USA        |
| GeneScript               | New Jersey, USA                |
| Gibco                    | Burlington, Ontario, Canada    |
| Invitrogen               | Burlington, Ontario, Canada    |
| New England Biolab (NEB) | Mississauga, Ontario, Canada   |
| Olympus                  | Richmond Hill, Ontario, Canada |
| Pall Corporation         | Washington, NY, USA            |
| PerkinElmer              | Boston, Massachusetts, USA     |
| Qiagen                   | Mississauga, Ontario, Canada   |
| Santa Cruz               | Santa Cruz, California, USA    |

|                          |                              |
|--------------------------|------------------------------|
| Sigma-Aldrich            | Oakville, Ontario, Canada    |
| Teknova                  | Hollister, California, USA   |
| Thermo Fisher Scientific | Whatham, Massachusetts, USA  |
| VWR                      | Mississauga, Ontario, Canada |
| Whatman                  | Piscataway, New Jersey, USA  |

### 3.2. Cell cultures

HEK293, BT20, MCF-10A, AU565, MDA-MB-231, MDA-MB-435, MDA-MB-468, T47D, HBL100, MCF7 and SKBR3 cells were originally obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in high glucose (4.5 g/l), Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (Thermo Scientific, Logan, Utah, USA) and containing 4 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich). MCF10A cells (ATCC, Manassas, VA, USA) were cultured in DME/F-12 1:1(1X) medium (Thermo scientific) containing 5% horse serum (Sigma-Aldrich, St Louis, USA), 20 ng/mL EGF (Upstate, Lake Placid, NY, USA), 0.5 µg/mL hydrocortisone (Sigma-Aldrich), 100 ng/mL cholera toxin (Sigma-Aldrich, St Louis, USA), 50 U/mL penicillin, and 50 mg/mL streptomycin as well as 10 ng/mL insulin (Sigma-Aldrich, St Louis, USA), as described by Debnath *et al.* (Debnath *et al.*, 2003).

### 3.3. Mammalian cell expression and immunoprecipitation

HEK293 cells were maintained in DMEM containing 10% fetal bovine serum, 50 U/mL of penicillin-streptomycin. Cells were rinsed and supplemented with a fresh serum-free culture medium just before transfection. The cells were transiently transfected with 1% Polyethylenimine “Max” (PEI) (Polysciences Inc., Warrington, PA, USA) at a ratio of 3:1 reagent to DNA with the total amount of DNA being 2.5 µg per well in six-well dishes. For each well, 2.5 µg of DNA was added to 107.5 µl of sterile 0.15 M NaCl in a microcentrifuge tube and vortexed gently for 10 sec. 15 µl 1% PEI was added to the DNA mixture and vortexed gently for 10 sec. The DNA-PEI complex was then incubated for 10 min at room temperature. The mixture was added dropwise to wells containing 2 mL of complete media and the plates incubated at 37



°C. Cells were washed 4 h after transfection, then cultured in complete media for an additional 16-48 h.

Whole cell lysates were directly prepared in 2x Laemmli buffer ((Sigma-Aldrich, St Louis, USA). For immunoprecipitation, cells were washed with cold 1x Phosphate-buffered saline (PBS), lysed with freshly prepared lysis buffer (20 mM Tris, pH 7.5, 1% triton (TX-100), 150 mM NaCl, protease inhibitors: Aprotinin 5 mg/L and 0.1 mM PMSF) containing 0.3 mM sodium orthovanadate (Enzo Life Sciences). Lysates were prepared by incubating the harvested cells in ice-cold lysis buffer for 30 min, followed by centrifugation for 10 min at 14 k rpm. Supernatants were collected and transferred into fresh tubes and incubated with 1 µg of the appropriate antibody and maintained on a gyrorotator for 1 h at 4 °C. 20 µl of Protein-A agarose beads were then added to the samples and incubated for another 40 min on the gyrotator at 4 °C. The beads were washed twice with ice-cold lysis buffer and 1x PBS and the immunoprecipitated proteins were resolved via SDS-PAGE.

### **3.4. Generation of stable cell lines**

We generated two sets of stable cell lines, BRK-overexpressing cells and BRK-knockdown cells. To the cells stably expressing BRK, an amphotropic HEK293 derived Phoenix packaging cells were used to package pBabe-puro retroviral system. The packaging cells were cultured on 10 cm, gelatin-coated plates in 10 mL of DMEM medium supplemented with 10% BCS. For viral production, the packaging cells in a 10 cm plate were transfected with 10 µg of retroviral DNA using 1% PEI (Polysciences, Inc). The transfection mix contained the plasmid and 60 µL of 1% PEI plus 430 µL of 0.15 M NaCl. Virus-containing supernatant was collected at 24 h and 48 h time points, filtered through 0.45 µm syringe filter, aliquoted and used immediately or stored at -80 °C. To infect MDA-MB-231 cells, the virus-containing supernatant was supplemented with 10 µL polybrene (Sigma-Aldrich, St Louis, USA), and overlaid on the target cells. After overnight incubation with the viral supernatant, this was changed to fresh culture medium. Pools of MDA-MB-231 cells stably expressing GFP alone, GFP-BRK-WT and GFP-BRK-YF fusions were selected with puromycin (Sigma-Aldrich, St Louis, USA). Expression of EGFP from the GFP-tagged BRK was detected by fluorescence microscopy 48 h - 72 h post-infection.

To produce a stable BRK knockdown cell line we used BRK-expressing parental breast cancer cell lines BT20 and SKBR3. This knockdown experiment was performed according to the manufacturer's protocol by using shRNA lentiviral vector plasmids from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The shRNA plasmids generally consist of a pool of three to five lentiviral vector plasmids, each encoding target-specific 19-25 nucleotides short hairpin RNAs (shRNAs) designed to knockdown gene expression. As controls, the cells were infected with a control shRNAs or with a GFP alone control plasmid. A set of three shRNAs was used to complete this knockdown process. 1) GFP-Control plasmid that allowed the confirmation of the transduction efficiency by expressing GFP, detectable by fluorescence microscopy. 2) Control-shRNA plasmid that encodes a scrambled shRNA sequence, which does not lead to the specific degradation of any mRNA. 3) BRK-shRNA lentiviral vector plasmids, which contains target-specific 19-25 nucleotides in shRNA, designed to knock down BRK gene expression. Transfected cells were selected using puromycin (Sigma-Aldrich, St Louis, USA).

### **3.5. RT-PCR and qPCR**

Total RNA was extracted using the RNeasy mini plus kit (Qiagen, Maryland, USA). Quantity and quality of RNA were determined spectrophotometrically. 1 µg of RNA was utilized for cDNA synthesis using the iScript cDNA Synthesis kit (Bio-Rad Inc., Hercules, CA) according to manufacturer's protocol. In brief, 1 µg of RNA was added to a mix containing 4 µL 5X iScript Reaction Mix and 1 µL iScript Reverse Transcriptase in a final volume of 20 µl. Then the cDNA synthesis reaction was completed by incubating at 25 °C for 5 min, followed by 30 min at 42 °C and 5 min at 85 °C. Samples were either used immediately or preserved at -20 °C. Total RNA was used as a template for the synthesis of cDNA using the iScript cDNA Synthesis kit (Bio-Rad) according to manufacturer's specifications. Briefly, 1 µg of RNA was added to a mix containing 4 µL 5X iScript Reaction Mix, 1 µL iScript Reverse Transcriptase and 1 µg RNA in a final volume of 20 µL. Synthesis of cDNA was completed by incubation at 25 °C for 5 min, followed by 30 min at 42 °C and 5 min at 85 °C. Samples were used immediately or stored at -20 °C.

Real Time PCR- quantification of cDNA was performed using a fluorescence-based detection system (Step One Plus, Applied Biosystems). Using Dok1 primers, PCR was performed in a final volume of 10 µL containing 0.3 µL cDNA, 33 ng of each primer and 5 µL of

SsoFast EvaGreen Supermix (Bio-Rad Inc., Hercules, CA). Cycling conditions were: 20 sec at 95 °C followed by 40 cycles of 95 °C for 3 sec, and 58.2 °C for 30 sec. Data was analyzed by the  $\Delta$ Ct method.

qPCR was performed using 50 ng of cDNA in 50  $\mu$ L reaction mixtures containing 0.02 mM deoxynucleoside triphosphate (dNTP) mix (GenScript, NJ, USA), 10 $\times$  Standard Taq buffer (New England Biolabs, MA, USA), 0.0125 U/ $\mu$ L of Taq DNA polymerase (New England Biolabs, MA, USA), and 0.4 mM each primer. Primers specific for human Dok1 and reference RPL 13A were the following: Dok-1 forward 5'-CTA CAA CCC TGC CAC TGA TGA CTA-3' and reverse primer 3'-CTA GAG AGC CCA CAG TCC CAG CTC-5'; RPL13A forward 5'-CAA GGT GTT TGA CGG CAT CC-3'; and reverse primer, 3' GCT TTC TCT TTC CTC TTC TCC 5'. Reaction mixtures containing cDNA prepared from HEK 293, HEK293-GFP-BRK-WT and HEK293-GFP-BRK-YF. The cycling program run was 95 °C for 2 min, followed by 35 PCR cycles (95 °C for 30 sec, 60 °C for 15 sec, 72 °C for 45 sec) and a final extension for 5 min at 72 °C. PCR products were run in a 1% agarose gels and visualized by GelRed staining using the AlphaDigiDocTM, Genetic Technologies, Inc, USA.

### 3.6. Dok1 expression vectors and mutagenesis

GFP-Dok1 construct, a gift from Dr. Bakary S. Scylla, Lyon, France, was used to generate GFP-Dok1 deletion mutants. Five pairs of primers were used to amplify five Dok1 cDNA variants of progressively differing lengths which were then cloned at the C-terminal of the GFP sequence in the *Eco*RI and *Sma*I sites of the pEGFP-C1 vector backbone: Dok $\Delta$ 1: 5'-AGT GAA TTC GGA CGG AGC AGT GAT GGA A-3' and 3'- ATT CCC GGG TCA AGT CTC AAC TGC CTG-5'; Dok $\Delta$ 2: 5'-AGT GAA TTC GGA CGG AGC AGT GAT GGA A -3' and 3'- ATT CCC GGG TCA CTT CCG TTG TAC TCC-5'; Dok $\Delta$ 3: 5'-AGT GAA TTC GGA CGG AGC AGT GAT GGA A-3' and 3'-ATT CCC GGG TCA CTT GGC CTT CAG CAA-5'; Dok $\Delta$ 4: 5'-AGT GAA TTC GGA CGG AGC AGT GAT GGA A and 3'-ATT CCC GGG TCA CTT CAC CCG AGC TTG-5'; Dok $\Delta$ 5: 5'-AGT GAA TTC GGA CGG AGC AGT GAT GGA A-3' and 3'-ATT CCC GGG TCA CTT GGG AGC AAG GAG-5'. The Dok1 C-terminal segment extending from the IRS-PTB and spanning 222 amino acids was cloned into the *Eco*RI - *Not*I sites of the pGEX-5-x-3 vector, using the primers: 5'-ATA GAA TTC CGA CGG AGC AGT GAT GGA A-3' and 5'-ATA GCG GCC GCT CAG GTA GAG CC-3'. The Dok1 cDNA

was cloned at the C-terminus of the mCherry (a generous gift from Dr. Scot Stone, University of Saskatchewan, Saskatoon, SK, Canada) sequence in the BglIII and SmaI restriction sites of the pmCherry-C1 vector backbone using the primers: 5'-AAA AGA TCT ATG GAC GGA GCA GTG ATG and 3'-ATT CCC GGG TCA GGT AGA GCC CTC TGA. The composite mcherry-Dok1 cDNA was subcloned into the KpnI and NotI sites of a pShuttle-CMV plasmid by using a set of primers 5'-AAA GGT ACC GTC GCC ACC ATG GTG AGC AAG GGC GAG and 3'-ATA GCG GCC GCT CAG GTA GAG CC. Site-directed mutations of human Dok1 were introduced using a Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All constructs were verified by sequencing.

### **3.7. Subcellular fractionation**

Cells were fractionated into four different subcellular parts: cytosolic, membrane, nuclear, and cytoskeletal matrix fractions, using ProteoExtract Subcellular Proteome Extraction Kit (EMD, 539790) following the manufacturer's protocol. In brief, cells were washed twice with wash buffer, then cells were subjected to extraction buffer I to elute cytosolic fraction, and extraction buffer II for the membrane fraction. Benzonase Nuclease was applied to collect the nuclear fractions and finally cytoskeletal matrix was obtained by using extraction buffer IV. All the fractionated proteins were resolved via SDS-PAGE and analyzed by immunoblotting.

### **3.8. Immunoblotting**

Total cell lysate prepared from transfected or non-transfected cells were subjected to sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoresed proteins were transferred to nitrocellulose membranes (Bio-Rad). In general, membranes were blocked for 30-45 min in a 5% non-fat dry milk or in 1.0% bovine serum albumin when phosphotyrosine antibodies were used. The membranes were incubated overnight at 4 °C with primary antibodies prepared according to manufacturer's instructions. Polyclonal goat HRP-conjugated secondary antibodies against mouse or rabbit IgG (Bio-Rad Inc., Hercules, CA, 1:10000 dilution) were incubated on membranes for 1 h at 4 °C, followed by chemiluminescence (ECL) detection using the ECL kit (DuPont, Wilmington, DE, USA) and protein bands visualized by autoradiography.

### **3.9. Cell migration (Wound healing or Scratch) assay**

Cells were seeded into 6 well plates at a density of  $1 \times 10^6$  cells /well and cultured until 80-90% confluent in the culture medium. A 1000  $\mu$ L sterile pipette tip was used to scratch a constant-diameter stripe or wound diagonally in the confluent monolayer. The medium and cell debris were aspirated away and replaced with a fresh culture medium. After wounding 0, 12, 24, 36 and 48 h later plates were imaged using Olympus 1X51 inverted microscope (Olympus America, Center Valley, PA) with a 10X phase contrast objective. These experiments were repeated at least three times. Values were means  $\pm$  SD from at least three independent experiments.

### **3.10. Transwell assay**

The cells were cultured in serum free-medium overnight, harvested and resuspended in serum-free medium. A suspension of cells ( $5 \times 10^5$  cells) was added to the upper chamber of 24-well Transwell plates (Corning Incorporated, Corning, NY, USA) and a complete medium (containing 10% FBS) was added into the bottom chamber of the Transwell plate (6.5 mm diameter and 8.0  $\mu$ m). The cells were then incubated at 37 °C and 5% CO<sub>2</sub> for 24 h and the non-migrated cells were removed by using a sterile cotton swab from the upper surface of the filter. The cells that migrated through the chamber onto the lower surface of the filter were fixed with paraformaldehyde and stained with crystal violet for 30 min. The number of migrating cells was counted (Five high power fields were counted per filter to score for migration) under an Olympus 1X51 microscope and the count was scored as migration in comparison to parental control cells.

### **3.11. Soft agar anchorage-independent growth assay**

MDA-MB-231 cells were suspended in a top layer of DMEM-10% calf serum containing 0.35% low melting point agarose (Sigma-Aldrich, St Louis, USA) at 42 °C and overlaid onto the solidified 0.6% agarose layer containing a DMEM-10% FBS. After 3 weeks of incubation at 37 °C, the numbers of colonies formed were counted in triplicate wells from five fields photographed with a 10 $\times$  objective.

### **3.12. Mouse tumorigenicity (Xenograft) assay**

Xenograft experiments were conducted in 6-7 week old female athymic nude mice, purchased from the National Cancer Institute, Frederick, MD. MDA-MB-231 cells expressing each of the GFP-BRK fusions including GFP alone as stable pools were harvested in PBS and resuspended in Matrigel (BD Biosciences). For each injection,  $2.5 \times 10^6$  MDA-MB-231 cells in a 100  $\mu$ L volume of Matrigel were injected subcutaneously bilaterally into mammary fat pad number 5 according to standard injection procedures, with 4 animals injected per cell line. Once tumors were palpable (about 2 weeks after injection of tumor cells), mammary primary tumor growth rates were monitored and analyzed by measuring tumor length (L) and width (W), for about eight weeks. Tumor size was assessed by measurements with an electronic caliper. Volume was calculated as  $0.50 \times \text{length} \times \text{width}^2$ . Nude mouse xenograft experiments were performed under an animal protocol approved by the Animal Care Unit and Committee of the University of Saskatchewan. Mice were sacrificed in a humane manner when the tumor size exceeded the approved limit by animal ethical authority.

### **3.13. Recombinant GST-fused protein expression and GST-pull-down assay**

GST pull-down assays were performed as previously described (Chen *et al.*, 1999). GST-tagged constructs (GST, GST-BRK-SH3 and GST-BRK-SH2 previously generated by the Lukong lab) were expressed in *E. coli* (BL21 strain), and cultured in 2XYT media. Protein induction was initiated by the addition of 1 mM IPTG to the bacterial cultures at an optical density (600 nm) of 0.6. Bacterial cells were then lysed by sonication in ice-cold 1x PBS buffer containing protease inhibitors: 1  $\mu$ g/mL aprotinin, and 0.01% phenylmethanesulfonyl fluoride (PMSF), supplemented with a protease inhibitor cocktail comprising 23 mM AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), 2 mM Bestatin, 100 mM EDTA (Ethylenediaminetetraacetic acid), E-64 0.3 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E 64), 0.3 mM Pepstatin A, in dimethylsulfoxide (DMSO) (P8465, Sigma-Aldrich Corporation, St. Louis, MO). Lysates were then incubated with Glutathione Sepharose beads (GST, Novagen, CA, USA.). In brief, the pull-down experiments were carried out using GST, GST-BRK-SH3 and GST-BRK-SH2 proteins immobilized on glutathione-Sepharose

beads, which were incubated with cell lysates followed by 3 times wash with ice cold PBS., The bound proteins were then resolved by SDS-PAGE as described above.

### **3.14. *In vitro* kinase assay**

*In vitro* kinase assays were performed using 100 ng GST-BRK and a 10  $\mu$ L volume of substrate (GST-C-terminus Dok1, residues 260-481) in a reaction volume of 50  $\mu$ l comprising 20  $\mu$ L kinase buffer (25 mM MOPS, pH 7.2, 2.5 mM DTT, 12.5 mM  $\beta$  glycerol-phosphate and 5 mM EGTA (Signalchem, Richmond, BC, Canada) with or without 200  $\mu$ M ATP. The reaction mixture was incubated at 30  $^{\circ}$ C for 30 min to complete the kinase reaction and eventually terminated by the addition of 2x Laemmli sample buffer. The samples were then boiled at 100  $^{\circ}$ C and resolved via SDS-PAGE (as described above).

### **3.15. *In vivo* ubiquitination assays**

GFP-BRK-YF expressing HEK293 stable cells were transfected with HA-tagged ubiquitin and/or Dok1 plasmids and the cells treated with 10  $\mu$ M MG132. The cell lysates were incubated with the primary rabbit anti-Dok1 antibody followed by protein-A agarose conjugation and immunoblotting with anti-HA antibody to detect ubiquitinated Dok1.

### **3.16. Statistical analysis**

For statistical analysis, one-way and two-way ANOVA followed by a post hoc Newman-Keuls test was used for multiple comparisons using GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com). The results are given as the means  $\pm$  SD.  $P \leq 0.05$  was considered statistically significant.

## 4. RESULTS

The results of this thesis are divided into two major parts under the following headings:

4.1 Constitutive activation of breast tumor kinase accelerates cell migration and tumor growth *in vivo*

4.2 BRK targets Dok1 for ubiquitin-mediated proteasomal degradation to promote cell proliferation and migration

### **4.1. Constitutive activation of breast tumor kinase accelerates cell migration and tumor growth *in vivo***

BRK is a non-receptor tyrosine kinase overexpressed in most human breast tumors and breast tumor cell lines. However, a positive or negative BRK activity regulator has not been identified. Studies have indicated that BRK is activated upon stimulation of the epidermal growth factor receptor (EGFR) and insulin-like growth factor-I receptor (IGF-IR) (Lukong *et al.*, 2003; Lukong *et al.*, 2005). Lukong *et al.* and others previously demonstrated that mutation of tyrosine 447 to phenylalanine results in a constitutively active variant of BRK whose activity is significantly higher than BRK-WT (Lukong *et al.*, 2005; Qiu and Miller 2002; Lukong *et al.*, 2009). During the preparation of this thesis, a newly published report showed that the activated form of BRK is found only in breast tumors and not in the normal mammary gland (Peng *et al.*, 2014a). This implies that the activation of BRK may play a prominent role in breast cancer tumorigenesis.

To understand the cellular and physiological significance of full activation of BRK, we first assessed the activity of various BRK mutants followed by the generation of stable cell lines expressing various BRK variants including the constitutively active form of BRK (BRK-Y447F). The stable cell lines were subjected to cell proliferation, migration and colony formation assays. Since the function of active BRK in tumors is not fully understood, we have also investigated the role of constitutively active BRK in tumor formation in Xenograft mice. We present evidence that full activation of BRK significantly enhances the cellular and physiological properties of BRK, which include cell proliferation, migration and tumor formation.



### 4.1.1. Results

#### 4.1.1.1. Tyr447Phe BRK mutant is significantly more active than the wild-type BRK

It was previously reported that some BRK mutants displayed varying degrees of activities compared to wild-type BRK when expressed in human embryonic kidney HEK293 cells (Qiu and Miller, 2004). These included W44A, SH2 and SH3 deletion mutants ( $\Delta$ SH2 and  $\Delta$ SH3), and Y342A, K219M, and Y447F mutants (Figure 4.1A). Trp44 is a conserved residue in the SH3 domain previously shown to make contacts with proline residues in the linker region and further stabilize the inactive conformation of BRK. Mutation of Trp44 to alanine (W44A) was shown to abolish the SH3–linker interaction (Kim *et al.*, 2007) and enhance enzyme activity (Qiu and Miller, 2004). Tyr342 is the major autophosphorylation site necessary for full activation of BRK, while mutation of Lys219, an essential active site residue, to methionine abrogates catalytic activity (Qiu and Miller, 2004). In order to understand the role of activated BRK in various cellular processes, we first generated GFP-tagged BRK wild-type (WT) and constitutively active BRK Y447F constructs. To ensure that the GFP tag did not interfere with the BRK enzymatic activity, we compared the activities of various non-tagged BRK constructs to those of GFP-tagged constructs in transfected HEK293 cell lysates (Figure 4.1B). The lysates were subjected to SDS-PAGE analysis followed by immunoblotting using anti-phosphotyrosine antibody, pY20, designed specifically to recognize phosphorylated tyrosine residues and using phospho-BRK antibody designed to recognize autophosphorylation of BRK at Tyr342. As previously shown, BRK mutants  $\Delta$ SH3, K219M and Y342A displayed significantly lower or no activity as compared to the BRK WT (Qiu and Miller, 2004) as demonstrated by the degree of staining by pY20 (Figure 4.1B, top panel, compare lanes 4, 5 and 7 to lane 1). The BRK mutant, W44A showed a slightly lower activity as compared to the WT. As anticipated, the  $\Delta$ SH2 (lane 3) and Y447F (lane 6) mutants displayed the highest levels of activity, confirming that docking of pY447 to the SH2 domain is equally important in BRK to stabilize an inactive conformation. In the context of the present work, it is important to note that both GFP-BRK-WT (lane 9) and GFP-BRK-Y447F (lane 10) displayed catalytic activity and that, as expected, GFP-BRK-Y447F showed a much higher level of substrate phosphorylation compared to GFP alone (lane 8) or GFP-BRK-WT (lane 9). These activity results using pY20 were corroborated with anti-phospho-BRK staining, although it is not clear why substrate phosphorylation by GFP-BRK  $\Delta$ SH2 (lane

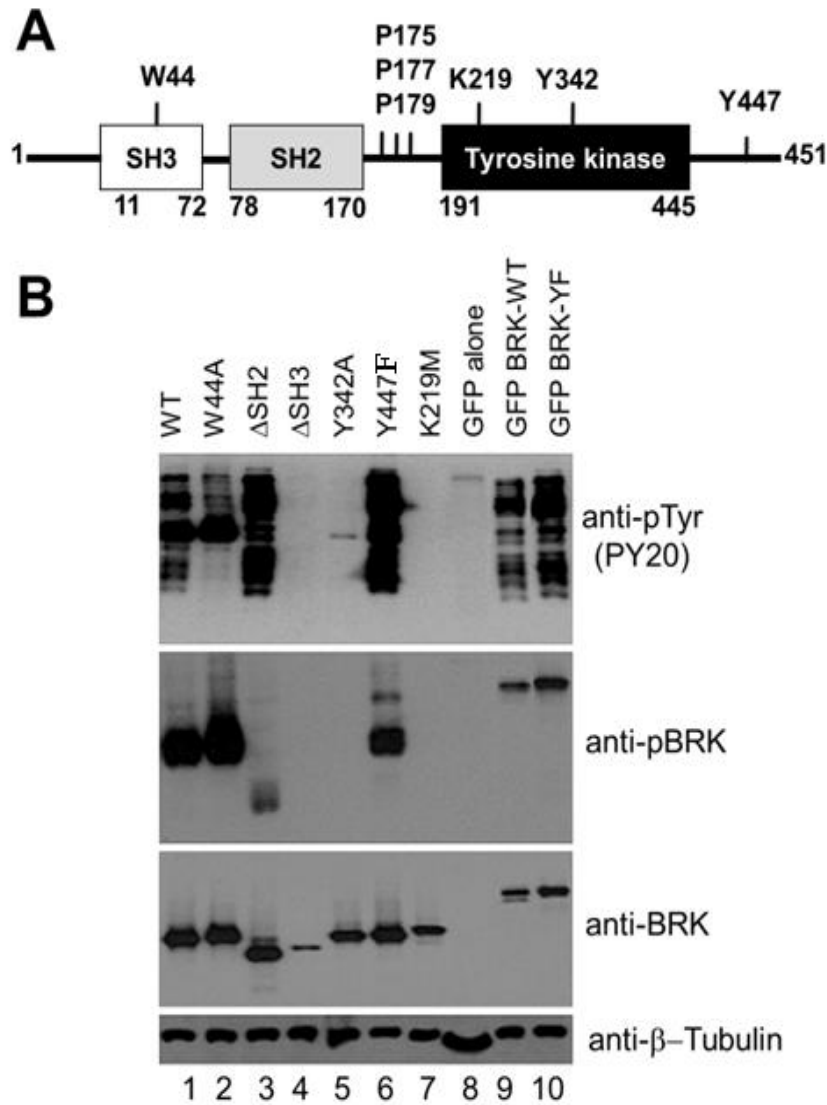


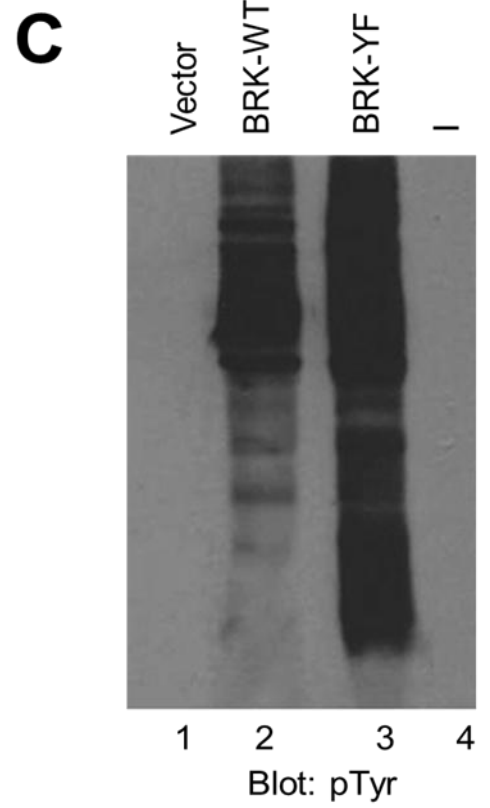
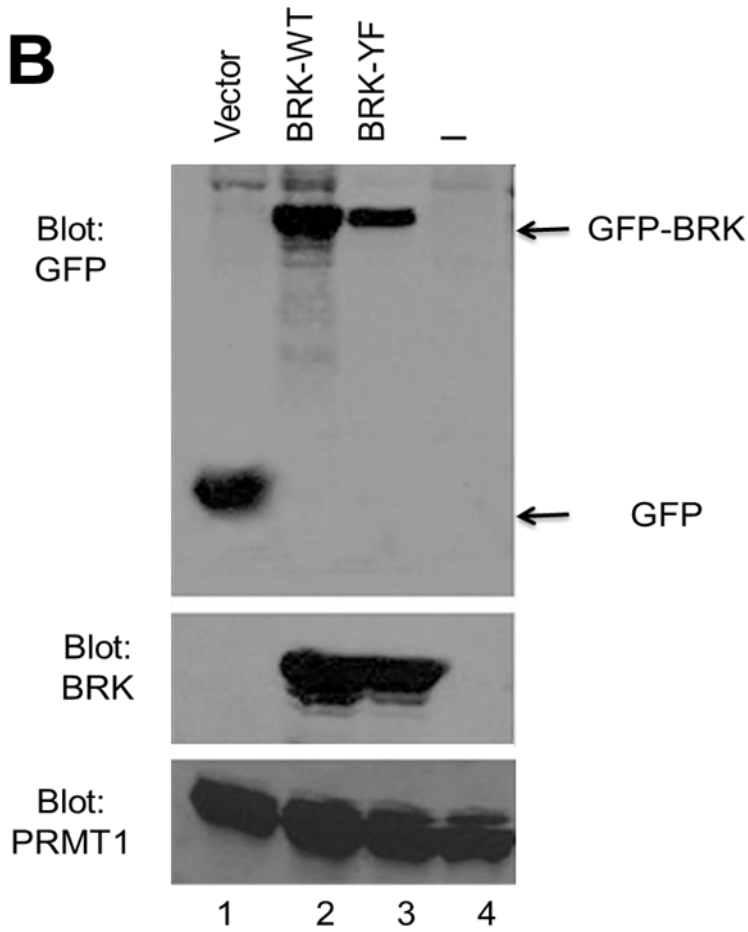
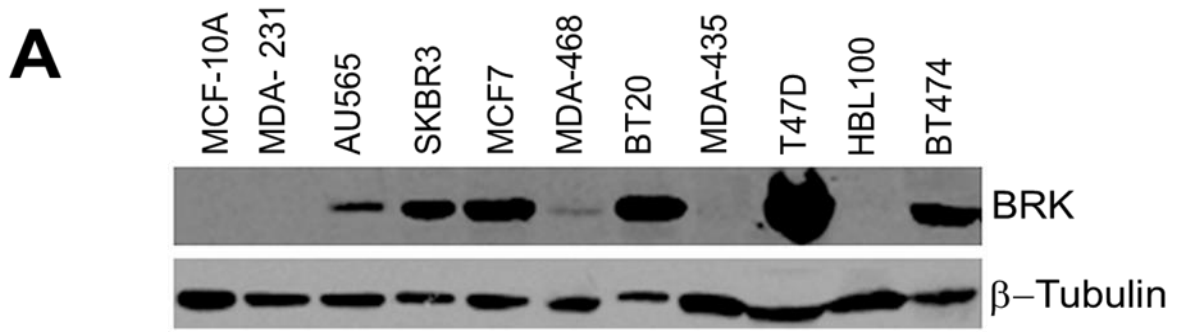
Figure 4.1. Tyr447Phe BRK mutant is notably more active than the wild-type BRK. (A) Schematic representation of BRK. The diagram shows the functional domains and the positions of some of the key residues mutated in this study. (B) The activity of BRK and BRK mutants in transfected HEK 293 cells. Wild-type (WT) BRK and BRK mutants, non-tagged and GFP-tagged, were transfected and expressed in HEK 293 cells as described in “Materials and methods”. Cell lysates were subjected to immunoblot analysis with anti-phosphotyrosine antibody (pY20) and anti-BRK and anti-phospho-BRK (pTyr342) antibodies. Anti-β-tubulin served as a loading control.

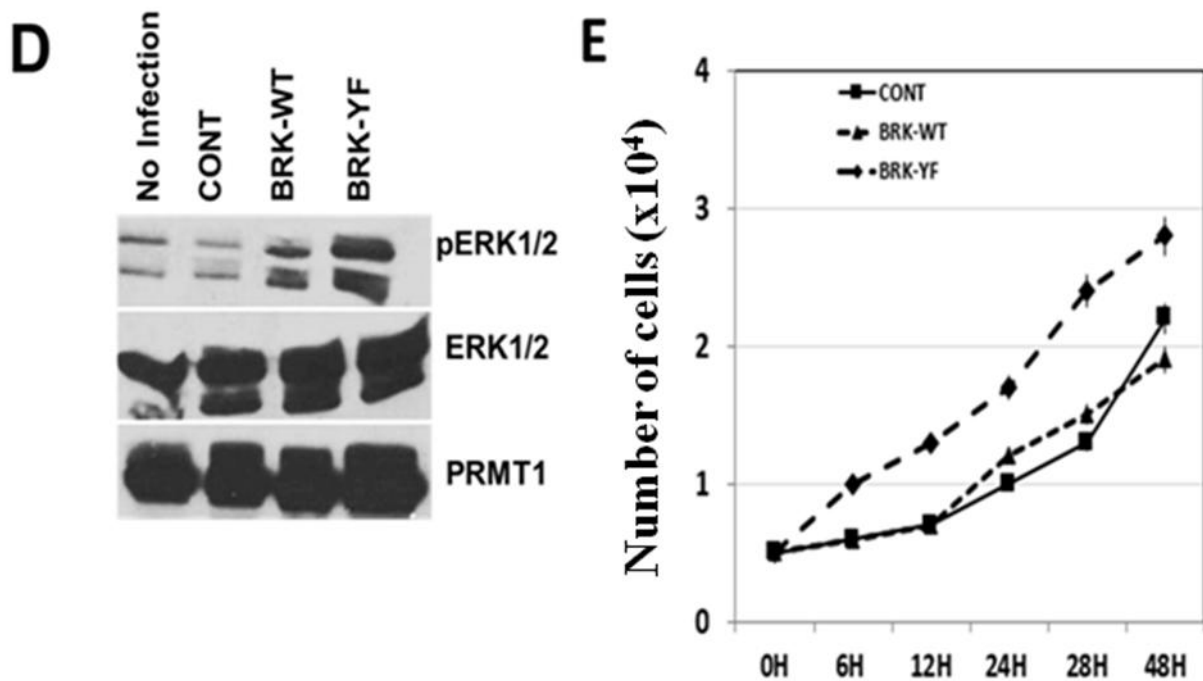
3) was not detected by this antibody. Taken together, these data substantiate that BRKY447F is notably more active than the wild-type and that a GFP tag did not interfere with the BRK’s catalytic activity.

#### 4.1.1.2. Constitutively active BRK enhances ERK activation and increases cell proliferation

In order to better understand the role of BRK in mammary gland tumorigenesis and to gain an insight into the role played by the constitutively active form of BRK in cell growth and proliferation, we generated three sets of stable cell lines by retroviral infections. Each cell line stably expressed GFP alone, GFP-BRK-WT or GFP-BRK-YF. We utilized BRK-negative cell lines, which include epithelial cell lines MCF10A and MDA-MB-231 (Figure 4.2A), as well as HEK293 cell line (Lukong *et al.*, 2005). MCF10A is an immortalized mammary epithelial cell line and MDA-MB-231 cells are estrogen receptor-negative, highly invasive breast cancer cell line. All of the cells were generated as pooled populations of puromycin-resistant cells to avoid any clonal variations. We subjected cell lysates from the stable cells to immunoblotting with anti-GFP and anti-BRK and demonstrated that stable MCF10A express equivalent levels of GFP-BRK-WT and GFP-BRK-YF (Figure 4.2B). More importantly, we analyzed the cell lysates for their relative levels of tyrosine phosphorylation using an anti-phosphotyrosine antibody to ensure that the activity in the BRK-YF stable cells was higher than the wild-type as expected. As shown in Figure 4.2C, overexpression of both BRK-WT and BRK-YF resulted in high phosphorylation of endogenous substrates as compared to the control cells. As expected, BRK-YF stable cells displayed a much higher level of tyrosine kinase activity as compared to BRK-WT samples (Figure 4.2C). Similar results were also obtained from both MDA-MB-231 and HEK293 stable cell lines (Figure 5.6).

Kamalati *et al.* demonstrated that exogenous expression of wild-type BRK in normal mammary epithelial cells enhanced mitogenic signaling (Kamalati *et al.*, 1996). Lukong *et al.* and others have shown that EGF-induced activation of BRK contributed to the phosphorylation of BRK substrates Sam68 and paxillin (Chen *et al.*, 2004; Lukong *et al.*, 2005). Using our MCF10A stable cell model system, we first investigated whether constitutive activation of BRK is accompanied by enhanced activation of mitogenic signaling in unstimulated cells. For this experiment, cell lysates from stable cell lines expressing GFP, GFP-BRK-WT or GFP-BRK-YF,





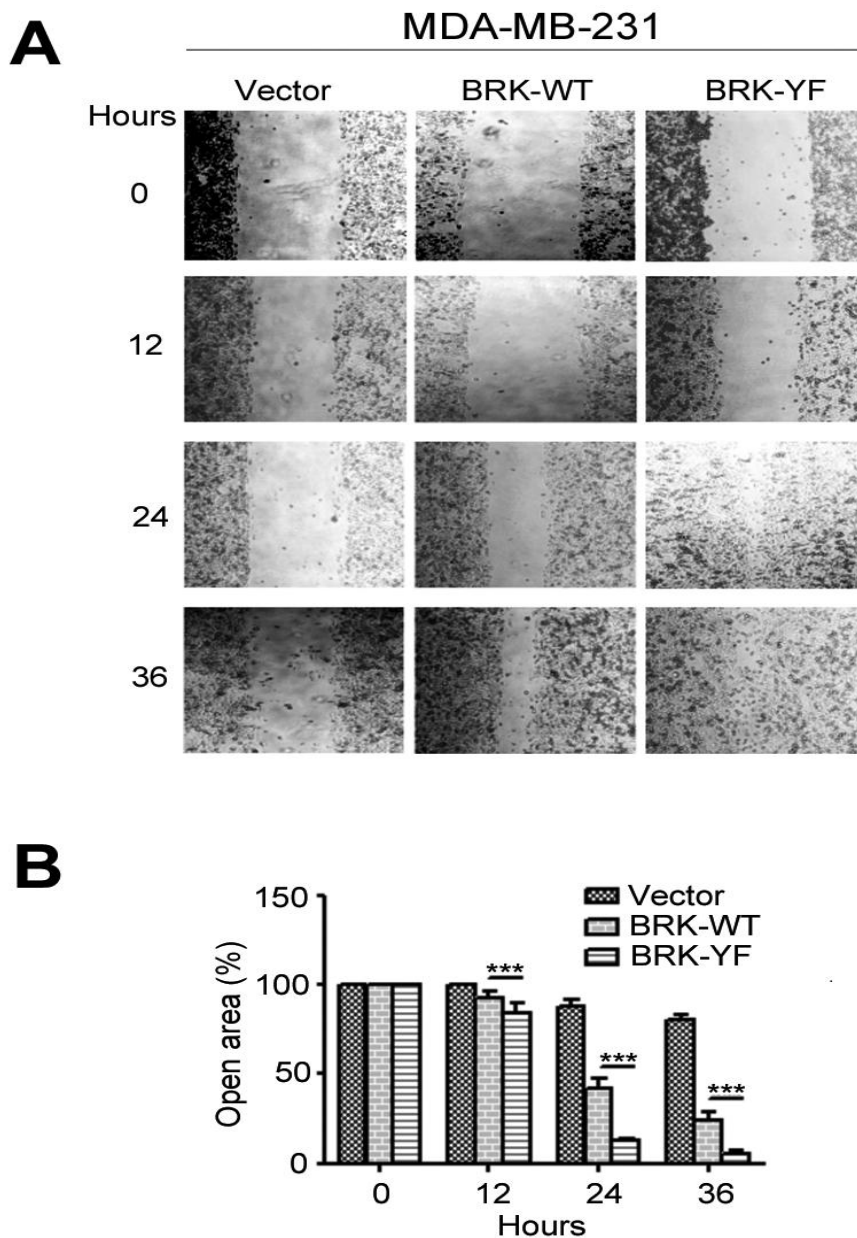
**Figure 4.2.** Constitutively active BRK enhances ERK activation and increases cell proliferation. (A) BRK is not expressed in normal mammary gland epithelial cell lines. BRK expression in the indicated breast cancer cell lines (lanes 3-11) and normal mammary epithelial cell lines (lanes 1 and 2) was detected by immunoblotting.  $\beta$ -actin was used as a loading control. (B) and (C) Stable cell lysates were analyzed by immunoblotting using an anti-BRK, anti-GFP and anti-phosphotyrosine antibody, pY20. Protein arginine methyltransferase 1 (PRMT1) served as a loading control. (D) and (E) Stable cell lysates were subjected to immunoblot to detect ERK1/2 and pERK1/2 by using anti-ERK1/2 and anti-pERK1/2 antibodies. PRMT1 served as a loading control. (E) Growth assay for MCF10A cells stably expressing BRK-YF, WT and vector alone control cell lines.

as well as parental cell line were analyzed by immunoblotting for Erk1/Erk2 activation (Figure 4.2D). Indeed, ERK activation, as demonstrated by the level of ERK1/2 phosphorylation was observed in both GFP-BRK-WT and GFP-BRK-YF samples (Figure 4.2D) and the presence of constitutive active BRK-YF resulted in a more pronounced activation of ERK compared to the WT counterpart. The GFP control cell lysates only showed background pERK as compared to lysates that were harvested from either the BRK-WT or YF stable cell lines. These data demonstrated that overexpression of BRK resulted in the activation of ERK signaling even in the

absence of EGF stimulation. Moreover, BRK activation was found to induce phosphorylation of ERK, thereby stimulating mitogenic signaling. Since activation of the Ras / ERK pathway plays a pivotal role in cell proliferation and is associated with a gain-of-function mechanism in breast carcinogenesis (Krishna and Narang, 2008), we next examined the effect of constitutive activation of BRK on cell growth. The MCF10A cell lines were plated at low density and cell number counted every 6 h for 48 h. Compared to the WT and control cell lines, we observed a significant increase in cell number in the pools of cells expressing BRK-YF as early as 6 h after the cells were plated (Figure 4.2E). A more modest ERK activation and increased cell growth was also observed in MDA-MB-231 cells stably expressing BRK-YF. No significant difference in cell viability was observed between the different cell types. Taken together, these data show that BRK is an upstream effector in the ERK pathway and constitutive activation of BRK results in increased ERK activation that corresponds with enhanced cell proliferation.

#### **4.1.1.3. Constitutive activation of BRK is associated with increased cell migration and invasion**

Several studies have shown that BRK contributes to the processes of migration and invasion that characterize the metastatic potential of breast cancers. For example, it was previously shown that BRK contributed to cell migration and proliferation by enhancing EGF-mediated phosphorylation of paxillin and activation of Rac1 via CrkII (Chen *et al.*, 2004). Similarly, BRK knockdown by RNA interference was shown to impair the migration of breast cancer cells (Harvey and Crompton, 2003; Lukong *et al.*, 2005; Ostrander *et al.*, 2007). In order to address the role of constitutive activation of BRK in cell migration, we employed both the wound-healing and Transwell migration assays. Cells were induced to migrate into a wound created by scratching confluent cultures with a pipette tip to examine the migration of MDA-MB-231 stable cell lines expressing GFP alone, GFP-BRK-WT or GFP-BRK-YF (Figure 4.3). MDA-MB-231 cells were selected for this experiment because of their characterized high migratory potential (Gruber and Pauli, 1999). Closure of wounded area was monitored for 36 h. As shown in Figure 4.3, the open area was rapidly covered by the BRK-WT and BRK-YF cells



**Figure 4.3.** Constitutive activation of BRK accelerates cell migration in wound healing assays. (A) MDA-MB-231 stable cells seeded into 6-well plates at 80-90% confluence. The wound of approximately 1 mm in width was scratched with a 200  $\mu$ L pipette tip. Wound closure was monitored at the indicated time intervals and imaged with phase contrast microscopy on an inverted microscope (Olympus 1X51 using a 10X phase contrast objective). The migration assay was performed in three independent experiments. (B) The open area (scratch) was quantified with TScratch software. The  $p$ -values were determined for control and stably transfected cells and set at  $p \leq 0.05$  for statistical significance.

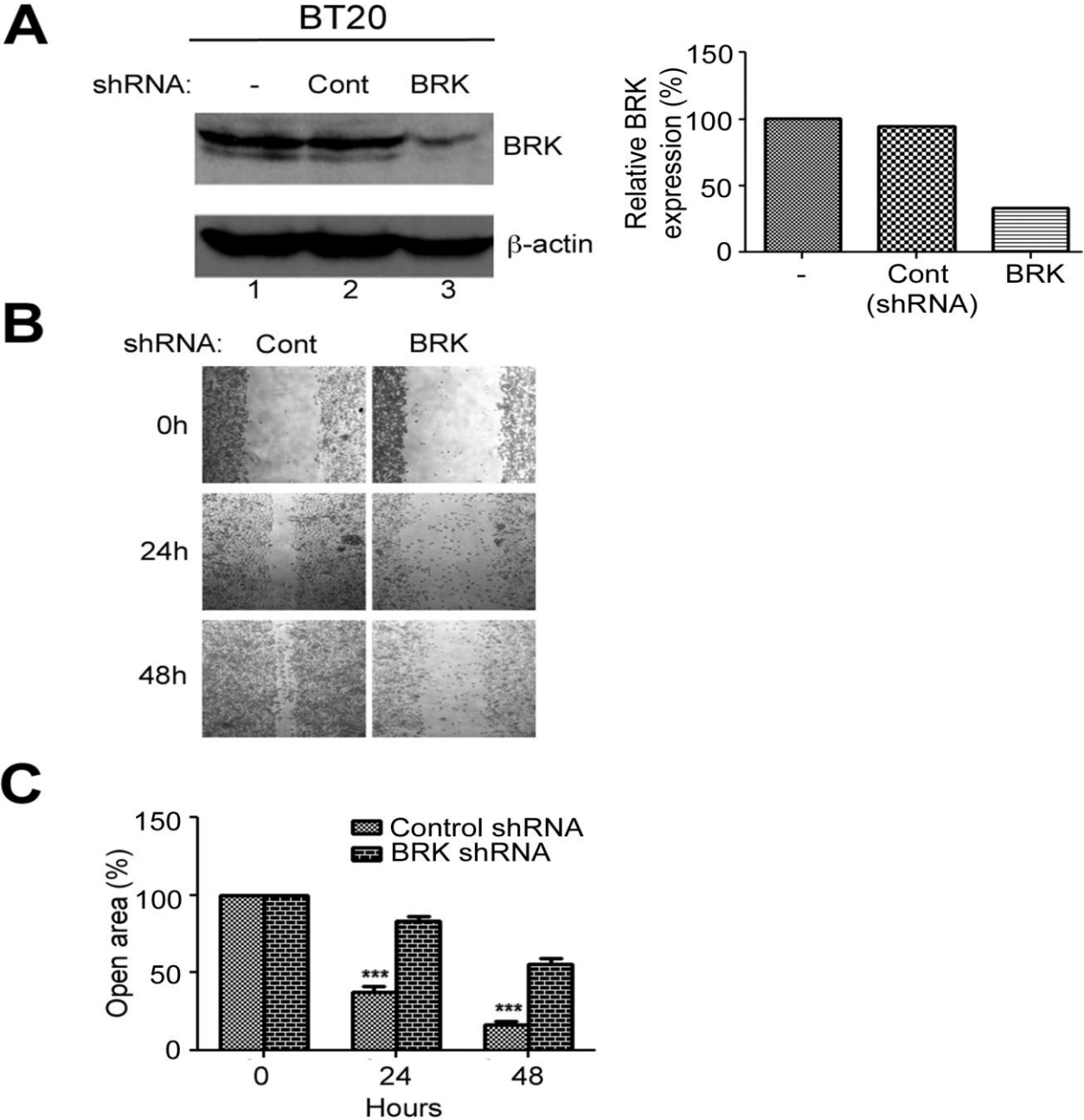
in comparison to control cells transduced with the empty vector. Moreover, constitutively active BRK-YF accelerated wound closure more efficiently at 24 h than the BRK-WT cells (Figure 4.3A). The BRK-YF cells migrated into the wounded area and almost completely closed the wound within 48 h. Quantification of wound closure is represented in bar diagram in Figure 4.3B. The quantified open area in vector control cells were reduced from 100% to only 81%, the open area of BRK-WT cells were shrunk from 100% to 24%, while the constitutively active BRK-YF cells were dramatically reduced from 100% to 6% (Figure 4.3B). These data suggest that activation of BRK significantly accelerates motility of the MDA-MB-231 cells.

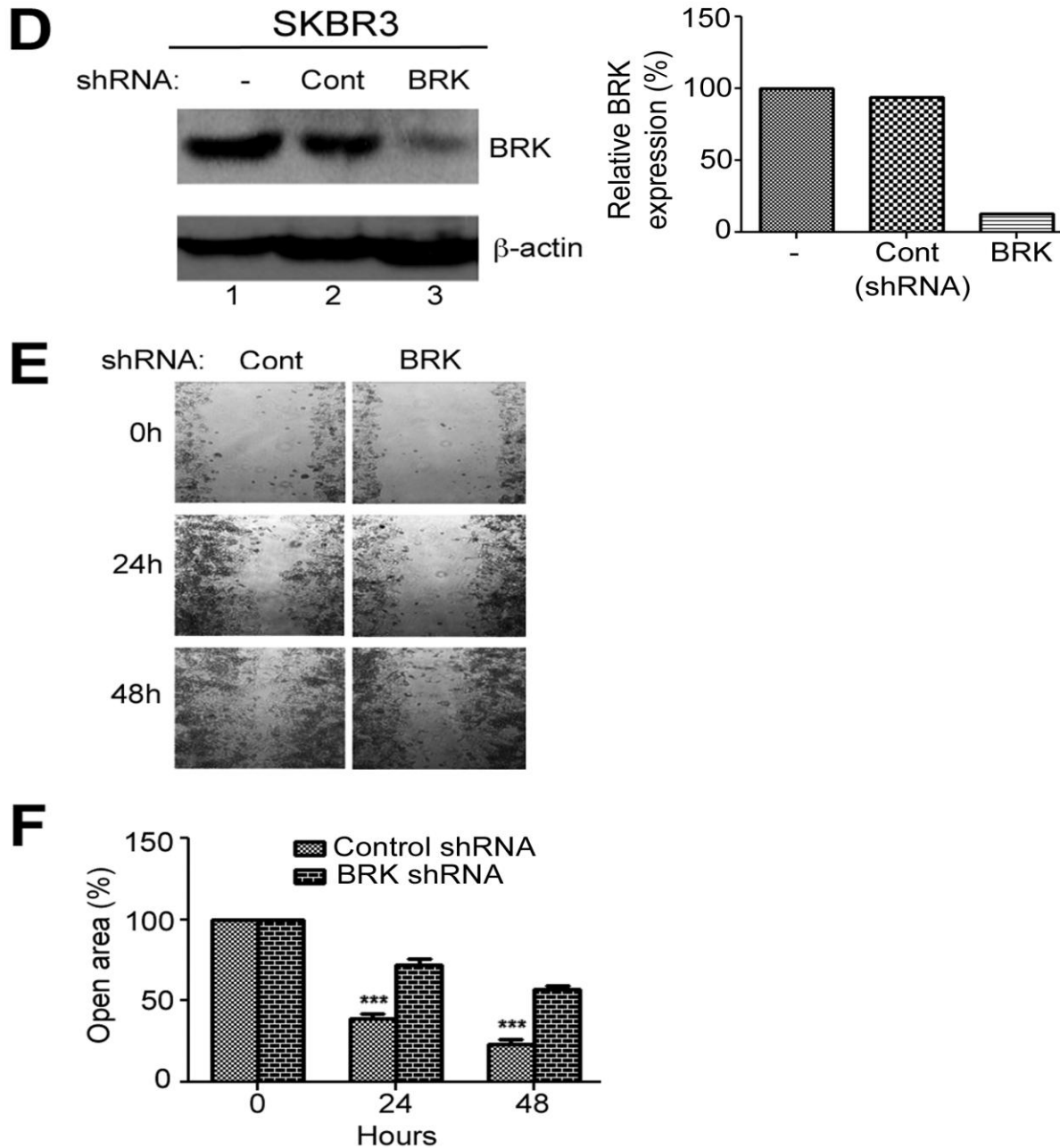
In order to validate the effects of BRK on cell migration by wound healing, we silenced BRK in two BRK-positive breast cancer cell lines BT20 and SKBR3. The BT20 is a triple negative breast cancer cell line, while SKBR3 is a HER2-positive breast cancer cell line (Neve *et al.*, 2006). To achieve stable BRK knockdown, the BT20 and SKBR3 cells were transfected with lentiviral vector plasmids encoding BRK-specific short hairpin RNAs (shRNAs) and cell lysates analyzed by immunoblotting using anti-BRK antibodies (Figure 4.4A and 4.4D). The degree of knockdown of BRK was quantified to 67% in BT20 cells and to 87% in SKBR3 cells as compared to their respective cell lines transfected with the shRNA, scrambles control or parental cell lines (Figure 4.4A and 4.4D, right panels). The confluent cell lines were scratched and wound healing examined over a period of 48 h. As shown in Figure 4.4B and 4.4E, BRK knockdown resulted in a significant delay in wound closure as compared to control cells after 48 h. The quantified score of open area showed that open area in the control cells was reduced from 100% to 17% after 48 h, whereas BRK knockdown in BT20 cells only reduced to 56% (Figure 4.4C). Similarly, in SKBR3 control cells the open area was reduced from 100 to 23% and only 57% in the knockdown cells (Figure 4.4E). These data suggest that silencing of BRK reduced the motility of breast cells irrespective of their molecular subtype.

As an independent means of measuring cell motility, we further investigated the contribution of activated BRK in cell migration *in vitro* by employing the Transwell migration assays. These assays were performed using MDA-MB-231 cells stably expressing BRK-WT and BRK-YF and breast cancer cell lines BT20 and SKBR3 in which BRK is stably knockeddown. For each assay, the stable cells including the controls were plated in the upper chamber in serum-free media. An 8  $\mu$ M polycarbonate membrane separated the upper chamber from a lower chamber containing complete media. After 24 h incubation cells on the top of the membrane



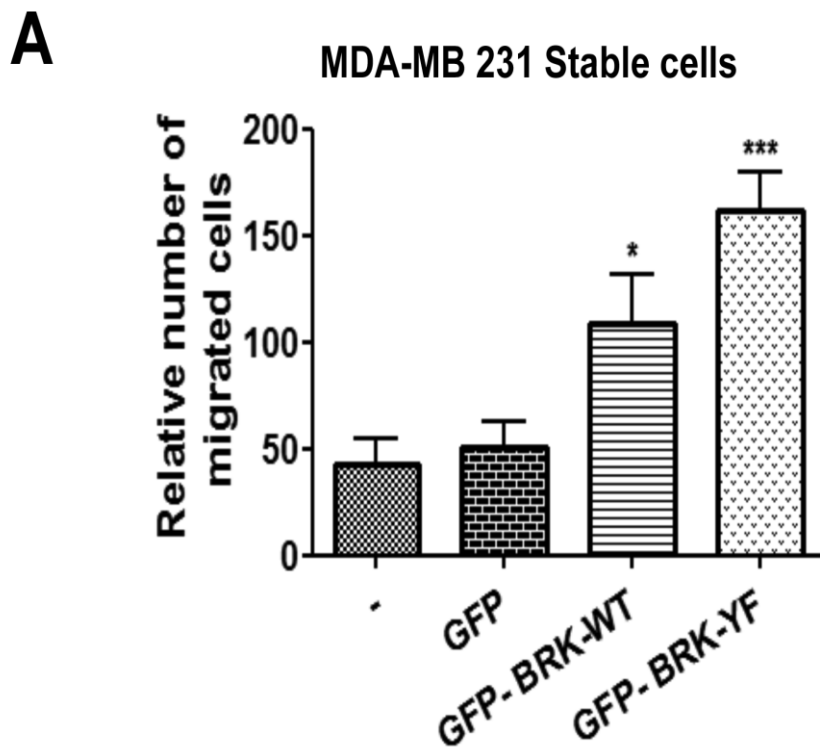
were removed by swiping and the membrane was rinsed and stained with trypan blue. Migrated cells

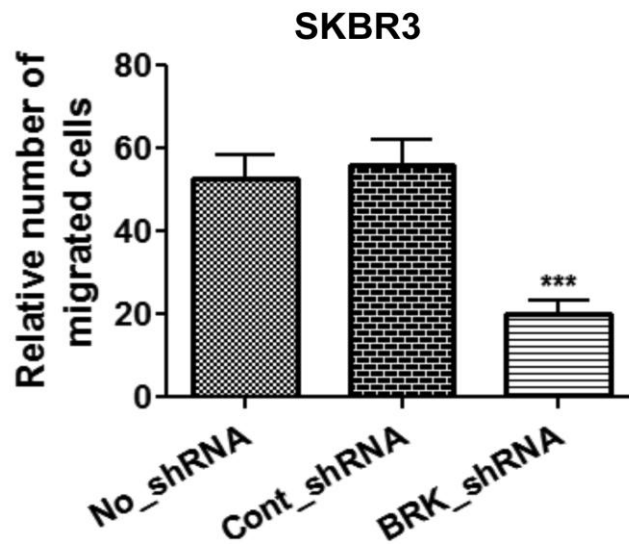
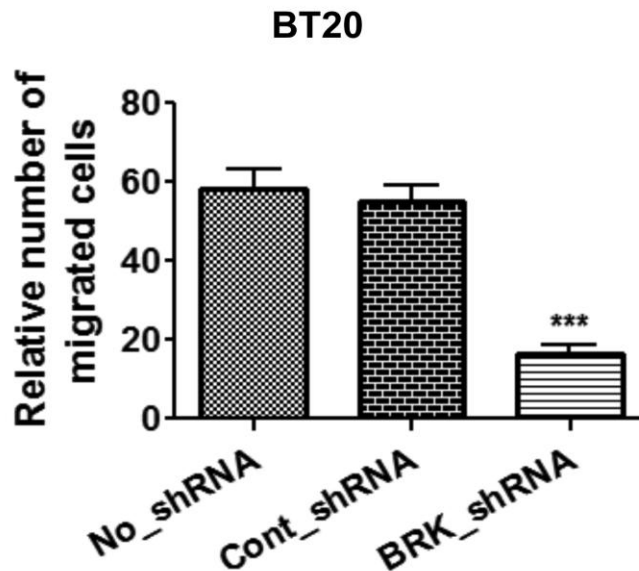




**Figure 4.4.** Stable knockdown of BRK significantly suppresses migration of breast cancer cells in wound healing assays. (A) and (D) Efficient knockdown of BRK in BT20 breast cancer cells. (B and C) and (E and F) BRK knockdown significantly suppresses migration of both BT20 and SKBR cells. The stable knockdown cells were analyzed for cell migration using the wound-healing assay in 6-well plates as described in Figure 3 legend. The open area (scratch) was quantified with TScratch software. The  $p$ -values were set at  $p \leq 0.05$  for statistical significance.

on the underside of the membrane were counted under a microscope, in four different viewing fields, at 20x magnification. As shown Figure 4.5A, both BRK-WT and BRK-YF induced a dramatic increase in cell migration compared to the GFP alone control or the parent cell line. BRK-WT enhanced migration as expected, in both BT20 and SKBR3 cell lines migration was attenuated by >50% in BRK-shRNA than the controls, while BRK-YF induced a marked increase in cell migration by over three-folds as compared to the control cells. To further validate the involvement of BRK in migration, we performed Transwell migration assays with BT20 and SKBR3 stably depleted of BRK by shRNA (Figure 4.5B and 4.5C). Cell migration across the Transwell was significantly decreased by the depletion of BRK in both the BT20 and SKBR3 cell lines using shRNA as compared to either the control shRNA transfected cell lines or the parental cell lines. Collectively, these data suggest that BRK contributes to the basal cell migration of BT20 and SKBR3 cells and also that full activation of BRK induces cell migration.



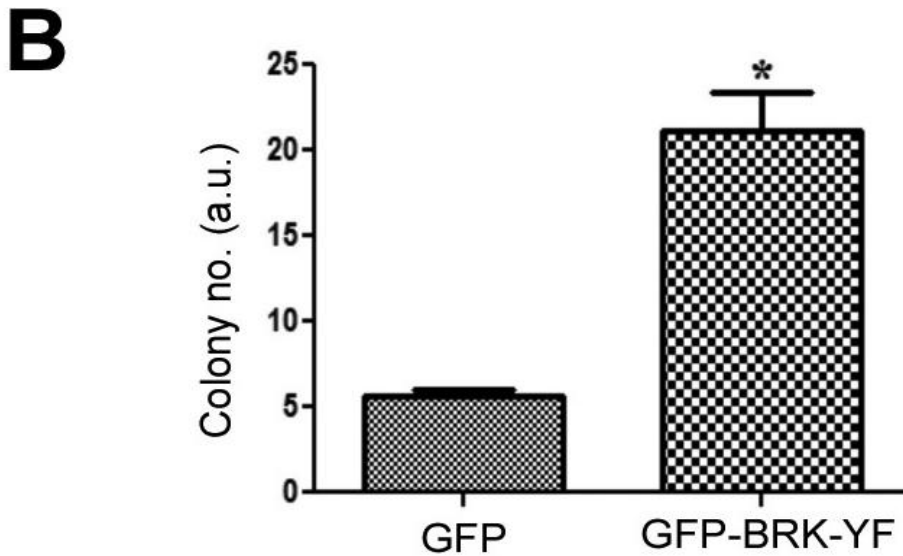
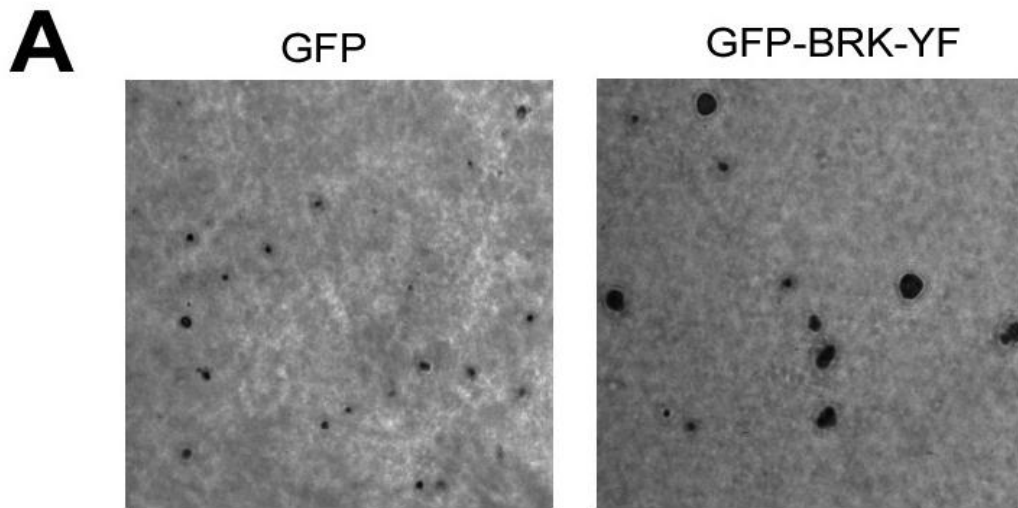
**B****C**

**Figure 4.5.** Transwell assays demonstrating the effect of BRK on cell migration. (A) Migration of BRK stable MDA-MB-231 cell lines expressing GFP-BRK-WT, or constitutively active GF-BRK-YF or GFP alone were evaluated in 24-well Transwell polystyrene membrane with 8 $\mu$ m size pores. Migrated cells were fixed with paraformaldehyde, stained with crystal violet for 30 min and the number of migrating cells was counted scored in relative units. (B) and (C) BRK knockdown significantly suppresses migration of BT20 and SKBR breast cancer cell lines. The stable knockdown BT20 and SKBR breast cancer cell lines were prepared as described in Figure 4 legend and analyzed for migration by Transwell assay as described above. All results are the mean ( $\pm$  SD) of more than three separate experiments. Statistical analysis \* $p \leq 0.05$  and \*\*\* $P \leq 0.0001$ .

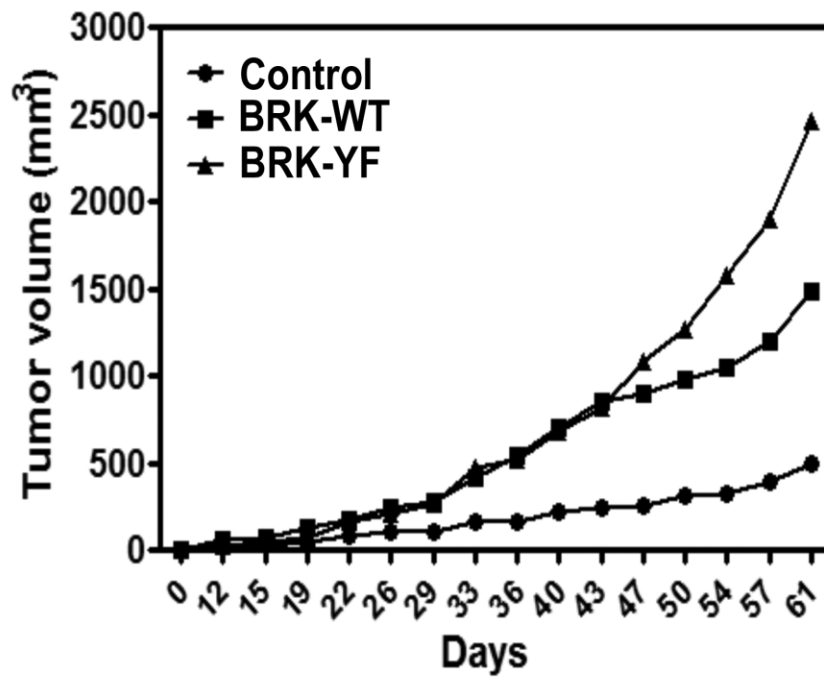
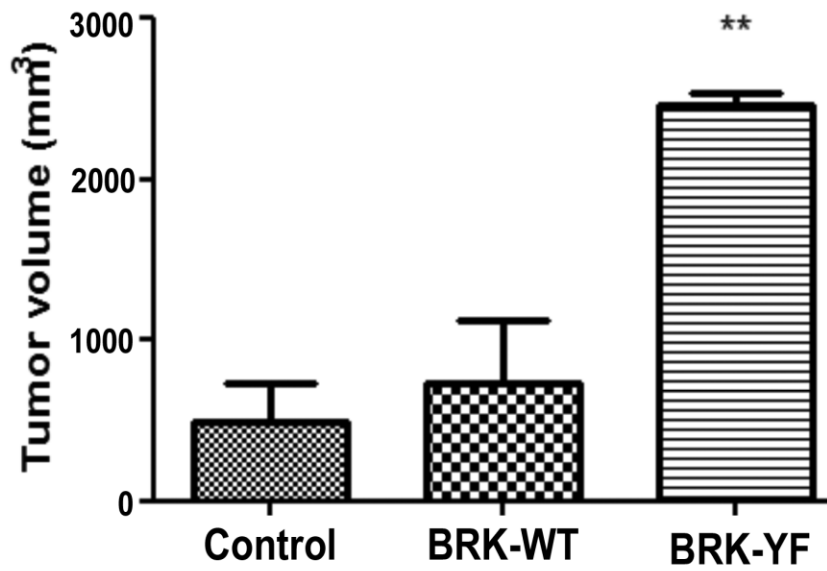
#### 4.1.1.4. Activated BRK promotes tumorigenicity *in vitro* and *in vivo*

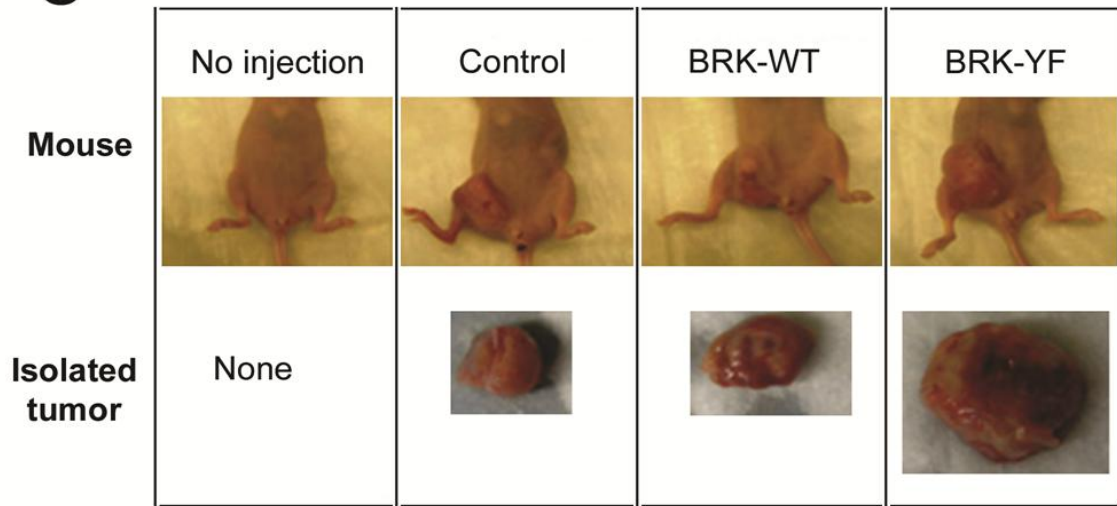
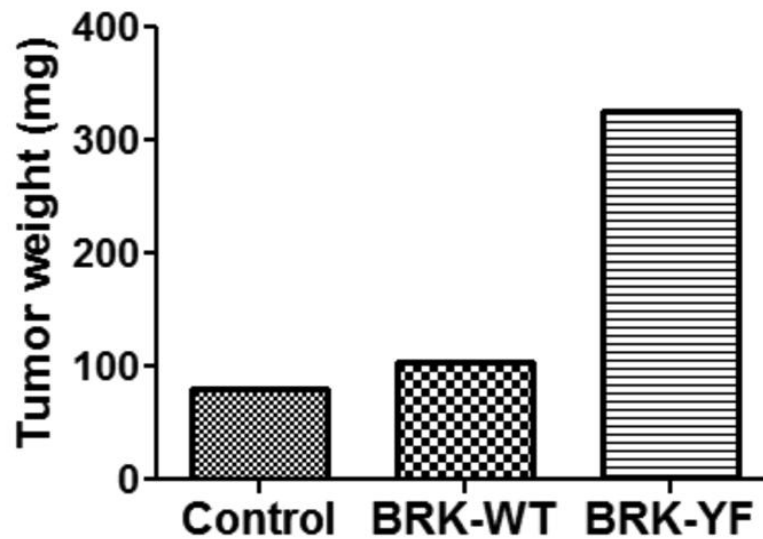
Growth in anchorage-independent conditions is a hallmark of tumorigenicity and invasiveness in several cancer cell types (Simpson *et al.*, 2008; Tsatsanis and Spandidos, 2004). Since we have shown that constitutively active BRK enhances proliferation and migration of stable MDA-MB-231 cells, we therefore considered whether BRK activation could cause an anchorage-independent growth advantage in soft agar in our MDA-MB-231 stable cell model (Figure 4.6). Notably, we observed that the ability of BRK-YF stable MDA-MB-231 cells to form colonies was five times greater than the parental MD-MB-231 (Figure 4.6A and B), indicating the importance of BRK activation in malignant transformation, hence tumorigenesis.

We corroborated these findings in *in vivo* studies using an athymic mouse model system. The mammary fat pads of these mice (n=4 mice per group) were injected with MDA-MB-231 cells stably expressing only one of the following genes: GFP alone, GFP-BRK-WT or GFP-BRK-YF. The mice were monitored for tumor formation and tumor volume measured every 7 days for 60 days (Figure 4.7A and B). All mice started developing palpable tumors 10 days after injection, and there was no significant difference in the latency period. However, in mice injected with GFP-BRK-YF-expressing cells, we observed a significantly faster growth rate compared to animals injected with the control cells (GFP alone) or the wild-type BRK (GFP-BRK-WT). 60 days post- injection, the average volume of tumors induced by GFP-BRK-YF-expressing cells was 2450 mm<sup>3</sup> compared to 1130 mm<sup>3</sup> for BRK-WT and 958 mm<sup>3</sup> for the control (GFP alone) group (Figure 4.7B). Primary tumors were excised at necropsy and weighed, then the tumor weights were compared across the groups (Figure 4.7C). In line with the final tumor volume data, we observed a significantly higher average weight of the BRK-YF-expressing tumors (3.25 gm) as compared to either BRK-WT (1.04 gm) or the control (0.81 gm) (Figure 4.7D). These results demonstrate that the activation of BRK significantly enhances tumorigenicity and suggest that the enzymatic activity of BRK is essential in BRK-regulated breast cancer tumor progression.



**Figure 4.6.** BRK activation promotes anchorage-independent growth of breast cancer cells. **(A)** Representative images of colony formation assay of MDA-MB-231 cells stably expressing control GFP or GFP-BRK-YF.  $1 \times 10^5$  cells were suspended in soft agar and photographed after 3-week incubation at 37 °C. **(B)** Cell colonies were counted in triplicate wells from five fields and mean of colonies were graphically represented. Standard deviations are indicated. Statistical analysis  $*p \leq 0.05$ .

**A****B**

**C****D**

**Figure 4.7.** Overexpression of constitutively active BRK significantly enhanced xenograft tumor growth as compared with wild-type or control vector. (A)  $2.5 \times 10^6$  MDA-MB-231 cells were injected subcutaneously bilaterally into mammary fat pads of 4 animals injected per cell line. Palpable tumors were monitored and measured bi-weekly for about eight weeks. Tumor volume was calculated as follows  $0.50 \times \text{length} \times \text{width}^2$ . (B) The average volume of tumors induced by GFP-BRK-YF-expressing cells was  $2450 \text{ mm}^3$  compared to  $1130 \text{ mm}^3$  for BRK-WT and  $958 \text{ mm}^3$  for the control (GFP alone) group. (C) A representative image of mice at endpoint showing the presence or absence of tumors at the site of injection in mammary. (D) The tumors of these mice at endpoint were isolated and weighed and the weights represented graphically. Statistical analysis  $**P \leq 0.001$ .



#### **4.1.1.5. Summary**

The activity of BRK, like Src family tyrosine kinase, is regulated negatively by phosphorylation of C-terminal tyrosine 447. It was previously shown that BRK-Y447F is a constitutively active variant because autoinhibition is disabled by the mutation of Y447. Overall the present study demonstrates that overexpression of constitutively active BRK highly correlates with increased cell proliferation and a greater transformation potential of epithelial cells. We demonstrated for the first time that full activation of BRK is an essential component in BRK-induced promotion of tumorigenesis. Using stable breast cancer cell MDA-MB-231 we observed significantly enhanced rates of cell proliferation, migration and transformation in BRK-Y447F stable cells compared to wild-type stable cell lines. Our results indicate full activation of BRK is an essential component in the tumorigenic role of BRK.

#### **4.2. BRK targets Dok1 for ubiquitin-mediated proteasomal degradation to promote cell proliferation and migration.**

The cellular roles of BRK in breast cancer have not been fully elucidated. We found that constitutive activation of BRK induces anchorage-independent growth and promotes tumorigenesis. Although no specific BRK signaling pathway has been delineated, BRK is implicated in several signaling cascades. Consistent with its potential role in tumorigenesis, BRK associates with EGFR, enhancing the mitogenic signals by promoting the recruitment of phosphatidylinositol 3-kinase (PI3K) and activating Akt as well as stimulating cell migration by activating signalling molecules such as Mitogen-activated protein kinase (MAPK) and paxillin (Chen *et al.*, 2004; Kamalati *et al.*, 2000; Kamalati *et al.*, 1996; Shen *et al.*, 2008).

More recently, it was demonstrated that depletion of BRK in breast cancer cells impairs the activation of EGFR-regulated signaling molecules (Ludyga *et al.*, 2011). Data from our group showed significantly increased ERK activity, cell proliferation and migration in breast cancer cells stably expressing BRK-Y447F, and decreased migration in breast cancer cells depleted of BRK (Miah *et al.*, 2012). These findings as a whole strongly suggest a role for BRK in promoting cell proliferation and migration.

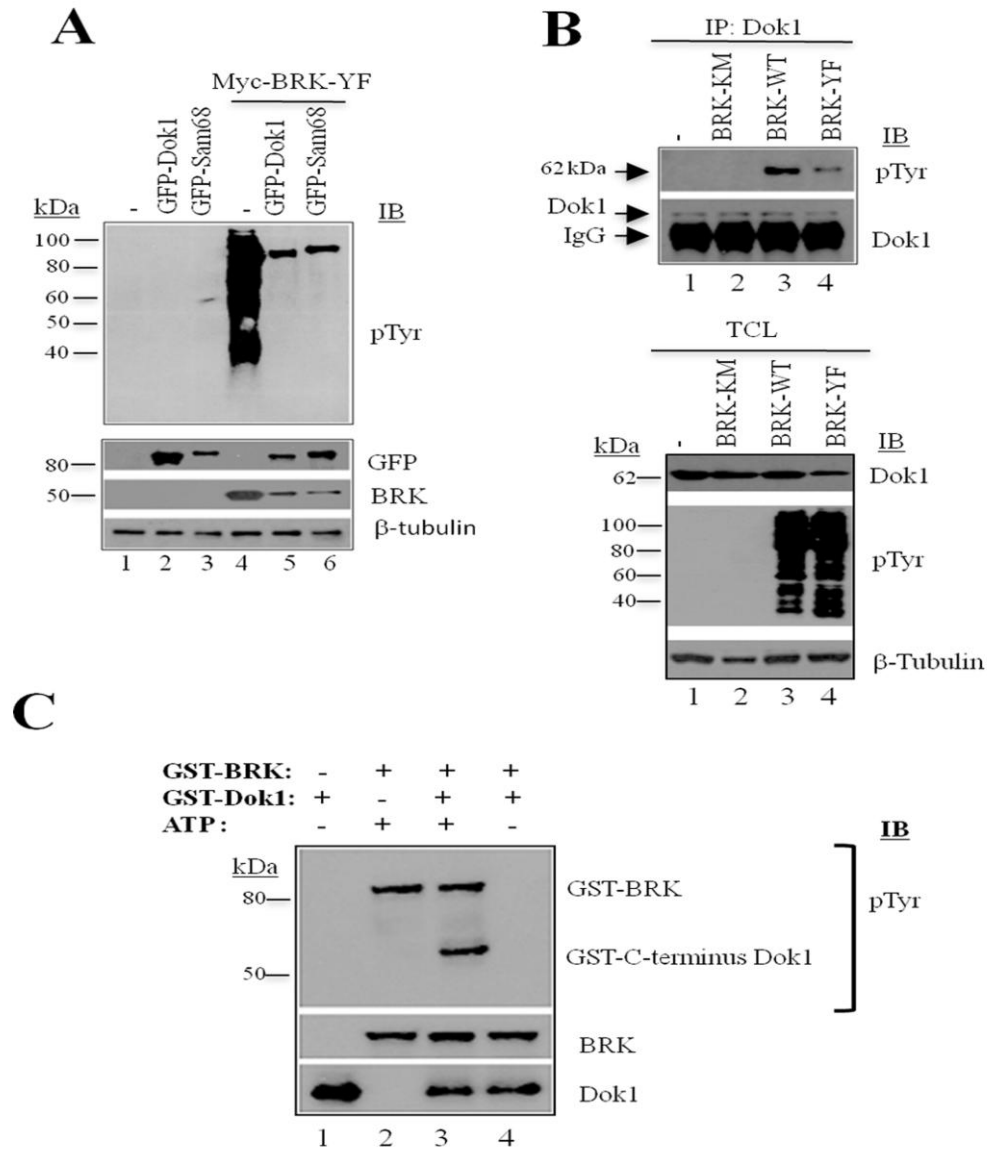
The identification and characterization of an expanding repertoire of BRK interacting proteins and substrates has significantly improved our understanding of the molecular and cellular functions of BRK. A recent proteomic study reported that downstream of tyrosine kinase 1 (Dok1), a tumor suppressor, is a potential substrate of BRK (Takeda *et al.*, 2010). Therefore, to further understand the cellular roles of BRK, we explored the functional link between BRK and Dok1. Dok1 is a scaffolding protein which mediates protein-protein interactions and has been shown to be phosphorylated by several tyrosine kinases including SRMS, v-Src, c-Abl and p210-Bcr-Abl (Goel *et al.*, 2013; Liang *et al.*, 2002; Mashima *et al.*, 2009; Murakami *et al.*, 2002; Niu *et al.*, 2006; Woodring *et al.*, 2004). Herein we show that BRK interacts with and phosphorylates Dok1 predominantly on Y362, promoting its proteasome-mediated degradation.

#### **4.2.1. Results**

##### **4.2.1.1. Dok1 is a substrate of BRK.**

In a recent report, it was suggested that Dok1 is a potential substrate of BRK (Takeda *et al.*, 2010). Therefore, we investigated whether Dok1 is an endogenous target of BRK. In the present study, we used a mutant BRK-Y447F that was previously reported to have a higher enzymatic activity than BRK-WT or BRK-K219M (Lukong *et al.*, 2005). HEK 293 cells were transiently transfected with GFP-Dok1 in the presence or absence of constitutively active myc-tagged BRK (BRK-Y447F or BRK-YF). As a positive control, we used GFP-Sam68, a characterized substrate of BRK (Lukong *et al.*, 2005). By immunoblotting with an anti-phosphotyrosine antibody pY20, we showed that BRK-YF triggered strong tyrosine phosphorylation of GFP-Dok1, (Figure 5.1A, lane 5). Likewise, GFP-Sam68, which migrates at a slower rate than GFP-Dok1, was also phosphorylated as expected (lane 6). The expression levels of GFP-Dok1 and GFP-Sam68 as well as those of myc-BRK-YF are shown in the bottom panels. These data show that overexpression of constitutively active BRK induces the phosphorylation of ectopically expressed GFP-Dok1.

We then examined whether ectopically expressed BRK could phosphorylate endogenous Dok1. To this end, we transiently transfected either the kinase-dead BRK-K219M, BRK wild type (BRK-WT) or the constitutively active BRK-YF into HEK293 cells followed by immunoprecipitation and immunoblotting (Figure 5.1B). Using a phosphotyrosine antibody, we



**Figure 5.1. Dok1 is a direct substrate of BRK.** (A) HEK293 cells were transiently transfected with empty control vector (-) or GFP-Dok1, GFP-Sam68, Myc-BRK or co-transfected with Myc-BRK+GFP-Sam68 and Myc-BRK+GFP-Dok1. Tyrosine phosphorylation of cellular proteins were detected in total cell lysates by immunoblot analysis (IB) with an anti-phosphotyrosine (anti-pTyr) antibody (pY20). The blots were reprobed with anti-GFP, anti-BRK and anti- $\beta$ -tubulin antibodies as a loading control. (B) Tyrosine phosphorylated endogenous Dok1 as confirmed by anti-Dok1 immunoprecipitation (IP) followed by immunoblot analysis with anti-phosphotyrosine antibody and anti-Dok1 (top panel). Immunoblot analysis of total cell lysates is showing the expression of Dok1, the kinase activity of BRK-WT and BRK-YF, and  $\beta$ -tubulin as a loading control (bottom panel). (C) An *in vitro* kinase assay was performed using the active kinase, GST-BRK, and the substrate, GST-C-terminus Dok1, in the presence (+) or absence (-) of ATP. Tyrosine phosphorylation was detected using the anti-phosphotyrosine antibody. The blots were reprobed with an anti-BRK and anti-Dok1 antibody (bottom panel).

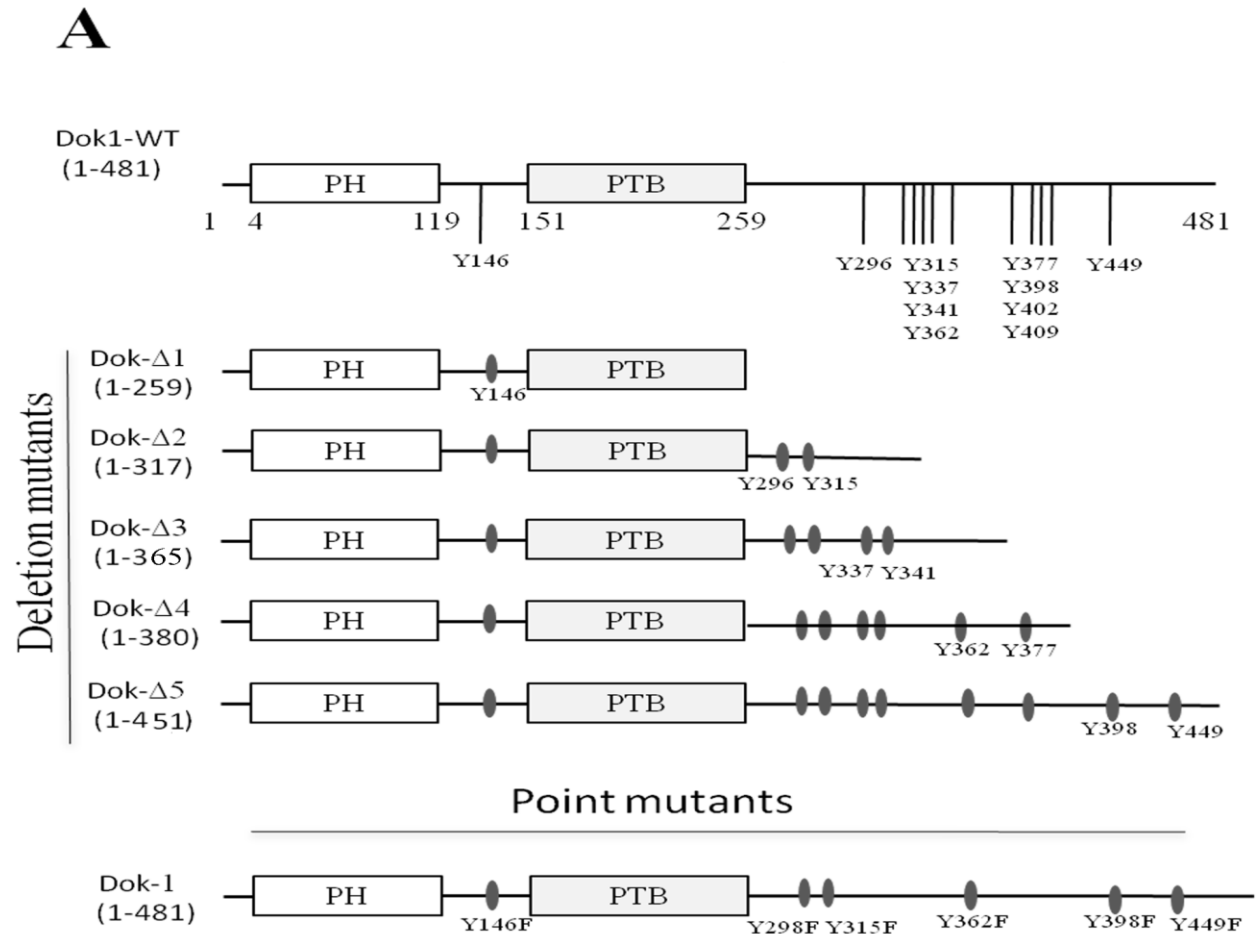
confirmed the phosphorylation of endogenous Dok1 in Dok1 immunoprecipitates from BRK-WT and BRK-YF cell lysates (Figure 5.1B, top panel, lanes 3 and 4). Strikingly, we observed a marked decrease in the levels of phosphorylation of the Dok1 protein in the immunoprecipitates from the BRKY447F-transfected cell lysates. No phosphorylation of Dok1 was detected in control cell lysates or lysates from BRK-KM-transfected cells (lanes 1 and 2, bottom), suggesting that BRK may directly phosphorylate Dok1 *in vivo*. The expression levels of Dok1 and activity of the transfected BRK variants (BRK-WT and BRK-YF) in the total cell lysates revealed strong phosphotyrosine staining as compared to either BRK-KM samples or the control lysates, as expected (Figure 5.1B, bottom). In light of these findings, we evaluated whether Dok1 was a direct substrate of BRK. In an *in vitro* kinase assay that was performed using glutathione S-transferase (GST)-tagged full-length BRK and the C-terminal region of Dok1 (GST-Dok1-CT)-terminal, we observed phosphorylation of GST-Dok1-CT in the presence of GST-BRK, indicating that Dok1 is a direct substrate of BRK (Figure 5.1C, lane 3, top panel). The activity of GST-BRK is shown by the presence of autophosphorylation (lanes 2 and 3). Together, these findings validate Dok1 as a bona fide BRK substrate.

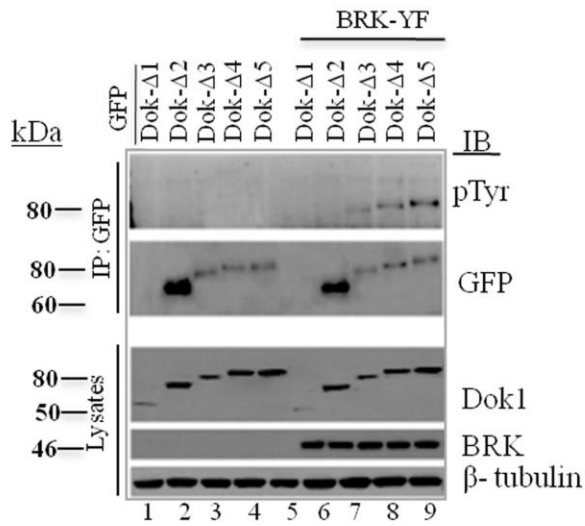
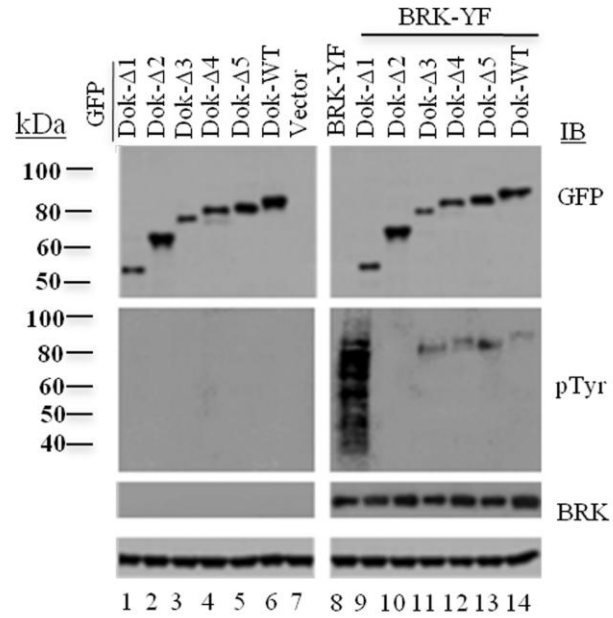
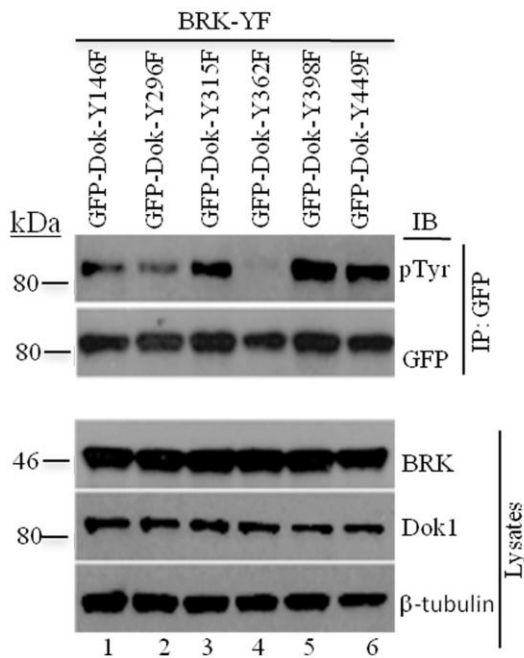
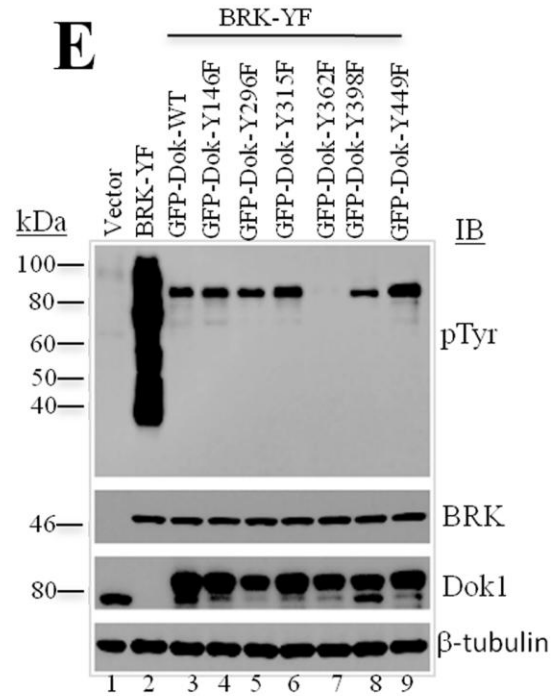
#### **4.2.1.2. BRK phosphorylates Dok1 at tyrosine 362.**

Dok1 is structurally composed of an N-terminal Pleckstrin Homology (PH) domain and an Insulin Receptor Substrate (IRS) Type PTB domain with a C-terminal segment rich in proline residues and several tyrosine residues (Carpino *et al.*, 1997) (Figure 5.2A). To map the tyrosine(s) on Dok1 phosphorylated by BRK, we first generated five GFP-tagged deletion mutants of Dok1 (Dok1- $\Delta$ 1 to  $\Delta$ 5) (Figure 5.2A). We transfected the plasmids containing the Dok1 deletion mutants in the presence or absence of BRK-YF into HEK293 cells and then immunoprecipitated the Dok1 variants using anti-GFP antibodies. The immunoprecipitates were then analyzed by immunoblotting using anti-phosphotyrosine antibodies (pY20) (Figure 5.2B). We observed that the presence of BRK-YF induced the phosphorylation of all GFP-Dok1 variants, except for the Dok- $\Delta$ 1 and Dok- $\Delta$ 2 fragments, which harbor respectively Y146 or Y146 together with Y296 and Y315 (Figure 5.2B). Analysis of total cell lysates also corroborated the

phosphorylation of the Dok1 mutants (Figure 5.2C). These data confirmed that BRK targets the tyrosine residues in the C-terminal of Dok1.

In order to determine which of the specific tyrosine residues along the C-terminal tail of Dok1 are targeted by BRK, we generated a series of 6 (Dok1- $\Delta$ 1 to  $\Delta$ 5) GFP-Dok1 mutants in which one of the following tyrosine residues, Y146, Y296, Y315, Y362, Y398 or Y449 was replaced by a phenylalanine (Figure 5.2A). Each construct was transiently transfected into HEK293 cells that stably expressed the BRK-YF mutant. The Dok1 mutants were then immunoprecipitated from the cell lysates with anti-GFP antibodies and analyzed by



**B****C****D****E**

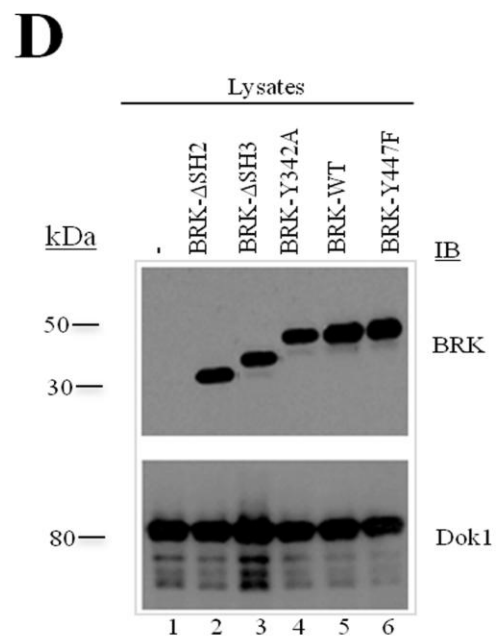
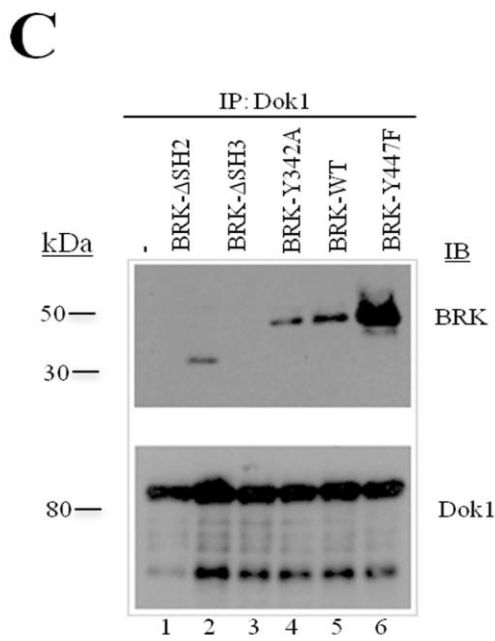
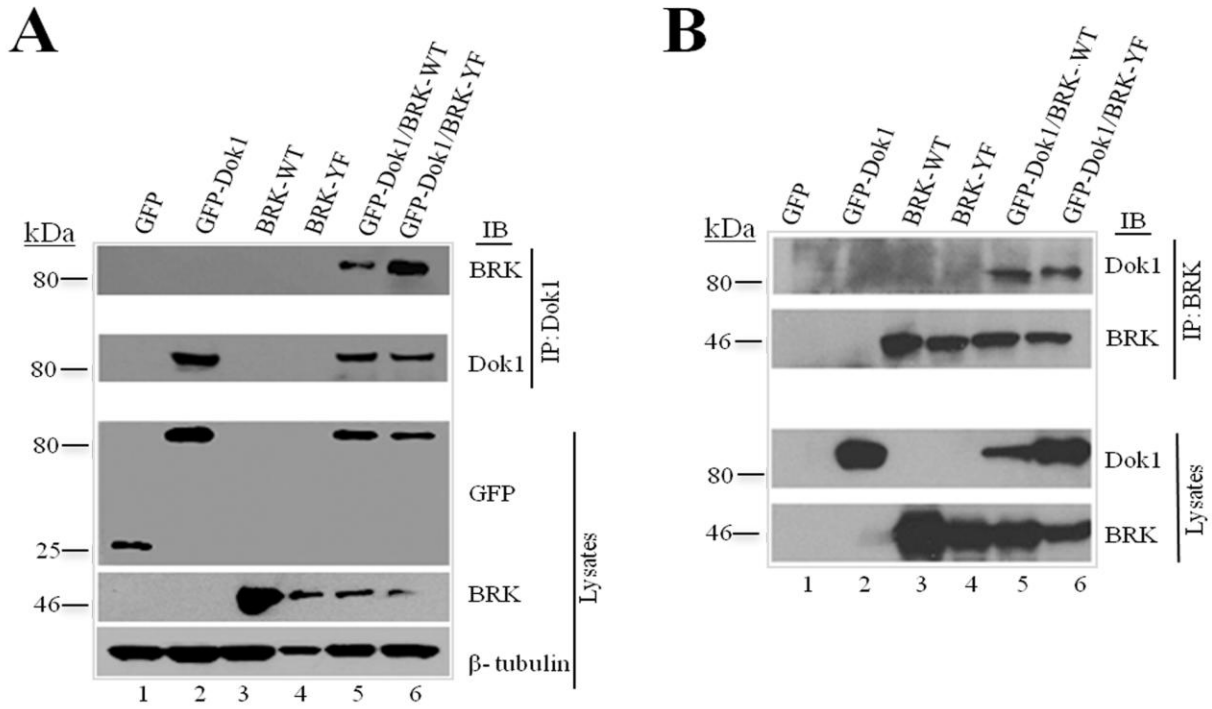
**Figure 5.2. Constitutively active BRK phosphorylates Dok1 at Y362.** (A) Schematic diagram of Dok1 showing different deletion and point mutants. (B) The Dok1 deletion mutants and BRK-YF were co-transfected into HEK 293 cells, the cell were then subjected to immunoprecipitation with anti-GFP antibody followed by immunoblotting analysis using anti-phosphotyrosine and anti-GFP antibodies (top panel). The lower panel shows the expression of different GFP-Dok1 deletion mutants, BRK (as input) and  $\beta$ -tubulin as a loading control. (C) Dok1 deletion mutants were transfected either alone or with BRK-YF into HEK293 cells, the cell lysates were then subjected to immunoblotting analysis using antibodies against Dok1, phosphotyrosines, BRK and  $\beta$ -tubulin as a loading control. (D) HEK293 cells were co-transfected with Dok1 point mutants and BRK-YF followed by immunoprecipitation with anti-Dok1 antibody and immunoblotting analysis using anti-phosphotyrosines and anti-Dok1 antibodies. Lower panel shows the expression of BRK, GFP-Dok1 mutants (as input) and  $\beta$ -tubulin as a loading control. (E) HEK293 cells were cotransfected with BRK-YF and Dok1 point mutants or transfected with BRK-YF alone. Total cell lysates were analyzed by immunoblotting analysis with antibodies against phosphotyrosines, BRK, Dok1 and  $\beta$ -tubulin as a loading control.

immunoblotting using pY20. As shown in Figure 5.2D, immunoblotting with the anti-phosphotyrosine antibodies revealed a robust phosphorylation of Dok1 wild type and all its mutants, except for GFP-Dok1 Y362F. The expression levels of phosphotyrosines, GFP Dok1 mutants and BRK-YF in the total cell lysates are shown in Figure 5.2E. As a whole, although transient co-transfection experiments showed a weak phosphorylation of GFP-Dok1 Y362F, our data support the notion that BRK induces the phosphorylation of Dok1 predominantly through tyrosine 362.

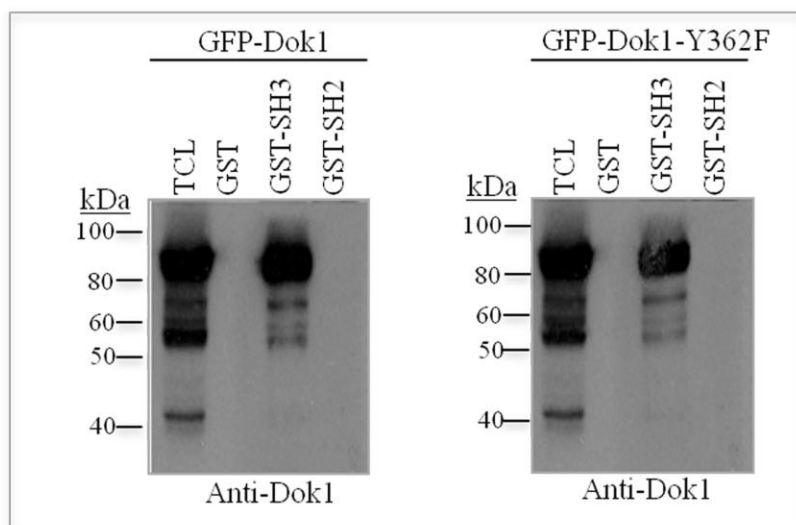
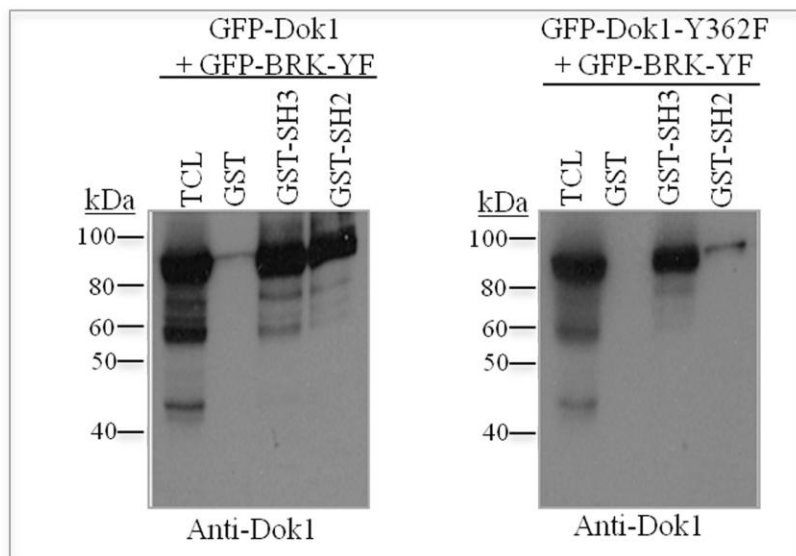
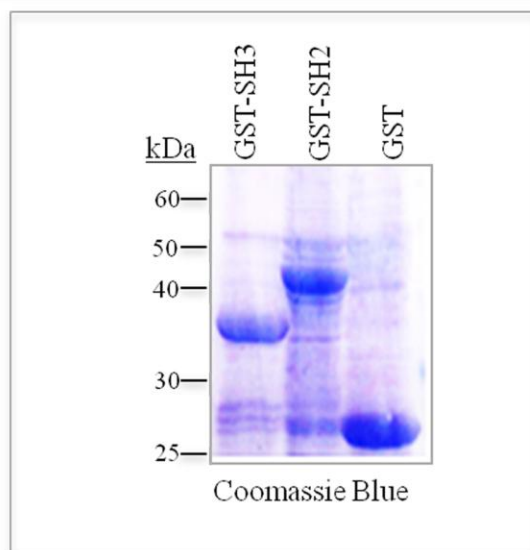
#### **4.2.1.3. BRK interacts with Dok1 via SH3 and SH2 binding.**

BRK possesses three functional domains: SH3, SH2, and a catalytic domain. The SH3 domain binds to proline-rich regions typically with the PXXP motif, while the SH2 domain tends to bind to phosphorylated tyrosine residues. Previous studies have shown that the SH3 domain of BRK plays a pivotal role in substrate recognition and that the SH2 domain interacts with phosphorylated residues of BRK substrates. The C-terminus of Dok1 contains several proline residues and the entire polypeptide contains eight PXXP motifs. We, therefore, examined whether Dok1 interacts with BRK and whether this interaction is SH3- and/or SH2-dependent and direct. First we transfected GFP-Dok1 in the presence or absence of either BRK-WT or

BRK-YF in HEK293 cells and subjected the cell lysates to immunoprecipitation with antibodies against Dok1 and BRK. We found that BRK associated with Dok1 and the strongest association





**E****F****G**

**Figure 5.3. BRK interacts with Dok1 through the SH3 domain *in vivo* and *in vitro*.** (A) HEK293 cells were transfected with empty vector, Myc-BRK-WT, Myc-BRK-YF, GFP-Dok1 or co-transfected with Myc-BRK-WT/GFP-Dok1 or Myc-BRK-YF/GFP-Dok1 and subjected to immunoprecipitation with anti-Dok1 and immunoblotted with BRK and Dok1 (top 2 panels). The expression of cellular proteins was determined in total cell lysates by immunoblotting for GFP, BRK and  $\beta$ -tubulin as a loading control. (B) BRK was immunoprecipitated with anti-BRK and subjected to immunoblotting analysis with anti-phosphotyrosines, anti-Dok1 and anti-BRK antibodies (top panels). Total cell lysates indicate the expression of BRK and Dok1 proteins. (C & D) HEK 293 cells were transfected with GFP-Dok1 alone or cotransfected with the indicated mutants of BRK and subjected to immunoprecipitation with anti-Dok1 followed by immunoblotting analysis with anti-BRK and anti-Dok1 antibodies. The cellular proteins were determined from the total cell lysates by immunoblotting analysis with anti-BRK and anti-Dok1 antibodies. (E) Overexpressed GFP-Dok1 or GFP-Dok1-Y362F in HEK 293 cell lysates from GFP-Dok1 or GFP-Dok1-Y362F expressing cells were subjected to pull-down assays with GST alone or recombinant GST-SH3 or GST-SH2 domain of BRK and immunoblotting analysis was performed with an anti-Dok1 antibody. (F) GFP-Dok1/BRK-YF or GFP-Dok1-Y362F/BRK-YF co-transfected cohorts of HEK 293 cell lysates were subjected to pull-down assays with GST alone or GST-SH3 or GST-SH2 domain of BRK followed by immunoblotting with an anti-Dok1 antibody. (G) Bacterially expressed GST, GST-SH3 and GST-SH2 domain of BRK proteins were detected via Coomassie blue staining.

was observed in GFP-Dok1/BRK-YF samples (Figure 5.3A, lane 6). We also observed a reciprocal association of GFP-Dok1 in anti-BRK immunoprecipitates (Figure 5.3B). Our data suggest that both BRK and GFP-Dok1 interact.

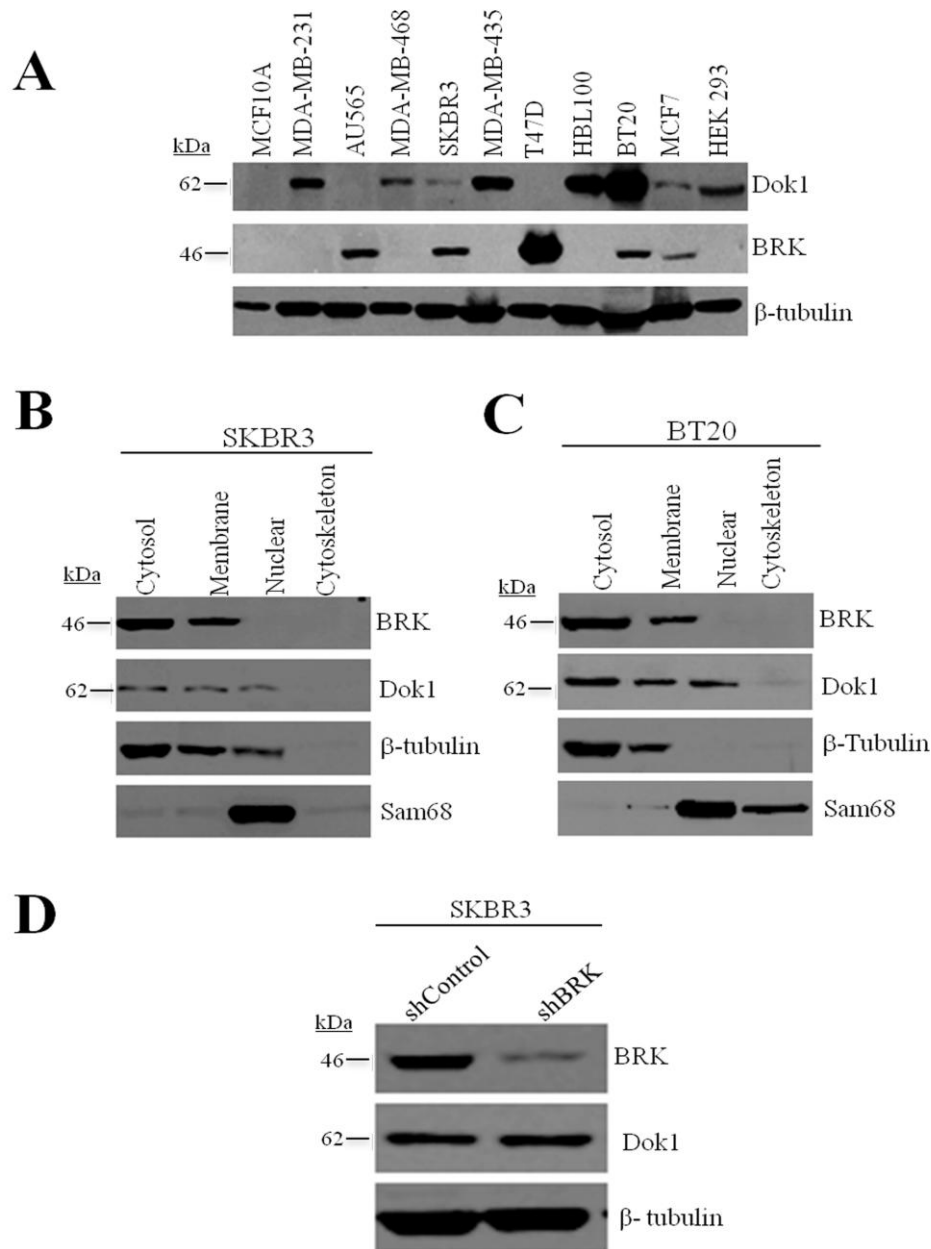
Next, to map the binding domain of BRK, we co-expressed the BRK-WT, constitutively active BRK-YF and kinase-inactive BRK-Y342A, as well as BRK mutants lacking an SH2 domain ( $\Delta$ SH2-BRK) or an SH3 domain ( $\Delta$ SH3-BRK) with GFP-Dok1 in 293 cells. We found that GFP-Dok1 co-precipitated with BRK-WT,  $\Delta$ SH2-BRK, BRK-Y342A and BRK-Y447F, but not with  $\Delta$ SH3-BRK (Figure 5.3C). Analysis of the total cell lysates are shown in Figure 5.3D. Together, these results suggest that recognition of GFP-Dok1 was mediated primarily by SH3 domain interactions. Interestingly, BRK-Y447F displayed a marked increase in GFP-Dok1 binding (Figure 5.3A, B and C, lane 6).

In order to further confirm that the binding of Dok1 to BRK was governed by the SH3 domain and to demonstrate whether the SH2 domain preferentially binds to tyrosine phosphorylated Dok1, we performed glutathione S-transferase (GST) pulldown assays on cell lysates from HEK 293 cells transfected with GFP-Dok1 WT or GFP-Dok1Y362F alone or co-transfected with BRKY447F. Probing with an antibody against Dok1 revealed that in the absence

of BRK Y447F, GFP-Dok1 WT and GFP-Dok1Y362F were able to interact with GST-BRK-SH3, but not with the GST-BRK-SH2 (Figure 5.3E). In the presence of BRK Y447F, in addition to SH3-binding, we also observed a strong interaction between GFP-Dok1 WT and BRK-SH2 domain (Figure 5.3F). However, the interaction between GFP-Dok1Y362F and BRK SH2 domain was markedly weaker than that of GFP-Dok1 WT (Figure 5.3F, right panel). This was predictable since we had showed in Figure 5.2D that BRK preferentially phosphorylates Dok1 on Y362. These data validate that BRK interacts with Dok1 through SH3 interactions and also suggest that the SH2 domain of BRK interacts predominantly with tyrosine-phosphorylated Dok1. Taken together, our data demonstrated the interaction between Dok1 and BRK under *in vivo* and *in vitro* conditions occurs via SH3 and also via SH2 binding on phosphorylated Y362 of Dok1.

#### **4.2.1.4. Inverse correlation between the levels of BRK and Dok1 in breast cancer cells**

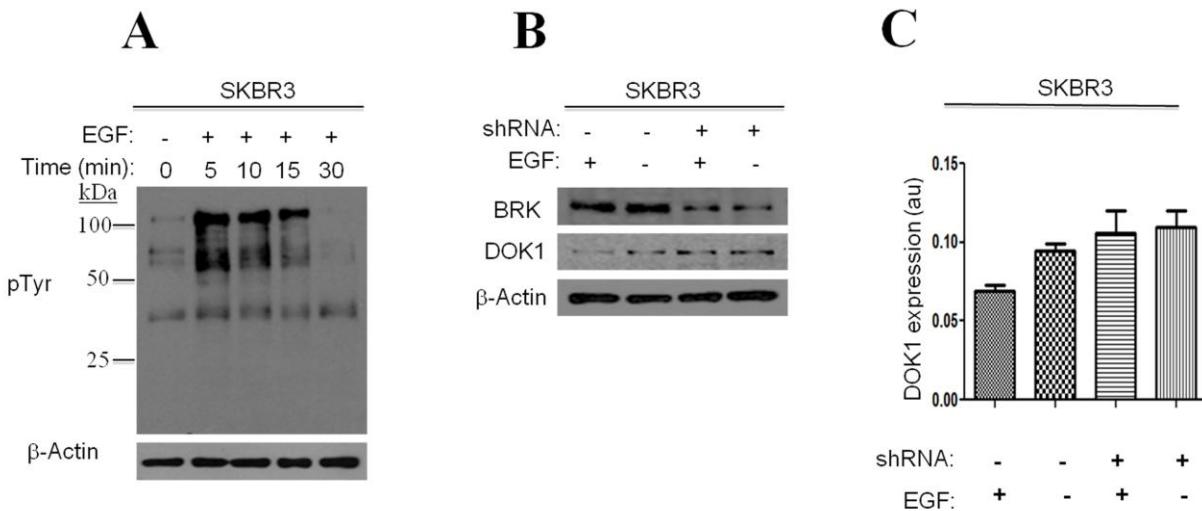
Since Dok1 has been described as a candidate tumor suppressor (Mashima *et al.*, 2009; Mercier *et al.*, 2011) and my data have shown that BRK has oncogenic properties (Brauer and Tyner, 2010; Miah *et al.*, 2012), we opted to investigate the functional link between BRK and Dok1. We began by evaluating the expression of Dok1 in breast cancer cells in order to determine if there was any correlation between the expression profiles of both proteins. Using immunoblotting analysis, we examined the expression of Dok1 and BRK in nine breast cancer cell lines and in an immortalized mammary epithelial cell line, MCF10A, as well as in the HEK293 cells. All the cell lines expressed detectable levels of Dok1, except for MCF10A, AU565 and T47D. The strongest expression was observed in BT20 cells while weaker expression levels occurred in SKBR3 and MCF7 (Figure 5.4A). BRK, on the other hand, was readily detectable in the breast cancer cell lines AU565, SKBR3, T47D, MCF7 and BT20, but not detectable in MCF10A, MDA-MB-231, MDA-MB-435, MDA-MB-468 and HBL100 (Figure 5.4A). The localization of BRK is predominantly cytoplasmic (Lukong *et al.*, 2005) and previous reports have shown that endogenous Dok1 was localized predominantly in the cytoplasm and plasma membrane (Niu *et al.*, 2006; Zhao *et al.*, 2001). Using sub-cellular fractionation studies, on Dok1 and BRK-positive breast cancer cell lines, SKBR3 and BT20, we found that both BRK and Dok1



**Figure 5.4. BRK and Dok1 are differentially overexpressed in the human breast cancer cell lines.** (A) Cellular proteins were detected in total cell lysates by immunoblotting analysis with anti-Dok1 and anti-BRK antibodies.  $\beta$ -tubulin expression served as a loading control. (B & C) SKBR3 and BT20 cells were fractionated into the cytosolic, membrane, nuclear and cytoskeleton fractions and subjected to immunoblotting analysis for the detection of BRK and Dok1.  $\beta$ -tubulin and Sam68 were used as controls for the cytosolic/membrane and nuclear compartments, respectively. (D) Stable BRK knockdown was performed on parental breast cancer cell lines SKBR3 using shRNA lentiviral vector plasmids against BRK and analyzed as indicated.

fractionated to the cytosolic and membrane fractions (Figure 5.4B and 5.4C). Since BRK and Dok1 were collected in the same cellular compartments and the expression levels of both proteins are inversely correlated, we investigated whether suppression of BRK expression by RNA interference could modulate the expression levels of Dok1 protein. As shown in Figure 5.4D, using short hairpin RNA (shRNA) against BRK in SKBR3 cells, we achieved a 60-70% knockdown of BRK; but the suppression of BRK did not have any significant effect on Dok1 re-expression.

Previously it has been shown that BRK is activated following EGF stimulation (Lukong *et al.*, 2005). We, therefore, investigated the effect of EGF stimulation on DOK1 expression in



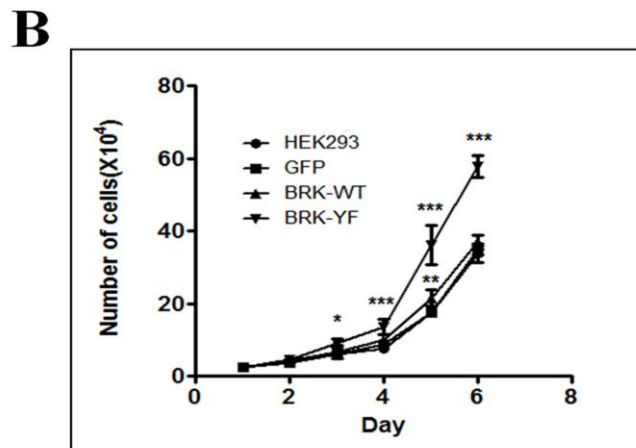
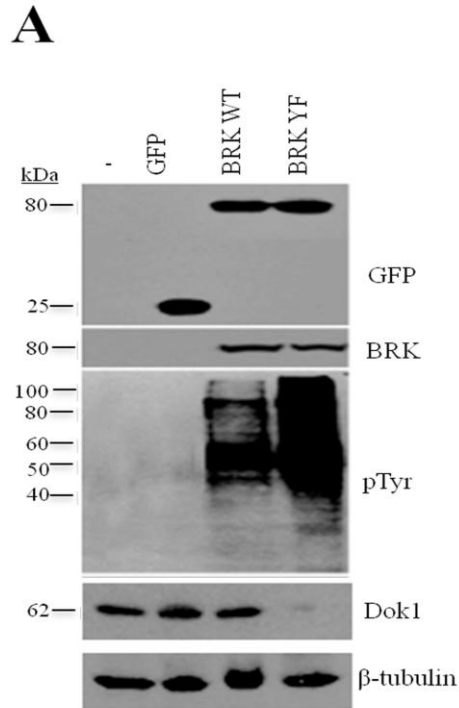
**Figure 5.5: The knock down of BRK in SKBR3 cells enhance DOK1 protein level.** (A) SKBR3 cells were treated with EGF (100 ng/mL) for 0, 5, 10, 15 and 30 min and then subjected to immunoblot analysis for the detection of phosphotyrosines and  $\beta$ -tubulin (as a loading control). (B and C) SKBR3 and stable BRK knock down SKBR3 cells were treated with or without EGF (100 ng/mL) for 15 min. Total cellular proteins were determined from the cell lysates by performing immunoblot analysis with anti-BRK and anti-DOK1 antibodies.  $\beta$ -actin served as a loading controls and the DOK1 expression was quantified and shown in a bar diagram.

the presence or absence of BRK (Figure 5.5). We stimulated SKBR3 breast cancer cell lines with EGF and observed peak activation of EGFR signalling at 15 min. We repeated the stimulation in BRK-positive as well as BRK negative (knockdown) SKBR3 cells and observed that while treatment with EGF suppressed endogenous Dok1 expression, a detectable increase in Dok1 levels was observed in BRK-knockdown cells (Figure 5.5B and C). Our data together indicate an

inverse correlation between the expression of Dok1 and BRK that is partly regulated through EGF stimulation.

#### **4.2.1.5. Activated BRK downregulates Dok1 protein expression**

The inverse correlation between BRK and Dok1 prompted us to further investigate whether BRK activation and overexpression could modulate the expression of Dok1 protein. In addition, previous studies have shown that oncogenic tyrosine kinases such as p210bcr-abl and v-Src downregulate Dok-1 in a kinase activity-dependent manner (Niu *et al.*, 2006). Since we recently reported that constitutively active BRK (BRK-Y447F) promotes tumor formation (Miah *et al.*, 2012), we examined whether BRK-Y447F, like oncogenic Src, could downregulate endogenous Dok1. We used HEK293 cells as a model to study the interaction between BRK and Dok1 since HEK293 cells express high levels of Dok1, but express no endogenous BRK (Figure 5.4A). We generated three HEK293 cell lines stably expressing GFP (empty control vector), GFP-BRK WT or GFP-BRK-YF by retroviral transduction. All stable cell lines expressed the transgene as determined by immunoblotting with the anti-GFP antibody (Figure 5.6A). Immunoblotting with anti-BRK confirmed the expression of GFP-BRK WT and Y447F and also validated the absence of BRK in HEK293 cells. The BRK-transduced cells displayed elevated levels of phosphorylation of cellular targets, as visualized with an anti-phosphotyrosine antibody, pY20. Furthermore, as expected BRK-Y447F-transduced cells displayed level of Try phosphorylation that were significantly higher than those of BRK-WT (Figure 5.6A). We therefore evaluated the expression of Dok1 in all transduced cell lines and the parental control cell line and observed a significant reduction in the levels of Dok1 protein in the cells transduced with constitutively active BRK- Y447F compared to those in the BRK-WT, GFP alone, and in the parental cells (Figure 5.6A, bottom panels). Since Dok1 is a tumor suppressor and we observed a dramatic difference between the effects of BRK-WT and BRK-Y447F on Dok1 expression, we evaluated the growth rates of the stable cell lines. We found that the BRK-Y447F-transduced cells displayed significantly higher growth rates than the cells transduced with either BRK-WT or GFP alone (Figure 5.6B). Taken together, our data indicate that the catalytic activation of BRK is critical for its ability to downregulate endogenous Dok1 and that the observed suppression of Dok1 may contribute to BRK-promoted cell proliferation.



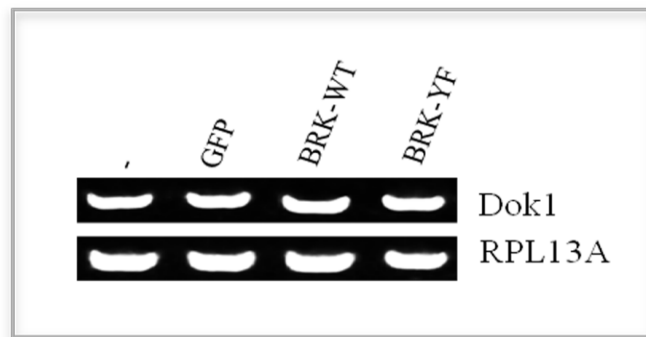
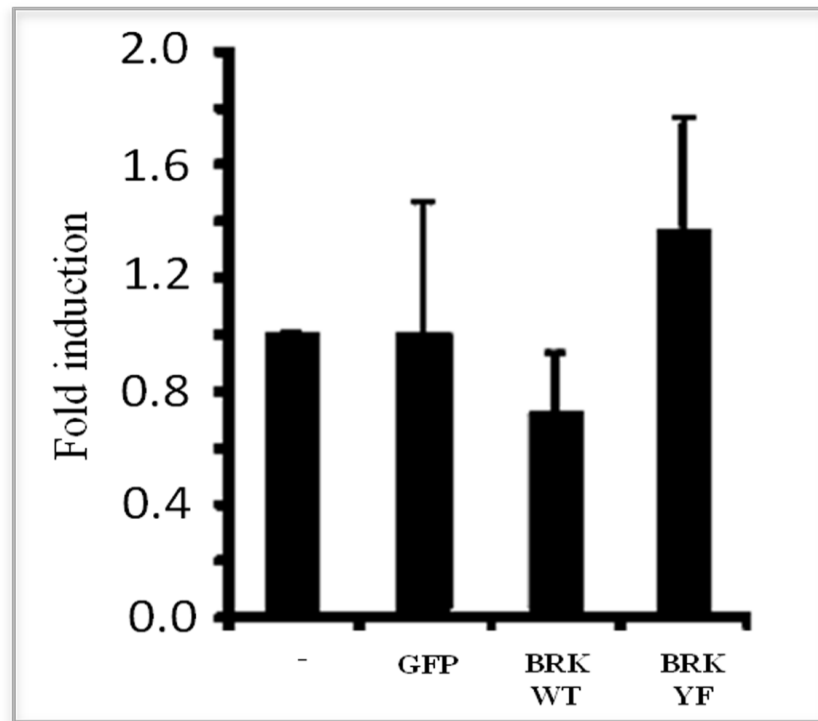
**Figure 5.6. Constitutively active BRK downregulates Dok1 protein expression.** (A) Immunoblotting analysis of total cell lysates from HEK-293 stable cell lines is showing the expression of GFP alone, GFP-BRK-WT and GFP-BRK-YF (top panel), BRK (middle panel) and phosphorylated tyrosines (bottom panel).  $\beta$ -tubulin served as a loading control. The bottom part shows the immunoblotting analysis of endogenous Dok1 in the stable HEK293 sublines. Expression of Dok1 was examined by immunoblotting analysis. (B) Characterization of cell proliferation in response to BRK-WT and BRK-YF. The P-values were determined for control and stably transfected cells and set at \*\*\*P  $\leq$  0.0001, \*\*P  $\leq$  0.001 and \*P  $\leq$  0.05 for statistical significance.

#### 4.2.1.5. Constitutively activated BRK diminishes the stability of Dok1 protein

Since BRK induces the downregulation of Dok1, we then investigated the mechanisms of action of BRK in the downregulation of Dok1. Dok1 is a tumor suppressor and there are several potential mechanisms that account for the inactivation of tumor suppressors in cancer including those pertaining to the regulation of gene expression and post-translational events targeting protein stability (Herman and Baylin, 2003; Oliveira *et al.*, 2005; Rodriguez-Paredes and Esteller, 2011; Yuan *et al.*, 2001). To identify the underlying mechanisms by which BRK-Y447F downregulates Dok1, we first evaluated the expression of Dok1 transcripts in BRK-transduced HEK293 cells by both semi-quantitative RT-PCR and quantitative real-time qRT-PCR using Dok1-specific primers. As shown in Figure 5.7A and 5.7 B, RT-PCR and qRT-PCR analyses revealed no significant difference between the levels of Dok1 mRNA levels in BRK-Y447F-transduced cells compared with cells stably expressing BRK-WT or the control cell lines. These results clearly indicate that BRK does not influence Dok1 mRNA levels, implying that BRK downregulates Dok-1 protein.

Our results in Figure 5.7 led us to the hypothesis that BRK downregulates Dok1 by reducing the stability of Dok1 protein. To investigate this possibility, we examined the relative half-life of endogenous Dok1 protein in the presence or absence of BRK-Y447F. To this end, we treated various BRK-Y447F-transduced HEK293 cells and control cells at various time points between 1 and 24 h with cycloheximide (CHX, an inhibitor of protein synthesis). The cells were harvested periodically as indicated in Figure 5.8A. Western blotting of BRK-Y447F cell lysates showed that Dok1 protein levels were reduced by more than 50%, 75% and 95% at 8, 12 and 24 h, respectively following treatment with CHX (Figure 5.8A, left panel). However, in the control samples (Figure 5.8A, right panel) the initial Dok1 protein level was reduced to half after 12 h of treatment with CHX and a residual level of about 25% after 24 h (Figure 5.8A, right panel). Statistical quantification of the levels of expression of Dok1 is provided in Figure 5.8A, lower panels. Our data thus indicate that Dok1 exhibited a relatively shorter half-life in cells transduced with BRK-Y447F compared with the control cells. These data indicate that Dok1 is a relatively stable protein with a physiological half-life of about 12 h and that the stability of the Dok1 protein is compromised in the presence of constitutively active BRK.

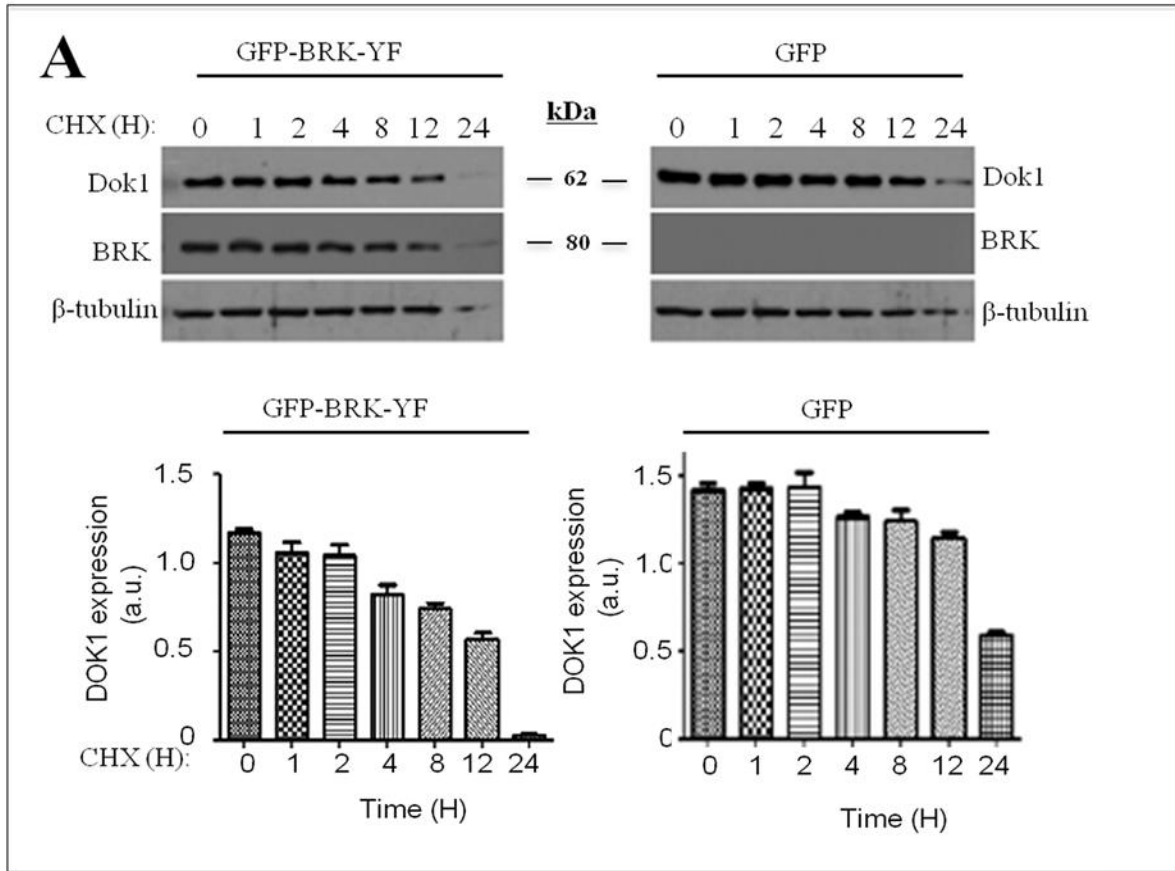


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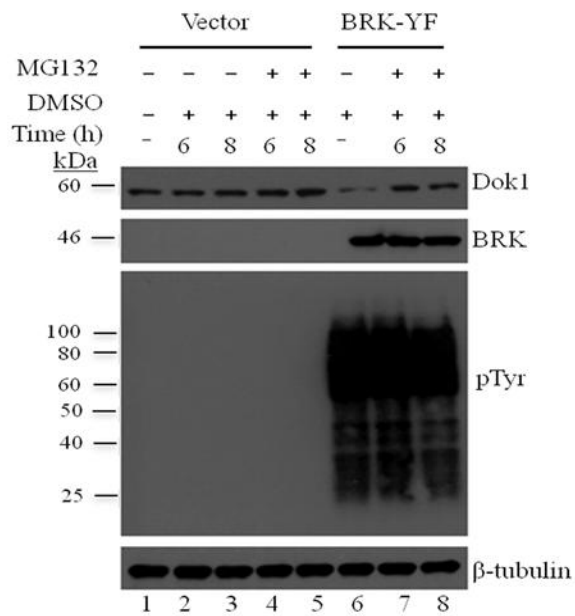
**Figure 5.7. Constitutively active BRK does not affect the levels of Dok1 mRNA.** (A & B) Total RNA was isolated from HEK293 cells stably transduced with empty vector, GFP, GFP-BRK-WT and GFP-BRK-YF. Levels of Dok1 mRNA were then analyzed using RT-PCR (A) and qPCR (B). RPL13A gene was used as internal control. Error bars are the mean  $\pm$  SEM of three biological repeats each having three technical repeats.

#### 4.2.1.6. BRK downregulates Dok1 via proteasomal degradation

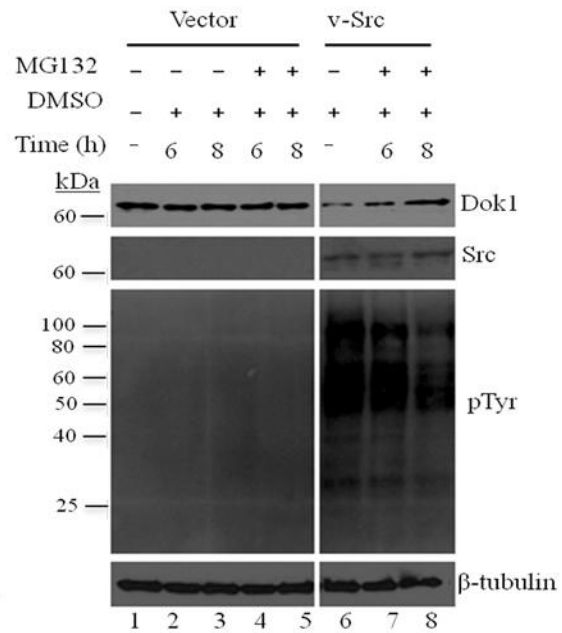
We next explored the possibility that the BRK-induced downregulation of Dok1 is mediated by the ubiquitin-proteasome pathway (UPP). UPP plays a well-characterized key role in protein stability by eliminating most of the intracellular proteins in eukaryotes via degradation (Kerscher *et al.*, 2006). To investigate whether Dok1 is regulated by BRK-Y447F via UPP associated degradation, we first examined Dok1 protein levels in the presence or absence of the peptide aldehyde proteasome inhibitor MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucine). MG132 is a specific proteasome inhibitor that binds reversibly to the N-terminal Thr residue of the  $\beta$ 1 subunit within the 26S proteasome (Myung *et al.*, 2001). We used the oncogenic v-Src as a positive control in these experiments since its effect on Dok1 stability in the presence of MG132 has been characterized (Janas and Van Aelst, 2011). First we examined the effect of MG132 on Dok1 stability in the control HEK 293 cells transduced with the empty vector. The cells were treated with or without MG132 for 6 or 8 h. Similar treatment with dimethyl sulfoxide (DMSO) served as a negative control (Figure 5.8B). We observed that MG132 treatment led to an increase in Dok1 protein levels in HEK293 cells (Figure 5.8B, lanes 4 and 5). The increase in Dok1 levels as a result of MG132 treatment indicates that the UPP associated degradation is indeed involved in regulating Dok1 protein turnover. We then transfected HEK 293 cells with plasmids expressing either BRK-Y447F or v-Src, and then treated the cells with MG132 or DMSO at the indicated intervals (Figure 5.8B and C). We observed a strong increase in the levels of the Dok1 protein following MG132 treatment in cells transfected with either BRK-Y447F or v-Src (Figure 5.8B and C). The presence of MG132 did not affect the activity of BRK-Y447F as indicated by comparable anti-phosphotyrosine staining (Figure 5.8B, lanes 6, 7 and 8). Surprisingly, the levels of tyrosine phosphorylation in v-Src-transfected cells were slightly lowered in the presence of MG132 (Figure 5.8C, lanes 6, 7 and 8). These findings suggest that the increase in Dok1 protein levels was the result of an inhibition of the proteasomal proteases by MG132. To corroborate these findings, we treated BRK-Y447F and v-Src-transfected cells for 8 h with lactacystin, a proteasome inhibitor that binds covalently to the 26S proteasome (Fenteany *et al.*, 1995). Immunoblot analysis revealed that treatment with lactacystin, akin to MG132, resulted in the stabilization of Dok1 protein in both BRK-Y447F and v-Src transfected cells (Figure 5.8D)

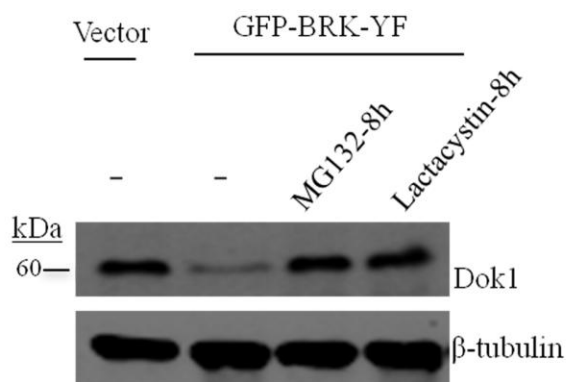
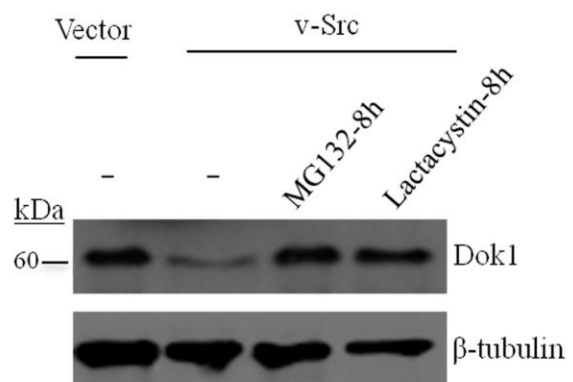
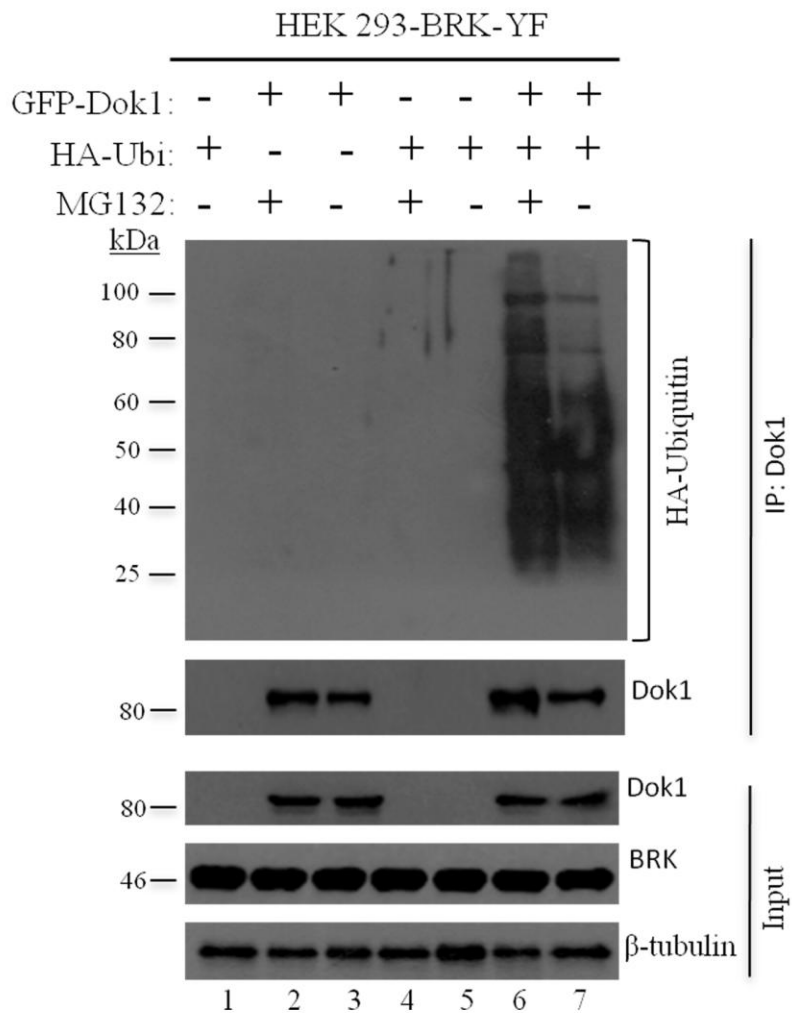


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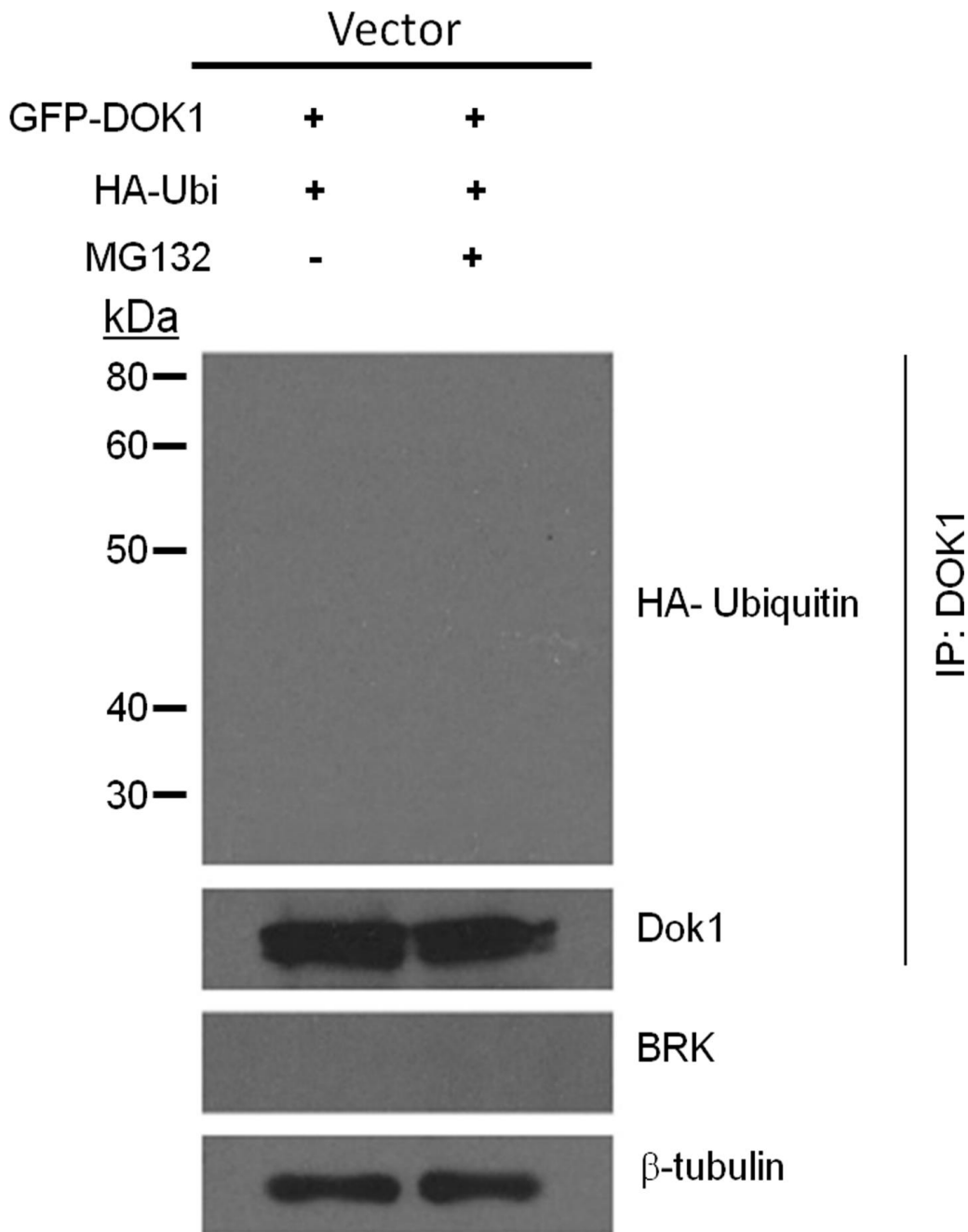


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**Figure 5.8. Activated BRK downregulates Dok1 by reducing its stability.** (A) HEK293 cells or HEK293-BRK-YF stable cell line were treated with a protein synthesis inhibitor cycloheximide (CHX: 200  $\mu\text{g}/\text{mL}$ ) for the indicated time points and then the cells were lysed and analyzed by immunoblotting for Dok1, BRK and  $\beta$ -tubulin as a loading control. (B) HEK293 cells were stably transduced with HEK293-BRK-YF and treated with either a proteasome inhibitor MG132 (10  $\mu\text{M}$ ) or the vehicle DMSO as the control, at different time points (above the plot). Cellular proteins were determined in total cell lysates by immunoblotting analysis with anti-Dok1, anti-BRK, anti-phosphotyrosines antibodies.  $\beta$ -tubulin was used as a loading control. (C) Empty vector or v-Src was transiently transfected into HEK293 cells and the cells treated with a proteasome inhibitor MG132 (10  $\mu\text{M}$ ) and vehicle control DMSO for the indicated time points. Immunoblotting analysis of total cell lysates was performed to detect Dok1, v-Src, phosphotyrosines and  $\beta$ -tubulin served as a loading control. (D & E) HEK293 cells were transfected with empty control vector or BRK-YF or v-Src and treated with MG132 (10  $\mu\text{M}$ ), Lactacystin (5  $\mu\text{M}$ ) or control vehicle for 8 h. Then the cell lysates were subjected to immunoblot analysis with anti-Dok1 antibody.  $\beta$ -tubulin is as a loading control. (F) HEK293-BRK-YF stable cells were transiently co-transfected with Dok1 and HA-Ubiquitin plasmids and after 12 h the cells were treated MG132 (10  $\mu\text{M}$ ) for an additional 8 h. The total cell lysates were subjected to immunoprecipitation with anti-Dok1 followed by immunoblotting analysis with anti-HA and anti-Dok1 antibodies. The inputs were analyzed as indicated.

and E). These findings support our notion that both BRK and v-Src render Dok1 unstable and increasingly prone to degradation via the UPP.

Finally, we examined whether the ubiquitination machinery directly mediates the BRK-induced downregulation of Dok1. Plasmids expressing GFP-Dok1, myc-BRK-Y447F and HA-ubiquitin were transfected into HEK293 cells. The cells were then treated with either the protease inhibitor, MG132 or DMSO as a negative control. We performed an immunoprecipitation assay using anti-Dok1 antibodies followed by immunoblotting using anti-HA antibodies. Our data showed a significantly enhanced high molecular weight smear of molecules conjugated to the GFP-Dok1 immunoprecipitated in the presence of MG132 (Figure 5.8F, lanes 6 and 7) compared with the controls in BRKY44F-expressing cells (lanes 1-5). In the presence of BRK-Y447F, ectopic of GFP-Dok1 and HA-Ub, in the presence or absence of MG132, resulted in no ubiquitination of Dok1 (Figure 5.9). It is worth noting that treatment with MG132 in the presence of GFP-BRK-Y447F had a greater impact on endogenous Dok1 levels (Figure 5.8D) than the ectopically expressed GFP-Dok1 levels (Figure 5.8F); however, the reason for these discrepancies is not obvious to us. Our findings as a whole strongly support the notion that BRK destabilizes Dok1 by promoting its ubiquitination, and an eventual degradation via the ubiquitin-proteasome pathway.



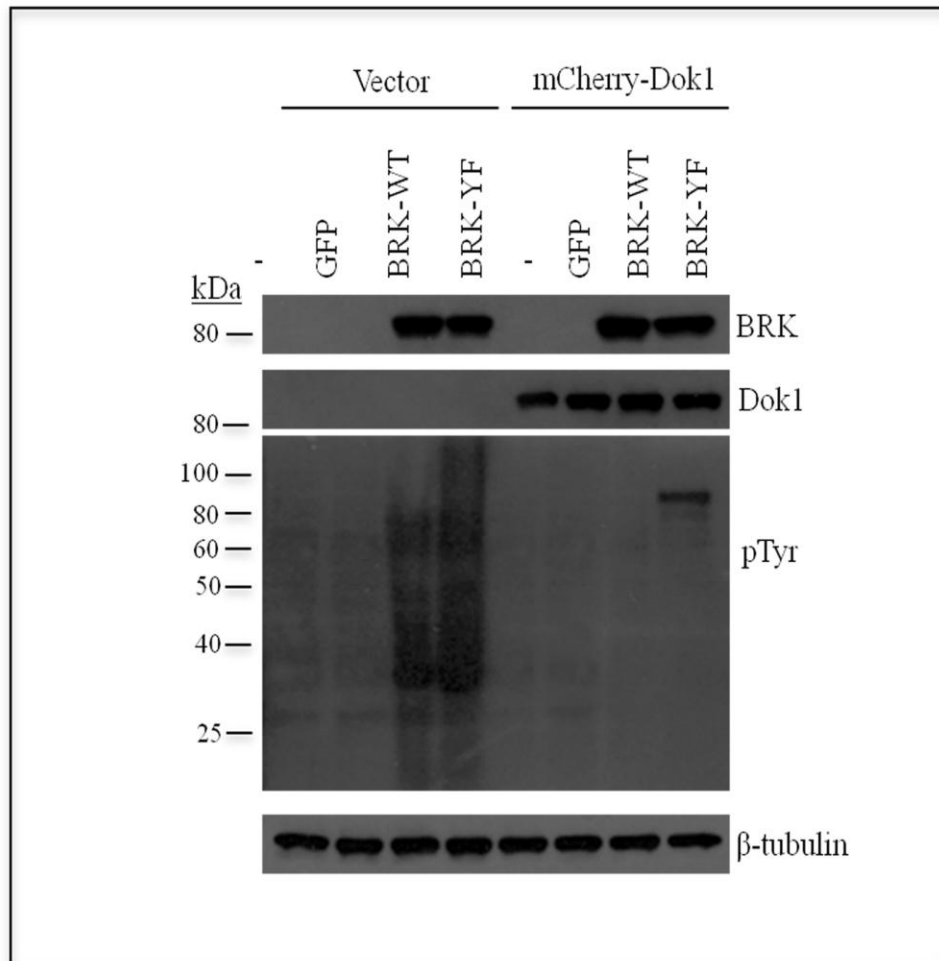
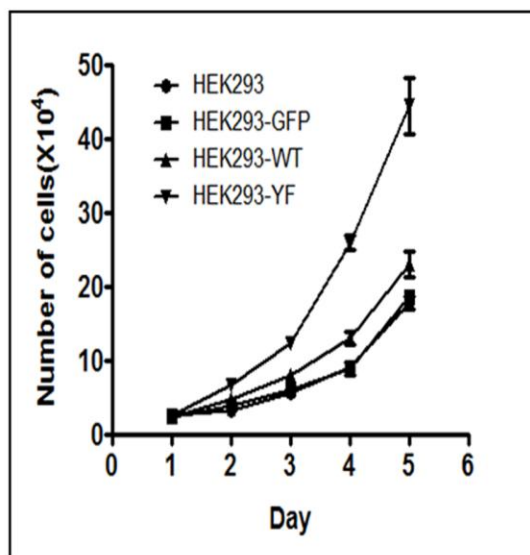
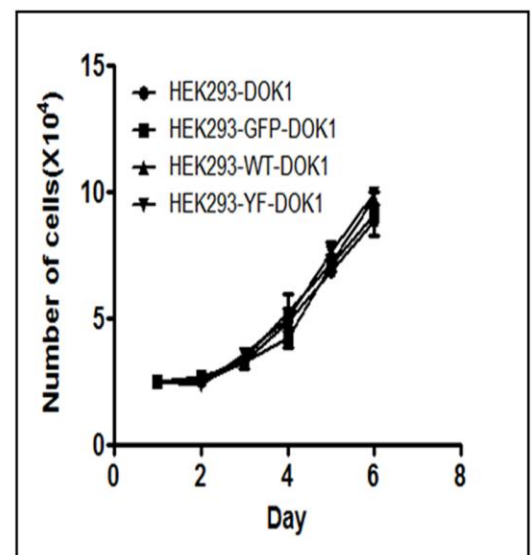
**Figure 5.9: Dok1 is not ubiquitinated in the absence of BRK.** HEK293 cells were transiently co-transfected with GFP-Dok1, HA-ubiquitin and empty Myc vector and incubated in the presence or absence of the proteasomal inhibitor, MG132 (10  $\mu$ M) for 8 h. Cell lysates were subjected to immunoprecipitation with anti-Dok1 antibody and immunoblotting was performed with antibodies against HA and Dok1 (top panel). Total cell lysates were subjected to immunoblotting with antibodies against Dok1, BRK and  $\beta$ -tubulin as a loading control.

#### 4.2.1.7. Overexpression of Dok1 suppresses BRK-induced cell proliferation and migration

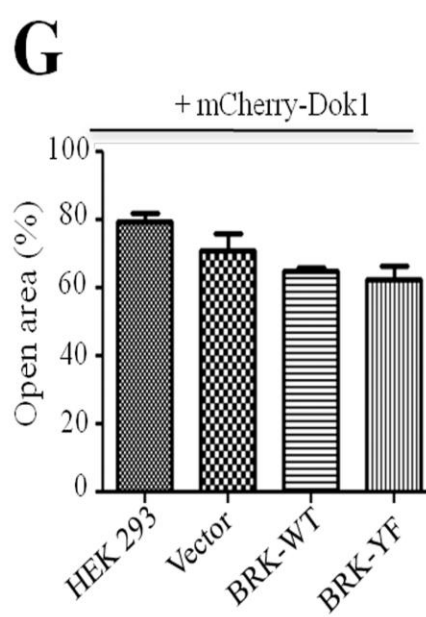
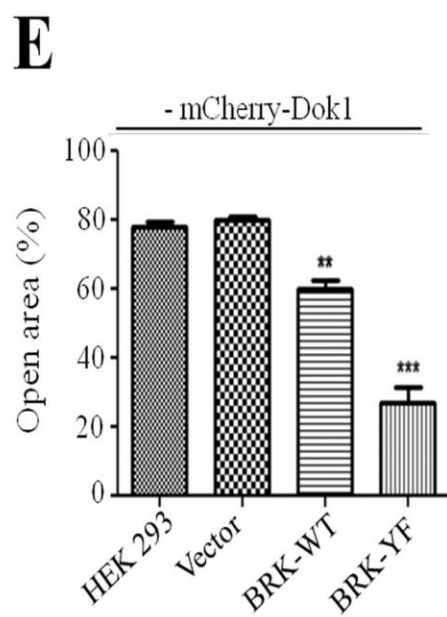
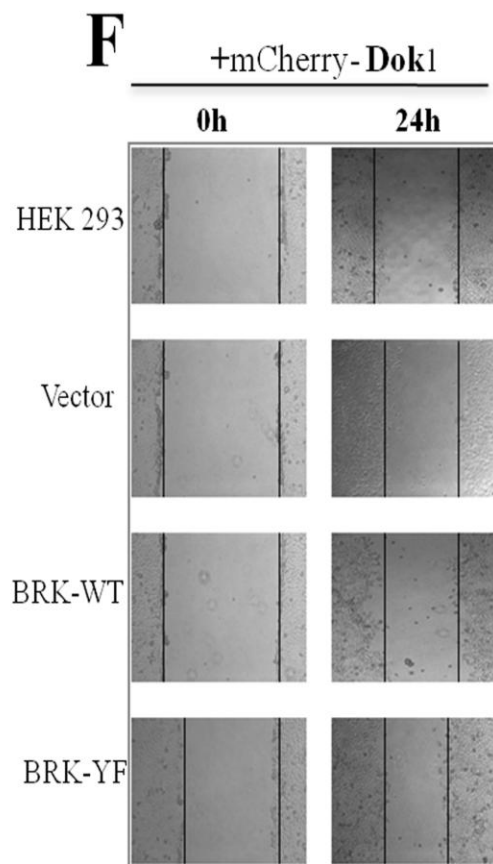
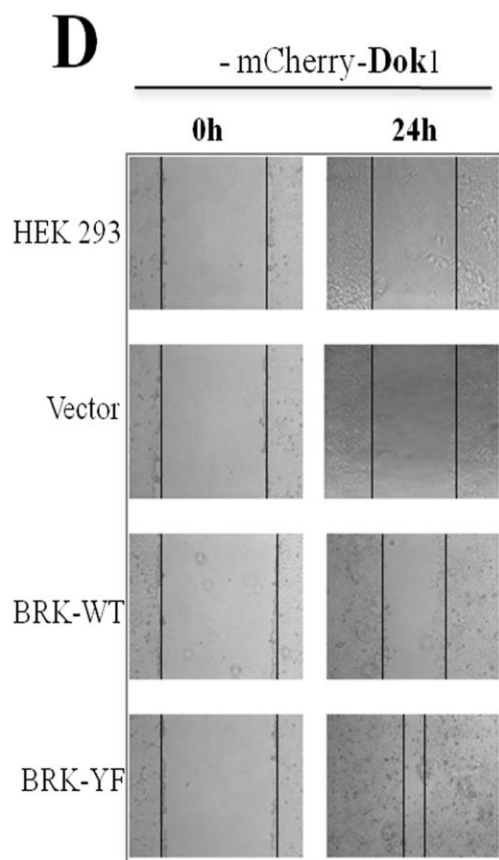
Dok1 is a tumor suppressor and several studies have concurred that the overexpression of Dok1 suppresses cell proliferation and migration (Mashima *et al.*, 2009; Mercier *et al.*, 2011; Niki *et al.*, 2004; Oki *et al.*, 2005). Ectopic expression of Dok1 has been shown to inhibit cell proliferation and transformation induced by oncogenic tyrosine kinases, including the 210bcr-abl and Src family kinases (Janas and Van Aelst, 2011; Oki *et al.*, 2005).

Previously, we along with others showed that BRK overexpression and activation enhanced cell proliferation, cell migration and tumor formation (Ie Kim and Lee, 2009; Ikeda *et al.*, 2010; Lofgren *et al.*, 2011; Lukong and Richard, 2008; Miah *et al.*, 2012; Xiang *et al.*, 2008). To test whether Dok1 can also modulate the oncogenic properties of BRK, we evaluated the effect of Dok1 on BRK-induced cell proliferation and migration. HEK 293 cells stably expressing GFP alone, GFP-BRK-WT and GFP-BRKY447F were infected with adenoviruses expressing mCherry-Dok1 (Figure 5.10A). In the absence of mCherry-Dok1, cells stably expressing GFP-BRKY447F displayed a significantly higher growth rate compared with GFP-BRK-WT expressing cells as well as the control cell lines (Figure 5.10B). Remarkably, the introduction of Dok1 resulted in a dramatic decrease in the rate of growth of the BRK-Y447F-transduced cells, similar to the levels exhibited by the control and BRK-WT cells (Figure 5.10C). Similar results were obtained with BRK-negative cells lines MDA-MB-231 stably expressing BRK variants BRK-Y447F or BRK-WT (Figure 5.11). These data indicate that the overexpression of Dok1 suppresses BRK-induced cell proliferation.

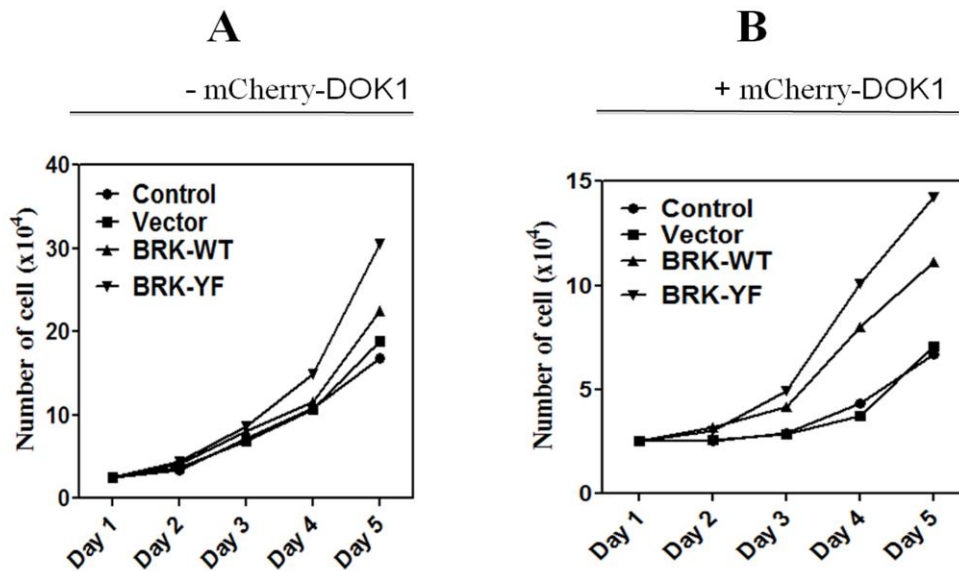
Finally, we employed wound healing assays to assess the effect of Dok1 on BRK-induced cell migration using the same set of cell lines described in Figure 5.10B and C. The cell surfaces were scratched and photo-micrographs taken at different time intervals between 0 and 24 h. Figure 5.10D and F show representative images taken at 0 and 24 h. The results showed that the overexpression of BRK-Y447F accelerated the wound healing process as observed by the reduced size of the wounded area after 24 h compared to either the BRK-WT or control samples (Figure 5.10D). Furthermore, the ectopic expression of Dok1 reduced the migration rates of the BRK-Y447F cells to near control rates (Figure 5.10F). The results are quantified in Figure 5.10E and G. These results were reproduced using MDA-MB-231 cells stably expressing the BRK

**A****B****C**

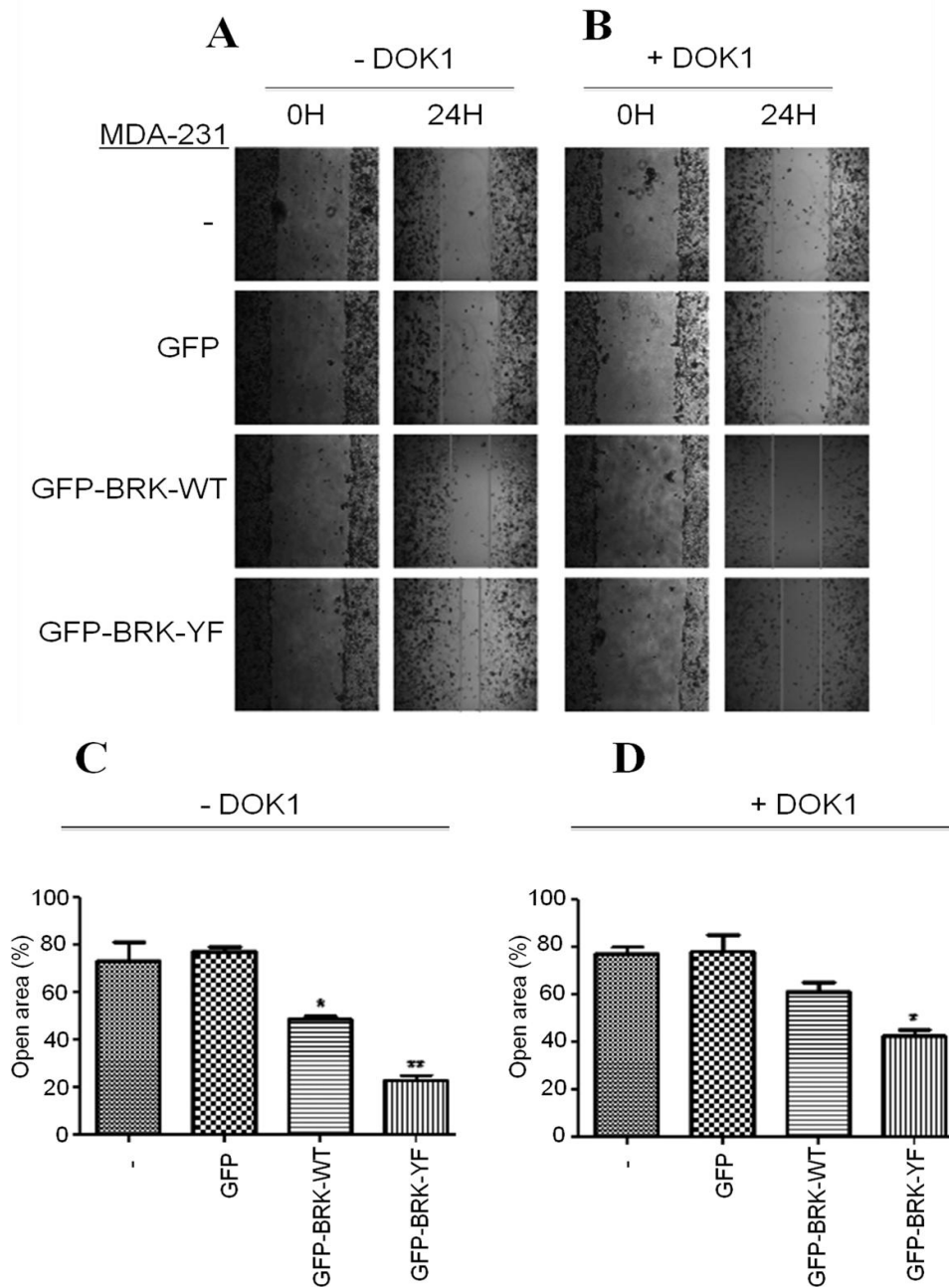




**Figure 5.10. Dok1 inhibits BRK-induced cell proliferation and migration.** (A) HEK293 stable sub-cell lines were transduced with mCherry-Dok1 using adenoviral vector. Cellular proteins were detected in total cell lysates by immunoblotting analysis with anti-BRK, anti-Dok1, and anti-phosphotyrosine antibodies.  $\beta$ -tubulin served as a loading control. (B & C) HEK293 stable cells were transduced with or without mCherry-Dok1 adenovector and were monitored for cell proliferation. (D & E) Cell migration determined by the healing of a fixed wound area induced in the different HEK293 stable cells. The percentage of open area at 24 h is plotted. (F & G) Cell migration analysis was performed with the indicated stable cell lines expressing mCherry-Dok1 or an empty vector. The assay was based on the rate of wound closure in the scratched cells. The percentage of open area at 24 h is plotted. The migration assay was performed in three independent experiments. Data were means  $\pm$  standard errors. Statistics: and  $**P \leq 0.001$  and  $***P \leq 0.0001$ .



**Figure 5.11: Dok1 inhibits BRK-induced cell proliferation in MDA-MB 231 cells.** (A and B) MDA-MB 231 stable cells (GFP alone, GFP-BRK-WT and GFP-BRK-YF) were transduced with or without mCherry-Dok1 adenovector and were monitored for cell proliferation by Coulter Counter.



**Figure 5.12: Dok1 inhibits BRK-induced cell migration in MDA-MB 231 cells.** (A & B) MDA-MB 231 stable cells were transduced with or without mCherry-Dok1adenovector and were monitored for cell migration based on the healing of the wound area. The percentage of open area at 24 h is plotted. (C & D) Cell migration analysis was performed with the indicated stable cell lines expressing mCherry-Dok1 or an empty vector. The assay was based on the rate of wound closure in the scratched cells. The percentage of open area at 24 h is plotted. The migration assay was performed in three independent experiments. Data were means  $\pm$  SD. Statistics: \* $P \leq 0.05$  and \*\* $P \leq 0.001$ .

variants (Figure 5.12). Taken together, these data confirm the anti-oncogenic properties of Dok1 and suggest that downregulation of Dok1 is one of the mechanisms by which BRK manifests its oncogenic function.

#### **4.2.1.8. Summary**

The overexpression of BRK has been shown to sensitize mammary epithelial cells to mitogenic signaling and to promote cell proliferation and tumor formation. The identification and characterization of BRK target proteins have helped in understanding of the potential molecular mechanisms of BRK. In this study, we demonstrate a functional link between Dok1 depletion and the regulation of BRK-induced cellular processes such as cell proliferation and migration. Downstream of tyrosine kinases 1 or Dok1 is a scaffolding protein and a substrate of several tyrosine kinases. Herein we show that BRK interacts with and phosphorylates Dok1 specifically on Y362. We demonstrate that this phosphorylation by BRK significantly downregulates Dok1 in a ubiquitin-proteasome-mediated mechanism. Together, these results suggest a novel mechanism of action of BRK in the promotion of tumor formation, which involves the targeting of tumor suppressor Dok1 for degradation through the ubiquitin-proteasomal pathway.

## 5. Discussion

### 5.1. Activation of breast tumor kinase (BRK) is essential for cell migration and tumor development.

BRK is a tyrosine kinase with a functional architecture and modes of regulation reminiscent of Src family kinases. Like Src kinases, BRK is regulated negatively by phosphorylation of C-terminal tyrosine 447 and positively by phosphorylation of tyrosine 342 in the catalytic domain (Derry *et al.*, 2000; Qiu and Miller, 2002). The BRK C-terminal tyrosine residue 447 and 342 are analogous to the human Src regulatory Y530 and Y419 respectively (Derry *et al.*, 2000; Qiu and Miller, 2002). Csk phosphorylates the C-terminal tyrosine of Src kinases, promoting intramolecular interactions that lock the Src kinases in an inactive conformation (Okada and Nakagawa, 1989). Therefore, Tyr530Phe mutation or dephosphorylation activates Src (Hunter, 1987). This dephosphorylation is often accompanied by autophosphorylation of pTyr419 within the activation loop, and results in a 10-fold increase in kinase activity (Boggon and Eck, 2004). Prevention of autophosphorylation by Tyr419-Phe mutation suppresses this activation by about 5 fold (Kmiecik and Shalloway, 1987; Piwnicka-Worms *et al.*, 1987), demonstrating the importance of synergy in Src activation. Therefore, the classical activation pathway of Src includes dephosphorylation of Y530 to initiate a conformational change of the protein (partial activation) that promotes full activation by autophosphorylation of tyrosine site 419. The transmembrane receptor protein tyrosine phosphatase (RPTP) $\alpha$  has been shown to activate Src by 5-fold by dephosphorylating both pTyr530 and pTyr419 *in vivo* (Pallen, 2003). Clinically, dephosphorylated Y530Src is associated with early stages of carcinogenesis in breast cancer patients (Elsberger *et al.*, 2010). However, the negative and positive regulators of BRK activity have not been identified.

In this study, in order to investigate the role of full activation of BRK on the oncogenic properties of BRK, we generated constitutively active mutant of BRK by mutating Tyr447 to Phe. We first evaluated the activities of various GFP-tagged and non-tagged BRK mutants and determined that in both cases certain mutant forms of BRK, including BRK-YF, displayed higher activity than wild-type BRK when transfected into HEK293 cells (Figure 4.1B) as has been previously demonstrated (Lukong *et al.*, 2009; Qiu and Miller, 2004). We next generated three sets of stable cell lines overexpressing GFP-BRK-WT and fully activated GFP-BRK-Y447F, as

well as a GFP-alone control (Figure 4.2B). The cell lines selected for these studies included HEK293 cells, immortalized mammary epithelial cell line, MCF10A and highly invasive breast cancer cell line, MDA-MB-231. These cell lines expressed little or no BRK (Figure 4.2A). We observed hyperactivation in BRK-YF stable cell lines compared to BRK-WT and controls (Figure 4.2C). Using our stable cell lines, we present evidence that support a critical role for BRK activity in the promotion of the oncogenic processes such as cell proliferation and migration, and tumor formation *in vivo*.

Mitogenic signaling involves the sequential activation of a MAPK (mitogen-activated protein kinase) kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and the MAPK (Krishna and Narang, 2008). There are four distinct MAPK signaling cascades, all of which have been implicated in breast cancer. They are the extracellular regulated kinase ERK 1/2 pathway, the ERK5 pathway, the p38 pathway and the c-Jun N-terminal kinase (JNK) pathway (Krishna and Narang, 2008; Wang and Tournier, 2006). ERK 1/2 is significantly activated in a large subset of mammary tumors (Mueller *et al.*, 2000) and persistent activation of ErbB2 oncogene in MCF-10A is associated with activation of ERK 1/2 (Seton-Rogers *et al.*, 2004). Previous studies have demonstrated that exogenous expression of BRK enhanced EGF-induced proliferation of normal mammary epithelial cells EGF (Kamalati *et al.*, 1996). We previously demonstrated that phosphorylation of BRK substrate Sam68 upon EGF stimulation is partly BRK-dependent (Lukong *et al.*, 2005). Here we show that full activation of BRK results in significantly higher cell growth rates is associated with hyperactivation of ERK 1/2 (Figure 2D and E), consistent with a previous study (Li *et al.*, 2012). We did not observed any effect of BRK on the activation of ERK5, p38 or JNK (data not shown) although Ostrander *et al* in their study showed that BRK regulates ERK5 and p38 activation albeit in heregulin-induced conditions (Ostrander *et al.*, 2007).

Accumulating evidence has highlighted the importance of BRK in cell migration. For instance, BRK activation downstream of the EGFR resulted in increased Rac1 activity, associated with migration and invasion programs in both skin and breast cancer cell lines (Chen *et al.*, 2004). A recent study indicated that silencing BRK in HER2-positive breast cancer cell lines BT20 and JIMT1 decreased HER2 activation and reduced cell migration (Ludyga *et al.*, 2011). In our study, using both the wound-healing and Transwell assays we showed that stable knockdown of BRK in both BT20 and SKBR3 cells which are HER2-positive and negative cell

lines respectively, cell migration was significantly reduced (Figure 4.4 and 4.5B and C). This implies that BRK can regulate cell migration in an EGFR-independent pathway. Similarly, stable expression of wild-type BRK and constitutively active BRK in HER2-negative MDA-MB-231 significantly increased cell migration (Figure 4.3 and 4.5A). We also confirmed the transforming potential of the Y447F mutant by evaluating the capacity our stable cell lines for anchorage-independent growth. Consistent with previous reports, stable expression of BRK-Y447F resulted in a significant increase in size of colonies formed in soft agar (Kamalati *et al.*, 1996; Kim and Lee, 2005).

Next we examined if activation of BRK was essential for tumor formation *in vivo*. We transplanted our engineered MDA-MB-231 stable cell lines into immune compromised mice and measured the tumor volume over time (Figure 4.7). We noted that the fully activated form of BRK promoted a more rapid tumor growth as compared to the wild-type BRK or the control. Overexpression of wild-type BRK was previously shown by Shen *et al* to promote tumor growth in animal (Shen *et al.*, 2008), but our data stresses the importance of BRK activity in the promotion of tumor growth. The exact mechanism by which BRK promotes migration and tumorigenesis is unknown. We have shown that BRK may regulate these processes via activation of the Raf1-MEK1/2 ERK1/2 cascade as demonstrated by hyperphosphorylation of ERK1/2 in the BRK stable cell lines (Figure 4.2E). In other studies, it was suggested that BRK promotes cell migration and invasion through p190RhoGAP phosphorylation that results in the activation of Ras to promote cell migration (Shen *et al.*, 2008).

Overall this study demonstrates that overexpression of constitutively active BRK highly correlates with exaggerated cell proliferation and ultimately, with increased transformation potential of epithelial cells. We have, however, demonstrated for the first time that full activation of BRK is an essential component in the promotion of tumorigenesis by BRK *in vivo*. Therefore, it can be predicted that BRK hyperactivation in human breast cancers might exhibit an aggressive clinical behavior. We know from a recent study that mammary-targeted expression of wild-type BRK promoted infrequent mammary tumors with delayed latency (Lofgren *et al.*, 2011). Based on our results, the question of whether activated BRK is capable of directly inducing mammary gland tumors is feasible. Furthermore, many studies have reported that elevation of Src activity in human tumors including breast cancer correlates with disease stage and poor prognosis (Elsberger *et al.*, 2010; Summy and Gallick, 2003). Hence future profiling of

the BRK activation in a large cohort of breast tumor samples may enable the use of BRK activation as a diagnostic or prognostic marker, and inhibition of BRK activity or activation as a viable therapeutic strategy in breast cancer treatment.

## **5.2. BRK induces Dok1 degradation via the ubiquitin proteasomal pathway to promote cell proliferation**

It has recently been shown that constitutive activation of BRK promotes cell proliferation and migration as well as tumor formation, validating the proto-oncogenic function of BRK (Miah *et al.*, 2012). However, the molecular mechanisms dictating the tumorigenic role of BRK are poorly understood. An increasing number of studies have reported an inhibitory function of oncogenic tyrosine kinases towards overcoming cellular and physiological constraints promoted by tumor suppressors (Blume-Jensen and Hunter, 2001; Dai *et al.*, 1998; Fry *et al.*, 2009; Kirisits *et al.*, 2007; Peng *et al.*, 2010). Members of the Dok1 family have been characterized as negative regulators of cell transformation induced by oncogenic tyrosine kinases (Mashima *et al.*, 2009). In the present study, we demonstrate for the first time that BRK mediates its oncogenic function at least in part by downregulating the tumor suppressor Dok1. We show that: 1) BRK interacts and phosphorylates Dok1 predominantly at tyrosine 362; 2) the levels of BRK and Dok1 in breast cancer cells are inversely correlated; 3) activated BRK promotes Dok1 protein downregulation via ubiquitin-proteasome degradation; and 4) Dok1 is a negative regulator of BRK-induced cell proliferation and migration.

The function of Dok1 is regulated upon phosphorylation by a variety of receptor and non-receptor tyrosine kinases including the Src tyrosine kinase family members Lck and Fyn (Nemorin and Duplay, 2000), as well as tyrosine kinases such as Tec and Bcr-Abl (Gerard *et al.*, 2004; Lee *et al.*, 2004; Liang *et al.*, 2002; Noguchi *et al.*, 1999; Woodring *et al.*, 2004). It has also been demonstrated that Src phosphorylates Dok1 and prevents its entry into the nucleus (Niu *et al.*, 2006). Recently, Takeda *et al.* identified Dok1 as a substrate of several tyrosine kinases including BRK (Takeda *et al.*, 2010). In the present study, we provide evidence that Dok1 interacts with and is a direct substrate of BRK. In cells stably expressing BRK, ectopically expressed Dok1 is phosphorylated preferentially on Y362. We noted that co-expression of BRK and Dok1 also resulted in the phosphorylation at other tyrosines, besides Y362, although the



phosphorylation signal was weak. The reasons for these discrepancies in the phosphorylation pattern of Dok1 by BRK were not obvious. It is tempting to speculate that at steady state levels, BRK is more specific in its phosphorylation of Dok1. It was previously shown that Y361, and Y450 of murine Dok1, equivalent to Y362, and Y449 of its human homologue, are all direct phosphorylation sites of Src (Niu *et al.*, 2006; Shah and Shokat, 2002). Phosphorylation at Y362 is not surprising since the Y<sub>361</sub>DEP motif in murine Dok1 fits the optimal consensus phosphorylation site for Src (Shah and Shokat, 2002). However, the LY<sub>450</sub>QSV motif is not optimal for Src phosphorylation, although the pYXXV motif has been shown to be an optimal binding site for the c-Src tyrosine kinase SH2 domain (Shah and Shokat, 2002). Like Src, it is, therefore, possible that BRK could also phosphorylate unpredictable sites, based on the apparent heterogeneity of the consensus motif.

*Dok1* was also shown to be repressed in other forms of cancer including head and neck cancer (HNC), lung, liver, and gastric cancers, likewise in Burkitt's lymphoma (Balassiano *et al.*, 2011; Lambert *et al.*, 2011; Saulnier *et al.*, 2012). Dok1 repression was suggested to be a consequence of aberrant promoter hypermethylation (Balassiano *et al.*, 2011; Lambert *et al.*, 2011; Saulnier *et al.*, 2012), although surprisingly no expression studies of Dok1 were found in the literature to support these findings. We evaluated the correlation between Dok1 and BRK in breast cancer cell lines, because of the overexpression of BRK in the majority of breast cancer (Brauer and Tyner, 2010; Miah *et al.*, 2012). We observed an inverse correlation between the expression patterns of the two proteins, although the knockdown of BRK resulted only in a slight restoration of Dok1 levels in SKBR3 cells. However, it is not yet known whether the expressions of Dok1 and BRK in breast cancers are regulated epigenetically via promoter hypermethylation. Interestingly, there is evidence of BRK promoter hypomethylation in cisplatin resistant ovarian cancer cells (Yu *et al.*, 2011).

In the present study, we also show that both BRK and Dok1 are predominantly expressed as cytoplasmic/membrane proteins (Figure 5.4B and C) as was previously reported (Brauer and Tyner, 2010; Lee *et al.*, 2004; Lukong *et al.*, 2005; Noguchi *et al.*, 1999). A proportion of both proteins also localize in the nucleus (Lukong *et al.*, 2005; Niu *et al.*, 2006). We and others reported that BRK phosphorylates nuclear Sam68 and promotes its subcellular relocalization (Brauer and Tyner, 2010; Lukong *et al.*, 2005). Dok1, on the other hand, was recently shown to shuttle between the nucleus and cytoplasm in a mechanism that is regulated by external stimuli

and Src phosphorylation (Niu *et al.*, 2006). Further research is needed to determine whether BRK also regulates the subcellular localization of Dok1.

Both p210bcr-abl and v-Src have been shown to downregulate Dok-1 in a kinase activity-dependent manner (Niu *et al.*, 2006). BRK was recently shown to phosphorylate Cbl, inducing its auto-ubiquitination and degradation through the ubiquitin-proteasome pathway (Kang and Lee, 2013). In the present work, we found that the constitutively active BRK also induced the degradation of Dok1, but had no effect on its transcript levels (Figures 5.6, 5.7 and 5.8). Intriguingly, in comparison to BRK-YF, BRK WT induced little to no effect on Dok1 protein levels. This is reflective of a kinase-dependent mechanism by which BRK downregulates Dok1 protein levels. This, however, raises the critical question as to how endogenous BRK is able to regulate Dok1 protein levels. It was previously shown that the ability of BRK to phosphorylate its endogenous substrate Sam68 in breast cancer cells was significantly enhanced by stimulation with epidermal growth factor (EGF) (Lukong *et al.*, 2005). Dok1 is a scaffolding protein and in the present study we have shown that BRK interacts with Dok1 via its SH3 domain (Figure 5.3E). It is conceivable that EGF stimulation may lead to BRK activation followed by its interaction, phosphorylation and therefore destabilization/degradation of Dok1 as shown in figure 5.5. It may be worth noting that the activation of protein tyrosine kinases via the transient treatment of fibroblast cells with platelet-derived growth factor (PDGF) did not induce a decrease in Dok1 expression (Janas and Van Aelst, 2011). Rather this effect of BRK on Dok1 may have been achieved through prolonged stimulation of BRK leading to its activation as demonstrated in the current work. It is not clear at this stage whether the effect of BRK was direct. We showed that tyrosine phosphorylated Dok1 interacts with the SH2 domain of BRK. How this interaction affects accessibility to the proteasomal machinery is not known. However, it is possible that activated BRK may have triggered other cellular events that culminated in other posttranslational modifications promoting Dok1 degradation. Usually, polyubiquitin chains are covalently bound to lysine residues in proteins targeted for degradation (Kerscher *et al.*, 2006). While we conclude that the BRK-induced degradation of Dok1 is via the ubiquitin-proteasomal pathway, at this stage we did not provide direct evidence that this is a lysine-dependent mechanism. Ideally, a Dok1 substrate in which all lysine residues have been mutated to arginines will serve as a better negative control for the study of BRK-induced ubiquitination of Dok1. We did not test this possibility since studies with other oncogenic tyrosine kinases have

observed only a modest increase in the expression levels of the lysine-less mutant (Janas and Van Aelst, 2011), raising the possibility that other potential posttranslational modifications might be involved in the ubiquitination process.

Finally, we provided a functional link between Dok1 inactivation and the regulation of BRK-induced cellular processes such as cell proliferation and migration. Dok1 itself has been shown to inhibit mitogenic signaling and cell proliferation, and to antagonize leukemogenesis, but paradoxically, it also promotes cell spreading, motility, and apoptosis (Di Cristofano *et al.*, 2001; Hosooka *et al.*, 2001; Niki *et al.*, 2004; Woodring *et al.*, 2004; Yasuda *et al.*, 2004). Inhibition of Dok1 expression has been associated with enhanced cell proliferation (Mercier *et al.*, 2011; Miao *et al.*, 2003). We showed in Figure 5.10 B and C that activated BRK-induced proliferation of stable HEK293 cells was inhibited in the presence of overexpressed Dok1. Similarly, Dok1 suppressed BRK-induced migration of these cells. Taken together, our findings suggest that Dok1 is a negative regulator of the BRK-promoted oncogenic cellular processes, in particular, cell migration and proliferation. Further studies are needed to comprehensively elucidate the physiological implications of Dok1 and other members of the DOK gene family in BRK-regulated mammary tumorigenesis using for instance xenograft mouse models.

In summary, our data show that Dok1 is phosphorylated and targeted for ubiquitin-proteasomal degradation, a process that is critical for the sustenance of BRK-induced cell migration and proliferation. Further *in vivo* studies are required to support a model in which Dok1 impacts BRK-driven tumorigenesis and metastasis.

## 6. Conclusion

BRK is a non-receptor protein tyrosine kinase and its kinase activity like Src is negatively regulated by a phosphorylatable regulatory C-terminal tyrosine 447. Mutation of this regulatory C-terminal tyrosine 447 to phenylalanine results in a constitutively active variant because BRK-Y447F significantly enhances the catalytic activity of the enzyme. I showed that constitutive activation of BRK promotes cell proliferation and cell migration, validating a proto-oncogenic function of BRK. Additionally we also demonstrated for the first time that full activation of BRK is an essential component in the promotion of tumorigenesis by BRK *in vivo*. However, the molecular mechanisms dictating the tumorigenic role of BRK are poorly understood. In the present study, we demonstrated for the first time that BRK mediates its oncogenic function at least in part by downregulating the tumor suppressor Dok1. We have shown that the level of BRK and Dok1 in breast cancer cells are inversely correlated and BRK-induced cell proliferation and migration by phosphorylating Dok1 and leads its degradation via ubiquitin-proteasomal pathway. Further studies are needed to comprehensively elucidate the physiological implications of Dok1 and other members of the *Dok* gene family in BRK regulated mammary tumorigenesis using xenograft mouse models for instance.

## 7. Future directions

### 7.1. Enzymatic activation is essential for BRK to induce mammary gland tumorigenesis and metastasis.

BRK is a member of a non-receptor protein tyrosine kinase family, which is evolutionarily conserved across species (Goel and Lukong 2015). It has been reported that more than 80% of invasive ductal carcinomas highly express BRK (Harvey *et al.*, 2013). This overwhelming presence of BRK in most of the mammary gland tumors implies a role for this non-receptor protein tyrosine kinase in the etiology of breast cancer. In fact, it is reported that BRK is involved in cell proliferation, differentiation, colony formation and also in tumor progression *in vivo* (Miah *et al.*, 2013). Additionally, the chromosomal location of BRK is 20q13.3, a region that is frequently amplified in breast cancer (Harvey and Crompton *et al.*, 2004). Moreover BRK contains multiple proteins interaction motifs by which it interacts with numerous signal transduction molecules (Goel and Lukong 2015), and regulates different signal transduction pathways including such as EGFR, PI3K/AKT, MAPK, MET and IGF-1R (Goel and Lukong 2015).

Transgenic mouse models have helped in the understanding the possible role of BRK in mammary tumorigenesis. Two transgenic mouse models generated thus far expressed wild-type BRK in mouse mammary glands (Lofgren *et al.*, 2011; Peng *et al.*, 2013). In both cases the penetrance was very low and the latency was very long compared to those of other oncogene including HER2 and polyoma middle T transgenic (Goel and Lukong 2015).

In my Ph.D. research work, I showed that constitutively active form of BRK significantly enhanced the cell proliferation, migration, colony formation as well as tumor formation in xenograft mouse model in comparison to wild type BRK indicating that the full activation of BRK is required for cellular transformation. In my study, I also revealed one of the molecular mechanisms of action of BRK by characterization of a BRK target protein Dok1. BRK phosphorylated tyrosine 362 of Dok1 for ubiquitin-mediated proteasomal degradation, which has tumor suppressor activity to promote cell proliferation, and migration, however, the oncogenic function of BRK can be nullified by overexpressing Dok1 in *in vitro* culture system. Together, these results suggest a novel mechanism of action of the constitutively active form of BRK in the

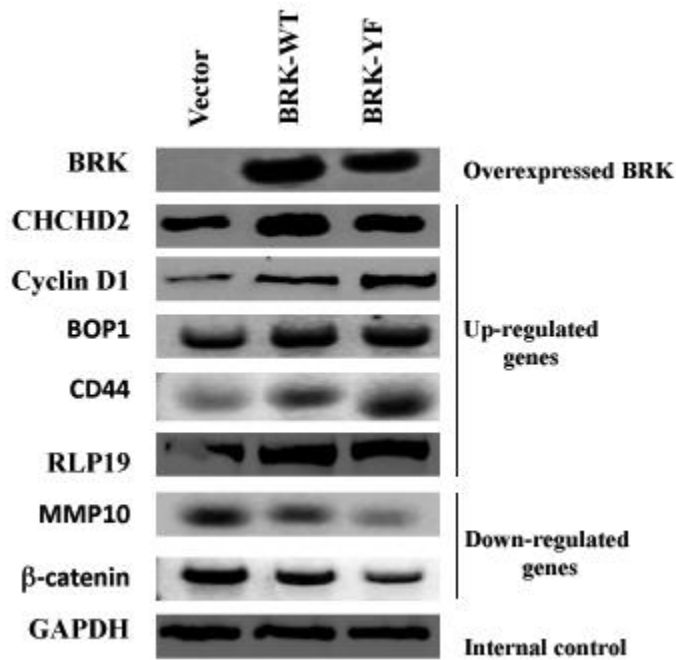
promotion of cell proliferation, migration and tumor formation by targeting Dok1 for degradation through the ubiquitin-proteasomal pathway.

To date, the oncogenic function of the constitutively active form of BRK has largely been studied in *in vitro* culture system. Despite significant progress in the characterization of BRK, the critical physiological role of the constitutively active form of BRK (BRK-Y447F) in the context of mammary gland tumorigenesis has not been addressed. Targeting the constitutively active BRK Y447F to the mouse mammary gland could provide a better model for the study of the role of BRK in mammary tumorigenesis. This mouse model can be crossed with an MMTV-Dok1 or MMTV-Dok1Y362F mice to validate that physiologically, BRK can promote Dok1 degradation and therefore enhance tumorigenesis. In this future study, the following question will be answered; A) Can constitutively active form of BRK (BRK-Y447F) potentiate mammary gland tumorigenesis in mice like oncogene HER2; and B) Can Dok1-Y362F expression nullify the BRK-Y447F induced mammary gland tumorigenesis and metastasis in mice.

## **7.2. To determine the effect of activated form of BRK on genes regulation**

Tumorigenesis is characterized by aberrant cell signaling culminating in increased cell proliferation, cell migration and prolonged cell survival. Likewise, in my studies I have shown that the aberrant activation of BRK results into the increased cell proliferation, migration and tumor formation. Tyrosine kinases are key regulators of various signaling cascades that control the transcription of various genes. It would, therefore, be interesting to examine if the aberrant activation of BRK has a direct or indirect influence on transcriptional regulation of genes. In order to examine the effect of BRK or its activated form on gene expression profiles, we performed microarray studies using lysates from various cell lines stably expressing BRK WT, BRK-Y447F or an empty vector as a control. We observed differential gene expression profiles between the control and the BRK-WT or BRK-YF. *CCD1*, *BOP1*, *CD44*, *CHCHD2* and *RLP19* were among the genes that were upregulated in the presence of BRK (WT and Y447F); whereas, *CTNNB1* and *MMP10* are examples of genes that were down-regulated (Figure 6.1.1). These results can be complemented with experiments using stably BRK knockdown cells to examine whether stably overexpressed BRK and stably knocked down BRK have opposing effects of

transcription. By corroborating these two sets of data, the genes regulated by BRK can be specified which will provide a better understanding the effect of BRK on gene regulation in breast cancer. In addition, it will be very helpful to further understand the mechanism of action of BRK and perhaps provide new ways of inhibiting the activity of BRK in BRK-positive breast cancer patients.



**Figure 6.1.1. Semi-quantitative RT-PCR analysis to validate the effect of BRK on the mRNA levels of some of the genes identified by microarray analysis.** Semi-quantitative RT-PCR was performed for the indicated differentially expressed genes with the cDNAs from HEK 293 cells stably expressing GFP, GFP-BRK-WT and GFP-BRK-YF. The reactions were conducted using the ProtoScript® M-MuLV Taq RT-PCR Kit as described by the manufacturer. The primers for GAPDH were supplied by the manufacturer and were used for internal control reactions.

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