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Cancer-associated mutations in the non-receptor tyrosine kinases Brk and Hck differentially affect enzyme activity and substrate recognition

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Abstract of the Dissertation

Cancer-associated mutations in the non-receptor tyrosine kinases Brk and Hck differentially affect enzyme activity and substrate recognitionby

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Tyrosine kinases play a vital role in proper cell growth and function. Aberrantly activated kinases often lead to diseases and have been recognized as important drivers of cancer. Aberrant kinase activity can occur by various mechanisms, such as by kinase overexpression or by activating mutations. This dissertation examines the effect of cancer associated mutations on two non-receptor tyrosine kinases. The first part of the dissertation will focus on breast tumor kinase (Brk, also known as PTK6), first identified in breast tumor samples. The second part of the dissertation focuses on hematopoietic cell kinase (Hck), which is implicated in drug resistance in chronic myelogenous leukemia (CML).

Overexpression of Brk has been implicated in several cancers. Recently, several cancer-associated mutations to Brk have been identified. Whether these mutations affect Brk activity and contribute to tumorigenic growth has not been determined. We have examined a panel of mutations, which were identified in different cancers and span different domains of Brk. We showed that several of the mutations activate Brk. Two of the mutations appear to activate Brk

by disrupting its autoinhibitory interactions. While the mutations led to increased Brk activation, expression of the mutants did not result in increased cell growth or transformation. We show that this may be due to differential effects on substrate recognition and activation by the different mutants.

We have also examined the effect of cancer-associated mutations on activation of Hck. While Hck is mainly expressed in cells of hematopoietic origin, several Hck mutations have been identified in various cancer samples derived from different cell types. The mutations we examined are all found in the kinase domain of Hck. One of these mutations, E389K, is adjacent to the autophosphorylation site of Hck and could potentially inactivate the protein. A previous study suggested that the E389K mutation could be linked to increased survival in ovarian cancer. The cancer-associated mutations we examined all decreased Hck activity to varying degrees as observed by measuring their effect on enzymatic activity, autophosphorylation, and activation of WASP, a known substrate of Hck. Given the role of Hck in diseases such as CML, these inactive variants of Hck may serve as tumor suppressors.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abl1	Abelson murine leukemia viral oncogene homolog 1
Akt	v-akt murine thymoma viral oncogene homolog 1
ATP	Adenosine triphosphate
Bcr	Breakpoint cluster region
Brk	Breast tumor kinase
BSA	Bovine serum albumin
c-Kit	kit oncogene
Cas	Crk-associated substrate
CML	Chronic myelogenous leukemia
Csk	C-terminal Src kinase
DTT	Dithiothreitol
EGFR	Epidermal growth factor receptor
ELMO	Eukaryotic engulfment and cell motility
ErbB2	Erb-B2 receptor tyrosine kinase 2
Erk5	Extracellular-signal-regulated kinase 5
ESCC	Esophageal squamous cell carcinoma
FAK	Focal Adhesion Kinase
Frk	Fyn-related Src family tyrosine kinase
HGF	Hepatocyte growth factor
Hif	Hypoxia-inducible factor
HRG	heregulin
Hck	Hematopoietic cell kinase
IGF-1R	Insulin-like growth factor I receptor
Jak3	Janus kinase 3
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MITF	Micrphthalmia-associated transcription factor
NaF	Sodium fluoride
Ni-NTA	Nickel-nitrilotriacetic acid
NSCLC	Non-small cell lung cancer
P130Cas	Crk-associated substrate
PI3K	Phosphoinositide 3-kinase
PTB	Phosphotyrosine binding
PTB1B	Protein-tyrosine phosphatase 1B
PTK6	Protein tyrosine kinase 6
PVDF	Polyvinylidene fluoride
Rac1	Ras-related C3 botulinum toxin substrate 1
RTK	Receptory tyrosine kinase
Sam68	Src-Associated substrate in Mitosis of 68 kDa

SH2	Src-homology 2
SH3	Src-homology 3
SH4	Src-homology 4
Sik	Src-related intestinal kinase
Src	SRC proto-oncogene, non-receptor tyrosine kinase
Srms	Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 3
TEV	Tobacco Etch virus
TNF	Tumor necrosis factor
WASP	Wiskott-Aldrich syndrome protein
YOPH	*Yersinia* protein phosphatase

Acknowledgments

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I would like to thank my committee for their advice and for pushing me to think about my thesis from a different perspective. I would like thank Dr. David Talmage, who served as the committee chair. I admire him greatly for his incredible wealth of knowledge and I thank him for his support and guidance during the qualifying exams and as part of my committee. I would also like to thank Dr. Markus Seeliger, who I have had the opportunity to work with during one of my rotations. I would like to thank him for his support and for the advice he has provided on my project. I would also like to thank Dr. Michael Hayman for taking time to meet with me to talk about my work and for his helpful suggestions during the committee meetings.

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the ErbB2, and β-catenin expression vectors. I would like to thank Chris Gordon for his advice on cell staining and imaging.

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Chapter 1

Introduction

Tyrosine kinases

Tyrosine kinases were first identified over 30 years ago with the discovery of Src kinase. The human genome encodes 90 tyrosine kinases. The post-translational modification carried out by these kinases, in which the gamma phosphate of ATP is transferred to the hydroxyl group of a tyrosine, affects all aspects of cellular function. Phosphorylated tyrosine residues can serve as binding sites for both intra- and inter-molecular interactions. These interactions alter the catalytic activities of proteins and allow for controlled propagation of signals that regulate proper cell growth and function. Non-receptor tyrosine kinases like Src, make up 32 of the 90 tyrosine kinases[1]. The remainder are receptor tyrosine kinases (RTKs), which span the cell membrane. RTKs can be activated by ligands such as growth factors, leading to trans-autophosphorylation of their cytoplasmic domains. These phosphorylation sites promote binding and activation of cytoplasmic non-receptor kinases either through the Src-homology 2 (SH2) or phosphotyrosine binding (PTB) domains[2].

Proper regulation of tyrosine kinase signaling is essential for cellular homeostasis. Aberrant expression or activation of tyrosine kinases leads to uncontrolled cell function that results in many diseases. Tyrosine kinases have long been implicated in the development of cancer. Cellular transformation involves the acquisition of a set of characteristics first proposed by Hanahan and Weinberg in 2000[3]. These characteristics include and are not limited to sustained proliferative signaling, activated invasion and metastasis, and evasion of cell death. Altered activation of tyrosine kinases has been shown to promote each of these characteristics.

Since its discovery, the role of Src in cancers has been thoroughly examined and is a prime example of the oncogenic potential of activated kinases. Src activation has been shown to

activate both the PI3K and MAPK signaling pathways[4], which regulate survival and cell proliferation. Src has also been shown to associate with adhesion proteins such as FAK, which leads to the dissociation of focal adhesions that connect the cell cytoskeleton with the extracellular matrix to promote cell migration[5]. Src has been found to be overexpressed in several cancers including breast[6], lung[7], and colon[8] cancer.

Given their role in cancer, strategies to target tyrosine kinases have been actively pursued in cancer therapy. Small molecule inhibitors have been successfully developed to target Src and other tyrosine kinases. Following the discovery of imatinib for the treatment of chronic myelogenous leukemia in 2001, over twenty small molecule kinase inhibitors have been approved by the FDA[9]. Small molecule inhibitors typically inhibit kinase activity by competing with ATP binding[10]. In addition, monoclonal antibodies targeting receptor tyrosine kinases have also been developed. This class of drugs binds the extracellular domains of the kinase and inhibits signaling by interfering with homo- and hetero-dimerization of the receptor which may be required for kinase activation, by blocking ligand binding, or by increasing internalization of the receptor[11].

Regulation of Src kinase

The Src family of kinases contains nine members in vertebrates. Each member has several domains: a Src-homology 4 (SH4) domain, a unique domain, a Src-homology 3 (SH3) domain, an SH2 domain, and the kinase domain (Fig. 1-1). During translation of Src, a myristoylation sequence is added to the SH4 domain[12]. Myristoylation of Src serves to localize the protein to the lipid membrane and thus in closer proximity to its substrates. The SH3 domain is composed of five β-strands that form a hydrophobic pocket that binds proline rich sequences[13,

[14]. The SH2 domain is composed of four β-strands flanked by two α-helices on either side[13, 14]. The kinase domain is composed of two lobes. The smaller N-lobe, which contains five β-strands and one α-helix, and the larger C-lobe, which contains only α-helices. The ATP and substrate binding sites reside in the cleft formed between the two lobes[13].

The intramolecular interactions facilitated by the SH3 and SH2 domains maintain Src in a downregulated conformation. In this conformation, the SH3 domain binds the poly-proline type II helix in the linker region between the SH2 domain and the kinase domain[13]. The SH2 domain binds to the phosphorylated tyrosine residue (Tyr 527) at the C-terminal tail[13]. Phosphorylation of Tyr 527 is carried out by C-terminal Src Kinase (CSK)[15]. In this state, the activation loop within the kinase domain is held in a restrained conformation that blocks the ATP and substrate binding site and prevents phosphorylation of Tyr 416 within the loop[16, 17].

The inhibited conformation of Src, can be released by three mechanisms: displacement of the SH3 domain, displacement of the SH2 domain, or dephosphorylation of the C-terminal tail (Fig. 1-2). The SH3 and SH2 domains that form the intramolecular interactions that maintain Src in its down regulated conformation are also responsible for interaction with Src substrates. These substrates, which contain sequences with greater affinity for the SH3 and SH2 domains, are able to disrupt the weaker intramolecular interactions[18]. Additionally, phosphatases such as PTP1B can dephosphorylate the C-terminal tail to disrupt its interaction with the SH2 domain[19]. The loss of these intramolecular interactions leads to activation of the enzyme, which involves autophosphorylation of Tyr 416 in the activation loop of the kinase domain[20]. Phosphorylation of the activation loop displaces it from the substrate binding pocket of the kinase domain to allow phosphorylation of its substrates.

Cancer-associated mutations

The activity of tyrosine kinases in normal cells is tightly regulated. It is the unchecked activation of these proteins that leads to uncontrolled cell growth and transformation. Aberrant activation of tyrosine kinases can occur through multiple mechanisms. This includes protein over expression due to gene amplification or mutational activation[21]. Recent sequencing efforts have identified 291 cancer genes, which make up more than 1% of the human genome[22]. Over 9% of the 291 cancer genes identified contain mutations in protein kinase domains[22]. While tyrosine kinases represent one quarter of protein kinases, they account for two-thirds of the protein kinases that carry cancer-associated mutations[22, 23]. Fifty-one of the 90 tyrosine kinases have been implicated in cancer, and have been recognized as important targets in cancer therapy[21].

Cancer-associated mutations include chromosomal alterations, single base-pair changes, and insertions or deletions. Chromosomal alterations are the most commonly occurring mutations in leukemia and lymphoma[22]. The most well-known mutation of this class is the Bcr-Abl fusion protein found in chronic myelogenous leukemia (CML). Bcr-Abl results from the fusion of the Abl1 gene on chromosome 9 to the Bcr gene on chromosome 22[24]. In the normal cellular form of Abl, an N-terminal myristoylated sequence participates in autoinhibitory interactions. The Bcr-Abl fusion results in a loss of N-terminal myristoylation and results in a constitutively active protein[24]. Additionally, the coiled-coiled domain gained from Bcr allows the formation of dimers and tetramers that trans-autophosphorylate[25]. Autophosphorylation of its combined multiple domains leads to activation of multiple signaling pathways. Janus kinase 3 (Jak3) kinase is a non-receptor tyrosine kinase that is essential for propagation of signals downstream of cytokine receptors[26]. Jak3 possesses a pseudo-kinase domain that binds the kinase domain and suppresses its catalytic activity[26]. Several single-base pair mutations have

been identified, many of them located within the Jak3 pseudo-kinase domain. Mutations to this domain potentially disrupt this inhibitory interaction leading to constitutive activation of the kinase in leukemia and lymphoma[27, 28]. Several cancer-associated mutations have also been identified in receptor tyrosine kinases. The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is over-expressed in several cancers including ovarian and esophageal cancers[29]. Somatic mutations identified in EGFR include both point mutations and insertions. An in-frame deletion of exon 19 and the point mutation L858R occur within the kinase domain of EGFR; both mutations dramatically increase its catalytic activity[30].

The presence of somatic mutations in cancer cells contributes to cancer growth and has led to the development of effective cancer therapies. Imantinib, which targets Bcr-Abl, has been successful in the treatment of CML[24]. Gefitinib is another small-molecule tyrosine kinase inhibitor that targets EGFR. Gefitinib was developed for the treatment of non-small cell lung cancer (NSCLC), in which EGFR is often overexpressed. The overall response to gefitinib treatment in clinical trials was modest, except for a subset of patients. Subsequent studies showed that the robust response to gefitinib in these patients was due to activating mutations within the kinase domain of EGFR[31].

While tyrosine kinase specific inhibitors have had great success, many patients also develop drug resistance. Several studies have found that resistance is often due to the presence of somatic mutations. In 50% of cases involving resistance to the EGFR inhibitor gefitinib, drug resistance occurs as a result of the T790M mutation within the kinase domain[32]. This mutation is equivalent to the T315I ‘gate-keeper’ mutation found in drug resistant Bcr-Abl; however, the mechanism of resistance is different. While the T315I mutation inhibits binding of the inhibitor imantinib, the T790M mutation increases the affinity of EGFR for ATP[33].

The identification of mutationally activated kinases has provided a deeper understanding of the molecular mechanisms that lead to cell transformation and cancer. Identification of these mutants has identified critical signaling pathways that confer cell growth advantages and has revealed valuable targets in cancer therapy. Thus, with the advances in genome sequencing technology, many studies have been initiated in order to fully characterize the genetic landscape of different cancers. Genome sequencing efforts may identify mutations that number into the thousands for a particular cancer genome. However, this information does not identify which of these mutations are 'driver mutations,' which are positively selected for because they confer a growth advantage to the cells, or 'passenger mutations', which do not contribute to cancer development[23].

To distinguish driver mutations from passenger mutations, several computational approaches have been developed. One commonly used strategy measures mutation recurrence, which is based on the assumption that driver mutations are selected for due to the growth advantage they provide and so should appear more frequently[34, 35]. Another approach is to select for mutations based on their predicted functional impact as determined by sequence conservation and the position of the mutation within specific protein domains. However, these approaches have shortfalls. Identification of potential driver mutations based on mutation frequency overlooks driver mutations that are present at low frequency[36]. For example, in a breast cancer study, over half of the mutations identified in 100 tumor samples examined were concentrated in six genes, while the rest were distributed among 34 genes[37]. Methods that identify potential driver mutations based on predicted functional impact may require protein structure information, which is not yet available for many proteins[34]. Therefore, experimental validation is necessary in order to confirm whether a particular mutation leads to malignant transformation of the cells and

to understand the mechanism by which the mutation affects kinase activity, so that effective inhibitors can be developed.

Brk structure and regulation

Brk is a non-receptor tyrosine kinase first isolated from human metastatic breast tumors[38]. Sequencing of the Brk gene revealed several motifs common among protein kinases such as Src, with which it has 46% sequence identity (Fig. 1-3). This includes an ATP binding motif as well as an autophosphorylation site at position 342, which when phosphorylated leads to increased enzymatic activity[38, 39]. Brk also contains SH3 and SH2 domains, and similar to Src kinase, contains a tyrosine residue at its C-terminus (Y447). Previous work from our laboratory has shown that the SH3 and SH2 domains and the C-terminal tyrosine residue regulate Brk activity[39]. The addition of SH2- and SH3-binding peptides to Brk, or mutations to these domains, increases Brk activity, which supports a role of these domains as intramolecular regulators of Brk[39, 40]. The SH3 domain interacts with proline residues P175, P177, and P179 within the linker region of Brk to down-regulate Brk[40, 41]. Mutation of the C-terminal tyrosine results in increased enzymatic activity as well as increased accessibility of the SH2 domain, which suggests that, like Src, the SH2 domain and C-terminal tail interact to down-regulate Brk activity[39]. The SH3 and SH2 domains are also responsible for recognition of its substrates. Studies from our laboratory have shown that the SH3 domain is essential for interaction with Sam68, an RNA-binding protein, one of the first substrates identified for Brk. The SH3 and SH2 domains have also been shown to be important for recognition of a multitude of substrates identified for Brk[42].

Despite the similarity in domain arrangement and regulation of kinase activity, Brk is not a Src kinase family member. Rather, Brk, along with Frk and Srms, form a distinct family of Src-

like kinases, which differ from Src in the arrangement of their intron and exon boundaries[38, 43, 44]. Additionally, kinases within this family lack a glycine at the second amino acid position, which in Src family kinases is essential for myristoylation[38]. Myristoylation of Src targets the kinase to the cell membrane. The absence of such a modification allows for localization of Brk to both the cytoplasm and nucleus. Additional biochemical studies from our lab and others have also discovered that a tryptophan residue within the linker region (W184), has an alternate function from its analogous residue (W260) in Src. While W260 in Src inhibits its activity, W184 in Brk is involved in activation of Brk[40, 45].

Both Brk and Src are down-regulated due in part to the intramolecular interaction between the SH2 domain and the C-terminal tail. This interaction is promoted through phosphorylation of Y447 at the C-terminal tail. Csk is responsible for phosphorylation of the analogous reside (Y527) in Src. However, Csk is unable to phosphorylate the inhibitory tyrosine in Brk. Instead, the reaction is catalyzed by Srms[46]. Brk and Src also diverge in the phosphatases that are involved in regulation of enzymatic activity. PTP1B dephosphorylates the C-terminal tail of Src, which leads to its activation. However, a recent study from our laboratory showed that PTP1B dephosphorylates Y342 in the activation loop causing inactivation of Brk[46, 47].

An alternatively spliced variant of Brk (Alt-PTK6) is a 15kDa protein with only the N-terminus and SH3 domain followed by a proline-rich C-terminal sequence[43]. Alt-PTK6 has been found to be expressed in breast, prostate, and colon cells[43, 48]. Expression of Alt-PTK6 has been shown to inhibit proliferation of prostate cells[48].

Brk expression and function in normal cells

Brk expression has been detected in a variety of epithelial cells including the skin[49], the digestive tract, which includes the oral epithelium[50], the colon[51], and small intestine[49], as well as in prostate cells[52]. Contrary to initial studies that only detected expression of Brk in transformed mammary cells, a recent study was able to detect Brk expression in the normal mammary epithelia as well, although its function in these cells is not clear[53]. Brk has also been detected in activated T-cells[54].

Brk is thought to play a role in the differentiation of normal cells. Expression of Brk is mainly restricted to non-dividing differentiated cells[51]. Calcium induced differentiation of mouse keratinocytes results in increased activation of Sik, the mouse ortholog of Brk, and increased expression of the differentiation marker, filaggrin[55]. An increase in Brk expression was also detected in differentiation of Caco-2 cells[51]. Knock-out of Brk in mice resulted in delayed differentiation of enterocyte cells, and increased villus length in the small intestine[56].

Brk expression and function in cancer

A tumorigenic role for Brk was first determined in breast cancer, where Brk was found to be overexpressed in over two-thirds of breast tumors examined[57]. Several additional studies also showed increased Brk expression in breast cancer, and furthermore correlated increased expression with higher grade tumors and decreased survival[53, 58, 59]. Increased expression of Brk has also been identified in NSCLC[60] and ovary carcinomas[61]. In normal prostate cells, a high level of Brk expression was detected in the nuclei. Closer analysis of normal and tumor samples revealed that nuclear localization of Brk correlated with tumor grade; decreased nuclear Brk was observed in less differentiated and more aggressive tumor samples[62]. The effect of altered Brk

localization on cell transformation was further demonstrated in another study that showed targeting of Brk to the cell membrane increased cell proliferation, survival, and migration[63]. Thus, in addition to increased expression, localization of Brk may play an important role in its ability to confer a growth advantage in cells.

Brk has been shown to mediate signaling in many pathways. Expression of Brk in human mammary epithelial cells promoted cell proliferation in response to EGF[64]. Expression of Brk also increased cell transformation as measured in soft-agar assays. Brk sustains signaling downstream of EGFR by inhibiting ubiquitination and degradation of EGFR[65]. Brk has also been shown to regulate signaling downstream of other EGFR family receptors. Using a modified chimeric ErbB2 construct that allowed specific activation of ErbB2 homodimers, it was shown that co-expression of Brk enhances ErbB2 induced cell proliferation via activation of Erk1/2[66]. In addition, Brk mediates signaling in response to the ErbB3 and ErbB4 ligand, heregulin (HRG). Knock-down of Brk results in decreased HRG induced activation of Erk5, p38MAPK, and Rac[58]. Other cell receptors that utilize Brk for signal transduction include the insulin-like growth factor 1 (IGF-1R) receptor and the hepatocyte growth factor receptor, c-Met. Knock-down of Brk reduces IGF-1R induced anchorage independent cell survival[67] and hepatocyte growth factor (HGF) induced migration[68].

A number of downstream substrates have been identified for Brk that are involved in cell migration. These include paxillin, a direct substrate for Brk, which localizes with Brk at the leading edge in migrating cells. Phosphorylation of paxillin by Brk activates Rac1 to promote migration[69]. Phosphorylation of p130Cas by Brk also leads to activation of Rac1 and cell migration. Brk also promotes cell migration through phosphorylation of p190RhoGAP-A, which

promotes interaction with p120RasGAP leading to activation of Ras. Thus, expression of Brk not only plays a role in tumor growth, but may also be important in metastasis of malignant cells.

Several studies in mice support the role of Brk in tumor growth *in vivo*. Co-expression of Brk and ErbB2 in mammary epithelial cells that were transplanted into mice resulted in decreased latency of tumor formation compared to wild-type mice[66]. Targeted expression of Brk in transgenic mice resulted in doubling of the number of tumors and delayed latency[70, 71]. In addition, injection of cells expressing constitutively activated Brk in SCID mice showed increased metastasis[72].

Recently, additional functions have been identified for Brk in cancer. Brk was found to be regulated by hypoxia inducible factors (HIFs)[73]. Over-expression of HIFs is associated with increases in cancer relapse and metastasis. HIF-1a and HIF-2a transcription factors directly regulate Brk transcription. Therefore, targeting of Brk could be an effective therapeutic strategy in cancers with increased HIF expression, such as the triple negative breast cancers. Brk has also been implicated in the epithelial to mesenchymal transition in which transformed cells acquire the abilities necessary for metastasis[74]. Targeting of Brk to the membrane of prostate cells resulted in decreased expression of the epithelial marker E-cadherin, and increased expression of mesenchymal markers such as vimentin[72].

In addition to promoting cell proliferation and migration, expression of Brk confers drug resistance. Co-expression of Brk and ErbB2 decreased the effectiveness of Lapatinib, an EGFR and ErbB2 dual inhibitor, in inhibiting cell proliferation[66]. Expression of Brk in human mammary epithelial cells conferred greater survival of cells treated with doxorubicin[70].

Hck structure and regulation

Hck is a member of the Src family of non-receptor tyrosine kinases. Unlike Src kinase, the expression of Hck is limited to a specific type of cells, namely hematopoietic cells of the myeloid and B-lymphocyte lineages[75]. Hck exists as two isoforms (Fig. 1-4). p61Hck contains an additional twenty-one amino acids at its N-terminus and is myristoylated[76]. The p59Hck isoform is both myristoylated and palmitoylated at the N-terminus[76, 77]. The distinct modifications at the N-terminus dictate the localization of each of these isoforms, and in turn their specific functions within the cell[76, 77]. The p61Hck isoform is mainly associated with lysosomes within the cell, whereas the p59Hck isoform is mostly found at the plasma membrane.

As a Src family kinase member, the auto-regulatory mechanisms for Hck are essentially the same as those described above for Src kinase. The SH3 and SH2 domains interact with the proline-rich linker region and the phosphorylated C-terminal tail, respectively, to maintain the protein in a down-regulated conformation[78]. Disengagement of these interactions by the presence of SH3 and SH2 ligands, or dephosphorylation of the C-terminal tail leads to autophosphorylation within the activation loop and increased kinase activity[78, 79].

Hck function in normal cells

Early Hck knockout studies showed impaired phagocytic activity and the two isoforms were shown to have distinct functions[80, 81]. p59Hck is involved early in formation of phagocytic cups, while p61Hck is involved in internalization of molecules in a later part of the process[81]. Hck has also been implicated in cell migration. Expression of constitutively active p59Hck led to formation of membrane protrusions[81] and expression of constitutively active p61Hck led to formation of podosomes, which are involved in cell migration and adhesion.

Hck is thought to regulate cell migration by facilitating actin rearrangement. This is achieved in part by activation of Hck substrates such as Wiskott-Aldrich syndrome protein (WASP). Previous work from our lab identified WASP as an Hck substrate with a pull-down assay utilizing the SH3 domain of Hck[82]. WASP is also specifically expressed in hematopoietic cells and is involved in phagocytosis and chemotaxis. Hck was shown to mediate phosphorylation of WASP at Y291, which promotes actin polymerization and filopodium formation[83, 84].

Hck function in cancer

Hck has been shown to be essential for Bcr-Abl transformation of myeloid cells, as expression of an inactive form of Hck suppressed Bcr-Abl induced cell proliferation[85]. Subsequent studies identified Hck as a mediator for activation of STAT5 by Bcr-Abl[86]. Activation of STAT5 downstream of Bcr-Abl causes the transcription factor to be retained within the cytoplasm, where it promotes activation of Akt and cell proliferation and survival[87]. Hck not only mediates Brc-Abl induced cell transformation, but is also able to confer resistance to Imatinib in CML cells[88].

Hck may also promote development of solid tumors which are often infiltrated by myeloid cells such as macrophages and neutrophils[74, 89]. The presence of such immune cells within the tumor microenvironment has been shown to promote cell growth and to facilitate cell migration through the secretion of signaling molecules such as growth factors and enzymes that promote degradation of the extracellular matrix[74]. Guiet et al., demonstrated that the presence of macrophages within tumor spheroids was able to promote invasion of tumor cells. However, this ability was compromised in Hck deficient macrophages[90].

Summary and overview of dissertation

Inhibition of tyrosine kinases has proven to be an effective strategy in the treatment of cancer. This is not surprising, given the impact that dysregulated tyrosine kinases have on cell proliferation, transformation, and migration. Dysregulated tyrosine kinase activity can result from overexpression of the kinase or by somatic mutations such as gene alterations or substitutions. Recent advances in sequencing technology have revealed the genetic landscape of several cancers. Over 9% of cancer-genes identified are protein kinases. Despite the availability of computational approaches to distinguish potential driver mutations from passenger mutations, the impact of somatic mutations must still be functionally validated.

Overexpression of the non-receptor tyrosine kinases Brk and Hck has been implicated in various cancers. Brk is overexpressed in epithelial cancers such as breast and colon cancer, and Hck expression is elevated in leukemias such as CML. Thus, both proteins could be potential targets for cancer therapy. Several somatic mutations to Brk and Hck have been identified; however, their effects on kinase activity and consequently in cancer development have not been examined. This dissertation examines the effect of somatic mutations identified in the non-receptor tyrosine kinases Brk (Chapter 3) and Hck (Chapter 4).

Figure 1-1 Regulation of Src family kinases. Top: domain structure of Src-family kinases. Bottom: the three-dimensional structure of the Src family kinase, Hck, in its inhibited conformation[20].

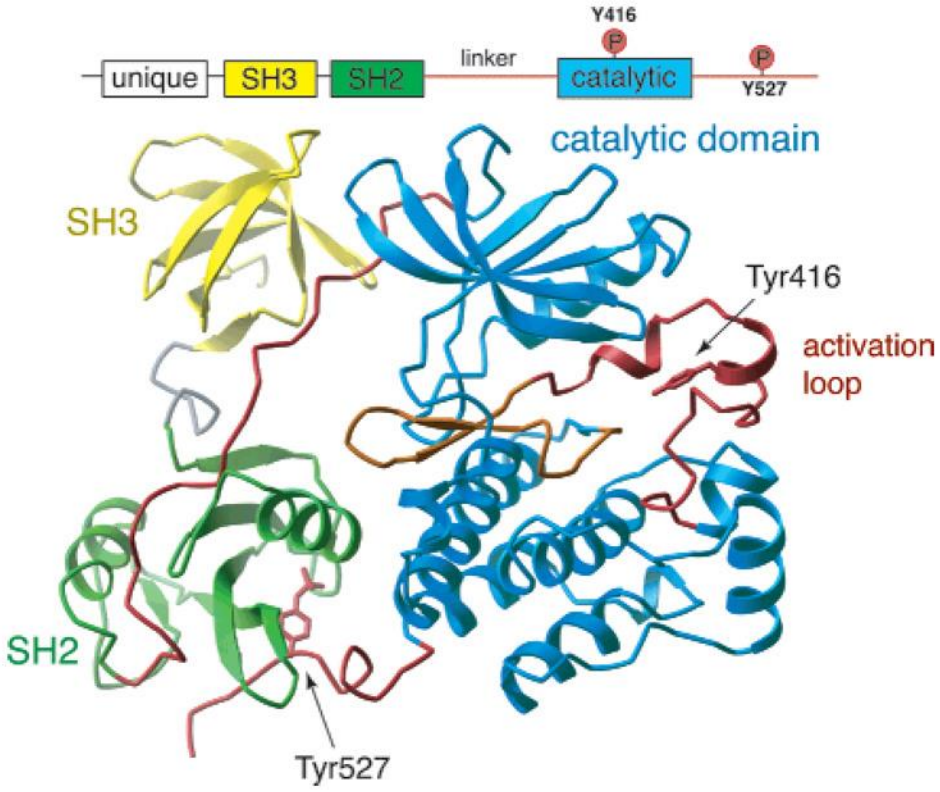

Y416
P
unique
SH3
SH2
linker
catalytic
P
Y527
catalytic domain
SH3
Tyr416
activation
loop
SH2
Tyr527

Figure 1-2 Activation of Src family kinases. SH3 ligation, SH2 ligation, or dephosphorylation of the C-terminal tyrosine lead to activation of Src family kinase. pY indicated a phosphorylated tyrosine. Adapted from (17).

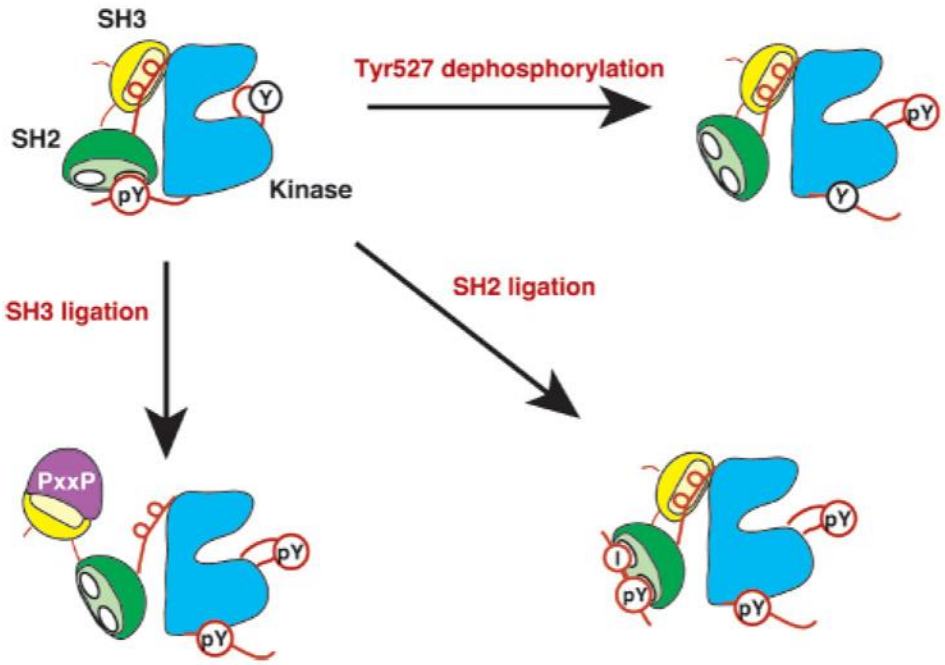

SH3
SH2
Y
pY
Kinase
Tyr527 dephosphorylation
pY
Y
SH3 ligation
SH2 ligation
PxxP
pY
pY
I
pY
pY
pY

Figure 1-3 Regulatory domains of Brk. Phosphorylation sites in the activation loop (Y342) and C-terminal tail (Y447) are indicated.

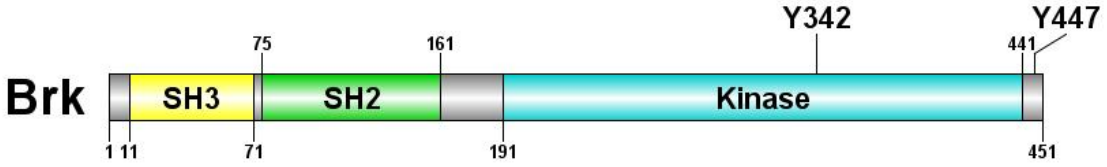
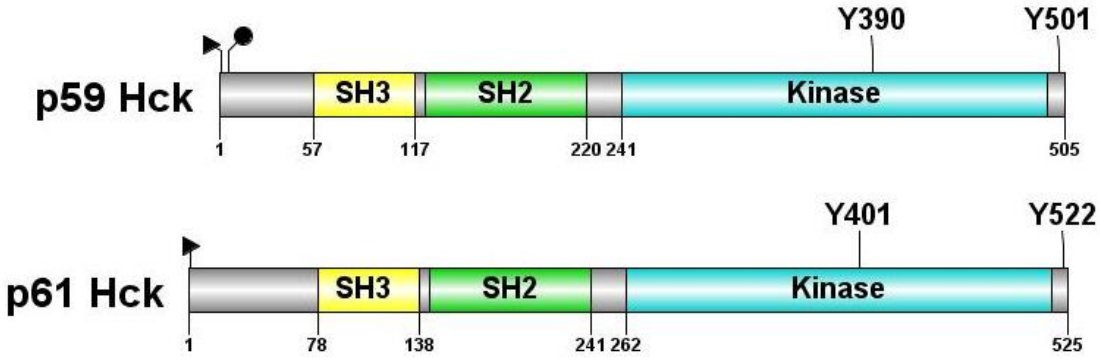

Brk
SH3
SH2
Kinase
Y342
Y447
75
161
441
1
11
71
191
451

Figure 1-4 Domain arrangement of Hck. The p61 and p59 isoforms are shown. Phosphorylation sites in the activation loop and C-terminal tail are indicated. Myristoylation and palmiotylation sites are represented by a triangle and circle, respectively.


Y390
Y501
p59 Hck
SH3
SH2
Kinase
1
57
117
220
241
505
Y401
Y522
p61 Hck
SH3
SH2
Kinase
1
78
138
241
262
525

Chapter 2

Materials and Methods

Reagents and antibodies. Bovine serum albumin (BSA) was obtained from Amresco. Leupeptin and aprotinin were from Roche. Phenylmethylsulfonyl fluoride, sodium vanadate, dithiothreitol (DTT), and polybrene were from Sigma, and NAF was purchased from JT Baker. Human epidermal growth factor (EGF) was purchased from Millipore. 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) was from Life Technologies. Primary antibodies were obtained from the following companies: anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-p130 Cas (Tyr249), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E), p44/42 MAPK (Erk1/2), and anti-phospho STAT3 (Tyr 705) were from Cell Signaling Technology, anti-ErbB2 (24B5), anti-phospho Brk (Tyr342), anti-phosphotyrosine, clone 4G10, anti-phospho N-wasp (Tyr256) were from Millipore, anti-p130 Cas (C-20), anti-GFP (B2), anti-Sam68 (c-20), anti-Stat3 (H-190), and anti-pTyr (pY99) were from Santa Cruz, anti-Beta-catenin and anti-Hck were from BD Biosciences, and Monoclonal ANTI-FLAG® M2-Peroxidase (HRP) was from Sigma. Horseradish peroxidase linked donkey anti-rabbit IgG and sheep anti-mouse antibodies were from GE Healthcare. Fluorescein (FITC) AffiniPure Goat Anti-Mouse IgG antibody was from Jackson ImmunoResearch Laboratories. SuperSignal West Femto Chemiluminescent Substrate and Pierce ECL Western Blotting Substrate were purchased from Thermo Scientific.

Cell lines. The mammalian cell lines HEK293T, $Src^{-}Yes^{-}Fyn^{-}$ null (SYF), NIH-3T3, and SkBr3 were cultured in Dulbecco's modified Eagle's medium (Corning) with 10% fetal bovine serum (Seradigm) and 1000 units/ml penicillin, 1 mg/ml streptomycin, and 2.5 µg/ml amphotericin B (Corning). Sf9 insect cells were maintained in Sf-900 medium (Gibco) with 5% FBS and1000 units/ml penicillin, 1 mg/ml streptomycin, and 2.5 µg/ml amphotericin B. 293-GPG cells used for generating retrovirus were maintained in DMEM with 10% FBS, 300 µg/mL L-glutamine,

1X Penicillin-Streptomycin (Corning) solution, 100 µg/mL tetracycline (Sigma), 2 µg/mL puromycin (Gibco), and 300 µg/mL G418 (Corning). MCF-10a cells were maintained in DMEM/F12 (Corning) containing 5% horse serum (Sigma), 20 ng/mL EGF (Millipore), 0.5 µg/mL hydrocortisone (MP Biomedical), 100 ng/mL cholera toxin, 10 µg/mL insulin (Sigma), and 1X Penicillin/Streptomycin.

Expression constructs and site-directed mutagenesis. The baculovirus vector for His-tagged Brk and the mammalian expression vector for Flag-tagged Brk were described previously[39, 40]. The pSKB-3 Hck kinase domain plasmid and the pCDF Duet-1 *Yersinia* phosphatase plasmid[91] were from Dr. Markus Seeliger of Stony Brook University. The pcDNA6 full length human Hck and pEGFP-C3 WASP-GFP mammalian expression vectors were described previously[82, 92]. Site-directed mutagenesis for Brk and Hck mutants were performed using the Stratagene QuikChange Kit, and mutations were confirmed by DNA sequencing. Full-length ErbB2 in pcDNA 3.1 neo and flag-tagged Beta-Catenin in pcDNA3.1 mammalian expression vectors were from Dr. Deborah Brown and Dr. Ken-Ichi Takermaru, respectively, of Stony Brook University. The expression vector for p130Cas in pcDNA3.1/V5-His B was described previously[93].

Protein expression and purification. His-tagged wild-type and mutant forms of Brk were expressed in Spodoptera frugiperda (Sf9) cells using the Bac-to-Bac baculovirus system (Invitrogen). Sf9 cells infected with recombinant Brk baculovirus were harvested 3 days post-infection and washed with PBS. The cells were lysed using a French pressure cell in buffer containing 20 mM Tris, pH 8, 10% glycerol, 5 mM β-mercaptoethanol, and protease inhibitors

(10 µg/ml aprotinin and leupeptin, 100 mM PMSF, 2 mM Na3VO4). The lysed cells were then centrifuged and filtered and incubated with Ni-NTA agarose (Qiagen) for 1 hr at 4°C. The protein bound beads were loaded onto a column then washed with Buffer A (20 mM Tris, pH 8, 500 mM NaCl, 10% glycerol, 10 mM imidazole, and 5 mM β-mercaptoethanol), then with Buffer B (Buffer A with 1 M NaCl), then again with Buffer A. Brk was eluted with buffer containing 20 mM Tris, pH 8, 10% glycerol, 5 mM β-mercaptoethanol, and 100 mM imidazole. Eluted fractions were resolved by SDS-PAGE. Peak fractions were combined and further purified by anion exchange chromatography. The Ni-NTA purified fractions were diluted with Buffer QA (20 mM Tris, pH 8, 100 mM NaCl, 5% glycerol, and 1 mM DTT) and loaded onto an anion exchange column (Mono Q 5/50 GL, GE Healthcare Life Sciences). Proteins were eluted over a linear gradient of 0-50% buffer QB (buffer QA with 1 M NaCl). Peak fractions were analyzed by SDS-PAGE.

To purify wild-type and mutant Hck kinase domain, Hck plasmid was transformed into BL21 (DE3) cells expressing Yersinia phosphatase (YOPH). Cell cultures were grown to OD_{600}=0.8 at 37°C, cooled to 18°C then induced with 0.4 mM IPTG for 16 hours. The cells were harvested by centrifugation then lysed with Buffer A (50 mM Tris, pH8, 500 mM NaCl, 5% glycerol, 25 mM imidazole) containing protease inhibitors (10 µg/mL aprotinin and leupeptin, 100 mM PMSF, and 1 mM Na_3VO_4) in a French pressure cell. The lysates were centrifuged and filtered then bound to Ni-NTA beads equilibrated with Buffer A for 45 minutes at 4°C. The protein bound beads were loaded onto a column and washed with Buffer A. The protein was eluted with Buffer B (Buffer A with 200 mM imidazole) and the eluted protein was analyzed by SDS-PAGE. The peak fractions were combined and cleaved with 1 mg TEV protease per 25 mg protein in a Spectra/Por membrane (12-14 kD cutoff, Spectrum Labs) in dialysis buffer (20 mM

Tris pH 8, 100 mM NaCl, 1 mM DTT, 5% glycerol, 0.1 mM EDTA) at 4°C. The TEV treated protein was loaded onto an anion exchange column (Mono Q 5/50 GL) equilibrated with Buffer QA (20 mM Tris, pH 8, 1 mM DTT, 5% glycerol). Proteins were eluted over a linear of gradient of 0-30% buffer QB (buffer QA with 1 M NaCl). Peak fractions were analyzed by SDS-PAGE.

Transient transfection, Western blotting, and immunoprecipitation. Cells (1 million) were transfected 24 hours after plating with 8 µL polyethylenimine per µg of DNA in 150 mM NaCl or with 2.5 µL TransIT LT1 reagent (Mirus) per µg DNA. In starvation studies, cells were starved in DMEM with 0.01% serum and 1% antibiotic. Cells were harvested 48 hours post-transfection using RIPA buffer (25 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% NP-40) supplemented with aprotinin, leupeptin, PMSF, NaF, DTT, and Na_3VO_4. The lysates were resolved by SDS-PAGE and transferred to PVDF membranes and probed with the appropriate antibodies, or were used in immunoprecipitation assays.

For immunoprecipitation studies, 1 mg/ml of cell lysates were pre-cleared with 15 µL of Protein G agarose beads (Thermo Scientific) for 1 hour at 4˚C on a rotator. The pre-cleared lystates were incubated with 1 µg of the appropriate antibody for 1 hour at 4˚C, then combined with 15 µL of fresh protein G agarose for 4 hours at 4˚C. The beads were washed three times with RIPA buffer, then eluted with SDS-PAGE buffer and resolved by SDS-PAGE. The proteins were transferred to PVDF membrane for Western blot analysis.

For immunoprecipitation of Flag-tagged Brk, cell lysates (1 mg protein) were incubated with 45 µL of anti-Flag M2 affinity gels (Sigma) on a rotator for 3 hours at 4˚C, then washed three times with Tris-buffered saline (TBS). The immunoprecipitated proteins were divided in three

tubes. Duplicate samples were used for a radioactive kinase assay with Src peptide (sequence: AEEEEIYGEFEAKKKKG).

Radioactive kinase assay. Immunoprecipitated Flag-tagged Brk proteins were incubated with 15 µL of reaction buffer (30 mM Tris, pH 7.5, 20 mM $MgCl_2$, 1 mg/mL BSA), 200 µM ATP, 1.2 mM Src peptide and 50 – 100 cpm/pmol of [γ^{32}-P] ATP at 30˚C for 10 minutes. To measure the activity of Brk purified from Sf9 cells, 1 µM of Brk protein was incubated with reaction buffer, 250 µM ATP, 1.2 mM Src peptide, and [γ^{32}-P] ATP at 30˚C for 10 minutes. To measure the activity of purified Hck kinase domain proteins, 100 nM of purified protein was incubated with reaction buffer 100 µM ATP, 250 µM Src peptide and [γ32-P] ATP. The reaction was incubated at 30˚C for 2 minutes. The reactions were quenched using 45 µL of 10% trichloroacetic acid. The samples were centrifuged and 30 µL of the reaction was spotted onto Whatman P81 cellulose phosphate paper, which was washed with 0.5% phosphoric acid. Transfer of radioactive phosphate to the peptide was measured by scintillation counting.

Autophosphorylation assay. Purified Hck protein was incubated with Glutathione beads bound with GST-tagged YOPH with 50 mM Tris, pH 8.0 and 50 mM NaCl for 45 minutes at RT. YOPH treated proteins (500 nM) were then incubated with 100 µM ATP, 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 1 mM Na_3VO_4. At each time point, 20 µL of the reaction was removed and added to 5 µL 5x SDS-PAGE buffer to stop the reaction. Untreated protein, YOPH treated protein, and autophosphorylated protein at each time point were resolved by SDS-PAGE and transferred to a PVDF membrane for Western blotting.

Peptide binding assay. SH2 ligand peptide (ETpYEEYGYDG) or SH3 ligand peptide (RGAAPPPPPVPRGRG) were coupled to Affi-gel 15 resin (Bio-rad) by incubation in 0.1 M Hepes (pH 7.5) overnight at 4˚C, then blocked with 100 mM ethanolamine. The peptide conjugated beads were washed with 0.1 M Hepes (pH 7.5) then equilibrated with binding buffer (5 mM EDTA, 50 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 0.5 mM Na_3VO_4). Lysates (500 µg) from transiently transfected cells were incubated with the beads for 1 hour at 4˚C. The bound protein was eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE and analyzed by Western blotting.

Stable cell expression. The MSCV-PIG vector and 293-GPG cells were a kind gift from Dr. Senthil Muthuswamy (University of Toronto). To generate retrovirus, 15 µg DNA was combined with 50 µL Lipofectamine 2000 (Life Technologies) in 2 mL serum-free DMEM. The mixture was added to 293-GPG cells in 4 mL of fresh serum-free DMEM. Following a 6 hour incubation, 5 mL of fresh media was added to the cells, and incubation continued overnight. The media was replaced the following day. Virus collected on the fifth day after transfection was filtered with a 0.45 µm filter and combined with 4 mL media and 8 µg/ml polybrene and added to NIH-3T3 cells. Following a 5 hour incubation, 6 mL of fresh media was added to the cells. Puromycin (2µg/mL) was added to the cells 48 hours post-infection to select for positively infected cells. Stable cells were monitored by GFP expression.

Growth assays. Cells (20,000) were plated in duplicate in 12-well plates. At each time point, the cells were trypsinized, then resuspended in media and counted with a hemacytometer. To measure non-anchored cell growth, 20,000 cells were plated in triplicate in 24-well low attachment plates (Corning). On day 6, the cells were centrifuged then resuspended using 100 µL trypsin and counted with a hemacytometer.

Migration Assay. MCF-10a cells were serum starved overnight (in 2% horse serum and no EGF), and plated in duplicate into 24-well Transwell plates with 0.8 uM polyester inserts (Corning). Media with or without EGF was added to the bottom chamber. After 8 hours, non-migrated cells were swabbed from the top of the chamber and the migrated cells were fixed with formaldehyde and stained with DAPI. Cells were counted and averaged for 5 different areas of each well.

Localization studies. 293T cells were plated on glass cover slips in 35 mm culture dishes. The next day, the cells were transfected with wild-type or mutant Brk DNA. Twenty-four hours post transfection, the coverslips were washed with 1X PBS and fixed with 3.7% formaldehyde in 1X PBS for 20 minutes at 4°C. The cells were permeabilized with 0.2% Triton-X for 5 minutes then blocked with buffer containing 3% BSA and 3% goat serum in 1X PBS for 1 hour. The cells were then incubated with anti-Brk for 1 hour then with FITC-anti-mouse for 30 minutes. The coverslips were mounted with Vectashield mounting medium with DAPI. Cells were imaged using an Olympus Flow View 1000 confocal microscope.

Isothermal titration calorimetry. The pET28 SAC SP vector encoding the Brk SH2 domain was a kind gift from Dr. John Engen (Northeastern University). The SH2 domain was expressed in BL21 (DE3) *E. coli* cells. Cells were lysed in a French pressure cell and the SH2 domain was purified by chromatography on Ni-NTA agarose. Purified protein was dialyzed overnight in ITC buffer (20 mM Hepes, 1 mM EDTA, 250 mM NaCl, 1 mM β-mercaptoethanol, and 5% glycerol). Synthetic peptides based on the wild-type or mutant Brk C-terminal tail were from Genemed Synthesis, Inc. Crude peptides were purified by reverse-phase HPLC and dialyzed in ITC buffer. The protein was diluted to 67.4 µM and the wild-type (FTS-Y(p)-ENLTG) and P450L (FTS-Y(p)-EN**P**TG) peptides were diluted to 1 mM and 827 µM, respectively. The protein and peptides were degassed at 4˚C and loaded into the sample cell and syringe, respectively. ITC experiments were performed on a MicroCal VP-ITC microcalorimeter at 25°C with 5 µL peptide added per injection. Origin 7.0 was used for data fitting.

Chapter 3

Cancer-associated mutations in Breast tumor kinase/PTK6 differentially affect enzyme activity and substrate recognition

The contents of this chapter have been published: Tiffany Tsui and W. Todd Miller, Biochemistry 2015, 54, 3173−3182.

Abstract

Brk (breast tumor kinase, also known as PTK6) is a non-receptor tyrosine kinase that is aberrantly expressed in several cancers and promotes cell proliferation and transformation. Genome sequencing studies have revealed a number of cancer-associated somatic mutations in the Brk gene; however, their effect on Brk activity has not been examined. We analyzed a panel of cancer-associated mutations and determined that several of the mutations activate Brk, while two eliminated enzymatic activity. Three of the mutations (L16F, R131L, and P450L) are located in important regulatory domains of Brk (the SH3, SH2 domains, and C-terminal tail, respectively). Biochemical data suggest that they activate Brk by disrupting intramolecular interactions that normally maintain Brk in an autoinhibited conformation. We also observed differential effects on recognition and phosphorylation of substrates, suggesting that the mutations can influence downstream Brk signaling by multiple mechanisms.

Introduction

Brk was first identified in a tyrosine kinase screen of metastatic breast cancers. It was found to be overexpressed in two-thirds of breast cancer samples and cell lines[57]. Aberrant expression of Brk is observed in several other cancers where it has been shown to mediate signaling of various pathways to promote cell growth, survival, and migration.

In addition to overexpression, tyrosine kinases can become hyperactivated in human cancer through somatic mutations. Several somatic mutations have been recently identified in the gene encoding PTK6/Brk. It has not been determined whether these cancer-associated mutations activate Brk or promote neoplastic growth. In this study, we have examined a panel of Brk somatic mutations to assess enzymatic activity and substrate binding. The mutations were identified in different cancer types and are located across the different domains of Brk (Fig. 3-1a). The L16F mutant, identified in clear cell renal cell carcinoma[94], is found in the SH3 domain. The R131L mutant, found in gastric cancer[95], is located in the SH2 domain. The V253M, N317S, and L343F mutants are found in the kinase domain. They were identified in head and neck squamous cell carcinoma[96], ovarian carcinoma[97], and cutaneous squamous cell carcinoma[98], respectively. The P450L mutant, identified in pancreatic cancer[99], is located adjacent to the inhibitory tyrosine residue at the C-terminal tail of Brk. The location of these mutations suggests the possibility that the autoinhibitory interactions of Brk could be disrupted, leading to increased activity. Sequence alignment with Src family kinases revealed that the L16F, V253M, and N317S mutations are to amino acids that are conserved among all Src kinases (Fig. 3-1b). The P450 residue is conserved with half of the Src kinases. Conservation of these amino acids with Src suggest that they may be essential for proper kinase function. In addition, several of these

mutations appear on analogous residues to other Src kinases. For example, the equivalent residue to L343 of Brk is also mutated in Src, Hck, and Blk, and the analogous residue to P450L of Brk is also mutated in Lyn, Fyn, and Fgr. The recurrence of these mutations indicates that mutations to these residues are selected for and thus may confer a growth advantage when present in cells. We show that the L16F, R131L, L343F, and P450L mutations increase Brk activity, while the V253M and N317S mutants inactivate Brk. Furthermore, as the SH3 and SH2 domains also mediate substrate interactions, we observe mutant-specific effects on substrate binding and phosphorylation.

Results

Cancer-associated mutations affect Brk activity. We introduced the six mutations into expression vectors encoding Flag-tagged full length Brk and expressed the proteins in HEK293T cells. We measured Brk phosphorylation in cell lysates using a general phosphotyrosine antibody and with an antibody specific for pY342, the major site of autophosphorylation in Brk[39]. The mutations located within important regulatory regions of Brk, L16F (SH3), R131L (SH2), and P450L (adjacent to the regulatory C-terminal phosphotyrosine), caused significant increases in Brk autophosphorylation (Fig. 3-2a). The L343F mutant, which is adjacent to the autophosphorylation site in the activation loop, also increased phosphorylation of tyrosine 342. The V253M and N317S mutations in the Brk kinase domain reduced autophosphorylation activity to undetectable levels. To confirm activation of Brk, we immunoprecipitated Brk from cell lysates and incubated the protein with a synthetic peptide substrate in the presence of [γ^{32}-P] ATP. The mutants that showed increased autophosphorylation also had increased activity against the peptide, although the degree of activation toward the exogenous substrate was not as high (Fig. 3-2b). The N317S mutant was inactive in this assay, consistent with its lack of autophosphorylation (Fig. 3-2a). We have also expressed wild-type Brk and two of the mutants (L16F and P450L) using the Sf9 baculovirus system. The activity of the purified proteins were measured against a synthetic peptide in the presence of [γ^{32}-P] ATP. Unexpectedly, no increase in activity of the P450L mutant was observed and the L16F mutant inactivated the protein (Fig. 3-2c).

Cancer-associated mutations disrupt Brk intermolecular and intramolecular interactions. The P450L mutation is located at the P+3 position relative to the regulatory tyrosine (Y447) at

the Brk C-terminal tail. The identity of the amino acid at the P+3 position often dictates the affinity and specificity of SH2 ligands[100]; thus, the P450L mutation could potentially affect autoinhibitory binding between the Brk SH2 domain and phosphorylated Y447. We tested this possibility first with a peptide affinity assay, using a peptide containing the pYEEY sequence that we previously showed binds to the SH2 domain of Brk[40]. The autoinhibited forms of Src and Brk bind weakly to immobilized SH2 ligands because their SH2 domains are engaged with their phosphorylated C-terminal tails, while activated forms bind more strongly[39, 101]. We incubated the immobilized pYEEY peptide with lysates from cells expressing wild-type or P450L Brk. The P450L mutant showed increased affinity for the SH2 ligand compared to wild-type Brk (Fig. 3-2d), suggesting that the P450L mutation may disrupt the inhibitory interaction between the SH2 domain and the C-terminal tail of Brk. We also measured binding of the SH2 domain to a synthetic peptide based on the phosphorylated C-terminal tail of Brk (FTS**pY**ENPTG) or to a similar peptide containing the P450L substitution (FTSpYEN**L**TG) by isothermal titration calorimetry. The dissociation constant of the Brk SH2 domain for a phosphorylated Brk C-terminal tail peptide increased from 15 μM to 53 μM with the introduction of the P450L mutation (Fig. 3-3). This is consistent with a decreased interaction between the SH2 domain and its phosphorylated C-terminal tail in the Brk P450L mutant.

Based on the autoinhibited structure of Src kinases, the Brk SH3 domain is predicted to bind to the SH2-kinase linker to stabilize the autoinhibited conformation. Mutations within the linker sequence strongly activate Brk[40]. A structural analysis of the Brk SH3 domain showed that Trp17 (the residue adjacent to Leu16) interacts with Pro179 in the linker region[102]. Thus, activation of Brk by the L16F mutation could be due to disruption of SH3-linker interactions. The SH3 domain of Brk plays a key role in the recognition of substrates, so the L16F mutation

could also interfere with substrate binding. We carried out a binding assay using an immobilized peptide based on the SH3 recognition sequence on Sam68, a confirmed substrate of Brk. The L16F mutant showed decreased affinity for the SH3 ligand compared to wild-type Brk, which suggests that the ability of the L16F mutant to recognize its substrates would be compromised (Fig. 3-2d).

Effect of Brk mutants on cell growth. Several studies have supported a role for Brk as an oncogenic driver of cancer. Expression of Brk increases cell proliferation and promotes anchorage independent growth[64], a measure of transforming potential. Brk also promotes tumorigenesis in the mouse mammary gland[71], and decreases tumor latency[70]. Knock-down of Brk inhibits cell growth[58, 103]. We measured anchored and non-anchored growth of NIH-3T3 cells stably expressing wild-type Brk or the cancer-associated mutants. We expressed Brk using a retroviral vector that contains an internal ribosomal entry site for expression of GFP. We selected Brk expressing cells by puromycin treatment, and analyzed cells with equivalent GFP expression (Fig. 3-4c). Cells expressing wild-type Brk showed increased cell growth compared to empty vector cells, with the greatest difference in cell number at Day 6 (Fig. 3-4a). All of the stable cells expressing Brk mutants showed reduced proliferation relative to wild-type Brk, and cells that expressed the P450L mutant did not survive past Day 6. We examined anchorage-independent growth using plates with a neutral surface to minimize cell attachment. We observed an increase in nonanchored growth in NIH3T3 cells expressing wild-type Brk as compared to control cells, but there were no significant differences between control cells and cells expressing Brk mutants (Fig. 3-4b).

The effect of Brk expression on cell growth is most often studied in the context of breast cancer. We stably expressed wild-type or mutant forms of Brk in MCF-10a non-transformed mammary epithelial cells, as well as in SKBR3 cells, which are a transformed mammary cell line. We chose theSKBR3 cell lineas it overexpresses ErbB2, which we have previously shown to cooperate with Brk to enhance cell growth[66]. Similarly to the results in NIH3T3 cells, MCF-10a cells expressing the P450L and L16F mutants showed reduced proliferation (anchored and non-anchored) relative to wild-type Brk (Fig. 3-5a, b). We also measured the effect of Brk mutants on EGF induced migration of MCF-10a cells. While, both wild-type and mutant Brk showed increased growth in response to EGF, both the L16F and P450L mutants showed decreased cell migration compared to wild-type Brk (Fig. 3-5c). In SKBr3 cells, we observed no difference in proliferation between control cells and cells expressing Brk (wild-type or mutant) at Day 6 (data not shown) or Day 14 (Fig. 3-5d). No significant difference was observed in anchorage independent growth of mutant expressing cells compared to wild-type expressing cells with exception for the L16F mutant, which showed decreased cell growth (Fig. 3-5e).

Effect of Brk mutants on signaling pathways. Several of the cancer-associated mutations increased Brk activity, yet reduced cell growth. To understand the basis of these effects, we examined signaling pathways that are often activated in cancer. Brk is a mediator of signaling downstream of the EGFR family of receptor kinases. Co-expression of Brk with ErbB2 leads to increase in phosphorylation of Erk1/2[66]. We first examined the effect of two of the mutations (L16F and P450L) on interaction with the ErbB2 receptor. Activation of ErbB2 leads to trans-phosphorylation of the intracellular domain, which provides binding sites for cellular proteins containing SH2 domains, including Brk[104]. Mutations to Brk that interfere with inhibitory

intramolecular interactions could allow greater access of Brk to the SH2 binding sites. We co-transfected ErbB2 and Brk into 293T cells, immunoprecipitated ErbB2, and examined Brk association by Western blotting. The L16F and P450L mutants showed increased interaction with ErbB2 compared to wild-type Brk (Fig. 3-6a).

Akt kinase has previously been identified as a binding partner and substrate for Brk[105]. We did not observe any differences in Akt phosphorylation between control 293T cells or cells expressing WT or mutant forms of Brk (Fig. 3-6b). The L16F mutant showed increased phosphorylation of Erk1/2 compared to wild-type Brk (Fig. 3-6b). In serum starved cells, no phosphorylation of Erk 1/2 was observed and there was no appreciable difference between wild-type and L16F Brk following stimulation with EGF (Fig. 3-6c). This suggests that the L16F mutation disrupts autoinhibitory constraints in Brk, but does not activate Brk to levels higher than those achieved by EGF stimulation. The increased phosphorylation of Erk 1/2 by the L16F mutant in cells that were not serum starved (Fig. 3-6b) suggests that the L16F mutant may be more sensitive to activation by other factors. Akt and Erk1/2 were still phosphorylated in cells expressing the catalytically inactive N317S mutant, suggesting that this mutant might potentiate phosphorylation of signaling molecules by another kinase (e.g., a Src family kinase), perhaps by acting as a scaffold protein. Both cell migration and cell growth are still observed even with expression of kinase inactive Brk[68, 103]. These results are consistent with observations of cell migration and cell growth upon expression of kinase inactive Brk. We also measured activation of Akt and Erk 1/2 in SkBr3 breast cancer cells (Fig. 3-6d). We observed increased activation of both Akt and Erk 1/2 in cells expressing the L343F mutant, but there was no increase in anchored of nonanchored cell growth.

A number of Brk substrates and interacting proteins have been identified in normal epithelial cells, as well as in tumors[42]. p130 CRK-associated substrate (CAS) is a substrate of Brk that is localized to focal adhesions and acts as an adaptor protein to facilitate cell migration[106]. Cas has been shown to be a direct substrate of Brk and mediates Brk-induced cell migration of prostate cells[107]. We cotransfected 293T cells with Brk and CAS, and probed the cell lysates with a phospho-specific Cas antibody (pY165). The R131L, L343F, and P450L mutants all showed greater phosphorylation of CAS compared to wild-type Brk (Fig. 3-6e). The L16F mutant showed decreased phosphorylation of Cas. This may be partly due to decreased expression of L16F Brk upon coexpression with Cas, an effect we observed consistently. Additionally or alternatively, decreased phosphorylation of Cas by the L16F mutant could result from decreased binding between the proteins. Src interacts with Cas through its SH3 domain[108]. The L16F mutation, which is within the SH3 domain of Brk and interferes with ligand binding (Fig. 3-2d); thus, the mutation may disrupt the Brk-Cas interaction.

The transcription factor, signal transducer and activator of transcription 3 (STAT3), is a substrate for Brk[109]. Constitutive activation of STAT3 has been observed in many types of cancer. Phosphorylation of STAT3 by Brk increases its transcriptional activity and coexpression of STAT3 and Brk promote cell proliferation[109]. We observed a decrease in phosphorylation of STAT3 in cells expressing the V253M and N317S mutants compared to control cells or cells expressing wild-type Brk (Fig 2-6f), suggesting that these mutants may exert a dominant-negative effect on STAT3 phosphorylation.

Sam68, an RNA binding protein, was one of the first substrates identified for Brk. Sam68 is upregulated in breast cancer[110] and prostate cancer[111], and expression of Brk relocalizes Sam68 to Sam68-SLM nuclear bodies (SNBs)[112], which are often observed in cancer cells[113]. We

transiently expressed Brk (wild-type or mutants) in 293T cells, immunoprecipitated endogenous Sam68, and examined phosphorylation by SDS-PAGE and anti-phosphotyrosine Western blotting. We also probed the membranes with Flag antibody to measure association of Brk. The R131L, L343F, and P450L mutants showed a small increase in Sam68 binding and phosphorylation compared to wild-type Brk (Fig. 3-6g). While the L16F mutant increased Brk activity (Fig. 3-2), there was decreased binding and phosphorylation of Sam68 by this mutant compared to wild-type Brk (Fig. 3-6g). This is likely due to the effect of the L16F mutation on the ligand-binding ability of the SH3 domain (Fig. 3-2d), which is critical for interaction with Sam68[40, 114]. The N317S mutation, which drastically reduced autophosphorylation (Fig. 3-2a), severely compromised Sam68 binding and tyrosine phosphorylation (Fig. 3-6g).

Phosphorylation of β-catenin by Brk inhibits its transcriptional activity[115]. We measured phosphorylation of β-catenin in cells co-expressing β-catenin and wild-type or mutant forms of Brk. We immunoprecipitated β-catenin from the lysates and probed with a general phosphotyrosine antibody. We reprobed the membrane with Flag antibody to assess Brk association. The R131L, L343F, and P450L mutants all showed greater binding and phosphorylation of β-catenin as compared to wild-type Brk (Fig. 3-6f). The L16F mutant was not able to phosphorylate β-catenin, likely due to its decreased interaction with the protein (Fig. 3-6f). While the N317S mutant bound normally to β-catenin, it was unable to phosphorylate the protein. The results suggest that increased phosphorylation of β-catenin by several of the cancer-associated mutants could underlie the reduced cell growth observed in NIH3T3 and MCF-10a cells.

Effect of cancer-associated mutations on localization of Brk. Studies in prostate cancer cells revealed that localization of Brk at the cell membrane correlated with severity of the disease[62]. Additionally, Kim et. al., demonstrated that targeting of Brk to the cell membrane increased cell proliferation and survival, and cell migration, while targeting of Brk to the nucleus had the opposite effect[63]. Unlike Src, Brk lacks a membrane-targeting myristoylation sequence at the N-terminus[38]. Brk also lacks a nuclear localization sequence[42]. Thus, the mechanism that dictates localization of Brk is unknown. We have shown that cancer-associated mutations to Brk differentially affect recognition of its substrates. We examined whether altered interaction with substrates such as Sam68, which is normally localized in the nucleus, and Cas, which is often detected at the cell membrane, affect localization of Brk.

We examined localization of wild-type and mutant forms of Brk transiently expressed in 293T cells by fluorescence microscopy. No significant difference in the localization of wild-type or mutant Brk was observed. Both wild-type and mutant Brk are localized in both the cytoplasm and nucleus (Fig. 3-7). There is some punctate staining of Brk in wild-type, L343F, and P450L cells in the cytoplasm. Further analysis is required to determine the significance of this localization, such as by staining for Brk substrates such as Cas.

DISCUSSION

Tyrosine kinases have emerged as one of the most frequently mutated gene families in cancer[22, 116]. In some cases (e.g., EGF receptor and c-Kit), the mutant kinases have increased enzymatic activity and transforming ability[21]. Brk was originally identified as a gene that is overexpressed in metastatic breast cancer, and several studies have supported a role for Brk as a driver of tumorigenesis. Brk is a key mediator in several signaling pathways, functioning downstream from the EGFR family of receptors[58, 64, 117, 118] as well as IGF-1R[67]. Aberrant expression of Brk promotes cell growth and migration, and more recently has been shown to mediate the epidermal to mesenchymal transition[72] and is involved in hypoxia induced growth in breast cancer[73]. Sequencing efforts to fully identify the genetic landscape of different cancers have revealed several cancer-associated mutations to Brk. It has not been determined whether these mutations are driver mutations that are critical for oncogenesis, or simply passenger mutations that do not contribute any growth advantage. In this study, we have shown that the L16F, R131L, L343F, and P450L mutants activate Brk as determined by autophosphorylation (Fig. 3-2a). The activation of Brk was corroborated by measuring the phosphorylation of a synthetic peptide substrate (Fig. 3-2b). In contrast, the V253M and N317S mutations eliminate the catalytic activity of Brk. We did not observe an increase in activity of the P450L mutant compared to WT protein purified from Sf9 cells and the L16F mutation inactivated the protein. Inactivation of Brk by the L16F mutant could be attributed to decreased stability of the mutant protein. Expression of the L16F mutant in Sf9 cells as well as in mammalian cells was often poor.

The P450L and L16F mutations are predicted to affect intramolecular and intermolecular interactions of Brk. Our results suggest that the P450L mutation weakens binding between the

SH2 domain and the C-terminal tail, an important interaction for maintaining Brk in its autoinhibited conformation; the mutation concomitantly increases accessibility of the SH2 domain for interaction with various substrates (Fig. 3-2d, 3-7, and 3-6f). The L16F mutation leads to decreased interaction with an SH3 ligand (Fig. 3-2d). The L16F mutation activated Brk (Fig. 3-2a) and promoted phosphorylation of Erk1/2 (Fig. 3-7b), but dramatically decreased the ability of Brk to bind and phosphorylate other substrates such as Sam68 and β-catenin. (Fig. 3-7f, g). This supports previous studies that showed the SH3 domain is essential for interaction with Sam68[40], and highlights the importance of this domain for interaction with β-catenin. This mutation may also inhibit interaction with other substrates such as paxillin, which Brk also phosphorylates to promote cell migration. Both the SH2 and SH3 domains were shown to be involved in interaction with paxillin[69].

The V253M and N317S mutations inactivate Brk (Figs. 3-2a,b) and expression of the N317S mutant in cells inhibits phosphorylation of several substrates including Sam68 and β-catenin (Fig. 3-7). Phosphorylation of Erk1/2and Akt is still present in cells expressing the N317S mutant (Fig. 3-7). These results show that the catalytic activity is important for activation of Sam68 and β-catenin, but imply that a catalytically inactive form of Brk is able to facilitate phosphorylation of other substrates. Previous studies have shown that a kinase-dead mutant of Brk is still able to promote proliferation of T-47D breast cancer cells[103], consistent with these observations. Additionally, recent studies have shown decreased Brk in some cancers to correlate with poor prognosis, suggesting that Brk may also act as a tumor suppressor[119-121]. Inactivating mutations to Brk could potentially disrupts its function as a tumor suppressor.

Expression of wild-type Brk showed an increase in anchored and non-anchored cell growth, as previously observed[64, 70, 71]. While several of the mutations increased Brk kinase

activity, expression unexpectedly led to decreased cell proliferation (Fig. 3-5, 3-6). We hypothesize that this effect may be due to differential interaction and activation of Brk substrates. We observed an increase in the interactions of the L16F and P450L mutants with ErbB2, relative to WT Brk (Fig. 3-7a). We have previously shown that co-expression of Brk and ErbB2 promotes cell proliferation mainly through activation of the Ras-MAPK pathway[66]. Thus, activation of Brk and increased interaction with ErbB2 by the L16F and P450L mutants would be expected to result in increased phosphorylation of Erk1/2, and potentially Akt, which is another key signaling protein downstream of ErbB2 and is a substrate of Brk[105, 118]. The L16F mutant (but not the P450L mutant) showed an increase in activation of Erk1/2, and we observed no significant difference between mutants compared to wild-type Brk for the activation of Akt. The lack of Akt activation by L16F Brk may be due to interference with SH3 ligand binding; the SH3 domain of Brk is important for interaction with Akt[105].

Differential phosphorylation of Brk substrates could result in activation of both pro- and anti-proliferative pathways. Activation of β-catenin has been implicated in colon cancer and NSCLC[122]. Expression of the R131L, L343F, and P450L mutants led to increased phosphorylation of β-catenin (Fig. 3-7g). Phosphorylation of β-catenin by Brk inhibits transcriptional activity[115]. Co-expression of an alternatively spliced variant of Brk (Alt-PTK6) enhances Brk downregulation of β-catenin and decreases colony formation of a prostate adenocarcinoma cell line[48]. Thus, the enhanced phosphorylation of β-catenin by the Brk mutants could partially explain the decrease in proliferation.

Whether Brk and Sam68 cooperate in a pro- or anti- tumorigenic manner is less clear. Sam68 may contribute to oncogenesis by promoting alternative splicing. For example, Sam68 promotes inclusion of a variable exon 5 in the *CD44* pre-mRNA, which is correlated with

tumorigenesis. Furthermore, expression of an RNA-binding deficient Sam68 inhibits cell growth[111]. When phosphorylated by Brk, Sam68 relocalizes to distinct nuclear bodies in breast and colon cancer cells; the presence of SNBs is correlated with tumorigenicity in some cancers[112]. On the other hand, phosphorylation of Sam68 by Brk inhibits its RNA-binding activity[114]. The R131L, L343F, and P450L mutants increased phosphorylation of Sam68 (Fig 3-7f), thus, expression of the mutants may inhibit the RNA-binding activity of Sam68 which is important for cell growth.

The localization of Brk is an additional factor that can modulate its tumorigenic potential. Brk lacks an N-terminal myristoylation sequence, which is important for membrane association in Src family kinases[38], and it also lacks a nuclear localization sequence[42]. In poorly differentiated prostate cell lines such as PC3, which form aggressive tumors in animals, Brk is mainly found in the cytoplasm[62]. Targeting of Brk to the cell membrane promotes cell growth and transformation[63]. We have shown that cancer-associated mutations can affect the interaction of Brk with its substrates, which may have indirect effects on the subcellular localization of the kinase. However, immunofluorescence analysis of wild-type and mutant Brk did not show any significant changes in localization of the protein (Fig. 3-7).

In summary, we have shown for the first time that cancer-associated mutations can affect Brk activity. Several of the mutations activate Brk, while the V253M and N317S mutations drastically reduce Brk activity. We have also shown that the mutations alter the set of substrates Brk is able to bind and phosphorylate, which may result in both positive and negative signals for cell growth and transformation. Expression of the mutants decreased cell proliferation, but did not result in overt changes in transformation (as measured by non-anchored cell growth or migration in the cell systems we studied (Fig. 3-5, 3-6). The tissues in which these mutations

arose could contain a different collection, or a different balance, of signaling components. Thus, Brk mutations could contribute to tumorigenesis by different mechanisms in different cell contexts. Cancer-associated somatic mutations can have important consequences in tumor growth and drug resistance. Because there is currently no reliable method to predict the consequence of somatic mutations, experimental testing and validation is essential.

Figure 3-1 Brk cancer-associated mutations. A) The domain arrangement of Brk is shown schematically. The autophosphorylation site (Tyr 342) and inhibitory tyrosine (Tyr 447) are in black. The cancer-associated mutations are indicated in red. B) Sequence alignment to Src family kinases. The SH3, SH2, and kinase domains are highlighted in yellow, green, and blue, respectively. Asterisks denote the autophosphorylation site (blue) and C-terminal inhibitory tyrosine (black). Red asterisks denote Brk cancer-associated mutations.

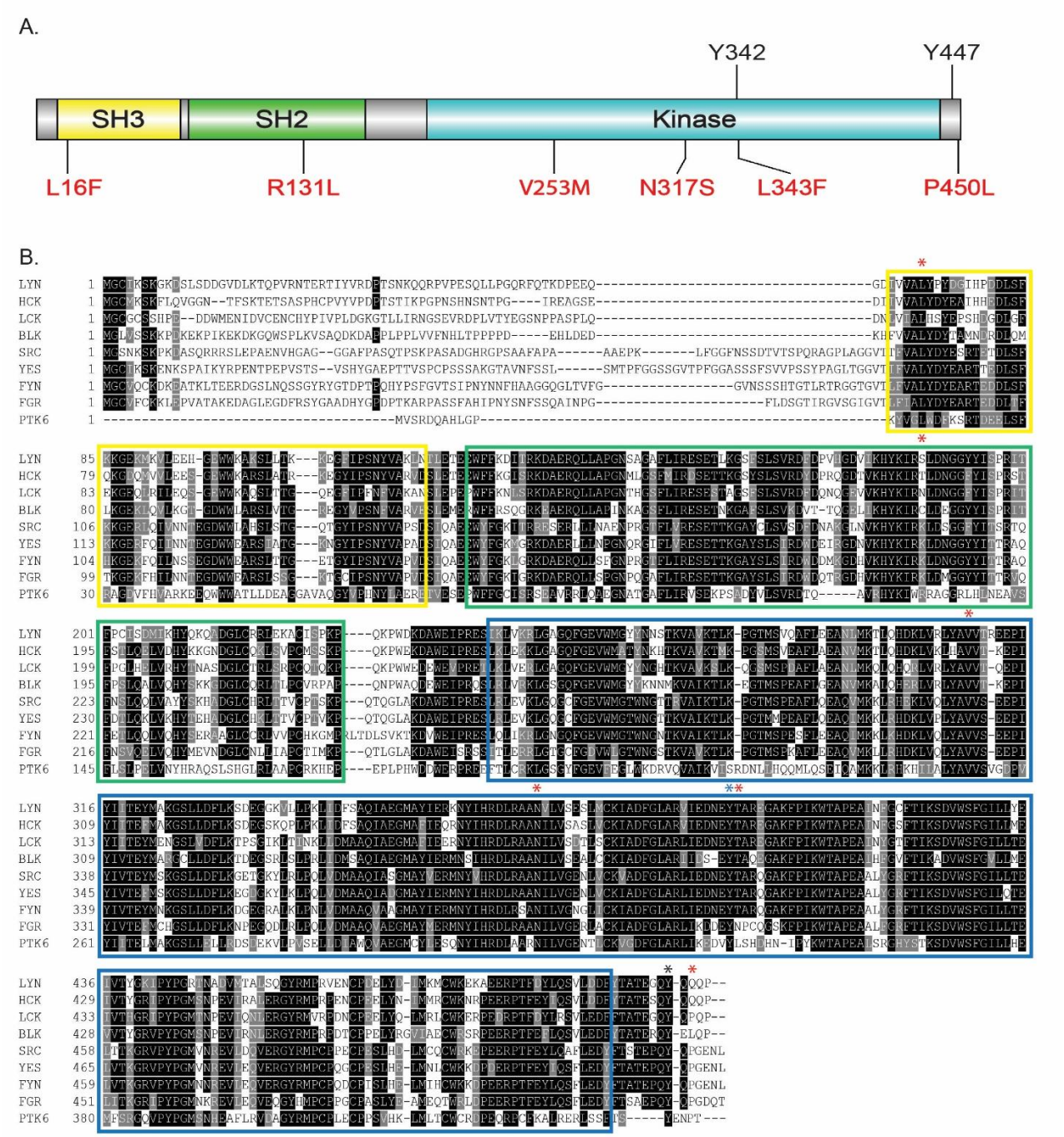

A.
Y342
Y447
SH3
SH2
Kinase
L16F
R131L
V253M
N317S
L343F
P450L
B.
LYN
HCK
LCK
BLK
SRC
YES
FYN
FGR
PTK6

Figure 3-2 Cancer-associated mutations affect Brk activation and intramolecular interactions. A) Lysates from 293T cells expressing Flag-tagged wild-type or mutant forms of Brk were probed with anti-phosphoBrk (pY342), phosphotyrosine, and Flag antibodies. The gel is representative of five similar experiments. B) Wild-type and mutant forms Brk were immunoprecipitated from transfected 293T cells using anti-Flag M2 affinity gel, then incubated with Src-substrate peptide in the presence of [γ32-P] ATP. The activities were measured in duplicate with the phosphocellulose paper assay. The results are representative of three experiments. The error bars show standard deviations. C) Wild-type and mutant forms of Brk purified from Sf9 cells were incubated with Src-substrate peptide in the presence of [γ32-P] ATP. The activities were measured in duplicate with the phosphocellulose paper assay. D) Lysates from Src-Yes-Fyn- null (SYF) cells expressing wild-type or mutant forms of Brk were incubated with SH2 ligand (left) or SH3 ligand (right) conjugated to agarose beads. Bound protein was eluted with SDS-PAGE sample buffer and resolved by SDS-PAGE, then probed with anti-Brk (left) and anti-Flag (right) antibodies. Affi-gel control samples contained lysates from wild-type Brk expressing cells incubated with non-conjugated beads. Untransfected controls (Un) contained untransfected cell lysates with peptide conjugated beads.

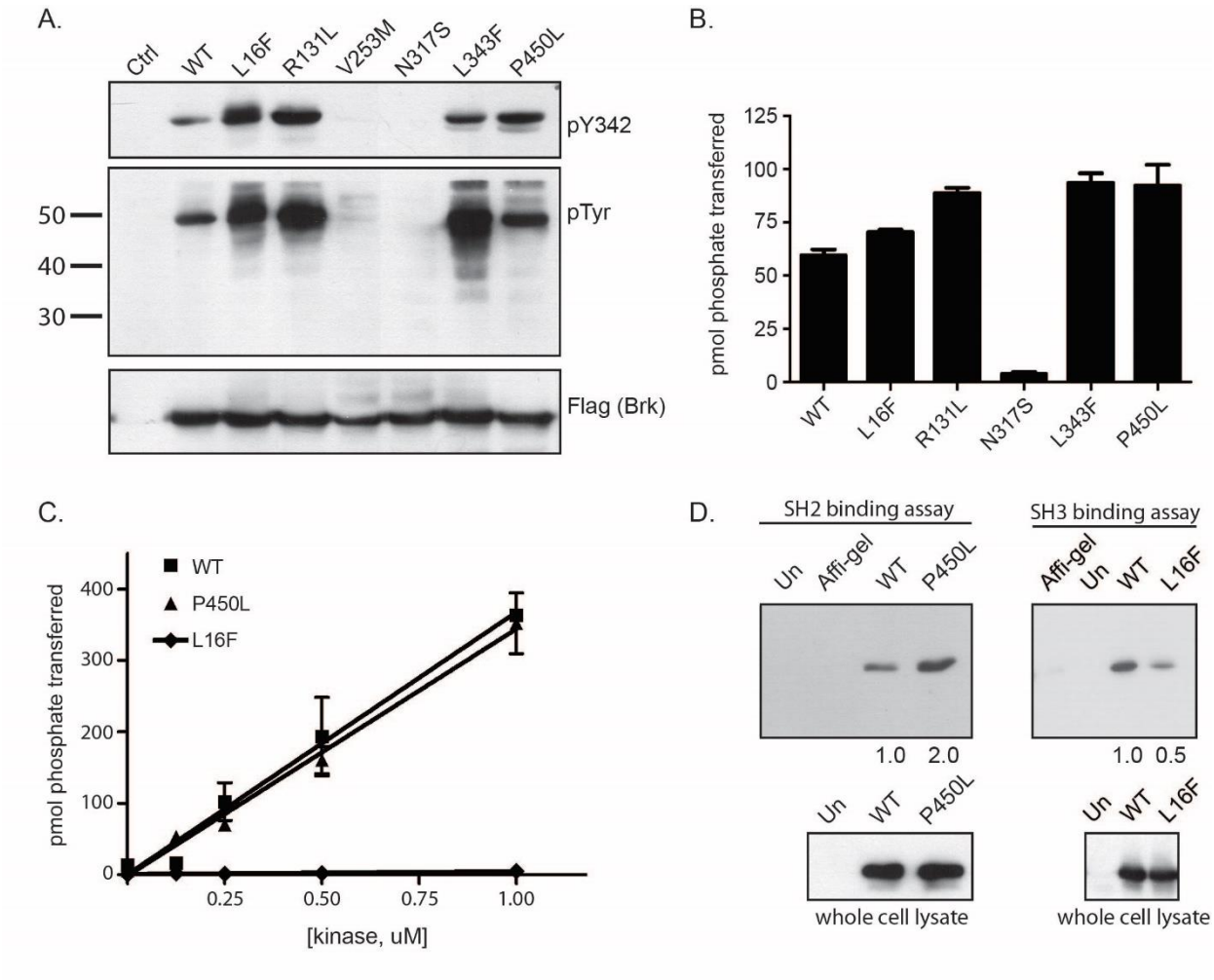

A.
Ctrl
WT
L16F
R131L
V253M
N317S
L343F
P450L
pY342
pTyr
Flag (Brk)
50
40
30
B.
pmol phosphate transferred
125
100
75
50
25
0
WT
L16F
R131L
N317S
L343F
P450L
C.
WT
P450L
L16F
pmol phosphate transferred
400
300
200
100
0
0.25
0.50
0.75
1.00
[kinase, uM]
D.
SH2 binding assay
Un
Affi-gel
WT
P450L
1.0
2.0
Un
WT
P450L
whole cell lysate
SH3 binding assay
Affi-gel
Un
WT
L16F
1.0
0.5
Un
WT
L16F
whole cell lysate

Figure 3-3 Interaction between the Brk SH2 domain and C-terminal peptides. Representative titrations of 5 μL injections of (A) wild-type peptide (FTS-Y(p)-ENLTG) (1 mM) and (B) P450L peptide (FTS-Y(p)-EN**P**TG) (0.83 mM) into 67.4 μM purified Brk SH2 domain. The dissociation constant calculated using Origin 7.0 was 15 μM and 53 μM, respectively. Note the difference in scale between the two panels; the P450L peptide produced smaller heat changes than the wild-type sequence.

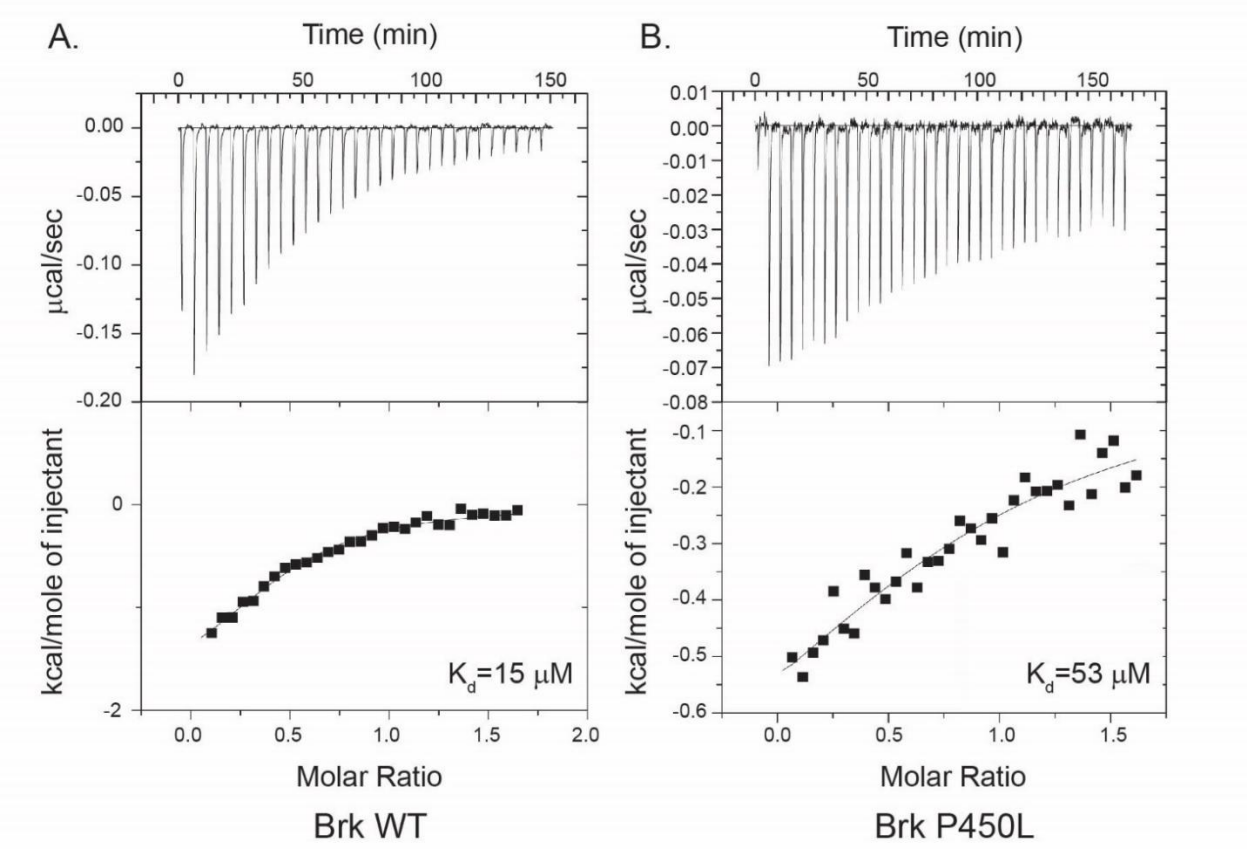

A.
Time (min)
µcal/sec
kcal/mole of injectant
K_d=15 µM
Molar Ratio
Brk WT
B.
Time (min)
µcal/sec
kcal/mole of injectant
K_d=53 µM
Molar Ratio
Brk P450L

Figure 3-4 Effect of Brk mutants on cell growth. A) Anchored cell growth: 20,000 NIH-3T3 cells stably expressing wild-type or mutant Brk were plated in duplicate in 12-well plates and counted over several days with a hemacytometer. The results are representative of three experiments. The error bars represent standard deviations. B) Non-anchored cell growth: 20,000 NIH-3T3 stable cells were plated in triplicate in 24-well low attachment plates. On day 6, the cells were collected and counted using a hemacytometer. The results are representative of two experiments. The error bars represent standard deviations. C) Lysates from NIH3T3 cells expressing wild type or mutant forms of Brk were probed with anti-Brk and anti-GFP antibodies.

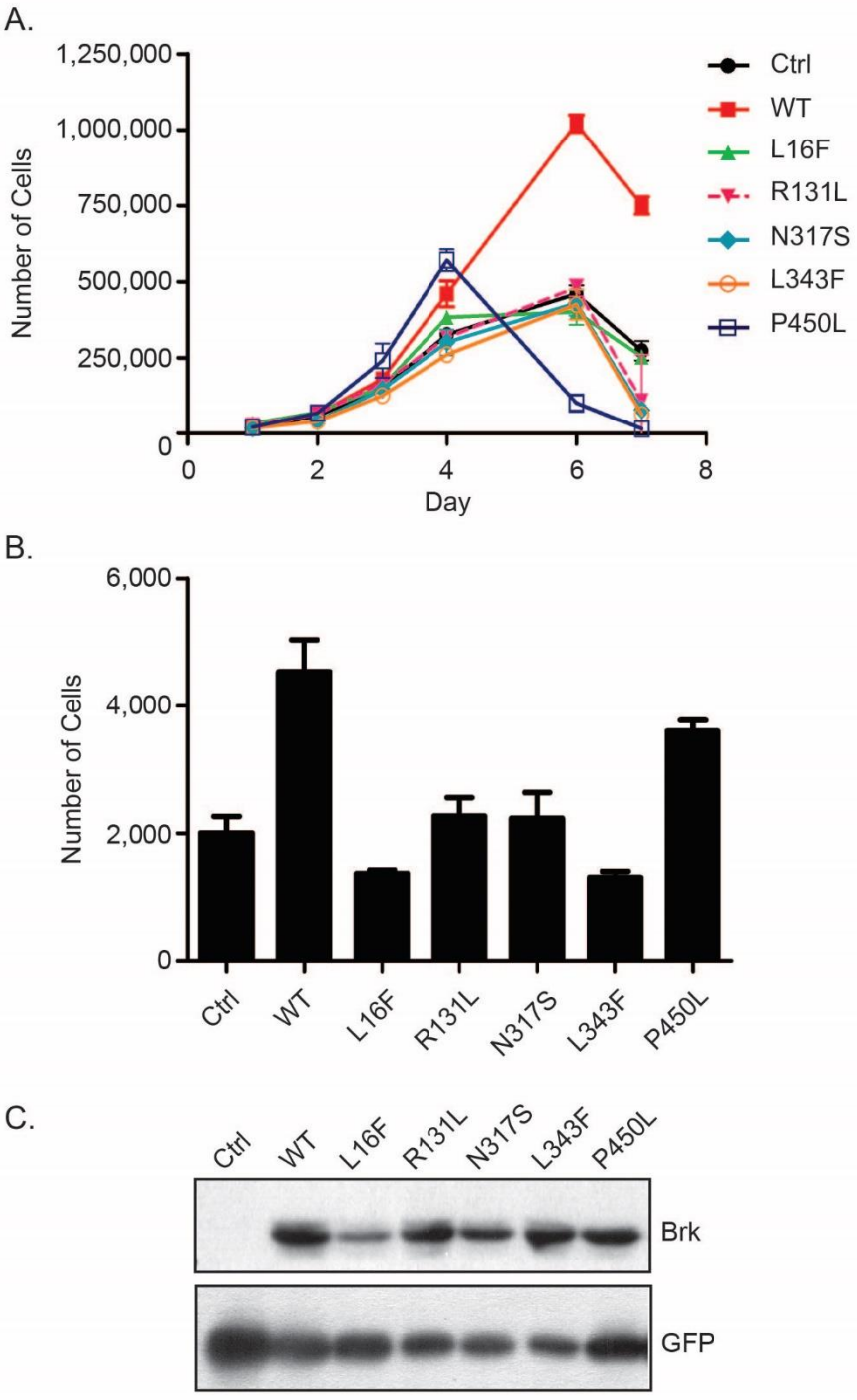

A.
1,250,000
1,000,000
750,000
500,000
250,000
0
Number of Cells
0
2
4
6
8
Day
Ctrl
WT
L16F
R131L
N317S
L343F
P450L
B.
6,000
4,000
2,000
0
Number of Cells
Ctrl
WT
L16F
R131L
N317S
L343F
P450L
C.
Ctrl
WT
L16F
R131L
N317S
L343F
P450L
Brk
GFP

Figure 3-5 Biological effects of Brk mutants in breast cells. A) MCF-10a cells (20,000) stably expressing wild-type or mutant forms of Brk were plated in 24-well plates in starvation media. 18 hours post-starvation, cells were stimulated with 2.5 ng/ml EGF or were left untreated. Cells were counted on a hemacytometer 6 days after EGF stimulation. The data are representative of three similar experiments. The error bars show standard deviations. *, $P < 0.05$ compared to Brk WT. B) MCF-10a cells (50,000) were plated in a 24-well low attachment plate. On days 3 and 6, the cells were collected and counted using a hemacytometer. The data are representative of three similar experiments. The error bars show standard deviations. *, $P < 0.05$ compared to Brk WT. C) MCF-10a cells (100,000) were serum starved for 24 hours then were plated in Transwell inserts of a 24-well plate. Media with or without 15 ng/mL EGF was added to the bottom chamber. After 8 hours, migrated cells were fixed then stained with DAPI, and imaged with a fluorescent microscope and counted with Image J software. The number of cells is the average of cells counted in 5 fields per well. The error bars show standard deviations. D) SKBr3 cells (20,000) stably expressing wild-type or mutant forms of Brk were plated in 24-well plates. Cells were counted on a hemacytometer on days 4, 8, 12, and 16. E) Stable cells (20,000) were plated in a 24-well low attachment plate. On day 14, the cells were collected and counted using a hemacytometer. The data are an average of two experiments. The error bars show standard deviations. *, $P < 0.05$ compared to Brk WT.

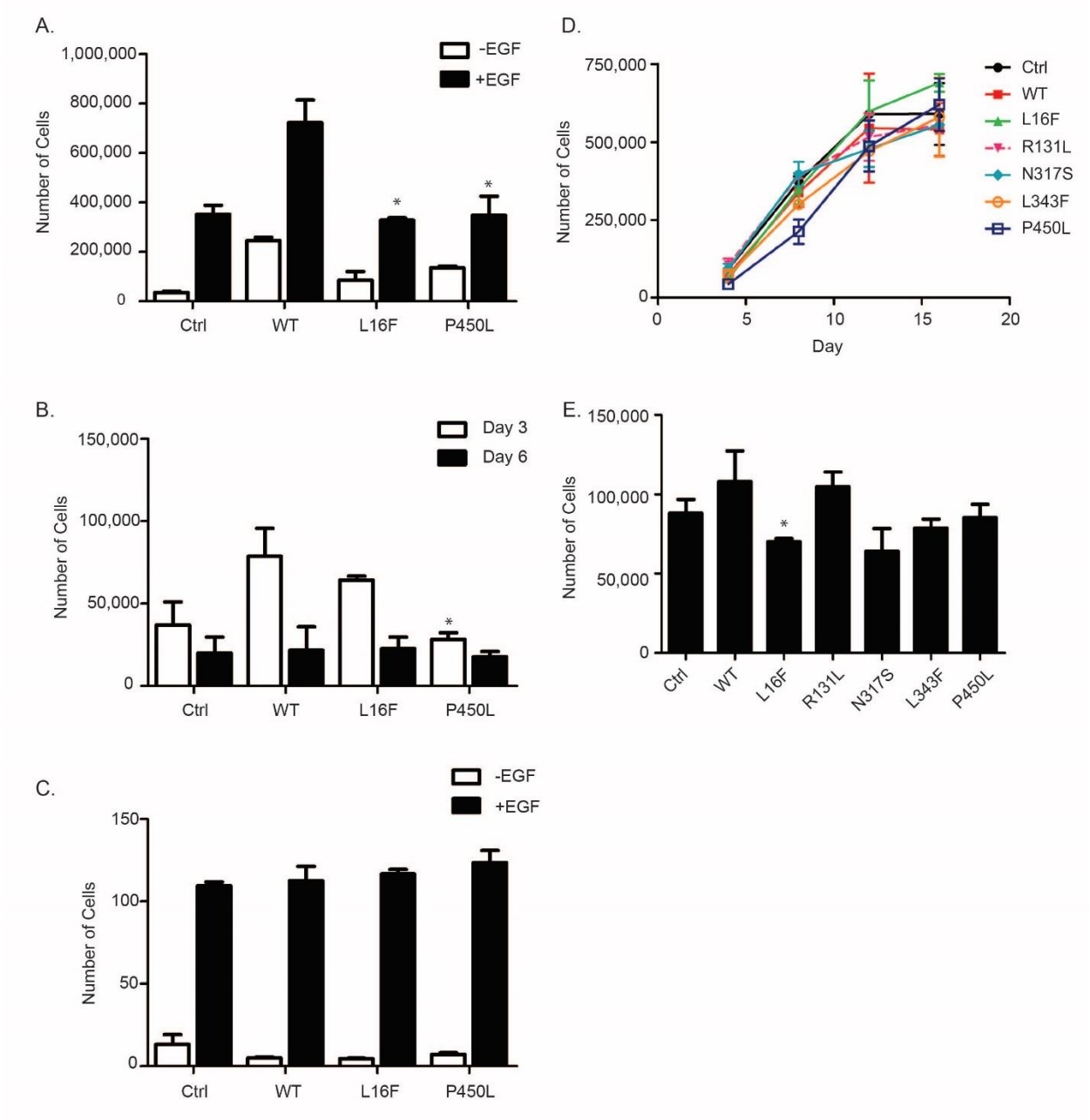

A.
1,000,000
800,000
600,000
400,000
200,000
0
Number of Cells
-EGF
+EGF
*
*
Ctrl
WT
L16F
P450L
D.
750,000
500,000
250,000
0
Number of Cells
0
5
10
15
20
Day
Ctrl
WT
L16F
R131L
N317S
L343F
P450L
B.
150,000
100,000
50,000
0
Number of Cells
Day 3
Day 6
*
Ctrl
WT
L16F
P450L
E.
150,000
100,000
50,000
0
Number of Cells
*
Ctrl
WT
L16F
R131L
N317S
L343F
P450L
C.
150
100
50
0
Number of Cells
-EGF
+EGF
Ctrl
WT
L16F
P450L

Figure 3-6 Effect of Brk mutants on signaling pathways. A) 293T cells co-expressing ErbB2 and wild-type or mutant forms of Brk were incubated with anti-ErbB2 antibody. Immunoprecipitated protein was eluted with SDS-PAGE sample buffer and probed with anti-ErbB2 and anti-Flag antibodies. B) 293T cells expressing wild-type or mutant forms of Brk were probed with anti-phosphoAkt (Ser473), anti-Akt, anti-phospho Erk1/2 (Thr202/Tyr204), anti-Erk 1/2, and anti-Flag antibodies. C) 293T cells were stimulated with 10ng/ml EGF for 2.5 minutes following overnight serum starvation. Lysates were probed with anti-phospho Erk1/2 (Thr202/Tyr204), anti-Erk 1/2, and anti-Flag antibodies. D) SkBr3 cells stably expressing wild-type or mutant forms of Brk were probed with anti-phosphoAkt (Ser473), anti-Akt, anti-phospho Erk1/2 (Thr202/Tyr204), anti-Erk 1/2, anti-Brk, and anti-GFP antibodies. E) Lysates from 293T cells expressing both p130Cas and wild-type or mutant forms of Brk were probed with anti-phospho p130Cas (Tyr 249), anti-p130Cas, and anti-Flag antibodies. F) 293T cells expressing wild-type or mutant forms of Brk were probed with anti-phosphoSTAT3 (Tyr 507), anti-STAT3, and anti-Flag antibodies. G) Endogenous Sam 68 protein was immunoprecipitated from 293T cells expressing wild-type or mutant forms of Brk using anti-Sam68 antibody. Immunoprecipitated proteins were eluted with SDS-PAGE sample buffer and probed with anti-phosphotyrosine, anti-Sam 68, and anti-Flag antibodies. H) β-Catenin was immunoprecipitated from 293T cells expressing β-catenin and wild-type or mutant forms of Brk. Immunoprecipitated proteins were eluted with SDS-PAGE sample buffer and probed with an anti-phosphotyrosine, anti-β-catenin, and anti-Flag antibodies.

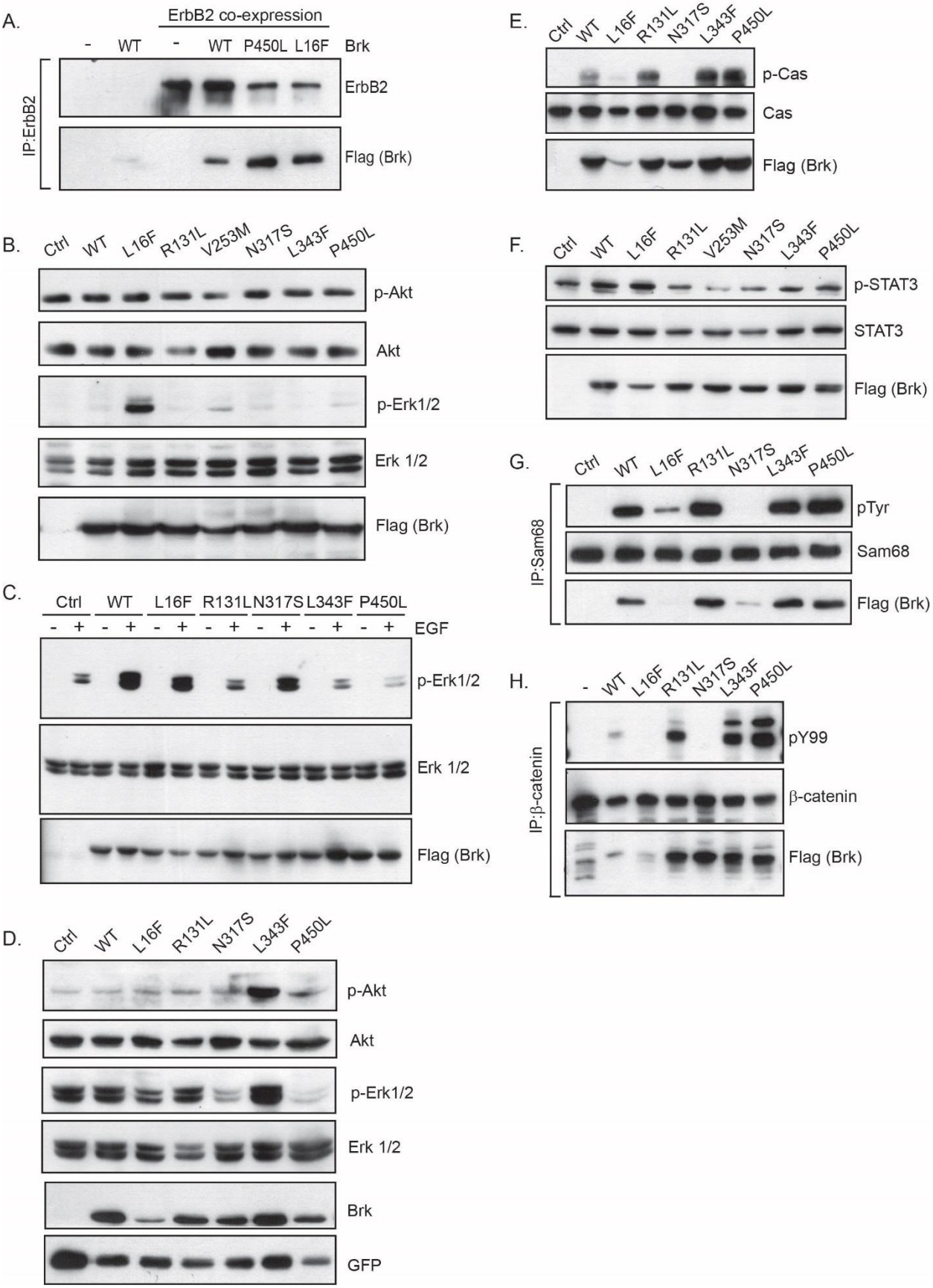

A.
ErbB2 co-expression
- WT - WT P450L L16F Brk
IP:ErbB2
ErbB2
Flag (Brk)
B.
Ctrl WT L16F R131L V253M N317S L343F P450L
p-Akt
Akt
p-Erk1/2
Erk 1/2
Flag (Brk)
C.
Ctrl WT L16F R131L N317S L343F P450L
- + - + - + - + - + - + EGF
p-Erk1/2
Erk 1/2
Flag (Brk)
D.
Ctrl WT L16F R131L N317S L343F P450L
p-Akt
Akt
p-Erk1/2
Erk 1/2
Brk
GFP
E.
Ctrl WT L16F R131L N317S L343F P450L
p-Cas
Cas
Flag (Brk)
F.
Ctrl WT L16F R131L V253M N317S L343F P450L
p-STAT3
STAT3
Flag (Brk)
G.
Ctrl WT L16F R131L N317S L343F P450L
IP:Sam68
pTyr
Sam68
Flag (Brk)
H.
- WT L16F R131L N317S L343F P450L
IP:β-catenin
pY99
β-catenin
Flag (Brk)

Figure 3-7 Effect of cancer-associated mutations on localization of Brk. 293T cells transiently transfected with wild-type or mutant forms of Brk were probed with an anti-Brk antibody (left column) and mounted with media containing DAPI (middle column). Merged images are shown in the right column. The cells were imaged at 63x using an Olympus Flow View 1000 confocal microscope.

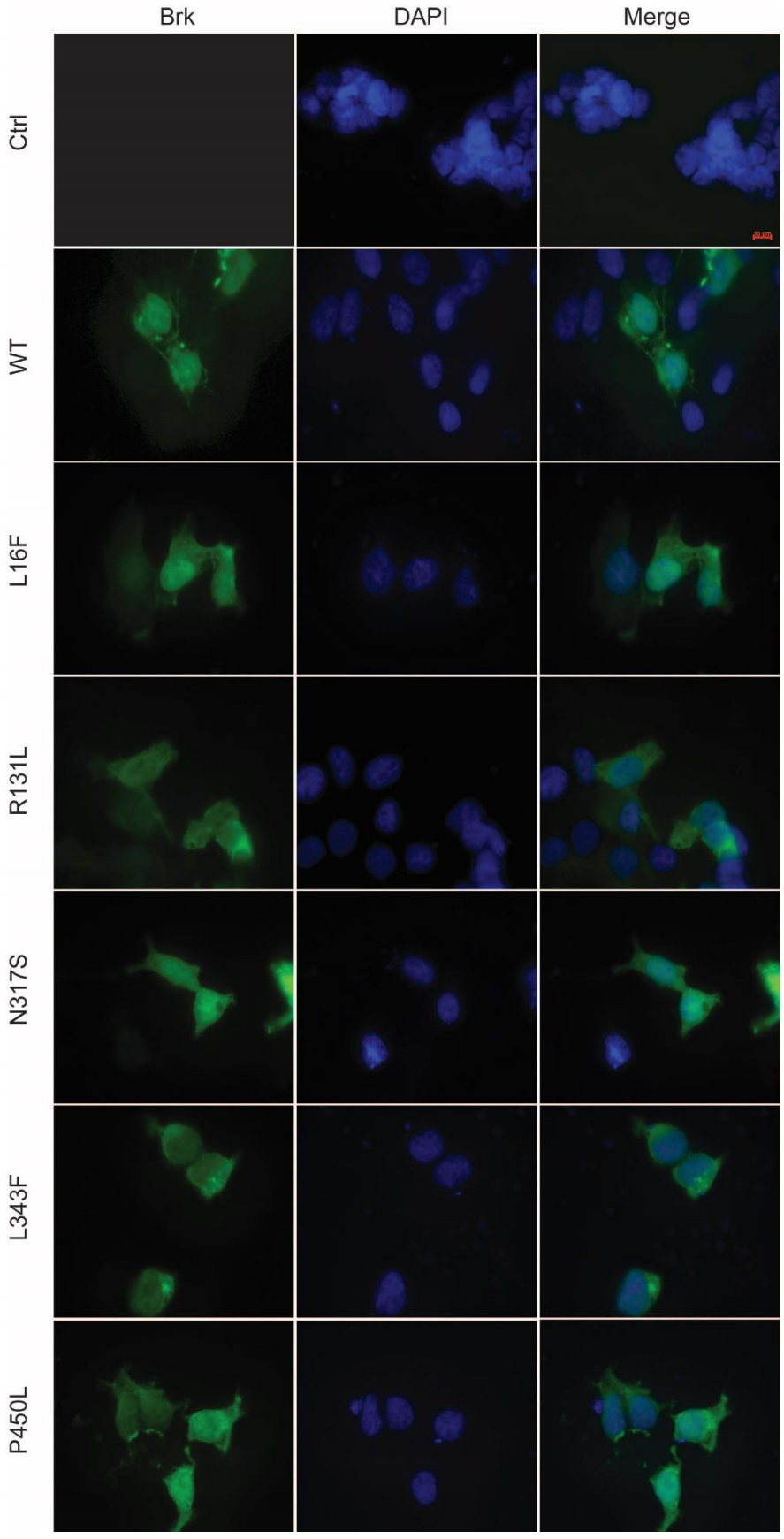

Brk
DAPI
Merge
Ctrl
WT
L16F
R131L
N317S
L343F
P450L

Table 3-1 Summary of mutant effects on Brk autophosphorylation and substrate activation. A minus sign (−) indicates a signal that was less than that of WT. A plus sign (+) indicates a signal that was similar to that of the WT, and increasing numbers of plus signs indicate signals stronger than that of the WT. ND, not determined.

	ctrl	WT	L16F	R131L	N317S	L343F	P450L
autophosphorylation	-	+	+++	+++	-	+++	++
ErbB2 Interaction	-	+	+++	ND	ND	ND	+++
p-Akt	+	+	+	+	+	+	+
p-Erk 1/2	+	+	+++	+	+	+	+
p-Erk 1/2 +EGF	-	+	+	-	+	-	-
p-Cas	-	+	-	++	-	+++	+++
p-STAT3	-	+	+	-	-	+	+
p-Sam68	-	+	-	++	-	++	++
p-β-Catenin	-	+	-	++	-	+++	+++

Chapter 4

Cancer-associated mutations in Hematopoietic Cell Kinase

Abstract

Hck (hematopoietic cell kinase) is a non-receptor tyrosine kinase that is a member of the Src kinase family. Expression of Hck is limited to myeloid cells, where it is involved in phagocytosis, adhesion, and migration. Studies have shown that, like many other tyrosine kinases, Hck is also involved in cancer. Hck mediates Bcr-Abl signaling in CML, and overexpression of Hck confers resistance to imatinib treatment. Recently, somatic mutations have been identified in the Hck gene in various cancers. The effect of these mutations on Hck enzymatic activity has not been examined. Here, we analyzed a panel of cancer-associated mutations located with the kinase domain of Hck. We found that all of the mutations decreased autophosphorylation of Hck *in vitro* and when expressed in mammalian cells. Two of the mutations (D378G and A392T) inhibited Hck mediated phosphorylation of WASP, a known substrate of Hck, while the T270M mutant is still able to facilitate WASP phosphorylation. The presence of somatic mutations that inhibit Hck enzymatic activity may have a growth-suppressive function.

Introduction

Hck is a non-receptor tyrosine kinase that is expressed in hematopoietic cells. It is involved in several processes within the cell that are important for immune function. Hck enhances production of cytokines such as tumor necrosis factor (TNF) and IL-6 downstream of lipopolysaccharide (LPS) induced activation of toll-like receptor 4 (TLR4)[123]. Hck is also important in phagocytosis of antibody coated pathogens mediated by the Fc-gamma receptor (FcγR)[124]. Previous work in our laboratory have identified several substrates for Hck including the eukaryotic engulfment and cell motility (ELMO) and Wiskott-Aldrich syndrome proteins (WASP), both of which are involved in regulation of actin rearrangement in cell migration[82, 125].

Overexpression of Hck has been implicated in CML where it interacts with Bcr-Abl to promote STAT5 activation and cell growth[85-87]. Hck has also been found to be overexpressed in gastric cancers and other solid tumors[89, 126]. As expression of Hck is limited to myeloid cells, the role of Hck in solid tumors is unclear. Given the role Hck plays in immune function and cell migration, Hck is thought to contribute to cancer progression as part of the tumor microenvironment, which is often infiltrated with immune cells[89]. Increasing studies have shown that immune cells in the tumor microenvironment can contribute to cell proliferation, angiogenesis, and metastasis[74].

Over 100 somatic mutations to the Hck gene have been reported to the COSMIC (Catalogue of Somatic Mutations in Cancer) database. The effects of these mutations on Hck activity have not been examined. Here, we have examined a panel of Hck somatic mutations, all of which are located in the kinase domain (Fig. 4-1). The T270M mutation was identified in a screen of B-cell lymphomas[127]. This mutation is adjacent to K269, a conserved amino acid

essential for coordinating ATP in the kinase domain. The E389K mutant was identified in ovarian and colon cancers[128, 129], and the A392T mutant was identified in lung adenocarcinoma[130]. The latter two mutations are located near Tyr390, the major autophosphorylation site involved in kinase activation. The D378G mutant was identified in ovarian and colon cancers[128, 129]. This mutation alters the aspartate residue that is part of the 'DFG' motif located at the beginning of the activation loop. This aspartate coordinates the Mg^{2+} ion that is essential for catalytic activity. In addition, the residues to which these mutations occur are highly conserved among the Src kinase family (Fig. 4-1). Several of these mutations also occur to analogous residues on other Src kinases, for example, the analogous residue to A392 of Hck is also mutated in Src, Lck, and Yes. The conservation of these residues and recurrence of the mutants on other Src kinases suggests that these residues may be crucial for proper function of Hck.

We showed that all of the mutations decrease autophosphorylation of the Hck kinase domain and of full-length Hck when expressed in mammalian cells. In addition, we showed that two of the mutations (D378G and A392T) inhibit phosphorylation of the Hck substrate WASP. Unexpectedly, the T270M mutant increases phosphorylation of WASP. Over expression of Hck has been shown to contribute to the development of cancer. The presence of inactivating mutations to Hck could potentially serve a tumor suppressor function.

Results

Cancer-associated mutations decrease Hck kinase activity *in vitro*. We introduced the different cancer-associated mutations into bacterial expression vectors encoding the kinase domain of Hck. Wild-type and mutant forms of Hck were expressed and purified from BL21 (DE3) *E. coli* cells. We measured the activities of wild-type and mutant forms of Hck against the Src substrate peptide in the presence of [γ^{32}-P] ATP (Fig. 2-2a). Both the T270M and A392 mutants had significantly decreased Hck kinase activity compared to wild-type Hck, while the D378G mutation completely eliminated Hck activity. The E389K mutant also showed a decrease in activity, but not to the same extent as the other mutants.

We also measured the effect of these mutations on autophosphorylation of Hck. We treated purified proteins with YOPH, then incubated with ATP for the indicated times (Fig. 4-2b). We analyzed tyrosine phosphorylation using a general phosphotyrosine antibody. The T270M, E389K, and A392T mutations all decreased Hck autophosphorylation, while no measurable levels of autophosphorylation was detectable for the D378G mutant. These results showed that the decreases in Hck activity towards exogenous substrates (Fig. 4-2a) were due to impaired Hck autophosphorylation.

Cancer-associated affect Hck activity in cells. To measure the effect of the cancer-associated mutations on Hck activity in cells, we transiently expressed wild-type and mutant forms of full-length Hck in 293T cells. We measured phosphorylation of Hck using a general phosphotyrosine antibody (Fig. 4-3a). The A392T mutant significantly decreased phosphorylation of Hck, and consistent with the *in vitro* assays, the D378G mutant completely inhibited phosphorylation of

Hck. The T270M mutation only showed a small decrease in phosphorylation compared to wild-type Hck.

The WASP protein was previously identified in our laboratory to be a substrate of Hck. We examined the effect of the Hck mutations on phosphorylation of WASP in 293T cells. We co-expressed wild-type or mutant Hck with GFP-tagged WASP. We measured phosphorylation of WASP was using an antibody specific for pY256, the phosphorylation site for WASP protein (Fig. 4-3b). The D378G mutation completely inhibits Hck mediated phosphorylation of WASP, and the A392T mutation decreases phosphorylation of WASP by Hck. However, the T270M mutant, which decreased phosphorylation of Hck *in vitro* and to a lesser extent in cells, showed greater phosphorylation of WASP compared to wild-type Hck.

Discussion

The role of Hck in cancer has been described in CML, where Hck interacts with Bcr-Abl to enhance activation of signaling pathways important for cell growth. Furthermore, expression of Hck in these cells leads to drug resistance. While Hck overexpression has also been detected in solid tumors, its role in mediating cancer growth in these cells is just beginning to be uncovered. Increasing evidence shows that the tumor microenvironment, in which immune cells are often present, plays an important part in cancer progression through its pro- and anti-inflammatory effects and through its role in migration. Given its role in cell migration and in eliciting an immune response, Hck may well play an important role in cancer as part of the tumor microenvironment.

A study by Reimand et. al. correlated somatic mutations in predicted functional regions of signaling proteins with clinical outcomes. By this method, they identified the Hck E389K mutation, and hypothesized that this mutation, which may affect autophosphorylation of the adjacent tyrosine, inactivates Hck activity to increase patient survival. The functional impact of the E389K and other Hck mutations have not been validated. In this study, we have shown that the T270M, D378G, E389K, and A392T mutants all decrease autophosphorylation of Hck to varying degrees. The impact of these mutations on Hck activity was further corroborated by measuring phosphorylation of the Hck substrate, WASP.

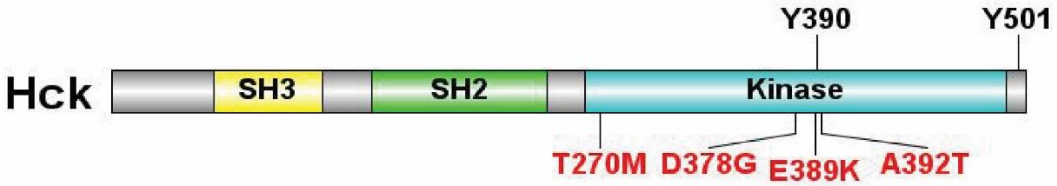
The D378G mutation, which alters the Asp residue within the DFG motif that is essential for Hck catalytic activity, had the greatest impact on Hck activity. The presence of this mutation completely inhibited autophosphorylation and activity of the kinase domain. This was also observed in cells, as the D378G mutant inhibits phosphorylation of both Hck and its substrate

WASP. The A392T mutant also decreased Hck activity, but not to the same extent as the D378G mutant. The T270M mutant decreased autophosphorylation of the kinase domain and activity against the substrate peptide. However, its impact on Hck phosphorylation in cells was not as significant; T270M is able to phosphorylate WASP to a greater extent than wild-type Hck. Given the effect of these mutations on phosphorylation of substrates such as WASP, it is likely that the mutations will have an effect on cell migration.

Taken together, the impact of these mutations on Hck activity suggests that Hck mediated cell functions such as migration may be inhibited. This may occur in a dominant negative manner by which Hck is able to interact with WASP but is not able to mediate its phosphorylation. Loss of function mutations to proteins such as p53 work in a similar manner, whereby mutant p53 proteins forms complexes with the wild-type protein to impair transcriptional activity[131]. While the T270M mutant affected Hck autophosphorylation, it was able to activate WASP, so unlike the other mutants examined, may have an oncogenic role. Clearly, additional studies are required to gain a more complete picture of the mechanism by which these mutations affect cell growth.

Figure 4-1 Hck cancer-associated mutations. A) The domain arrangement of Hck is shown schematically. The autophosphorylation site (Tyr 390) and inhibitory tyrosine (Tyr 501) are in black. The cancer-associated mutations are in red. The amino acid numbering follows that of the human p59Hck isoform. B) Sequence alignment to Src family kinases. The SH3, SH2, and kinase domains are highlighted in yellow, green, and blue, respectively. Important residues are noted: K269 (black asterisk), DFG motif (black bar), and Y390 (blue asterisk). Red asterisks denote Hck cancer-associated mutations.

A.


Y390
Y501
Hck
SH3
SH2
Kinase
T270M
D378G
E389K
A392T

B.

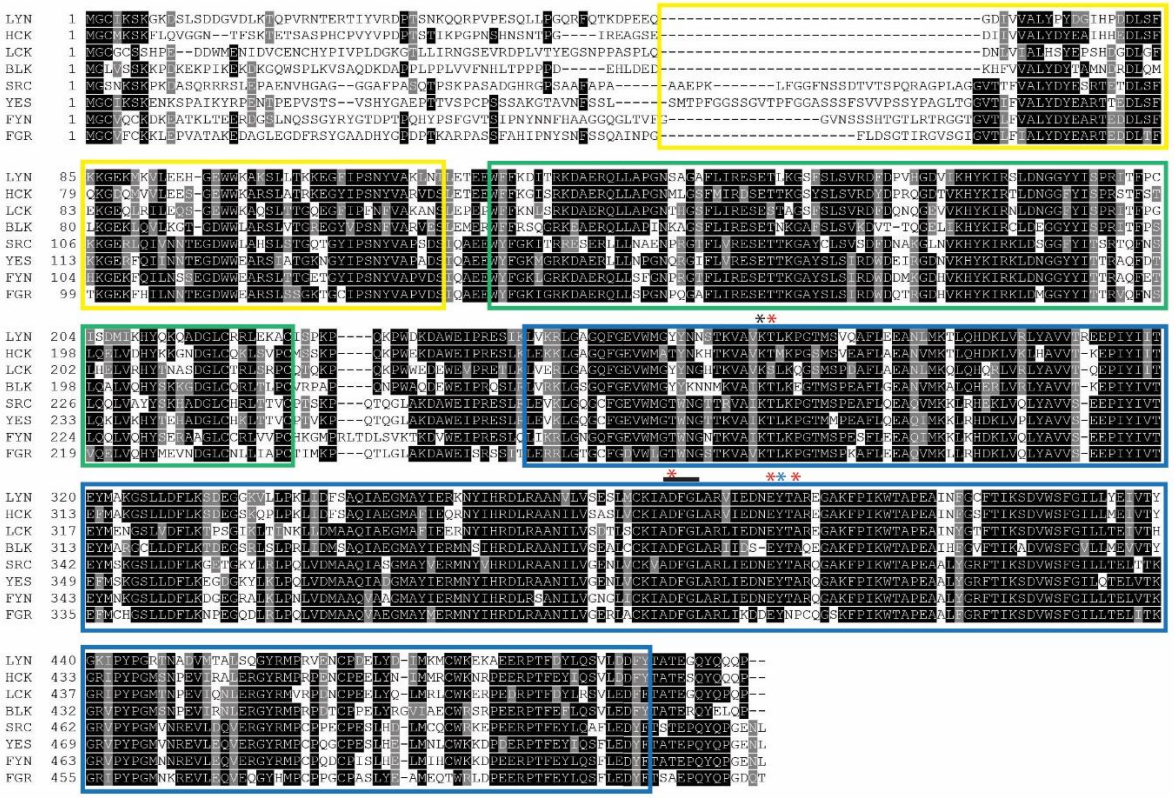
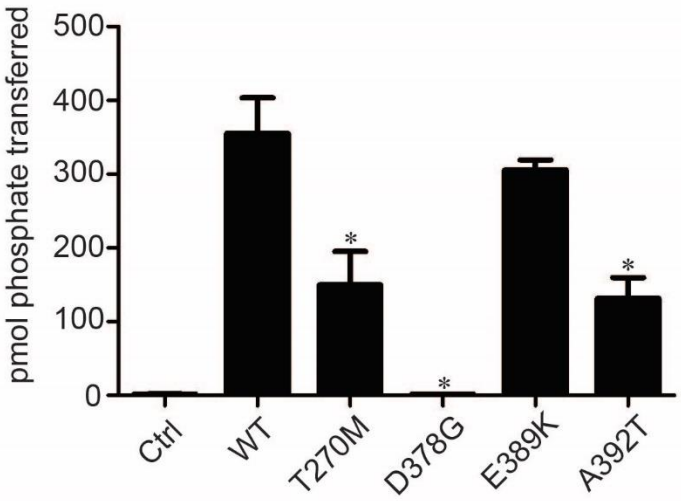

LYN
HCK
LCK
BLK
SRC
YES
FYN
FGR

Figure 4-2 Hck cancer-associated mutations affect autophosphorylation of Hck *in vitro.* A) Purified wild-type and mutant forms of the Hck kinase domain were incubated with Src-substrate peptide in the presence of [γ32-P] ATP. The activities were measured in duplicate with the phosphocellulose paper assay. The results are representative of two experiments. The error bars show standard deviations. *P< 0.05 compared to Hck WT. B) Purified wild-type and mutant forms of Hck kinase domain were treated with YOPH phosphatase then incubated with ATP for the indicated amount of time. Protein with and without ATP incubation were resolved by SDS-PAGE and probed with a general phosphotyrosine antibody.

A.


pmol phosphate transferred
500
400
300
200
100
0
*
*
*
Ctrl
WT
T270M
D378G
E389K
A392T

B.

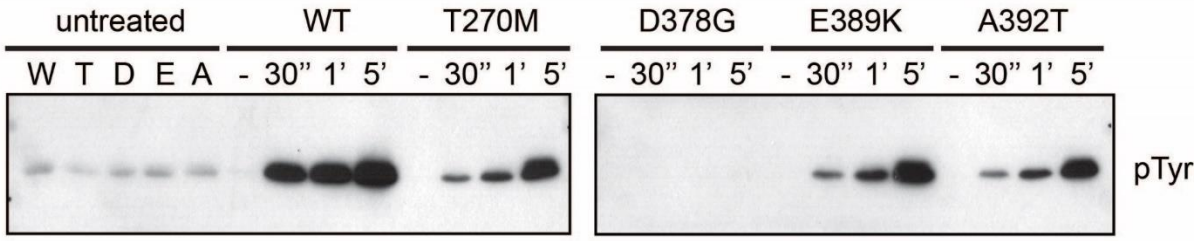
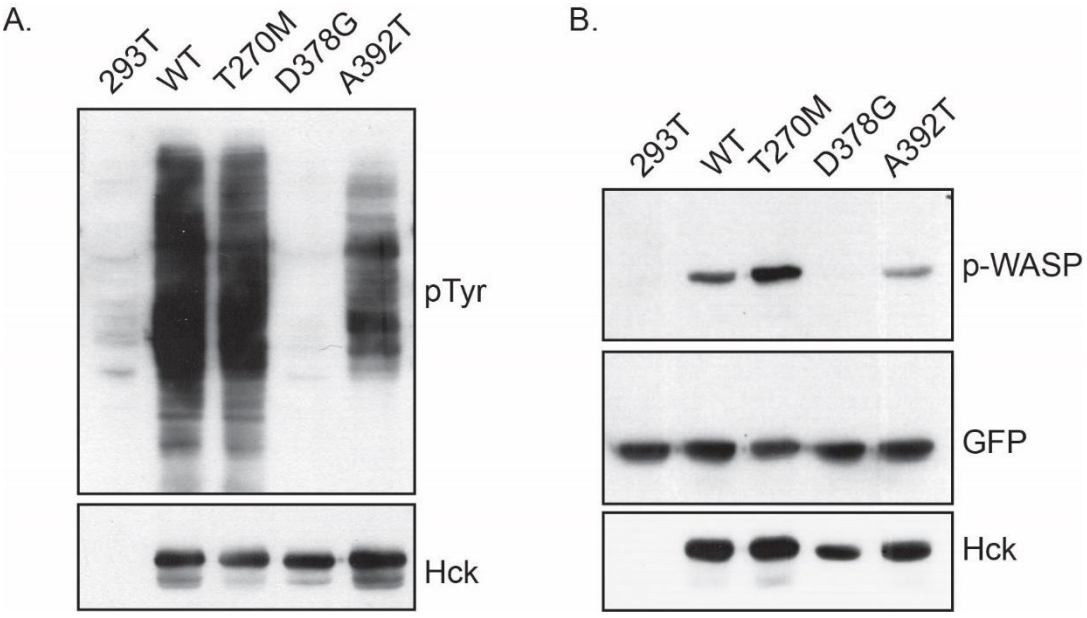

untreated
WT
T270M
D378G
E389K
A392T
W T D E A
- 30" 1' 5'
- 30" 1' 5'
- 30" 1' 5'
- 30" 1' 5'
- 30" 1' 5'
pTyr

Figure 4-3 Hck cancer-associated mutations affect Hck activity in cells. A) Lysates from 293T cells expressing full-length wild-type or mutant forms of Brk were probed with anti-phosphotyrosine, and anti-Hck antibodies. The gel is representative of three similar experiments. B) Lysates from 293T cells expressing both Wasp-GFP and wild-type or mutant forms of Brk were probed with anti-phospho Wasp (Tyr 256), anti-GFP, and anti-Hck antibodies.


A.
293T
WT
T270M
D378G
A392T
pTyr
Hck
B.
293T
WT
T270M
D378G
A392T
p-WASP
GFP
Hck

Chapter 5

Concluding discussion and future directions

Discussion

The work presented in this thesis describes for the first time the effect of cancer-associated mutations on the non-receptor tyrosine kinases Brk and Hck. We show that the mutations can differentially affect kinase activity and alter the recognition and activation of its substrates.

In Chapter 3, we describe the effects of a panel of somatic mutations to Brk. The L16F, R131L, L343F, and P450L all activated Brk, while the V253M and N317S mutant did not. We showed through peptide binding assays that the P450L mutant activates the kinase by interfering with inhibitory interactions between the SH2 domain and C-terminal tail. In addition, the L16F mutant decreases affinity for SH3 ligands, which may interfere with recognition and interaction with Brk substrates.

We showed that while some of the Brk mutants led to kinase activation, the increase in Brk activity did not translate to increased cell growth, transformation, or migration. Our results suggest that this is due to altered recognition and activation of substrates and signaling pathways by the Brk mutants. Overexpression of Brk leads to activation of various signaling pathways. However, activation of these pathways is dependent on the stimulating factor. For example, activation of Brk by ErbB2 dimerization leads to activation of Erk1/2 but not Akt[66]. Additionally, Brk was shown to mediate activation of Erk5 and p38MAPK downstream of heregulin, but not Akt and Erk1/2[58]. Thus, the function of Brk mutants may be dependent on cell type and the set of signaling pathways activated in those cells.

A tumor-suppressor function has recently been demonstrated for Brk. Studies published by two groups showed that Brk is down-regulated in esophageal squamous cell carcinoma

(ESCC), and lower expression of Brk in these cells is correlated with poor survival[119, 120]. Low expression of Brk was also shown to be correlated with poor outcome in laryngeal cancer[121]. In ESCC, decreased expression of Brk resulted in increased β-catenin activity[119]. Activation of β-catenin downstream of the Wnt signaling pathway has been shown to contribute to cancer growth. We showed that the R131L, L343F, and P450L mutants all increase phosphorylation of β-catenin, which has previously been shown to inhibit its transcriptional activity[115]. Increased phosphorylation of Sam68 was also observed. Phosphorylation of Sam68 by Brk inhibits RNA binding. Sam68 is thought to contribute to tumorigenesis via its splicing functions[111]. Thus, while the R131L, L343F, and P450L mutants activated Brk, they do not function as drivers of oncogenic growth as they were incapable of activating the MAPK and Akt signaling pathways to promote cell growth, and their increased activity may inhibit the oncogenic functions of substrates such as β-catenin and Sam68.

Two of the mutations we examined (V253M, N317S) eliminated Brk activity. However, phosphorylation of Brk substrates such as Akt is still evident, which suggests that Brk has a kinase-independent function. This is consistent with previous observations that Brk can promote cell growth and affect substrate function in a kinase-independent manner[103, 115]. The Brk inactivating mutants were also shown to decrease phosphorylation of Brk substrates such as STAT3 and β-catenin, although interaction of the N317S mutant with β-catenin is not affected. These results suggest that cancer-associated mutations may alter Brk signaling in a dominant-negative manner.

We have demonstrated in our studies that cancer-associated mutations alter Brk activity and substrate activation and ultimately affect both Brk pro- and anti-tumorigenic functions. The mechanisms that dictate whether Brk acts as an oncogene to promote cell growth or as a tumor

suppressor are unclear. Evidence from other studies suggests that localization of Brk may regulate its function by restricting the range of substrates Brk can activate. In prostate cells, localization of Brk at the cell membrane was correlated with more aggressive forms of the cancer[62]. Additionally, artificially targeting of Brk to the cytoplasm with a myristoylation signal led to increased cell proliferation, migration, and survival[63]. We examined localization of the different Brk mutants by immunofluorescence but did not observe any shifts in nuclear or cytoplasmic expression of Brk. Altered Brk localization has only been observed in prostate cells, therefore, this mechanism of Brk regulation may be dependent on cell type.

As a cancer develops, many mutations are accumulated and the set of mutations that drive cancer growth may differ between cancer types. Previous studies have shown that growth promoting functions of cancer-associated mutations may require the presence of other cancer-related genes[36]. For example, the microphthalmia-associated transcription factor (MITF) is only able to affect cell proliferation of melanoma cells when expressed with the BRAF V600E mutant, which is often present in melanomas[132]. We analyzed sequencing data from the COSMIC database to identify additional somatic mutations present in the tissues from which the Brk mutants were discovered. Some of the mutations such as L343F were among hundreds of other somatic mutations identified in the tissue sample. On the other hand, the L16F mutant was only one of two mutations present in the sample. We compared the mutations present in the tissue samples and discovered that mutations to the p53 tumor suppressor were also present in samples that contained the R131L, N317S, and L343F mutants. A relationship between Brk and p53 has not been established. It would be interesting to see if presence of mutant p53 alters the function of Brk mutants in cells.

In Chapter 4, we performed a similar analysis on Hck somatic mutations. All of the Hck mutants we examined decreased activity of Hck. The T270M mutant decreased Hck autophosphorylation *in vitro* and in cells, but with the least effect compared to the other mutants. Interestingly, the T270M mutant increased phosphorylation of WASP.

Given the differing effects of the Hck mutants on activation of WASP it would be interesting to examine the effect of these mutations on cell migration. Expression of WT Hck promotes migration of cells by regulating actin rearrangement through activation of substrates such as WASP. We would expect the D378G and A392T mutants to inhibit cell migration, but the T270M mutant to increase cell migration compared to wild-type Hck. These studies are ongoing.

As described in Chapter 4, Reimand et. al. developed a computational approach to determine if interaction of a mutated phosphoprotein with other proteins in its signaling network is correlated with a clinical outcomes such as patient survival. Through this method they determined that the Hck E389K mutant correlated with increased survival in ovarian cancer, and hypothesized that inactivation of Hck signaling by this mutant increased patient survival. Our results confirms that the E389K mutant does lead to decreased Hck activity. Additionally, our work shows that the inactivating effect of Hck mutants may inhibit tumor growth by preventing phosphorylation of Hck substrates such as WASP. Whether these mutants also affect other Hck partners, such as Bcr-Abl, remains to be seen. Loss of Hck activity could interfere with Bcr-Abl signaling in CML.

Hck is over expressed in CML, however, a decreased level of Hck is observed in acute promyeolytic leukemia and in Bcr-Abl negative CML[89]. Interaction of Hck with the guanine

nucleotide exchange factor, C3G, has been shown to promote apoptosis[133]. Thus, inactivation of Hck could facilitate tumorigenesis by inhibiting apoptosis and increasing cell survival.

Whether Hck inactivating mutations promote or inhibit tumor growth may depend on the set of substrates Hck is able to activate. The set of substrates that Hck can interact with may depend on cell type given that Hck may contribute to tumorigenesis by different mechanisms in different cancers. While Hck influences signaling mediated by Bcr-Abl in myelogenous cells, Hck is thought to contribute to growth of solid tumors as part of the tumor microenvironment potentially by promoting matrix degradation to influence metastasis of the tumor. Therefore, careful consideration must be made in which cell type is chosen when exploring the effect of Hck mutants on cancer development.

Taken together, the work presented in this thesis highlights the importance of functional validation of somatic mutations. While many of the Brk mutants activated the kinase, we did not observe increases in cell growth or transformation as expected. Our results suggest that this is due to the altered effect on substrate recognition and signaling that promote both pro- and anti-tumorigenic signals. This may depend on the collection of, or balance of signaling molecules present in the cells from which the mutations arise. For example, Brk enhances signaling downstream of ErbB2 receptors to promote cell growth. Thus, Brk mutants may function differently in triple negative breast cancers, which lack estrogen, progesterone, and ErbB2 receptors compared to cells cancers overexpress ErbB2.

In addition, the association of somatic mutations with functional regions of the protein (such as phosphorylation sites) does not always accurately predict the effect of these mutations on kinase activity. Sequence conservation is often utilized as part of computational algorithms in

identifying important mutations. The L343F mutant in Brk and the E389K mutant in Hck are both located adjacent to the activating tyrosine in the catalytic domain of each kinase; while the L343F mutant activated Brk, the E389K mutant decreased Hck activity. Important strides have been made in the effort identify driver mutations present in various cancers, and several methods have been developed to parse through the dense genetic information. However, biological experimentation is still essential to validate mutations as drivers of oncogenic disease.

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