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Characterization of Oncogenic Fusion Protein BCR-FGFR1

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in

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By

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The thesis of Malalage Nicole Peiris is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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2019

DEDICATION

This thesis is dedicated to my parents, Swarnakanthie Peiris who passed away in 2005 after a hard battle with cancer, and my father, Malalage Chandrawansa Peiris. Although the memory of my mother lives on, this work is a constant reminder to never give up hope in finding a cure for cancer. I would especially also like to thank my father for his constant support and his push for me to develop my critical thinking skills. Tatha, I'll always remember our conversations in the car when you would drive me to school in the mornings and would talk about a variety of topics always including politics, religion, and science. Thank you for encouraging me to think outside the box, and to consider other points of view. I'd like to think that I first developed my research skills through those conversations, and for that I'll be forever grateful. I'm lucky and thankful to have had amazing parents who were not only supportive of my endeavors, but also enthusiastic about my work.

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ABSTRACT OF THE THESIS

Characterization of Oncogenic Fusion Protein BCR-FGFR1

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Fibroblast Growth Factor Receptors (FGFRs) are part of the Receptor Tyrosine Kinase (RTK) family and are essential in the activation of various downstream signaling pathways, which are necessary for cell differentiation and proliferation. However, mutation and translocation of FGFRs leads to aberrant activation of signaling, which often results in cancer. With the emergence of personalized medicine, cancer genome sequencing is vital in order to determine the appropriate therapies for patients. This work focuses on the t(8;22)(p11;q11) chromosomal translocation, which results in the fusion protein Breakpoint Cluster Region (BCR)-FGFR1 (BCR-FGFR1). BCR-FGFR1 is poorly characterized, resulting in few therapies and clinical advancements for patients positive for this fusion protein. This work focuses on the

biochemical and biological characterization of BCR-FGFR1 along with the analysis of therapeutic options. The BCR-FGFR1 fusion shows transformation ability in NIH3T3 cells, and shows heavy activation of MAPK, STAT, and phospho-FGFR1 receptor. Additional phosphorylation sites on BCR-FGFR1 were identified through titanium dioxide based phosphopeptide-enriched Liquid chromatography/ mass spectrometry (LC/MS) analysis. Additionally, BCR contributes a coiled-coil dimerization domain to BCR-FGFR1; the importance of the dimerization domain is shown, as when disrupted, BCR-FGFR1 is unable to retain transforming ability. Lastly, BCR-FGFR1 is shown to be a client of the chaperone protein Hsp90 and is sensitive to Ganetespib (STA-9090), a potent Hsp90 inhibitor suggesting that BCR-FGFR1 relies on the Hsp90 complex to evade proteasomal degradation.

Chapter 1

Receptor Tyrosine Kinases: Translocation Partners in Hematopoietic Disorders

Review

Receptor Tyrosine Kinases: Translocation Partners in Hematopoietic Disorders

Katelyn N. Nelson,[1] Malalage N. Peiris,[1] April N. Meyer,[1] Asma Siari,[2] and Daniel J. Donoghue[1,3,*]

Receptor tyrosine kinases (RTKs) activate various signaling pathways and regulate cellular proliferation, survival, migration, and angiogenesis. Malignant neoplasms often circumvent or subjugate these pathways by promoting RTK overactivation through mutation or chromosomal translocation. RTK translocations create a fusion protein containing a dimerizing partner fused to an RTK kinase domain, resulting in constitutive kinase domain activation, altered RTK cellular localization, upregulation of downstream signaling, and novel pathway activation. While RTK translocations in hematological malignancies are relatively rare, clinical evidence suggests that patients with these genetic abnormalities benefit from RTK-targeted inhibitors. Here, we present a timely review of an exciting field by examining RTK chromosomal translocations in hematological cancers, such as Anaplastic Lymphoma Kinase (ALK), Fibroblast Growth Factor Receptor (FGFR), Platelet-Derived Growth Factor Receptor (PDGFR), REarranged during Transfection (RET), Colony Stimulating Factor 1 Receptor (CSF1R), and Neurotrophic Tyrosine Kinase Receptor Type 3 (NTRK3) fusions, and discuss current therapeutic options.

Trends

RTK overactivation by chro translocation and fusion prote tion can result in aberrant cel and cancer progression.

In hematological cancers, the domains in RTK fusion prot plays the RTK-derived doma C-terminal fusion partner, fu variety of other proteins as the inal fusion partner. This diff RTK fusion proteins in solid which typically display the RT N-terminal fusion partner.

RTK translocations involvir CSF1R, and NTRK3 in patient novel potential therapeutic ta

The use of tyrosine kinase (TKIs) is increasingly effective i patients with hematological driven by RTK fusion Although TKI clinical trials ar lent, the use of TKIs often drug resistance.

Cancer genome sequen becoming increasingly imp the determination of approp administration for patients.

Receptor Tyrosine Kinase Translocations in Cancer

Malignant genetic events can often be sorted in two categories: gene inactivation and gene activation or deregulation. Chromosomal **translocations** (see Glossary) have been detected in all cancer types and account for approximately 20% of all malignant **neoplasms** [1]. Moreover, there is a close correlation between the translocations and the tumor phenotypes in which they occur [1].

Translocations usually arise by multiple erroneous double-stranded breaks (DSB) in chromosomes, which may occur for various reasons. A translocation also relies on the spatial proximity of the DSB and the ability of the damaged region to rearrange in the nucleus, which can allow chromosomes to incorrectly repair [2,3]. These translocations can result in a translatable fusion protein, some of which have oncogenic potential. While the percentage of chromosomal translocations in hematological disorders is generally lower than in solid tumors (1.4% of all hematological cancers), their occurrence is nevertheless significant, especially in diseases such as **chronic myeloid leukemia (CML)**, where 100% of cases harbor the t(9;22)(q34;q11) translocation, resulting in the gene fusion of breakpoint cluster region (*BCR*) and *ABL1*, a nonreceptor tyrosine kinase [1]. CML is a classic example of a translocation-driven disease that is amenable to treatment with a **tyrosine kinase inhibitor (TKI)**. Imatinib, also known as Gleevec, has been widely used to treat CML and diseases presented by some of the fusion proteins discussed in this review. CML treatment with imatinib has ushered in a new era of rational drug development to identify TKIs with therapeutic value.

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Box 1. RTK Fusion Proteins in Hematological Malignancies

Structurally, all RTKs discussed here contain an extracellular ligand-binding domain, transmembrane (TM) domain, and intracellular tyrosine kinase domain. Ligand binding results in dimerization of the receptors, allowing for *trans*-autophosphorylation of cytoplasmic kinase domain tyrosine residues and kinase activation [14,47,120–122] (Figure 1, main text). Within the cell, ALK, FGFRs, and PDGFRs have been implicated in proliferation, survival, and migration. PDGFRs and FGFRs are also important in angiogenesis, inflammation, and wound healing [14,47,123]. However, normal hematopoiesis does not require expression of PDGFR, ALK, or FGFR receptors [11], which makes their overactivated expression by chromosomal translocation even more interesting.

ALK: A Member of the Insulin Receptor Family

Domains: glycine-rich, low-density lipoprotein class A (LDLa) and meprin/A5-protein/PTPmu (MAM) extracellular domains, TM and intracellular tyrosine kinase domains [4,122].

Ligands: recently discovered FAM150B (Augmentor-α, or AUG-α), and FAM150A (AUG-β) [124,125]; additionally, heparin and heparin-binding growth factors Pleiotrophin (PTN) and Midkine (MK) [4,126].

FGFR1 and FGFR3: Members of the FGFR Family

Domains: extracellular immunoglobulin (Ig)-like domains, a TM-spanning segment, and an intracellular tyrosine kinase domain [57].

Ligands: 18 fibroblast growth factors (FGFs) are receptor ligands and require heparin sulfate proteoglycans (HSPGs) [57].

PDGFRα and PDGFRβ: Members of the PDGFR Family

Domains: five immunoglobulin-like extracellular domains, a TM domain, and an intracellular tyrosine kinase domain [47,127].

Ligands: PDGFs (A, B, C, and D); A and B ligands can heterodimerize [47,127].

Fusion proteins in hematopoietic cancers are usually found with an N-terminal protein partner fused to a C-terminal RTK kinase domain (Figure 1, main text) The transcription of such a fusion is dependent on the partner gene promoter. This is strikingly different from most oncogenic RTK fusion proteins identified in solid cancers, which typically contain the RTK domains (extracellular, TM, and intracellular domains) in the N-terminal domain. However, exceptions to this general rule have been observed, as in the case of ALK fusion proteins in solid tumors, where ALK is usually in the C-terminal domain.

The N-terminal domains of fusion proteins in hematopoietic cancers contribute a dimerization domain, allowing the RTK kinase domains to autophosphorylate and become constitutively active. This can result in: (i) a gain-of-function fusion protein, with overstimulation of downstream signaling; (ii) a loss of normal regulatory mechanisms for wildtype (WT) receptors; (iii) abnormal localization of a constitutively activated kinase; and (iv) altered interactions with novel proteins and pathways.

Glossary

32Dcl3 cells: a murine hematopoietic progenitor cell line dependent on the cytokine IL-3 for proliferation.
8p11 myeloproliferative syndrome (EMS): a blood cancer containing the genetic translocation of the *FGFR1* gene, location at position p11 of chromosome 8.
Acute myeloid leukemia (AML): cancer of myeloid cells, most often nonlymphocytic white blood cells, but sometimes red blood cells or megakaryocytes.
Anaplastic large cell lymphomas (ALCL): a non-Hodgkin lymphoma considered to be a subtype of peripheral T cell lymphoma.
B/myeloid mixed phenotype leukemia: a B cell lineage mixed phenotype acute leukemia (MPAL), resulting from the combination of two forms of leukemia.
B9 cells: murine B cell hybridoma line dependent on the cytokine interleukin-6 (IL-6) for growth.
Ba/F3 cell line: a murine IL-3-dependent pro-B cell line frequently used as a model system for determining the oncogenicity of proteins.
Chronic eosinophilic leukemia (CEL): cancer resulting in an overproduction of eosinophils, a type of white blood cell.
Chronic lymphocytic leukemia (CLL): cancer of mature lymphocytes originating in the bone marrow.
Chronic myeloid leukemia (CML): cancer of white blood cells frequently associated with the Philadelphia chromosomal translocation encoding BCR-ABL.
Diffuse large B cell lymphoma (DLBCL): cancer of B cells and the most common type of non-Hodgkin lymphoma.
Fluorescence *in situ* hybridization (FISH) analysis: fluorescent probes that hybridize to specific chromosomal regions allowing their identification; used to determine genetic translocations.
FMS-Like tyrosine Kinase 3 (FLT3) receptor: one of 58 RTKs belonging to the PDGFR superfamily.
Gatekeeper mutation: mutation in the ATP-binding pocket of an RTK, which reduces binding of inhibitors; results in inhibitor-resistant cancers, often arising after initial treatment with a TKI.

In this review, we focus on translocations involving **RTKs** in hematological cancers (Boxes 1–3; Figure 1). Of the 58 known human RTKs, the following have been identified as fusion partners resulting from chromosomal translocations in hematopoietic cancer cells: ALK, FGFR, and PDGFR, RET, CSF1R, and NTRK3. As discussed here, the most common hematopoietic cancer RTK translocations include genes that encode ALK, FGFR, and PDGFR. These translocations result in cancers that present with different proliferative effects and treatment options, highlighting the importance of determining cancer-causing genetic alterations in patients.

ALK Translocations: Fusion Proteins of the Only RTK Named after a Disease

ALK regulation normally occurs by ligand binding to its extracellular domain. ALK expression occurs in the central and peripheral nervous system, primarily during development, as shown in multiple species, including humans [4]. After birth, as found in mouse studies, ALK mRNA and protein levels reach a minimum in all tissues and remain at low levels in adult animals [4]. As such, *Alk*-knockout (KO) mice display only mild behavioral phenotypes and ALK inhibitors appear to be

Box 2. Other RTK Translocations Linked to Hematopoietic Diseases

Although rare, other RTK translocations have been identified in hematopoietic diseases. They exhibit similar characteristics of having a C-terminal RTK fusion partner activated by an N-terminal dimerization domain from another protein. This leads to activation of RTK kinase activity, cellular proliferation, and activation of signaling pathways (Table 1, main text). As more oncogenic chromosomal translocations are identified and molecularly characterized, new inhibitors can potentially be developed for clinical use.

RET is an RTK activated by binding of glial-derived neurotrophic factor (GNDF) ligands, which induce receptor dimerization and tyrosine phosphorylation [119]. Recently, RET was identified in translocations with *FGFR1OP* [t(6;10)(q27;q11)] and *BCR* [t(10;22)(q11;q11)] in chronic myelomonocytic leukemia (CMML) [119].

CSF1R is an RTK activated by colony stimulating factor 1 that controls the production, differentiation, and function of macrophages [128]. In a sample from a patient with acute lymphoblastic leukemia (ALL), the translocation t(1;5)(q21;q33) was identified between *CSF1R* and myocyte enhancer factor 2D (*MEF2D*) [92].

NTRK3 is an RTK activated upon **neurotrophin** binding and signals through the MAPK pathway. *NTRK3* was identified as fused to *ETV6* in AML in the t(12;15)(p13;q25) translocation [98].

Box 3. Dimerization Domains: Partners in Oncogenicity

The contribution of a dimerization domain appears to be essential for the oncogenicity of RTK fusion proteins. Dimerization domains are provided by a partner gene fused to the RTK gene, giving rise to a fusion protein with a constitutively activated kinase domain. A further understanding of RTK fusion proteins and their respective dimerization domains would be beneficial, because inhibition of the dimerizing potential of these domains could serve as a possible therapeutic approach. Below are the dimerization domains that have been identified in the RTK fusions discussed in this review:

Ankyrin protein motif: characterized by approximately 33 amino acids forming two alpha helices separated by loops; common motifs that mediate protein–protein interactions.

Coiled-coil: this domain comprises two to five alpha helices wrapped around each other to form a super coil. A heptad repeat occurs every two turns of this helix.

(Four point one) 4.1 protein Ezrin Radixin Moesin (FERM): this domain comprises three modules located at the N terminus of many membrane-associated proteins, and is responsible for localization to the membrane.

Helix-loop-helix (HLH): this domain is characterized by two alpha helices connected by a loop.

Leucine-rich domain: a Leucine-rich structural motif comprising 20–30 amino acids, which forms an alpha/beta horseshoe fold.

Leucine zipper: a structural motif that comprises approximately 30 amino acids in an alpha helical confirmation with a repetition of Leu residues at every 7th position.

MCM1 Agamous Deficiens SRF (MADS) box: this domain binds to DNA sequences of high similarity to the CArG-box. MADS domain-containing proteins are generally transcription factors.

Phox and Bem1 (PB1): this domain contains two alpha helices and a mixed five-stranded beta sheet. It usually comprises around 80 amino acids and adopts a ubiquitin-like beta grasp fold.

Really Interesting New Gene (RING) finger: a structural domain of zinc finger type, which binds to two zinc cations.

RNA recognition motif: RNA-binding domain of around 90 amino acids. It typically contains four antiparallel beta strands and two alpha helices.

WD-40: a short motif of approximately 40 amino acids, also known as the beta-transducin repeat, terminating in a tryptophan-aspartic (WD) acid dipeptide. Tandem copies of these domains fold together to form a circular solenoid protein domain.

Zinc finger motif: a family of small protein motifs characterized by the coordination of one or more zinc ions.

KIT receptor: one of 58 RTKs, belonging to the PDGFR receptor superfamily.
Leukemia: cancer of blood-forming tissue; different types exist depending on the type of cancerous blood cell.
Lymphoma: cancer of lymphocytes, a type of white blood cell; separable into two main categories: Hodgkin disease and non-Hodgkin lymphoma (NHL).
Multiple myeloma (MM): a cancer of plasma cells, which are antibody-producing white blood cells.
Neoplasm: new or abnormal tissue growth; malignant neoplasms are also referred to as cancer.
Neurotrophin: a member of a family of growth factors that signal cell survival, growth, or differentiation.
Receptor tyrosine kinase (RTK): cell surface receptors with intrinsic tyrosine protein kinase activity; activated by growth factors, hormones, and cytokines (also see Boxes 1 and 2).
T acute lymphoblastic leukemia (T-ALL): cancer of immature lymphocytes in the bone marrow.
Translocation: rearrangement of nonhomologous chromosome parts resulting in the joining of two genes to create a gene fusion.
Tyrosine kinase inhibitors (TKIs): bioactive small molecules that inhibit tyrosine kinase activity (see Table S1 in the supplemental information online).
WW-domain: inhibitory domain in the juxtamembrane domain of PDGFR containing two conserved tryptophan residues.

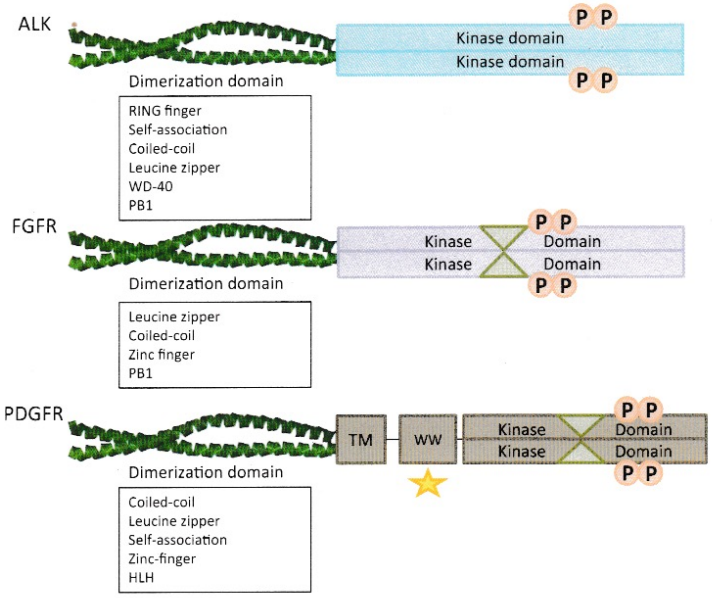

Figure 1. General Structure of Receptor Tyrosine Kinase (RTK) Fusion Proteins. Depicted in the schematic are the Anaplastic Lymphoma Kinase (ALK), Fibroblast Growth Factor Receptor (FGFR), Platelet-Derived Growth Factor Receptor (PDGFR) fusion proteins, showing a generic dimerization domain for each. A star indicates an alternate breakpoint; a triangle indicates a kinase insert domain; TM is a transmembrane domain; WW is a WW-like domain; P is a phosphorylation site. Each of these RTK fusion proteins displays a dimerization domain fused to a C-terminal kinase domain provided by the respective RTK. The dimerization domains commonly associated with each RTK fusion protein are shown in the outlined box. For a complete list of dimerization domains, please see Box 3 (main text).

well tolerated in patients presenting ALK-positive **lymphoma**[4]. ALK was initially identified in a human t(2;5)(p23;q35) translocation, fusing Nucleophosmin (*NPM1*) to *ALK*, expressing the fusion protein NPM-ALK leading to overexpression and constitutive activation of NPM-ALK kinase activity [5]. This fusion protein occurs in 50–60% of **anaplastic large cell lymphomas (ALCLs)** [6]. The two main forms of ALCL are primary cutaneous, affecting the skin, and systemic, and can be divided into ALK-positive and ALK-negative subgroups. ALK fusion-positive ALCL tends to occur in younger patients and has greater disease-free and overall survival rates than ALK fusion-negative ALCL [7].

ALK fusion proteins are a recurring abnormality in ALCL, accounting for 2% of adult non-Hodgkin's lymphomas (NHL) and 13% of pediatric NHL [8]. Some of the N-terminal ALK fusion partners in ALCL include clathrin heavy chain gene (CLTC), nucleophosmin (NPM), tropomyosin 3 (TPM3), TPM4, and tumor necrosis factor (TNF) receptor-associated factor 1 (TRAF1) [4,5,7,9]. A complete list is shown in Table 1. All ALK fusion partners contain dimerization domains in the N-terminal fusion partner fused to the C-terminal ALK kinase domain [4] (Figure 1). While NPM-ALK is the most common translocation, 15–28% of ALK fusion-positive cases display an alternative ALK fusion protein [5]. ALK fusion proteins have also been detected in **diffuse large B cell lymphoma (DLBCL)**, a rare but aggressive B cell lymphoma. The most

Table 1. RTK Fusion Proteins in Hematological Cancers[a]

C-terminal fusion partner	N-terminal fusion partner	Protein name	N-terminal domain	Cancer type	Pathway	Refs
ALK; kinase domain	ATIC	Aminoimidazole Carboxamide Ribonucleotide Transformylase	Self-association	ALCL	STAT	[55]
	CLTC	Clathrin, Heavy Chain	Self-association	ALCL, DLBCL, BPDCN, LBCL	STAT	[80–82]
	MSN	Moesin	FERM	ALCL	TBD	[83]
	MYH9	Myosin, Heavy Chain 9	Coiled-coil	ALCL	TBD	[84]
	NPM	Nucleophosmin	Self-association	ALCL, DLBCL	PI3K, AKT, STAT, JUNB	[4,10,55]
	RANBP2	RAN Binding Protein 2	Leucine zipper	AMMoL, AML, DLBCL	TBD	[85–87]
	RNF213/ALO17	Ring Finger Protein 213/ALK Lymphoma Oligomerization partner on chromosome 17	Ring finger	ALCL	TBD	[80]
	SEC31A	SEC31 Homolog A	WD-40	DLBCL	AKT, MAPK, STAT	[88]
	SQSTM1	Sequestosome 1	PB1	LBCL	STAT	[89]
	TFG	TRK-Fused Gene	Coiled-coil	ALCL	STAT, PLCγ	[55]
	TPM3	Tropomyosin 3	Coiled-coil	ALCL	PI3K, AKT, STAT	[55]
	TPM4	Tropomyosin 4	Coiled-coil	ALCL	TBD	[90]
	TRAF1	TNF Receptor-Associated Factor 1	Coiled-coil	ALCL	NFκB	[91]
CSF1R; kinase domain	MEF2D	Myocyte Enhancer Factor 2D	MADS-box	ALL	TBD	[92]
FGFR1; kinase domain	BCR	Breakpoint Cluster Region	Ser/Thr kinase, coiled-coil	EMS	STAT, MAPK	[29,33]
	CEP110	Centrolin	Leucine zipper, coiled-coil	EMS	TBD	[14]
	CPSF6	Cleavage and Polyadenylation Specific Factor 6	RNA recognition motif	EMS	TBD	[14]

Table 1. (continued)

C-terminal fusion partner	N-terminal fusion partner	Protein name	N-terminal domain	Cancer type	Pathway	Refs
	CUX1	Cut Like Homeobox 1	Coiled-coil	EMS	STAT, RPS6K	[14,93]
	FGFR1OP	Fibroblast Growth Factor 1 Oncogenic Partner	Leucine-rich	EMS	STAT, MAPK	[14,17]
	HERV-K	Human Endogenous Retrovirus Group K 6	HERV-K Rec open reading frame	EMS	TBD	[14]
	LRRFIP1	Leucine-Rich Repeat (in FLII) Interacting Protein 1	Coiled-coil	EMS	TBD	[14,94]
	MYO18A	Myosin XVIIIA	Coiled-coil	EMS	TBD	[14]
	NUP98	Nucleoporin 98kDa	Coiled-coil	EMS	TBD	[14]
	RANBP2	RAN Binding Protein 2	Leucine zipper	EMS	TBD	[14,95]
	SQSTM1	Sequestosome 1	PB1	EMS	TBD	[96,97]
	TPR	Translocated Promoter Region, Nuclear Basket Protein	Coiled-coil	EMS	TBD	[14]
	TRIM24	Tripartite Motif Containing 24	Coiled-coil	EMS	TBD	[14]
	ZNF198	Zinc Finger MYM-Type Containing 2	Zinc finger motif, proline-rich domain	EMS	STAT, PLCγ, Notch, PI3K/AKT	[17,27,28]
FGFR3: TM kinase domain	ETV6	Ets Variant 6	HLH	AML	STAT, PI3K, MAPK	[98]
NTRK3: kinase domain	ETV6	Ets Variant 6	HLH	AML	MAPK	[98]
PDGFRA: kinase domain	BCR	Breakpoint Cluster Region	Ser/Thr kinase, coiled-coil	aCML/T-ALL/CEL	TBD	[39,40]
	CDK5RAP2	CDK5 Regulatory Subunit Associated Protein 2	Coiled-coil	CEL	TBD	[99]
	EVT6/TEL	Ets Variant 6	HLH	CEL	TBD	[41]
	FIP1L1	FIP1 Like 1	Self association	CEL, HES, Eos-MPN, AML, T-cell NHL	MAPK, STAT, NFκB	[50,59,61,100]
	FOXP1	Forkhead Box P1	Zinc-finger	MPN	TBD	[101]
	KI5B	Kinesin family member 5B	Coiled-coil	HES	TBD	[102]
	STRN	Striatin	Coiled-coil	CEL	TBD	[41]

Table 1. (continued)

C-terminal fusion partner	N-terminal fusion partner	Protein name	N-terminal domain	Cancer type	Pathway	Refs
PDGFRB: kinase domain	c6orf204/ CEP85L	Centrosomal Protein 85 kDa-Like	Coiled-coil	Precursor T lymphoblastic lymphoma/MPN	TBD	[103,104]
	NIN	Ninein	Coiled-coil	MPN	TBD	[105]
	PRKG2	Protein Kinase, CGMP-Dependent, Type II	Coiled-coil	MPN	TBD	[106]
PDGFRB: TM kinase domain	CEV14/TRIP11	Thyroid Hormone Receptor Interactor 11	Leucine zipper	CMML, AML, T-ALL, APL	TBD	[107,108]
	DTD1	D-Tyrosyl-TRNA Deacylase 1	Unknown	MPN	TBD	[109]
	ERC1	ELKS/RAB6-Interacting/CAST Family Member 1	Coiled-coil	AML	TBD	[110]
	EVT6/TEL	Ets Variant 6	HLH	CMML, HES, aCML, AML (APL)	STAT, MAPK, PI3K, NFκB	[42,43,59]
	GIT2	G-Protein-Coupled Receptor Kinase Interacting ArfGAP 2	Ankyrin protein interaction motif	MPN	TBD	[106]
	GPIAP1/ CAPRIN1	Cell Cycle Associated Protein 1	Coiled-coil	MPN	TBD	[106]
	H4/D10S170/ CCDC6	Coiled-Coil Domain Containing 6	Leucine zipper	aCML, CMML	TBD	[111,112]
	HCMOGHT1/ SPECC1	Sperm Antigen with Calponin Homology and Coiled-Coil Domains 1	Coiled-coil	MPN	TBD	[113]
	HIP1	Huntingtin Interacting Protein 1	Leucine zipper	CMML	SHIP1, STAT	[64,65]
	KANK1	KN Motif And Ankyrin Repeat Domains 1	Coiled-coil; KOD	MPN	STAT, MAPK, PLCγ	[42,63]
	KIAA1509/ CCDC88C	Coiled-Coil Domain Containing 88C	Coiled-coil	MPN	TBD	[109]
	MYO18A	Myosin 18A	Coiled-coil	MPN	TBD	[114]

Table 1. (continued)

C-terminal fusion partner	N-terminal fusion partner	Protein name	N-terminal domain	Cancer type	Pathway	Refs
	NDE1	NudE Neurodevelopment Protein 1	Coiled-coil	CML	TBD	[115]
	PDE4DIP/ myomegalin	Phosphodiesterase 4D Interacting Protein	Coiled-coil	MPN	TBD	[116]
	RAB5A	RAS-Associated Protein RAB5A	Coiled-coil	CMML	TBD	[117]
	RABEP1	Rabaptin, RAB GTPase Binding Effector Protein 1	Coiled-coil	CMML	TBD	[117]
	TP53BP1	Tumor Protein P53 Binding Protein 1	Coiled-coil	aCML	TBD	[118]
RET: kinase domain	FGFR1OP	Fibroblast Growth Factor 1 Oncogenic Partner	Leucine-rich domain	CMML	PI3K, STAT	[119]
	BCR	Breakpoint Cluster Region	Ser/Thr kinase, coiled-coil	CMML	MAPK, STAT, AKT	[119]

[a]Abbreviations: aCML, atypical chronic myeloid leukemia; AML, acute myeloid leukemia; AMMoL, acute myelomonocytic leukemia; APL, acute promyelocytic leukemia; BPDCN, blastic plasmacytoid dendritic cell neoplasm; CEL, chronic eosinophilic leukemia; CMML, chronic myelomonocytic leukemia; DLBCL, diffuse large B cell lymphoma; Eos-MPD, eosinophilia-associated myeloproliferative disorders; HES, hypereosinophilic syndrome; HLH, helix-loop-helix; IHES, idiopathic HES; JUNB, Jun B proto-oncogene; LBCL, large B cell lymphoma; MAPK, mitogen-activated protein kinase; MPN, myeloproliferative neoplasms; NF-κB, nuclear factor kappa B; PI3K, phosphatidylinositide 3-kinase; PLCγ, phospholipase C gamma 1; RPS6K, ribosomal protein S6 kinase beta-1; SHIP1, inositol polyphosphate-5 phosphatase D; STAT, signal transducer and activator of transcription; T cell NHL, lymphoblastic T cell non-Hoogkin lymphoma; TM, transmembrane domain.

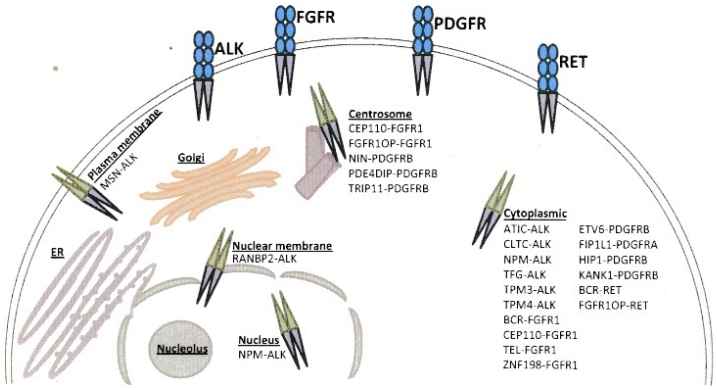

Figure 2. Cellular Localization of Various Receptor Tyrosine Kinase (RTK) Fusion Proteins. The identified localization of the parent RTKs and the resulting fusion proteins are shown. The RTK fusion proteins are depicted in their corresponding localization site. These RTK fusions may localize in the plasma membrane, centrosome, nuclear membrane, nucleus, or cytoplasm. For a complete list of RTK fusion proteins identified in this review and their definitions, please see Table 1.

common *ALK* translocation partner in this disease is *CLTC* [4]. In addition, the translocation partner must exhibit active promoter activity, because *ALK* is not typically expressed outside of the nervous system or after birth [4,6]. Thus, the initiation of transcription of the fusion protein relies on the promoter sequence of the 5′ fusion gene.

The most common hematological ALK fusion, NPM-ALK, arises from the translocation t(2;5)(p23;q35) between *ALK* on human chromosome 2 and *NPM1* on chromosome 5. The ALK tyrosine kinase domain becomes constitutively activated by formation of homodimers mediated by the self-associating domain of NPM. This dimerization is essential for oncogenic transformation by NPM-ALK, which is capable of the transformation of various cell types, IL-3 independent proliferation of **Ba/F3** lymphocytes by interaction with PLCγ, and activation of PI3K, AKT, and STAT5. Additionally, a human lymphoblastic Jurkat T cell line stably expressing NPM-ALK displays PI3K and PLCγ-independent inhibition of doxorubicin-induced apoptosis [5].

Although the NPM1 domain is essential for oncogenic activity, it is also responsible for the nuclear localization of the fusion protein, because its normal role is an RNA-binding nucleolar phosphoprotein. NPM-ALK is the only ALK fusion protein identified so far that displays nuclear localization [5,10] (Figure 2). While NPM-ALK is detected in the cytoplasm and the nucleus, only the cytoplasmic fusion protein exhibits an active ALK kinase domain [10]. The nuclear population is inactivated by dimerization with wildtype (WT) NPM1, which includes nuclear (NLS) and nucleolar localization signals (NuLS) not included in the NPM-ALK fusion protein. Formation of NPM-ALK/NPM1 heterodimers does not allow ALK kinase to become activated by *trans*-phosphorylation, but does result in nuclear localization. Cytoplasmic expression appears to be a requirement for cell transformation, because this is the location of many other ALK fusion proteins [11] (Figure 2). Altered localization of a strongly activated tyrosine kinase may result in interaction with, and phosphorylation of, novel proteins and pathways.

Studies have emerged identifying the spatial organization of the genome as a cause of recurring translocations in lymphomas [12]. Specifically, in ALCL, there are several dysregulated genes

surrounding the chromosomal breakpoints for *ALK* and *NPM1*. In ALK fusion-negative cells, the breakpoint regions of the t(2;5) translocation are in close proximity within the nucleus, but not yet fused. This allows for the experimental generation of this translocation. The spatial proximity of *NPM* and *ALK* genes does not exist in non-ALCL cells, such as Jurkat and KE-37 (T cell leukemia) cell lines [13]. The t(2;5) translocation may not be the initial transformation event for the development of ALCL, a hypothesis supported by the fact that not all ALCL cases bear this NPM-ALK fusion protein [13]. Nevertheless, the presence of ALK fusion proteins in cancer cells leads to increased proliferation and cancer viability, thus representing a potential therapeutic target.

FGFR Translocations: Relatively Rare But Providing Important Insights

FGFRs are often aberrantly activated in cancer by overexpression, mutation, or translocation [14]. In early hematopoietic cells, FGFRs are usually poorly expressed, but, as cells mature, FGFR expression generally increases. Human **leukemia** cells have been shown to express at least one type of receptor (FGFR1, FGFR3, or FGFR4) [15,16].

FGFR1 is involved in **8p11 myeloproliferative syndrome (EMS)**, also known as stem cell leukemia-lymphoma syndrome (SCLL). EMS involves a chromosomal translocation that produces a dimerizing protein partner fused N-terminally to the kinase domain of FGFR1, normally encoded at the 8p11 locus. EMS is a rare, aggressive myeloproliferative disorder that can quickly progress into **acute myeloid leukemia (AML)** [17].

FGFR1 fusion partners in EMS are many and varied (Table 1), some of which include breakpoint cluster region (BCR), cut-like homeobox 1 (CUX1), FGFR1 oncogenic partner (FGFR1OP), and zinc finger 198 (ZNF198) [14]. Interestingly, many of these partners also contain leucine zipper, leucine-rich, and coiled-coil domains. The contribution of a dimerization domain by each fusion partner is necessary for the phosphorylation and activation of the FGFR1 kinase domain, resulting in a gain-of-function fusion protein. Additionally, given that biologically active translocations result from the in-frame fusion of two coding sequences that are normally distinct, this dictates that expression of the FGFR1 kinase domain in these fusions is reliant on the promoter sequence of the partner N-terminal protein.

In patients with EMS, the presence of an 8p11 translocation does not always mean an *FGFR1* rearrangement. Studies have identified a small subset of 8p11 translocations as rearrangements of the histone lysine acetyltransferase KAT6A (*KAT6A* gene), also located at the same chromosomal region as *FGFR1*. *KAT6A* has several translocation partners occurring in 2% of AML cases [18]. Diagnostic **fluorescent *in situ* hybridization (FISH) analysis** is recommended for patients with EMS and 8p11 rearrangements to correctly identify the translocation. This then allows for treatment with TKI therapeutics (e.g., ponatinib and dovitinib), for patients expressing FGFR1 fusion proteins [19–21].

Although not as common, FGFR3 is also involved in translocations in hematopoietic disorders. Ets variant 6 (ETV6, previously known as TEL, translocation-ets-leukemia) is fused to FGFR3, and is found in T cell lymphomas that progress to AML. WT ETV6 contains a helix-loop-helix (HLH) domain and serves as a transcription factor. The fusion of ETV6 to FGFR3 arises from the t(4;12)(p16;p13) translocation and leads to the HLH domain of ETV6 fused to the transmembrane (TM) domain of FGFR3. The HLH domain is a dimerization domain, allowing constitutive activation of the FGFR3 kinase domain. The ETV6-FGFR3 fusion leads to IL-3-independent growth in Ba/F3 cells, activation of STAT3, STAT5, MAPK, and PI3K, and exhibits cytoplasmic localization [22].

Multiple myeloma (MM)commonly contains a t(4;14) translocation between *IgH* promoter to the *MMSET* and *FGFR3* genes, a translocation that does not result in a novel FGFR3 fusion

protein but in overexpression instead. MMSET overexpression is observed in all translocation-positive cases and FGFR3 overexpression in 70% of translocation-positive cases, which often also exhibit activating point mutations in FGFR3 [14,23]. This overexpression leads to IL-6-independent growth in murine **B9 cells**, upregulated MAPK and PI3K signaling, and induced lymphoid malignancies in mice [15,23]. In **chronic lymphocytic leukemia (CLL)**, rare translocations between FGFR3 and IgH [t(4;14)(p16;q32)] and IgL [t(4;22)(p16;q11.2)] have been identified [14,24]. These types of translocation, which result in altered FGFR3 expression, are medically important, yet they are distinct from other translocations discussed here where two distinct reading frames fuse to create a novel fusion protein.

The most commonly identified FGFR1 fusion protein is ZNF198-FGFR1, found in 48% of EMS cases [17]. Endogenous ZNF198, also known as ZMYM2, contains a zinc finger-related motif, a proline-rich domain, and a MYM domain, and is suggested to serve as a transcription factor [17,25]. The fusion of ZNF198 and FGFR1 arises from the t(8;13)(p11;q12) human translocation, in which *ZMYM2*, the gene encoding ZNF198 on chromosome 13, is fused 5′ to *FGFR1* on chromosome 8. This fusion occurs in both myeloid and lymphoid cells, suggesting a multipotent hematopoietic progenitor cell origin. The N-terminal ZNF198 domain, particularly the proline-rich domain, facilitates dimerization and activation of the FGFR1 kinase domain [17]. The ZNF198-FGFR1 fusion is oncogenic, as shown by IL-3-independent Ba/F3 cell proliferation, increased tyrosine phosphorylation of STAT1 and STAT5, as well as activation of PLC-γ, PI3K/AKT, and Notch signaling pathways [26–28]. While WT ZNF198 displays nucleolar localization, the fusion protein exhibits cytoplasmic localization [25] (Figure 2).

BCR-FGFR1 is another commonly identified fusion protein in EMS. BCR contains a coiled-coil domain, has serine/threonine kinase activity, and is a GTPase-activating protein for Rac1 [29]. *BCR* is more commonly found fused to *ABL* to form the *BCR-ABL* oncogene, where ABL encodes a nonreceptor tyrosine kinase. This *BCR-ABL* fusion results from the Philadelphia chromosome, where exon 1 of *BCR* is fused to exon 2 of *ABL*, found in 95% of patients with CML [30,31]. Cases positive for other fusion proteins, including BCR-FGFR1 fusion, are considered atypical CML (aCML). Both CML and aCML share similar phenotypes, because both are myeloproliferative disorders of hematopoietic stem cells, characterized by leukocytosis and a high number of immature granulocytes [30].

The fusion of *BCR* and *FGFR1*, resulting from a t(8;22)(p11;q11) translocation, occurs commonly in EMS but is also observed in AML and B cell lymphomas. The BCR-FGFR1 fusion differs from the BCR-ABL fusion, because *BCR* exon 4 is fused to *FGFR* exon 9 [32]. This fusion gives rise to a kinase-kinase fusion product, with the serine-threonine kinase domain of BCR fused to the tyrosine kinase domain of FGFR1. The kinase domain of FGFR1 becomes constitutively activated as a result of this fusion, leading to activation of STAT3, STAT5, and MAPK3/1 pathways, and IL-3-independent proliferation of Ba/F3 cells [33]. The BCR-FGFR1 fusion protein localizes to the cytoplasm, but it is unknown what effect this has on its oncogenicity [34] (Figure 2). The discovery and further characterization of FGFR fusion proteins arising from translocations is vital to determine the extent of cell signaling and proliferation that occurs from different fusion partners.

PDGFR Translocations: Fusion Proteins and Their Cancers

Similar to other hematopoietic translocations, PDGFR fusion proteins express the RTK kinase domain as the C-terminal fusion protein partner whose expression is now reliant on the promoter of the gene encoding the N-terminal fusion protein. Unlike ALK receptors, WT PDGFRs are expressed at constant low levels in hematopoietic human and mouse cells [35]. However, as shown using murine hematopoietic chimeras reconstituted with *pdgfrb*$^{-/-}$ fetal liver cells, PDGFR expression is not required for normal hematopoiesis [36].

Although translocations creating PDGFR fusion proteins are low, several different fusion protein partners have been reported. Translocations have been reported that result in PDGFRA fused to BCR, FIP1-like 1 (FIP1L1), and striatin (STRN). For fusions with PDGFRB, many fusion partners have been reported, including myosin 18A (MYO18A), Rab5A, tropomyosin 3 (TPM3), and others (Table 1). Both PDGFRA and PDGFRB have been found fused to ETV6. In myelodysplastic/myeloproliferative neoplasms (MDS/MPNs), 1.8% of cases appear to contain translocations encoding PDGFRB fusions proteins [37]. As with ALK and FGFR translocations, most of these fusion partners contain dimerization domains that are essential for constitutive activation of the PDGFR receptor, an exception being FIP1L1-PDGFRα.

For WT PDGFR, dimerization alone is not enough to constitute receptor activation. Activation of the kinase domain also relies on reorganization and homotypic interaction of the extracellular Ig-like domain D4 between PDGFR receptors [38]. However, in PDGFR fusion proteins, the extracellular domains are no longer present, yet the kinase domain is constitutively active (Figure 1). This indicates that an altered mechanism of activation relying on the fused N-terminal dimerization domain is taking place.

One potentially interesting rearrangement results in the kinase domain of BCR fused to the kinase domain of PDGFRα, similar to the BCR-FGFR1 and BCR-ABL fusion proteins. This t (17;13) translocation between *BCR* and *PDGFRA* was reported in aCML, CEL, **B/myeloid mixed phenotype leukemia** and **T acute lymphoblastic leukemia (T-ALL)**. Only a few cases of BCR-PDGFRα have been reported with varying breakpoints: exon 7, 12, or 17 for *BCR* fused to exon 12 or 13 of *PDGFRA* [39,40]. To determine the extent of activation, signaling, and proliferative differences contributed by different fusion partners, a molecular analysis comparing the relative extent of BCR-PDGFRα, BCR-FGFR1, or BCR-ABL oncogenicity and/or clinical disease may prove interesting.

The most common *PDGFRB* fusion partner is *ETV6*, defined by t(5;12)(q33;p13) translocations and identified in chronic myelomonocytic leukemia (CMML). *ETV6* has also been found fused to *PDGFRA* in one patient [41]. The ETV6 domain contains a HLH dimerization domain that allows for ligand-independent activation of PDGFR. Increased cell proliferation and transformation demonstrated by ETV6-PDGFRβ is reliant on increased fusion protein stability by reduced ubiquitination and increased STAT5 activation in Ba/F3 cells and mouse models [42]. Murine stem cell differentiation is also induced by the ETV6-PDGFRβ fusion protein through MAPK and STAT5 pathway activation [43].

The ETV6-PDGFRβ fusion protein, along with FIP1L1-PDGFRα and ZNF198-FGFR1, displays increased stability by evading ubiquitination and degradation [44]. To prevent overactivation, RTKs are often controlled by proteosomal degradation and negative feedback signals and, upon ligand binding, the complex is internalized and degraded. Additionally, the PDGFR juxtamembrane domain acts as an inhibitory domain by interacting with, and inhibiting, the kinase domain when ligand is not present [42,45]. The C-terminal tail of PDGFR also functions as an allosteric inhibitor of the kinase domain [46]. Despite these processes, overactivation occurs through PDGFR translocations in myeloid malignancies [47].

Most of the PDGFR fusion proteins, including ETV6-PDGFRβ, involve a breakpoint occurring just before the TM domain of PDGFRβ, although some contain a breakpoint in between the TM and kinase domains (Table 1, Figure 1). Experimental deletion of the TM domain in the ETV6-PDGFRβ fusion does not hinder dimerization or kinase domain activation, but does result in a decrease in cell proliferation and STAT5 and MAPK activation in Ba/F3 cells, suggesting that cell transformation relies not only on activation, but also proper alignment of the kinase domain [42].

The inhibitory effects that the intracellular-juxtamembrane domain and C-terminal tail have on the WT receptor are lost or subdued in this fusion protein.

Another common PDGFRα fusion protein is FIP1L1-PDGFRα, discovered in myeloproliferative diseases associated with hypereosinophilia, sometimes referred to as **chronic eosinophilic leukemia (CEL)**. This fusion protein is estimated to occur in 10–20% of eosinophilia cases [37]. This chromosomal rearrangement is caused by a 800-kb deletion in chromosome 4 [del(4)(q12g12)], a segment including the cysteine-rich hydrophobic domain 2 (*CHIC2*) locus [48]. This fusion protein poses an exception to previously discussed RTK fusion proteins, because FIP1L1 is dispensable for PDGFRα dimerization, as shown in Ba/F3 cell transformation assays, as well as in murine bone marrow transplantation assays using FIP1L1-PDGFRα deletion constructs in transduced bone marrow cells, where all or most of the FIP1L1 was deleted [49]. However, the FIP1 motif is involved in protein–protein interactions and is essential for homodimer formation of a fusion protein between FIP1L1 and retinoic acid receptor α (FIP1L1-RARA) in leukemia. The FIP1L1 domain does have a role in human progenitor cell proliferation and contains two phosphotyrosine sites that may provide protein-binding sites [50]. The IL-3-independent proliferation of Ba/F3 cells and the dispensability of the FIP1L1 domain was also recently confirmed by CRISPR/Cas9 genome editing in Ba/F3 cells where the fusion protein was created at endogenous levels [51].

The breakpoint of FIP1L1-PDGFRα lies within the juxtamembrane domain of PDGFRα and disrupts an inhibitory **WW-like domain**, which may be the key to constitutive receptor activation and transforming potential. The WW-like domain contains two conserved tryptophan residues in the juxtamembrane domain. When truncated by fusion protein formation, absence of one of the tryptophan residues results in constitutive receptor activation [49]. The disruption of this domain has been noted in BCR-PDGFRα and STRN-PDGFRα [41,49]. Fusion proteins with the TM and juxtamembrane domains intact most likely require an alternative dimerization and activation mechanism provided by the N-terminal fusion partner. Although PDGFR translocations are relatively rare compared with other hematological translocations, their existence may lead to potential therapeutic targeting in patients with certain cancers.

Signaling Alterations Resulting from RTK Translocations

ALK Fusions

Aberrant expression of highly active RTK kinases in tissues can result in pathway activation, and may present novel therapeutic possibilities. For instance, WT ALK results in the activation of multiple pathways, including PLCγ, JAK/STAT, PI3K/AKT, JUNB, MAPK, and MYCN. ALK activation of ERK and PI3K can lead to MYCN expression, and high MYCN levels have been linked to neuroblastoma oncogenesis [52,53]. The NPM-ALK fusion protein specifically activates JUNB, Y-box transcription factor (YBX1), BCL2A1, matrix metalloproteinase 9 (MMP9), CDKN2A, and hypoxia-inducible factor 1α (HIF1A), as shown in various studies using either Ba/F3 cells or ALK-positive ALCL human cell lines [4].

NPM-ALK downregulates STAT1 in ALCL cells. STAT1 is known to function as a tumor suppressor in some cancer cell types and phosphorylation of STAT1 at Y701 leads to its proteasomal degradation. Tumor suppression in ALCL cells can be restored by increasing STAT1 upon transfection with a constitutively activated STAT1 expression plasmid [54]. A correlation has been seen between invasive cell ability and PI3K/AKT pathway activation, implicated in cell migration. For the fusion proteins NPM-ALK, TPM3-ALK, TFG-ALK, CLTC-ALK, and ATIC-ALK, their ability to stimulate PI3K and AKT phosphorylation (as shown by immunoblotting) correlates with their transendothelial migration ability [55]. Among these fusion proteins, ATIC-ALK displays the highest phosphorylation of STAT3 in mouse NIH3T3 cells [55].

NPM-ALK, TPM3-ALK, TFG-ALK, CLTC-ALK, and ATIC-ALK fusion proteins result in cell transformation, proliferation, invasion, transendothelial cell migration, and tumor development in nude mice [55,56]. In general, the oncogenic effects of these proteins increase as expression levels increase; an exception is provided by the TPM3-ALK fusion protein, for which increased expression results in lower proliferation rates in NIH3T3 cells, but increased invasiveness [55]. Confocal microscopy and fractionation of NIH3T3 cells has shown that TPM3-ALK fusion proteins localize to the cytoskeleton; thus, this effect may be due to the role of TPM3 as an actin filament stabilizer, potentially altering cell shape and movement [56]. Of note, TPM3-ALK, TFG-ALK, CLTC-ALK, and ATIC-ALK all display cytoplasmic localization, while NPM-ALK displays both nuclear and cytoplasmic localization [55,56] (Figure 2).

FGFR Fusions

WT FGFRs result in the activation of multiple signaling pathways, including PLCγ, PI3K/AKT, MAPK, and STAT, and are important in cell proliferation and differentiation as demonstrated in mouse models [57]. However, the signaling differences between WT FGFRs and FGFR fusion proteins are not completely understood. Both the ZNF198-FGFR1 and BCR-FGFR1 fusion proteins induce aberrant signaling through the dimerization of the kinase domain of FGFR1. Activation of FGFR1 through the ZNF198-FGFR1 fusion leads to phosphorylation or activation of FGFR1 targets, such as STATs, PI3K, PLC-γ, AKT, and MAPK, as shown in Ba/F3 cells *in vitro*. In addition, ZNF198-FGFR1 is able to activate a pathway involving plasminogen activator inhibitor 2 gene (PAI-2/SERPINB2), which is not observed in native FGFR1 signaling. The *PAI2* gene induces resistance to TNFα, which could suggest an alternative pathway contributing to the oncogenic potential of the ZNF198-FGFR1 fusion, as shown in HEK293 and Ba/F3 cells in *in vitro* assays [58]. The BCR-FGFR1 fusion is dependent on adaptor protein Grb2. This translocation binds Grb2 through BCR Y177, and has been shown to induce CML-like leukemia in mice. However, BCR-FGFR1 with a mutated Y177 lacks Grb2 binding and causes an EMS-like disease [34].

PDGFR Fusions

Upon activation by ligand binding, PDGFRs bind various signal transduction molecules via phosphotyrosine interaction motifs, such as SH2 or PTB, resulting in activation of downstream signaling. Some key interacting proteins include PI3K, PLCγ, Src family tyrosine kinases, SHP2 tyrosine phosphatase, and STAT proteins [47].

Although few PDGFR fusion proteins have been analyzed for biological function, a study analyzing ETV6-PDGFRβ and FIP1L1-PDGFRα found that NFκB activation was required for human $CD34^+$ cell proliferation and differentiation with a bias towards the eosinophil lineage [59]. These fusions have been found to have a large role in human hypereosinophilia development in the absence of growth factors IL-3 and IL-5, whose expression usually supports hematopoietic stem cell differentiation to form eosinophils [59]. IL-5 expression is increased in cells expressing these PDGFR fusions and, in such patients, an *IL5* gene polymorphism has been linked to more severe disease development, as determined from eosinophil counts and increased tissue infiltration [59].

Multiple tyrosine phosphorylation sites (Y579/581) in PDGFRβ of ETV6-PDGFRβ are responsible for myeloproliferative neoplasm (MPN) development in mice. Mutation to phenylalanine in Y579F/Y581F mutants results in the development of T cell lymphoma, but not MPN [60]. For FIP1L1-PDGFRα, it was identified that tyrosine 720 of PDGFRα is critical for SHP2 recruitment, which results in MAPK activation and Ba/F3 hematopoietic cell transformation. Interestingly, SHP2 recruitment represents an altered mechanism compared with WT PDGFR, because cell proliferation and MAPK activation occurs regardless of SHP2 interaction with the WT receptor, as shown by expression of the human FIP1L1-PDGFRα fusion protein in murine

Ba/F3 cells [61]. Indeed, SHP2 is involved in JAK/STAT, PI3K, MAPK, and other signaling pathway regulation, and has been implicated in leukemogenesis caused by mutations in the **KIT** and **FLT3 receptors** [61].

Both ETV6-PDGFRβ and FIP1L1-PDGFRα display cytosolic expression and result in the activation of STAT1, STAT3, and STAT5 (Figures 2 and 3). STAT5 has an important role in myeloproliferation by PDGFR fusion proteins, as shown in both human and murine cell lines [59,61,62]. STAT5 activation was also demonstrated with the KANK1-PDGFRβ fusion protein, despite an inactivity of JAK2 and an inability of a JAK inhibitor to affect cell growth. This fusion protein is found in MPN and arises from a t(5;9) translocation that results in the KN Motif and Ankyrin Repeat Domains (*KANK1*) fused to *PDGFRB*. KANK1 contributes three coiled-coil domains and an oligomerization domain, both of which are required for cell proliferation and upregulation of signaling [63]. Interestingly, this fusion protein has been shown to exist as a homotrimer, of which either the coiled-coil or the oligomerization domain may be present to allow for this motif formation [63]. KANK1-PDGFRβ also activates PLCγ and MAPK pathways, and displays cytosolic expression, as shown in human and murine cell lines [63] (Figures 2 and 3).

STAT5 activation was also found to be essential for Ba/F3 cell transformation by the fusion protein Huntingtin Interacting Protein (HIP1)-PDGFRβ [64]. This fusion protein also colocalizes with Src Homology 2-containing Inositol 5-Phosphatase (SHIP1) and displays cytosolic

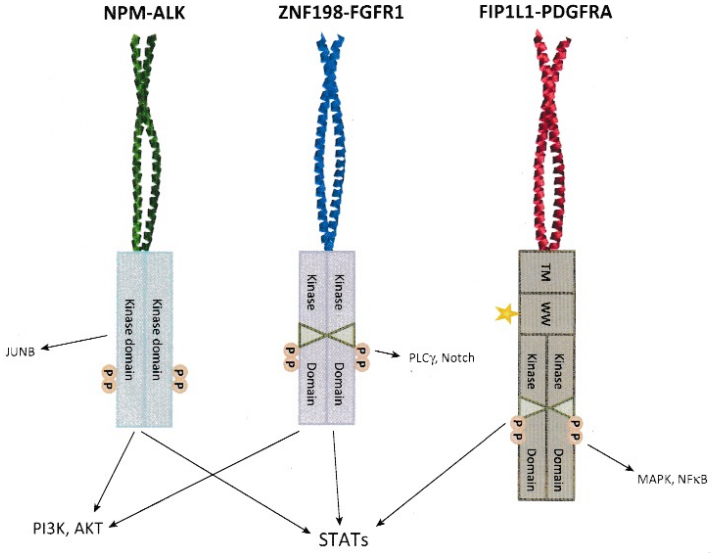

Figure 3. Major Signaling Pathways Activated by Common Receptor Tyrosine Kinase (RTK) Fusion Proteins. Activation of Signal Transducer and Activator of Transcription (STAT) signaling from RTK fusion proteins is a common occurrence in hematological malignancies. The arrows indicate activated pathways; the activation of these pathways leads to cell survival and proliferation. A star indicates an alternate breakpoint; a triangle indicates a kinase insert domain; TM is a transmembrane domain; WW is a WW-like domain; P is a phosphorylation site. Abbreviations: ALK, Anaplastic Lymphoma Kinase; FGFR, Fibroblast Growth Factor Receptor; FIP1L1, FIP1-Like 1; JUNB, Jun B proto-oncogene; MAPK, Mitogen-Activated Protein Kinase; NFκB, nuclear factor kappa B; NPM, Nucleophosmin; PDGFR, Platelet-Derived Growth Factor Receptor; PI3K, Phosphatidylinositide 3-Kinase; PLCγ, Phospholipase C Gamma 1; ZNF, Zinc Finger.

localization, as shown in human HEK293T cells [64] (Figure 2). Given that SHIP1 is only expressed in hematopoietic tissues and developing spermatogonia, SHIP1 might serve as a potential therapeutic target [65].

Therapeutics for Hematopoietic Cancers with RTK Translocations

There are several drugs that have been characterized for their potential to inhibit the fusion proteins discussed in this review (Table S1 in the supplemental information online). These function to inhibit or reduce the kinase activity of the RTK fusion partner, leading to reduced proliferation, increased apoptosis, and altered downstream signaling.

ALK Fusions

Crizotinib, the first ALK inhibitor to be clinically tested, is a potent, ATP-competitive, small-molecule inhibitor initially designed against the hepatocyte growth factor receptor (c-Met) to inhibit phosphorylation. It inhibits ALK phosphorylation and signal transduction, leading to apoptosis in lymphoma cell lines that express the NPM-ALK fusion protein [4,66]. This ALK inhibitor, by inhibiting c-Met and ALK downstream signaling, also exhibits antitumor activity in ALCL Karpas299 mouse xenograft models expressing the NPM-ALK fusion, resulting in the reduction of tumor growth [66]. Crizotinib has been extensively used to treat solid tumors containing EML4-ALK and STRN-ALK fusions in thyroid cancer and EML4-ALK rearrangements in non-small cell lung cancer (NSCLC) [67–69]. Crizotinib is currently in multiple clinical trials in patients with ALCL (Table S1 in the supplemental information online).

Unfortunately, resistance and relapse can occur with crizotinib treatment leading to secondary mutations in ALK, rendering the drug ineffective [70]. For instance, after treating human cell lines expressing the NPM-ALK fusion with high doses of crizotinib, the mutations L1196Q and I1171N, identified in the ALK kinase domain, were shown to confer resistance to crizotinib in NPM-ALK expressing Ba/F3 cells [71]. L1196Q is a **gatekeeper mutation** within the ATP-binding pocket, in the hinge region between the N and C lobes. Point mutations in this region prevent or reduce the binding of inhibitory molecules, a common occurrence in inhibitor-resistant cancers [71]. The I1171N mutation is part of the hydrophobic spine of the kinase domain critical for tyrosine kinase activity [71]. In RANBP2-ALK, the kinase domain mutation G1269A has been found in patients with AML and NSCLS after crizotinib treatment [70] and, again, this occurs in the ATP-binding pocket, decreasing TKI affinity [70].

An alternative selective ALK inhibitor, ceritinib, has been approved for treatment of NSCLC with the NPM-ALK fusion and is in a Phase 2 trial for relapsed/refractory ALK+ hematologic malignancies (Table S1 in the supplemental information online) [72]. Another alternative, brigatinib, also leads to resistance through point mutations in the ALK kinase domain in NPM-ALK-amplified ALCL cells [73]. One study reported that removal of the kinase inhibitor led to apoptosis of brigatinib-resistant ALCL cells by hyperactivation of the MAPK pathway [10]. This suggests that a periodic suspension of drug treatment could be beneficial for patients with cancer and ALK translocations and/or amplifications. Additionally, since NPM-ALK fusion proteins are only active in the cytoplasm, blocking nuclear export of the fusion with selective inhibitors of nuclear export (SINE), such as selinexor, is currently under investigation in clinical trials for hematological cancers (Table S1 in the supplemental information online) [10]. Interestingly, silibinin, a nontoxic naturally occurring compound found in extracts from seeds of the plant *Silybum marianum* (milk thistle) that has known antitumor effects, is able to inhibit NPM-ALK activation, leading to reduced proliferation and increased apoptosis of Karpas299 and SupM2 cell lines [74].

FGFR Fusions

Inhibiting aberrant FGFR signaling in FGFR-dependent malignancies is a well-established therapeutic strategy; however, specific FGFR inhibitors have been elusive [75]. The classic

FGFR inhibitor, dovitinib, is a multitargeted RTK inhibitor that targets FGFR, PDGFR, VEGFR, FLT3, and c-KIT. When the fusion proteins ZNF198-FGFR1 and BCR-FGFR1 are expressed in Ba/F3 cells, treatment with dovitinib results in the inhibition of STAT5, MAPK, IL-3 independence, and phosphorylation of these fusion proteins [20]. Proliferation of FGFR1OP2-FGFR1 cell lines is also inhibited by dovitinib [20]. A Phase 2 trial for dovitinib was recently completed in 2015 for patients with solid or hematologic malignancies with mutations or translocations of FGFR and other RTKs (Table S1 in the supplemental information online). The US Food and Drug Administration (FDA)-approved FGFR inhibitor, ponatinib, is also a multi-RTK inhibitor currently being tested in multiple trials for AML and CML [75]. Ponatinib has shown potential for EMS treatment when tested against the murine Baf3 cell lines expressing the ZNF198-FGFR1 and BCR-FGFR1 fusions, and in the human KG1A cell line expressing the FGFR1OP2-FGFR1 fusion; these fusion proteins are phosphorylated and their expression also leads to reduced cell proliferation and survival, and induction of apoptosis [76]. In addition, cells from patients with EMS showed reduced colony growth when treated with ponatinib [21]. The specific pan-FGFR inhibitor, infigratinib, also shows potential for EMS treatment because it has been demonstrated to reduce the survival and proliferation of TPR-FGFR1-expressing murine **32Dcl3 cells** [77]. It is currently in clinical trials for patients with FGFR genetic alterations (Table S1 in the supplemental information online).

To overcome the resistance that can occur with kinase inhibitors, FGFR irreversible inhibitors 2 (FIIN-2) and 3 (FIIN-3) have recently been developed that target cysteines in the ATP-binding pocket. They inhibit the proliferation of transformed Ba/F3 cells and are dependent on FGFR1 or FGFR2 gatekeeper mutants, which often lead to drug resistance [78].

PDGFR Fusions

Imatinib is a multikinase inhibitor selective for ABL, PDGFR, and c-Kit, and is the most common treatment for malignancies associated with activated PDGFR. Hematolymphoid neoplasms associated with PDGFRα and PDGFRβ fusions, such as FIP1L1-PDGFRα and ETV6-PDGFRβ, respond well to treatment with imatinib, with secondary resistance being uncommon. By contrast, patients with rare and aggressive neoplasms containing FGFR1 fusions tend not be responsive to imatinib treatment [37]. BCR-PDGFRα fusions found in aCML become undetectable when treated with imatinib. Diagnosing the difference between CML and aCML, both of which display highly similar phenotypes, is important to prevent treatment with an inadequate TKI [39].

When resistance does occur, mutations have been found in the ATP-binding site gatekeeper residue, T674I, of FIP1L1-PDGFRα. A novel TKI, S116836, was recently found to be effective in inhibiting both FIP1L1-PDGFRα and FIP1L1-PDGFRα T674I downstream signaling, and reducing xenograft tumors in nude mice formed in response to BaF3 cells expressing FIP1L1-PDGFRα T674I [79]. These fusion proteins drive hematopoietic cancers that often becoming resistant, leading to additional mutation; thus, this highlights the need to potentially administer multiple types of drug at various times during treatment.

Concluding Remarks

Factors that influence translocations include chromosome position, DNA damage response pathways, transcription frequency, and epigenetic factors. Transcription can be a driver of translocations, possibly due to DNA supercoiling and torsional stress leading to topoisomerase-induced breaks [6]. In this review, we have discussed translocations involving RTKs in hematopoietic disorders, including ALK, FGFR, PDGFR, RET, CSF1R, and NTRK3. Although many translocations have been identified, activation pathways and mechanistic insight for many of these RTK fusions in cancer pathogenesis have yet to be elucidated (see Outstanding Questions and Box 4).

Outstanding Questions

Does any individual RTK fusion protein constitute a precise driver mutation for the cancer to which it is associated?

How do each of the translocations described in this review compare with each other in terms of oncogenicity? Do certain RTK fusions lead to more aggressive cancers than others? If so, by which mechanisms and pathways?

Are DNA damage pathways impaired in most cells harboring RTK translocations?

Considering that many patients develop resistance to TKIs, can personalized therapies be used to induce or inhibit certain cell signaling pathways, thus leading to cancer cell death?

Why do hematological RTK translocations typically result in the fusion of an N-terminal partner gene to a C-terminal RTK kinase domain? Is this random? Considering that RTK fusion domains in solid cancers typically occur in the reverse order, which factors determine these structural differences in RTK fusions between cancer types? What functional insight could thus be provided? Why are ALK fusion proteins in solid cancers the exception to this general rule?

Is the frequency of occurrence of a specific translocation influenced by cell type, developmental stage, chromatin modifications, or other factors?

Box 4. The Clinician's Corner

RTKs are responsible for cellular proliferation, survival, development, angiogenesis, and activation of downstream signal transduction pathways. The progression of cancer takes advantage of the overactivation of an RTK due to chromosomal translocations and fusion protein formation, amplifying such cellular processes.

TKIs have proved very effective in the treatment of many patients with RTK fusion proteins. However, the occurrence of secondary mutations, specifically gatekeeper mutations, is common in inhibitor-resistant cancers. These gatekeeper mutations occur in the ATP-binding pocket of the RTK and reduce binding of inhibitory molecules. This leads to the need for second-generation TKIs.

Once inhibitor resistance has occurred in cancers with RTK fusion proteins, additional therapies are often necessary. This can include a drug suspension, a nuclear export inhibitor, cord blood transplants, or combination therapies with TKIs.

As the existence of RTK fusion proteins becomes known, it enforces the importance of cancer genome sequencing to identify the type of fusion protein present and, thus, of accurately selecting TKI(s). Given the occurrence of inhibitor resistance by secondary mutations in RTK fusion proteins, post-treatment sequencing may prove valid and helpful in selecting the next course of treatment. Sequencing of various cancer types should become a standard in the course of treatment, allowing the identification of unique genetic alterations in patients, and ideally, personalized medical treatments.

The discovery of these translocations has already facilitated the use of novel RTK inhibitor therapies to treat patients who are positive for translocation-induced cancers. While some RTK-targeted therapies have proven to be beneficial in various malignancies, many challenges remain. Indeed, many cases result in drug resistance or relapse. Therefore, there is an urgent need to develop additional approaches to the characterization and treatment of RTK-translocation induced cancers. The identification of chromosomal translocations occurring in different cancers will be essential, and the utilization of multiple drug types during different treatment stages might prove to be efficacious. The robust efforts in drug discovery and further characterization of RTK fusions must continue to facilitate the development of finely tuned therapies for hematopoietic disorders.

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The thesis author was a co-author of this review, but did not perform the research described by the review. The thesis author was responsible for the FGFR section of this review in its entirety. The thesis author also assisted with figures, tables, the conclusion, and other sections as well. Co-authors include Katelyn N Nelson, April N Meyer, Asma Siari, and Daniel J Donoghue.

Chapter 2

Characterization of the Oncogenic Fusion Protein BCR-FGFR1, a Client of the Molecular Chaperone HSP90.

ABSTRACT

Fibroblast Growth Factor Receptors (FGFRs) are part of the Receptor Tyrosine Kinase (RTK) family and are essential in the activation of various downstream signaling pathways, which are necessary for cell differentiation and proliferation. However, mutation and translocation of FGFRs leads to aberrant activation of signaling, which often results in cancer. This work focuses on the t(8;22)(p11;q11) chromosomal translocation, which results in the fusion protein Breakpoint Cluster Region (BCR)-FGFR1 (BCR-FGFR1). Patients who harbor this translocation are usually diagnosed with 8p11 myeloproliferative syndrome (EMS), which can progress to atypical Chronic Myeloid Leukemia (aCML), or Acute Myeloid Leukemia (AML). Unlike BCR-ABL, BCR-FGFR1 is poorly characterized, resulting in few therapies and clinical advancements for patients positive for this fusion protein. This work focuses on the biochemical and biological characterization of BCR-FGFR1 along with the analysis of therapeutic options. BCR-FGFR1 gives rise to a kinase-kinase fusion product with the serine/threonine kinase domain of BCR fused to the tyrosine kinase domain of FGFR1. BCR-FGFR1, along with kinase dead and kinase activated mutants were assayed for transformation of NIH3T3 cells, and activation of STAT and MAPK signaling. This work shows the reliance of the

fusion protein on the tyrosine kinase activity of FGFR1. Additionally, BCR contributes a coiled-coil dimerization domain to BCR-FGFR1. Various salt bridge mutations and a proline mutant were assayed for cell transformation and the activation of signaling pathways. The importance of the dimerization domain is shown, as when disrupted, BCR-FGFR1 is unable to retain transforming ability. Lastly, BCR-FGFR1 is shown to be a client of the chaperone protein Hsp90, suggesting that BCR-FGFR1 relies on the Hsp90 complex to evade proteasomal degradation. Additionally, BCR-FGFR1 is sensitive to the Hsp90 inhibitor Ganetespib (STA-9090), proposing novel clinical treatment options for patients who are positive for this fusion.

INTRODUCTION

Fibroblast growth factor receptors (FGFRs) are part of the receptor tyrosine kinase (RTK) family, and are responsible for cell growth and proliferation. The FGFR family is composed of 4 homologous receptors, FGFR1, FGFR2, FGFR3, and FGFR4. These receptors all contain three extracellular immunoglobulin like domains, a transmembrane domain, and a split kinase domain. When these receptors are bound to fibroblast growth factor (FGF) and heparin sulfate proteoyglycans, they are able to dimerize, which leads to auto-phosphorylation of the kinase domain and activation of downstream cell signaling pathways such as STAT, MAPK, AKT, and PLCγ. (1)

FGFRs are often aberrantly activated in cancer by overexpression , mutation, or translocation. Specifically, FGFR1 is involved in 8p11 myeloproliferative syndrome (EMS), which is also known as stem cell leukemia-lymphoma (SCLL). EMS is characterized by a chromosomal translocation that produces a dimerizing protein partner fused to the kinase domain

of FGFR1. Although EMS is rare, is can aggressively progress to atypical chronic myeloid leukemia (aCML) or acute myeloid leukemia (AML). (2)

This work focuses on the t(8;22)(p11;q11) chromosomal translocation, which results in the breakpoint cluster region- FGFR1 (BCR-FGFR1) fusion protein where exon 4 of BCR is fused to exon 9 of FGFR1. Although BCR was first identified fused to Abelson murine leukemia viral oncogene homolog-1 (ABL), also known as the Philadelphia chromosome, BCR has since then been identified fused to ret proto-oncognene (RET), janus kinase2 (JAK2), and placental derived growth factor receptor alpha (PDGFRA). Although a common fusion partner, the endogenous function of the BCR gene remains unknown. BCR contains a coiled coil dimerization domain, has serine/threonine kinase activity, and is a GTPase activating protein for p21rac (3).

The BCR-FGFR1 fusion is not well characterized, and the pathways of oncogenesis for this fusion are poorly understood. This work seeks to analyze how the BCR-FGFR1 fusion leads to cancer, through it's biochemical and biological characterization. Although tyrosine kinase inhibitor therapies (TKI) can be used to treat patients with hematological cancers, the use of TKIs often results in drug resistance(2). Thus, it is crucial to establish additional therapeutic strategies in treating hematological cancers. Here we investigate the regulation of BCR-FGFR1 in the cell to establish novel therapeutic targets for patients who are positive for this fusion protein.

RESULTS

Downstream signaling activation by BCR-FGFR1

It is crucial to uncover the signaling cascade used by BCR-FGFR1 in order to reveal which pathways are used by this fusion to activate cell growth and proliferation. It is unclear what the role is of BCR is in the BCR-FGFR1 fusion, or if BCR solely is able to activate downstream cell signaling pathways. In order to elucidate these mechanisms, both a kinase dead and a kinase activated constructs were employed in the FGFR1 and BCR-FGFR1 backgrounds. The kinase dead mutation contained the K514A mutation in the FGFR1 kinase domain, where as the kinase activated mutation contained the K656E mutation in the FGFR1 kinase domain, as described previously (4). HEK293T cells were transfected with either the respective full-length FGFR1 constructs, or the BCR-FGFR1 constructs, and immunoblotting was performed. Activation of mitogen activated protein kinase (MAPK) shows little to no difference between the FGFR1 wildtype and the BCR-FGFR1 fusion. However an increase in phosphorylation is observed from the wildtype to the kinase activated mutants. A strong increase in signal transducer and activator of transcription (STAT) signaling is seen between the FGFR1 WT and BCR-FGFR1 (Figure 4). An activation of both STAT3 and heavy activation of STAT5 is observed. Additionally, these lysates were immunoprecipitated with FGFR1 anti-sera, and were immunoblotted for tyrosine phosphorylation. Heavy tyrosine phosphorylation is seen in BCR-FGFR1, indicating that the contribution of the BCR to the fusion increases the constitutive phosphorylation of FGFR1. Interestingly BCR-FGFR1 (K514A), which contains BCR fused to a kinase dead FGFR1 does not activate either MAPK or STAT pathways nor phosphorylation on the receptor, suggesting that BCR relies on the constitutive kinase activity of FGFR1 for activation of downstream cell signaling.

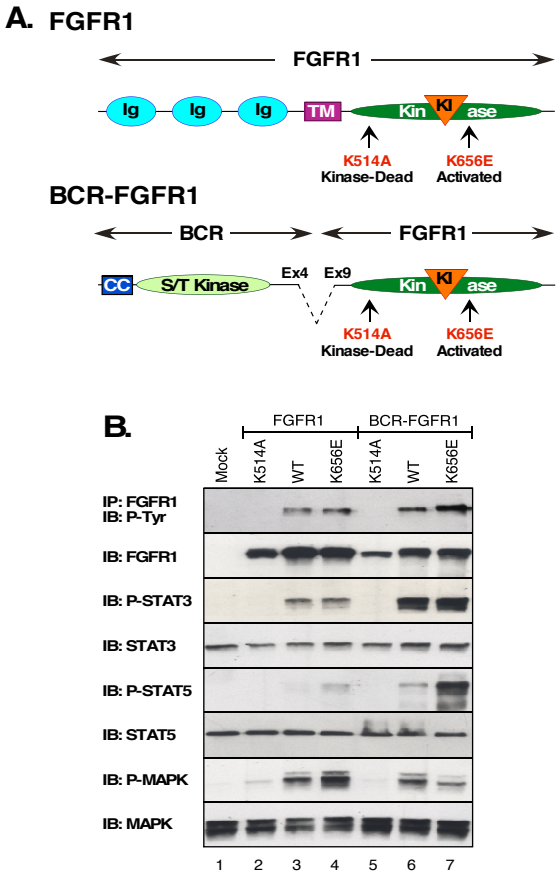


Figure 4. Activation of downstream cell signaling pathways by BCR-FGFR1. (A) Schematic of FGFR1 and BCR-FGFR1 with 514A kinase dead, and K656E kinase activating mutations. WT FGFR1 contains an extracellular ligand binding domain, a transmembrane domain, and a split kinase domain. BCR-FGFR1 contains BCR exon 4 at the N-terminus fused to the kinase domain of FGFR1 at exon 9 through a glycine serine (GS) linker region. BCR contributes a coiled-coil and a serine/threonine kinase domain to the BCR-FGFR1 fusion (B) Lysates of HEK293T cells expressing either FGFR1 or BCR-FGFR1 derivatives were immunoprecipitated with anti-FGFR1 antibody and immunolbotted with phospho-tyrosine antibody (1st panel). These lysates were also immunoblotted for total FGFR1 expression (2nd panel), and were also immunoblotted for phospho-STAT3 (Y705) (3rd panel), phospho-STAT5 (Y694) (5th panel) and phospho-MAPK (T202, Y204) (7th panel).

Cell Transforming ability of BCR-FGFR1 by Focus Assay

In order to investigate the transforming ability of BCR-FGFR1 and subsequent mutants, these constructs were placed in a NIH3T3 cell transforming assay. NIH3T3 cells are a murine cell line, which under normal conditions grow in a monolayer and express contact inhibition; however, these cells form foci when expressing oncogenic proteins (5). BCR-FGFR1, BCR-FGFR1(K656E), FGFR1(K656E) all expressed high levels of foci formation (Figure 5). Foci formation results were normalized to FGFR3-TACC3 as this fusion has previously demonstrated transforming ability. (6). BCR-FGFR1, BCR-FGFR1(K656E) and FGFR1(K656E) produced nearly 3 times as many foci as FGFR3-TACC3. BCR-FGFR1(K514A) which contains the kinase dead mutation in the kinase domain of FGFR1 was not transforming, indicating that the kinase activity of FGFR1 is critical in the transforming ability of this fusion. Additionally, lack of foci for BCR-FGFR1(K514A) suggests that the kinase activity of BCR is insignificant for cell transforming ability.

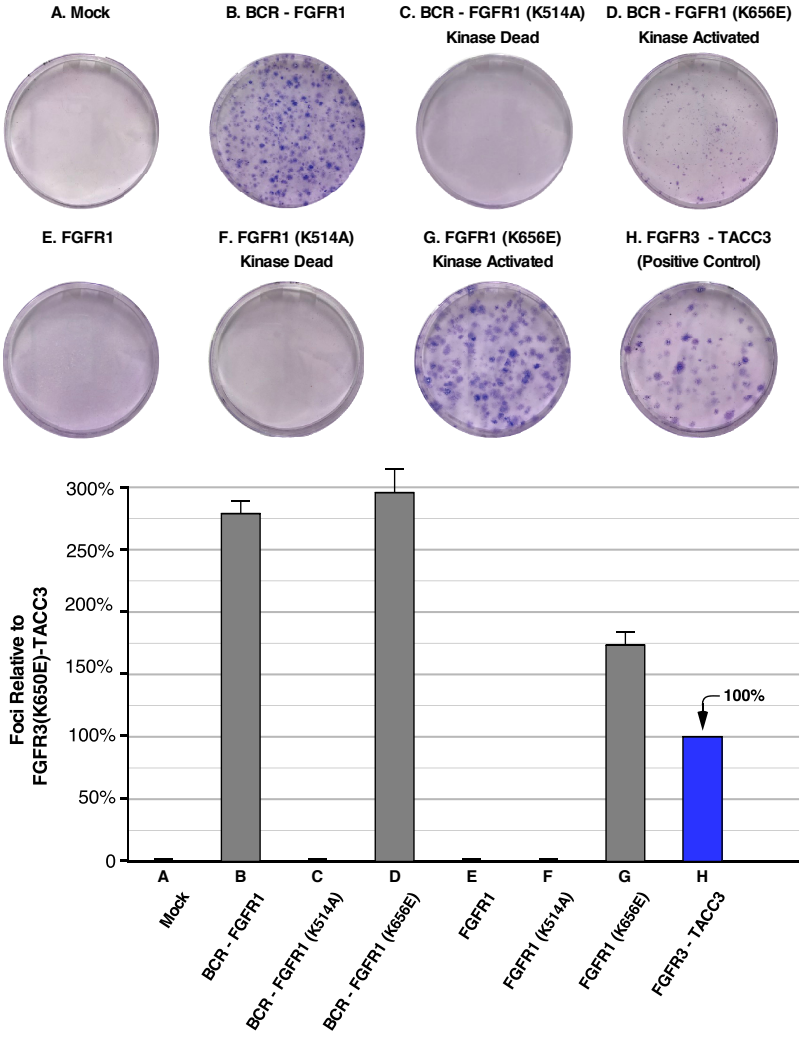


Figure 5. Cell transformation of NIH3T3 cells by BCR-FGFR1 and other derivatives. Plates from a focus assay are shown, with transfected constructs indicated. The number of foci were scored, normalized for transfection efficiency and calculated as a percentage of transformation relative to FGFR3-TACC3. Error is indicated as a relative error of the mean. Each assay was performed a minimum of 3 times.

LC/MS Analysis Identifies Novel Phosphorylation Sites

The strong tyrosine phosphorylation signal seen in BCR-FGFR1 and BCR-FGFR1(K636E) lysates though immunoblotting (Figure 4) lead to the inquiry of whether BCR-FGFR1 contains a constitutively active FGFR1 kinase, and if there were any novel phosphorylation sites found in this fusion. To further investigate this question, HEK293T cell lysate expressing either FGFR1 or BCR-FGFR1 derivatives were immunoprecipitated, followed by an on-bead trypsin digest. These samples were then analyzed via liquid chromatography-tandem mass spectroscopy (LC/MS) with titanium dioxide phospho-peptide enrichment (TiO_2) .

The LC/MS data shows that both BCR-FGFR1, and BCR-FGFR1(K656E), WT and kinase activated fusion, respectively, have robust phosphorylation levels, where as BCR-FGFR1(K514A), only sees a slight phosphorylation on Y558 in the kinase domain of FGFR1 (Figure 6). Y463, Y558, Y563, Y605, Y653, Y654 are all phosphorylated in BCR-FGFR1, and additional sites Y572, Y583, Y585, Y613 are detected in BCR-FGFR1 (K656E). This data suggests that the contribution of a coiled-coil dimerization domain by BCR leads to higher phosphorylation levels in the FGFR1 receptor. Conversely, the lack of phosphorylation on the activation loop tyrosines in BCR-FGFR1(K514A) indicates that FGFR1 kinase activity is critical for activation of the BCR-FGFR1 fusion.

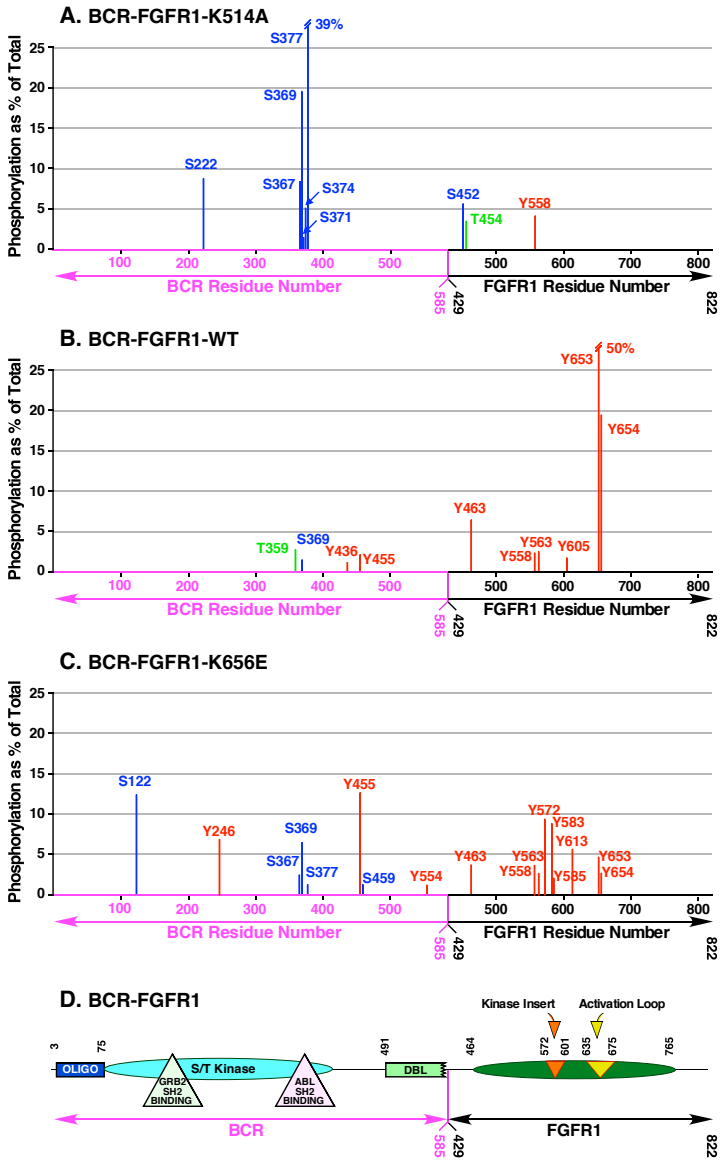


Figure 6. Phosphorylated sites on BCR-FGFR1 and derivatives. Only phosphorylation sites containing > 1% of total phosphorylation were graphed. (A) Phosphorylation sites on BCR-FGFR1(K514A), containing a kinase dead FGFR1. (B) Phosphorylation sites on BCR-FGFR1. (C) Phosphorylation sites on BCR-FGFR1 (K656E) containing a kinase activated FGFR1. (D) Schematic indicating domains on BCR-FGFR1 fusion protein. Amino acid positions labeled.

Although an increase in tyrosine phosphorylation is detected in FGFR1 for BCR-FGFR1 and BCR-FGFR1(K656E), additional tyrosine phosphorylation sites are also detected in BCR. Y436 and Y455 are both phosphorylated in BCR, when fused to either WT or kinase activated FGFR1 (Figure 6). These residues have not been previously reported in the literature, and what role phosphorylation plays on these residues is unknown. Residues Y436 and Y455 on BCR were mutated to phenylalanine in order to investigate the role of these residues in the oncogenic activity of this fusion protein. In addition to these mutations, a BCR Y177F mutant was also used as it mutates away the Grb2 binding site, which has previously been shown to reduce activation of the BCR-FGFR1 fusion protein (7). The Y177 site is not shown in the phosphorylation data since it showed less than 1% of the total phosphorylation for all samples.

The BCR(Y436F)-FGFR1, BCR(Y455F)-FGFR1, along with BCR-FGFR1, and BCR(Y177F)-FGFR1, were all assayed via NIH3T3 cell focus assay as described previously. Although all constructs displayed transformation ability, the Y177 Grb2 mutation shows a 50% decrease in transforming ability when compared to BCR-FGFR1. However, neither the Y436F, nor the Y455F mutation in BCR showed any decrease in foci formation when compared to BCR-FGFR1 (Figure 7).

Taken together, the LC/MS data and focus formation assay suggest that BCR-FGFR1 relies on an active FGFR1 kinase domain for transformation, and that BCR, although having serine/threonine kinase activity cannot act alone for the oncogenic activation of this fusion.

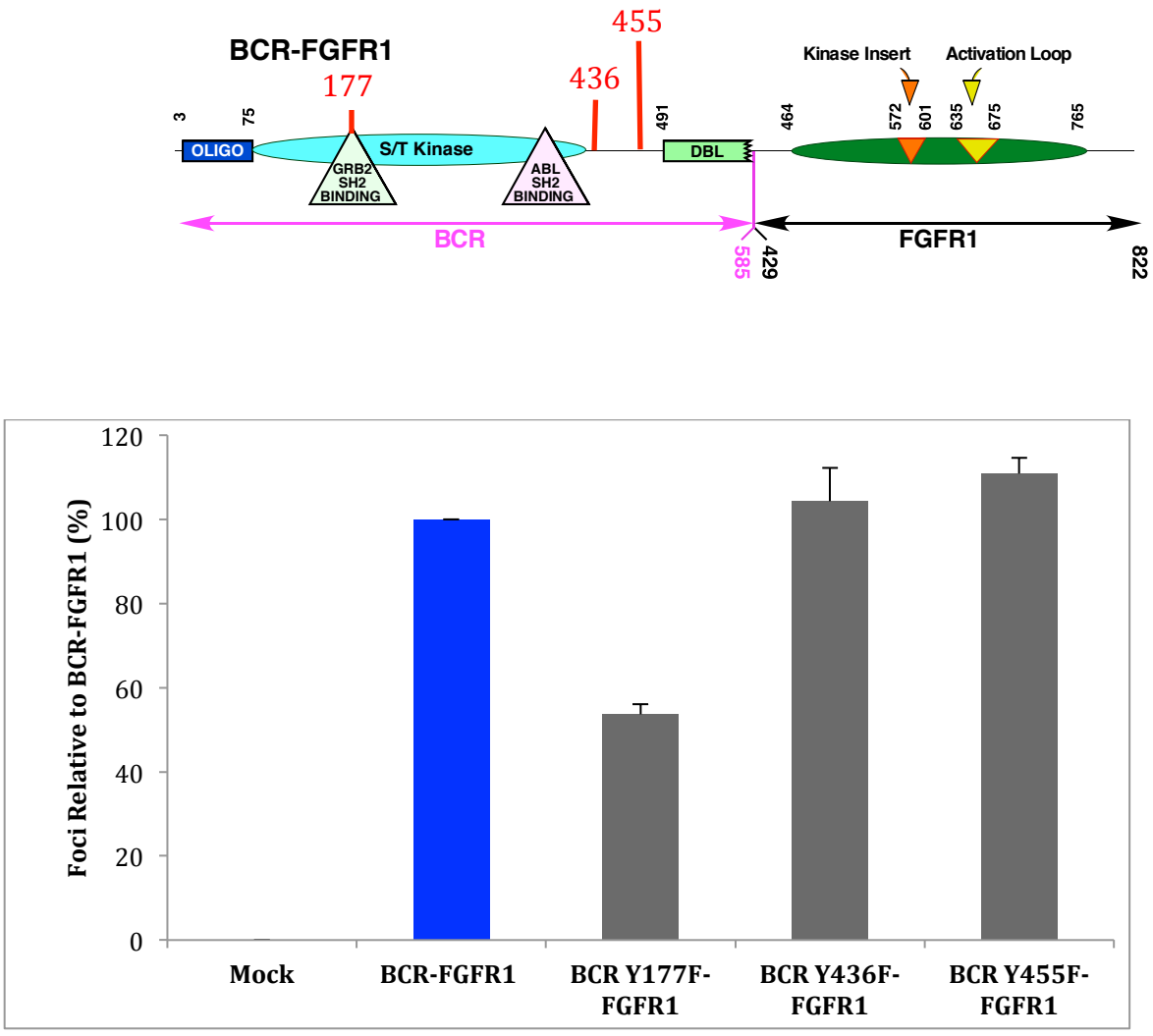


Figure 7. Focus assay results with BCR Y177F, BCR Y436F, BCRY455F mutations in BCR-FGFR1. Top panel shows schematic of BCR-FGFR1, with phospho-Tyrosine sites highlighted. These sites were mutated to phenylalanine in BCR-FGFR1 and assayed for foci formation. Bottom panel is a graph of the amount of foci produced by each mutation, compared to BCR-FGFR1 set to 100%.

Salt Bridge Disruption in BCR Dimerization Domain Abrogates Cell Transforming Ability

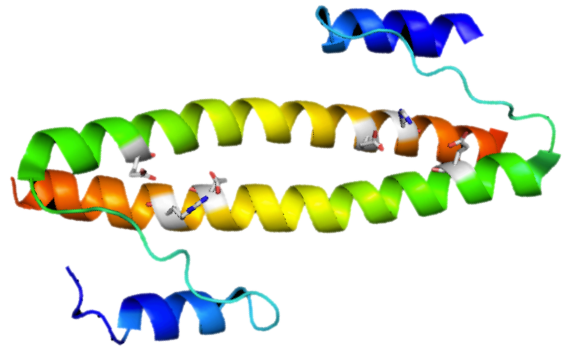
The BCR gene contains an anti-parallel coiled coil dimerization domain, a serine/threonine kinase domain, a guanine nucleotide exchange factor (GEF) domain, and a Rac GTPase activating protein domain (RacGAP) domain. The BCR-FGFR1 fusion protein contains BCR coiled coil domain, serine/threonine kinase domain and part of the GEF domain of BCR fused to the kinase domain of FGFR1 (Figure 6,7). The anti-parallel coiled-coil dimerization domain in BCR has previously been shown to be essential for cell transformation as demonstrated with assays done in BCR-ABL (8,9). Although the disruption of the dimerization domain has been completed with either an insertion of a 5 amino acid beta-proline turn sequence, or complete deletion of the dimerization domain, little is known about the necessity of biochemical interactions between amino acids in the dimerization domain. Here we investigate the importance of salt bridge formation in the BCR coiled-coil domain as a potential requirement of dimerization for BCR-FGFR1.

The coiled-coil region of BCR spans from amino acid residues 3-75 (Figure 7,8). Although BCR contains the typical heptad repeat pattern $(abcdefg)_n$ for coiled coils, where positions a and d correspond to hydrophobic residues, and positions e and g are charged, it also contains a charged E52 at position d, which is unusual. Previous work has shown that the E52 may be essential for stability of the coiled-coil domain (10). We hypothesize that E52 may interact and provide stability for a nearby salt bridge formed between residues E34 and R55 in BCR.

To further investigate the role of residues E34, E52, and R55 in BCR, various constructs were made which either mutated these residues to their respective opposite charges, mutated all

three charged residues to the opposite charge (BCR E34R/E52R/E55E), or mutated all three residues to proline (BCR E34P/E52P/E55P), these mutants were assayed for transformation ability in NIH3T3 cells (Figure 8). Here it is seen that the single point mutation of either BCR(E34R)-FGFR1, or BCR(E52R)-FGFR1 lowers the amount of foci observed by 10-30%. A 30% reduction in foci formation is seen when Arg55 is mutated to Glu (BCR R55E-FGFR1), thus creating opposite charges in the predicted salt bridge and stabilizing residue. Interestingly, a 85% reduction in foci is seen when BCR Glu34 is mutated to Arg along with the Glu52 to Arg mutation (BCR E34R/ E52R-FGFR1). This "all R" mutant displays opposing charges in the predicted salt bridge, thereby abolishing salt bridge formation. Additionally, a loss of transforming ability in NIH3T3 cells is also observed through the "all proline" mutant, where BCR residues E34, 352, and R55 were all mutated to proline. Lastly, when all charges are reversed to the opposite charge (BCR E34R/E52R/E55E), the salt bridge is potentially re-established as this mutant retains 100% transforming ability when compared to BCR-FGFR1.

The focus assay data demonstrates that salt bridge formation between residues E34 and R55, along with potential interaction of the stabilizing residue at E52 in BCR are all critical in the transforming ability of BCR-FGFR1. The loss of foci formation as seen through the BCR(E34R/E52R)-FGFR1 mutant confirms the necessity of salt bridge interaction for dimerization in BCR-FGFR1. This data together suggests that targeting the dimerization domain of BCR could be a therapeutic target in patients positive for the BCR-FGFR1 fusion.



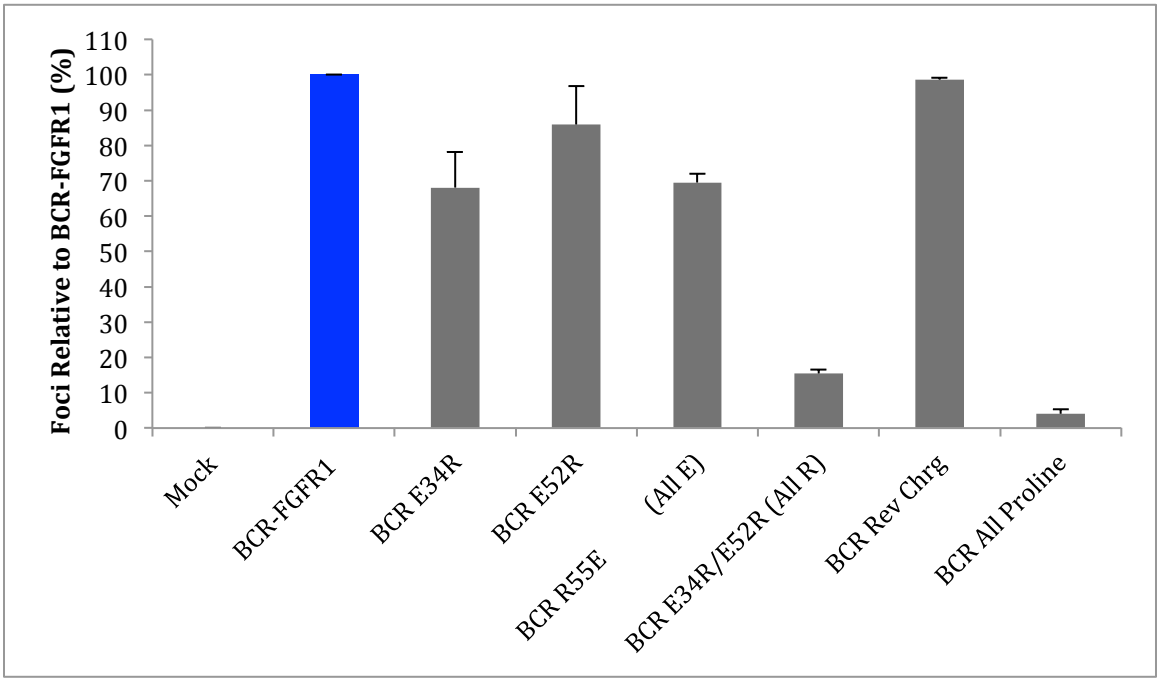


Figure 8. Focus assay results with potential salt bridge mutations made in BCR coiled coiled-coil domain. All amino acid numbers correspond to residues in BCR. Top figure is solved crystal structure of BCR oligomerization domain (PDB 1K1F), with residues of interest shown in stick model. Bottom panel shows focus assay results with BCR-FGFR1 set to 100%.

BCR-FGFR1 is an HSP90 Addicted Oncoprotein

Heat shock protein 90 (Hsp90) is a highly conserved, ubiquitously expressed molecular chaperone that controls the stability of certain proteins (11, 12). Prior work has shown that Hsp90 is overexpressed in certain cancers, and the Hsp90 complex provides stability for various oncogenic proteins, which are necessary for cancer cell survival (13). Many of these oncogenes, such as mutated P53 or BCR-ABL take advantage of the Hsp90 chaperone system to avoid ubiqitination and proteasomal degradation (14). Specifically, Hsp90 and co-chaperones cell division cycle 37 (Cdc37), and cyclin dependent kinase 4 (cdk4) have been shown to act in complex to aid the maturation and development of various client kinases, showing association with roughly half of the human kinome (15). This work demonstrates that BCR-FGFR1 is a client of Hsp90 and possibly relies on the Hsp90 complex for stability. Additionally, BCR-FGFR1 is sensitive to Ganatespib (STA-9090), a potent Hsp90 inhibitor, which could serve as a potential therapeutic target in patients positive for the BCR-FGFR1 fusion.

HEK293T lysates expressing either FGFR1 or BCR-FGFR1 derivatives were immunoprecipitated with FGFR1 antisera and immunoblotted for Hsp90. A strong signal is observed via western blot for BCR-FGFR1 and its derivatives, were as a faint signal is seen for FGFR1 (Figure 9). This result indicates that Hsp90 could have a strong interaction with BCR-FGFR1, and FGFR1 as well. To further analyze if BCR-FGFR1 is dependent on Hsp90 for cellular stability, assays with Hsp90 inhibitor, Ganatespib, were performed. HEK293T cells expressing either FGFR1 or BCR-FGFR1 derivatives were analyzed for overall FGFR1 expression, and activation of MAPK and STAT3 pathways, and phospho-tyrosine both with and without addition of Ganatespib. HEK293T cells without treatment with Ganatespib see similar

levels of FGFR1 expression, MAPK, STAT3 and phosphorylated FGFR1 receptor similar to Figure 1. However, when HEK293T cells were treated with 200nM Ganatespib for 4 hours, a significant reduction in FGFR1 expression is observed through western blot(Figure 9). Furthermore, a decrease of MAPK and STAT3 activation is also seen, as well as a loss of phosphorylated FGFR1 receptor (Figure 6). The dramatic decrease in FGFR1 expression with the addition of Ganatespib suggests that BCR-FGFR1 is a client protein of Hsp90, and could potentially use the Hsp90 complex for protein stability within the cell.

To investigate if BCR-FGFR1 relied on Hsp90 for cell transformation, NIH3T3 cells expressing FGFR1 or BCR-FGFR1 derivatives were assayed for foci formation with increasing concentrations of Ganatespib. Here it is observed that increasing concentrations of Ganatespib reduces foci formation when compared to control cells that were dosed with dimethyl sulfoxide (DMSO) (Figure 9). Therefore, this data suggests that BCR-FGFR1 is dependent on the molecular chaperone Hsp90 for cellular transformation.

Taken together, this data suggests that BCR-FGFR1 is a client of Hsp90, and that this fusion protein relies on the Hsp90 complex for protein stability within the cell. When treated with potent Hsp90 inhibitor, Ganatespib, over all expression of BCR-FGFR1 decreases, along with a decrease in activation of MAPK and STAT3 pathways. Furthermore, this data suggests that BCR-FGFR1 depends on Hsp90 for cell transformation and foci formation, indicating therapies that target Hsp90 in BCR-FGFR1 driven cancers could be therapeutically beneficial for patients positive for this fusion.

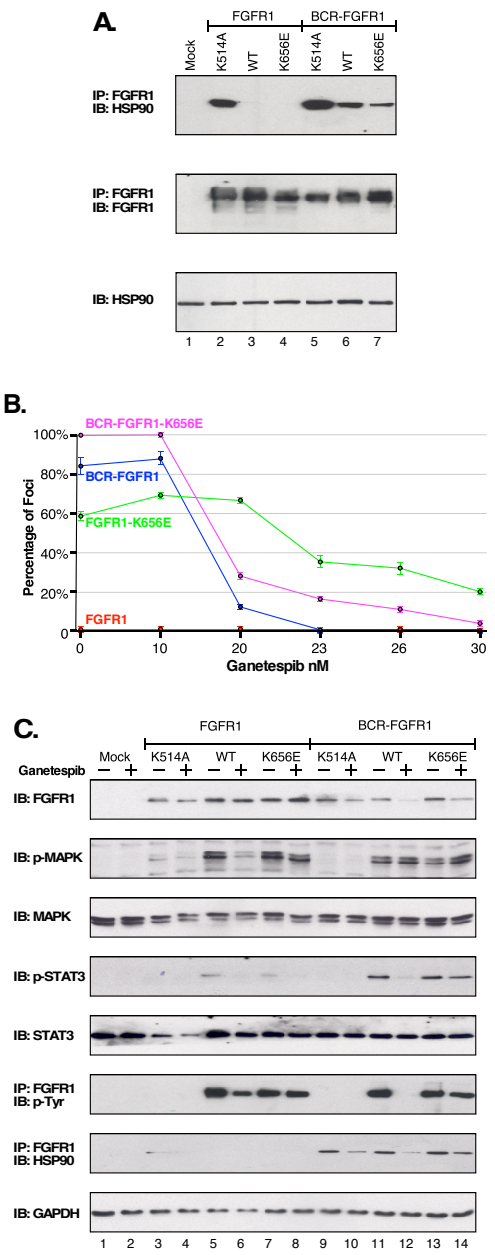


Figure 9. BCR-FGFR1 shows Hsp90 addiction. (A) Immunoprecipitation with FGFR1 and blotted for Hsp90 to show interaction between FGFR1 and Hsp90. (B) Graph of foci formation with addition of Hsp90 inhibitor, Ganatespib, performed in NIH3T3 cells. BCR-FGFR1(K656E) set to 100%. (C) Western blots with HEK293T cells. Assayed for FGFR1 expression, phospho-MAPK, phosphor-STAT3, and phosphor-Tyrosine. GAPDH expression is used as control.

DISCUSSION

Through the data presented, we were able to extensively characterize the fusion protein BCR-FGFR1. With the emergence of personalized medicine and cancer tumor sequencing, characterization and analysis of mutations such as the described chromosomal translocation is vital. We demonstrate that the N-terminal introduction of BCR results in constitutive activation of FGFR1 on key tyrosine residues. Through our cell signaling studies, we demonstrate that BCR-FGFR1 over activates crucial downstream cell signaling pathways MAPK, STAT3 and STAT5. The loss of both FGFR1 receptor phosphorylation and activation of MAPK and STAT pathways by BCR-FGFR1(K514A) kinase dead mutant indicates that FGFR1 kinase activity is necessary for gain of function and cancer progression. This result is furthermore confirmed through cell transformation and focus formation assays. Both BCR-FGFR1 and BCR-FGFR1(K656E) displayed cell transformation and foci formation, however the kinase dead BCR-FGFR1(K514A) was not transforming. The high oncogenic potential of BCR-FGFR1 is characterized through its activation of downstream cell signaling pathways and nearly three-fold increase in foci formation when compared to FGFR3-TACC3, a fusion protein previously characterized in our lab.

We have also described a novel inhibition of the BCR dimerization domain through disruption of salt-bridge formation of the anti-parallel coiled-coil. The lack of foci formation and cell transformation when residues E34 and E55 are mutated to Arg suggest the disruption of the salt bridge between these residues has a causal effect on the dimerization ability of BCR. The

potential loss of dimerization and near absence in foci formation suggests that the coiled-coil dimerization domain can be used as a potential therapeutic target.

The interaction and dependence on Hsp90 for the stability of BCR-FGFR1 is shown through both foci formation assays as well as investigation of overall BCR-FGFR1 protein expression. We see a large decrease in expression of BCR-FGFR1 with the addition of Ganatespib, a potent Hsp90 inhibitor. Furthermore, a decrease in phospho-FGFR1, along with a decrease in MAPK and STAT signaling when cells expressing BCR-FGFR1 are treated with Ganatespib indicates that BCR-FGFR1 is sensitive to this drug treatment. Additionally, the loss of foci formation as seen through cell transformation assay confirms the dependence of BCR-FGFR1 on the Hsp90 molecular chaperone complex to avoid proteasomal degradation.

We have presented overwhelming evidence for the oncogenicity of the BCR-FGFR1 fusion protein. With personalized medicine becoming more commonplace, the characterization of mutations such as this fusion is essential in providing proper treatment. Although tyrosine kinase inhibitor therapy has shown to be beneficial for patients harboring certain mutations, there are currently no known cures for patients positive for the BCR-FGFR1 translocation. Here we also describe novel therapeutic strategies for patients who are positive for this fusion, suggesting that BCR dimerization inhibitors, Hsp90 inhibitors, and chemotherapy in combination may be a beneficial therapeutic strategy in patients.

MATERIALS AND METHODS

DNA Constructs

The BCR gene was purchased from Addgene (pSG65-Bcr) and was subcloned into pcDNA3. FGFR1 and FGFR1(K656E) were developed as previously described (4). FGFR1 (K514A) was made through PCR based site directed mutagenesis. All PCR reactions used Pfu Turbo polymerase (Agilent). To construct BCR-FGFR, a BamHI site was introduced through PCR based site directed mutagenesis after amino acid L584 in BCR and before amino acid V429 in FGFR1. This unique BamHI site was used to subclone 5' BCR into FGFR1 pCDNA3, creating a fusion breakpoint of BCR exon 4 fused to FGFR1 exon 9. The BamHI site contained a GS linker region which fuses 5' BCR to 3' FGFR1.

DNA fragments containing the K656E mutation or the K514A mutation were either subcloned or were introduced through PCR based site directed mutagenesis, the same technique was used for all pLXSN constructs as well. All single and multiple mutations for assays with phosphorylation site mutations, or dimerization domain mutants were made with PCR based site directed mutagenesis.

pcDNA3 vector was used for all experiments with HEK293T cells for western blotting. pLXSN vector was used for all experiments with NIH3T3 cell focus assays. All DNA constructs were fully sequenced.

Cell Culture

HEK293T cells were maintained in 10% Fetal Bovine Serum (FBS) in DMEM media with 1% penicillin/streptomycin in 10% CO_2, 37 °C. NIH3T3 cells were maintained in 10% Fetal Calf Serum (CS) in DMEM media with 1% penicillin/streptomycin 10% CO_2, 37 °C.

Cell Transfection, Immunoprecipitation, Immunobloting

For HEK293T cell work, cells were first plated to a density of 1x 10^6 cells per 100mm plate. These cells were then transfected with 3μg pcDNA3 constructs as described with calcium phosphate transfection protocol. Cells were then incubated at 3% CO_2 37 °C for 17 hours and then recovered via incubation at 10% CO_2, 37 °C for 6-8 hours. These cells were then serum deprived (starved) in 0% FBS/DMEM for 18 hours. Cells were washed in 1x ice-cold PBS and then were lysed in radioimmunoprecipitation assay buffer [RIPA; 50 mmol/L Tris-HCl (pH 8.0), 150 54 mmol/L NaCl, 1% TritionX-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSF, and 10 μg/mL aprotinin]. Bradford assay or Lowry assay was used to measure total protein concentration. Antibodies were added to lysates for overnight incubation at 4°C with rocking, followed by immunoprecipitation, as described previously. Samples were separated by 10% or 12.5% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were blocked in 3% milk/TBS/0.05% Tween 20 or 3% bovine serum albumin (BSA)/TBS/0.05% Tween 20 (for anti-phosphotyrosine, anti–phosphoSTAT1, and anti–phospho-STAT3 blots).

For NIH3T3 cells, cells were plated to a density of 4x 10^5 cells per 60mm plate. These cells were then transfected with 10μg of pLXSN constructs are described with Lipofectamine 2000 reagent from Invitrogen. 16 hours following transfection, Lipofectamine reagent was aspirated off, and cells were allowed to recover in 10% CS/DMEM. 48 hours following transfection, cells were split 1:10 onto 100mm plates containing either 2.5% CS/DMEM or 500μg/mL Geneticin. (G418) The cells split onto the 2.5% CS/DMEM plates were used as focus

assay plates, whereas cells on the G418 plates were used to as a control for transfection efficiency. 18 days following transfection, both focus and G418 plates were fixed with methanol, stained with Giemsa stain, and scored. The foci were normalized against the G418 plates for transfection efficiency.

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