UNIVERSITY OF CALIFORNIA, SAN DIEGO

Oncogenic Potential of Fibroblast Growth Factor Receptor Translocations

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Chemistry

by

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The Thesis of Katelyn N. Nelson is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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2015

DEDICATION

This thesis is dedicated to my parents and grandparents, without whom college and graduate school would have never been possible. Thank you for encouraging me always and teaching me the importance of hard work. This thesis is also dedicated to Randy Au. He may have lost his battle with cancer, but in his memory we persevere.

EPIGRAPH

Science is simply the word we use to describe a method of organizing our curiosity.

-Tim Minchin

It's not important how many times you fall, but how many times you rise.

-Nelson Mandela

The expert in anything was once a beginner.

-Helen Hayes

Play is the highest form of research.

-Albert Einstein

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Chapter 2, in part is currently being prepared for submission for publication of the material, with the authors of Nelson KN, Meyer AN, Siari A, Donoghue DJ. The thesis author was the primary investigator and author of this material.

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PUBLICATIONS

Gallo LH, Meyer AN, Motamedchaboki K, Nelson KN, Haas M, Donoghue DJ. Novel Lys63-linked ubiquitination of IKKβ induces STAT3 signaling. Cell Cycle 2014;13(24):3964-76.

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ABSTRACT OF THE THESIS

Oncogenic Potential of Fibroblast Growth Factor Receptor Translocations

by

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Master of Science in Chemistry

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Professor Daniel J. Donoghue, Chair

Fibroblast Growth Factor Receptors (FGFRs) are critical for cell proliferation and differentiation. Mutation and/or translocation of FGFRs lead to aberrant signaling that often results in developmental syndromes or cancer growth. As sequencing of human tumors becomes more frequent, so does the emergence of FGFR translocations and fusion proteins. The research conducted in this work will focus on a frequently identified fusion protein between FGFR3 and transforming acidic coiled-coil containing protein 3 (TACC3). Through titanium dioxide-based phosphopeptide enrichment (TiO2)-liquid chromatography (LC)-high mass accuracy tandem mass spectrometry

(MS/MS), it is apparent that the fused coiled-coil TACC3 domain results in constitutive phosphorylation of key activating FGFR3 tyrosine residues. Fusion of FGFR3 and TACC3 also results in MAPK pathway activation, nuclear localization, and cell transformation. Introduction of K508R FGFR3 kinase dead mutation abrogates these effects, except for nuclear localization which is due solely to the TACC3 domain. FGFR3-TACC3 also produces IL-3 independent growth and cell proliferation. Taken together, these results suggest that FGFR3 kinase activity is essential for the oncogenic effects of the FGFR3-TACC3 fusion protein and could serve as a therapeutic target.

Chapter 1

Functions of Fibroblast Growth Factor Receptors in Cancer Defined by Novel Translocations and Mutations

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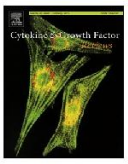

Mini review

Functions of Fibroblast Growth Factor Receptors in cancer defined by novel translocations and mutations

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ABSTRACT

The four receptor tyrosine kinases (RTKs) within the family of Fibroblast Growth Factor Receptors (FGFRs) are critical for normal development but also play an enormous role in oncogenesis. Mutations and/or abnormal expression often lead to constitutive dimerization and kinase activation of FGFRs, and represent the primary mechanism for aberrant signaling. Sequencing of human tumors has revealed a plethora of somatic mutations in FGFRs that are frequently identical to germline mutations in developmental syndromes, and has also identified novel FGFR fusion proteins arising from chromosomal rearrangements that contribute to malignancy. This review details approximately 200 specific point mutations in FGFRs and 40 different fusion proteins created by translocations involving FGFRs that have been identified in human cancer. This review discusses the effects of these genetic alterations on downstream signaling cascades, and the challenge of drug resistance in cancer treatment with antagonists of FGFRs.

1. Overview of canonical FGFR signaling

Receptor tyrosine kinases (RTKs) represent important signal transducers in the cell membrane and are comprised of nearly twenty families of homologous proteins in humans, with almost 60 distinct members [1]. In the FGFR family, four homologous human receptors have been identified: FGFR1, FGFR2, FGFR3 and FGFR4. All of the FGFRs exhibit three extracellular immunoglobulin (Ig)-like domains, a membrane-spanning segment and a split tyrosine kinase domain. Fibroblast Growth Factors (FGFs), a large family of related growth factors, act in concert with heparin sulfate proteoglycans (HSPGs) as high-affinity FGFR agonists [2,3]. The splicing of FGFRs results in further distinction of ligand specificity accompanied by altered biological properties, in which the most studied splicing isoforms involve the third immunoglobulin-like domain of the receptors [4]. For FGFR2 and FGFR3, the first half of third Ig domain consists of an invariant exon (IIIa), and splicing of the second half of third Ig domain results in either IIIb isoform (exons 7 and 8) or IIIc isoform (exons 7 and 9). Generally, the IIIb isoforms of FGFRs are expressed in tissues of epithelial origin whereas the IIIc isoforms are expressed in mesenchymal tissues [5].

Binding of FGF/HSPG to FGFR induces the dimerization of receptor monomers in the plasma membrane, followed by trans-autophosphorylation of tyrosine residues located in the cytoplasmic kinase domain. This tyrosine phosphorylation triggers the binding of Src homology (SH2) domain of phospholipase C gamma (PLCγ) to the receptor, resulting in the activation of PKC. Activation also induces RAS–MAPK and PI3K–AKT signaling *via* FRS2 and GRB2 adaptor proteins. Additional pathways activated by FGFRs include Jun N-terminal kinase and JAK/STAT pathways. FGFR signaling results in cellular proliferation and migration, anti-apoptosis, angiogenesis and wound healing (Fig. 1) [6].

Abbreviations: ARMS, alveolar rhabdomyosarcoma; BSS, Beare Stevenson cutis gyrata syndrome; CFS, chromosomal fragile site; CC, coiled coil domain; EMS, 8p11 myeloproliferative syndrome (EMS); ERMS, embryonal rhabdomyosarcoma; FN, fibronectin domain; Ig, immunoglobulin-like domain; IMD, IRSp53/MIM domain; ITD, internal tandem duplication; JM, juxtamembrane domain; LIsH, LIS1-homologous domain; LZ, leucine zipper domain; KD, kinase domain; KI, kinase insert domain; LADD, lacrimo auriculo dento digital syndrome; ORF, open reading frame; RMS, rhabdomyosarcoma; SAM, sterile alpha motif; SADDAN, severe achondroplasia with delayed development and acanthosis nigricans; SP, signal peptide; SPFH, stomatin/prohibitin/flotillin/HflK/C domain; TK domain, tyrosine kinase domain; TD, thanatophoric dysplasia; TM, transmembrane domain; ZF, zinc finger domain.

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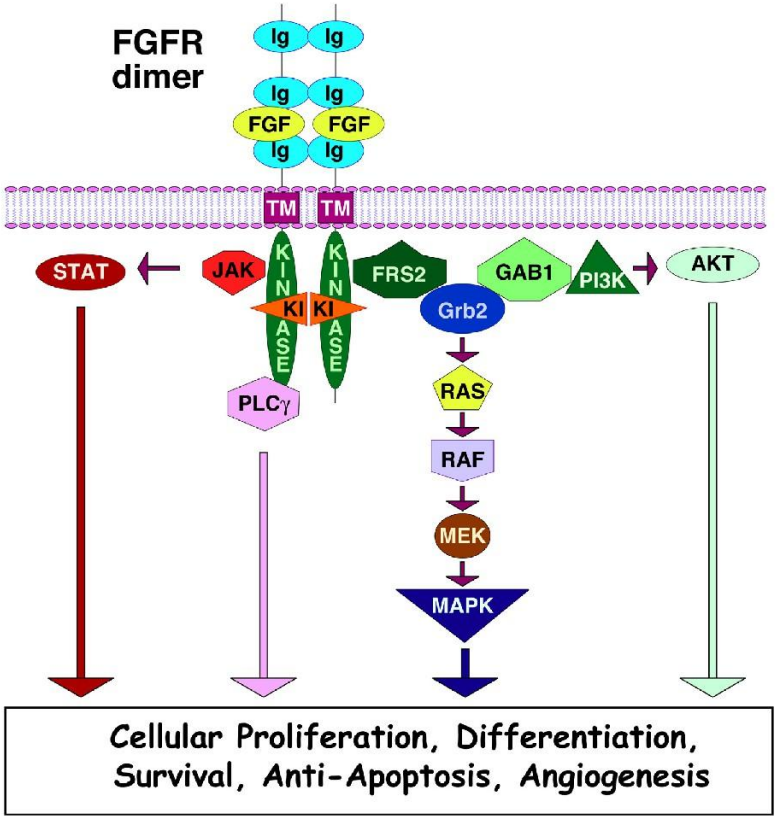

Fig. 1. FGFR signaling pathways. FGF ligand binds to FGFR monomers, leading to the dimerization and subsequent tyrosine autophosphorylation of the receptor. This event leads to activation of FGFRs and various downstream proteins, resulting in cellular proliferation, differentiation, survival, anti-apoptosis and angiogenesis.

2. FGFRs are mutated in human syndromes and cancers

2.1. Nomenclature of mutations with respect to isoforms

The alternatively spliced isoforms of FGFR1, FGFR2 and FGFR3 result in considerable confusion in numbering specific mutations, depending upon the convention employed by the original authors. In Table 1, we have presented the residue numbers in FGFR1 for both the αA1 and αB1 isoforms, in FGFR2 for the IIIb and IIIc isoforms, in FGFR3 for the IIIb and IIIc isoforms, and FGFR4 for the Uniprot P22455-1 and P22455-2 isoforms. Throughout this manuscript, we will refer to the numbering for the isoforms FGFR1 αA1, FGFR2 IIIb, FGFR3 IIIb, and full-length FGFR4 (Uniprot P22455-1), although a specific mutation may have been described initially in the other isoform. Rarely, a mutation may occur at a residue that is not present in either of the most common isoforms; in these unusual cases, this other isoform is identified in Table 1.

2.2. Cysteine mutations in the extracellular domain lead to aberrant activation of FGFRs

Many mutations in the extracellular domains of FGFRs induce tyrosine kinase activation by disulfide bond disruption. For instance, each Ig domain of FGFR2 is stabilized by a disulfide bond between pairs of cysteine residues: Cys62 and Cys107 in Ig-I, Cys179 and Cys231 in Ig-II, Cys278 and Cys340 in Ig-III [7]. Mutations in FGFR2 that perturb a disulfide bond in the extracellular domain result in increased receptor activation, such as the C278F mutation in Crouzon and Pfeiffer Syndromes, or the mutation of C340 to S or Y in Crouzon Syndrome. These are examples of craniosynostosis syndromes exhibiting premature closure of cranial sutures, accompanied by defects in chondrocyte signaling and brain development [8]. This same theme is recapitulated in somatic mutations involved in human cancer as exemplified by the C278F mutation and the mutations C340F/R/S/W/Y identified in spermatocytic seminoma [9]. Conceptually similar mutations that remove a critical Cys residue also occur in FGFR3 and FGFR4 (Table 1, Fig. 2).

Conversely, the *addition* of a single cysteine mutation creates an unpaired cysteine that can participate in abnormal intermolecular disulfide bond formation leading to receptor activation. One such example is FGFR2 W290C, a mutation causing Pfeiffer Syndrome, which has also been identified in lung squamous cell carcinoma and spermatocytic seminoma (Table 1). A conceptually similar mutation is that of FGFR2 S352C in Crouzon Syndrome [10,11], also identified in spermatocytic seminoma. Other examples of FGFR2 mutations that introduce a new cysteine residue in the extracellular domain include R203C, Y281C, S320C, Y338C, and S373C, which have been identified in various cancers including breast cancer, endometrial carcinoma, lung squamous cell carcinoma and

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Table 1
Mutations in FGFRs identified in diverse human cancers.

FGFR1						
Disease	Mutation/isoform			Location in receptor	Role in developmental syndromes	Reference
	Residue in αA1	Residue in αB1	Other isoform			
Breast cancer	S125L	S125L		IgI–IgII		[141]
Colorectal cancer	P150S	P150S	βA1: P61S	IgI–IgII		[142]
	A268S	A268S		IgII–IgIII		[34]
	S430F	S428F	A	JM		[142]
	A431S	A429S		JM		[143]
	G610D	G608D	A	KD2		[142]
Esophageal adenocarcinoma	K598N	K596N		KD2		[144]
Gallbladder cancer	S125L	S125L		IgI–IgII		[107]
Gastric cancer	A268S	A268S		IgII–IgIII		[34]
Glioblastoma	N546K	N544K		KD1	Analogous to FGFR2 N549K in Crouzon and Pfeiffer Syndromes; Analogous to FGFR3 N540K in Hypochondroplasia	[145]
	R576W	R574W		KD1		[145]
	K656E	K654E		KD2	Analogous to FGFR2 mutation K659E in Syndromic Craniosynostosis; Analogous to FGFR3 mutation K650E in TDII	[65]
Head and neck squamous cell carcinoma	n/a	n/a	P11362-21 G33R	N-term		[146]
	E334Q	E334Q		IgIII		[70]
Lung adenocarcinoma	P252T	P252T		IgII–IgIII	Mutation causes Pfeiffer Syndrome; Analogous to FGFR2 mutations at P253 in Pfeiffer and Apert Syndromes; Analogous to FGFR3 mutation at P250 in Syndromic Craniosynostosis	[141,147]
Lung large cell carcinoma	V664L	V662L		KD2		[141]
Lung squamous cell carcinoma	G70R	G70R		IgI		[148]
	T141R	T141R		IgI–IgII		[148]
Melanoma	P252S	P252S		IgII–IgIII	Mutation causes Pfeiffer Syndrome; Analogous to FGFR2 mutations at P253 in Pfeiffer and Apert Syndromes; Analogous to FGFR3 mutation at P250 in Syndromic Craniosynostosis	[34,149]
Pilocytic astrocytoma	N546K	N544K		KD1	Analogous to FGFR2 N549K in Crouzon and Pfeiffer Syndromes; Analogous to FGFR3 N540K in Hypochondroplasia	[60]
	K655I	K653I		KD2		[60]
	K656D/E/M/N	K654D/E/M/N		KD2	Analogous to FGFR2 mutation K659E in Syndromic Craniosynostosis; Analogous to FGFR3 mutations: K650E in TDII, K650M in SADDAN, K650N in Hypochondroplasia	[60]
	T658P	T656P		KD2		[60]
Prostate cancer	R78H	R78H		IgI		[34]
Rosette forming glioneuronal tumor	N546K	N544K		KD1	Analogous to FGFR2 N549K in Crouzon and Pfeiffer Syndromes; Analogous to FGFR3 N540K in Hypochondroplasia	[64]
	K656E	K654E		KD2	Analogous to FGFR2 mutation K659E in Syndromic Craniosynostosis; Analogous to FGFR3 mutation K650E in TDII	[64]

Table 1 (*Continued*)

FGFR1

Disease	Mutation/isoform			Location in receptor	Role in developmental syndromes	Reference
	Residue in αA1	Residue in αB1	Other isoform			
Spermatocytic seminoma	P252R/T	P252R/T		IgII–IgIII	Mutation causes Pfeiffer Syndrome; Analogous to FGFR2 mutations at P253 in Pfeiffer and Apert Syndromes; Analogous to FGFR3 mutation at P250 in Syndromic Craniosynostosis	[9]
	N330I	N330I		IgIII	Mutation causes Osteoglophonic Dysplasia	[9]
	Y374C	Y374C		TM	Mutation causes Osteoglophonic Dysplasia; Analogous to FGFR2 Y375C in Beare–Stevenson Cutis Gyrata Syndrome; Analogous to FGFR3 Y373C in TDI	[9]
	C381R	C381R		TM	Mutation causes Osteoglophonic Dysplasia	[9]
	R576W	R574W		KD1		[9]

FGFR2

Disease	Mutation/isoform			Location in receptor	Role in developmental syndromes	Reference
	Residue in IIIb	Residue in IIIc	Other isoform			
Adenoid cystic carcinoma	Y376C	Y375C		TM	Mutation causes Beare–Stevenson Cutis Gyrata Syndrome; Analogous to FGFR1 Y374C in Osteoglophonic Dysplasia; Analogous to FGFR3 Y373C in TDI	[150]
	K642R	K641R		KD2	Mutation causes Pfeiffer Syndrome	[150]
Bladder cancer	M186T	M186T	P21802-20 M71T	IgII	Mutation causes Apert Syndrome	[34]
Breast cancer	R203C	R203C		IgII		[12,141]
	N550K	N549K		KD1	Mutation causes Crouzon and Pfeiffer Syndromes; Analogous to FGFR3 N540K in Hypochondroplasia	[92]
	S588C	S587C		KI		[92]
	K660N	K659N		KD2	Mutation causes Syndromic Craniosynostosis; Analogous to FGFR3 mutation K650N in Hypochondroplasia	[12]
Cervical squamous cell carcinoma	A97T	A97T		IgI		[23]
	S252L	S252L		IgII–IgIII	Mutation causes Apert Syndrome; Mutations in FGFR3 at S249 cause TDI	[58]
	P256S	P256S		IgII–IgIII		[58]
	K406E	K405E		JM		[58]
	M585V	M584V		KI		[58]
	Y589D	Y588D		KI		[58]
	K660M	K659M		KD2	Mutation causes Syndromic Craniosynostosis; Analogous to FGFR3 mutation K650M in SADDAN	[23]
Colorectal cancer	R203H	R203H	P21802-20 R88H	IgII		[142]
	R210Q	R210Q	P21802-20 R95Q	IgII		[142]
	D334N	D336N	P21802-20 D221N	IgIII		[142]
	Q361R	n/a		IgIII-TM		[151]
	L552I	L551I		KD1		[142]
	P583L	P582L		KI		[151]
	R665W	R664W		KD2		[142]
	E778K	E777K		C-term		[142]

Endometrial carcinoma	D101Y	D101Y		IgI		[23]
	S252W	S252W		IgII–IgIII	Mutation causes Apert Syndrome; FGFR3 mutation at S249 causes TDI	[23,32,152]
	P253R	P253R		IgII–IgIII	Mutation causes Apert Syndrome; Analogous to FGFR1 mutations at P252 in Pfeiffer Syndrome; Analogous to FGFR3 mutation at P250 in Muenke Syndrome and Nonsyndromic Craniosynostosis	[23,152]
	K310R	K310R		IgIII		[23,32]
	n/a	A314D		IgIII	Mutation causes Pfeiffer Syndrome and Unicoronal Non-Syndromic Craniosynostosis	[23]
	n/a	A315T		IgIII	Mutation causes Crouzon Syndrome and Unicoronal Non-Syndromic Craniosynostosis	[32]
	S373C	S372C		IgIII–TM	Mutation causes Beare–Stevenson Cutis Gyrata Syndrome	[32,152]
	Y376C	Y375C		TM	Mutation causes Beare–Stevenson Cutis Gyrata Syndrome; Analogous to FGFR1 Y374C in Osteoglophonic Dysplasia; Analogous to FGFR3 Y373C in TDI	[32,152]
	C383R	C382R		TM	Analogous to FGFR1 C381R in Osteoglophonic Dysplasia	[23,32,152]
	A390T	A389T		TM		[23]
	M392R	M391R		TM		[32,152]
	V396D	V395D		TM		[152]
	L398M	L397M		TM		[152]
	I548D/V	I547D/V		KD1		[32,152]
	N550H/K	N549H/K		KD1	Mutation causes Crouzon Syndrome; Analogous to FGFR3 N540K in Hypochondroplasia	[23,32,152]
	K660E/M/N	K659E/M/N		KD2	Mutation causes Syndromic Craniosynostosis; Analogous to FGFR3 mutations: K650E in TDII, K650M in SADDAN, K650N in Hypochondroplasia	[23,32,152]
Esophageal adenocarcinoma	C383R	C382R		TM	Analogous to FGFR1 C381R in Osteoglophonic Dysplasia	[144]
Gallbladder cancer	S252W	S252W		IgII–IgIII	Mutation causes Apert Syndrome; FGFR3 mutation at S249 causes TDI	[107]
	N550K	N549K		KD1	Mutation causes Crouzon and Pfeiffer Syndromes; Analogous to FGFR3 N540K in Hypochondroplasia	[107]
Gastric cancer	S267P	S267P		IgII–IgIII	Mutation causes Crouzon Syndrome, Pfeiffer Syndrome, Apert Syndrome	[153]
Glioblastoma	Q212K	Q212K		IgII		[65]
	G463E	G462E		JM		[151]
Head and neck squamous cell carcinoma	N550D/K	N549D/K		KD1	Mutation causes Crouzon and Pfeiffer Syndromes; Analogous to FGFR3 N540K in Hypochondroplasia	[70]
Lung adenocarcinoma	E116K	E116K		IgI–IgII		[154]
	P253L	P253L		IgII–IgIII	Mutation causes Apert Syndrome; Analogous to FGFR1 mutations at P252 in Pfeiffer Syndrome; Analogous to FGFR3 mutation at P250 in Muenke Syndrome and Nonsyndromic Craniosynostosis	[154]
	I381V	I380V		TM		[148]
	K421I	K420I		JM		[154]
	D480N	D479N		JM		[154]
	H545Q	H544Q		KD1		[148]
	G584V	G583V		KI		[154]
	I591M	I590M		KI		[154]
	Q621K	Q620K		KD2		[154]
	R626T	R625T	p.R496T	KD2		[141]
Lung squamous cell carcinoma	D138N	D138N		IgI–IgII		[154]
	N211I	N211I		IgII		[23]
	D247Y	D247Y		IgII–IgIII		[154]
	D283N	D283N		IgIII		[141,147]
	W290C	W290C		IgIII	Mutation causes Pfeiffer Syndrome	[13,23,98,147,154]
	G302W	G302W		IgIII		[154]
	S320C	n/a		IgIII		[13,154]
	C383R	C382R		TM	Analogous to FGFR1 C381R in Osteoglophonic Dysplasia	[154]
	E471Q	E470Q		JM		[13,98,154]
	M538I	M537I		KD1		[154]

Table 1 (*Continued*)

FGFR2						
Disease	Mutation/isoform			Location in receptor	Role in developmental syndromes	Reference
	Residue in IIIb	Residue in IIIc	Other isoform			
	G584W	G583W		KI		[98,154]
	D603E	D602E		KD2		[154]
	K660E/N	K659E/N		KD2	Mutation causes Syndromic Craniosynostosis; Analogous to FGFR3 mutations: K650E in TDII, K650N in Hypochondroplasia	[13,98,154]
	L773F	L772F		C-term		[154]
	T787K	T786K		C-term		[13,98,154]
Lymphoma	M186T	M186T	P21802-20 M71T	IgI	Mutation causes Apert Syndrome	[34]
Medulloblastoma	K660E	K659E		KD2	Mutation causes Syndromic Craniosynostosis; Analogous to FGFR3 mutation K650E in TDII	[155]
Melanoma	S24F	S24F		N-term		[75]
	V77M	V77M		IgI		[75]
	H213Y	H213Y		IgII		[75]
	E219K	E219K		IgII		[75]
	G227E	G227E		IgII		[75]
	V248D	V248D		IgII–IgIII		[75]
	R251Q	R251Q		IgII–IgIII		[75]
	G271E	G271E		IgII–IgIII		[75]
	G305R	G305R		IgIII		[75]
	T371R	T370R		IgIII-TM		[75]
	E476K	E475K		JM		[75]
	D531N	D530N		KD1		[75]
	E575K	E574K		KD1		[75]
	E637K	E636K		KD2		[75]
	M641I	M640I		KD2		[75]
	I643V	I642V		KD2		[75]
	A649T	A648T		KD2	Mutation causes Lacrimo-Auriculo-Dento-Digital Syndrome	[75]
	S689F	S688F		KD2		[75]
	G702S	G701S		KD2		[75]
	P709S	P708S		KD2		[75]
	R760Q	R759Q		C-term		[75]
	L771V	L770V		C-term		[75]
Oral squamous cell carcinoma	P253R	P253R		IgII–IgIII	Mutation causes Apert Syndrome; Analogous to FGFR1 mutations at P252 in Pfeiffer Syndrome; Analogous to FGFR3 mutation at P250 in Muenke Syndrome and Nonsyndromic Craniosynostosis	[13]
	V393A	V392A		TM		[156]
Ovarian serous carcinoma	G272V	G272V		IgII–IgIII		[141]
Pilocytic astrocytoma	K660E	K659E		KD2	Mutation causes Syndromic Craniosynostosis; Analogous to FGFR3 mutation K650E in TDII	[60]

Spermatocytic seminoma	S252F/W	S252F/W		IgII–IgIII	Mutation causes Apert Syndrome; Mutations in FGFR3 at S249 cause TDI	[9]
	P253R/S	P253R/S		IgII–IgIII	Mutation causes Apert Syndrome; Analogous to FGFR1 mutations at P252 in Pfeiffer Syndrome; Analogous to FGFR3 mutation at P250 in Muenke Syndrome and Nonsyndromic Craniosynostosis	[9]
	S267P	S267P		IgII–IgIII	Mutation causes Crouzon Syndrome	[9]
	F276V	F276V		IgII–IgIII	Mutation causes Crouzon Syndrome	[9]
	C278F	C278F		IgIII	Mutation causes Crouzon and Pfeiffer Syndromes	[9]
	Y281C	Y281C		IgIII	Mutation causes Crouzon Syndrome	[9]
	Q289P	Q289P		IgIII	Mutation causes Crouzon Syndrome	[9]
	W290C	W290C		IgIII	Mutation causes Pfeiffer Syndrome	[9]
	n/a	A315S		IgIII	Mutation causes Crouzon Syndrome and Unicoronal Non-Syndromic Craniosynostosis	[9]
	G336R	G338R		IgIII	Mutation causes Crouzon Syndrome	[9]
	Y338C/H	Y340C/H		IgIII	Mutation causes Crouzon and Pfeiffer Syndromes	[9]
	n/a	T341P		IgIII	Mutation causes Pfeiffer Syndrome	[9]
	C340F/R/S/W/Y	C342F/R/S/W/Y		IgIII	Mutation causes Crouzon and Pfeiffer Syndromes	[9]
	n/a	A344G/P		IgIII–TM	Mutation causes Crouzon Syndrome	[9]
	n/a	S347C		IgIII–TM	Mutation causes Crouzon Syndrome	[9]
	S352C	S354C		IgIII–TM	Mutation causes Crouzon Syndrome	[9]
	Y376C	Y375C		TM	Mutation causes Beare–Stevenson Cutis Gyrata Syndrome; Analogous to FGFR1 Y374C in Osteoglophonic Dysplasia; Analogous to FGFR3 Y373C in TDI	[9]
	K527E	K526E		KD1	Mutation causes Crouzon Syndrome	[9]
	N550K	N549K		KD1	Mutation causes Crouzon and Pfeiffer Syndromes; Analogous to FGFR3 N540K in Hypochondroplasia	[9]
	K642R	K641R		KD2	Mutation causes Pfeiffer Syndrome	[9]
	K660E	K659E		KD2	Mutation causes Syndromic Craniosynostosis; Analogous to FGFR3 mutation K650E in TDII	[9]

FGFR3

Disease	Mutation/isoform			Location in receptor	Role in developmental syndromes	Reference
	Residue in IIIb	Residue in IIIc	Other isoform			
Bladder cancer	E216K	E216K		IgII		[157]
	D222N	D222N		IgII		[157]
	G235D	G235D		IgII–IgIII		[157]
	R248C	R248C		IgII–IgIII	Mutation causes TDI	[49,53,101, 157–164]
	S249C	S249C		IgII–IgIII	Mutation causes TDI; Mutations in FGFR2 at S252 cause Apert Syndrome	[49,53,101, 157–166]
	P283S	P283S		IgIII		[101]
	V306I	V306I		IgIII		[157]
	n/a	H349Y		IgIII–TM		[157]
	G372C	G370C		IgIII–TM	Mutation causes TDI	[49,101, 157–163,166]
	S373C	S371C		IgIII–TM	Mutation causes TDI	[101,158,164]
	Y375C	Y373C		TM	Mutation causes TDI; Analogous to FGFR1 Y374C in Osteoglophonic Dysplasia; Analogous to FGFR2 Y375C in Beare–Stevenson Cutis Gyrata Syndrome	[49,53,101, 157–160,163, 164,166,167]
	I378C	I376C		TM		[164]
	Y381C	Y379C		TM		[163]
	G382R	G380R		TM	Mutation causes Achondroplasia	[49,157,158, 161,164,165]
	F386L	F384L		TM		[164]
	A393E	A391E		TM	Mutation causes Crouzon Syndrome with Acanthosis Nigricans	[158,159,161, 163,164,167]
	N542S	N540S		KD1	Mutation causes Hypochondroplasia; Analogous to FGFR2 mutations at N549 in Crouzon Syndrome	[165]
	K652E/M/Q/T	K650E/M/Q/T		KD2	Mutation to E causes TDII; Mutation to M causes SADDAN; Mutation to Q/T causes Hypochondroplasia	[101,141, 158–162,166]

Table 1 (*Continued*)

FGFR3						
Disease	Mutation/isoform			Location in receptor	Role in developmental syndromes	Reference
	Residue in IIIb	Residue in IIIc	Other isoform			
Breast cancer	n/a	n/a	P22607-4 P688S	KD2		[92]
Cervical cancer	S249C	S249C		IgII–IgIII	Mutation causes TDI; Mutations in FGFR2 at S252 cause Apert Syndrome	[162]
Colorectal cancer	C228R	C228R		IgII		[141]
	n/a	E322K		IgIII		[153]
	R401C	R399C		JM		[142]
	V679I	V677I		KD2		[142]
Esophageal adenocarcinoma	n/a	A341T		IgIII–TM		[144]
Gallbladder cancer	R248C	R248C		IgII–IgIII	Mutation causes TDI	[107]
	S249C	S249C		IgII–IgIII	Mutation causes TDI; Mutations in FGFR2 at S252 cause Apert Syndrome	[107]
	G372C	G370C		IgIII–TM	Mutation causes TDI	[107]
	Y375C	Y373C		TM	Mutation causes TDI; Analogous to FGFR1 Y374C in Osteoglophonic Dysplasia; Analogous to FGFR2 Y375C in Beare–Stevenson Cutis Gyrata Syndrome	[107]
	G382R	G380R		TM	Mutation causes Achondroplasia	[107]
	K652M	K650M		KD2	Mutation causes SADDAN	[107]
	G699C	G697C		KD2		[107]
Glioblastoma	E468K	E466K		JM		[168]
	R605Q	R603Q		KD2		[169]
Head and neck squamous cell carcinoma	Q209H	Q209H		IgII		[170]
	S249C	S249C		IgII–IgIII	Mutation causes TDI; Mutations in FGFR2 at S252 cause Apert Syndrome	[70] [171]
	F388L	F386L		TM		[146]
	K415N	K413N		JM		[70]
	K652N	K650N		KD2	Mutation causes Hypochondroplasia	[171]
Lung adenocarcinoma	T79S	T79S		IgI		[141]
Lung squamous cell carcinoma	R248C/H	R248C/H		IgII–IgIII	Mutation causes TDI	[13,98,172]
	S249C	S249C		IgII–IgIII	Mutation causes TDI; Mutations in FGFR2 at S252 cause Apert Syndrome	[13,98]
	S435C	S433C		JM		[13]
	K717M	K715M		KD2		[13,98]
Mesothelioma	D648Y	D646Y		KD2		[173]
Multiple myeloma	G197S	G197S		IgII		[174]
	Y241C	Y241C		IgII–IgIII		[175]
	R248C	R248C		IgII–IgIII	Mutation causes TDI	[176,177]
	Y375C	Y373C		TM	Mutation causes TDI; Analogous to FGFR1 Y374C in Osteoglophonic Dysplasia; Analogous to FGFR2 Y375C in Beare–Stevenson Cutis Gyrata Syndrome	[43,178]
	G384D	G382D		TM		[178]
	F386L	F384L		TM		[43,179]
	S435C	S433C		JM		[174]
	K652E/M	K650E/M		KD2	Mutation to E causes TDII; Mutation to M causes SADDAN	[43,174,178]
	L796R	L794R		C-term		[180]
	P797A	P795A		C-term		[180]
Oral squamous cell carcinoma	G699C	G697C		KD2		[181]

Seborrheic keratosis	R248C	R248C	IgII–IgIII	Mutation causes TDI	[182]
	S249C	S249C	IgII–IgIII	Mutation causes TDI; Mutations in FGFR2 at S252 cause Apert Syndrome	[182]
	G372C	G370C	IgIII–TM	Mutation causes TDI	[182]
	S373C	S371C	IgIII–TM	Mutation causes TDI	[182]
	A393E	A391E	TM	Mutation causes Crouzon Syndrome with Acanthosis Nigricans	[182]
	K652E/M	K650E/M	KD2	Mutation to E causes TDII; Mutation to M causes SADDAN	[182]
Spermatocytic seminoma	R248C	R248C	IgII–IgIII	Mutation causes TDI	[9]
	S249C	S249C	IgII–IgIII	Mutation causes TDI; FGFR2 S252F/W causes Apert Syndrome	[9]
	P250R	P250R	IgII–IgIII	Mutation causes Muenke Syndrome and Nonsyndromic Craniosynostosis; FGFR1 mutation at P252 causes Pfeiffer Syndrome; FGFR2 mutation at P253 causes Apert and Pfeiffer Syndromes	[9]
	E370K	E368K	IgIII–TM		[9]
	G372C	G370C	IgIII–TM	Mutation causes TDI	[9]
	S373C	S371C	IgIII–TM	Mutation causes TDI	[9]
	Y375C	Y373C	TM	Mutation causes TDI; Analogous to FGFR1 Y374C in Osteoglophonic Dysplasia; Analogous to FGFR2 Y375C in Beare–Stevenson Cutis Gyrata Syndrome	[9]
	G377C	G375C	TM	Mutation causes Achondroplasia	[9]
	G382R	G380R	TM	Mutation causes Achondroplasia	[9]
	A393E	A391E	TM	Mutation causes Crouzon Syndrome with Acanthosis Nigricans	[9]
	N542K/S/T/V	N540K/S/T/V	KD1	Mutation causes Hypochondroplasia; Analogous to FGFR2 mutations at N549 in Crouzon and Pfeiffer Syndromes	[9]
	K652E/M/N/Q/T	K650E/M/N/Q/T	KD2	Mutation to E causes TDII; Mutation to M causes SADDAN; Mutation to N/Q/T causes Hypochondroplasia	[9]
	G699C	G697C	KD2		[9]
	X809C/G/R/T	X807C/G/R/T	C-term	Mutation causes TDI	[9]

FGFR4

Disease	Mutation/isoform			Location in receptor	Role in developmental syndromes	Reference
	Residue in P22455-1	Residue in P22455-2	Other isoform			
Breast cancer	E326K	E326K		IgIII		[92]
	Y367C	n/a		TM	Analogous to FGFR1 Y374C in Osteoglophonic Dysplasia; Analogous to FGFR2 Y375C in Beare–Stevenson Cutis Gyrata Syndrome; Analogous to FGFR3 Y373C in TDI	[34]
	A484T	A444T		KD1		[92]
	V550M	V510M		KD1		[141]
Colorectal cancer	P583Q	P543Q		KI		[142]
	A614S	A574S		KD2		[142]
Glioblastoma	Q144E	Q144E		IgI–IgII		[65]
	R434Q	R394Q		JM		[65]
Head and neck squamous cell carcinoma	D671N	D631N		KD2		[70]
Lung adenocarcinoma	R183S	R183S		IgII		[148]
	S232I	S232I		IgII–IgIII		[148]
	R616G	R576G		KD2		[148]
	E681K	E641K		KD2		[148,183]
	P712T	P672T		KD2		[141,147]
	A729G	A689G		KD2		[148]
Lung neuroendocrine carcinoma	S772N	S732N		C-term		[141]
Lung squamous cell carcinoma	Q144E	Q144E		IgI–IgII		[98]
	R434Q	R394Q		JM		[98]

Table 1 (*Continued*)

FGFR4						
Disease	Mutation/isoform			Location in receptor	Role in developmental syndromes	Reference
	Residue in P22455-1	Residue in P22455-2	Other isoform			
Rhabdomyosarcoma	C56S	C56S		N-term		[68]
	R72L	R72L		IgI		[68]
	T122A	T122A		IgI–IgII		[68]
	A175T	A175T		IgII		[68]
	R234H	R234H		IgII–IgIII		[68]
	N535D/K	N495D/K		KD1	Mutations in FGFR2 at N549 cause Crouzon and Pfeiffer Syndromes. Analogous to FGFR3 N540K in Hypochondroplasia	[68]
	V550E/L/M	V510E/L/M		KD1		[68,71]
	A554V	A514V		KD1		[68]
	G576D	G536D		KI		[68]

Note: References for germline mutations in developmental disorders are: [2,18,29,77,78,93,184–190].

spermatocytic seminoma (Table 1, Fig. 2). Some of these mutations have been shown to result in increased receptor autophosphorylation and elevated phosphorylation of FRS2, MAPK and STAT3 [12]. The FGFR2 W290C and S320C mutants have been shown to contribute to tumor formation in xenograft models, and such tumors were sensitive to a selective FGFR inhibitor BJG398, which caused dramatic tumor shrinkage [12]. Overall, these gain-of-function mutations lead to constitutive FGFR activation and drive cellular proliferation and tumor progression. Note that in Fig. 2, all mutations such as W290C that have been identified in human cancer and as developmental syndromes are shown in red. Mutations identified only in human cancer are shown in black.

Mutations that introduce a novel cysteine residue also occur in FGFR3, such as R248C and S249C which, as congenital mutations, cause Thanatophoric Dysplasia Type I (TDI), a severe achondroplasia typically causing neonatal lethality. As somatic mutations, they have been identified in bladder cancer, cervical cancer, gallbladder cancer, head and neck squamous cell carcinoma, lung squamous cell carcinoma, multiple myeloma, sebborheic keratosis, and spermatocytic seminoma (Table 1). These mutations are able to induce colony formation in NIH3T3 anchorage-independent assays, transform myeloid Ba/F3 cells to IL-3 independence [13] and cause ligand-independent receptor activation [14]. Treating IL-3-independent Ba/F3 cells expressing these FGFR mutants with the multikinase inhibitor ponatinib (AP24534) inhibited proliferation [13].

2.3. Non-cysteine mutations in the extracellular domain lead to aberrant activation

Non-cysteine mutations in the extracellular domain are also able to activate FGFRs, such as P252R/S/T mutations in FGFR1 in melanoma, lung adenocarcinoma and spermatocytic seminoma (Table 1). Autosomal dominant mutations at this codon lead to Pfeiffer Syndrome [15]. This residue is located in the IgII–IgIII linker region and contributes to increased receptor activation by decreasing the dissociation rate of the receptor and the FGF ligand [16]. Mutations in the analogous FGFR2 residue, P253R/S, are associated with Apert Syndrome [17,18] and have been frequently identified in cancers, including endometrial carcinoma, lung adenocarcinoma, oral squamous cell carcinoma and spermatocytic seminoma (Table 1). Cells expressing FGFR2 P253R exhibit increased FRS2 phosphorylation and increased FGF2- and FGF9-induced activation of MAPK signaling [19]. A patient with oral squamous cell carcinoma expressing FGFR2 P253R responded to the multikinase inhibitor pazopanib (GW786034B), which effectively reversed cellular transformation and contributed to tumor shrinkage [13].

Another well-studied non-cysteine substitution is S252W in FGFR2, which occurs in approximately 67% of patients with Apert Syndrome, a developmental syndrome characterized by craniosynostosis and syndactyly [20,21]. The pathophysiological effect of this mutation comes from the higher affinity of FGFR2 for a greater repertoire of FGF ligands due to the formation of a hydrophobic patch that stabilizes the ligand-receptor interaction [16]. This mutation leads to aberrant activation of MAPK signaling and interferes with proper endochondral bone development [22]. Mutations at this position, either S252W, S252F, or S252L, have been identified in cervical squamous cell carcinoma, endometrial carcinoma, gallbladder cancer and spermatocytic seminoma (Table 1). FGFR2 S252W leads to colony formation and anchorage-independent proliferation of endometrial carcinoma cells, whereas treatment with FGFR inhibitor PD173074 results in decreased FRS2 phosphorylation, colony formation and tumor cell proliferation [23].

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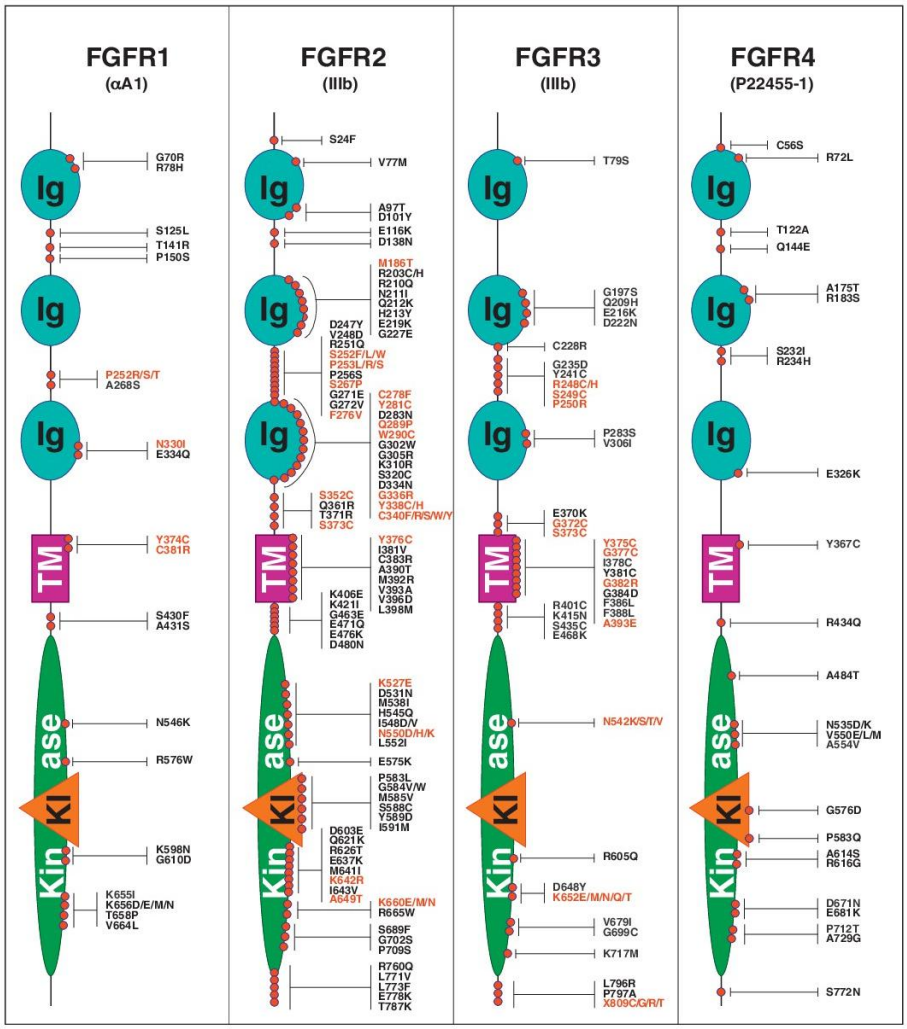

Fig. 2. FGFR mutations identified in human cancer. Mutations present in both developmental syndromes and cancers are highlighted in red. Mutations present only in cancer are shown in black. The residue numbers are based on the following isoforms according to Table 1: αA1 for FGFR1, IIIb for FGFR2, IIIb for FGFR3, and full-length form FGFR4 (Uniprot P22455-1).

Overall, mutations in the extracellular domains of FGFRs, especially numerous for FGFR2 (Fig. 2), have been overwhelmingly detected in both developmental syndromes and cancers. These mutations lead to aberrant receptor signaling either by abnormal disulfide bond formation and receptor dimerization or by increasing the affinity of the receptor for an expanded repertoire of FGF ligands.

2.4. Activating mutations in the transmembrane domain of FGFRs

Biochemical studies suggest that activating mutations adjacent to or within the transmembrane domain of RTKs induce a rotation in the dimer interface of receptor monomers, contributing to increased receptor activation [24]. Recently, the juxtamembrane domain has been shown to synergize with the transmembrane domain to stabilize the unliganded FGFR3 dimer [25].

The introduction of an abnormal Cys residue in the transmembrane domain of FGFRs represents one category of activating mutation. For instance, the Y376C mutation in the transmembrane domain of FGFR2 has been identified in adenoid cystic carcinoma, endometrial carcinoma and spermatocytic seminoma (Table 1). Ba/F3 cells, normally IL-3 dependent, exhibit proliferation and survival in the absence of IL-3 when expressing FGFR2 Y376C [26]. As a congenital mutation, it causes Beare–Stevenson Cutis Gyrata Syndrome (BSS), a severe craniosynostosis syndrome with a high risk of infant death due to respiratory complications [27]. In FGFR3, the introduction of a cysteine residue adjacent to or within the transmembrane domain, such as G372C, S373C, Y375C, G377C, I378C or Y381C, also leads to ligand-independent receptor

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activation [28]. Such mutations have been identified in a variety of cancers, including bladder cancer, gallbladder cancer, multiple myeloma, sebborheic keratosis and spermatocytic seminoma (Table 1). Some of these same mutations have been identified congenitally as causing TDI [29].

Other mutations within the transmembrane domain do not involve the creation of a novel cysteine residue, such as the I379V mutation in FGFR2 identified in lung adenocarcinoma (Table 1). Another example in FGFR3 is G382R that leads to achondroplasia, the most common rhizomelic dwarfism, originally identified in the IIIc isoform as the famous G380R mutation [30,31]. This mutation leads to abnormal localization of FGFR3 to the plasma membrane, including a slower rate of internalization and degradation. In addition, this mutation leads to increased receptor activation [28], dimerization and MAPK activation [32]. A mutation at the paralogous site in FGFR2, C383R, has been identified in endometrial carcinoma, esophageal adenocarcinoma and lung squamous cell carcinomas. Expression of FGFR2 C383R has been shown to transform NIH3T3 cells [33].

A similar mutation in the transmembrane domain of FGFR4, G388R, is a common single nucleotide polymorphism (SNP) which has been examined in many cancers, including bladder, breast, colon, head and neck, kidney, liver, lung and ovarian cancers, and neuroblastoma [34]. It is still unclear whether the G388R mutation is a reliable marker for cancer risk and prognosis. Genomic analysis of breast epithelial cells revealed that roughly half of the patients (53%) exhibited a heterozygous FGFR4 G388R variant [35]. Breast cancer cells expressing FGFR4 G388R exhibit increased motility and proliferation [36] and acquire resistance to adjuvant therapy [37]. In contrast, another report found that this SNP is not a relevant prognostic marker for both node-positive and node-negative breast cancers [38]. In prostate cancer, cells expressing FGFR4 G388R display increased proliferation, motility, invasion and metastasis [39]. In pituitary tumors, the G388R mutation changes hormone secretion by enhancing growth hormone (GH) production and leads to S727 phosphorylation of STAT3 that translocates to the mitochondria and modulates changes in cellular metabolism [40]. The long term significance of this common polymorphism in human cancer will require further study.

2.5. Mutations in the kinase domain of FGFRs

The kinase domain of FGFRs is the site of several mutations with significant impact both in human cancer and developmental syndromes. Of particular importance are mutations within the activation loop containing the signature motif YYKK and the major autophosphorylation site, present in all FGFR family members. First identified in FGFR3, mutations within this motif profoundly increase kinase activation, receptor autophosphorylation and downstream signaling [41–43]. These mutations were initially identified in the IIIc isoform as K650E or K650M, with the former causing the neonatal lethal syndrome TDII, and the latter causing SADDAN (Severe Achondroplasia with Delayed Development and Acanthosis Nigricans) [29,44,45]. The homologous mutations K655I and K656D/E/M/N in FGFR1 have been identified in pilocytic astrocytoma, glioblastoma and rosette forming glioneural tumor (Table 1). Similarly, in FGFR2, the mutations K660E/M/N have been identified in breast cancer, cervical squamous cell carcinoma, endometrial carcinoma, lung squamous cell carcinoma, medulloblastoma, pilocytic astrocytoma and spermatocytic seminoma (Table 1). In FGFR3, the mutations K652E/M/N/Q/T have been identified in bladder cancer, gallbladder cancer, head and neck squamous cell carcinoma, multiple myeloma, seborrheic keratosis and spermatocytic seminoma (Table 1). Curiously, somatic mutations in the YYKK motif of FGFR4 have not yet been identified in human cancer, but this seems only a matter of time.

These activating mutations lead to different processing and trafficking of the receptors through the secretory pathway [46,47]. For instance, the FGFR3 mutations K652E/M lead to intense receptor phosphorylation and also defects in glycosylation and maturation, causing intracellular localization within the endoplasmic reticulum (ER). The high level of tyrosine phosphorylation associated with the receptors in the ER induces activation of STAT1, STAT3 and STAT5 through the direct recruitment of JAK1 to the ER and/or Golgi [46]. Nordihydroguaiaretic acid (NDGA), a compound that inhibits protein trafficking from the ER to Golgi, was found to inhibit tyrosine phosphorylation of FGFR3 K650E resulting in impairment of STAT1, STAT3 and MAPK signaling [48].

Although activating mutations in the kinase domain of FGFR3 have been overwhelmingly detected in aggressive cancers, these mutations are often present in low-grade papillary urothelial bladder cancers [49,50]. Generation of a mouse model with the murine equivalent of FGFR3 K652E expressed in the urothelium did not induce the onset of bladder tumors in mice, but when combined with deletion of PTEN (a negative regulator of PI3K–AKT), urothelial tumorigenesis occurred. Combining the activated FGFR3 with mutations in KRAS or Beta-catenin led to tumor formation in skin and lung through upregulation of PI3K–AKT signaling [51]. Thus, in these microenvironmental contexts, it appears that activating mutations in FGFR3 may synergize with other mutations that activate PI3K–AKT signaling in these cancers [52,53].

Another mutation originally identified as a human developmental syndrome deserves special mention. As a congenital mutation, the FGFR3 mutation N542K, originally described in the IIIc isoform as N540K, causes the mild dwarfing syndrome hypochondroplasia [54]. Biochemical studies have shown that this mutation provides constitutive kinase activation, but much less than mutations in the YYKK motif [41,55]. This site is located in the loop between the αC helix and the β4 strand in the kinase hinge region. This residue participates in a network of hydrogen bonds that functions as a molecular brake to inhibit FGFR2. This activating mutation disengages this inhibitory network in the hinge region and constitutively activates the kinase activity of the receptor [56]. Somatic mutations at this site commonly occur in human cancer. In FGFR1, the mutation N546K occurs in glioblastoma, pilocytic astrocytoma and rosette forming glioneural tumors. In FGFR2, the mutations N550D/H/K have been identified in breast cancer, endometrial carcinoma, gallbladder cancer, head and neck squamous cell carcinoma and spermatocytic seminoma. In FGFR3, the mutations N542K/S/T/V have been found in bladder cancer and spermatocytic seminoma. Lastly, in FGFR4, the mutations N535D/K have been identified in rhabdomyosarcoma (Table 1).

Recently, the importance of the kinase insert (KI) domain in the functionality of RTK families was described [57]. In comparison with other RTKs, FGFRs exhibit a short 15-amino acid kinase insert domain. The KI domain of each FGFR contains possible phosphoacceptor sites, such as Y583 and Y585 in FGFR1. Interestingly, a phosphomimic mutation of Y589D has been identified in the kinase insert domain of FGFR2 in cervical carcinoma [58]. This residue is analogous to Y585 in FGFR1, in which phosphorylation of Y583 and Y585 has been shown to be critical for mitogenesis, transformation of Ba/F3 cells to IL3-independence and cellular proliferation [59]. Mutations in this region may provide a conformational change that increases kinase activation. In FGFR2, these mutations include P583L in colorectal cancer, G584V/W in lung adenocarcinoma and lung squamous cell carcinoma, M585V in cervical squamous cell carcinoma, S588C in breast cancer and I591M in lung adenocarcinoma. These mutations collectively

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define a patch from P583-I591 in the KI domain of FGFR2 which must be involved in the regulation of normal receptor activity and, when disturbed by mutation, participates in oncogenesis. In FGFR4, KI domain mutations include G576D in rhabdomyosarcoma and P583Q in colorectal cancer (Table 1).

2.6. FGFR mutations in cancers of the central nervous system

Pilocytic astrocytoma (PA) is a common central nervous system neoplasm that accounts for approximately 20% of pediatric tumors [60] and usually involves alterations within the MAPK pathway; frequent mutations occur in BRAF such as V600E, or translocations resulting in a KIAA1549-BRAF fusion protein [61,62]. Genetic sequencing of non-cerebellar PA tumors identified mutations in the kinase domain of FGFR1, including N546K and K656E/M/N [63] (Table 1). These N546K and K656E mutations have recently been identified in rosette-forming glioneuronal tumors (RFNTs), which are rare cerebellar parenchyma-derived tumors histologically similar to PA [64]. These mutations, interestingly, have been found in RFNT occurring in the fourth ventricle, a rare site for PA. These studies indicate that FGFR1 plays a critical and active role in the tumorigenesis of a subset of extracerebellar tumors in the absence of activated BRAF.

Several mutations in each FGFR have been identified in glioblastoma. In FGFR1, these mutations include N546K, R576W and K656E; in FGFR2, Q212K and G463K; in FGFR3, E468K and R605Q; and in FGFR4, Q144E and R434Q (Table 1) [65]. Except for the FGFR1 N546K and K656E mutations, analogous to the hypochondroplasia and TDII mutations in FGFR3, the mode of action for most of these mutations is not well understood. Of much greater significance in glioblastoma are translocations involving FGFRs, which will be discussed later.

2.7. FGFR4 activation in rhabdomyosarcoma (RMS)

Activating mutations in FGFR4 have not, so far, been linked to developmental syndromes. However, it is clear that activating mutations in FGFR4 play a direct role in the aggressiveness of some pediatric tumors. Rhabdomyosarcoma (RMS), a soft-tissue sarcoma, is a relatively common type of pediatric tumor histologically divided into two subtypes: embryonal (ERMS) that occurs in the head, neck and trunk, and alveolar (ARMS) often found in the extremities [66,67].

Activating mutations in the kinase domain of FGFR4 have been identified in approximately 7.5% of primary RMS tumors, including N535D/K, V550E/L, A554V and G576D mutations (Tables 1 and 2) [68]. FGFR4 N535K and V550E mutants lead to increased STAT3 activation and drive *in vivo* pulmonary metastasis in xenograft models. Dose-dependent inhibition of mutated FGFR4 signaling by the multi-kinase inhibitor ponatinib leads to ablation of STAT3 signaling resulting in decreased RMS tumor growth *in vivo* [69]. This small-molecule inhibitor may be a promising candidate to treat other cancers harboring these FGFR4 mutations [70]. Additionally, recent genomic sequencing of RMS tumors has revealed the V550L/M mutations in the kinase domain of FGFR4 in ERMS tumors (Table 1) [71]. The Val residue at codon 550 is a gatekeeper residue that controls the accessibility of ATP in the FGFR catalytic pocket [72].

ARMS is the more severe subtype and 75–80% of ARMS tumors exhibit a t(2;13) or t(1;13) chromosomal translocation that generates PAX3-FKHR or PAX7-FKHR fusion proteins, respectively. FGFR4 is a direct transcriptional target of PAX3-FKHR in which the enhancer PAX motif is downstream of the *FGFR4* gene, and PAX3-FKHR binding leads to increased expression of FGFR4 in ARMS. Inhibition of PAX3-FKHR with shRNA leads to reduced expression in ARMS tumors [73]. PAX3-FKHR-mediated increased transcription of FGFR4 supports cell survival *via* the increased expression of antiapoptotic protein BCL2L1, as shown by shRNA-mediated suppression of FGFR4 that decreases BCL2L1 expression [74] (Fig. 3).

The existence of activating mutations in FGFR4, more commonly found in ERMS, suggests a similar functional role as fulfilled by the fusion protein PAX-FKHR in ARMS; both pathways result in increased FGFR4 activation.

2.8. Loss-of-function mutations in FGFR2 in melanoma

Thus far, mutations in FGFRs have been described as resulting in increased kinase activation. In contrast, all FGFR2 mutations in melanoma – including mutations located in the kinase domain – lead to loss-of-function of the receptor (Table 1). Of particular interest is A649T in FGFR2 detected in a patient homozygous for this mutation in melanoma [75]. This residue is located in the FGFR2 activation loop. The introduction of the Thr side chain hinders the phosphotransfer reaction that is critical for proper receptor activation, thus resulting in loss-of-function [76]. Ba/F3 cells expressing FGFR2 A649T exhibit decreased cellular proliferation compared to wild-type FGFR2, and this mutation blocks FGF2-induced activation of MAPK signaling. This mutation was first detected as an autosomal dominant mutation in lacrimo-auriculo-dento-digital (LADD) Syndrome [77]. These patients exhibit congenital anomalies affecting the salivary glands, lacrimal glands, teeth and ears. In contrast with activating mutations in FGFR2 that generally lead to Pfeiffer, Apert, Crouzon and Beare-Stevenson Syndromes marked by severe craniosynostosis, LADD patients do not exhibit such severe phenotypes.

2.9. Stop codon read-through mutations in FGFR3

Thanatophoric Dysplasia (TD) is the most common lethal form of chondrodysplasia. The K652E activation loop mutation in the FGFR3 causes TDII, and mutations in the extracellular domains are usually responsible for TDI. However, TDI can also be caused

Table 2
Mutations in FGFR4 and PAX-FKHR Fusions in rhabdomyosarcoma.

Mutation	Histology	PAX-FKHR	Pathway activation	Phenotype	Target drug/outcome	Refs.
N535D	ERMS	Absent	FGFR4 autophosphorylation; STAT3 activation; DNA replication	Pulmonary lesions; pulmonary Metastases; decreases survival	Ponitinib/Apoptosis	[68,69]
N535K	Unknown	Absent				
V550E	ERMS	Absent	FGFR4 autophosphorylation; STAT3 activation; DNA replication	Pulmonary lesions; pulmonary Metastases; decreases survival	Ponitinib/Apoptosis	
V550L	ARMS	Present				
V550L	ERMS	Absent				
A554V	ARMS	Present				
G576D	ARMS	Present				

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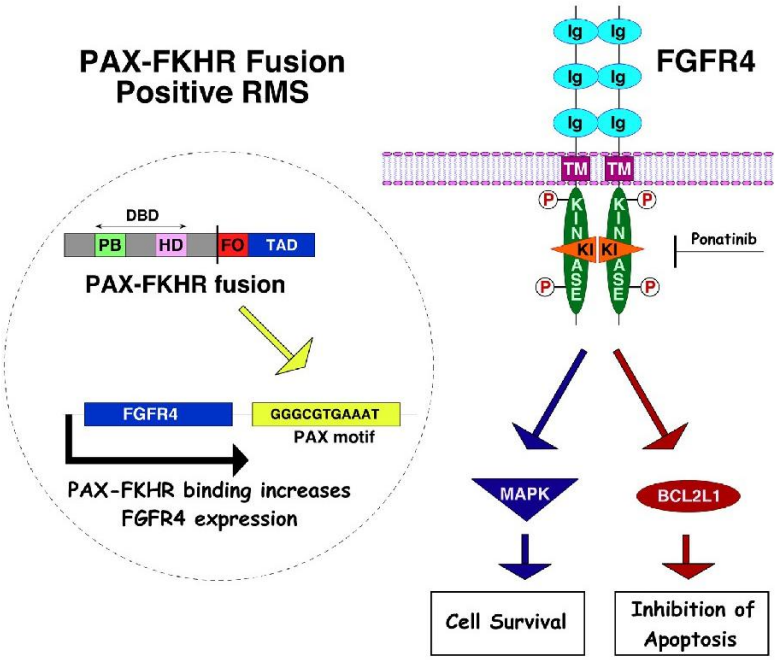

Fig. 3. FGFR4 signaling contributes to progression of RMS. In ARMS, the PAX-FKHR fusion protein functions as a transcriptional factor to stimulate FGFR4 expression, which upregulates proliferative and anti-apoptotic pathways. Inhibition of FGFR4 with ponatinib suppresses these effects.

by unusual mutations at the stop codon of FGFR3, allowing additional in-frame translation of an additional 423 nt. These read-through mutations can result in the introduction of a Cys, Gly or Arg (X809C/G/R). The resulting FGFR3 is 114 amino acids longer, and the elongated hydrophobic C-terminal region adopts an α-helix conformation suggested to serve as a second transmembrane domain that activates FGFR3 [78,79]. Recently, additional mutations to Leu, Ser, or Trp at the stop codon have been detected in patients with TDI [80]. Remarkably, in spermatocytic seminoma, stop codon mutations X809C/G/R/T in FGFR3 have been observed in 21/29 (72%) of patients [9] (Table 1). These mutations clearly support cellular proliferation and clonal expansion of spermatogonia *via* FGFR3 activation in these tumors.

2.10. The challenges of drug resistance in tumors with mutated FGFRs

The overall efficacy of tyrosine kinase inhibitors can be limited due to acquired mechanisms of chemotherapy resistance, which impedes treatments and leads to tumor relapse [81]. A well-documented drug resistance mechanism may include the emergence of secondary mutations in gatekeeper residues, such as T790M in EGFR in 50% of erlotinib- and gefitinib-resistant tumors [82,83] and T315I mutation in BCR-ABL in imatinib-resistant Chronic Myelogenous Leukemia (CML) [84,85]. Similarly, mutation of the gatekeeper residue V561M in FGFR1 has been shown to confer *in vitro* drug resistance to pyrido-[2,3-d]pyrimidine multi-kinase inhibitor PP58 [86].

Dovitinib (TKI-258) is an ATP-competitive multikinase inhibitor with activity against FGFRs [87]. This drug has shown promising pre-clinical anti-tumor activity in cancers driven by FGFR activation such as multiple myeloma and acute myelogenous leukemia [88–90]. In response to dovitinib, FGFR2 mutations were observed including N550H/K/S/T, V565I, E566A/G and K642N, which resulted in increased receptor kinase activity. Residues N550, E566 and K642 are part of a triad that forms a network of autoinhibitory hydrogen bonds termed the molecular brake. Drug-resistant mutations at these sites disrupt this molecular brake in the kinase hinge region of FGFR2 [91]. Furthermore, FGFR2 mutations at N550 and K642 have been identified in adenoid cystic carcinoma, breast cancer, endometrial carcinoma, gallbladder cancer, head and neck squamous cell carcinoma and spermatocytic seminoma (Table 1) [23,32,92,93].

Furthermore, the use of FGFR inhibitors AZD4547 and AZ8010 for diseases such as multiple myeloma, gastric cancer and urothelial cancer, has been shown to decrease tumor proliferation [94]. In order to identify potential mechanisms of resistance to FGFR inhibitor AZ8010 in multiple myeloma, AZ8010-resistant multiple myeloma KMS11 cells (KMS11-R) were generated. KMS11-R cells, which express FGFR3 Y375C, exhibited elevated levels of phosphorylated FGFR and FRS2, and increased STAT3 and MAPK signaling [94]. Genomic sequencing of KMS11-R cells revealed a secondary mutation in the gatekeeper residue V557M in FGFR3, which is analogous to the V561M mutation in FGFR1 in PP58-resistant cells. Molecular simulation predicts that the Met side chain is approximately 25% bulkier than the Val at position 561 in FGFR1, and it restricts the binding of FGFR inhibitor PD173074 to the catalytic domain [86].

Effective cancer treatments depend upon the prediction of drug-resistance mechanisms evolved in tumor cells. Without such predictions, the efficacy of chemotherapy agents is compromised, contributing to a decrease in treatment progression resulting in tumor relapse.

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3. FGFR translocations and fusion proteins in cancer

3.1. FGFR fusion protein discovery across a variety of cancers

Fusion proteins are continually being discovered in a variety of human cancers. Particularly, fusions involving FGFRs are prevalent in hematological cancers and solid tumors. The existence of translocations involving FGFRs has been known since the late 1990s, when a patient with T-cell lymphoblastic lymphoma was found to harbor a ZNF198-FGFR1 fusion, now also referred to as ZMYM2-FGFR1. Lymphoma or leukemia cases from the 1970s and 1980s described disease characteristics similar to the now well-defined disease, 8p11 myeloproliferative syndrome (EMS). This correlation may arise because FGFR1 fusions in leukemia and lymphoma often originate as EMS. According to the World Health Organization, EMS is classified as "myeloid and lymphoid neoplasms with FGFR1 abnormalities," and has also been called "stem cell leukemia/lymphoma" [95].

In EMS, FGFR1 located at 8p11.22 is often disrupted by chromosomal translocation, resulting in a fused coding region. The fusions in EMS consistently result in FGFR1 fused to an N-terminal dimerization domain (Fig. 4), an alteration that has also been found in breast cancer, lung squamous cell carcinoma, phosphaturic mesenchymal tumor, rhabdomyosarcoma and leukemia (Table 3) [96–99]. With FGFR as the 3′ partner, the ligand-binding extracellular domain and transmembrane domain are excluded from the fusion protein, with only the FGFR kinase domain attached to the 5′ protein partner. Dimerization of this fusion type would result only from the N-terminal oligomerization domain, not FGF ligand binding. In solid tumors, it is more common to find FGFR as the 5′ fusion gene, with the breakpoint consistently found in exons 17, 18, or 19, leaving the extracellular, transmembrane and kinase domains intact. When the extracellular domain is present, dimerization is thought to increase with the addition of FGF ligand. Although the domains present in fusion proteins vary, the intact FGFR kinase domain is always retained, indicating this domain is critical for a functioning fusion protein and cancer progression. It is rare to see an FGFR fusion protein with an additional FGFR activating mutation. The reason may be that either event alone may be sufficient for cancer to progress, although the dual activation of an FGFR both by mutation and translocation could provide additional oncogenic potential. Additionally, while some FGFR fusions occur with high tissue specificity, others occur across many cancer types [100].

3.2. Dimerization of FGFR induced by the fusion partner

In FGFR fusion proteins, almost all fusion partners contribute a known dimerization domain which allows the FGFR to dimerize and autophosphorylate the kinase domain, leading to activation and downstream signaling, increased cell proliferation and cancer progression (Fig. 1). Recently, an FGFR3 fused to transforming acidic coiled-coil containing 3 (TACC3) has been discovered in glioblastoma, bladder cancer, lung cancer, oral cancer, head and neck squamous cell carcinoma and gallbladder cancer [96,97,101–107] (Table 3). Additionally, FGFR1-TACC1 has been identified in glioblastoma [106,108]. The coiled-coil domain of TACC3 is assumed to bring the FGFR3 portion of the fusion proteins close together, inducing activation. FGFR3-TACC3, FGFR3-BAIAP2L1, and FGFR2-CCDC6 have been shown to dimerize presumably through their coiled-coil domains [96]. The FGFR2-BICC1 gene fusion has been found in cholangiocarcinoma, colorectal cancer and hepatocellular carcinoma [96,109–111]. The self-associating sterile alpha

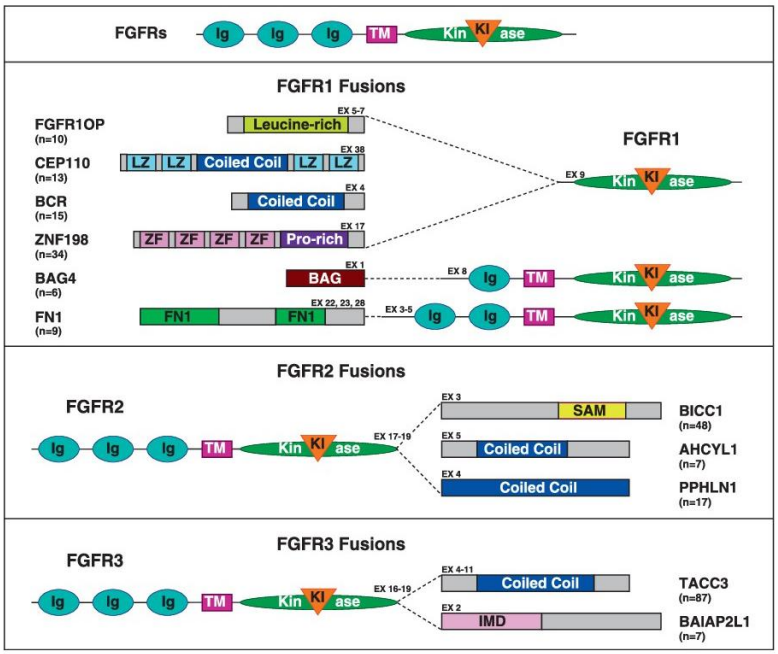

Fig. 4. Structural organization of select FGFR fusion proteins. Schematic representations are presented for the more common ($n > 5$) FGFR fusions identified in human cancers and cell lines. The most common breakpoint of each fusion is shown. Occurrence numbers (n) indicate the total number of times the fusion has been identified, including breakpoints not shown in the figure. See Table 3 for full list of FGFR fusions and translocations.

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Table 3
FGFR Fusion Proteins Arising from Translocations.

5′ gene	Disease	FGFR isoform	5′ gene exon fusion point	3′ gene	3′ gene exon fusion point	Occurrences	Translocation	Normal biological function/ pathway of FGFR fusion partner	Fusion description	Refs.
Fusions with FGFRs as 5′ gene										
FGFR1	Glioblastoma		17	TACC1	7	2	t(8,8) (p11;p11)	Microtubule interaction	TK domain: coiled coil domain	[106,108]
	Pilocytic astrocytoma	αA1	18	FGFR1	11	1	duplication	Regulation of various cellular processes	ITD of TK domain	[60]
FGFR2	Breast cancer	IIIc	19	AFF3	8	1	t(2;10) (q11;q26)	Nuclear transcriptional activator	TK domain: AFF3 domain	[96]
		IIIc	19	CASP7	4	1	t(10;10) (q25;q26)	Caspase involved in apoptosis, inflammation	TK domain: self association domain	[96]
		IIIc	19	CCDC6	2	1	t(10;10) (q21;q26)	Coiled-coil domain containing protein	TK domain: coiled coil domain	[96]
	Cholangiocarcinoma		19	AHCYL1	5	7	t(10;1) (q26.1;p13.2)	Adenosylhomocysteinase activity/IP3 binding	TK domain: coiled coil domain	[109]
		IIIb	17	BICC1	1	1	t(10;10) (q21.1;q26.1)	RNA binding protein	TK domain: SAM	[111]
			17	BICC1	3	1	t(10;10) (q21.1;q26.1)	RNA binding protein	TK domain: SAM	[110]
		IIIb	19	BICC1	3	4	t(10;10) (q21.1;q26.1)	RNA binding protein	TK domain: SAM	[96,109]
			Not ID'd	BICC1	Not ID'd	40	t(10;10) (q21.1;q26.1)	RNA binding protein	TK domain: SAM	[118]
			18	KIAA1598/SHOOTIN1	7	1	t(10;10) (q25;q26)	Needed for neuronal polarization	TK domain: coiled coil domain	[110]
		IIIb	17	MGEA5	12	1	t(10;10) (q24;q26)	O-GlcNAc transferase	Unknown	[111]
		IIIb	19	PPHLN1	4	17	t(10;12) (q26;q12)	Epithelial differentiation	TK domain: coiled coil domain	[118]
		IIIb	17	TACC3	11	2	t(4;10) (p16;q26)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[110,111]
	Colorectal cancer		19	BICC1	3	1	t(10;10) (q26.1;q21.1)	RNA binding protein	TK domain: SAM	[109]
	Hepatocellular carcinoma		19	BICC1	3	1	t(10;10) (q26.1;q21.1)	RNA binding protein	TK domain: SAM	[109]
	Lung adenocarcinoma	IIIb	17	CIT	23	1	t(10;12) (q26;q24)	Cell division	TK domain: coiled coil domain	[116]
	Lung squamous cell carcinoma	IIIc	19	KIAA1967/CCAR2	5	1	t(8;10) (p21;q26)	Cell cycle and apoptosis regulator	TK domain: coiled coil domain	[96]
		IIIc	Not ID'd	KIAA1967/CCAR2	Not ID'd	1	t(8;10) (p21;q26)	Cell cycle and apoptosis regulator	TK domain: coiled coil domain	[98]
	Ovarian cancer		17	FAM76A	2	1	t(1;10) (p35;q26)	Unknown	TK domain: coiled coil domain	[117]
	Thyroid cancer	IIIc	19	OFD1	3	1	t(10;X) (q26;p22)	Centriolar component, regulates Wnt signaling	TK domain: coiled coil; LisH domain	[96]

Table 3 (*Continued*)

5′ gene	Disease	FGFR isoform	5′ gene exon fusion point	3′ gene	3′ gene exon fusion point	Occurrences	Translocation	Normal biological function/ pathway of FGFR fusion partner	Fusion description	Refs.
FGFR3	Bladder cancer	IIIb	18	BAIAP2L1	2	2	t(4;7)(p16;q22)	Formation of actin	TK domain: coiled coil; IMD domain	[102,191]
			Not ID'd	BAIAP2L1	Not ID'd	3	t(4;7)(p16;q22)	Formation of actin	TK domain: coiled coil; IMD domain	[191]
			16	TACC3	11	3	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[157]
			17	TACC3	4	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[101]
			17	TACC3	11	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[101]
		IIIb	18	TACC3	4	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[102]
		IIIb	18	TACC3	8	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[102]
		IIIb/IIIc	18	TACC3	11	5	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[96,102]
	Gallbladder cancer		17	TACC3	11	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[107]
	Glioblastoma		16	TACC3	8	3	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[106]
			16	TACC3	9	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[106]
			16	TACC3	10	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[106]
			16	TACC3	11	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[106]
			17	TACC3	6	2	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[108]
			17	TACC3	8	5	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[108,192]
			17	TACC3	10	4	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[108,192]
			17	TACC3	11	7	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[108,192]
			18	TACC3	4	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[108]
			18	TACC3	5	2	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[108]
			18	TACC3	9	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[108]
			18	TACC3	10	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[105]
			18	TACC3	11	4	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[96,105]
			18	TACC3	13	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[108]
		IIIc	19	TACC3	4	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[105]
	Head and neck squamous cell carcinoma		18	TACC3	6	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[193]
		IIIb	18	TACC3	10	2	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[96]
			18	TACC3	14	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[193]
			19	TACC3	11	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[193]
	Lung adenocarcinoma		Not ID'd	BAIAP2L1	Not ID'd	1	t(4;7)(p16;q22)	Formation of actin	TK domain: coiled coil; IMD domain	[191]
			17	TACC3	4	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[194]
			17	TACC3	8	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[194]
			17	TACC3	10	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[194]
			17	TACC3	11	8	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[97,194]
	Lung squamous cell carcinoma		Not ID'd	BAIAP2L1	Not ID'd	1	t(4;7)(p16;q22)	Formation of actin	TK domain: coiled coil; IMD domain	[191]
			17	TACC3	5	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[97]
			17	TACC3	7	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[103]
			17	TACC3	8	2	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[97,103]
			17	TACC3	10	2	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[97]
			17	TACC3	11	7	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[97,103]
			18	TACC3	9	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[103]
			18	TACC3	10	2	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[104]
		IIIb	18	TACC3	11	6	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[96,97]
	Oral cancer	IIIb	18	TACC3	10	1	t(4;4)(p16;p16)	Stabilization of mitotic spindle	TK domain: coiled coil domain	[96]
Fusions with FGFRs as 3′ gene										
BAG4	Lung squamous cell carcinoma		1	FGFR1	8	2	t(8;8)(p11;p11)	Anti-apoptotic protein	BAG domain: Ig,TM,TK domains	[97]
		IIIc	2	FGFR1	9	1	t(8;8)(p11;p11)	Anti-apoptotic protein	BAG domain: Ig,TM,TK domains	[96]
			Not ID'd	FGFR1	Not ID'd	3	t(8;8)(p11;p11)	Anti-apoptotic protein	BAG domain: Ig,TM,TK domains	[98]
ERLIN2	Breast cancer	IIIc	10	FGFR1	4	1	t(8;8)(p11;p11)	Lipid raft associated protein family	SPFH domain: Ig, TM, TK domain	[96]
FN1	Phosphaturic mesenchymal tumor		22	FGFR1	3,4	1	t(2;8)(q35;p11)	Cell adhesion	FN domain: Ig, TM, TK domain	[137]
			23	FGFR1	3,4	1	t(2;8)(q35;p11)	Cell adhesion	FN domain: Ig, TM, TK domain	[137]
			28	FGFR1	5	1	t(2;8)(q35;p11)	Cell adhesion	FN domain: Ig, TM, TK domain	[137]
			Not ID'd	FGFR1	Not ID'd	6	t(2;8)(q35;p11)	Cell adhesion	FN domain: Ig, TM, TK domain	[137]

Table 3 (*Continued*)

5′ gene	Disease	FGFR isoform	5′ gene exon fusion point	3′ gene	3′ gene exon fusion point	Occurrences	Translocation	Normal biological function/ pathway of FGFR fusion partner	Fusion description	Refs.
FOXO1	Rhabdomyosarcoma		Not ID'd	FGFR1	Not ID'd	1	t(8;13;9) (p11.2;q14; q32)	Transcription factor	Unknown	[136]
SQSTM1	Leukemia		9	FGFR1	9	1	t(5;8) (q35;p11)	Ubiquitin binding, NFκB regulation	PB1-ZF: TK domain	[138]
TEL/ETV6	Lymphoma		5	FGFR3	10	1	t(4;12) (p16;p13)	ETS family of transcription regulators	SAM: TK domain	[99,195]
8p11 myeloproliferative syndrome (EMS) resulting from fusions of FGFR1										
BCR	8p11 myeloproliferative syndrome (EMS)		4	FGFR1	9	10	t(8;22) (p11;q11)	Serine/Threonine kinase	Coiled coil domain: TK domain	[95,196–201]
			Not ID'd	FGFR1	Not ID'd	5	t(8;22) (p11;q11)	Serine/Threonine kinase	Coiled coil domain: TK domain	[95,113,202, 203]
CEP110/ centriolin			Not ID'd	FGFR1	8	2	t(8;9) (p11;q33)	Required for centrosome function	LZ/coiled coil domain: TK domain	[123,204]
			38(15)(40)	FGFR1	9	4	t(8;9) (p11;q33)	Required for centrosome function	LZ/coiled coil domain: TK domain	[95]
			Not ID'd	FGFR1	Not ID'd	7	t(8;9) (p11;q33)	Required for centrosome function	LZ/coiled coil domain: TK domain	[95]
CPSF6			8	FGFR1	9	1	t(8;12) (p11;q15)	RNA processing	RNA recognition motif: TK domain	[95]
			Not ID'd	FGFR1	Not ID'd	2	t(8;12) (p11;q15)/ dic(8;12) (p11;p11)	RNA processing	RNA recognition motif: TK domain	[95]
CUX1			11	FGFR1	10	1	t(7;8) (q22;p11)	Homeodomain family of DNA binding proteins	Coiled coil domain: TK domain	[114]
FGFR1OP (FOP)			5	FGFR1	9	1	t(6;8) (q27;p11-12)	Microtubule anchoring	Leu rich domain: TK domain	[95]
			6	FGFR1	9	4	t(6;8) (q27;p11-12)	Microtubule anchoring	Leu rich domain: TK domain	[95]
			7	FGFR1	9	2	t(6;8) (q27;p11-12)	Microtubule anchoring	Leu rich domain: TK domain	[95]
			Not ID'd	FGFR1	Not ID'd	3	t(6;8) (q27;p11-12)	Microtubule anchoring	Leu rich domain: TK domain	[95]
FGFR1OP2			4	FGFR1	9	3	t(8;12) (p11;p12)/ ins(12;8) (p11;p11p22)	Wound healing	Coiled coil domain: TK domain	[95,115,205]
HERV-K			3	FGFR1	9	1	t(8;19) (p12; q13.3)	Retroviral sequence	LTR: TK domain	[206]
			Not ID'd	FGFR1	Not ID'd	1	t(8;19) (p12; q13.3)	Retroviral sequence	LTR: TK domain	[139]
LRRFIP1			9	FGFR1	9	1	t(2;8) (q37;p11)	Transcriptional repressor	Coiled coil domain: TK domain	[95]
MYO18A			32	FGFR1	9	1	t(8;17) (p11;q23)	Golgi membrane trafficking and shape	Coiled coil domain: TK domain	[95]
NUP98			Not ID'd	FGFR1	Not ID'd	1	t(8;11) (p11;p15)	Nuclear pore complex component	Unknown	[95]
RANBP2/ NUP358			20	FGFR1	9	1	t(2;8) (q12;p11)	Nuclear pore complex component	LZ: TK domain	[128]
TRIM24 (TIF1)			12	FGFR1	9	1	t(7;8) (q34;p11)	Transcription control	Coiled coil domain: TK domain	[95]
TPR			22	FGFR1	13	1	t(1;8) (q25;p11.2)	Nuclear pore complex component	Coiled coil domain: TK domain	[207]
			23	FGFR1	13	1	t(1;8) (q25;p11.2)	Nuclear pore complex component	Coiled coil domain: TK domain	[119]
			Not ID'd	FGFR1	Not ID'd	1	t(1;8) (q25;p11.2)	Nuclear pore complex component	Coiled coil domain: TK domain	[208]
ZNF198/ ZMYM2			17	FGFR1	9	34	t(8;13) (p11;q12)	Transcription factor	ZF: TK domain	[95,209]

Note: Several fusions are described in an earlier review article, Ref [95].

motif domain (SAM) of BICC1, containing a helix-loop-helix domain, fused 3′ to FGFR2, is believed to instigate constitutive dimerization of FGFR2 in order to produce an active receptor [100] (Fig. 4).

Other dimerization domains found in FGFR fusion proteins are believed to have the same function. FGFR fusion partner domains include zinc-finger, leucine zipper, coiled-coil, SAM, LIS1-homologous (LIsH), IRSp53/MIM (IMD), BAG, FN1, AFF3, and stomatin/prohibitin/flotillin/HflK/C (SPFH) domains (also known as the prohibitin PHB domain) (Table 3). Other fusions thought to dimerize by self-association domain include FGFR2-CASP7 in breast cancer, which dimerizes through active site loops, and CPSF6-FGFR1, which dimerizes through a RNA recognition motif [95,96,112]. The most frequent fusion partner domain is the coiled-coil, occurring in the proteins mentioned above; in addition, the other coiled-coil fusion proteins are BCR-FGFR1 [113], CEP110-FGFR1 [95], CUX1-FGFR1 [114], FGFR1OP2-FGFR1 [115], FGFR2-AHCYL1 [109], FGFR2-CIT [116], FGFR2-FAM76A [117], FGFR2-KIAA1598 [110], FGFR2-KIAA1967 [96], FGFR2-OFD1 [96], FGFR2-PPHLN1 [118], FGFR2-TACC3 [111], LRRFIP1-FGFR1 [95], MYO18A-FGFR1 [95], TRIM24-FGFR1 [95], and TPR-FGFR1 [119].

In order for autophosphorylation to occur, RTKs need to be exactly aligned. It has been shown that dimerization of the intracellular domain alone will not activate the receptor. Ligand binding rotates and aligns the extracellular juxtamembrane domain and intramembrane α-helices, leading to intracellular kinase domain alignment, dimerization and activation [24]. To create an active FGFR fusion protein, the dimerization domain must provide the correct alignment. The most common FGFR1 fusion in EMS is ZNF198-FGFR1, which contains either 4 or 10 zinc finger domains and a proline-rich domain from ZNF198, followed by the tyrosine kinase domain of FGFR1 (Fig. 4) [95]. The proline-rich domain is a self association domain and is essential for dimerization and activation of FGFR1 [120].

An exception to the activation-by-oligomerization theme is an internal tandem duplication (ITD) of FGFR1 in a patient with pilocytic astrocytoma [60], resulting in a duplication of the FGFR1 kinase domain. ITD has previously been observed in Acute Myeloid Leukemia with FLT3, another receptor tyrosine kinase. This ITD, which occurs in the juxtamembrane domain of FLT3, leads to enhanced receptor activation and increased downstream signaling of MAPK and STAT5 [121].

3.3. Altered cellular localization of FGFR by the fusion partner

Often, the creation of FGFR fusion proteins not only activates FGFR and its canonical pathways, but results in an incongruous FGFR localization as well. Some partner proteins can lead to localization of FGFR to a cellular compartment other than the plasma membrane. Fusion proteins that have been shown to have irregular localization include FGFR1OP-FGFR1, CEP110-FGFR1, ZNF198-FGFR1, and TEL-FGFR3 in lymphoma and FGFR3-TACC3 in glioblastoma. Wild-type FGFR1OP (FGFR1 oncogenic partner) and CEP110 (centriolin) are centrosomal proteins. Once engaged in a fusion with FGFR1, FGFR1OP localizes the kinase domain to the centrosome through a CAP350 interaction [122]. CEP110 is involved in centriole maturation and localizes to the centrosome *via* an 170-amino acid region in the C-terminus, a region retained in the CEP110-FGFR1 fusion. Instead of the expected localization to the centrosome, cytoplasmic expression of the fusion protein was observed [123]. Continuous kinase activity and inappropriate cytoplasmic localization due to CEP110-FGFR1 fusion formation may result in increased cell viability and hematopoietic stem cell growth. The fusion proteins ZNF198-FGFR1 and TEL-FGFR1 have been identified as cytoplasmic proteins [95,99]. The translocation of ZNF198 and FGFR1 genes removes the FGFR1 transmembrane domain and the C-terminal nuclear localization signal of ZNF198, which most likely leads to cytoplasmic localization.

Expressed FGFR3-TACC3 has been shown to localize to the mitotic spindle poles in dividing mouse astrocytes, most likely due to recruiting effects of TACC3. In addition, the fusion protein increased the percentage of aneuploidy by greater than 2.5-fold [106]. As TACC3 is an important component of mitotic spindle assembly and is involved with the attachment of chromosomes to microtubules, it is most likely playing a role in chromosomal segregation errors. During mitosis, wild-type TACC3 is strongly diffused around centrosomes, due to the localizing effects of the C-terminal coiled-coil [124]. As this domain is present in the FGFR3 fusion, multiple effects could be implicated by the fusion protein such as localization of FGFR3-TACC3 to the centrosome or a novel biochemical activity. During interphase, wild-type TACC3 has been found to be concentrated in the nucleus [124]. The location of the FGFR3-TACC3 fusion in non-dividing cells has not yet been identified.

Although the localization of ERLIN2-FGFR1 has not yet been investigated, wild-type ERLIN2 anchors to the ER membrane *via* an N-terminal binding motif. This motif is still present when ERLIN2 is fused to FGFR1, and may be affecting fusion protein location [125]. The fusion results in the SPFH oligomerization domain of ERLIN2 fused 5′ to exon 4 of FGFR1, and was detected in breast cancer.

Thus, for these and other FGFR fusion proteins discussed: is the salient biological feature the localization of the FGFR kinase domain to a novel cellular compartment? Or, is it the constitutive dimerization and activation of the FGFR kinase domain, regardless of the localization of the normal fusion partner, that is determinative? Much further experimental research will be required to arrive at a definitive answer.

3.4. Downstream signaling impacts of fusion proteins

FGFR fusion proteins have been shown to activate the normal FGFR pathways, specifically the PI3K/AKT, MAPK, and JAK/STAT pathways (Fig. 1). FGFR3-TACC3, FGFR3-BAIAP2L1, and FGFR2-CCDC6 increase activation of PI3K/AKT and MAPK pathways [100]. FGFR2-TACC3 has also been shown to increase MAPK activation, but only a moderate increase of FRS2 phosphorylation of the PI3K pathway has been seen [111]. In wild-type FGFR1, FRS2 normally binds to the juxtamembrane domain between amino acids 407 and 433. In many FGFR1 fusions, this domain is either fully or partially disrupted by translocation of the fusion partner, which results in an inability to recruit FRS2. This has been shown to occur in ZNF198-FGFR1, but may occur in other fusion proteins with FGFR as the 3′ partner. However, although FRS2 interaction with ZNF198-FGFR1 was undetectable, the PI3K pathway remained active [95].

In addition to the activation of MAPK and PI3K pathways, cells expressing FGFR1OP-FGFR1 exhibit increased phosphorylation of STAT1 and STAT3, but not STAT5 [126]. Furthermore, ZNF198-FGFR1 activates STAT5, FGFR3-TACC3 activates STAT3, and FGFR3-BAIAP2L1 and FGFR2-CCDC6 increase STAT1 activation [100,122]. ERLIN2-FGFR1 and CEP110-FGFR1 have been shown to be biologically active through tyrosine phosphorylation of the respective fusion proteins, but further downstream signaling activation has not been explored [96,123]. Despite an overall increase in cell proliferation pathway activation, a contrasting study reports a failure to over-activate MAPK and AKT by FGFR3-TACC3 [106]. Studies exploring FGFR2-AHCYL1 and FGFR2-BICC1 fusions report an absence of AKT and STAT3 phosphorylation, although the MAPK pathway remained active [109]. Additionally, TEL-FGFR3 directly interacts with and activates STAT3 and STAT5, presumably through the FGFR3 portion of the protein, an

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interaction that has not been shown with other fusion proteins [99].

Fusions with FGFR as the 5′ partner usually result in a deletion of the last exon of FGFR, which includes the tyrosine residue important for PLCγ binding [127]. In bladder cancer, cells transfected with FGFR3-TACC3 or FGFR3-BAIAP2L1 were unable to activate PLCγ, due to a deletion of the last exon of FGFR3 in both fusion proteins [102] (Fig. 4) Chromosomal rearrangements such as these also result in the loss of the 3′ UTR (untranslated region) of FGFR, significant as a region that contains various microRNA (miRNA) regulation sites. MiR-99a is normally present at high levels in the brain and results in a downregulation of FGFR3 translation. The formation of FGFR3-TACC3 fusion in glioblastoma results in a loss of the miR-99a site, which leads to the overexpression of FGFR3-TACC3. This miRNA site is unique to FGFR3, but overexpression due to a loss of miRNA regulation could occur in any FGFR fusion where the 3′ UTR region contains a regulatory miRNA site [105].

Interestingly, nuclear pore complex proteins have been identified in fusion proteins with FGFR1. RANBP2-FGFR1, TPR-FGFR1, and NUP98-FGFR1 have all been identified in EMS [95,119,128]. Mechanistically, these may be similar to other fusion proteins discussed previously in that two of these possess dimerization domains, with RANBP2 (RAN binding protein 2, also NUP358) containing a leucine zipper domain and TPR (Translocated Promoter Region) containing a coiled-coil domain (Table 3). A dimerization motif in NUP98 has not yet been identified, however. Also mechanistically unclear is the fusion partner AFF3 (AF4/FMR2 Family, Member 3, also known as LAF4), a nuclear transcriptional activator, which has been identified as the 3′ fusion partner with FGFR2 (Table 3). AFF3 has also been found fused to the MLL gene in acute lymphoblastic leukemia [129]. It is unclear whether the significant biochemical consequence of these fusion proteins is manifested in the dimerization and activation of the FGFR partner, or whether the abnormal nuclear localization of the FGFR component represents the key event.

All EMS cases with FGFR1 fusions have thus far been negative for the BCR-ABL fusion gene, which occurs in 85–90% of CML. The remaining cases either contain other translocations or are classified as BCR-ABL negative CML, or atypical CML. Some of these atypical CML cases are now linked to the broad spectrum of EMS cases, due to the presence of a translocation involving the 8p11 region [130]. Patients with BCR-FGFR1 [t(8;22)(p11;q11)] fusion are often referred to as CML-like due to their greater resemblance to CML than to EMS. BCR has been shown to interact with Grb2 by phosphorylation of Y177 [95]. This interaction is thought to be important for BCR-ABL signaling in CML patients, and may be playing a role in EMS patients with BCR-FGFR1 as well.

3.5. Inhibition of FGFR fusion proteins

Through the use of various drug treatments, a reduction of cell proliferation and FGFR fusion protein activity has been accomplished. Studies indicate that an active FGFR kinase domain drives cancer progression, thus the goal of many cancer treatments is to inhibit the FGFR portion of the fusion [106,109]. FGFR inhibitors have been used *in vitro* to reduce phosphorylation of FGFR and subsequent downstream signaling proteins. FGFR kinase inhibitors AZD4547, BGJ398, and PD173074 have inhibited growth of FGFR3-TACC3-expressing Rat1A and glioma stem-like cells (GSC-1123). PD173074 and AZD4547 both resulted in tumor shrinkage during *in vivo* mouse xenograft studies as well [106]. For fusions FGFR2-AHCYL1 and FGFR2-BHCC1, both BGJ398 and PD173074 were successful in reducing *in vitro* fusion activity and cell growth [109]. In bladder cancer, sensitivity of FGFR3-TACC3 and FGFR3-BAIAP2L1 to the kinase inhibitors PD173074, dovitinib, SU5402, and BGJ398 has been reported [102]. BGJ398 and dovitinib are currently involved in numerous clinical trials (clinicaltrials.gov).

FGFR3 translocations were also targeted using the heat shock protein 90 (HSP90) inhibitor, ganetespib (STA-9090). By inhibiting HSP90, hundreds of proteins soon become degraded, which disrupts oncogenic signaling pathways. Ganetespib treatment of bladder cancer cell line RT112, which contains FGFR3-TACC3, resulted in a decrease of fusion protein expression and cell viability. Expression of the apoptosis facilitator protein BIM (BCL2-Like 11, or BLC2L11) was induced, indicative of apoptotic pathway activation. Combination of ganetespib with BGJ398 proved to be the most effective in causing cell death. However, ganetespib had differential effects on protein expression and cell viability in RT4 and SW780 cell lines, which contain FGFR3-TACC3 and FGFR3-BAI1AP2L1, respectively. While HSP90 inhibitors 17-AAG and 17-DMAG reduced cell viability, resistance to ganetespib was exhibited. This discrepancy may be due to differences in drug movement or metabolism [131]. Other HSP90 inhibitory compounds were effective in killing cells expressing BCR-ABL *in vitro* [122]. These results collectively indicate the potential of HSP90 inhibitors against fusion positive cases.

In cholangiocarcinoma, pazopanib (GW786034B) followed by ponatinib (AP24534) treatment, both RTK inhibitors, induced anti-tumor activity in a patient with FGFR2-TACC3. Ponatinib treatment also led to anti-tumor activity in a patient exhibiting FGFR2-MGEA5 fusion. Ponatinib has been FDA approved for treatment of the drug resistant T315I mutation in BCR-ABL fusion protein in CML [111].

In EMS, the small number of patients who have achieved long term remission have received hematopoietic stem cell transplantation. Many therapies used for acute lymphoblastic leukemia, acute myeloid leukemia, and myeloproliferative neoplasms have proven unsuccessful or display only short term remission against EMS. FGFR1 kinase inhibitor SU5402 has shown promise, demonstrating inhibitory effects in cells expressing BCR-FGFR1 or ZNF198-FGFR1. Interestingly, PI3K, farnesyltransferase, and p38 inhibitors were also successful in reducing growth of these cells, whereas MEK inhibitor PD98059 was not [130]. This is distinct from the MEK inhibitor U0126, which was shown to inhibit growth of cells expressing FGFR3-TACC3 [105]. While dovitinib has been successful in inhibiting the proliferation of Ba/F3 cells transfected with ZNF198-FGFR1 or BCR-FGFR1 and cell lines expressing FGFR1OP2-FGFR1, a push for effective FGFR1 inhibitors is needed for EMS cases [132].

3.6. Translocations leading to FGFR overexpression without creation of a fusion protein

Some translocations do not create a novel fusion protein; rather, these result in overexpression of FGFR. In the translocations of SLC45A3-FGFR2 in prostate cancer and IgH-MMSET-FGFR3 in Multiple myeloma (MM), the partner gene promoter now controls FGFR transcription, which alters the expression levels of the receptor. SLC45A3-FGFR2 translocation results in the endogenous promoter and exon 1 noncoding region of SLC45A3 attached 5′ to the FGFR2 gene, which places FGFR2 transcription under the control of an androgen-regulated promoter. This leads to FGFR2 overexpression and oncogenicity [96].

Multiple myeloma (MM) is characterized by a growth of malignant cells in the bone marrow. In approximately 20% of MM cases, a t(4;14) (p16.3;q32) translocation places MMSET and FGFR3 under the control of the IgH promoter, leading to overexpression of FGFR3 [133]. The overexpressed FGFR3 often contains an additional mutation, resulting in functional changes such as resistance to tyrosine kinase inhibitors (V557M),

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constitutive dimerization (Y375C), or constitutive kinase activation (K652E) [94]. However, one third of cases with this translocation lose FGFR3 expression while IgH is overexpressed. Additionally, although rare, translocations between FGFR3 and an immunoglobulin gene enhancer have been found in chronic lymphocytic leukemia (CLL), including t(4;14) (p16;q32) between FGFR3 and IgH, and t(4;22) (p16;q11.2) involving FGFR3 and IgL [134,135].

MM cases with the t(4;14) translocation have shown partial responsiveness to the FGFR3 inhibitor PD173074 and RTK inhibitor sunitinib (SU-11248). During *in vitro* studies, both inhibitors halted cell growth and inhibited FGFR3 activity, inducing an apoptotic response. However, during *in vivo* studies, tumor growth in the translocation-positive model was not inhibited by sunitinib, even though sunitinib was active in the translocation-negative tumors. The difference between the *in vitro* and *in vivo* data may be due to a difference in tumor microenvironment [133]. These studies also revealed that RTK inhibitors PD173074, sunitinib, and vandetanib (ZD6474) inhibited viability of Ba/F3 cells transformed with ZNF198-FGFR1. Sunitinib, which inhibits many RTKs, is approved for metastatic renal cell carcinoma treatment [133], and is being examined in clinical trials for relapsed multiple myeloma patients. Additionally, masitinib (AB1010, a TK inhibitor) has entered phase II clinical trials for MM patients with the t(4;14) translocation [clinicaltrials.gov].

3.7. Genomic events that contribute to FGFR fusion proteins

Although the occurrence of FGFR fusion proteins may be rare, there are similarities between fusions. Fusions with FGFR as the 5′ partner have only been found in solid tumors so far. In contrast, fusions with FGFR as the 3′ partner have consistently been found in EMS, which predisposes patients to either lymphoma, leukemia, or both. A few exceptions have been ERLIN2-FGFR1 found in breast cancer [96], BAG4-FGFR1 in lung squamous cell carcinoma (LUSC) [96], FOXO1-FGFR1 in rhabdomyosarcoma [136], TEL-FGFR3 in lymphoma [99], FN1-FGFR1 in phosphaturic mesenchymal tumor [137], and SQSTM1-FGFR1 in leukemia [138] (Table 3).

While the mechanism and cause of gene rearrangements is unknown, both intrachromosomal and interchromosomal rearrangements have been identified. Rearrangements in the form of tandem duplication, inversion, deletion, or translocation have all been identified as FGFR fusion formation events. Translocations occur when two double stranded breaks on different chromosomes rearrange and repair [100]. Fusion genes joined by a translocation can result in the formation of a reciprocal gene (*i.e.* FGFR2-BICC1 and BICC1-FGFR2 genes). This has been reported in some cases, such as BCR-FGFR1, CEP110-FGFR1, FGFR1OP-FGFR1, FGFR2-AHCYL1, FGFR2-BICC1, HERVK-FGFR1, LRRFIP1-FGFR1, RANBP2-FGFR1, SQSTM1-FGFR1, TIF1-FGFR1, and ZNF198-FGFR1 fusions [95,109–111,123,128,138,139]. However, reciprocal translocations have not been shown to be translated into functional proteins. The majority of these studies do not report the presence of a reciprocal fusion gene, and this may be indicative of another genetic alteration, such as an insertion or complex rearrangement, which would preclude the formation of the reciprocal gene [95].

The formation of these chromosomal rearrangements may occur due to common chromosomal fragile sites (CFSs). An increasing number of studies have identified CFSs as areas commonly affected by deletions, amplifications, and rearrangements in cancer [140]. CFSs have become linked to genomic instability, the driving force of cancer. Chromosomal breakpoints identified in cancer match to 67% of fragile sites induced *in vitro* [140]. All individuals posses CFSs, and these regions have been identified as evolutionarily conserved. CFSs contain tandem repeat sequences, often flexible AT-rich repeats and the formation of non-B-DNA secondary structures. Additionally, the fragile nature of CFSs has been linked to a lack of replicating origins within the CFS region, which may lead to incomplete replication. CFS expression is also specific to tissue or cell type. An investigation should be made into the correlation between CFS and tumor-specific gene rearrangements, as seen with some FGFR fusion protein expression. Mutagens and carcinogens often target CFS regions. Regulation of CFS occurs by DNA damage response proteins, including the ataxia telangiectasia mutated (ATM) pathway. This pathway is downregulated in cholangiocarcinoma patients with FGFR2 fusions [118].

CFS FRA10F has been identified at 10q26, a region which contains the FGFR2 gene [111], though some indicate FGFR2 is proximal to FRA10F [140]. FGFR2 is also surrounded by ribosomal protein pseudogenes (RPS15AP5 and RPL19P16), which contain repetitive bases, leading to genomic instability [111]. Although not thoroughly investigated, these factors could be an indication of the high level of genomic rearrangements seen in the FGFR2 region. In this regard, it may be noteworthy that 10 of 107 cholangiocarcinoma patients simultaneously exhibited two different fusions, FGFR2-BICC1 and FGFR2-PPHLN1 [118]. CFS regions have also been identified on the X chromosome, in regions flanking the ODF1 gene, which has been identified in a FGFR2-ODF1 fusion in thyroid cancer [96,140]. As seen (Table 3), FGFR1, FGFR2, and FGFR3 rearrangements predominate while, for unknown reasons, FGFR4 fusions are strikingly absent.

4. Concluding remarks

Aberrant FGFR signaling, either due to activating mutations or the presence of fusion proteins, supports cellular proliferation, tumorigenesis, and cancer progression. Although extensive research has shown that targeting FGFRs with small molecule inhibitors halts receptor activation, downstream signaling and results in tumor shrinkage, secondary mutations that contribute to drug resistance in tumors are challenges to successful clinical treatment. In addition, FGFRs fused to dimerizing partners brings a new level of complexity in terms of receptor activation and the specificity of small-molecule inhibitors. The development of FGFR therapeutics with personalized specificity will advance treatments of patients whose tumors harbor activated FGFRs *via* mutation or fusion protein.

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The thesis author was a co-author of this review, but not the research described by the review. The thesis author was responsible for section 3 in its entirety as well as assisting with editing and formatting all other figures, tables, and sections. Co-authors include Leandro H. Gallo, April N. Meyer, and Daniel J. Donoghue.

Chapter 2

Oncogenic Gene Fusion FGFR3-TACC3 Regulated by Tyrosine Phosphorylation

ABSTRACT

The discovery of translocations and fusion proteins involving Fibroblast Growth Factor Receptors (FGFRs) are becoming increasingly common in human cancers. Their presence leads to aberrant signaling that contributes to cell proliferation and cancer growth. A fusion protein between FGFR3 and transforming acidic coiled-coil containing protein 3 (TACC3) has become frequently identified in glioblastoma, lung cancer, bladder cancer, oral cancer, head and neck squamous cell carcinoma, gallbladder cancer, and cervical cancer. Through extensive analysis of the FGFR3-TACC3 fusion protein by titanium dioxide-based phosphopeptide enrichment (TiO2)-liquid chromatography (LC)-high mass accuracy tandem mass spectrometry (MS/MS), constitutive activation of the FGFR3 through altered phosphorylation of tyrosine residues can be seen due to the fused TACC3 coiled-coil domain. The FGFR3-TACC3 fusion protein also displays nuclear localization dependent on the fused TACC3, as well as altered cellular function, such as MAPK pathway activation, and cell transformation. These effects can be abrogated by introduction of K508R FGFR3 kinase dead mutation,

indicating kinase activity is required for oncogenicity of FGFR3-TACC3. IL-3 independent growth and cell proliferation assays confirmed the oncogenic potential of the fusion protein. These results demonstrate the oncogenic effects of FGFR3-TACC3 in human cancers due to an over-stimulated FGFR3 kinase domain.

INTRODUCTION

A subset of the Receptor Tyrosine Kinase (RTK) family is the Fibroblast Growth Factor Receptor (FGFR) family, which contains four homologous receptors: FGFR1, FGFR2, FGFR3, and FGFR4. FGFR activation results in changes in cellular proliferation and migration, anti-apoptosis, angiogenesis, and wound healing. All FGFRs contain three immunoglobulin-like (Ig) domains, a transmembrane (TM) domain, and a split tyrosine kinase (TK) domain. Binding of Fibroblast Growth Factors (FGFs) and heparin sulfate proteoglycans (HSPGs) to the extracellular Ig domains collectively induces FGFR activation through dimerization of receptor monomers and trans-autophosphorylation of kinase domain activation loop tyrosine residues. Tyrosine phosphorylation of the kinase domain initiates activation of RAS-MAPK, PI3K-AKT, and JAK/STAT pathways (1).

Mutations in FGFRs have been linked to numerous human cancers and somatic disorders, many of which have been extensively studied. More recently, FGFR fusion proteins have also begun to emerge in multiple cases of human cancers (1). Since their initial discovery in the late 1990s, the detection of these fusion proteins has steadily increased at an alarming rate. The focus of this paper is a fusion protein consisting of FGFR3 fused to transforming acidic coiled-coil containing protein 3 (TACC3) that has

been identified in glioblastoma, lung cancer, bladder cancer, oral cancer, head and neck squamous cell carcinoma, gallbladder cancer, and cervical cancer (1,2). The FGFR3-TACC3 fusion protein is a consequence of a 70 kb tandem duplication at 4p16.3 (3). This causes a reversal of the two genes, as TACC3 is normally upstream of FGFR3. TACC3 is a member of the TACC family, which consists of 3 known human proteins, TACC1, TACC2, and TACC3, all of which are involved in key roles of microtubule organization during mitosis. TACC3 is believed to be essential for the stabilization of kinetochore fibers and the mitotic spindle. A particularly important domain of this family is the C-terminal coiled coil domain (named TACC domain), which is highly conserved in all family members. This domain is believed to play an important role in localization of the protein during mitosis (4).

The frequent occurrence of this fusion protein across many cancer types leads to the question of how this protein is contributing to cancer progression. Is FGFR3 becoming constitutively activated due to the presence of the TACC domain? Is the presence of the coiled-coil domain able to stimulate activation loop phosphorylation in the FGFR3 kinase domain? Does the TACC3 domain play an important role in advancing cancer progression, or is its key role to activate the tyrosine kinase? While studies have investigated FGFR3 and TACC3 as separate entities, little has been defined about the FGFR3-TACC3 fusion protein. This paper investigates various properties of this fusion protein and its contribution to cancer progression, including mass spectrometry analysis of phosphorylation of key tyrosine residues, downstream signaling, cell transformation, and localization.

RESULTS

Constitutive phosphorylation of FGFR3-TACC3 fusion protein

In the FGFR3-TACC3 fusion protein, tyrosine kinase domain dimerization and autophosphorylation may be elevated by the presence of the TACC3 coiled coil domain, which could be crucial to cancer progression. To investigate changes in phosphorylation and biological activity, various FGFR3-TACC3 DNA derivatives were constructed. All fusion constructs contain the breakpoint between exon 18 of FGFR3 to exon 11 of TACC3 as shown in figure 5, chosen due to the high occurrence of this particular fusion breakpoint (3,5). This fusion is predicted to contain the extracellular, transmembrane, and intracellular kinase domains of FGFR3 fused 5' to the coiled-coil domain of TACC3 (6). Constitutively activated FGFR3 clones were produced by mutation of K650 to E. This mutation is known to cause Thanatophoric Dysplasia type II (TDII), a lethal form of achondroplasia, and is a highly activating and pathogenic FGFR3 mutation (1). The kinase activity of FGFR3 was abrogated by K508 to R mutation, known as the "kinase-dead" (KD) mutant (figure 5A).

To examine the phosphorylation of each fusion construct compared to FGFR3 WT, FGFR3(K650E), and FGFR3(K508R), constructs were expressed in HEK293 cells, collected and immunoprecipitated with an N-terminal FGFR3 antibody (figure 5B, top panel). An increase in tyrosine phosphorylation was seen in FGFR3-TACC3 compared to FGFR3 WT (lanes 2 and 6). No phosphorylation signal could be detected for the kinase-dead FGFR3 with or without the fused TACC3 (figure 5B). These results show that tyrosine phosphorylation of the fusion protein was increased by the presence of dimerizing TACC3 coiled coil and can be amplified by the presence of the activating

K650E mutation. Quantitation of phosphorylation levels shows a 2-fold increase in tyrosine phsophorylation on the FGFR3-TACC3 fusion protein compared to FGFR3 WT (figure 5C).

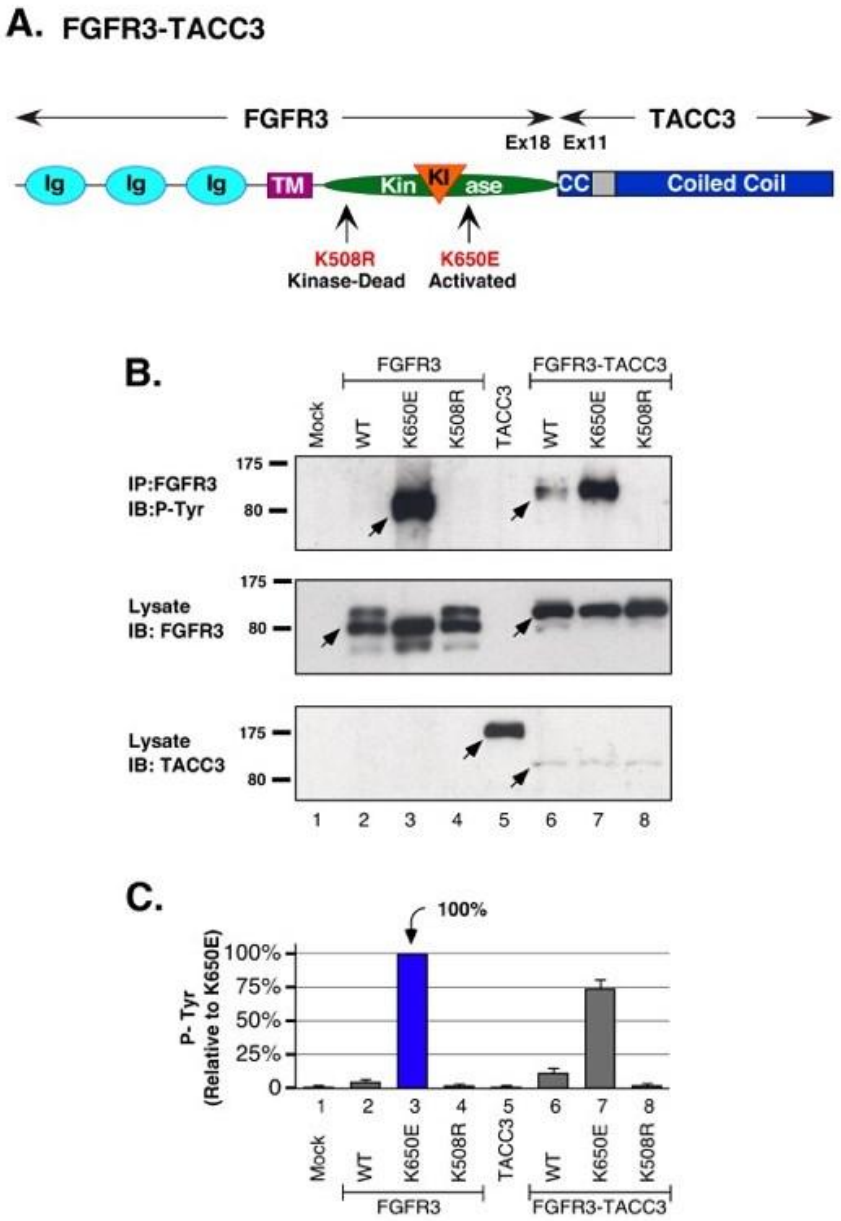

Figure 5. Increase in tyrosine phosphorylation by introduction of the TACC domain. (**A**) Schematic of FGFR3-TACC3 fusion protein. The N-terminal extracellular ligand-binding domain, transmembrane (TM), kinase, and kinase insert (KI) domains of FGFR3 are followed by a 3 amino acid linker (residues ASM), and fused to TACC3 starting at exon 11, which contains a coiled-coil domain. The location of K508 and K650 are shown. (**B**) Various mutation and fusions with FGFR3 were expressed in HEK293 cells, immunoprecipitated with FGFR3 antibody, and immunoblotted with phosphotyrosine antibody (top panel). Expression of the constructs were visualized in the lysates by immunoblotting with FGFR3 antisera (middle panel) and TACC3 antisera (bottom panel). (**C**) Quantification of tyrosine phosphorylation showing the standard error of the mean for 3 independent repeats, normalized to FGFR3(K650E).

LC-MS/MS analysis identifies elevated and novel phosphorylation sites

The strong increase in tyrosine phosphorylation seen by Western blot led to the question of whether TACC3 leads to a constitutively phosphorylated FGFR3 kinase and if additional or novel phosphorylation sites exist on the fusion protein. In order to explore this possibility, titanium dioxide-based phosphopeptide enrichment (TiO_2)-liquid chromatography (LC)-high mass accuracy tandem mass spectrometry (MS/MS) was used with samples from HEK293T cells expressing FGFR3 or FGFR3-TACC3 derivatives to identify significant phosphorylation sites. Immunoprecipitation with the FGFR3 N-terminal antibody and on-bead tryptic digestion revealed strong FGFR3 activation loop phosphorylation at residues Y647 and Y648 in both fusion proteins and non-fused FGFR3 and FGFR3(K650E) (figure 6B), indicating the receptor was constitutively active in all samples. Mass spectrometry analysis performed on the FGFR3 (K508R) derivatives detected no phosphorylated tyrosine residues (data not shown). All tyrosine phosphorylation sites detected on the fusion protein are indicated in figure 6C.

By comparing non-fused FGFR3 to FGFR3-TACC3, the effect of the coiled-coil domain on receptor phosphorylation and activation can be seen (figure 6A, 6B). Not only are phosphorylation levels more robust, but additional phosphorylation sites can be detected in the FGFR3 portion of the fusion, such as Y577, Y599, and Y607 (figure 6B, 1st and 3rd panels), indicating that receptor phosphorylation is over-stimulated in a ligand independent manner due to the presence of the TACC domain. The presence of the activating mutation K650E in FGFR3-TACC3 shows that the presence of the TACC

domain leads to higher phosphorylation intensity levels of the receptor (figure 6A, 2nd and 4th panels).

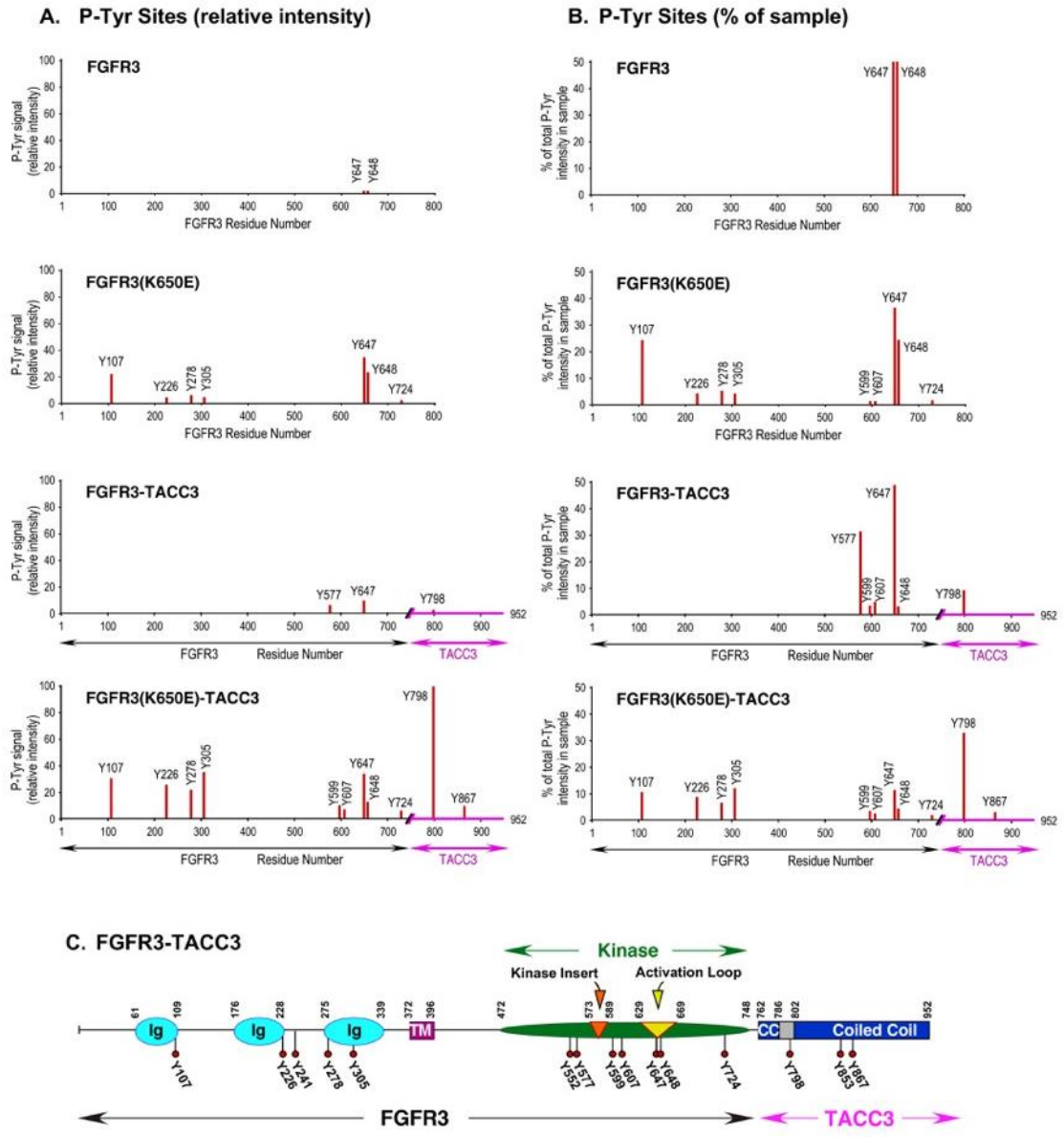

Figure 6. Phosphorylated tyrosine residues in FGFR3, FGFR3(K650E), FGFR3-TACC3, and FGFR3(K650E)-TACC3 identified by mass spectrometry analysis. (A) The intensity of the phosphotyrosine residues detected are presented normalized to 2 phosphoserine residues (S424 and S444) which were found to be constitutively phosphorylated across all samples. Duplicate, independent samples were subjected to mass spectrometry analysis. (B) For each phosphotyrosine residue detected, the percentage of intensity within the total protein is presented. (C) Schematic of FGFR3-TACC3 with the location of all tyrosine phosphorylation sites identified by LC-MS/MS.

Representative phosphorylated spectra are shown in figure 7. A commonly identified FGFR3 WT peptide containing double phosphorylation of Y647 and Y648 in the activation loop is shown in panel A. In FGFR3-TACC3 fusion protein constructs, this double phosphorylated peptide becomes less frequent, with detection of peptides containing single Y647 phosphorylation becoming more common (figure 6, figure 7B, 7C). Also shown are spectra containing primary phosphorylation sites Y577, Y798, and Y867 in FGFR3-TACC3 and FGFR3(K650E)-TACC3 (figure 7D, 7E, 7F).

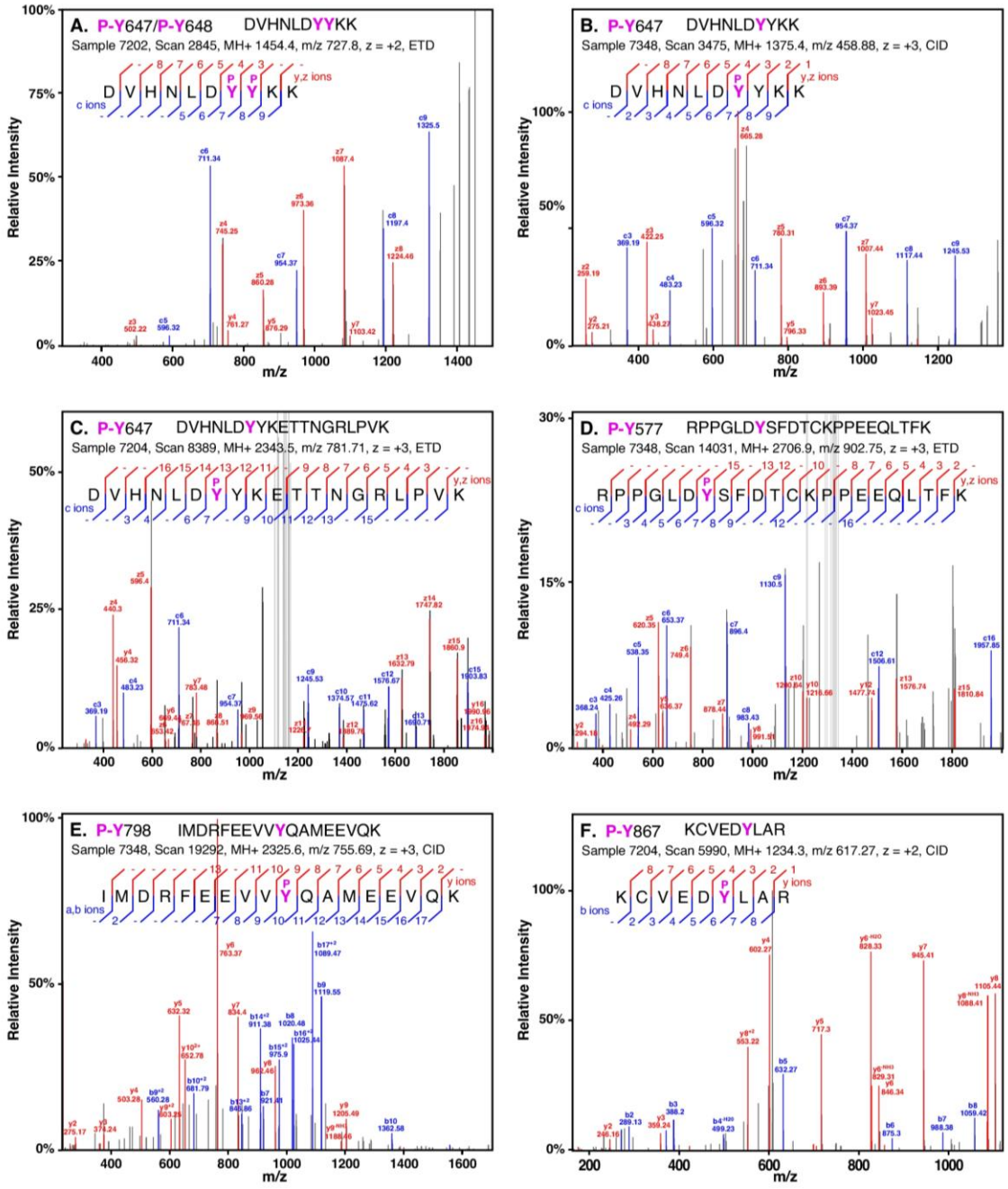

Figure 7. Representative spectra of selected peptides. The relative intensity of select ions of major phosphorylation sites are shown. Due to space constraints, not all identified ions are labeled. Identification of samples are as follows: A) FGFR3 WT (B) FGFR3-TACC3 (C) FGFR3(K650E)-TACC3 (D) FGFR3-TACC3 (E) FGFR3-TACC3 (F) FGFR3(K650E)-TACC3.

There are four tyrosine residues in the TACC3 portion of the FGFR3-TACC3 fusion protein: Y798, Y853, Y867, and Y878, corresponding to residues Y684, Y739, Y753, and Y764 in TACC3 WT. In FGFR3-TACC3, it was previously unknown if these tyrosine residues were also phosphorylated, possibly by the fused kinase domain, and if they play a role in cancer development. Through MS analysis, phosphorylation sites Y798, Y853, and Y867 were identified in FGFR3-TACC3 (figure 6C). Due to tryptic digest peptide size, Y853 was only recovered by a peptide miscleavage and Y878 was unable to be recovered. Increasing receptor activation by K650E mutation led to an increase in intensity levels of TACC3 tyrosine phosphorylation (figure 6A, 3rd and 4th panels).

Of the phosphorylation sites detected in the TACC3 portion of the fusion protein, Y798 and Y853 have been previously identified as a phosphorylation sites in TACC3 WT. The function of these sites is unclear and these residues are not conserved in the TACC family (7,8). However, Y867 is a conserved tyrosine residue in the TACC family and our data has identified it as a novel phosphorylation site for the FGFR3(K650E)-TACC3 fusion protein.

As mentioned above, mass spectroscopy of HEK293T cells expressing FGFR3(K508R)-TACC3 (kinase dead mutation) revealed no phosphorylated peptides within the FGFR3 or TACC3 domains. This indicates that receptor activation is required for tyrosine phosphorylation of the fusion proteins, and the TACC domain is most likely phosphorylated by the FGFR3 kinase domain, not another tyrosine kinase.

Cell transforming ability of FGFR3-TACC3 by focus assay

To examine the transforming activity of FGFR3-TACC3 and subsequent mutants, focus-forming assays with NIH3T3 cells were performed. FGFR3-TACC3 and FGFR3(K650E)-TACC3 produced extremely high foci formation and cell transformation compared to FGFR3 WT or FGFR3(K650E) (figure 8). Expression of PR/neu*, a focus assay positive control, displayed less transformation than FGFR3-TACC3, the latter of which also consistently produced much larger foci. PR/neu* is a Platelet-Derived Growth Factor Receptor, Beta (PDGFR-β) with a Neu receptor transmembrane domain with the activating V664E mutation ($p185^{neu*}$) (9). Despite the previously demonstrated elevated activation of PR/neu*, its transforming ability was dwarfed by the foci formation seen by FGFR3(K650E)-TACC3. As a result, samples were normalized to FGFR3(K650E)-TACC3 (figure 8). Expression of FGFR3(K508R)-TACC3 (kinase-dead mutation) and TACC3 WT in NIH3T3 cells did not produce significant foci formation, indicating that an active FGFR3 kinase domain is essential for cell transforming ability of FGFR3-TACC3.

Within the coiled-coil domain in FGFR3-TACC3, there are four tyrosine residues. Three of these residues were found to be phosphorylated by MS analysis, as discussed above, and the fourth tyrosine, Y878, undetectable by trypic digest, is believed to be phosphorylated as well (10). In order to assess the importance of these FGFR3-TACC3 phosphorylation sites, all four TACC3 tyrosine residues were mutated to phenylalanine (Y798F, Y853F, Y867F, Y878F) with and without the activating FGFR3 K650E mutation by site-directed mutagenesis and analyzed for focus forming ability. NIH3T3 cells expressing the fusion constructs with all four tyrosine mutations,

FGFR3-TACC3 4xYF and FGFR3(K650E)-TACC3 4xYF, displayed high foci formation when compared to FGFR3-TACC3 or FGFR3(K650E)-TACC3 with no additional mutations (figure 8).

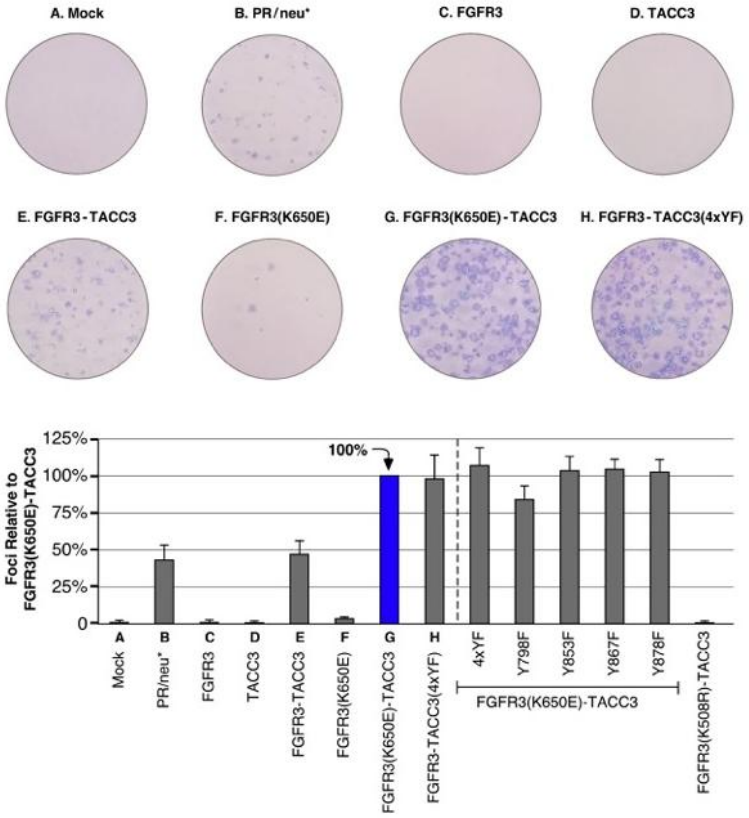

Figure 8. Transformation of NIH3T3 cells by FGFR3 and FGFR3-TACC3 derivatives. Representative plates from a focus assay are shown, with transfected constructs indicated. Number of foci were scored, normalized by transfection efficiency, and quantitated relative to FGFR3(K650E)-TACC3 +/- standard error of the mean. PR/neu* is a positive control. Assays were performed a minimum of three times per DNA construct.

To assess the effects of each individual phosphorylation site, single Y to F mutants were made in combination with activating mutation K650E. As shown in figure 8, three of the mutations (Y853F, Y867F, Y878F) increased foci formation at an even higher rate than K650E mutation alone, indicating an inhibitory role on cell growth

when phosphorylated in the FGFR3-TACC3 fusion. An exception may be FGFR3(K650E)-TACC3(Y798F), which displayed a slightly lower transformation ability than FGFR3(K650E)-TACC3, indicating this phosphorylation site may be important to cell proliferation.

FGFR3-TACC3 promotes IL-3 independent cell growth

The transforming potential of select fusion proteins was also examined in the murine myeloid cell line 32D which is dependent on Interluekin-3 (IL-3) for growth (11-13). FGFR3 WT, FGFR3-TACC3, FGFR3(K650E), FGFR3(K650E)-TACC3, FGFR3-TACC3(4xYF) and PR/neu* were electroporated into the 32D cell line and selected as described in the Materials and Methods. As seen in figure 9A, in the absence of IL-3 all the clones expressed were able to lead to IL-3 independent growth indicating their transforming potential. Interestingly, the FGFR3-TACC3(4xYF) clone had the highest proliferation even without the activating K650E mutation. This could support the suggestion of the TACC3 tyrosine residues as being inhibitory. In addition, even in the presence of IL-3 (figure 9B) the expression some of the clones enhanced the proliferation of the 32D cells compared to nonexpressing cells. The viability assays performed on days 3 and 7 shown in figure 9C support the cell population assay results. All transfected constructs display cell viability, whereas 32D control cells do not, indicating that FGFR3-TACC3 and other constructs promote cell proliferation.

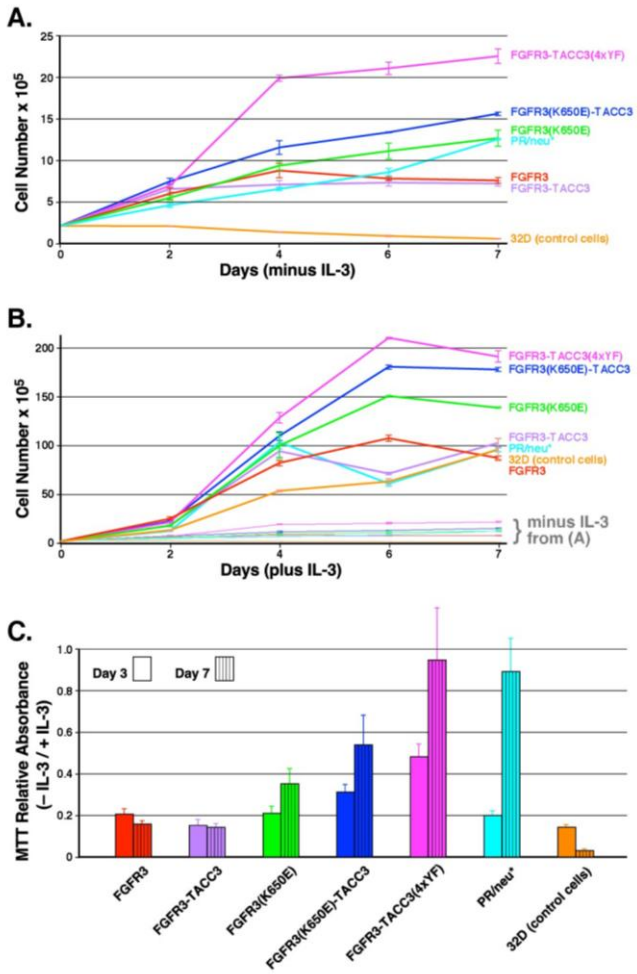

Figure 9. IL-3 independent growth and MTT viability assay in 32D cells expressing FGFR3 or FGFR3-TACC3 derivatives. PR/neu* is a positive control. (A) 32D cells selectively expressing FGFR3, FGFR3-TACC3, FGFR3(K650E), FGFR3(K650E)-TACC3, FGFR3-TACC3(4xYF), or PR/neu* were cultered in the absence of IL-3. The total number of viable cells were determined by trypan blue exclusion. Experiments were performed in triplicate, standard deviation is shown. (B) Cell counts of cultures in (A) in the presence of IL-3. Experiments were performed in triplicate, standard deviation is shown. Inset of growth from (A) without IL-3 is shown for comparison. (C) Cell viability as determined by MTT assay by 3 independent repeats on days 3 and 7. Relative absorbance was obtained by ratio of –IL-3 to +IL-3 absorbances read at 570 nm. Standard deviation is shown.

FGFR3-TACC3 displays nuclear localization

The presence of TACC3, a nuclear localizing protein (4), led to the question of whether a delocalization of the over activated FGFR3 kinase to the nucleus was

occurring. Indeed, fractionation of MCF7 cells expressing FGFR3 WT, FGFR3(K650E), FGFR3(K508R), and their fusion counterparts displayed a clear difference in localization (figure 10A). All three fusions, FGFR3-TACC3, FGFR3(K650E)-TACC3, FGFR3(K508R)-TACC3 (nuclear fraction, lanes 5, 6 & 7) displayed strong nuclear localization. The non-fused tyrosine kinase domains (lanes 2, 3 & 4) were present mainly in the cytoplasmic fraction. Perinuclear localization of FGFR3(K650E) has been demonstrated previously (14), but fusion of FGFR3(K650E) to TACC3 dramatically increased nuclear localization. These results indicate the presence of the TACC3 coiled coil domain is responsible for nuclear localization of the FGFR3 kinase, regardless of receptor activation. Immunoblotting for nuclear localizing mSin3A and cytoplasmic β-tubulin confirmed separation of nuclear and cytoplasmic fractions.

Downstream signaling activation by FGFR3-TACC3

It has been shown previously that FGFR3 WT and FGFR3(K650E) activate the signal transducer and activator of transcription (STAT) pathway and mitogen activated protein kinase (MAPK) pathway, but it is not clear how this activation compares to our constructed FGFR3-TACC3 or FGFR3(K650E)-TACC3 fusions. HEK293 cells expressing these fusions and their non-fused counterparts were analyzed for STAT1 and STAT3 activation. Both FGFR3(K650E) and FGFR3(K650E)-TACC3 led to phosphorylation of STAT1 and STAT3, but a significant increase in phosphorylation was not seen for the fusion constructs (figure 10B). However, MAPK phosphorylation was strongly elevated by FGFR3-TACC3 and FGFR3(K650E)-TACC3 (figure 10C,

lanes 6 & 7), compared to non-fused FGFR3 WT and FGFR3(K650E) (lanes 2 & 3), indicating that FGFR3-TACC3 induces MAPK pathway activation. The kinase-dead FGFR3(K508R)-TACC3 did not display this activation (lane 8), indicating that FGFR3 kinase activity in the fusion protein is essential to downstream signaling activation.

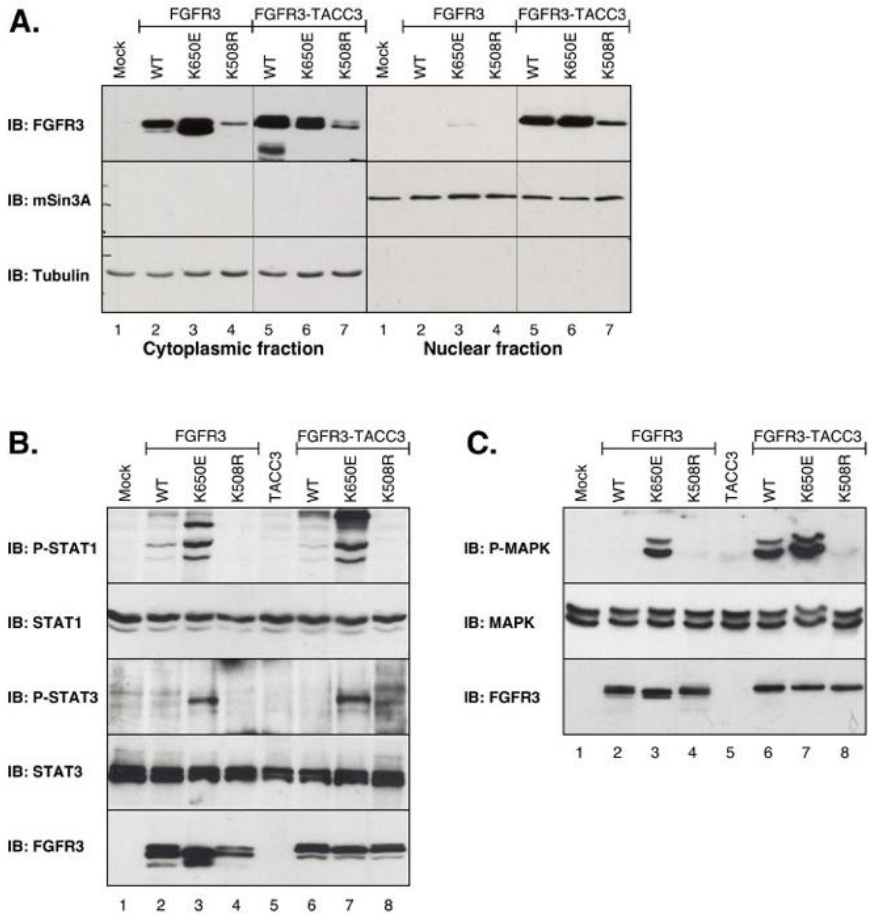

Figure 10. Localization and signaling of FGFR3-TACC3 fusions. (A) Fractionation of MCF7 cells expressing FGFR3 or FGFR3-TACC3 derivatives. Cells were separated into cytoplasmic (left) and nuclear (right) fractions. Immunoblotting with FGFR3 antibody shows nuclear localization of FGFR3-TACC3 fusions (top panels). Immunoblotting for mSin3A and β-Tubulin confirm fractionation (2nd and 3rd panels). (B) Lysates of HEK293 cells expressing FGFR3 or FGFR3-TACC3 derivatives were immunoblotted for Phospho-STAT1 (Y701) (top), STAT1 (2nd panel), Phospho-STAT3 (Y705) (3rd panel), STAT3 (4th panel), and FGFR3 (bottom). (C) HEK293 cell lysates expressing FGFR3 or FGFR3-TACC3 derivatives were immunoblotted for Phospho-MAPK (T202/Y204) (top), MAPK (2nd panel), and FGFR3 (bottom).

DISCUSSION

We extensively analyzed the FGFR3-TACC3 fusion protein by tyrosine residue phosphorylation changes and the impacts on cancer progression. We demonstrate that introduction of a 3' TACC3 coiled-coil domain results in constitutive activation and phosphorylation of key residues in FGFR3. Clearly the TACC domain over-stimulates kinase activity, as shown by the additional phosphorylation sites detected by LC-MS/MS. Activation by this coiled coil domain has a more severe impact on cell transformation and downstream signaling than the activating K650E mutation alone, which causes the lethal syndrome Thanatophoric Dysplasia type II. By focus, proliferation, and viability assay, the high cell transformation, proliferation, and oncogenic potential of the fusion protein was demonstrated. The absence of activity shown by FGFR3(K508R)-TACC3 kinase dead mutant indicates that kinase activity is required for gain of function and cancer progression, but not required for nuclear localization of the fusion protein, as shown by cellular fractionation.

The analysis by LC-MS/MS indicates key FGFR3 residues are being phosphorylated, such as Y647, Y648, and Y724. Residues Y647 and Y648 are part of the YYKK activation loop motif that has been proven essential to FGFR kinase activity (15). Interestingly, the intensity of the activation loop phosphorylation varies greatly between samples, with double phosphorylation of Y647 and Y648 becoming less common in the fusion protein constructs, indicating that presence of the TACC domain alters the mechanism of receptor activation. Residue Y724 has been shown to be critical for activation of downstream signaling pathways, such as MAPK, STAT, and PI3 Kinase, and cell transformation (16).

Although the function has not been thoroughly explored for all the phosphotyrosine sites detected by MS analysis (figure 6C), all sites are highly conserved in the four FGFRs, with the exception of Y577 which is not conserved in FGFR4. Interestingly, it has been suggested that Y577 is a key residue for the activation of FGFR3(K650E). Upon phosphorylation at Y577 the active state confirmation of the receptor is stabilized, independent of activation loop phosphorylation. Contrastingly, FGFR3 WT activation is dependent on ligand binding and activation loop auto-phosphorylation (17). The strong peak intensity seen for Y577 in FGFR3-TACC3 could indicate a change in the mechanism of activation in a ligand independent manner due to the TACC domain (figure 6B).

The fusion breakpoint of exon 18 in FGFR3 excludes the binding site for PLCγ (Y760), thus PLCγ is no longer recruited by FGFR3-TACC3, as previously shown (5). Additionally, Y760 may contribute to maximal STAT activation (16). The removal of this site from FGFR3-TACC3 may be contributing to the absence of STAT pathway overactivation. However, significant increase of downstream signaling activation was seen in the MAPK pathway independent of FGF ligand stimulation, which correlates with previous findings and further indicates ligand-independent activation and cell growth (3,5,18).

Overexpression of TACC3 WT has been shown to increase activation of MAPK signaling pathway and contribute to the epithelial-mesenchymal transition (EMT) (19). However, we found that overexpression of TACC3 alone does not lead to increased MAPK activity (HEK293) or cell transformation (NIH3T3). Our results indicate that the fusion of FGFR3 and TACC3 is required for gain of oncogenic function.

Also missing from this fusion breakpoint is the Aurora-A phosphorylation sites on TACC3. Aurora-A has been shown to phosphorylate TACC3 WT at S552 and S558 which is required for the localization of a TACC3-chTOG-clathrin complex to mitotic spindle microtubules and spindle poles (6,20,21). Localization of TACC3 to kinetochore fibers in complex with chTOG and clathrin is believed to assist with stabilization and formation of the mitotic spindle (21). However, previous studies have found the FGFR3-TACC3 fusion protein localized only to the mitotic spindle poles during mitosis, and relocated during late stage mitosis to the midbody. A mechanism for this change in recruitment and the role of FGFR3-TACC3 during interphase remains unclear (22).

Although not analyzed in regards to the cell cycle, we show a strong indication of nuclear localization for the fusion protein. Additionally, localization of FGFR3-TACC3 to the nucleus is not dependent on kinase activity as shown by K508R mutation, indicating that this localization is solely due to the fused TACC domain. Since the Aurora A phosphorylation sites are no longer present in the fusion protein, there must be another nuclear recruitment mechanism occurring. This delocalized kinase could be recruiting unknown proteins that lead to cancer progression, a topic for further study.

The detection of phosphorylated TACC3 residues Y798, Y853, and Y867 could indicate the ability of a highly activated kinase to self-phosphorylate the TACC domain and potentially lead to increased downstream signaling. Phosphorylation of Y878 was unable to be recovered by MS, but is presumably phosphorylated as it is located in a conserved 9 amino acid tyrosine phosphorylation motif within the TACC family (10).

Recently, it has been shown that chTOG (colonic and hepatic tumor overexpressed gene), a centrosomal localizing protein, recruits TACC3 to microtubule plus-ends during interphase. This localization is dependent on chTOG, not TACC3, and is independent of Aurora A phosphorylation (21). TACC3 residues 672-688 contain the binding site of ch-TOG and are present in the FGFR3-TACC3 fusion protein (at residues 786-802). Within this region is Y798, which we have found to be highly phosphorylated in the FGFR3-TACC3 and FGFR3(K650E)-TACC3 fusion proteins by LC-MS/MS. In the K650E background, when the phosphorylation site is removed by mutation Y798F, foci formation decreases compared to FGFR3(K650E)-TACC3 activity, indicating this region may be assisting fusion protein function, cell growth, and cancer progression.

Introduction of single mutations Y853F, Y867F, or Y878F lead to a slight increase in foci formation, indicating that phosphorylation at these three sites may have a negative impact on the oncogenic function of FGFR3-TACC3. An inhibitory phosphorylation site has been shown to occur in FGFR3 WT at Y770 which, upon phosphorylation, inhibits cell transformation (16). Residue Y770 has been removed from the FGFR3-TACC3 fusion, but the presence of C-terminal phosphorylation sites in the TACC domain may have a similar role.

We have presented overwhelming evidence of the high oncogenicity of the FGFR3-TACC3 fusion protein. The presence of the TACC coiled-coil domain leads to increased and altered levels of FGFR3 activation, fusion protein phosphorylation, downstream signaling, and cellular transformation, proliferation, and viability. This brings up the question of what causes the fusion protein to be oncogenic. Further study

of possible novel pathways becoming activated by the FGFR3-TACC3 fusion protein could lead to potential drug treatments which would prove beneficial. We hope the information presented here will be useful in interpreting future research in this field.

MATERIALS AND METHODS

DNA constructs

The TACC3 gene was purchased from Sino Biological Inc (pMD-TACC3) and was subcloned into pcDNA3. FGFR3, FGFR3(K650E), and FGFR3(K508R) were developed as previously described (23). To construct FGFR3-TACC3 fusion gene a unique ClaI site was introduced by PCR based site directed mutagenesis after residue 758 in FGFR3 and before residue 648 in TACC3. This unique site was used to subclone TACC3 3' of FGFR3 in pcDNA3, creating a fusion breakpoint of FGFR3 exon 18 to TACC3 exon 11 with a 3 amino acid linker of residues ASM containing the ClaI site.

Fragments containing K650E or K508R mutations were subcloned into the FGFR3-TACC3 fusion gene. Single and multiple tyrosine mutations in the TACC3 region (Y798F, Y853F, Y867F, Y878F) were introduced by PCR based site directed mutagenesis. DNA constructs were then subcloned into pLXSN vector (24) for focus, proliferation, and MTT assays. All clones were confirmed by DNA sequencing.

Cell culture

HEK293, HEK293T, and NIH3T3 cells were maintained in DMEM plus 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 10% CO_2, 37°C. MCF7 cells were maintained at 5% CO2 in DMEM plus 10% FBS and 1%

penicillin/streptomycin in 37°C. 32D clone 3 (ATCC CRL-11346) cells were maintained in RPMI 1640 medium with 10% FBS, 1% penicillin/streptomycin, and 5 ng/mL mouse IL-3 in 5% CO_2 37°C.

Mass Spectrometry Sample Preparation

HEK293T cells were plated one day prior to transfection at 3.0 x 10^6 cells per 15-cm tissue culture plate. 10 plates per sample were transfected by calcium phosphate precipitation with 9μg of FGFR3 or FGFR3-TACC3 derivatives. After 18-20 hr, cells were treated with 10 μM MG132 for 4-6 hr, washed once in 1xPBS + 1mM Na_3VO_4 before being lysed in RIPA. Clarified lysates were immunoprecipitated with FGFR3 antisera overnight at 4°C with rocking. Immune complexes were collected with Pierce protein A/G magnetic beads as per manufactures directions. Samples were taken to The Sanford Burnham Prebys Medical Discovery Institute mass spectrometry facility for proteasome on bead digestion and liquid chromatography (LC)-high mass accuracy tandem mass spectrometry (MS/MS) analysis.

Following immunoprecipitation, proteins were digested directly on-beads using Trypsin/Lys-C mix. Briefly, the samples (IP's and controls) were washed with 50 mM ammonium bicarbonate, and then resuspended with 8M urea, 50 mM ammonium bicarbonate, and cysteine disulfide bonds were reduced with 10 mM tris(2-carboxyethyl)phosphine (TCEP) at 30°C for 60 min followed by cysteine alkylation with 30 mM iodoacetamide (IAA) in the dark at room temperature for 30 min. Following alkylation, urea was diluted to 1 M urea using 50 mM ammonium bicarbonate. The samples were finally subjected to overnight digestion with mass spec

grade Trypsin/Lys-C mix (Promega, Madison, WI). Finally, peptides were collected into a new tube, and the magnetic beads were washed once with 50mM ammonium bicarbonate to increase peptide recovery. The digested samples were partially dried to approximately 50% of the total volume, and desalted using a C_{18} TopTip (PolyLC) according to the manufacturer's recommendations. The desalted peptide sample was split into 2 aliquots, 'Total' and 'Phospho' containing 10% and 90% of the sample, respectively. Both aliquots were then dried using a SpeedVac system.

The 'Phospho' aliquot was resuspended in 80% acetonitrile, 5% trifluoroacetic acid in 1M glycolic acid and incubated with TiO_2 magnetic beads (GE) for 30 min in a Thermomix at room temperature and 900 rpm. The unbound peptides were removed and the magnetic beads were washed twice with 80% acetonitrile, 5% trifluoroacetic acid to remove non-phosphorylated peptides. Finally, phosphopeptides were eluted with 5% ammonium hydroxide and dried down using a SpeedVac system.

LC-MS/MS Analysis

Both the 'Total' and 'Phospho' were analyzed by LC-MS/MS. Fifty percent of each sample was used for LC-MS/MS, a 0.180 x 20 mm C_{18} trap Symmetry column (Waters corp., Milford, MA) connected to an analytical C_{18} BEH130 PicoChip column 0.075 x 100 mm, 1.7µm particles (NewObjective, MA) mounted on a nanoACQUITY Ultra Performance Liquid Chromatography system (Waters corp., Milford, MA), directly coupled to an Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific). The peptides were separated with a 90-min non-linear gradient of 2-35% solvent B at a flow rate of 400nL/min. The mass spectrometer was operated in positive

data-dependent acquisition mode. MS1 spectra were measured with a resolution of 60,000, an AGC target of 10^6 and a mass range from 350 to 1400 m/z. Up to 5 MS2 spectra per duty cycle were triggered, and each precursor was fragmented twice by collisium-induced dissociation (with multiple stage activation enabled) and electron transfer dissociation (ETD), and acquired in the ion trap with an AGC target of 10^4, an isolation window of 2.0 m/z and a normalized collision energy of 35. Dynamic exclusion was set to 5 seconds to allow multiple fragmentation of phosphopeptides.

Proteomics data analysis

All mass spectra from were analyzed with MaxQuant software version 1.5.2.8 (Cox et al). Briefly, MS/MS spectra were searched against the cRAP protein sequence database (http://www.thegpm.org/crap/) indexed with corresponding FGFR3 or FGFR3-TACC3 derivative sequences. Precursor mass tolerance was set to 20ppm and 4.5ppm for the first search where initial mass recalibration was completed and for the main search, respectively. Product ions were searched with a mass tolerance 0.5 Da. The maximum precursor ion charge state used for searching was 7. Carbamidomethylation of cysteines was searched as a fixed modification, while phosphorylation of serines, threonines and tyrosines, and oxidation of methionines was searched as variable modifications. Enzyme was set to trypsin in specific mode and a maximum of two missed cleavages was allowed for searching. The target-decoy-based false discovery rate (FDR) filter for spectrum and protein identification was set to 1%. Second peptide mode of MaxQuant software was also enabled.

Antibodies and Reagents

Antibodies were obtained from the following sources: FGFR3 (B-9), mSin3A (K-20), β-tubulin (H-235), STAT1 (E-23), STAT3 (C-20) from Santa Cruz Biotechnology; phosphotyrosine (4G10) from Millipore; TACC3 C-terminal (SAB4500103) from Sigma; Phospho-STAT1 (Tyr701) (9171), Phospho-STAT3 (Tyr705) (D3A7), Phospho-p44/42 MAPK (Erk1/2) (T202/Y204) (E10), p44/42 MAPK (Erk1/2) (9102) from Cell Signaling Technology; horseradish peroxidase (HRP) anti-mouse, HPR anti-rabbit from GE Healthcare. Enhanced chemiluminence (ECL and Prime-ECL) reagents were from GE Healthcare. MG132, aFGF, and recombinant mouse Interleukin-3 (IL-3) were obtained from R&D systems; Heparin was from Sigma; Geneticin (G418) was from Gibco. Lipofectamine 2000 Reagent was from Invitrogen.

Transfection, Immunoprecipitation, Immunoblot

HEK293 were plated at a density of 1 x 10^6 cells/100-mm plate and transfected with 3 μg plasmid DNA using calcium phosphate transfection in 3% CO_2 as previously described (25). 20 to 24 hr after transfection, media was changed to DMEM with 0% FBS. Cells were starved for 20 hr before collecting and lysis.

Transfected HEK293 cells were collected, washed once in PBS, and lysed in 1% NP40 Lysis Buffer [20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 1% Nonidet P-40, 5 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 10 μg/mL aprotinin] or radioimmunoprecipitation assay buffer [RIPA; 50 mmol/L Tris-HCl (pH 8.0), 150

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 (24). 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–phospho-STAT1, and anti–phospho-STAT3 blots). Immunoblotting was performed as previously described (26).

Focus Assay

Focus assays were performed using NIH3T3 cells plated at a density of 2 x 10^5 cells/60-mm plates in DMEM with 10% FBS 24 hr before transfection. Cells were transfected by Lipofectamine 2000 Reagent per manufacturer directions with 10 μg plasmid DNA. Between 22 and 24 hr after transfection cells were re-fed with DMEM 10% FBS. Cells were split 1:12 onto 100-mm plates between 22 and 24 hr later. Foci were scored at 12-14 days, fixed in methanol, stained with Geimsa stain, and photographed. Efficiency of transfection was determined by Geneticin (G418, 0.5 mg/ml)-resistant colonies plated at a dilution of 1:240.

Fractionation

MCF7 cells were plated at a density of 1.5 x 10^6 cells/100-mm plates 24 hr before transfection. Immediately prior to transfection, media was changed to DMEM 0% FBS with no antibiotic. Cells were transfected with 8 μg of plasmid DNA using Lipofectamine 2000 Reagent, per manufacturer's directions. 23 hr after transfection cells were collected in PBS and 1 mM EDTA for fractionation as described previously (27). Separated fractions were analyzed for protein content by Bradford assay, separated by 10% SDS-PAGE, and transferred to Immobilon-P membrane for Western Blot analysis.

IL-3 independent growth in 32D cells

1x10^6 exponentially growing 32D cells were electroporated (1500 V, 10 ms, 3 pulse) by the Neon Transfection System (Invitrogen) using 30 μg of FGFR3, FGFR3-TACC3 or PR/neu* derivatives in pLXSN in triplicate. Twenty-four hours after transfection cells were selected with 1.5 mg/ml Geneticin (G418) sulfate for 10 days to generate stable cell lines. For IL-3 independent proliferation assays, 2x10^5 cells were seeded in 12 well plates in the absence of IL-3 or 6 well plates in the presence of IL-3. The media also contained 1 nM aFGF and 30 μg/ml heparin (28). Cell numbers were determined in triplicate, with a hemocytometer and trypan blue exclusion on days 2, 4, 6 and 7. Media was added to cultures when cell numbers reached ~1x10^6 cells/mL during the assays to maintain at viable concentrations. To measure cell viability MTT assays were performed. A stock solution of 5mg/ml in PBS of MTT 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was added at 1:10 to

the cultures. After incubation at 37°C, 5% CO_2 for approximately 4 hrs equal volume of 0.04 M HCl in isopropanol was added and mixed well and incubated again for at least 30 min (29). Cultures were transferred to microfuge tubes, spun for 30 sec at room temperature and supernatant absorbance was measured in a Beckman DU 350 UV/Vis spectrophotometer at 570 nm. $5x10^4$ cells per well were plated in triplicate in 24-well plates in the presence or absence of IL-3 and 1nM aFGF and 30ug/ml heparin and assayed 3 days later. The cell viability at day 7 was measured using the cultures from the proliferation assay. In triplicate, 0.5 ml of the cultures were transferred to 24 well plates and treated with the MTT reagent.

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Chapter 2, in part is currently being prepared for submission for publication of the material, with the authors of Nelson KN, Meyer AN, Siari A, Donoghue DJ. The thesis author was the primary investigator and author of this material.

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