

**IDENTIFICATION OF NOVEL COMPONENTS IN
FIBROBLAST GROWTH FACTOR SIGNALLING**

by

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Abstract

Fibroblast growth factor receptors play many roles in development, cell proliferation and differentiation. They possess intrinsic tyrosine kinase activity which enables activation of other signalling proteins, formation of multiprotein signalling complexes and activation of downstream cascades. The goal of this project was to identify novel FGFR1 interacting proteins. The strategy employed for mapping novel partners on the basis of peptide-protein interaction was peptide pull-down. Using synthetic pairs of phosphorylated and unphosphorylated peptides, pull-down experiments were performed to enrich phospho-specific binding partners which then were identified by mass spectrometry. Experiments carried out using FGFR1 peptides revealed novel proteins associated with receptor. Signal transducer and activator of transcription 3 (STAT3) was identified as a phospho-dependent partner for Tyr677 of FGFR1. Mutation of this tyrosine to phenylalanine eliminated the binding of STAT3 to FGFR1. Furthermore, it was presented that STAT3 tyrosine phosphorylation required over-expression of FGFRs, as shown in the breast cancer cell line, SUM-52PE. The inhibition of Src and Janus non-receptor tyrosine kinases decreased FGF1-induced tyrosine STAT3 phosphorylation. The findings suggested that FGFR kinase activity was mandatory for physical association between FGFR and STAT3 and its subsequent tyrosine activation by Src and Jak kinases. Moreover, Src and Jak2 were demonstrated to form a complex with kinase active FGFR1. Finally, STAT3 was serine phosphorylated by JNK and ERK kinases, which were activated by FGF1 stimulation. Since over-expression of FGFRs is correlated with tumour development and STAT3 is a well known oncogene, it is possible that the FGFR-STAT3 signalling pathway is up-regulated in cancer cells and therefore merits consideration as a therapeutic target.

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Abbreviations:

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

ERK - Mitogen-activated protein kinase kinase

FGF – Fibroblast growth factor

FGFR – Fibroblast growth factor receptor

FRS2 – FGFR substrate 2

GEF - guanine nucleotide exchange factor

GFRs – Growth factor receptors

HSPGs - Heparan sulphate proteoglycans

IGFR - Insulin growth factor receptor

IP - Immunoprecipitation

Jak – Janus kinase

KGF - Keratinocyte growth factor

KGFR – Keratinocyte growth factor receptor

MAPK - Mitogen-activated protein kinase

MS/MS – Mass spectrometry

PDGF – Platelet derived growth factor receptor

PI3K – Phosphatidylinositol 3-kinase

PKC – Protein kinase C

PPD – Peptide pull-down

PTB - Phosphotyrosine binding domain

RTKs – Receptor tyrosine kinases

SH2 – Src homology domain

SILAC – Stable isotope labelling by amino acids in cell culture cell

STAT – Signal transducer and activator of transcription

VEGFR - Vascular endothelial growth factor receptor

WCLs – Whole cell lysates

CHAPTER 1

Introduction

Introduction

A number of pathway-specific proteins are involved in the efficient processing of signals from the extracellular matrix to the nucleus of the cell. Thanks to signal transduction, cells are capable to modulate their activity in response to their surrounding environment. The point of contact for external signals is provided by cell surface receptors. Receptor tyrosine kinases (RTKs) are a large family of receptors that are normally activated in a ligand-dependent manner. They possess intrinsic tyrosine kinase activity which enables activation of other signalling proteins and the formation of multiprotein complexes (Schlessinger 2000). RTKs catalyse transfer of the phosphate of ATP to hydroxyl groups of tyrosine residue on adaptor proteins and thus activate numerous signalling pathways (Hunter 1998). The interaction between adaptor proteins and RTKs is mediated by specific phospho-motifs on C-terminal parts of the receptors (Pawson 2004).

There are many members of the RTK family, including epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), insulin growth factor receptor (IGFR) and fibroblast growth factor receptor (FGFR) (van der Geer, Hunter et al. 1994; Schlessinger 2000). Each type of cell can be stimulated by many different ligands which activate distinct signalling pathways leading to various cellular responses. Furthermore, oncogenic activity of RTKs has been

implicated in tumour progression of various cancer types (Zandi, Larsen et al. 2007; Hynes and MacDonald 2009). The complexity of signal transduction makes it incredibly interesting and at the same time difficult to examine the mechanisms responsible for these processes. This review focuses on FGFR signalling pathway, its adaptor proteins and implications in cancer progression.

1. Structure of FGFs and FGFRs

Fibroblast growth factors (FGFs) constitute a large family of 22 human distinct polypeptide growth factors varying in size from 17 to 34 kDa (Ornitz and Itoh 2001). Their genes have been described in other vertebrate and invertebrate organisms including *Drosophila* and *C. elegans* (Ornitz and Itoh 2001). They play a vital role in regulation of embryogenesis (Martin 1998). In the adult, they are responsible for cell growth, differentiation, proliferation and cell migration (Eswarakumar, Lax et al. 2005; Mohammadi, Olsen et al. 2005). All members possess a conserved sequence (16-65% identity) within a central core domain of 120 amino acids that interacts with FGF receptors (FGFRs) (Ornitz and Itoh 2001). Most FGFs have amino terminal signal peptide sequence and are secreted into the extracellular environment where they bind heparan sulphate proteoglycans (HSPGs) in the extracellular matrix (ECM) (Powers, McLeskey et al. 2000; Ornitz and Itoh 2001; Bottcher and Niehrs 2005). FGF1-FGF10 all bind specific FGFRs (Ornitz and Itoh 2001). FGF1 and FGF2 are known as acidic and basic fibroblast growth factor, respectively. FGF11-FGF14 are FGF homologous factors which possess similar sequence homology but they do not bind FGFRs and have been shown to have distinct functions unrelated to rest of FGFs (Olsen, Garbi et al. 2003). Newer members of FGFs family, FGF16-23, are not as well characterized.

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FGFs activate cellular responses by binding to FGFRs. The FGF receptor family consists of five distinct receptors which are cell surface receptor tyrosine kinases. In general, FGFRs are composed of an extracellular ligand binding domain, a single spanning transmembrane domain and intracellular domain (Figure 1). The extracellular domain consists of three immunoglobulin-like domains, called D1, D2, D3; an acidic box between D1 and D2, and a positively charged heparin binding region that regulates the interaction between the ECM and cell adhesion molecules (Powers, McLeskey et al. 2000; Schlessinger 2000; Eswarakumar, Lax et al. 2005).

FGFR5 was identified as highly homologous to FGFR1-4 with 32% identity in extracellular domain and two immunoglobulin-like domains (Kim, Moon et al. 2001; Sleeman, Fraser et al. 2001). However, in contrast to other FGFRs, it lacks a cytoplasmic tyrosine kinase domain and its functionality needs to be further investigated (Kim, Moon et al. 2001; Sleeman, Fraser et al. 2001).

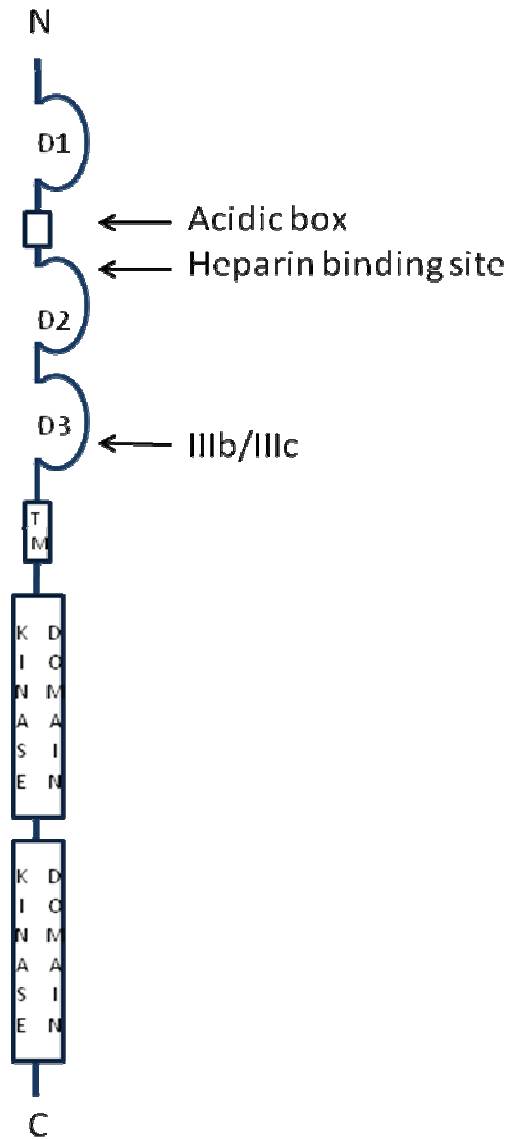


Figure 1. The structure of FGF receptors.

Schematic diagram of FGFR. FGFRs consist of extracellular domains (immunoglobulin-like domains D1-D3, acidic box), a transmembrane domain (TM) and a cytoplasmic kinase domains.

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The Ig-like domains of FGFRs are responsible for ligand binding and specificity, as well as they have an auto-regulatory function (Schlessinger 2000). Ligand specificity is accomplished by interaction between N-terminal and central regions of FGFs and two loop regions in D3, which undergo alternative splicing (Plotnikov, Hubbard et al. 2000). Alternative splicing of D3 creates different FGFR isoforms that have varying ligand binding specificity (Table 1) (Eswarakumar, Lax et al. 2005). FGF interacts with D2 and D3 and with the linker between them. This is enhanced by interaction between FGF and D2 of the second receptor in the complex and receptor:receptor interactions (Schlessinger 2000). Neither D1 nor acidic box is necessary for the interaction with FGF (Schlessinger 2000). In fact, this extracellular region of FGFRs was proposed to be responsible for auto-inhibition of the receptor (Plotnikov, Schlessinger et al. 1999). It was suggested that D1 and acidic box interact with the basic region of D2 which inhibits HSPGs binding. Ligand stimulation unlocks the confirmation and induces receptor activation (Plotnikov, Schlessinger et al. 1999).

Table 1. FGFRs ligand specificity (based on Eswarakumar et al. 2005).

| FGFR isoforms | Ligand specificity |
|----------------------|-------------------------------------|
| FGFR1b | FGF1, 2, 3, 10 |
| FGFR1c | FGF1, 2, 4, 5, 6 |
| FGFR2b | FGF1, 3, 7, 10, 22 |
| FGFR2c | FGF1, 2, 4, 6, 9, 17, 18 |
| FGFR3b | FGF1, 9 |
| FGFR3c | FGF1, 2, 4, 8, 9, 17, 18, 23 |
| FGFR4 | FGF1, 2, 4, 6, 8, 9, 16, 17, 18, 19 |

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The intracellular region of FGFRs contains a juxtamembrane domain and a split tyrosine kinase region with catalytic activity (Powers, McLeskey et al. 2000; Bottcher and Niehrs 2005). At least seven autophosphorylation sites in the cytoplasmic tail of FGFR1 have been identified: Tyr463, Tyr583/585, Tyr653/654, Tyr730, Tyr766 (Mohammadi, Dikic et al. 1996). Tyr463 is located in the juxtamembrane and is responsible for binding CrkII (Larsson, Klint et al. 1999). Tyr583/585 are located in the kinase insert region of the receptor (Mohammadi, Dikic et al. 1996). Tyr653/654 are conserved in all FGFRs and are critical for kinase activity (Mohammadi, Dikic et al. 1996). Tyr653 could be mutated to phenylalanine and still retain activity of the receptor, thus Tyr654 is essential for maintaining FGFR signalling (Mohammadi, Dikic et al. 1996). Tyr730 is required for FGF2-mediated urokinase-type plasminogen activator induction (Dell'Era, Mohammadi et al. 1999) and Tyr766 is the binding site for PLC γ 1 and Shb (Mohammadi, Honegger et al. 1991; Mohammadi, Dionne et al. 1992). Additionally, site-directed mutagenesis in PC12 cells revealed that Tyr677 and Tyr701 are required for PC12 cell differentiation (Foehr, Raffioni et al. 2001). Thus, apart from Tyr654 that was previously identified by Mohammadi *et al.* (Mohammadi, Dikic et al. 1996), Tyr677 and Tyr701 were also essential for the bioactivity of FGFRs in PC12 cells (Foehr, Raffioni et al. 2001). Substitution of Tyr463, Tyr558, Tyr563, Tyr572, Tyr583, Tyr585, Tyr605 and Tyr613 to phenylalanine revealed that these residues are not necessary for receptor activity in PC12 cells (Foehr, Raffioni et al. 2001). Larsson and colleagues showed that substitution of Tyr463 decreased proliferation rate of endothelial cells (Larsson, Klint et al. 1999).

FGFRs dimerisation generates trans-phosphorylation that takes place in an exact order (Furdui, Lew et al. 2006; Lew, Furdui et al. 2009). The first stage is phosphorylation of Tyr653 in the activation loop which increases kinase activity of the receptor. The second stage

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is activation of Tyr583, Tyr463, Tyr766 and Tyr585. Phosphorylation of these tyrosines provides docking sites for adaptor proteins, including PLC γ 1. The last stage is phosphorylation of Tyr654 that further increases tyrosine kinase activity and leads to phosphorylation of adaptor proteins (Furdui, Lew et al. 2006). It was demonstrated that introduction of oncogenic and activating mutations in FGFR results in a disrupted sequence of phosphorylation and thus aberrant assembly of signalling molecules (Lew, Furdui et al. 2009). The disturbances in FGFR signalling may have serious consequences leading to development of disease or cancer, as shown in glioblastoma (Lew, Furdui et al. 2009).

2. Alternative splicing of FGFRs.

Alternative splicing of the FGFRs transcripts gives rise to multiple isoforms of receptors which vary mainly in the structure of their Ig-like domains (Johnson and Williams 1993). As mentioned above, alternative splicing of the D3 domain gives rise to different FGFR isoforms resulting in different ligand binding specificity (Plotnikov, Hubbard et al. 2000). D3 domain exists in three versions; D1-3, known as IIIa, IIIb and IIIc (Johnson, Lu et al. 1991). For example, isoform FGFR2a is encoded by exon 7, FGFRb by exon 8 and FGFRc is encoded by 9 (Eswarakumar, Lax et al. 2005). The type of FGFR2 isoform can influence the expression pattern and so FGFRIIIb is expressed in epithelial lineages, whereas the IIIc in mesenchymal lineages. Moreover, truncated versions of the receptor are created by introduction of early stop codons (Johnson and Williams 1993). FGFR that express IIIa domain are examples of truncated receptors and do not transmit the signal (Johnson, Lu et al. 1991).

Alternative splicing of the juxtamembrane region of FGFR regulates FRS2 binding (Burgar, Burns et al. 2002). Two residues, valine and threonine, in juxtamembrane region of

FGFR are essential for FRS2 recruitment (Burgar, Burns et al. 2002). Both forms are found to be expressed during embryonic development and their inclusion or exclusion (VT+ or VT-) determines the signalling potential (Gillespie, Chen et al. 1995; Burgar, Burns et al. 2002).

3. Role of HSPGs in FGFR activation

An important biological feature of FGFs is their interaction between FGFRs and heparin or heparan sulphate proteoglycans (HSPGs) (Harmer 2006). HSPGs are glycosaminoglycans that are highly negatively charged. FGFs bind FGFR with high affinity, however, HSPGs are necessary to provide effective activation of FGFRs (Yayon, Klagsbrun et al. 1991; Spivak-Kroizman, Lemmon et al. 1994). The positively charged region of D2 serves as a HSPG binding site (Schlessinger, Plotnikov et al. 2000). HSPGs are bound to a low affinity site on FGFs core region. Binding of proteoglycans and the formation of a ternary complex between FGF/FGFR/HSPGs is required to activate FGFRs and stabilise the complex, as well as facilitate FGF-mediated signalling (Schlessinger 2000; Harmer 2006). Besides coupling receptors and ligands together, HSPGs protect FGFs from denaturation and proteolysis in the extracellular matrix (Powers, McLeskey et al. 2000). Furthermore, HSPGs regulate the kinetics and thermodynamics of ternary complex formation and it was shown that FGF-FGFR pairs preferentially bind heparin comparing to individual FGFs (Mohammadi, Olsen et al. 2005; Harmer 2006).

The crystal structure of FGFRs and two models has been proposed (Plotnikov, Schlessinger et al. 1999; Pellegrini, Burke et al. 2000; Plotnikov, Hubbard et al. 2000; Schlessinger, Plotnikov et al. 2000). First symmetric model was suggested by Schlessinger's group with 2:2:2 (FGF:FGFR:HSPG) stoichiometry (Schlessinger, Plotnikov et al. 2000). Activated FGFRs are stabilized by three events: ligand binding to D2 and D3 region,

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receptors dimerization and binding of HSPGs to D2 region (Schlessinger, Plotnikov et al. 2000). Simultaneous binding of HSPGs helps dimerization of FGF:FGFR complexes, it stabilises the formation of the complex between ligand and receptor (1:1 FGF:FGFR complex), as well as formation of the complex between two dimers (2:2 FGF:FGFR) (Schlessinger, Plotnikov et al. 2000). Alternative asymmetric model was proposed with 2:2:1 stoichiometry (FGF:FGFR:HSPG), where only one heparin molecule is present in the complex (Pellegrini, Burke et al. 2000). The crystal structure was obtained for FGF1 and FGFR2 in the presence of heparin decasaccharid, which is located between two halves of the protein complex. Moreover, heparin binds only one receptor and two ligands, while second receptor is left unoccupied (Pellegrini, Burke et al. 2000).

4. Signalling through FGFRs

Binding of ligand to FGFRs activates multiple signal transduction pathways through a series of phosphorylation events. The first step following FGF/FGFR/HSPGs complex formation is receptor dimerization (Schlessinger 2000). This triggers tyrosine kinase activity leading to autophosphorylation of the intracellular domains of both receptors. The key event is activation of FGF receptors which serves as a mechanism for recruitment of adaptor proteins. Formation of multiprotein complexes is an essential step in signal transduction from the cell surface into the nucleus (Schlessinger 2000).

4.1. SH2 and PTB domains

The phospho-tyrosine residues on FGFRs form binding sites for proteins which contain SH2 (Src homology domains 2) or PTB (phosphotyrosine binding domain) domains (Bottcher and Niehrs 2005). Proteins with SH2 or PTB domains recognise specific tyrosine motifs on the receptors which facilitate protein-protein interactions. SH2- and PTB-containing proteins couple signals from tyrosine kinases to the effectors that are located inside the cells. Importantly, different SH2 and PTB domains bind tyrosine motifs with distinct specificity which determines recruitment of signalling proteins to the receptor and subsequently influences the specificity of the signal transduction (Pawson 2004; Huang, Li et al. 2008). The SH2 domain, found in PLC γ 1, Src, STATs, PI3K, Grb2 and Shb, binds a phosphorylated tyrosine residue in the context of a longer peptide motif within a target protein containing carboxy-terminal residues (Pawson 2004). The mechanism of SH2 domain binding is based on the interaction between phospho-tyrosine residue of the receptor and arginine residue in the SH2 domain. Phospho-tyrosine, which is located within the specific motif on the receptor, fits into the conserved binding pocket of the SH2 domain where it is captured by the invariant arginine (Pawson 2004). The PTB domain also binds phospho-tyrosine motifs but in the context of amino-terminal residues (Pawson 2004). For example, the PTB domain distinguishes the consensus sequence NPXpYX and is found in adaptor proteins, such as FRS2, IRS1, IRS2 and Shc (Liu, Jablonowski et al. 2006). Identification of novel binding motifs for proteins containing SH2 domain is crucial in order to comprehend proteins' function. Phospho-tyrosine consensus binding motifs for multiple proteins built of SH2 domain have been proposed by screening orientated peptide library against all SH2 domains identified in human genome (Huang, Li et al. 2008).

4.2. FGFRs' adaptor proteins

4.2.1. PLC γ 1

There have been a number of proteins described to interact with FGFRs. PLC γ 1 was the first protein identified to directly associate with FGFR1 and its role was extensively studied (Burgess, Dionne et al. 1990; Mohammadi, Honegger et al. 1991). Activation of PLC γ 1 by FGFR and its association with the receptor was reported in 1990 (Burgess, Dionne et al. 1990; Dionne, Crumley et al. 1990). The C-terminal site of FGFR1 was demonstrated as a binding site for PLC γ 1 by its SH2 domain. This association was described as phospho-dependent and mediated by Tyr766 of FGFR1 (Mohammadi, Honegger et al. 1991). Mutation of Tyr766 to phenylalanine prevents PLC γ 1 binding and phosphatidylinositol hydrolysis, however, it does not inhibit FGF-induced mitogenesis (Mohammadi, Dionne et al. 1992). Although Tyr766 seems to be the main binding site for PLC γ 1, additional residues, Tyr677 and Tyr701, are needed for maximal activation of PLC γ 1 (Foehr, Raffioni et al. 2001).

4.2.2. FRS2

FRS2, also called SNT-1, is an adaptor protein linking the FGFR to downstream signalling proteins (Kouhara, Hadari et al. 1997; Xu, Lee et al. 1998). There are two isoforms of FRS2: FRS2 α (SNT1) and FRS2 β (SNT2) which have very similar structure and the sequence identity between them is 49%. The PTB domain of FRS2 is located at the N-terminal part of the protein and the C-terminal tail of FRS2 contains multiple tyrosine

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residues that become phosphorylated upon FGF stimulation (Figure 2) (Xu, Lee et al. 1998; Burgar, Burns et al. 2002). FRS2 is associated with non-canonical segment of juxtamembrane region of FGF receptor via its' PTB domain (Kouhara, Hadari et al. 1997; Xu, Lee et al. 1998). This association is constitutive and is independent of phospho-tyrosine residues on FGFRs, unlike FRS2 interaction with nerve growth factor receptor, TrkA, that is only observed upon stimulation (Xu, Lee et al. 1998; Ong, Guy et al. 2000). FRS2 is anchored to the membrane by myristylation on consensus sequence MGSCCS (Figure 2).

The C-terminal region of FRS2 is rich in phosphorylation sites and consequently FRS2 is able to bind many signalling proteins (unpublished data, Leila Fares PhD Thesis 2009) (Ong, Guy et al. 2000). The disruption of FRS2 α gene leads to severe defects in mouse development and embryonal lethality at E7.0-E7.5 (Hadari, Gotoh et al. 2001). Moreover, it has been demonstrated that FRS2 α deficient fibroblasts show impairment in MAPK stimulation, PI3K activation and cell proliferation (Hadari, Gotoh et al. 2001).

FRS2 has been shown to recruit both positive and negative regulators of signalling to modulate the balance between FGF signal transduction and attenuation. It plays an important role in Ras/MAPK pathway signal transduction via association with Grb2, another adaptor protein that links FGFR signalling to the MAP kinase pathway (Kouhara, Hadari et al. 1997). The FGFR lacks a consensus binding site for Grb2 and thus relies on the adaptor protein, FRS2, for its recruitment. Grb2 contains one SH2 and two SH3 domains. The SH2 domain of Grb2 can bind four of the determined phosphorylated tyrosine residues of FRS2 (Y196, Y306, Y349, Y392) whilst the SH3 domains of Grb2 constitutively bind Sos, which is guanine nucleotide exchange factor. Upon ligand binding FRS2 becomes phosphorylated and recruits Grb2/Sos complex, which in turn initiates the Ras/MAPK pathway activation (Kouhara, Hadari et al. 1997).

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Furthermore, indirect Grb2 association with FRS2 is mediated via the protein tyrosine phosphatase Shp2 (Hadari, Kouhara et al. 1998). Shp2 is a positive regulator of FGFR signalling (Hanafusa, Torii et al. 2004). FRS2 directly interacts with Shp2 via two tyrosine residues, Tyr436 and Tyr471 and SH2 domain of Shp2 (Hadari, Kouhara et al. 1998). Shp2 as a phosphatase is responsible for de-phosphorylation of Sprouty, negative regulator of FGFR signalling and it was shown to be required for sustained Ras/MAPK pathway activation in PC12 cells (Hadari, Kouhara et al. 1998; Hanafusa, Torii et al. 2004). Moreover, Shp2 was described as constitutively associated with Shb, a recently described adaptor protein bound to phosphoTyr766 of FGFR1 via its SH2 domain (Cross, Lu et al. 2002). The interaction of Shp2 with Shb regulates FRS2 phosphorylation and is required for FGF-mediated mitogenicity via the Ras/MAPK pathway (Cross, Lu et al. 2002).

FRS2 is also responsible for indirect activation of PI3K pathway by recruitment of Gab1 (Grb2-associated binder). Gab1 is bound to Grb2, which is a part of multiprotein complex formed by phosphorylated FRS2 (Hadari, Gotoh et al. 2001). Recruitment of Gab1 leads to subsequent phosphorylation and interaction with p85 subunit of PI3K (Schaeper, Gehring et al. 2000).



Figure 2. Structure of FRS2.

FRS2 is composed of an N-terminal myristylation site, PTB domain and C-terminal domain containing tyrosine residues.

4.2.3. Src family kinases

Src is a cellular counterpart of v-Src, the first identified oncogene. Src is a member of membrane-associated non-receptor tyrosine kinases. All Src family members (Src, Yes, Fyn, Fgr, Lyn, Hck, Lck, Blk, Yrc) are composed of multiple functional domains: SH2, SH3 domain, kinase domain, myristylation domain, a unique domain and C-terminal regulatory region (Thomas and Brugge 1997; Sandilands and Frame 2008). Src is auto-inhibited by phosphorylation of Tyr527 in the C-terminal region, which upon stimulation is dephosphorylated together with phosphorylation Tyr416 in the activation loop (Bjorge, Pang et al. 2000; Harrison 2003; Sandilands and Frame 2008). Src family proteins take part in multiple cellular functions, including cell adhesion, motility, proliferation and survival. Moreover, Src family kinases play a vital role in signal transduction from receptor tyrosine kinases (Morgan, Nicholson et al. 2008).

Src activation upon FGF stimulation was previously described (Sandilands, Akbarzadeh et al. 2007), although an association between FGFR and Src is often controversial. It was demonstrated that the Src SH2 domain binds phosphorylated FGFR1 protein (Zhan, Plourde et al. 1994). Moreover, the phospho-specific interaction between FGFR1 and Src influences tyrosine phosphorylation of cortactin (Zhan, Plourde et al. 1994). Similarly, the direct interaction between Yes and FGFR-1 β was revealed by Zhang *et al.* who reported that FGFR-1 β -induced phosphorylation of Yes was linked with cortactin activation (Zhang, Greendorfer et al. 2006). In contrast, Landgren *et al.* did not detect a Src/Fyn-FGFR interaction (Landgren, Blume-Jensen et al. 1995). Recently a link between FGFR and Src mediated via FRS2 was demonstrated (Li, Brunton et al. 2004). The interaction between Src/FRS2 and FGFR leads to activation of Sprouty by direct binding to Src. Formation of this

complex triggers inhibition of MAPK pathway and attenuates signal transduction (Li, Brunton et al. 2004).

Furthermore, FGFR and Src activation is inter-dependent; FGFR activation induces Src phosphorylation whereas, Src controls FGFR activity and regulates its trafficking and signalling dynamics (Sandilands, Akbarzadeh et al. 2007). Phosphorylated Src was shown to co-localise with active FGFR at the plasma membrane and in endosomal vesicles (Sandilands, Akbarzadeh et al. 2007). Because Src can regulate endosomal trafficking of RTKs via RhoB- and Rab11-associated endosomes (Sandilands, Cans et al. 2004; Sandilands, Brunton et al. 2007), FGFR endosomal trafficking is impaired in the absence of RhoB (Sandilands, Akbarzadeh et al. 2007).

4.2.4. CrkII

CrkII is another example of an FGFR binding protein. It was isolated as an oncogene product of the CT10 chicken retrovirus. CrkII is a member of adaptor protein group that contains both SH2 and SH3 domains, thus it can interact with phospho-tyrosine motifs or proline-rich regions, respectively (Mayer, Hamaguchi et al. 1988). CrkII has been shown to interact with multiple proteins: paxilin, growth factor receptors, C3G (a guanine nucleotide exchange factor for Rap1), Sos (GEF for Ras), c-Abl, PTP1B, p85 subunit of PI3K and JNK (Feller, Posern et al. 1998; Takino, Tamura et al. 2003). CrkII can be phosphorylated upon stimulation of different ligands and its activity is regulated by various kinases and phosphatases (Takino, Tamura et al. 2003).

The association between CrkII and FGFR1 is facilitated by the SH2 domain and Tyr463 of activated FGFR1 (Larsson, Klint et al. 1999). Initially, it was thought that Tyr463

is not essential for FGFR mitotic response (Mohammadi, Dikic et al. 1996), however, later it was demonstrated that CrkII links FGF receptors to the downstream signalling. CrkII couples molecules like Sos to the MAPK cascade and C3G to the Jun kinase cascade. Therefore, its role is crucial in FGF1-induced endothelial cell proliferation (Larsson, Klint et al. 1999). Substitution of Tyr463 in the juxtamembrane region of FGFR1 and subsequent impaired CrkII recruitment results in reduction of both MAPK and Jun kinase activity and decreased proliferation rate (Larsson, Klint et al. 1999).

4.2.5. Shc family

FGFRs interact with the family of docking proteins Shc (SH2 domain containing transforming protein). There are three isoforms of Shc: 46 kDa, 52 kDa and 66 kDa. They are composed of an SH2 domain, a proline-rich CH1 domain and a C-terminal PTB domain (Pelicci, Lanfrancone et al. 1992). Shc proteins bind and become phosphorylated by EGFR. Constitutive over-expression of Shc in NIH3T3 mouse fibroblasts leads to transformation and tumour development (Pelicci, Lanfrancone et al. 1992).

Shc activation was described upon FGF2 stimulation and also direct recruitment of Shc to FGFR1 was presented (Klint, Kanda et al. 1995). The proposed role of Shc in FGFR signalling was to recruit Grb2 and Sos to the activated receptor and couple the Grb2-Sos complex to the Ras pathway (Klint, Kanda et al. 1995). Association between Shc/Grb2/Sos and FGFR1 was later confirmed in v-Src-transformed cells (Curto, Frankel et al. 1998). The site for the direct Shc-FGFR interaction was suggested as Tyr730 of FGFR1 (Dunican, Williams et al. 2001). A cell permeable FGFR1 peptide containing Tyr730 and flanking residues was able to recruit Shc protein and the association between them was maintained by

PTB domain of Shc (Dunican, Williams et al. 2001). However, recently Shc has been described as an indirect binding partner for FGFR2 (Schuller, Ahmed et al. 2008). Results from fluorescence lifetime imaging microscopy (FLIM) showed that Shc was a part of signalling complex recruited to the receptor but it did not bind receptor directly. It was proposed that the Shc SH2 domain interacted with Src to form a ternary complex with FGFR2, whereas the Shc PTB domain was responsible for membrane localization (Schuller, Ahmed et al. 2008).

4.2.6. Grb14

Grb14 belongs to a small family of adapter proteins (Grb7 family) that are known to interact with a number of receptor tyrosine kinases and signaling molecules. It is built of pleckstrin homology domain-containing central region, SH2 domain, a conserved N-terminal motif which is characteristic for Grb7 family (Daly, Sanderson et al. 1996).

Grb14 was identified as a novel FGFR1 binding partner by yeast two hybrid system (Reilly, Mickey et al. 2000). It was suggested that Grb14 interacts with FGFR1 at multiple sites; the C-tail domain of the receptor containing tyrosine residues is required for binding of Grb14 as well as the juxtamembrane domain. The SH2 domain of Grb14 takes part in FGFR binding. Additionally, a region of Grb14 upstream of the SH2 is crucial for FGFR specificity. The interaction between Grb14 and FGFR1 is ligand-dependent and it plays important role in FGF signalling (Reilly, Mickey et al. 2000).

4.3. FGFR signalling pathways

4.3.1. Ras/MAPK pathway

The Ras/mitogen-activated protein kinase (MAPK) pathway is common mechanism of signal transduction in eukaryotic cells. It is composed of multiple serine/threonine kinases that react to extracellular stimuli and regulate developmental changes in organisms (Pearson, Robinson et al. 2001). They are involved in RTK-dependent signal transduction that regulates cell proliferation, differentiation and metabolism (Pearson, Robinson et al. 2001; Bottcher and Niehrs 2005; Eswarakumar, Lax et al. 2005).

The activation mechanism relies on successive phosphorylation events of downstream kinases, including RAF-1, MEK and ERK (Marshall 1995). Activation of FGF receptor leads to phosphorylation of FRS2 which then recruits Grb2 and Sos. Formation of a FRS2/Grb2/Sos complex activates Ras which interacts with several proteins leading to activation of the MAPK pathway (Figure 3). Ras is a membrane-bound guanine nucleotide binding protein with intrinsic GTPase activity. It is inactive in the GDP-bound state and active in the GTP-bound state. Sos leads to the exchange of GDP to GTP on Ras and its subsequent activation. Active Ras triggers recruitment of the serine/threonine kinase RAF-1 (MAPKKK) to the plasma membrane (Marais, Light et al. 1995). Activation of RAF is the first step in phosphorylation cascade, starting from MEK. Once MEK (MAPKK) is activated, it phosphorylates ERK (MAPK). ERK directly phosphorylates a variety of targets, such as transcription factors, kinases and enzymes which result in cell proliferation and differentiation via changes to gene expression (Schlessinger 2000; Bottcher and Niehrs 2005; Eswarakumar, Lax et al. 2005).

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There are three forms of RAF: ARAF, BRAF and CRAF (RAF-1), which share similar structure with two N-terminal regulatory domains and a C-terminal kinase domain, as well as multiple phosphorylation sites (Wellbrock, Karasarides et al. 2004). BRAF has been described as strong activator of ERKs due to its constitutive activation at Ser445 resulting in high basal activity (Mason, Springer et al. 1999). Moreover, V599E mutation in BRAF is commonly found in cancer, especially melanoma (Davies, Bignell et al. 2002; Wellbrock, Ogilvie et al. 2004). It was suggested that this mutation mimics phosphorylation of Thr598 and Ser601, becoming active and leads to sustained ERK activation (Mercer and Pritchard 2003).

Rap1 is another small GTPase that can be activated upon growth factor stimulation (Raaijmakers and Bos 2009). Similarly to Ras, it was demonstrated that Rap1 induces MAPK pathway activation (Vossler, Yao et al. 1997; York, Yao et al. 1998). Rap1 and Ras are regulated by different GEFs; for example classical Ras GEF is Sos, whereas Rap1 GEFs are Epac or Vav2 (Raaijmakers and Bos 2009). Rap1 can be activated by cAMP-dependent kinase PKA, which in turn leads to BRAF binding and phosphorylation (Vossler, Yao et al. 1997). This interaction is only specific for BRAF, not for CRAF; moreover, Rap1 inhibits CRAF activation (Vossler, Yao et al. 1997). Furthermore, activation of MAPK pathway by NGF-induced is triggered by Ras and sustained by Rap1 (York, Yao et al. 1998). Recently, Rap1 has been identified as FGFR1 binding partner in proteomic screen (Vecchione, Cooper et al. 2007). Interestingly, Rap1 is a key regulator in FGF-induced angiogenesis (Yan, Li et al. 2008). Rap1 depletion results in reduction of signalling levels of ERK, p38 and Rac that are crucial in the process of FGF-mediated angiogenesis (Yan, Li et al. 2008). Thus, Rap1 can be an important mediator of FGF-induced ERK activation.

4.3.2. PLC γ 1 signalling pathway

Activation of PLC γ 1 involves binding to phosphorylated Tyr766 of FGFR through its SH2 domain (Mohammadi, Honegger et al. 1991). Phosphorylation of PLC γ 1 enables hydrolysis of phosphatidyl-inositol-4,5-biphosphate (PIP₂) to two second messengers: diacylglycerol (DAG) and phosphate-1,4,5-inositol (IP₃). IP₃ then binds the IP₃ receptor on the surface of endoplasmic reticulum and induces opening of the calcium channel and release of the intracellular calcium from the endoplasmic reticulum. This is the mechanism by which PLC γ 1 activates of calcium-dependent kinases, like calmodulin-dependent protein kinases. The second messenger, DAG, works together with calcium to activate protein kinase C (PKC) (Figure 3) (Bottcher and Niehrs 2005).

Activation of PLC γ 1 by FGFR1 affects cytoskeletal reorganization in endothelial cells, however, it is not required for mitogenesis in L-6 myoblasts and BaF3 cells, neither for neurite outgrowth in PC12 cells nor for induction of chemotaxis by FGF2 in certain cell types (Mohammadi, Dionne et al. 1992; Mohammadi, Dikic et al. 1996).

4.3.3. PI3K/Akt pathway

As previously mentioned, FRS2 takes part in activation of the PI3K/Akt pathway by formation of complex with Grb2 and Gab1 (Grb2-associated binder) (Figure 3). This complex phosphorylates the p85 subunit of PI3K and allows signal transmission to the nucleus. Another mechanism of PI3K/Akt activation can be utilised by cells, which involves direct binding of p85 to the C-terminal region of FGFR and activation of downstream proteins.

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Moreover, Ras can associate with the p110 subunit of PI3K, which also results in activation of PI3K/Akt signalling (Powers, McLeskey et al. 2000; Bottcher and Niehrs 2005).

Activation of the PI3K/Akt pathway by FGF regulates the cell survival. Phosphoinositol 3 kinase (PI3K) is a heterodimer composed of a 110 kDa catalytic subunit and a regulatory subunit of 85kDa (Vanhaesebroeck, Leever et al. 2001). PI3K generates the second messenger PtdIns(3,4,5)P₃ by phosphorylation of PtdIns(4,5)P₃ at the plasma membrane (Vanhaesebroeck, Leever et al. 2001). PtdIns(3,4,5)P₃ interacts with Akt (also called PKB), which is a PH domain containing serine/threonine kinase. PtdIns(3,4,5)P₃ couples Akt with its activator phosphoinositide-dependent protein kinase 1 (PDK1). Thus, PtdIns(3,4,5)P₃ facilitates Akt/PDK1 binding by bringing them to the same compartment, the plasma membrane (Andjelkovic, Alessi et al. 1997; Milburn, Deak et al. 2003). The movement of Akt to the plasma membrane induces a conformational change where its activation domain becomes exposed to PDK1 (Milburn, Deak et al. 2003). Phosphorylation of Akt by PDK1 activates Akt and induces Akt-dependent cell survival (Lawlor and Alessi 2001).

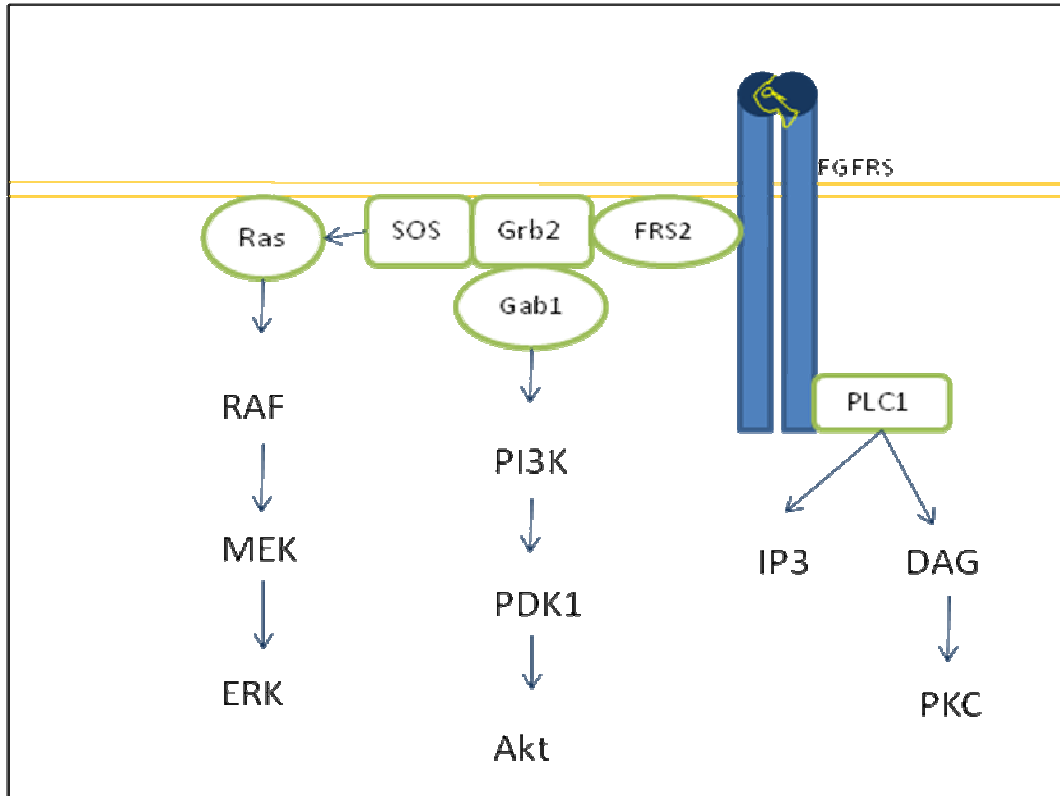


Figure 3. Intracellular signalling pathways activated through FGFRs.

A simplified schematic representation of FGFR downstream signalling pathways. Upon FGF binding receptors dimerize, become phosphorylated and induce formation of multiprotein complex leading to activation of Ras/MAPK, PLC β 1/Ca²⁺ and PI3K/Akt.

5. Negative regulation of FGFR signalling

Negative regulators provide a balance between activation of signalling pathways and signal inhibition to ensure a suitable physiological outcome. The inhibition of FGFR signalling might be targeted at the level of the receptor as well as at the level of signalling pathways. Receptor activation can be switched off by tyrosine phosphatases (PTPs) that are capable of de-phosphorylation of the receptor. Human phosphatases that regulate FGFR signalling are still unknown, however, the *C. elegans* phosphatase, CLR-1, was proved to attenuate the activity of EGL-15 FGFR (Kokel, Borland et al. 1998).

Inhibition at the level of the receptor can be regulated by the transmembrane proteins SEF (Similar Expression to FGF genes). FGFR1 and SEF interact directly at the cell membrane via the cytoplasmic domain of SEF. Tyrosine 330 of SEF was shown to be critical for SEF localization and its inhibitory function (Ren, Li et al. 2007). Over-expression of SEF decreases the activation of FGFR1 and FRS2 as well as inhibits the activation of MAPK pathway (Kovalenko, Yang et al. 2003). Furthermore, down-regulation of SEF is linked with enhanced tumorigenic potential of FGFR (Darby, Sahadevan et al. 2006; Darby, Murphy et al. 2009).

The prominent negative regulator, not only for FGFR signalling but also for several other RTKs, is Sprouty. There are four human and mouse homologues of Sprouty; Spry1-4. All of them possess a cysteine-rich Spry translocation domain which targets Spry to the membrane. Human Spry is often localized in vesicles inside the cell and on the plasma membrane (Kim and Bar-Sagi 2004). Sprouty was first identified in *Drosophila* as an inhibitor of EGFR-mediated MAPK pathway activation (Casci, Vinos et al. 1999). The exact

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mechanism of Sprouty function is not known yet but it is becoming obvious that it is a rather complex and dynamic process. The action of Sprouty as negative regulator is thought to be downstream of RTKs but upstream of members of MAPK pathway (Kim and Bar-Sagi 2004). A conserved tyrosine residue, Tyr55, of Spry is phosphorylated upon ligand binding. In the case of FGF signalling, it was demonstrated that Spry2 can be directly tyrosine phosphorylated by Src in a FRS2-dependent manner which leads to inhibition of the MAPK pathway (Li, Brunton et al. 2004). Additionally, phosphorylated Spry competes and blocks Grb2 interaction with FRS2 and Shp2. Because formation of FRS2/Grb2/Shp2 is essential step in signal transduction, interaction between Spry and Grb2 subsequently decreases MAPK pathway activation (Kim and Bar-Sagi 2004). De-phosphorylation of Spry and its subsequent release from the complex with Grb2 is mediated by Shp2 (Hanafusa, Torii et al. 2004).

On the other hand, the role of Spry in EGFR signalling in mammalian cells is distinct to FGFR. While Spry attenuates activation of the MAPK pathway in FGFR signalling, it potentiates EGFR-mediated signal transduction. Upon EGF stimulation Spry is phosphorylated and directly interacts with c-Cbl. This interaction inhibits ubiquitin-dependent degradation of EGFR. As a consequence it sustains MAPK activation that results in differentiation of PC12 cells (Guy, Wong et al. 2003; Kim and Bar-Sagi 2004). The complex formation between Spry and c-Cbl influences the duration of the signal and finally leads to proteolytic degradation of Spry. More recently, the link between Spry activity and EGFR trafficking was shown where Spry controls EGFR signalling by regulation of endocytosis (Kim, Taylor et al. 2007). By interaction with Hrs (hepatocyte growth factor-regulatory protein), Spry inhibits the progression of early endosomes to late endosomes. Thus, the ERK activation is sustained in early endosomes. It suggests that Spry-mediated regulation of MAPK activity is spatio-temporal (Kim, Taylor et al. 2007).

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Furthermore, SPREDs (Spry related EVH1 domain proteins) have a similar function to Spry. They are localized at the membrane by their cysteine-rich domain and inhibit the MAPK pathway induced by GFR. Additionally, they are composed of EVH1 (ENA/Vasodilator-stimulated phosphoprotein homology-1) domain which contributes to its inhibitory effects. SPRED changes the dynamics of Raf distribution which alters its accessibility to activators (Kim and Bar-Sagi 2004). Recently, SPRED2 has been reported to take part in regulation of FGFR trafficking through its direct interaction with NBR1. SPRED2 targets phosphorylated FGFR to the lysosomal degradation which implies the role in negative regulation of RTK signalling (Mardakheh, Yekezare et al. 2009).

C-Cbl is an E3 ubiquitin ligase that plays an important role in receptor ubiquitination and degradation. In case of EGFRs, upon ligand binding receptors undergo autophosphorylation and recruit c-Cbl. EGFRs and c-Cbl are internalised to early endosomes where c-Cbl mediates polyubiquitination of the receptor tail and concomitant sorting of the receptor to late endosomal/lysosomal compartments which leads to receptor degradation (Levkowitz, Waterman et al. 1999). Ubiquitin facilitates lysosomal degradation of many receptors by serving as an endosomal sorting signal therefore, the loss of ubiquitin from the receptor tail is a signal for recycling (Haglund and Dikic 2005). It is still controversial as to whether ubiquitin is essential for internalization as it was demonstrated that it is not needed *in vivo* (Haglund and Dikic 2005). Recently, ubiquitination of FGFR1 was shown to be necessary for degradation but not for the early steps of endocytosis (Haugsten, Malecki et al. 2008). Overall, ubiquitination plays an important role in regulation of trafficking and signalling due to its ability to target receptors into specific subcellular compartments leading to modulation of the strength and duration of the signal.

6. Endocytosis and trafficking of growth factor receptors

Endocytosis of membrane receptors is a complex and dynamic process essential for maintaining homeostasis. In the traditional view, signalling is based on the binding of the ligand to the receptor on the cell surface, activation of secondary messengers in the cytosol which transmit signals to the nucleus, whereas endocytosis was considered as a way to attenuate the signalling by degradation of receptors. However, receptor trafficking is not only important for deactivation of receptor but it also plays a role in signal transduction and many studies revealed that RTKs stay active while in endosomes (Wiley and Burke 2001; Miaczynska, Pelkmans et al. 2004). There are several advantages of signalling during receptor trafficking, mainly in terms of spatio-temporal regulation. Firstly, endocytosis regulates signalling specificity by manipulating the pattern of substrate activation. This hypothesis is based on the observation that different substrates are located in distinct places inside the cell; from apical to basolateral distribution in case of polarized cells or from membrane to cell compartments. Since the signalling might be slightly different from the membrane than from endosomes, it is clear that trafficking regulates the quality of the signal. Moreover, by interaction with many effector proteins, endocytosis influences on the duration of the signal (Wiley and Burke 2001; Miaczynska, Pelkmans et al. 2004).

Receptor trafficking can be constitutive or ligand-induced, and it can occur in a clathrin-dependent or -independent manner. Receptor internalization and transport into the cell is regulated by protein modifications and many protein-protein and protein-lipid interactions (Szymkiewicz, Shupliakov et al. 2004). The most studied system in this field is endocytosis of epidermal growth factor receptors (EGFRs). After stimulation with EGF or TGF- α , EGFRs are internalized through clathrin-coated vesicles (CCV) within several

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minutes. Formation of the CCV involves many specific proteins, like dynamin, clathrin, Eps-15 and AP2. Next EGFRs are moved through the series of endosomal compartments (Figure 4). Initially, receptors are delivered to early endosomes from where they are either recycled to the plasma membrane in recycling endosomes or are moved to lysosomes via late endosomes. Acid-dependent protease degradation of EGFRs in lysosomes attenuates receptor signalling. Furthermore, EGFRs can be endocytosed prior to stimulation. Ligand-free receptors are almost exclusively recycled to the cell surface whereas ligand-occupied receptors are sorted mainly to lysosomes (Wiley and Burke 2001; Sorkin and Von Zastrow 2002). In addition to removing receptors from the cell surface, endocytosis efficiently removes growth factors from extracellular fluids, which contributes to the regulation of ligand concentration in the extracellular matrix. For example, exogenous FGF1 and FGF2 are internalized via a clathrin-dependent and clathrin-independent endocytosis (Wiedlocha and Sorensen 2004).

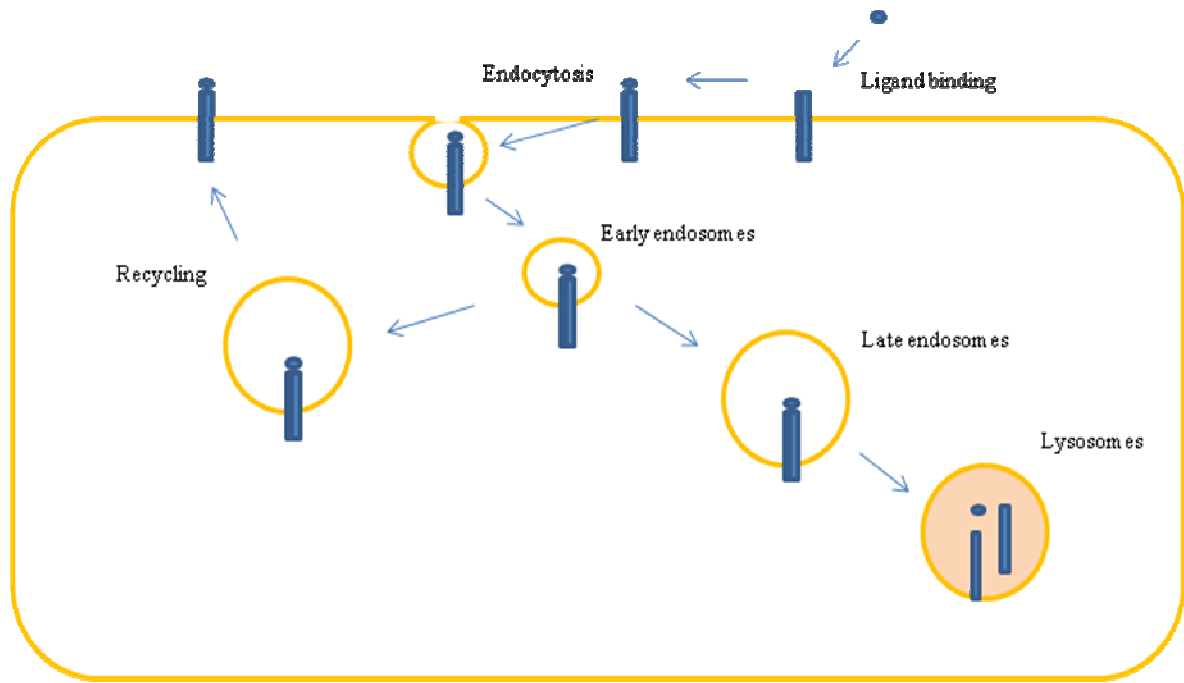


Figure 4. Ligand-induced EGFR trafficking.

Schematic representation of EGFR endocytosis. Upon ligand binding receptors are internalized into early endosomes, next they are targeted into lysosomes for degradation or to recycling endosomes.

6.1. Proteins involved in growth factor receptors endocytosis

In recent publications, a number of proteins are described as either direct or indirect binding partners required for efficient endocytosis of growth factor receptors. Protein-protein interactions that occur at the surface of endosomes are the key mechanism in signal transduction activated by receptor trafficking. Endosome-bound receptor couples effector molecules which induce signal transduction. Thus, it is very likely that the localization of the receptor in the cell can determine and specify the signalling outcome.

Moreover, the duration of the signal depends on the balance between recycled receptors and receptors undergoing degradation. Therefore, kinetics of the receptor trafficking provides temporal regulation of signalling.

6.1.1. Rab GTPase family

One of the largest subfamily of proteins implicated in regulation of endocytosis is the Rab (Ras-related proteins in Brain) GTPase family. Rab GTPases constitute a diverse group of Ras-like small G proteins that control cellular process, such as budding, tethering, fusion and motility at various sites within cells (Seachrist and Ferguson 2003). Rab proteins cycle between an active GTP-bound and an inactive GDP-bound form. Nucleotide exchange is regulated by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). Each isoform of Rab proteins is located to the surface of distinct membrane organelles (Seachrist and Ferguson 2003). Rab5 plays a role in formation of clathrin-coated vesicles, endocytosis of clathrin-coated vesicles, fusion of clathrin-coated vesicles with early

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endosomes and homotypic fusion between early endosomes. Rab9 and Rab7 are located on late endosomes and they control late endosome-to-Golgi and late endosome-to-lysosome trafficking, respectively. Rab4 regulates traffic from recycling endosomes to the plasma membrane, whereas Rab11 controls transport from early endosomes to recycling endosomes, slow recycling from the perinuclear recycling endosomes and traffic to the trans-Golgi network. Finally, Rab1 and Rab2 take part in transport from the rough endoplasmic reticulum to the Golgi (Seachrist and Ferguson 2003). Furthermore, Rab5 has been shown to take part in regulation of the trafficking of several growth factor receptors coupling endocytosis to signal transduction (Barbieri, Roberts et al. 2000; Lanzetti, Rybin et al. 2000; Miaczynska, Christoforidis et al. 2004).

6.1.2. Signalling molecules in growth factor receptors endocytosis

It is becoming more obvious that trafficking of the receptors facilitates signal transduction and the output of the signal transmitted from the membranes might be different than from endosome compartments (Miaczynska, Pelkmans et al. 2004). Studies have shown that RTKs are internalized into endosomes together with other signalling proteins. For example, Grb2, Shc, and Sos were observed in endosomes that contain activated EGFRs (Di Guglielmo, Baass et al. 1994). The Grb2/Sos complex induces Ras/MAP kinase pathway activation (Baass, Di Guglielmo et al. 1995) and it is likely that this complex in endosomes sustains the activity of Ras (Sorkin and Von Zastrow 2002). Furthermore, CRAF, MEK and ERK were observed in endosomes as a result of ligand activation (Pol, Calvo et al. 1998; Rizzo, Shome et al. 2000). The presence of these kinases on the endosomes might facilitate kinase-substrate interactions during receptor trafficking. PLC1, PI3K, c-Src and several other

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proteins involved in signalling were also found in endosomal compartments together with GFRs (Sorkin and Von Zastrow 2002). The recognition of many signalling proteins on endosomes suggests that signal transduction might be mediated by endocytic machinery. Some of the kinases that are recruited to the endosomes might take part in regulation of clathrin-coated vesicle formation and assembly. Since phosphorylation of AP-2 proteins, clathrin heavy chain and dynamin is essential for the appropriate assembly of CCV, kinases like c-Src, casein kinase 2 (CK2) and PKC play an essential role in internalization. They phosphorylate the components of CCV machinery and facilitate their activation (Korolchuk and Banting 2003). Importantly, it was demonstrated that inhibition of endocytosis blocks the activation of the MAPK pathway. Viera *et al* showed that impairment of clathrin-dependent EGFR endocytosis by use of dominant negative dynamin reduced the activity of downstream signalling components (Vieira, Lamaze et al. 1996). Moreover, endocytosis of TGF- β receptors supports downstream signalling from these receptors. Signal transduction during TGF- β receptor trafficking depends on SARA (Smad anchor for receptor activation) activity, whereas down-regulation of TGF- β receptors is linked with Smad7-Smurf activity (Di Guglielmo, Le Roy et al. 2003). Importantly, SARA and Smad7-Smurf vesicles have distinct localization in the cells. TGF- β receptors which are localized with SARA in EEA1 endosomes, actively transmit the signal. TGF- β receptors localized in Smad7-Smurf-positive endosomes are destined for degradation (Di Guglielmo, Le Roy et al. 2003).

6.2. FGFR trafficking

The mechanism of FGFR trafficking is still elusive, however, there are several reports demonstrating the involvement of receptor endocytosis in signal transduction. One of the first

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study regarding FGFR endocytosis was performed by Sorokin et al (Sorokin, Mohammadi et al. 1994). It was demonstrated that deletion of C-terminal region of FGFR1 containing 58 amino acids impaired the endocytosis of the receptor. Moreover, point mutation of Tyr766 abolished FGFR internalization (Sorokin, Mohammadi et al. 1994). As it was mentioned above, Tyr766 is a binding site for PLC γ 1 (Mohammadi, Honegger et al. 1991), thus it was suggested that PLC γ 1 could associate directly with proteins driving endocytosis, for example with adaptor proteins. Alternatively, receptor trafficking could be activated by downstream components of PLC γ 1 pathway, like PKC (Sorokin, Mohammadi et al. 1994).

The analysis of KGFR endocytosis, a FGFR2 splice variant, was presented by Marchese and colleagues (Marchese, Mancini et al. 1998). They showed that KGFR and its ligand KGF are internalised together through clathrin-coated pits (Marchese, Mancini et al. 1998). Subsequently, investigation of the intracellular localisation of four FGFRs and their ligands showed a distinct trafficking pattern for each receptor (Haugsten, Sorensen et al. 2005). Similar early endosomal localisation of each of the four FGF receptors was shown shortly after stimulation with FGF1. After a longer incubation with ligand, FGFR1 was found mainly in late endosomes/lysosomes, FGFR2 and FGFR3 were localised to lysosomes but to a lesser extent than FGFR1, whereas, FGFR4 was detected in recycling endosomes. Degradation of FGFR4 was much slower than other receptors which was linked with stronger ubiquitination of FGFR1-3 comparing with FGFR4 (Haugsten, Sorensen et al. 2005). Thus, the dynamics of FGFRs trafficking differs for each receptor.

Bryant *et al.* showed co-localisation of FGFR1 with E-cadherin during endocytosis which regulates adhesion and cell morphology as well as influences signalling cascades (Bryant, Wylie et al. 2005). FGFR1 and E-cadherin internalise together into Rab5/EEA1-endosomes. E-cadherin, as a major element of adherent cell-cell junctions, is responsible for

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adhesion. As a result of endocytosis of E-cadherin, connection between cells is released, which induces cell migration and changes cell shape. Over-expression of E-cadherin causes inhibition of co-endocytosis of FGFR1 and E-cadherin and blocks FGF-induced signalling to the MAP kinase pathway (Bryant, Wylie et al. 2005).

More recently, Rab5 and Rab1 were identified as novel FGFR1 binding proteins (Vecchione, Cooper et al. 2007). Moreover, knock-down of Rab5 results in a significant attenuation of FGF-activated ERK phosphorylation (Vecchione, Cooper et al. 2007). It suggests that FGFR internalisation is coupled to ERK activation. However, Rab5 knock-down does not alter Akt activation, which might be explained by the fact that Akt activation takes place at the plasma membrane (Sandilands, Akbarzadeh et al. 2007), whereas ERK is activated in the cytosol and at the plasma membrane (Harding, Tian et al. 2005). Moreover, CRAF and BRAF do not co-localise with FGFR1 suggesting that at some point these two pathways become spatially distinct (unpublished data, Kimberley Trim PhD Thesis 2009).

Furthermore, several mutants of FGFR1, where lysine residues were substituted with arginine, were created to test the role of ubiquitination in FGFR1 trafficking (Haugsten, Malecki et al. 2008). Studies showed that ubiquitin was dispensable for endocytosis of FGFR1 but necessary for degradation. Mutants lacking lysines residues could not be targeted by ubiquitin and as a result could not be sorted into the lysosomes. Thus, ubiquitination of the receptor plays an important role in maintaining the duration of the signal and sorting to the lysosome. Moreover, clathrin-dependent endocytosis of FGFR1 was confirmed (Haugsten, Malecki et al. 2008).

The connection between FGFR2 and Hrs (hepatocyte growth factor regulated tyrosine kinase substrate) in endocytic sorting was presented by Belleudi *et al* (Belleudi, Leone et al. 2009). Hrs is an adaptor molecule involved in endosomal sorting which can be activated by a

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variety of membrane receptors, including EGFR, Met receptors for GM-CSF and interleukin 2 (Clague and Urbe 2001). Hrs consists of VHS domain and FYVE-finger motif which is responsible for recruitment to early endosomes. Because Hrs possesses a ubiquitin binding domain, it is implicated in the degradation of ubiquitinated tyrosine kinase receptors. A role for Hrs- containing endosomes in EGFR trafficking has been previously described (Clague and Urbe 2001). Later, the role of Hrs in FGFR2 trafficking was investigated (Belleudi, Leone et al. 2009). Activation of KGFR by FGF7 targets the receptor to the degradation pathway, whereas stimulation with FGF10 induces recycling of the receptor. It was shown that, when Hrs activity is blocked, KGFR stimulated by FGF7 is not degraded but it recycles back on the plasma membrane. On the other hand, inhibition of Hrs in cells stimulated with FGF10 does not change the recycling of the receptor. Thus, Hrs is involved in sorting KGFR endocytosis towards the degradation pathway (Belleudi, Leone et al. 2009).

7. Fibroblast growth factor signalling in disease and tumourigenesis

7.1. Alterations of FGFRs in human diseases

Aberrant FGFR signalling has been implicated in several human diseases and a variety of cancers (Katoh 2008). The most common causes are activating mutations in FGFRs or over-expression of the receptors. Also gain-of-function or loss-of-function mutations in the ligands can lead to disease. Examples of these are loss-of-function of FGF3 which results in deafness, FGF8 loss-of-function contributes to Kallmann syndrome, whereas loss-of-function of FGF10 leads to LADD (lacrimo-auriculo-dento-digital) syndrome (Beenken and Mohammadi 2009). Furthermore, gain-of-function of FGF23 results in autosomal dominant hypophosphataemic rickets (Beenken and Mohammadi 2009). Usually, mutations of the ligands exist together with mutations of the receptors. And so abnormalities in FGFRs signalling contribute to Kallmann syndrome and LADD syndrome (Beenken and Mohammadi 2009).

Kallmann syndrome is a hypogonadism that is a result of a deficiency of gonadotropin-releasing hormone. Initially, the gene responsible for Kallmann syndrome was recognized as KAL1 (Eswarakumar, Lax et al. 2005). It encodes anosmin-1 which is glycoprotein present in extracellular matrix. Anosmin-1 has been shown to enhance FGFR signalling (Gonzalez-Martinez, Kim et al. 2004; Hu, Guimond et al. 2009). Interaction between anosmin-1 and FGFR1:FGF:heparin complex leads to increased MAPK pathway phosphorylation and Cdc/Rac1 activation (Gonzalez-Martinez, Kim et al. 2004). Moreover, association between anosmin-1 and FGFR 1 is specific for IIIc isoform and involves N-

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terminal domain of anosmin-1 and the D2 and D3 domains of FGFR1 (Hu, Guimond et al. 2009).

LAAD (Lacrimo-Auriculo-Dento-Digital) syndrome is very rare genetic disorder characterised by renal and respiratory anomalies. Also congenital malformations of lacrimal and salivary glands, ducts, ears, teeth and limbs have been reported. The mutations within catalytic loop of the kinase domain of FGFR1-3 and loss-of-function of FGF10 were detected as a cause of LAAD syndrome (Rohmann et al. 2006; Beenken and Mohammadi 2009). It was demonstrated that A628T mutation in FGFR2 in catalytic loop disturbs the recruitment of adaptor proteins and results in reduced kinase activity (Lew, Bae et al. 2007).

Point mutations in FGFR1, FGFR2 and FGFR3 are linked with several human skeletal dysplasias, including craniosynostosis syndrome and dwarfism. Multiple activating mutations in FGFR1 and FGFR2 cause Pfeiffer syndrome which is characterised by a craniosynostosis disorder and limb defects (Eswarakumar, Lax et al. 2005; Beenken and Mohammadi 2009). Craniosynostosis results from the premature closure of sutures in a developing skull before the completion of brain growth. A single mutation (P252R) within the linker region of D2 and D3 of FGFR1 leads to mild form of Pfeiffer syndrome (Muenke, Schell et al. 1994; Eswarakumar, Lax et al. 2005). The most common cause of syndromic craniosynostosis is Crouzon syndrome and Apert syndrome, both induced by mutations in FGFR2. Multiple substitutions have been reported in Crouzon syndrome, e.g. C278F, C342Y, S347C, while in Apert syndrome there are two main mutations, S252T and P253R (Eswarakumar, Lax et al. 2005; Katoh 2008). Apert syndrome is associated with malformations of feet and hands, and abnormalities of the cardiovascular, respiratory and urogenital systems. Crouzon syndrome is also known as branchial arch syndrome and it is connected with disturbances in development of the embryo, creating widespread effects. Jackson-Weiss syndrome is caused by A344G or

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C342S/R mutations in FGFR2 and results in craniosynostosis with feet abnormalities (Eswarakumar, Lax et al. 2005; Katoh 2008). Apart from missense mutations, small insertions, deletions due to alternative splicing of FGFR2 have been shown (Meyers, Day et al. 1996).

Most of the mutations in FGFR2 are located in the extracellular part of the receptor where they cause impairment of FGFR auto-inhibition, which leads to constitutive activation. Mutations can also promote receptor dimerization. Receptor dimerization is usually connected with mutation of a cysteine residue in the D3 region that results in an unpaired cysteine creating a disulfide bridge with a cysteine residue on a neighbouring receptor, causing dimerization (Eswarakumar, Lax et al. 2005; Beenken and Mohammadi 2009). Other mutations in these syndromes that do not involve cysteine residues, lead to changes in conformation that result in receptor dimerization or change ligand specificity. Increased ligand affinity is linked with P252R mutation of FGFR1 in Pfeiffer syndrome (Muenke, Schell et al. 1994).

Several mutations in FGFR3 often cause achondroplasia, a genetic form of dwarfism. In general, there are three types of mutations of FGFR3 leading to achondroplasia; transmembrane mutations promote non-covalent interactions between receptors; extracellular mutations and kinase-domain mutations that enhance catalytic activity of the receptor in ligand-independent manner (Beenken and Mohammadi 2009). Thanatophoric dysplasia type II is caused by an activating K650G mutation, and SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans) is also triggered by an activating mutation on the same residue, K650M. Hypochondroplasia is caused by multiple mutations, also mutations in transmembrane domain of FGFR3 (Beenken and Mohammadi 2009). Because FGFR3 is a negative regulator of bone growth, constitutive activation of FGFR3 leads to

retardation in bone development. Homozygous fetuses are characterized by severe bone abnormalities and they often die soon after birth, while heterozygous develop achondroplasia.

7.2. FGFR signalling in cancer

Over-expression of FGFRs is often linked with development and progression of a variety of human cancers (Katoh 2008; Beenken and Mohammadi 2009). FGFR gene amplifications, protein over-expression or expression of a constitutively active form of the receptor have been described in several tumour types, including breast cancer (Tannheimer, Rehemtulla et al. 2000; Reis-Filho, Simpson et al. 2006; Xian, Pappas et al. 2009), gastric cancer (Takeda, Arao et al. 2007; Kunii, Davis et al. 2008), rhabdomyosarcoma (Goldstein, Meller et al. 2007), prostate cancer (Giri, Ropiquet et al. 1999; Sahadevan, Darby et al. 2007), endometrial cancer (Byron, Gartside et al. 2008), and urothelial cancer (Tomlinson, L'Hote et al. 2005; Tomlinson, Baldo et al. 2007). As a result of this, and other oncogenic manifestations of FGFR function, the FGFR pathway has elicited significant interest as a target for the development of therapeutic interventions (Beenken and Mohammadi 2009; Knights and Cook 2009).

7.2.1. Breast cancer

Gene amplification is an important event in oncogene activation in breast cancer cells. FGFRs are often over-expressed in breast tumour specimens together with altered expression of FGFs (Adnane, Gaudray et al. 1991; Penault-Llorca, Bertucci et al. 1995). Mouse models of FGFR signalling in breast cancer development demonstrated that disturbed FGFR

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activation leads to cell proliferation and increased cell survival, promotes expression of matrix metalloproteinase 3, and changes cell polarity (Welm, Freeman et al. 2002; Xian, Schwertfeger et al. 2005). The amplification of the chromosome 8p11-p12 coding FGFR1 is observed in 10-15% breast cancer patients (Ray, Yang et al. 2004). This amplicon harbours several other oncogenes that could together influence cancer cell progression. FGFR1 amplification was demonstrated as a predictor of poor outcome, especially in patients with ER-positive breast cancer (Elbauomy Elsheikh, Green et al. 2007). Often 8p11-p12 amplification is important for the survival of lobular breasts carcinoma cells (Reis-Filho, Simpson et al. 2006). Inhibition of FGFR1 in cells expressing the receptor results in cell death which suggests that FGFR1 could be targeted during therapy (Reis-Filho, Simpson et al. 2006). Recently, FGFR1 transforming activity in lobular carcinomas was reported to be dependent on RSK (ribosomal S6 kinase) indicating that RSK could also be a therapeutic target in cells over-expressing FGFR1 (Xian, Pappas et al. 2009).

FGFR2 over-expression is also observed in breast cancer cells. Interestingly, small nucleotide polymorphisms (SNPs) that are located within intron 2 of FGFR2 were correlated with an increased risk of breast cancer development (Easton, Pooley et al. 2007; Hunter, Kraft et al. 2007). Moreover, alternative splicing of FGFR2 contributes to the transforming potential of breast cancer cells (Tannheimer, Rehemtulla et al. 2000). Three different variants of FGFR2 can be produced; C1, C2 and C3 carboxyl terminus type. C3 is a shorter variant of FGFR2 and it utilises a different exon than the C1 and C2 isoforms. It lacks the PLC γ 1 binding site but instead it leads to increased FRS2 activation (Moffa, Tannheimer et al. 2004). Expression of the C3 variant was demonstrated in tumourigenic samples showing a greater transforming activity than C1 isoform, whereas C3 expression in normal epithelial cells was

not detected (Moffa, Tannheimer et al. 2004). Thus, alternative splicing of FGFR2 isoforms might contribute to the aggressive potential of breast cancer cells.

7.2.2. Prostate cancer

An increased level of expression of FGFR1,4 and FGF1,2,6,8 is found in prostate cancer cells (Kwabi-Addo, Ozen et al. 2004). The abnormal expression of FGFR1 in prostate cancer cells is linked with transformation and loss of differentiation of these cells (Kwabi-Addo, Ozen et al. 2004). There is evidence suggesting that FGFR1 promotes prostate cancer development (Kwabi-Addo, Ozen et al. 2004). The germline polymorphism in the FGFR4 gene, which as a consequence, leads to expression of FGFR4 containing Gly388 or Arg388, is correlated with the increased aggressiveness of prostate cancer cells. Over-expression of FGFR1 and -4 in prostate cancers is a potential target for therapy (Gowardhan, Douglas et al. 2005; Sahadevan, Darby et al. 2007). Moreover, down-regulation of the negative regulator Sprouty was also observed, which might potentiate the effects of FGFR over-expression (Kwabi-Addo, Wang et al. 2004). The biological effects of the changes in prostate cancer cells include increased proliferation, decreased apoptosis, increased motility, invasiveness and angiogenesis (Kwabi-Addo, Ozen et al. 2004).

7.2.3. Gastric cancer

Multiple alternations have been reported in gastric cancer, including Ras mutation, loss-of-function of E-cadherin, amplification of the receptor her2, Met and FGFR2 (Kunii, Davis et al. 2008). Several gastric cancer cell lines express high levels of constitutively

phosphorylated FGFR2 together with EGFR family members. Inhibition of FGFR2 induces EGFR inactivation, growth inhibition and apoptosis in these cells, however, inhibition of EGFR has no effect on FGFR2 activation. This reveals interesting mechanism in gastric cancer cell lines where FGFR2 is upstream of EGFR and FGFR2-mediated trans-activation of EGFR is necessary for proliferation (Kunii, Davis et al. 2008). Over-expression of FGFR2 merits consideration as an attractive therapeutic target in gastric cancer cells. Moreover, AZD2171, which is mixed VEGFR/FGFR inhibitor, was shown to inhibit FGFR2 activity and downstream signalling proteins in gastric cancer cell lines (Takeda, Arao et al. 2007).

7.2.4. Urothelial carcinoma

Urothelial carcinoma, a common type of bladder cancer, is characterised by deletions in chromosome 9 and mutations of FGFR3. These activating mutations in FGFR3 are assessed as a predictive biomarker in tumours and as a diagnostic biomarker in urine (Knowles 2007). Over-expression of FGFR3 takes place in low grade and low stage tumours as well as in invasive bladder cancers. Normal urothelial cells express FGFR3IIIb, whereas bladder cancer cells change the expression pattern from FGFR3IIIb to mesenchymal isoform FGFR3IIIc which induces epithelial-to-mesenchymal transition so it could potentially increase cell migration (Tomlinson, L'Hote et al. 2005). Additionally, the expression of a truncated isoform of FGFR3 that acts as a negative regulator of FGF signalling in normal urothelial cells, is significantly decreased in bladder cancer cells (Tomlinson, L'Hote et al. 2005). This mechanism could explain increased FGFR3 activation in urothelial carcinomas.

Recently, the effect of three mutations in FGFR3 (S249C, Y375C and K652E) in immortalized normal human urothelial cells (TERT-NHUC) was studied (di Martino, L'Hote

C et al. 2009). Phosphorylation of FRS2 and activation of MAPK pathway were achieved in cells harbouring each mutation, whereas PLC γ 1 was activated by S249C and Y375C but not K652E. Src and Akt were not phosphorylated by any of the mutants (di Martino, L'Hote C et al. 2009). Interestingly, the same three FGFR3 mutants expressed in NIH-3T3 cells induced activation of all downstream proteins, FRS2, PLC γ 1, ERK, Src and Akt suggesting that effects of expression of FGFR3 mutants are cell-type specific (di Martino, L'Hote C et al. 2009). Moreover, cells expressing FGFR3 with S249C and Y375C mutations were characterised by increased proliferation and survival rate and this effect is correlated with PLC γ 1 activation (di Martino, L'Hote C et al. 2009).

7.2.5. 8p11 Myeloproliferative Syndrome (EMS)

8p11 Myeloproliferative Syndrome (EMS) is a result of a gene fusion formed from two previously separate genes due to chromosomal translocation. EMS is caused by uncontrolled proliferation of cells of the myeloid lineage and it is an aggressive disease characterised as a chronic myeloproliferative disorder that can rapidly transform into acute leukaemia. Chimeric proteins are produced as a consequence of fusion between the kinase domain of FGFR1 and a domain of one of many potential partners. So far, several partner genes have been identified to form chimeric proteins with FGFR1 (ZNF198, BCR, CPSF6, MYO18A, HERV-K, LRRFIP1, CEP1, FOP, FGFR1OP2, TIF1) (Eswarakumar, Lax et al. 2005; Soler, Nusbaum et al. 2009). The chimeric protein is activated in a ligand-independent manner by dimerization of two fusion proteins and the subsequent trans-phosphorylation of the kinase domain of FGFR1. ZNF198-FGFR1 and BCR-FGFR1 are the most common fusions of FGFR1. ZNF198-FGFR1 and BCR-FGFR1 fusion proteins transmit signals via

PLC γ 1, but not FRS2 due to the lack of juxtamembrane domain of FGFR1. Cells expressing ZNF198-FGFR1 stimulate STAT activation which plays an important role in ZNF198-FGFR1-induced transformation (Heath and Cross 2004). Disease caused by BCR-FGFR1 resembles BCR-ABL chronic myeloid leukemia rather than EMS. BCR-FGFR1 additionally activates Grb2 through tyrosine residue on BCR (Eswarakumar, Lax et al. 2005).

7.3. Therapeutic strategies

Since aberrant expression of FGFRs is common in various cancer cells, FGFRs are attractive therapeutic targets. Constitutive activation of FGFRs, caused by over-expression or activating mutations, disturbs FGF-mediated signal transduction and deregulates gene expression. Inhibition of the FGFR signalling pathway could decrease proliferation and induce apoptosis of tumour cells. FGFR inhibition can be targeted in two ways, by using small kinase inhibitors or antagonistic antibodies (Knights and Cook 2009). There are several FGFR-specific kinase inhibitors, including SU5402 and PD173074, which are ATP-competitive and associate with the ATP-binding pocket of the receptor. PD173074 was shown to inhibit FGFR2-dependent growth in gastric cancer cells (Kunii, Davis et al. 2008) as well as induce cell cycle arrest and apoptosis in FGFR2-expressing endometrial cancer cells (Byron, Gartside et al. 2008). Also studies on small cell lung cancer showed that PD173074 inhibits tumour growth *in vitro* and *in vivo* (Pardo, Latigo et al. 2009). Furthermore, AZD2171 (mixed VEGFR/FGFR inhibitor) also proved to be beneficial in anti-tumour activity (Takeda, Arao et al. 2007). The use of SU5402 on breast cancer cells over-expressing FGFR1 induced apoptosis proving the potential of FGFRs inhibitors in therapy of lobular carcinomas (Reis-Filho, Simpson et al. 2006). Therapeutic antibodies can target FGFRs in

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very specific manner, including defined splice variants. Their advantage over kinase inhibitors is that they induce less off-target effects and are more specific. However, the production is more expensive and delivery to the cell can be problematic. The use of a therapeutic antibody against FGFR3 in bladder cancer cells was presented by Martinez-Torrecuadrada *et al.* A ligand-competitive antibody blocked proliferation of cells in a dose- and FGF-dependent manner (Martinez-Torrecuadrada, Cifuentes et al. 2005). Recently, another antibody against FGFR3 was used to inhibit proliferation of urothelial cells (Qing, Du et al. 2009).

8. Jak/STATs signalling pathway

8.1. Structure of STATs

The Signal Transducers and Activators of Transcription (STATs) were described as transcription factors in cytokine signalling in the early 1990's (Darnell, Kerr et al. 1994; Darnell 1997). Seven mammalian STATs have been identified; STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6. STATs take part in the regulation of many processes in cell, including development, differentiation, proliferation and apoptosis (Lim and Cao 2006; Schindler, Levy et al. 2007). In the human genome STAT1 and STAT4 are located on chromosome 2, STAT3, STAT5a, STAT5b are clustered on chromosome 17 and STAT2 and STAT6 on chromosome 12. Several different STAT isoforms are produced due to alternative splicing: full length (STAT α) and shorter isoforms (STAT β , γ , δ) (Lim and Cao 2006; Schindler, Levy et al. 2007).

The STATs vary in size from 750 to 900 amino acids. They are built of several conserved domains: amino-terminal, coiled-coil, DNA-binding, linker domain, SH2 and transcriptional activation domains (Figure 5) (Schindler, Levy et al. 2007).

The SH2 domain is the most conserved domain among all STATs. It is built of an anti-parallel β -sheet flanked by two α -helices, which form a pocket. In the base of the pocket lies a conserved arginine, which is essential for the interaction with phosphate (Kisseleva, Bhattacharya et al. 2002). The SH2 domain plays a crucial role in recognition of specific receptor phosphotyrosine motifs, in the interaction with the cytoplasmic domain of receptors, and during dimerization of STAT molecules. All STATs, apart from STAT2, were

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demonstrated to form stable homodimers *in vivo* and *in vitro*. Moreover, STATs heterodimerize with each other, including STAT2 (Kisseleva, Bhattacharya et al. 2002).

The N-terminal region of STATs is a highly conserved stable domain (Kisseleva, Bhattacharya et al. 2002). It is involved in DNA binding to tandem ISRE/GAS (IFN type I-stimulated response element/gamma interferon activation site) elements (McBride and Reich 2003). It was also demonstrated to take part in dephosphorylation of STAT in the nucleus due to the ability to re-orientate STAT dimers into a conformation that becomes accessible to phosphatases (Mertens, Zhong et al. 2006).

The coiled-coil domain is involved in protein-protein interactions by providing a hydrophilic domain. The coiled-coil domain is crucial for receptor binding and its deletion impairs SH2 domain-mediated STAT3 interaction with receptors and subsequent phosphorylation of tyrosine residue of STAT3 (Zhang, Kee et al. 2000). It is also required for nuclear import of unphosphorylated STAT3 and recognition by the specific carrier importin- α 3 (Liu, McBride et al. 2005).

The DNA binding domain consists of a β -barrel with an immunoglobulin fold. It associates with the GAS family of the enhancers and ISRE enhancer family (Schindler, Levy et al. 2007). Its co-operation in DNA binding is likely to be essential for efficient transcriptional activity (Kisseleva, Bhattacharya et al. 2002).

The transcriptional activation domain is the least conserved among STATs due to its ability to regulate unique transcriptional responses (Kisseleva, Bhattacharya et al. 2002). A conserved serine residue within the transcriptional activation domain seems to enhance transcriptional activity for some but not for all target genes (Decker and Kovarik 2000).

8.2. Structure of Janus kinases

Four members of the Jak (Janus kinase) family have been described in mammals: Jak1, Jak2, Jak3 and Tyk2 (Schindler, Levy et al. 2007). The expression of Jak1, Jak2 and Tyk2 is ubiquitous, whereas Jak3 is expressed only in myeloid and lymphoid lineages (Kisseleva, Bhattacharya et al. 2002). Jak proteins range in size from 120 to 130 kDa and are over 1000 amino acids long.

There are seven regions of high homology between Jaks (JH1-JH7) and only some of them are well characterized (Figure 5). JH1 is a kinase domain, JH2 represents a pseudokinase domain, and JH3-JH7 form the N-terminal part of Jak (Kisseleva, Bhattacharya et al. 2002; Schindler, Levy et al. 2007).

The N-terminal part, JH3-JH7, is involved in receptor association and is responsible for its binding specificity. The JH3 region shares similarity with the SH2 domain, whereas JH4-JH7 comprise of a FERM (Four-point-one, Ezrin, Radixin, Moesin) homology domain known for involvement in protein-protein interactions. It was shown that this region of Jak might contribute to the interaction with the gp130 receptor (Hilkens, Is'harc et al. 2001).

The pseudokinase domain (also called kinase-like domain, JH2) possesses structural features of a tyrosine kinase but it lacks catalytic activity. Its exact role is elusive but its activity is crucial for regulation of the kinase domain, JH1, and also the interaction between Jak and STATs (Kisseleva, Bhattacharya et al. 2002). Unlike JH2, JH1 contains a typical tyrosine kinase domain which is well conserved. Tyrosine residues that is located in the activation loop can be phosphorylated, which is a critical step in Jak activation.

A.



B.

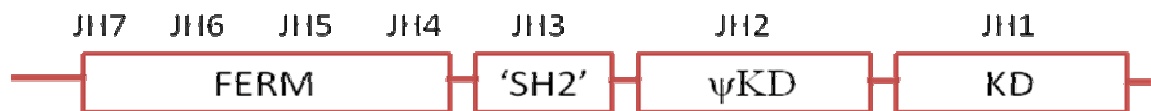


Figure 5. STAT and Jak structure.

- A. STAT structure: NH2 (N-terminal domain), CC (coiled-coil domain), DBD (DNA binding domain), Lk (linker domain), SH2 domain, Y (tyrosine residue), TAD (transcription activation domain).
- B. Jak structure FERM (Four-point-one, Ezrin, Radixin, Moesin homology domain), 'SH2' (SH2 homology domain), ψ KD (pseudokinase domain), KD (kinase domain). JH1-JH7 represent Jak Homology regions.

8.3. Activation of Jak/STAT pathway

STATs are activated by various ligands: cytokines, including interferons and interleukins, growth factors and hormones (Leaman, Leung et al. 1996; Lim and Cao 2006; Schindler, Levy et al. 2007). Activation of STATs depends on the type of ligand. One or more STATs may be activated in one cell by a given ligand and the output effect depends on identity of the ligand, time of stimulation and also concentration of the ligand. Moreover, the cell maturation and the state of cell differentiation also contribute to the final STAT activation. Some STATs can only be activated by specific ligands (STAT2 and STAT4), whereas others are activated by multiple ligands (STAT1, STAT3, STAT5a STAT5b) (Pellegrini and Dusanter-Fourt 1997). Furthermore, specificity of cytokine-STAT signalling depends on receptor preferences (Hennighausen and Robinson 2008).

The classical Jak/STAT pathway is well studied and takes place in cytokine signalling. Cytokine receptors, e.g. gp130, lack intrinsic tyrosine kinase activity but are associated with Janus kinases via key residues near their juxtamembrane region. Upon ligand binding, gp130 receptors dimerize which brings Jaks into proximity that is sufficient to induce cross-phosphorylation of their catalytic domains. Activated Jaks phosphorylate conserved tyrosine residues within the intracellular domain of the receptor. Phosphorylation of the receptor provides docking sites for STATs, for example, the gp130 receptor possesses a consensus binding motif, pY₇₅₉XXQ for STAT3. Recruited STATs are phosphorylated by Jak leading to homo- or heterodimerization via the SH2 domains of one monomer and the phospho-tyrosine of the partner dimer (Figure 6) (Pellegrini and Dusanter-Fourt 1997; Kisseleva, Bhattacharya et al. 2002; Schindler, Levy et al. 2007).

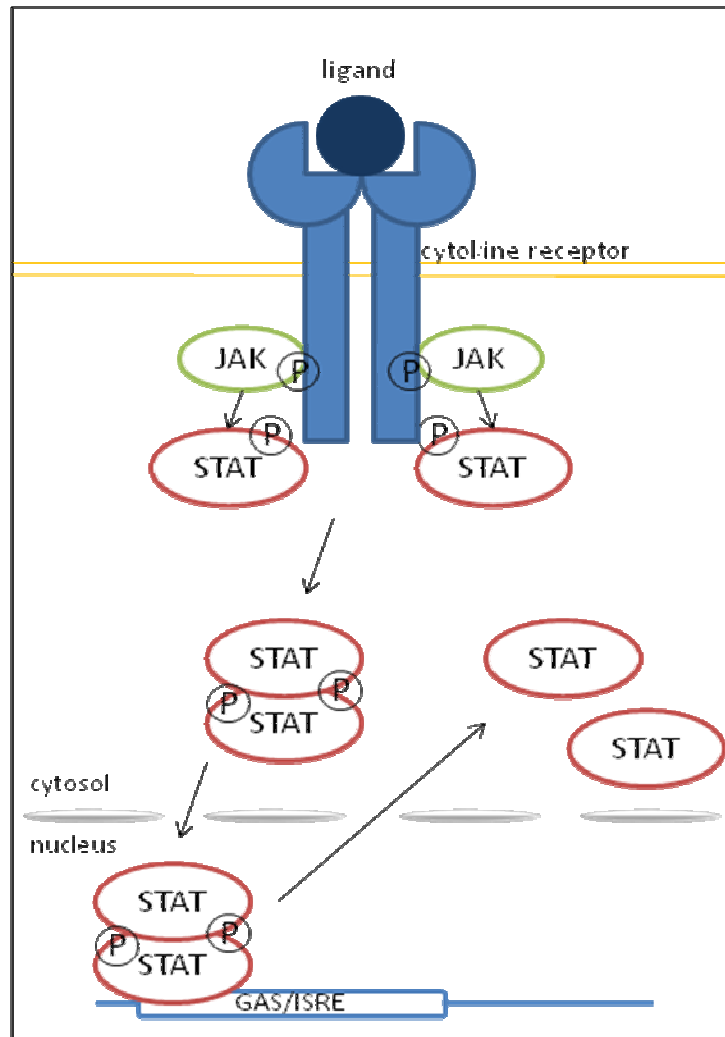


Figure 6. Jak/STAT signalling pathway.

A schematic representation of the events leading to activation of STATs. Upon ligand binding receptor becomes phosphorylated by Jak, which in turn induces recruitment of STAT to the receptor and its subsequent phosphorylation by Jak. Phosphorylated STATs form dimers and translocated into nucleus where they regulate gene expression. P represents phosphorylation.

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STATs can also be phosphorylated by non-receptor kinases like v-Src, v-Fps, v-Sis (Garcia, Yu et al. 1997). Aberrant activation of these kinases results in constitutive STAT phosphorylation that is observed in many cancers (Bowman, Garcia et al. 2000; Garcia, Bowman et al. 2001). Furthermore, STATs are activated by growth factors receptors, such as EGFR, PDGFR and VEGFR, by tyrosine phosphorylation of STATs in response to stimulation (David, Wong et al. 1996; Park, Schaefer et al. 1996; Vignais, Sadowski et al. 1996; Paukku, Valgeirsdottir et al. 2000). STATs activation by various RTKs might be JAK-dependent or JAK-independent and both models of STATs activation are presented in the literature (Olayioye, Beuvink et al. 1999; Vignais and Gilman 1999; Paukku, Valgeirsdottir et al. 2000; Ren and Schaefer 2002). Because EGFR, VEGFR and PDGFR possess an intrinsic tyrosine kinase domain, they might directly phosphorylate STAT (Vignais and Gilman 1999; Paukku, Valgeirsdottir et al. 2000). However, in some conditions RTKs require the presence of Jak or Src for STAT phosphorylation (Olayioye, Beuvink et al. 1999; Vignais and Gilman 1999; Ren and Schaefer 2002).

Dimers of tyrosine phosphorylated STATs are accumulated in the nucleus where they regulate gene expression. There are two transcriptional enhancers within the promoter elements of STAT target genes; ISRE/GAS elements. ISRE sites contain two tandem sequences (AGTTTN₃TTTC), whereas, GAS elements have consensus palindromic elements (TTTCCNGGAAA). The ISRE sites are non-classical sites recognized by complex of STAT1, STAT2 and p48/IRF9 (ISGF3 complex), whereas STATs dimers bind GAS elements (Kisseleva, Bhattacharya et al. 2002).

STATs are capable of interacting with other transcription factor that are in the nucleus, for example p48, Sp1, CBP/p300, c-Jun, CREB (Pellegrini and Dusanter-Fourt 1997). It is speculated that the interaction with other transcription factors leads to recognition of DNA

motifs in the non-conventional GAS/ISRE elements. It may contribute to the regulation of transcription of a large number of genes.

8.4. Regulation of the Jak/STAT pathway

The Jak/STAT signalling pathway influences a variety of cell functions, like differentiation, proliferation, cell migration, apoptosis, and wound healing, as well as processes involved in oncogenesis (Bromberg, Wrzeszczynska et al. 1999; Schindler, Levy et al. 2007). Thus, there are several modes of positive and negative regulation of Jak/STAT pathway activation.

The Jak/STAT pathway can be regulated at many levels, including modifications of STATs, as well as regulation on the receptor or Jak level. Post-translational modifications of STATs, like tyrosine and serine phosphorylation/dephosphorylation, are well characterized. As mentioned above, the most critical modification for STAT function is tyrosine phosphorylation. It triggers dimerization of STATs, accumulation in the nucleus and regulation of gene expression. Tyrosine phosphorylation of STATs is induced by cytokine and growth factor receptors and non-receptor kinases, like the Src family.

All STATs, except for STAT2 and STAT6, are also phosphorylated on a conserved serine residue within the transcriptional domain which regulates the activity of STATs. This conserved site consists of a LPMSP motif (Ser⁷²⁷ in STAT1, 3 and Ser⁷²¹ in STAT4) or LPSP motif (Ser⁷²⁵ in STAT5a and Ser⁷³⁰ in STAT5b). Serine phosphorylation is thought to be mediated by a number of different Ser/Thr kinases including MAPK kinases (ERK, JNK, p38), PKC δ , mTOR, NLK, JKK ϵ and CaMKII (Lim and Cao 2006). The choice of Ser/Thr kinase depends on the cell-type, type of ligand and STAT member. It was shown that growth

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factor-induced STAT3 serine phosphorylation is mediated by ERK (Chung, Uchida et al. 1997), p38 and PKC are involved in IL-6-induced SerSTAT3 activation (Jain, Zhang et al. 1999; Zauberman, Zipori et al. 1999), whereas JNK takes part in serine STAT3 phosphorylation in stress-induced responses or by activation of their upstream kinases (Lim and Cao 1999).

The role of serine phosphorylation of STAT1 and STAT3 is controversial. The use of STAT1 or STAT3 mutants lacking serine residue (Ser⁷²⁷) showed that serine phosphorylation is necessary for increased and complete transcriptional activity (Wen, Zhong et al. 1995) but has no effect on DNA binding (Wen and Darnell 1997). Furthermore, tyrosine phosphorylated STAT1 gains its complete transcriptional activation after serine phosphorylation which takes place in nucleus (Sadzak, Schiff et al. 2008). Moreover, STAT1 needs to be pre-assembled with chromatin in order to become serine phosphorylated, which is the final activation step (Sadzak, Schiff et al. 2008).

On the other hand, a negative effect of serine STAT activation on tyrosine phosphorylation was demonstrated; serine phosphorylated STAT3 decreased tyrosine STAT3 activation (Chung, Uchida et al. 1997). STAT3 serine phosphorylation by PKC has a negative influence on DNA binding and transcription activity (Jain, Zhang et al. 1999). There is also evidence that STAT serine phosphorylation can occur independently from tyrosine phosphorylation (Beuvink, Hess et al. 2000; Decker and Kovarik 2000). Recently, it was shown that STAT3 phosphorylated only on serine residue leads to gene expression in response to TrkA receptor activated by nerve growth factor (Ng, Cheung et al. 2006). Thus, it is likely that serine phosphorylation regulates gene transcription by altering the affinity for transcriptional regulators or by driving the transcription of other genes than tyrosine phosphorylated STAT3 (Decker and Kovarik 2000).

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Negative regulation of the Jak/STAT pathway is achieved by de-phosphorylation which attenuates STAT or Jak activity. Several phosphatases taking part in this process have been described, including Shp2, SHP1, CD45, PTP1B, TC-PTP (Xu and Qu 2008).

Shp2 is a ubiquitously expressed phosphatase that contains SH2 domain. It has been reported as a positive and negative regulator of Jak/STAT signalling, depending on the stimuli. IFN-induced activation of the pathway is negatively regulated by Shp2 via direct de-phosphorylation of STAT1, STAT3 and STAT5 (Xu and Qu 2008). Shp2 can also regulate the Jak/STAT pathway at the receptor level by direct de-phosphorylation of Jak or receptor tyrosine residues (Kisseleva, Bhattacharya et al. 2002; Lim and Cao 2006). Studies where Shp2 activation was impaired (by the loss of the receptor tyrosine motif that recruits Shp2 or by the mutations in Shp2 itself) have shown that the signal from Jak/STAT signalling pathway was prolonged (Klingmuller, Lorenz et al. 1995; Ohtani, Ishihara et al. 2000; Xu and Qu 2008). On the other hand, a positive effect of Shp2 on prolactin-induced Jak/STAT5 pathway activation was presented; Shp2 acts by limiting negative feedback mediated by SOCS. It was demonstrated that Shp2 blocks association between Jak2 and SOCS1 and therefore prevents from Jak2 lysosomal degradation facilitating STAT5 activation (Ali, Nouhi et al. 2003).

The PIAS (Protein Inhibitor of Activated STATs) family has been identified as STAT interacting proteins. They promote SUMOylation of target proteins that resembles the action of the RING-type ubiquitin E3 ligase, however their action is not restricted to SUMOylation (Palvimo 2007). The PIAS associate directly with phosphorylated STAT molecules to negatively regulate them. PIAS-STAT interaction takes place in the nucleus in a ligand-dependent manner. Each PIAS possesses some specificity towards STATs, for example, PIAS1 and PIAS3 inhibit STAT1 and STAT3, respectively (Chung, Liao et al. 1997). STAT

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activity is attenuated by this interaction and STAT-DNA association is prevented (Chung, Liao et al. 1997; Liao, Fu et al. 2000).

A classic negative feedback loop in the Jak/STAT pathway is mediated by activation of SOCS (Suppressors of Cytokine Signalling), which are STAT target genes. The SOCS family consists of 8 members, including CIS (cytokine-inducible SH2-containing protein), that share the SH2 domain and a C-terminal SOCS box. The members of SOCS family are rapidly expressed in cells where STATs are activated and they can directly antagonise STAT/Jak signalling pathway (Kisseleva, Bhattacharya et al. 2002; Croker, Kiu et al. 2008). The importance of this negative feedback loop was proved by gene targeting studies. SOCS1 null mice die between their second and third week of life due to a constitutively active Jak/STAT pathway (Naka, Matsumoto et al. 1998; Starr, Metcalf et al. 1998). The mechanism of action by which SOCS facilitates attenuation of the Jak/STAT pathway is not fully understood. Several different models of action have been described. STAT attenuation by CIS can be achieved by blocking the interaction between STAT and the receptor (Yoshimura, Ohkubo et al. 1995; Matsumoto, Seki et al. 1999). In this case, SOCS competes with STAT for the recruitment to the receptor. For example, CIS can be recruited to EPO receptor; SOCS1 bind IFNGR1; SOCS2 targets GH and leptin receptors; SOCS3 binds gp130, IL-12, leptin receptors; whereas SOCS4 interacts with EGFR and SOCS6/7 associate with IRS2 and IRS4 (Croker, Kiu et al. 2008). Also the interaction between FGFR3 and SOCS1 has been shown to lead to STAT1 attenuation (Ben-Zvi, Yayon et al. 2006). Another model for regulation of the Jak/STAT pathway by SOCS was suggested in which a direct interaction between CIS3 and Jak2 inhibits Jak/STAT signalling (Sasaki, Yasukawa et al. 1999). Alternatively, attenuation of the signal can be achieved by coupling ubiquitin ligase to the

Jak/STAT complex causing targeting to the lysosomal degradation pathway (Kamura, Sato et al. 1998; Kisseleva, Bhattacharya et al. 2002).

8.5. STATs dimers and phosphorylation status of STATs

Formation of STATs dimers after ligand stimulation is established, whereas latent STATs were considered to be monomeric in the cytoplasm (Shuai, Horvath et al. 1994). Recently, it was discovered that latent STATs can also form homodimers, heterodimers or even multimeric complexes called ‘stratosomes’ in phospho-independent manner (Stancato, David et al. 1996; Novak, Ji et al. 1998; Haan, Kortylewski et al. 2000). Pre-association between STAT1 and STAT3 proteins prior to stimulation was shown with no effect on nuclear translocation and DNA binding (Haan, Kortylewski et al. 2000). The binding affinity between two unphosphorylated STAT proteins is weaker than between phosphorylated molecules. It is speculated that there are two forms of unphosphorylated dimer – parallel and anti-parallel. The anti-parallel version is thought to be formed by latent STATs prior to ligand stimulation. The parallel form of unphosphorylated STAT is proposed to be bound to the receptor. The benefit of the parallel conformation is that the SH2 domain and tyrosine residue of STAT proteins are in close proximity with the receptor which makes phosphorylation easier. The conformation of unphosphorylated STAT dimer is then transformed into a phosphorylated dimer that is capable of DNA binding (Lim and Cao 2006).

The emerging role of unphosphorylated cytoplasmic STATs points out that it is equally important as phosphorylated STATs molecules. For example, cytoplasmic STAT3 was described as a microtubule-stabilizer which implicates its role in microtubule dynamics and cell migration. It interacts with stathmin, which is a microtubule-destabilising protein and

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therefore it prevents its function (Ng, Lin et al. 2006). Moreover, STAT3 has also been demonstrated to play a role in cell invasion and motility (Teng, Lin et al. 2009). It was suggested that cytoplasmic STAT3 regulates the activity of Rac1 by binding to β PIX, a Rac activator. In this way STAT3 modulates the organisation of actin cytoskeleton and controls directional migration (Teng, Lin et al. 2009).

The unphosphorylated monomers of STATs are also found in the nucleus suggesting that they may influence gene expression, however, the choice of gene targets is likely to be different from phosphorylated STAT. Due to the fact that mutants of STATs lacking tyrosine 705 residue could not bind DNA, it is believed that unphosphorylated STAT molecules interact with other transcription factors rather than induce gene expression by themselves. The role of unphosphorylated STATs as transcription co-regulators might be another mechanism of gene regulation existing in cells. Furthermore, unphosphorylated STAT1, together with IRF1 (interferon regulatory factor 1), induces constitutive expression of several genes, including LMP2 (low molecular mass polypeptide 2) (Chatterjee-Kishore, Wright et al. 2000). A similar function was presented for unphosphorylated STAT3 where a distinct set of genes is expressed by unphosphorylated STAT3 versus tyrosine phosphorylated STAT3 (Yang, Chatterjee-Kishore et al. 2005). Some of the genes regulated by unphosphorylated STAT3 (for example MET and MRAS) are over-expressed in cancer cells (Yang, Chatterjee-Kishore et al. 2005). The model was proposed where ligand-induced phosphorylated STAT3 induces gene expression including STAT3 itself. Then, an increased level of unphosphorylated STAT3 regulates expression of other genes.

The non-canonical Jak/STAT pathway was described in *Drosophila*. It was shown that unphosphorylated STAT translocates into the nucleus where it interacts with heterochromatin.

The proposed role for unphosphorylated STATs in the nucleus is to maintain the stability of the chromatin-remodelling machinery (Brown and Zeidler 2008; Li 2008).

8.6. Nucleocytoplasmic shuttling of STATs

The processes of nuclear import and export of proteins are directed by amino acid sequences named nuclear localization signals (NLS) or nuclear export signals (NES), respectively. Nucleus translocation of molecules through a nuclear pore complex (NPC) might be carrier-dependent or carrier-independent. Carrier-independent import is possible for proteins that possess regions enabling an interaction with nucleoporins, whereas proteins that lack such regions are transported by chaperones called importins. Importins recognise NLS motifs and mediate the transport of the cargo into the nucleus (Wozniak, Rout et al. 1998). This process is energy-dependent and requires hydrolysis of GTP by Ran (a Ras-like small GTPase). Analogously, the export receptor, CRM1 (transporter chromosome region maintenance 1, known also as exportin 1), recognizes NES and translocates proteins outside the nucleus in Ran-GTP dependent manner. Leptomycin B can block nuclear export by binding CRM1 directly and inhibiting its interaction with NES elements.

The classical model of STAT activation is recently being challenged. As mentioned above, unphosphorylated STATs have been observed to dimerize and even form oligomers prior to stimulation. Even though STATs are predominantly localized in the cytoplasm in resting cells, unphosphorylated STATs are believed to translocate to the nucleus. The mechanisms of nuclear import of phosphorylated and unphosphorylated STATs are different. It was demonstrated that latent STAT1, STAT2, STAT3 and STAT5 are constantly shuttling between the cytosol and nucleus in resting cells and this translocation is energy-independent

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and carrier-independent (Meyer, Begitt et al. 2002; Meyer, Gavenis et al. 2002; Banninger and Reich 2004; Marg, Shan et al. 2004). It was demonstrated that STATs directly interact with NPC (Marg, Shan et al. 2004). Furthermore, constitutive shuttling between the cytoplasm and nucleus does not depend on the phosphorylation of tyrosine of STATs and requires a balance between nuclear import and export (Pranada, Metz et al. 2004).

Nuclear accumulation of STATs is observed in response to ligand stimulation (Schindler, Shuai et al. 1992). Phosphorylated STATs are present in the nuclei up to several hours before gradually returning to their resting distribution. Ligand induced dimerization of STATs prevents carrier-free nuclear import, therefore, another mechanism of nuclear transport is used for phosphorylated STAT dimers. Carrier-dependent translocation is facilitated by the importin- α and - β family that recognise the NLS of dimeric STATs. This process also requires energy from Ran-GTPase (Vinkemeier 2004). NLS elements have been recognised in various regions of STATs depending on the type of STATs, including the DNA binding region or coiled-coil domain (McBride, Banninger et al. 2002; Ma, Zhang et al. 2003; Melen, Fagerlund et al. 2003). Tyrosine phosphorylated dimers of STAT3 are recognized by importin- α 5 and importin- α 7 (Ma and Cao 2006), the STAT1 dimer is translocated by importin- α 5 (McBride, Banninger et al. 2002), whereas importin- α 5 recognizes STAT1-STAT2 dimers (Melen, Fagerlund et al. 2003). Interestingly, unphosphorylated STAT3 can use importin- α 3 and importin- α 6 in a phosphorylation-independent shuttling between cytoplasm and nucleus (Liu, McBride et al. 2005). It means that STAT3 also employs carrier-dependent nuclear import in contrast to unphosphorylated STAT1, which is translocated into the nucleus by direct contact with the NPC.

The CRMI export is induced in a ligand-dependent manner, as well it controls constitutively shuttling of STATs (Vinkemeier 2004). The export of STAT1, STAT3 and

STAT5 occurs via CRMI receptors that recognise the NES in STAT proteins (Begitt, Meyer et al. 2000; McBride, McDonald et al. 2000; Zeng, Aoki et al. 2002; Bhattacharya and Schindler 2003). Additionally, it was also demonstrated for STAT3 that a CRMI-independent mechanism exists, because inhibition of CRMI with leptomycin B does not completely block STAT3 export (Liu, McBride et al. 2005).

The rate of nuclear import, whether it is carrier-dependent or carrier-independent, is not changed by phosphorylation of STATs. So what is the cause of nuclear accumulation of phosphorylated STATs? The phenomenon of ligand-induced STATs accumulation in the nucleus is explained by the rate of STATs/DNA dissociation and dephosphorylation of STATs. STATs are incapable of leaving the nucleus in a phosphorylated state. As long as STATs are phosphorylated they bind DNA and are trapped in the nucleus. The major parameter is the STAT/DNA dissociation off-rate which is proportional to the rate of dephosphorylation. For example, phosphorylated STAT1 binds DNA until it is dephosphorylated which then leads to nuclear export (Meyer, Marg et al. 2003). Thus, the dynamic status of the STAT3/Jak pathway is regulated by two key determinants: STAT3 dephosphorylation and nuclear export (Guerriero, Dudka et al. 2009).

8.7. STAT3 as an oncogene

STAT3 is a member of STAT family that was identified as a DNA-binding factor which is phosphorylated by IL-6 in the acute-phase inflammatory response (Wegenka, Buschmann et al. 1993; Akira, Nishio et al. 1994; Luttkien, Wegenka et al. 1994). STAT3 is expressed early during post-implantation and is expressed in most tissues. STAT3^{-/-} mice are characterized by embryonic lethality. As soon as embryos reach egg cylinder stage, they lack properly developed mesoderm which induce rapid regression and death around day E7.5

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(Takeda, Noguchi et al. 1997). Moreover, tissue-specific knockout shows impaired apoptosis and response to pathogens (Takeda, Kaisho et al. 1998). Deletion of STAT3 in mouse keratinocytes induced defects in skin and hair development (Sano, Kira et al. 2000).

STAT3 shares the same structure as other members of STAT family; N-terminal domain, coiled-coil domain, DNA binding domain, linker domain, SH2 domain and transcription activation domain (Figure 5). There are two isoforms of STAT3, α and β . They originate from a single gene by alternative splicing (Huang, Qiu et al. 2007). Isoform β is shorter and lacks C-terminal transcriptional activation domain, which is substituted by seven residues (Huang, Qiu et al. 2007). Different isoforms of STAT3, α and β , are characterised by distinct intracellular dynamics (Huang, Qiu et al. 2007). STAT3 α nucleocytoplasmic shuttling is rapid and following ligand stimulation STAT3 α quickly accumulates in the nucleus. In contrast, STAT3 β shuttling is slower and STAT3 β shows prolonged nuclear accumulation which was proved to be due to seven unique C-terminal residues on STAT3 β (Huang, Qiu et al. 2007).

STAT3 is involved in promoting cell growth and constitutively active STAT3 is able to potentiate tumourigenesis (Bromberg and Darnell 2000; Yu, Pardoll et al. 2009). Persistent phosphorylation of STAT3 can be induced by up-regulated upstream kinases, such as over-expression of EGFR or v-Src (Bromberg, Horvath et al. 1998; Bromberg, Wrzeszczynska et al. 1999; Garcia, Bowman et al. 2001; Herrmann, Vogt et al. 2007). STAT3 is often expressed at a high level and constitutively activated in many human tumour specimens and cells, including prostate cancer (Mora, Buettner et al. 2002), breast cancer (Garcia, Bowman et al. 2001), myeloid leukemias (Coffer, Koenderman et al. 2000), head and neck cancer (Song and Grandis 2000), brain cancer (Schaefer, Ren et al. 2002).

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The first observation of oncogenic STAT3 was made in cells transformed by v-Src (Yu, Meyer et al. 1995). Oncogenic Src activation leads to permanent STAT3 phosphorylation and subsequently results in DNA binding, which implicates that STAT3 contributes to tumourigenesis (Yu, Meyer et al. 1995). Furthermore, a constitutively active form of STAT3 was shown to transform fibroblasts (Bromberg, Horvath et al. 1998; Bromberg, Wrzeszczynska et al. 1999). Colony formation in soft agar by v-Src-transformed cells was enhanced by STAT3, however, the inactive mutant forms of STAT did not show that effect (Bromberg, Horvath et al. 1998). In 1999 STAT3 was formally established as an oncoprotein (Bromberg, Wrzeszczynska et al. 1999). Constitutively active form of STAT3 (STAT3C) was obtained by cysteine residues inserted in the SH2 region (Bromberg, Wrzeszczynska et al. 1999). This mutation leads to spontaneous dimerisation, phosphorylation and nuclear accumulation of STAT3 and contributes to tumourigenesis. Expression of STAT3C is linked with increased cell proliferation, anti-apoptotic responses and cellular transformation (Bromberg, Wrzeszczynska et al. 1999).

Persistent STAT3 activation induced by v-Src has also been shown to have an effect on STAT3 nucleocytoplasmic shuttling (Herrmann, Vogt et al. 2007). STAT3 in v-Src transformed fibroblasts is linked with increased STAT3 shuttling (Herrmann, Vogt et al. 2007). V-Src-activated STAT3 cycles between nucleus and cytoplasm much faster than non-activated STAT3. The phosphorylation of STAT3 by v-Src is independent of Jak2 and it prevents STAT3 inhibition by SOCS3 (Herrmann, Vogt et al. 2007). Blockade of nuclear export showed reduction of STAT3 phosphorylation, slower translocation of STAT3 and decreased expression of gene targets (Herrmann, Vogt et al. 2007). Moreover, inhibition of nuclear export induced apoptosis of v-Src transformed cells, thus targeting nuclear export of STAT3 might be an interesting therapeutic approach (Herrmann, Vogt et al. 2007).

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Pro-oncogenic STAT3 activity is linked with gene expression that are known to promote proliferation and inhibit apoptosis (Regis, Pensa et al. 2008). Activation of STAT3 in cancer cells is correlated with the expression of genes resulting in endothelial-mesenchymal transition (EMT) (Lo, Hsu et al. 2007). During EMT the level of E-cadherin is reduced by transcription repressors, including TWIST, Slug or Snail. Lo *et al* demonstrated that EGF-induced STAT3 up-regulates TWIST expression and therefore contributes to EMT in cancer cells (Lo, Hsu et al. 2007). Inhibitors of Jak2/STAT3 pathway suppressed STAT3 phosphorylation and TWIST expression (Lo, Hsu et al. 2007). Recently, comparative genomics was used to identify novel direct targets for STAT3 (Vallania, Schiavone et al. 2009). Most of the newly detected genes regulated by STAT3 play a role in tumour transformation, metastasis, angiogenesis and growth, thus they may function as mediators of STAT3 pro-oncogenic activity (Vallania, Schiavone et al. 2009).

The role of STAT3 in cancer inflammation and immunity has been extensively studied. STAT3 can be activated by IL-10 which is a major anti-inflammatory cytokine and the role of STAT3 as an inhibitor of inflammation has been reviewed (Regis, Pensa et al. 2008). On the other hand, pro-inflammatory of STAT3 is well known (Pensa, Watson et al. 2009; Yu, Pardoll et al. 2009). IL-6-mediated activation of STAT3 is important for induction of pro-inflammatory pathway (Yu, Pardoll et al. 2009). Extrinsic inflammatory factors, like UV radiation, chemical compounds, stress and infection, also contribute to STAT3 activation (Yu, Pardoll et al. 2009).

The opposing roles of STAT3 in inflammatory responses implicate that the choice of response varies depending on conditions. It suggests that STAT3 biological function is different according to physiological and pathological conditions (Regis, Pensa et al. 2008). There are several aspects that influence the choice of STAT3 response, including combination

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of ligands, cell type and the subset of STAT3 target genes that can be distinct in cancer and normal cells (Regis, Pensa et al. 2008). Moreover, the mode of STAT3 phosphorylation is crucial; ligand-induced STAT3 phosphorylation is usually strong and weakens in time, while, oncogenic activation of STAT3 is constitutive but weaker (Pensa, Watson et al. 2009).

An interesting hypothesis about the role of serine phosphorylated STAT3 in mitochondria was recently proposed (Gough, Corlett et al. 2009; Wegrzyn, Potla et al. 2009). The activity of SerSTAT3 was shown to induce Ras-dependent malignant transformation (Gough, Corlett et al. 2009). This STAT3 activity is not dependent on tyrosine phosphorylation or STAT3 transcriptional activity. It was proposed that STAT3 accumulation in mitochondria sustains altered glycolytic and oxidative phosphorylation activities which are typical for cancer cells and thus, STAT3 facilitates Ras-mediated transformation (Gough, Corlett et al. 2009). Moreover, involvement of serine STAT3 in oncogenesis was suggested and its activity in cancer cells might be as important as tyrosine phosphorylated STAT3 (Bowman, Garcia et al. 2000).

STAT3 is an important molecule that plays multiple roles in cells at many levels. It has a dual role as a mediator of signalling and regulator of gene expression. Moreover, STAT3 has been described as an oncogene correlated with tumour progression and upregulated in many tumour specimens. Therefore, STAT3 signalling pathway has emerged of a major interest as a promising therapeutic target.

9. Strategies of studying protein-protein interactions

Reconstruction of signalling pathways has been extensively studied over the last few years (Papin, Hunter et al. 2005). High throughput technologies allow descriptions of signalling mechanisms on a broader scale. However, it is still a challenge to explain the complexity and dynamics of a signal transduction network. Thus, studies on reconstruction of signalling pathways are undertaken on many levels – protein-protein interactions, dynamics of signalling and output of gene expression. Moreover, the trend in large-scale research has changed from qualitative to quantitative analysis and the integration of data received from different sources improves our understanding of the spatiotemporal signalling events (Papin, Hunter et al. 2005).

Variety of techniques analysing protein-protein interactions are continually being developed to understand more precisely the nature of these interactions (Kuroda, Kato et al. 2006). Moreover, data obtained by from several sources are integrated and analysed by a relatively new field, known as systems biology. Systems biology is an interdisciplinary study that describes the entirety of biological events occurring in cells and tries to understand biological processes at systems levels. Furthermore, employment of mathematical models can be useful in description of the properties of signalling networks (Guerriero, Dudka et al. 2009).

Recently, proteomics strategies have been developed along with mass spectrometry technique. Proteomics facilitates the observation of quantitative changes of the proteome in space and time, as well as determining even subtle changes in protein expression levels or post-translational modifications. A mass spectrometry-based approach is useful not only for

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identification of novel binding partners but it also creates dynamic maps of entire phospho-events occurring in cells. Thus, it has become an attractive field in reconstruction of signalling networks.

In order to obtain quantitative data, these techniques take advantage of unique amino-specific stable isotopic labelling. One of the most popular proteomics methodologies to study signalling networks is Stable Isotope Labelling by Amino acids in Cell culture (SILAC) (Blagoev and Mann 2006). It uses amino acids containing non-radioactive stable isotopes that are metabolically incorporated into new synthesized proteins in the cells, so that entire proteomes are encoded with either the light or heavy version of the same amino acid. After encoding, samples are mixed together to minimize quantification errors. This makes labelled samples easily distinguishable via mass spectrometry from the control samples (Blagoev and Mann 2006). The SILAC approach is useful in identifying novel components of signalling networks and has already contributed to the description of numerous proteins involved in signal transduction (Hinsby, Olsen et al. 2004; Schulze and Mann 2004; Schulze, Deng et al. 2005; Blagoev and Mann 2006; Zhou, Galan et al. 2007).

Alternative strategy mapping novel partners on the basis of protein-protein interactions is the peptide pull-down. Using synthetic peptide pairs in phosphorylated and unphosphorylated forms, pull-down experiments can be performed to enrich specific binding partners to the phosphorylated bait peptides. These proteins can be identified and quantified by mass spectrometry. The purpose of peptide pull-downs is to screen for direct binding partners that recognise specific motifs on peptides. For example, peptide pull-down technique together with SILAC has been successfully used in finding interacting partners for ErbB2 receptor (Schulze and Mann 2004). Also screening for primary interaction partners based on

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peptide-protein interactions was employed also for EGFR and ErbB4 (Schulze, Deng et al. 2005).

Due to the fact that signal transduction depends on protein-protein interactions, it is crucial to identify proteins taking part in FGFR signalling pathways. The proteomics approach has already been used to identify novel FGFR1 interacting proteins (Vecchione, Cooper et al. 2007) and protein partners associated with FRS2 upon FGF1 stimulation (Leila Fares PhD Thesis 2009). Nevertheless, further studies are necessary to comprehend the nature of FGFR signalling as well as abnormalities in FGF-mediated signal transduction.

AIMS

The goal of this study was to identify proteins involved in the FGF signalling pathway. There were three main objectives:

- First objective was to generate the method that would be used in proteomics screen for FGFR1 novel interacting partners; in this work it was peptide pull-down experiment followed by mass spectrometry (Chapter 3).
- Second objective was to investigate the interaction between FGFR1 and potential binding proteins identified by proteomics; in this work STAT3-FGFR1 interaction was further analysed (Chapter 4).
- Third and final objective was to study the mechanism for STAT3 activation induced by FGFR (Chapter 5 and 6).

CHAPTER 2

Materials and methods

Materials and methods

1. Buffers and solutions

Bacterial culture

Luria Bertani Broth (LB): 10 µg/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.4

DNA manipulation

DNA loading buffer (6x): 0.25 % bromophenol blue (w/v), 0.25 % xylene cyanol FF (w/v), 15 % Ficol (w/v)

TBE buffer (5x): 54 g/l Tris, 27.5 g/l orthoboric acid, 10 mM EDTA pH 8.0

Protein manipulation

Triton X-100 lysis buffer: 120 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100 (v/v) and 1 tablet of protease inhibitor cocktail (Roche Diagnostics) per 10ml of buffer

NP-40 buffer: 50 mM Tris pH 7.4, 1 mM EDTA, 1% NP-40 (v/v), 0.1 % SDS (w/v), 1 mM Na₃VO₄, 50 mM NaF, 100 mM PMSF and 1 tablet of protease inhibitor cocktail (Roche Diagnostics) per 10 ml of buffer

SDS sample buffer (2x): 125 mM Tris-HCl pH 6.8, 20 % glycerol (v/v), 4 % SDS (w/v), 0.1 % bromophenol blue (w/v), 10 % β-mercaptoethanol (v/v)

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SDS running buffer: 25 mM Tris, 0.2 M glycine, 0.1 % SDS (w/v)

MES running buffer (20x) (Invitrogen)

NuPAGE 4-12% Bis-Tris pre-cast 10-, 12- and 20 wells gels (Invitrogen)

Western blot transfer buffer: 25 mM Tris, 0.2 M glycine, 10 % methanol (v/v)

Phosphate buffered saline (PBS) pH 7.3: PBS tablets (Oxoid) made to manufacturers' instruction

Tris buffered saline (TBS): 10 mM Tris pH 7.4, 75 mM NaCl

TBS-T: 10 mM Tris-HCl pH 7.4 75 mM NaCl, 0.1% Tween 20 (v/v)

TBS-E: 10 mM Tris-HCl pH 7.4 150 mM NaCl, 1 mM EDTA

Ponceau-S stain: 0.1 % Ponceau-S sodium salt (w/v), 5 % acetic acid (v/v)

Stripping buffer: 0.5 mM Tris pH 6.7, 2 % SDS (w/v) and 0.7 % β -mercaptoethanol

PVDF Stripping buffer (Li-cor)

Flg C15-conjugated agarose beads (Santa Cruz)

Sepharose protein-G fast flow slurries (Amersham Bioscience Inc.)

Protein pull-down assay

Dynabeads® MyOne™ Streptavidin T₁ (Invitrogen)

Washing buffer - PBS, 0.01% Tween 20 (v/v)

In-gel digestion

25 mM ammonium bicarbonate

50 % acetonitrile, 25 mM ammonium bicarbonate

10 mM DTT, 25 mM ammonium bicarbonate

55 mM iodoacetamid, 25 mM ammonium bicarbonate

Trypsin 1 μ g/ μ l in 50 mM acetic acid re-suspension buffer (Promega)

Immunofluorescence

Saponin protocol:

Fixation solution: 4% paraformaldehyde in PBS (v/v)

Tris buffered saline (TBS): 10 mM Tris pH 7.4, 75mM NaCl

Permeabilization solution: 0.02 M glycine (w/v), 0.1 % saponin (v/v), 0.1 M Tris/HCl pH 8.5

Blocking buffer: 2.5 % FCS (v/v), 0.1 % saponin (v/v), 0.1 M Tris/HCl pH 8.5

Methanol protocol:

Fixation solution: 4 % paraformaldehyde in PBS (w/v)

Methanol 100%

Phosphate buffered saline (PBS) pH 7.3: PBS tablets (Oxoid) made to manufacturers' instruction

Blocking buffer: 4 % BSA in PBS (w/v)

Mowiol solution: 10 % Mowiol 4-88 (w/v), 25 % glycerol (v/v), 0.1 M Tris/HCl pH 8.5 dissolved at 50⁰C. The solution was centrifugated at 5,000g, for 15 minutes. To 5 ml of supernatant 2 grains of p-phenylendiamine were added and the solution was stored at -70⁰C.

2. Antibodies

IB is abbreviated for immunoblotting, IP is abbreviated for immunoprecipitation, IF is abbreviated for immunofluorescence.

| Antibody (anti-) | Species | Manufacturer | Dilution |
|-------------------------------------|----------------|--------------------------------------|----------------------------|
| Secondary mouse IgG HRP conjugated | sheep | Amersham Pharmacia Biotech. (NA9310) | 1:10000 (IB) |
| Secondary rabbit IgG HRP conjugated | donkey | Amersham Pharmacia Biotech. (NA9340) | 1:5000 (IB) |
| pSer727STAT3 | mouse | BD Transduction Lab | 1:1000 (IB) 1:100 (IF) |
| pSer727STAT3 | rabbit | Cell Signaling Technology | 1:1000 (IB) |
| c-myc | rabbit | Cell Signaling Technology | 1:1000 (IB) |
| Myc-tag (9B11) | mouse | Cell Signaling Technology | 1:1000 (IB) 1:1000 (IP) |
| p416Src family | rabbit | Cell Signaling Technology | 1:1000 (IB) |
| p653/654 FGFR (55H2) | mouse | Cell Signaling Technology | 1:1000 (IB) |
| pTyr1007/1008 Jak2 (C80C3) | rabbit | Cell Signaling Technology | 1:1000 (IB) |
| pTyr705STAT3 (D3A7) | rabbit | Cell Signaling Technology | 1:1000 (IB) 1:25 (IF) |
| pTyr705STAT3 (M9C6) | mouse | Cell Signaling Technology | 1:1000 (IB) 1:25 (IF) |
| Src (L4A1) | mouse | Cell Signaling Technology | 1:1000 (IB) 1:100 (IP) |

Materials and methods

| | | | |
|---|--------|---------------------------|---|
| STAT3 (124H6) | mouse | Cell Signaling Technology | 1:1000 (IB) 1:200 (IF) 1:100 (IP) |
| Secondary mouse IRDye-conjugated | goat | Li-cor | 1:15000 (IB) |
| Secondary rabbit IRDye-conjugated | goat | Li-cor | 1:15000 (IB) |
| Secondary mouse IgG (H+L) FITC conjugated | goat | Molecular Probes | 1:300 (IF) |
| Secondary mouse IgG (H+L) Texas Red conjugated | goat | Molecular Probes | 1:300 (IF) |
| Secondary rabbit IgG (H+L) Alexa Fluor 495 conjugated | goat | Molecular Probes | 1:300 (IF) |
| Secondary rabbit IgG (H+L) FITC conjugated | goat | Molecular Probes | 1:300 (IF) |
| Human IgG Fc horseradish peroxidase (HRP) | goat | Pierce | 1:10000 (IB) |
| ERK1 (K-23) | rabbit | Santa Cruz Biotechnology | 1:1000 (IB) |
| FGFR1 (Flg, C-15) | rabbit | Santa Cruz Biotechnology | 1:1000 (IB) 1:100 (IF) 1:100 (IP) |
| c-fos (H-125) | rabbit | Santa Cruz Biotechnology | 1:1000 (IB) |
| FGFR2 (Bek, C-17) | rabbit | Santa Cruz Biotechnology | 1:1000 (IB) 1:100 (IP) |
| JunB (210) | rabbit | Santa Cruz Biotechnology | 1:1000 (IB) |
| pERK1 (E-4) | mouse | Santa Cruz Biotechnology | 1:1000 (IB) |
| α -tubulin | mouse | Sigma | 1:10000 (IB) |

3. Molecular cloning

3.1. DNA constructs

FGFR1 WT in pcDNA3.1 coding full length human was provided by Pamela Maher (Department of Cell Biology, The Scripps Research Institute, La Jolla, USA). Ss-Fc-3C-FGFR1 ires Tpz pEF-BOS was previously described by Burgar *et al.* 2002. GFP-FGFR2 construct coding full length human protein was a gift from John Ladbury (University College London, London, UK). STAT3 WT in pZeoSVZ coding full length mouse was kindly provided by Valeria Poli (Department of Genetics, Biology and Biochemistry, Molecular Biotechnology Centre, University of Turin, Italy). N-terminal myc-Jak2 was cloned by Susanne Brewer using Gateway system (Invitrogen). Src WT was provided by Margaret Frame (Edinburgh Cancer Centre, University of Edinburgh, UK).

3.2. siRNA oligonucleotides

Set of siRNA ON-TARGET oligonucleotides against FGFR2 were purchased from Thermo Fishers Scientific. siRNA oligonucleotides against human Src and Jak2 were purchase from Santa Cruz. Non-targeting control siRNA were purchased from Qiagen. Oligonucleotides were diluted in RNase-free water (Santa Cruz) to 20 μ M concentration and stored in -20°C .

3.3. Transformation of competent cells

To 100 µl of *E.coli* DH5α competent cells 1 µg of DNA was added, followed by incubation on ice for 20 minutes. Cells were heat-shocked for 45 seconds at 42⁰C and returned to ice for 2 minutes. 1 ml LB was added and followed by incubation at 37⁰C for 1 hour. Cells were pelleted by short centrifugation, most of the supernatant was removed and the pellet was re-suspended in the remaining volume. Cells were incubated overnight at 37⁰C on LB plates.

3.4. Production of constructs

Single colonies of transformed *E.coli* were grown in 5 ml of LB with 100 µg/ml ampicillin at 37⁰C, 225 rpm for 6-8 hours. The 5 ml pre-culture was added to 200 ml LB and ampicillin (100 µg/ml) and grown at 37⁰C overnight. Then Maxi Prep conducted to extract DNA from the cells.

3.5. Maxiprep

Cultures were pelleted by centrifugation at 4000g for 10 minutes at 4⁰C in a Beckman centrifuge (JLA 10.5 rotor). The pellet was re-suspended in 10 ml solution I, it was lysed in 10 ml solution II, mixed and incubated for 5 minutes at room temperature. After neutralization with 10 ml solution III, the lysed cells were cleared by centrifugation at 8000g for 10 minutes in a tabletop Beckman centrifuge. Excess isopropanol was added to the supernatant and centrifuged for 30 minutes at 15000g. Pellet was washed with 70 % ethanol, centrifugated for 10 minutes at 15000g, air-dried and re-dissolved in 1 ml TE or water.

3.6. DNA electrophoresis

DNA was analysed on 1 % agarose in TBE containing 0.2 µg/ml ethidium bromide, run at 100 V. The DNA was visualized under UV illumination.

3.7. Quantifying DNA

The concentration of DNA was determined by measuring the absorbance at OD_{260nm}. Distilled water was used as a blank.

3.8. Site-directed mutagenesis

The QuickChange site directed mutagenesis method (Stratagene) was used to make point mutations in FGFR1 WT (Y677 to F677) and STAT3 WT (R609 to L609). Primers were designed for FGFR1 WT (forward primer 5'GCATTATTTGACCGGATCTTCACCCACCAGAGTGATG3', reverse primer 5'CATCACTCTGGTGGGTGAAGATCCGGTCAAATAATGC3') and STAT3 WT (forward primer 5'GGAACCGGTTCTCCCGACATTCCCAAGGAGG3', reverse primer 5'AGAACCGGTTCTTCATTCCAAAGGGCCAAG3').

The mixture for PCR contained:

Template DNA – 50 ng (STAT3WT) or 500 ng (FGFR1 WT)

Primers – 300 ng each

dNTPs – 500 µM

10x Pfu buffer – 5 µl

Materials and methods

PfuTurbo DNA polymerase - 1 µl

Distilled water - up to a total volume of 50 µl

The PCR program was performed:

95⁰C 1 minute, 1 cycle

95⁰C 50 seconds, 60⁰C 50 seconds, 72⁰C 18 minutes, 18 cycles

72⁰C 5 minutes, 1 cycle

Product from PCR was reacted with 2 µl of DpNI (New England Biolabs Inc) over-night at 37⁰C. Highly-competent DH5α cells (Invitrogen) were transformed with 2 µl of DpNI-treated PCR product. LB media was added to transformed cells and cells were grown in 37⁰C for 1 hour. After that time cells were plated on LB plate and were grown overnight in 37⁰C. Single colonies from plates were taken for MiniPrep.

3.9. Miniprep

DNA mini-preparations were made by inoculating 5 ml of LB media with ampicillin with a single colony from the transformations and growing it shaking at 37⁰C and 220rpm overnight. Miniprep purifications were performed using Qiaprep Spin Mini-prep Kits (Qiagen Ltd) in accordance with their recommended protocol.

3.10. DNA sequencing

DNA sequencing was performed by the Functional Genomics Laboratory (School of Biosciences, University of Birmingham). Samples for sequencing were prepared:

DNA template – 500 ng

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Primers – 3.2 pmol

H₂O – up to 10 µl of total volume

25 cycles of sequencing in Genomics Lab Thermal Cycler – PCR conditions used:

96⁰C – 10 seconds

50⁰C – 5 seconds

60⁰C – 4 minutes

Sequencing results and chromatograms were analysed using Sequencher 4.9.

4. Cell culture

4.1. Culturing cells

HEK293T (human embryonic kidney epithelial cell line transformed with the SV40 large T antigen 293T), HeLa (cervical cancer cell line), MCF7 (human breast adenocarcinoma) and MFM-223 (human breast cancer cell line) were cultured in Dulbecco's Modified Eagle Media (DMEM, Gibco, Invitrogen) supplemented with 10% foetal bovine serum (FBS), 0.2 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. Cells were grown at 37⁰C in 5% CO₂ and atmospheric O₂. ZR-75-1 and SUM-52PE breast cancer cell lines were cultured in RPMI1640 (Lonza) supplemented with 10% foetal bovine serum (FBS), 0.2 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. Cells were grown at 37⁰C in 5% CO₂ and atmospheric O₂. NIH3T3 cells (mouse embryonic fibroblasts) were cultured in Dulbecco's Modified Eagle Media (DMEM, Gibco, Invitrogen) supplemented with 10% donor calf serum (DCS), 0.2 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. Cells were grown at 37⁰C in 5% CO₂ and atmospheric O₂. MDA-MB-134 and MDA-MB-453 breast

Materials and methods

cancer cell lines were cultured in ATCC-formulated Leibovitz' L-15 medium with 10% foetal bovine serum (FBS), 0.2 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. Cells were grown at 37⁰C in atmospheric O₂ and without CO₂. MEF (mouse embryonic fibroblasts) and bac16 cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Gibco, Invitrogen) supplemented with 10% foetal bovine serum (FBS), 0.2 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. Cells were grown at 37⁰C in 5% CO₂ and hypoxic conditions. TERT-NHUC (immortalized normal human urothelial cells) cells were cultured in Keratinocyte Growth Medium 2 (Promocell) supplemented with all the provided growth factors but only 0.09 mM CaCl₂. Cells were grown at 37⁰C in 5% CO₂ and atmospheric O₂. All cell lines were split at the appropriate dilution into 25 cm², 75 cm² flasks or 6-well plates with fresh media.

4.2. Transient transfection using Gene Juice

Gene Juice reagent (Novagen, MERCK Bioscience) was used to transfect HEK293T and HeLa cells with DNA constructs. Cells were split into 6 well-plates and transfected when they reached 50-80% of confluency. 3 µl of Gene Juice was added to 100 µl of serum-free DMEM and incubated for 5 minutes at room temperature. Then 1 µg of DNA was added to the mixture and incubated for another 20 minutes at room temperature to allow the formation of GeneJuice-DNA complexes. The GeneJuice-DNA mixture was added dropwise to well containing cells. Transfected cells were grown at 37⁰C in 5% CO₂ for 48 hours before analysis.

4.3. Transient transfection using Lipofectamine2000

Lipofectamine2000 (Invitrogen) was used to co-transfect HEK293T cells with DNA constructs and siRNA oligos. Cells were split into 6 well-plates and transfected when they reached 80% of confluency. 6 μ l of Lipofectamine2000 was added to 250 μ l of serum-free media. 0.5 μ g of DNA and 50 pmole of siRNA oligos were added to 250 μ l of serum-free media. After 5 minutes of incubation at room temperature both samples were mixed and incubated for another 20 minutes at room temperature to allow the formation of Lipofectamine-DNA complexes. The mixture was added dropwise to wells containing cells. Transfected cells were grown at 37⁰C in 5% CO₂ for 48 hours in antibiotic-free media.

4.4. Transient transfection using DharmaFECT3

SUM-52PE cells were transfected using DharmaFECT3 (Thermo Fisher Scientific) with siRNA against FGFR2 or control siRNA. Reverse transfection was performed at the same time as cells were plated into 12-well plate. In separate tubes with 97 μ l of serum-free media dilutions of 100 nM siRNA oligos and 3 μ l DharmaFECT3 were prepared. After 5 minutes of incubation both samples were mixed and incubated for another 20 minutes at room temperature. After that time samples were added to cells. After 24 and 48 hours forward transfections were performed according to the same protocol. Transfected cells were grown at 37⁰C in 5% CO₂ in antibiotic-free media.

4.5. Cell stimulation

Cells were grown to 90% confluency, growth media was aspirated and the cells were washed carefully with PBS. The media was then replaced with serum-free media. Cells were stimulated with 20 ng/ml FGF1 or FGF7 and 10 µg/ml heparin or 50 ng/ml oncostatin M at 37⁰C, 5% CO₂ for indicated times. Stimulation was terminated upon removal of the media and washing with cold PBS. Cells were placed on ice before lysis.

4.6. Pharmacological inhibitors

SU5402, SU6656, SP600125, SB203580, JAK inhibitor I, calphostin C, Cucurbitacin I, AG490 (Calbiochem), PD173074 (Sigma), U0126 (Cell Signaling Technology) and Dasatinib (US Biological) were dissolved in DMSO and stored in -20⁰C. After serum starvation cells were pre-incubated with inhibitors (or DMSO as control) at indicated concentrations for 30 minutes at 37⁰C, 5% CO₂ prior to stimulation.

5. Protein analysis

5.1. Cell lysis

Cells were lysed at 4⁰C. Cells were washed twice in cold PBS before addition of cold lysis buffer Triton-X (for Western blotting analysis of whole cell lysates and immunoprecipitation) or NP-40 (for peptide pull-down experiments). The whole cell lysates were removed from the bottom of the flask or well with a scraper and transferred to a tube, and then it was incubated

for 30 minutes on ice. Whole cell lysates were spun at 15000g for 30 minutes at 4⁰C before the supernatant was transferred to new tubes.

5.2. Measurement of protein concentration by Coomassie assay

BSA standards were prepared at concentrations of 25, 20, 15, 10, 5 and 2.5 µg/ml in distilled water with 0.05% azide. Samples were diluted at 1:300 or 1:500 in distilled water and 150 µl of each was added to 150 µl of Coomassie reagent (Pierce). The absorbance at 590 nm was measured and unknown sample concentrations were calculated from the standard curve of BSA samples. Concentrations in samples used in single immunoprecipitation were equalized by addition of lysis buffer subsequently.

5.3. Immunoprecipitation and SDS-PAGE

For immunoprecipitation primary antibody was directly added to cell lysates in dilution according to Manufacturer's recommendation. After 1 hour incubation at 4⁰C, 30 µl of 50% washed Sepharose protein-G fast flow slurries (Amersham Bioscience Inc.) was added to each sample and incubated for another 1 hour with rotation. Alternatively, agarose beads conjugated with antibody were added to cell lysates and incubated for 2 hours. Beads were separated by centrifugation, washed 5 times with cold lysis buffer and re-suspended in 2x sample buffer. Proteins were boiled for 5 minutes and separated by SDS-PAGE.

5.4. Western blotting

Proteins separated by SDS-PAGE were transferred to PVDF membrane (Milipore) prior to its activation in methanol and in transfer buffer. Primary antibodies were diluted in TBST with 5% BSA (w/v) and incubated with the membrane overnight at 4⁰C. The membrane was washed with TBST (4 x 15 minutes) and probed with the HRP-conjugated secondary antibody for 1 hour at room temperature, dilute in TBST with 5% BSA (w/v). The membrane was washed with TBST (4 x 15 minutes) followed by a final wash with TBS. The membrane was incubated for 2 minutes in enzyme chemioluminescence (ECL) substrate (Pierce) and exposed to hyperfilm (Scientific Laboratory Supplies). Alternatively, Odyssey system was used. Following incubation with primary antibody diluted in Odyssey blocking buffer, membrane was washed with TBSE buffer for 30 minutes and PBS for 5 minutes. Membrane was incubated with InfraRedDye-conjugated secondary antibody (Li-cor), washed with TBSE buffer for 30 minutes and PBS for 5 minutes and then proteins were revealed by scanning using Infrared Imaging System Odyssey. Membranes were stripped for re-probing with stripping buffer (0.5 mM Tris pH 6.7, 2% SDS and 0.7% β -mercaptoethanol) for 30 minutes at 50⁰C or Odyssey PVDF Stripping Buffer (Li-cor) for 20 minutes at room temperature.

6. Peptide pull-down assay and trypsin digestion

Dynabeads® MyOne™ were washed with 4 times with peptide binding/wash buffer (PBS-T) before re-suspending in the same. 0.1 μ M desthiobiotinylated peptides for FGFR1 were added and incubated on the shaker at room temperature for 1 hour, followed by washes with PBS-T buffer on a shaker. Final wash solution was removed and 10 mg of whole cell lysate was

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added for overnight incubation in 4⁰C on the rotator. Whole cell lysate was removed and beads were extensively washed with PBS-T and finally 2x sample buffer was added to samples which were then boiled for 5 minutes. Samples were resolved by SDS PAGE and stained with Coomassie for 1 hour. The gel tracks were divided into 6 gel pieces and then destained with 30% acetonitrile (15 minutes incubation with agitation) followed by 50% acetonitrile, 25 mM ammonium bicarbonate (15 minutes incubation with agitation). Destained gel pieces were dried by vacuum centrifugation for 5 minutes and rehydrated in 10 mM DTT, 25 mM ammonium bicarbonate at 56⁰C for 45 minutes. The liquid was replaced with 55 mM iodoacetamid, 25 mM ammonium bicarbonate for 45 minutes incubation in the dark. Samples were washed with 25 mM ammonium bicarbonate (10 minutes incubation with agitation) and 50% acetonitrile, 25 mM ammonium bicarbonate (2 x 5 minutes incubation with agitation) and dried by vacuum centrifugation for 10 minutes. 1 µg/µl trypsin was diluted with 25 mM ammonium bicarbonate for final concentration of 6.25 ng/µl and added to dried samples for 10 minutes on ice. Excess trypsin was removed, gel pieces were covered with 25 mM ammonium bicarbonate and hydrolysis was allowed to occur overnight at 37⁰C. Formic acid was added to 0.5% and the supernatant was removed. Remaining peptides were extracted from the gel pieces using 50% acetonitrile with vigorous agitation. This was repeated, followed by a final neat acetonitrile extraction. All supernatants were pooled and the volume reduced by vacuum centrifugation.

Mass spectrometry run was performed by Dr Steve Sweet. Typical conditions for MS/MS were as described below. On-line liquid chromatography was performed by use of a Micro AS autosampler and Surveyor MS pump (Thermo Electron, Bremen, Germany). Peptides were loaded onto a 75 µm (internal diameter) Integragrit (New Objective, USA) C8 resolving column (length 10 cm) and separated over a 40 minute gradient from 0% to 40% acetonitrile

Materials and methods

(Baker, Holland). Peptides eluted directly (~350 nL/min) via a Triversa nanospray source (Advion Biosciences, NY, USA) into a 7 Tesla LTQ FT mass spectrometer (Thermo Electron). The mass spectrometer alternated between a full FT-MS scan (m/z 395-1600), subsequent CID MS/MS scans of the five most abundant ions. Survey scans were acquired in the ICR cell with a resolution of 100,000 at m/z 400. Precursor ions were isolated and subjected to CID in the linear ion trap. Isolation width was 3 Th. Only multiply-charged precursor ions were selected MS/MS. CID was performed with helium gas at normalized collision energy of 35%. Precursor ions were activated for 30 ms. Data acquisition was controlled by Xcalibur 2.0 software. Data were searched against the Swissprot database using the SEQUEST (Thermo Electron).

7. Immunofluorescence

Cell growing on coverslips were serum starved and stimulated with FGF1. The stimulation was terminated with by washing three times with TBS. Cells were fixed with 4% paraformaldehyde for 10 minutes. Two protocols were used depending on primary antibody used for staining.

Saponin protocol: fixed cells washed three times with 0.1 M glycine and three times with TBS. Cells were permeabilized using saponin permeabilization buffer for 20 minutes, then washed three times with TBS and incubated for 1 hour with saponin blocking buffer. Cells were incubated with primary antibody diluted in blocking buffer for 1 hour followed by three time washes for 5 minutes with blocking buffer with quick rinse in TBS between them. Cells were incubated with fluorescence-conjugated secondary antibody for 45 minutes at dark.

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Cells were washed three times for 5 minutes with blocking buffer with quick rinse in TBS between them.

Methanol protocol: fixed cells were washed with PBST and permeabilized using methanol for 5 minutes at -20°C . Coverslips were incubated with 4% BSA-PBS blocking buffer for 1 hour. Cells were incubated with primary antibody followed by 3 washes with PBS and incubation with fluorescence-conjugated secondary antibody. The coverslips were mounted with 5 μl of Mowiol solution on the slide and were observed under confocal microscope.

CHAPTER 3

Identification of novel FGFR1 binding partners

Introduction

Protein-protein interactions contribute to a large number of intracellular signal transduction pathways (Papin, Hunter et al. 2005). This complex network affects all biological processes occurring in cells. Many of these interactions are regulated by post-translational modifications, like phosphorylation, acetylation, and methylation, which therefore provide a general mechanism to link the dynamic state of the proteome to cellular responses. Signal transduction depends on interactions between proteins and formation of multiprotein complexes. Signalling pathways are activated by recruitment of a variety of proteins to growth factor receptors. Formation of such complexes is possible due to specific motifs on GFRs that facilitate association between proteins (Pawson 2004). To fully comprehend signalling processes, as well as the differences between signalling in a healthy organism and an organism affected by disease, it is crucial to understand the architecture of the interactions between receptors and adaptor proteins and to identify potential binding partners.

Several techniques have been developed to screen for protein-protein interactions. The yeast two-hybrid system is a relevant approach that has been effectively used to identify protein-protein interactions on a large scale (Kuroda, Kato et al. 2006). Additionally, the immense role of proteomics in this field is undeniable; phospho-proteomics, a technology platform widely used in describing protein-protein interactions, has contributed significantly to characterization of phosphorylation events in stimulated cells (Blagoev, Kratchmarova et al. 2003; Schulze, Deng et al. 2005; Blagoev and Mann 2006). There are two main strategies in signalling-related proteomics. First is mapping of tyrosine-phosphorylated proteins involved in a particular signalling pathway, second is detection of binding partners for RTKs.

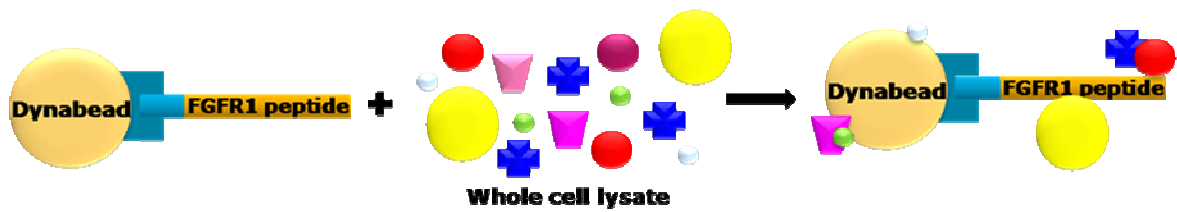
The aim of the work described here is to identify novel binding partners for FGFR1 which was performed by peptide pull-down (PPD) experiments. Peptide pull-down is an *in vitro* methodology that is based on two distinct interactions: protein-peptide and peptide-Dynabeads. The basics of PPD are shown in Figure 7. Synthetic peptides were designed against different segments of FGFR1. Each peptide contained a different C-terminal tyrosine from FGFR1 that was either phosphorylated or non-phosphorylated ('active' or 'inactive'). FGFR1 peptides worked as 'baits' to pull down specific proteins from whole cell lysates. Peptides were labelled with desthiobiotin that enabled reversible binding to streptavidin coated Dynabeads. Protein-peptide-bead complexes were easily separated by a magnet due to magnetic properties of the Dynabeads. Elution with biotin, which has irreversible binding to streptavidin, allowed separation of peptide-protein complexes from Dynabeads. Pulled-down proteins were subjected to identification by mass spectrometry.

Several autophosphorylation sites in the cytoplasmic tail of FGFR1 have been identified (Mohammadi, Dikic et al. 1996; Foehr, Raffioni et al. 2001). The presence of other tyrosines on FGFR1 suggests that they might also become phosphorylated in response to FGF stimulation and interact with adaptor proteins. The list of all FGFR1 peptides used in this study, as well as their position in cytoplasmic region of FGFR1 is presented in Table 2.

A.



B.



C.

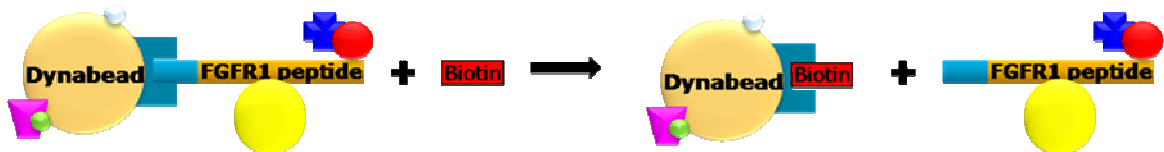


Figure 7. The principles of peptide pull-down.

A. Incubation of desthiobiotin labelled FGFR1 peptide with streptavidin coated Dynabeads.

B. Incubation of peptide bound beads with whole cell lysate which contains protein targets that specifically bind FGFR1 peptides.

C. Elution of peptides off the beads using biotin.

Table 2. FGFR1 synthetic peptides used in peptide pull-down.

| Tyrosine residue | FGFR1 peptide sequence |
|-------------------------|-------------------------------|
| Tyr 558/563 | BGSGS-TQDGPL(p)YVIVEYASKG |
| Tyr 572 | BGSGS-SKGNLRE(p)YLQARRPPG |
| Tyr 583/585 | BGSGS-RPPGLE(p)YC(p)YNPSHNPE |
| Tyr 605 | BGSGS-DLVSCA(p)YQVARGMEY |
| Tyr 613 | BGSGS-VARGME(p)YLASKKCIH |
| Tyr 653/654 | BGSGS-ARDIHHID(p)Y(p)YKKTNG |
| Tyr 677 | BGSGS-EALFDRI(p)YTHQSDVWS |
| Tyr 701 | BGSGS-FTLGGSP(p)YPGVPVEEL |
| Tyr 730 | BGSGS-SNCTNEL(p)YMMMRDCWH |
| Tyr 766 | BGSGS-ALTSNQE(p)YLDLSMPLD |
| Tyr 776 | BGSGS-LSMPLDQ(p)YSPSPDTR |

FGFR1 sequence.

The positions of peptides used in the PPD experiment are bolded.

1 mswkellfw avlvttatlet arpsptlpeq aqpwgapvev esflvhpgdl lqlrcrlrdd
61 vqsinwlrldg vqlaesnrtr itgeevevqd svpadsglya cvtsspsgsd ttyfsvnvsd
121 alpssedddd dddssseeke tdntkpnrmv vapywtspek mekkhavlpa aktvkfkcpv
181 sgtpnptlrw lkngkefkpd hriggykvry atwsiimsv vpsdkgnytc iveneygsin
241 htyqldvver sphrpilqag lpanktvalg snvefmckvy sdpqphiqwl khievngski
301 gpdnlpyvqi lktagvnttd kemevlhlrn vsfedageyt clagnsigls hhsawltvle
361 aleerpavmt splyleiiiy ctgafliscm vgsvivymk sgtkksdfhs qmavhklaks
421 iplrrqvtvs adssasmnsg vllvrpsrls ssgtpmlagv seyelpedpr welprdrvl
481 gkplgegcfg qvvlaeaigl dkdnpnrvtk vavkmlksda tekdlldlis ememmkmigk
541 hkniinllga ct**qdgplyvi veyaskgnlr eylqarrppg leycynpshn** peeqlsskdl
601 **vsayqvarg meylaskkci** hrdlaarnvl vtednvmkia dfglardihh idyykktng
661 rlpvkwmape **alldriythq sdvwsfgvll weiftlggsp ypgvpveelf** kllkeghrmd
721 kpsnctnely **mmmrdewhav psqrptfkql vedldrival tsnqeyldls mpldqyspsf**
781 **pdtrsstcsc** gedsvfshep lpeepclprh paqlangglk rr

1. Optimisation of peptide pull-down

To use peptide pull-down for detection of novel binding proteins for FGFR1 optimisation of the method was required. A major challenge was to obtain more specific results in the peptide pull-down. Optimisation of the method was performed by choosing the most suitable conditions for the experiment: including concentration of WCLs, amount of Dynabeads and peptides, concentration of elution buffer, time of elution and numbers of washes. For example, if the concentration of whole cell lysate was too low, signalling molecules could not be detected in eluted fractions. On the other hand, high concentration of WCL could increase unspecific bindings. Moreover, the number of washes, as well as time of each wash, seemed to be essential in the peptide pull-down. The most difficult step to optimise was elution and several experiments were repeated under slightly different conditions to obtain the best results.

All optimisation experiments were undertaken using desthiobiotinylated peptides for FGFR1 with the Tyr766 phosphorylation site. The reason for choosing phosphorylated and unphosphorylated Tyr766 peptides in optimisation step was that Tyr766 of FGFR1 is a known binding site for PLC γ 1 and Shb (Mohammadi, Dionne et al. 1992; Cross, Lu et al. 2002) so it acts as a positive control of the experiment. The consensus binding motif for the SH2 domains of PLC γ 1 and Shb fits with the sequence of FGFR1 (Table 3) (Liu, Jablonowski et al. 2006).

Table 3. FGFR1 binding motif for SH2 domains of PLC γ 1 and Shb.

| Sequence of FGFR1 (pTyr766) | | | | |
|----------------------------------|----|---|---|---|
| FGFR1 | pY | L | D | L |
| Consensus motifs for SH2 domains | | | | |
| PLC γ 1 | pY | L | D | L |
| Shb | pY | L | X | L |

Firstly, an experiment was carried out to compare direct and indirect peptide pull-down. The principles of the direct method are described above (Figure 7). In an indirect approach, the FGFR1 peptides were first added to whole cell lysates to allow complex formation between peptides and target proteins. Then, Dynabeads were added for a short time followed by washes and an elution step similar to the direct method (Figure 8). The reason for using indirect capture was to minimise unspecific binding of proteins to the Dynabeads. Samples from both approaches were eluted with biotin and probed for PLC γ 1 together with whole cell lysates (Figure 8).

Results obtained from this experiment showed that the indirect method was not efficient enough to pull down PLC γ 1 using the phosphorylated Tyr766 FGFR1 peptide, however, PLC γ 1 was detected in the equivalent sample using the direct method (Figure 8). The amount of PLC γ 1 pulled down in the direct PPD was low, which was probably due to an inefficient elution step. Based on this result it was decided to use the direct peptide pull-down method for further experiments.

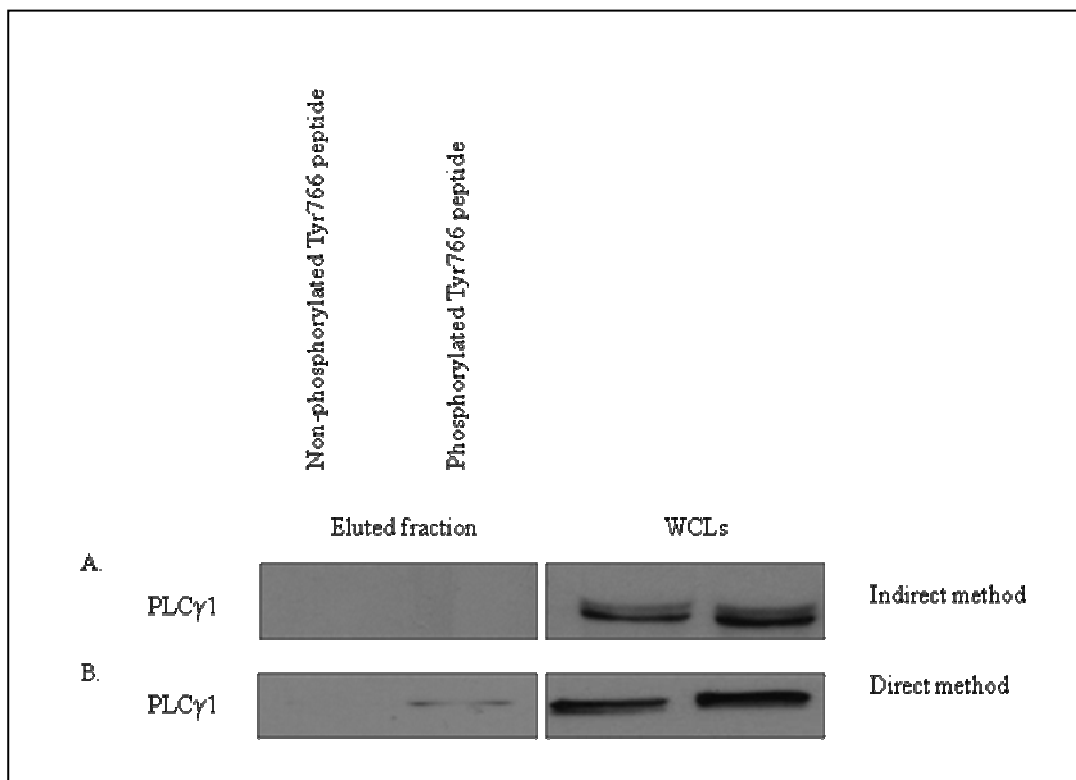


Figure 8. Comparison between direct and indirect PPD.

- A. Indirect PPD method – 0.1 μ M of Tyr677 and phospho-Tyr677 FGFR1 peptides were incubated with 10 mg of whole cell lysate from HEK293T cells overnight. Samples were washed with PBST and 50 μ l of Dynabeads was added. After 3 minutes of incubation, samples were washed with PBST and 30 μ l of 20 mM biotin was added for 15 minutes. SDS sample buffer was added to eluted fractions. Whole cell lysates and eluted fractions were resolved by SDS-PAGE, transferred to PVDF membrane and probed for PLC γ 1.
- B. Direct PPD method – 0.1 μ M of Tyr677 and phospho-Tyr677 FGFR1 peptides were incubated with 50 μ l of Dynabeads for 1 hour. Samples were washed with PBST and 10 mg of whole cell lysate from HEK293T cells was added to all samples. Samples were incubated overnight followed by washes with PBST and elution with 30 μ l of 20 mM biotin for 15 minutes. SDS sample buffer was added to eluted fractions. Whole cell lysates and eluted fractions were resolved by SDS-PAGE, transferred to PVDF membrane and probed for PLC γ 1.

To check the efficiency of biotin elution, beads fractions were probed for PLC γ 1 and Shb along with eluted fractions (Figure 9). The experiment was carried out using the direct peptide pull-down. After incubation, samples were washed 5 times, each time for 15 minutes, in order to minimise unspecific bindings. The elution lasted 1 hour and was performed using a larger volume of 20 mM biotin. Additionally, more Dynabeads were used which allowed more PLC γ 1 and Shb to bind.

PLC γ 1 and Shb were present in the eluted fractions for phosphorylated Tyr766 peptide but not in non-phosphorylated peptide or negative control (Figure 9). The efficiency of elution was enhanced compared with the first experiment (Figure 8 and Figure 9). Bead fractions were analysed by eluting off the remained proteins in reducing SDS sample buffer which were separated by SDS-PAGE. PLC γ 1 and Shb were present in all beads fractions – phosphorylated, non-phosphorylated Tyr766 peptides and negative control (Figure 9). However, the amount of PLC γ 1 was much higher in the phospho-Tyr766 bead fraction. Comparing the amount of PLC γ 1 in eluted fractions with beads fractions for phosphorylated Tyr766 peptide, it was concluded that most of PLC γ 1 was still bound to beads rather than being eluted with biotin. Even though the efficiency of biotin elution was improved compared with the previous experiment, it was not efficient enough to elute all target proteins from beads. This might be crucial during identification of novel binding partners for FGFR1, especially for detection of low abundant proteins. In order to elute all proteins that were pulled down by FGFR1 peptides, the final PPD experiment was performed by addition of reducing SDS sample buffer directly to beads after extensive washes.

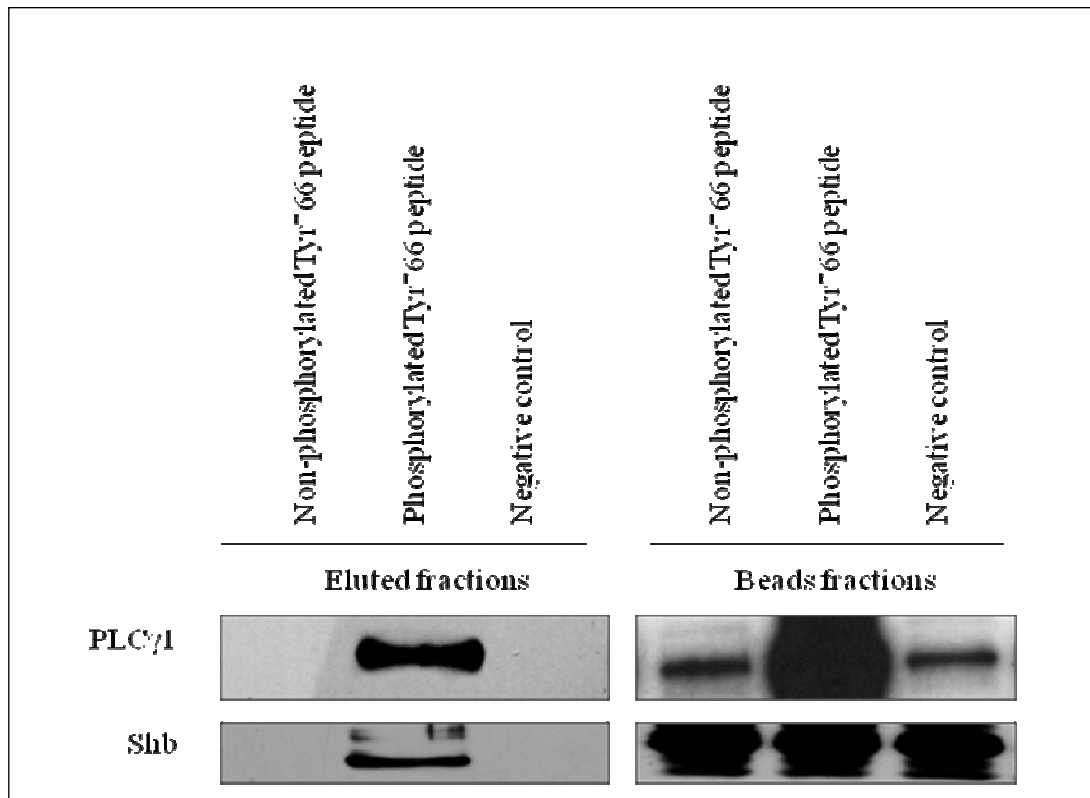


Figure 9. Verification of efficiency of biotin elution.

0.1 μ M of Tyr677 and phospho-Tyr677 FGFR1 peptides were incubated with 100 μ l of Dynabeads for 1 hour. Samples were washed with PBST and 10 mg of whole cell lysate from HEK293T cells was added to all samples.

Samples were incubated overnight followed by 5 washes with PBST, 15 minutes each, and elution for 1 hour with 120 μ l of 20 mM biotin. SDS sample buffer was added to eluted fractions and to bead fractions. Samples were resolved by SDS-PAGE, transferred to PVDF membrane and probed for PLC γ 1 and Shb. Negative control sample did not contain any peptide.

2. Identification of novel binding partners by proteomics

The main goal of the project was to identify novel binding partners for FGFR1 which was achieved by using PPD followed by mass spectrometry. The first step of PPD was 1 hour incubation of FGFR1 peptides with Dynabeads. Peptides-beads complexes were washed and incubated overnight with whole cell lysates. During incubation proteins from WCLs bound FGFR1 peptides. The following day, samples were extensively washed to remove non-specific background proteins (15 times, 15 minutes each). Concomitantly, sample without any peptide was prepared as a negative control. Reducing SDS sample buffer was added to elute proteins from the beads. Proteins were resolved by SDS-PAGE, stained using Coomassie (Figure 10) and subjected to trypsin digestion, prior to analysis using MS/MS. The MS/MS data obtained were searched against the Swissprot database using SEQUEST (Thermo Electron) in order to identify the proteins originally present. The results for each of phospho-FGFR1 peptides are presented in Table 4.

An important part of proteomics was data analysis. Here, it was carried out in several steps allowing final selection of specific proteins. Firstly, peptides detected by MS/MS were filtered and verified under the same parameters in BioWorks 3.3.1. Two basic parameters used to filter the data and reject unlikely hits were probability and x-score. Probability is a parameter that shows the probability of random match. If the hit is smaller than $1e-5$, then it is likely to be correct, values bigger than $1e-3$ specifies a false match, values between $1e-3$ and $1e-5$ are borderline. X-score (cross correlation) is a value for the quality of the match that is calculated by comparing predicted spectrum for peptide with experimental spectrum. Secondly, control sample and samples containing phospho-peptides were compared. This eliminated proteins bound in unspecific manner to the beads. Next, bioinformatics' tools were

applied in order to link protein hits obtained by PPD. An application called ‘String’ (www.string.embl.de) is a database that shows a network of direct or indirect protein-protein interactions that were derived from several sources (eg. genomic context, high-throughput experiment, biochemical experimental data, Pubmed publications) (Jensen, Kuhn et al. 2009). The network accomplished by ‘String’ forms clusters of proteins with similar functions which helps integrating the data and analysing interactions on a broader level. The network of proteins obtained by PPD is presented in Figure 11. Lastly, validation of the PPD was done by immunoprecipitation (IP) experiments. FcFGFR1 constructs (kinase active, kinase dead and truncated form of the receptor) were transiently transfected into HEK293T cells, immunoprecipitated and probed using several antibodies to investigate the interactions between FGFR1 and protein hits from PPD (Figure 12). The majority of proteins, which were checked by IP, formed complexes with FGFR1 in a phospho-specific manner (Figure 12). Nevertheless, not every protein hit from Table 4 was verified by IP due to lack of antibodies.

The principle of PPD is to detect proteins recruited directly to peptides containing phospho-tyrosine motifs, rather than multi-protein complexes. PPD was successfully used for identification of EGFR binding partners (Schulze and Mann 2004; Schulze, Deng et al. 2005) where direct association between peptides and detected proteins was suggested. However, it cannot be ruled out that peptide-protein complex were mediated by other proteins which were not identified by mass spectrometry. Overall, the number of identified proteins proved that PPD is powerful method successfully used to screen for protein-protein interactions.

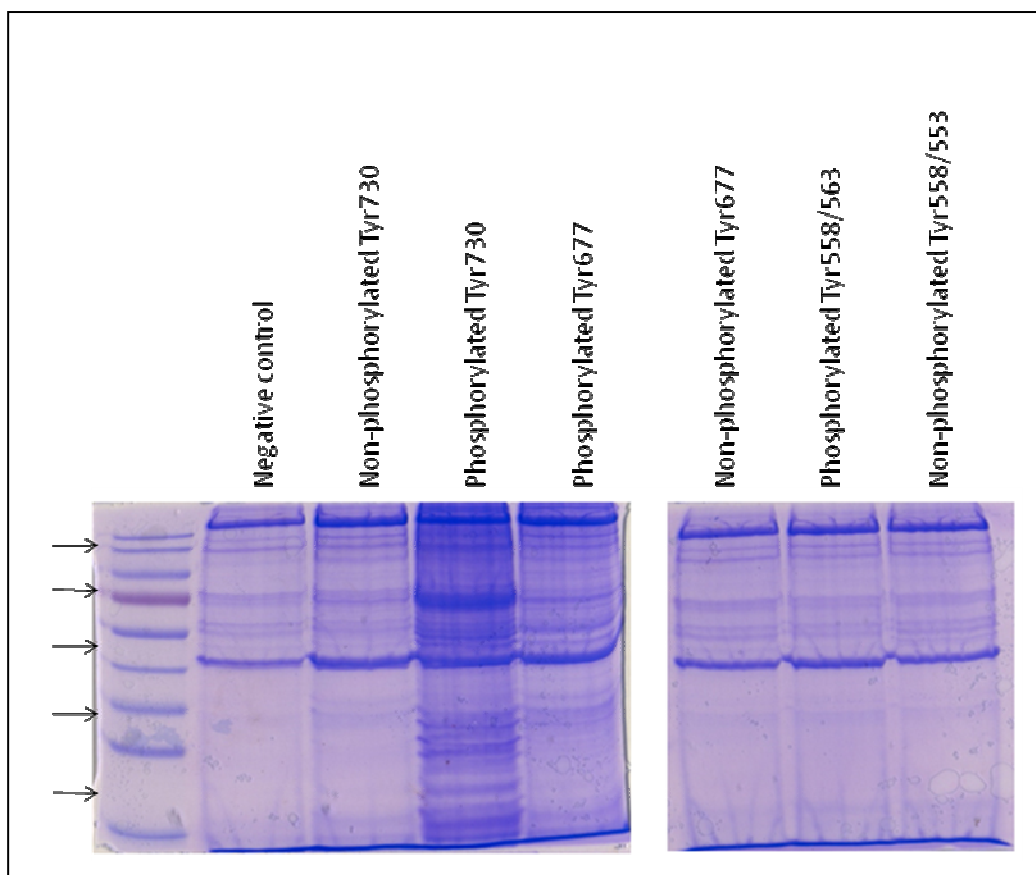


Figure 10. Coomassie stained gel for PPD samples.

Representative Coomassie stained gel after PPD experiment for FGFR1 peptides Tyr730, Tyr677 and Tyr 558/553 (phosphorylated and non-phosphorylated). 0.1 μ M of paired FGFR1 peptides were incubated with 100 μ l of Dynabeads for 1 hour. Samples were washed 5 times with PBST and 10 mg of whole cell lysate from HEK293T cells was added to all samples. Samples were incubated overnight followed by 15 extensive washes, each 15 minutes with PBST. SDS sample buffer was added to elute of the proteins from the beads. The negative control sample did not contain any peptide. Samples were resolved by SDS-PAGE and Coomassie stained. Each lane on the gel was cut into 6 pieces (arrows indicate places where lanes were cut) and each piece was trypsin digested then analysed by MS/MS (Table 4).

Table 4. Proteins identified as FGFR1 binding partners.

0.1 μ M of paired phosphorylated and non-phosphorylated FGFR1 peptides were incubated with 100 μ l of Dynabeads for 1 hour. Samples were washed 5 times with PBST and 10 mg of whole cell lysate from HEK293T cells was added to all samples. Samples were incubated overnight followed by 15 extensive washes, each 15 minutes with PBST. SDS sample buffer was added to elute of the proteins from the beads. The negative control sample did not contain any peptide. Samples were resolved by SDS-PAGE, Coomassie stained (Figure 10) and trypsin digested then analysed by MS/MS. The MS/MS data were searched against the Swissprot database using SEQUEST (Thermo Electron). Results were verified by Bioworks 3.3.1. P is probability of random hit, x-score is the quality of match. Peptide list of the identified proteins is shown in Appendix

I.

| Protein hits | P (probability) | Score (XC) | MW (kDa) | Accession number |
|---|-----------------|------------|-----------|------------------|
| Tyr 558/563 | | | | |
| KPCI_HUMAN Protein kinase C iota type (EC 2.7.11.13) (nPKC-iota) (Atypical protein kinase C-lambda/iota) (aPKC-lambda/iota) (PRKC-lambda/iota) - Homo sapiens (Human) | 1.7e-010 | 2.02E+01 | 67215.02 | P41743 |
| TRAF2_HUMAN TNF receptor-associated factor 2 (Tumor necrosis factor type 2 receptor-associated protein 3) - Homo sapiens (Human) | 3.2e-008 | 2.01E+01 | 55822.80 | Q12933 |
| Tyr 572 | | | | |
| PI4KA_HUMAN Phosphatidylinositol 4-kinase alpha (EC 2.7.1.67) (PI4-kinase alpha) (PtdIns-4-kinase alpha) (PI4K-alpha) - Homo sapiens (Human) | 3.6e-008 | 2.02E+01 | 231141.20 | P42356 |
| Tyr 583/585 | | | | |
| IQGA3_HUMAN Ras GTPase-activating-like protein IQGAP3 - Homo sapiens (Human) | 1.4e-014 | 2.04E+01 | 184430.70 | Q86VI3 |

Identification of novel FGFR1 binding partners

| | | | | |
|--|----------|----------|-----------|--------|
| PERQ2_HUMAN PERQ amino acid-rich with GYF domain-containing protein 2 (Grb10-interacting) (GYF protein 2) - Homo sapiens (Human) | 8.8e-015 | 8.03E+01 | 149977.80 | Q6Y7W6 |
| CKP1_HUMAN Nck-associated protein 1 - Homo sapiens (Human) | 3.8e-013 | 2.02E+01 | 128706.60 | Q9Y2A7 |
| DUS3_HUMAN Dual specificity protein phosphatase 3 (EC 3.1.3.48) (EC 3.1.3.16) - Homo sapiens | 7.1e-010 | 5.82E+01 | 20465.32 | P51452 |
| PTN1_HUMAN Tyrosine-protein phosphatase non-receptor type 1 (EC 3.1.3.48) (Protein-tyrosine phosphatase 1B) (PTP-1B) - Homo sapiens (Human) | 5.2e-006 | 2.02E+01 | 49934.87 | P18031 |
| Tyr605 | | | | |
| CRKL_HUMAN Crk-like protein (Human) | 2.8e-006 | 2.02E+01 | 33755.93 | P46109 |
| Tyr 613 | | | | |
| Tyr 653/654 | | | | |
| CSK_HUMAN Tyrosine-protein kinase CSK (EC 2.7.10.2) (C-SRC kinase) (Protein-tyrosine kinase CYL) - Homo sapiens (Human) | 4.8e-011 | 2.03E+01 | 50671.81 | P41240 |
| RAB1A_HUMAN Ras-related protein Rab-1A (YPT1-related protein) - Homo sapiens (Human) | 2.1e-008 | 2.02E+01 | 22663.38 | P62820 |
| Tyr 677 | | | | |
| AP2A2_HUMAN AP-2 complex subunit alpha-2 (Adapter-related protein complex 2 alpha-2 subunit) (Alpha-adaptin C) (Adaptor protein complex AP-2 alpha-2 subunit) (Human) | 1e-030 | 2.02E+02 | 103895.10 | O94973 |
| AP2B1_HUMAN AP-2 complex subunit beta-1 (Adapter-related protein complex 2 beta-1 subunit) (Beta-adaptin) (Plasma membrane adaptor HA2/AP2 adaptin beta subunit) (Human) | 1.1e-015 | 2.68E+02 | 104486.20 | P63010 |
| CLH1_HUMAN Clathrin heavy chain 1 (CLH-17) - Homo sapiens (Human) | 1.7e-014 | 8.23E+01 | 191491.70 | Q00610 |
| AP2A1_HUMAN AP-2 complex subunit alpha-1 (Adapter-related protein complex 2 alpha-1 subunit) (Alpha-adaptin A) (Adaptor protein complex AP-2 alpha-1 subunit) (Human) | 2.9e-014 | 4.10E+02 | 107487.00 | O95782 |

Identification of novel FGFR1 binding partners

| | | | | |
|--|----------|----------|-----------|----------|
| STAT3_HUMAN Signal transducer and activator of transcription 3 (Acute-phase response factor) - Homo sapiens (Human) | 3.1e-013 | 1.88E+02 | 88011.44 | P40763 |
| AP2M1_HUMAN AP-2 complex subunit mu-1 (Adaptin mu-1) (AP-2 mu-2 chain) (Clathrin coat assembly protein AP50) (Clathrin coat-associated protein AP50) (Human) | 6.1e-012 | 1.90E+02 | 49623.09 | Q96CW1 |
| AP2S1_HUMAN AP-2 complex subunit sigma-1 (Adapter-related protein complex 2 sigma-1 subunit) (Clathrin coat assembly protein AP17) (Clathrin coat-associated protein AP17) (Human) | 2.6e-008 | 4.02E+01 | 17006.70 | P53680 |
| EPN4_HUMAN Clathrin interactor 1 (Epsin-4) (Epsin-related protein) (EpsinR) (Clathrin-interacting protein localized in the trans-Golgi region) (Human) | 1.4e-007 | 6.02E+01 | 68215.83 | Q14677 |
| CLCB_HUMAN Clathrin light chain B (Lcb) - Homo sapiens (Human) | 3.8e-005 | 1.42E+01 | 25175.14 | P09497 |
| Tyr 701 | | | | |
| RAB1B_HUMAN Ras-related protein Rab-1B - Homo sapiens (Human) | 1.7e-009 | 1.82E+01 | 22157.23 | Q9H0U4 |
| Tyr 730 | | | | |
| SRC_HUMAN Proto-oncogene tyrosine-protein kinase Src (p60-Src) (c-Src) (pp60c-src) (Human) | 3.3e-015 | 8.03E+01 | 59797.09 | P12931 |
| SHC1_HUMAN SHC-transforming protein 1 (SH2 domain protein C1) (Src homology 2 domain-containing-transforming protein C1) (Human) | 6.5e-014 | 1.90E+02 | 62782.63 | P29353.4 |
| P85A_HUMAN Phosphatidylinositol 3-kinase regulatory subunit alpha (PI3-kinase p85 subunit alpha) (PtdIns-3-kinase p85-alpha) (Human) | 2.2e-013 | 8.03E+01 | 83546.53 | P27986.2 |
| P85B_HUMAN Phosphatidylinositol 3-kinase regulatory subunit beta (PI3-kinase p85 subunit beta) (PtdIns-3-kinase p85-beta) (Human) | 9.7e-012 | 7.02E+01 | 81574.09 | O00459.1 |
| PK3CB_HUMAN Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform (EC 2.7.1.153) (PI3-kinase p110 subunit beta) (PtdIns-3-kinase p110) (Human) | 9.9e-011 | 2.02E+01 | 122683.30 | P42338 |
| RSU1_HUMAN Ras suppressor protein 1 (Rsu-1) (RSP-1) (Human) | 3.3e-009 | 2.02E+01 | 31520.70 | Q15404 |

Identification of novel FGFR1 binding partners

| | | | | |
|--|----------|----------|-----------|--------|
| GRB10_HUMAN Growth factor receptor-bound protein 10 (GRB10 adaptor protein) (Insulin receptor-binding protein GRB-IR) (Human) | 2.9e-008 | 7.02E+01 | 67188.91 | Q13322 |
| ARAF_HUMAN A-Raf proto-oncogene serine/threonine-protein kinase (A-raf-1) (Proto-oncogene Pks) (Human) | 3.0e-008 | 2.02E+01 | 67542.38 | P10398 |
| FYN_HUMAN Proto-oncogene tyrosine-protein kinase Fyn (p59-Fyn) (Protooncogene Syn) (Human) | 6.9e-008 | 3.02E+01 | 60723.48 | P06241 |
| RAB5A_HUMAN Ras-related protein Rab-5A - Homo sapiens (Human) | 2.5e-007 | 2.01E+01 | 23643.82 | P20339 |
| VAV2_HUMAN Protein vav-2 - Homo sapiens (Human) | 7.1e-007 | 3.02E+01 | 101191.80 | P52735 |
| YES_HUMAN Proto-oncogene tyrosine-protein kinase Yes (p61-Yes) (c-Yes) (Human) | 4.7e-005 | 2.02E+01 | 60762.63 | P07947 |
| Tyr 766 | | | | |
| PLCG1_HUMAN 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-1 (Phosphoinositide phospholipase C) (PLC-gamma-1) (Phospholipase C-gamma-1) (Human) | 2.0e-012 | 2.70E+02 | 148437.50 | P19174 |
| Tyr 776 | | | | |
| RAB10_HUMAN Ras-related protein Rab-10 - Homo sapiens (Human) | 3.4e-006 | 1.42E+01 | 22526.59 | P61026 |
| RUFY1_HUMAN RUN and FYVE domain-containing protein 1 (FYVE-finger protein EIP1) (Zinc finger FYVE domain-containing protein 12) – Homo sapiens (Human) | 2.7e-007 | 2.02E+01 | 79767.31 | Q96T51 |

Identification of novel FGFR1 binding partners

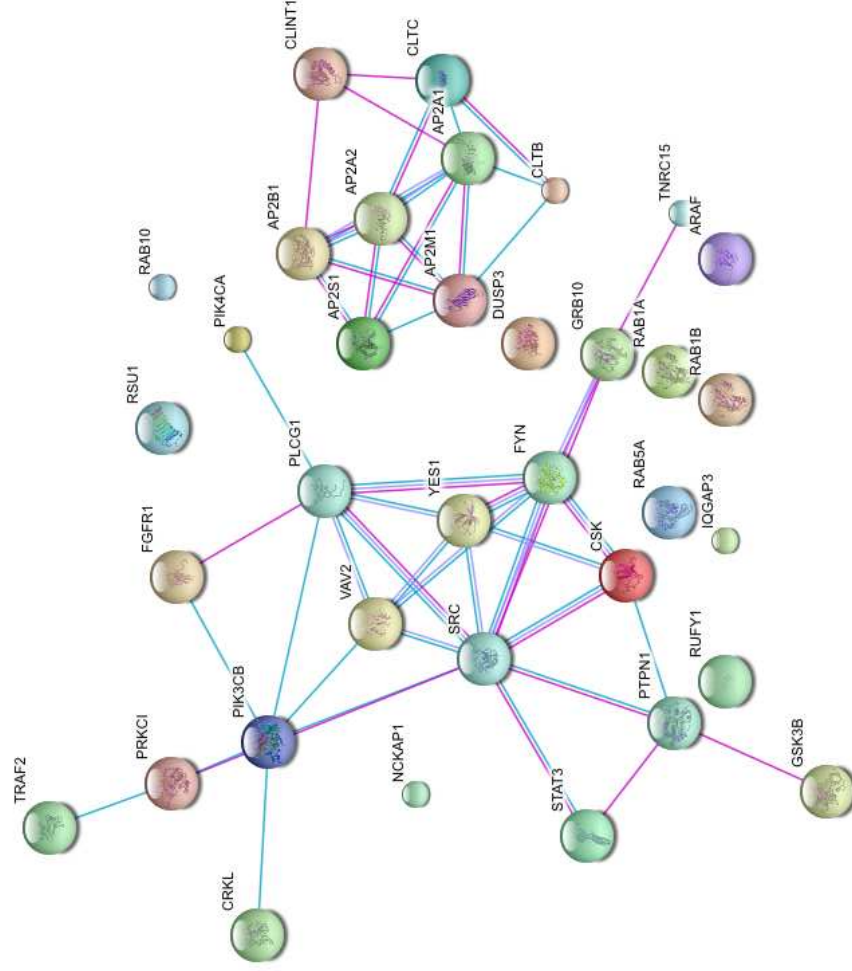


Figure 11. String analysis of protein-protein interactions.

Data obtained in the PPD experiments were loaded into String database. Prediction methods used: experiments (pink line), database (blue line). Medium confidence (0.4).

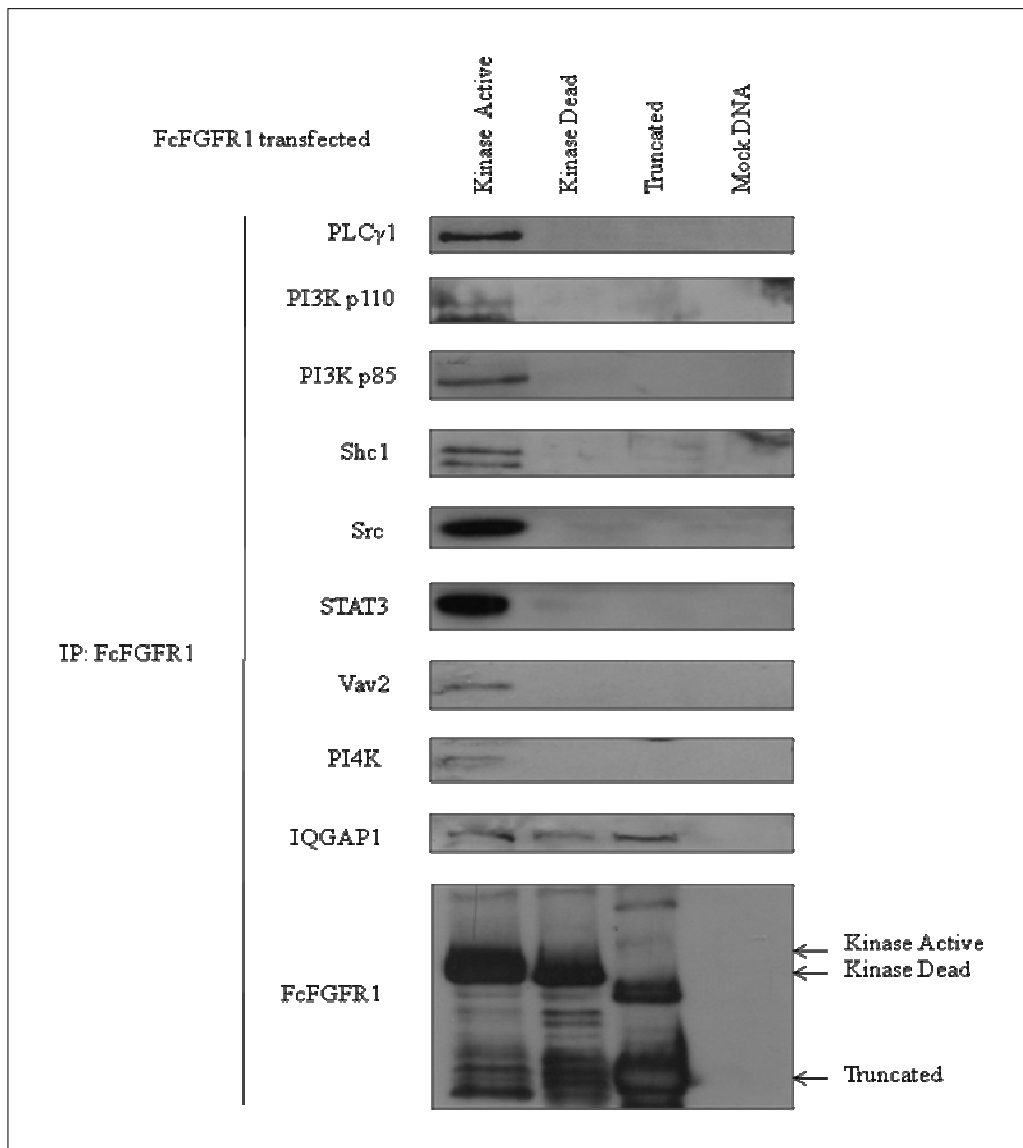


Figure 12. Validation of PPD by FcFGFR1 immunoprecipitation.

FcFGFR1 constructs (kinase active, kinase dead, truncated) were transiently transfected into HEK293T cells. After 48 hours equal amounts of protein were immunoprecipitated using protein G-Sepharose. Samples were resolved by SDS-PAGE and transferred to PVDF membrane. FGFR1 complex formation with particular proteins was investigated by probing with specific antibodies.

2.1. Signalling proteins

PLC γ 1

PLC γ 1 was the first protein described to bind directly to FGFR1. Previous data showed that activated FGFR1 recruits PLC γ 1 by its SH2 domain to Tyr766 in the C-terminal tail of FGFR1 (Mohammadi, Honegger et al. 1991; Mohammadi, Dionne et al. 1992). Here, PLC γ 1 was identified by PPD as a binding partner for Tyr766 of FGFR1 and its interaction with FGFR1 was validated by immunoprecipitation (Table 4 and Figure 12). Detection of this known FGFR1 binding partner proved the specificity of the PPD experiment.

Shc1

Shc1 was previously described as an interacting partner for FGFR1 but the site of interaction was not known. In this study, Shc1 was detected as binding protein for the phospho-Tyr730 FGFR1 peptide (Table 4) and it was confirmed on a protein level by immunoprecipitation (Figure 12). SH2 and PTB domain consensus binding motifs for Shc family are presented in Table 5 (Liu, Jablonowski et al. 2006).

Table 5. FGFR1 binding motif for SH2 and PTB domains of Shc proteins

| Sequence of FGFR1 (pTyr730) | | | | | | | |
|---------------------------------|---|---|---|----|-----------|-----|-----------|
| FGFR1 | N | E | L | pY | M | M | M |
| Consensus motifs for SH2 domain | | | | | | | |
| Shc1 | X | X | X | pY | I/E/T | X | I/L/M |
| Shc2 | X | X | X | pY | I/M/T/D/L | M/I | M/F/I/Y/V |
| Shc3 | X | X | X | pY | L/M/I/Q | M/Y | I/L/M/V |
| Consensus motif for PTB domain | | | | | | | |
| Shc1 | N | P | X | pY | X | X | X |

According to recent reports, Shc proteins bind activated RTKs directly through either the SH2 domain or PTB domain, which induces MAPK activation via Grb2/Sos (Klint, Kanda et al. 1995). Shc1 links Grb2 to FGFR1, which is another mechanism of recruitment of Grb2 to FGFR1 and subsequent activation of Grb2/Sos complex (Klint, Kanda et al. 1995). Moreover, Tyr730 of FGFR1 was suggested as a direct the site for Shc-FGFR interaction by use of cell permeable FGFR1 peptide that was able to associate with Shc1 via its PTB (Dunican, Williams et al. 2001). On the contrary, Shc1 has been recently described as indirect Src-dependent binding partner for FGFR2 (Schuller, Ahmed et al. 2008).

Results obtained by PPD indicated rather direct association of Shc with FGFR1 via Tyr730 of the receptor. However, it is possible that the binding between Shc and another protein, like Src, is strong enough to be pulled down as a ternary complex in the PPD. Association of Shc with FGFR1 proved to be specific, however, further investigation is needed in order to address the questions about direct/indirect interaction and Shc domain involved in FGFR binding.

PI3K

PI3K is another protein identified by PPD as a potential binding partner for Tyr730 of FGFR1 (Table 4). The binding motif for SH2 domain of PI3Kp85 fits for Tyr730 of FGFR1 (Table 6).

Table 6. FGFR1 binding motif for SH2 domain of PI3K

| Sequence of FGFR1 (pTyr730) | | | | |
|---------------------------------|----|---------|---|---|
| FGFR1 | pY | M | M | M |
| Consensus motifs for SH2 domain | | | | |
| PI3K p85 | pY | M/I/V/E | X | M |

The PI3K-FGFR interaction was previously studied but there has never been shown direct interaction between these proteins. Similarly to Shc, data obtained using a FGFR1 peptide suggested that the SH2 domain of PI3Kp85 can bind a motif surrounding Tyr730 of FGFR1 (Dunican, Williams et al. 2001). Furthermore, developmental studies in *Xenopus* showed that p85 subunit of PI3K bound phosphorylated tyrosine on FGFR1 (Ryan, Paterno et al. 1998). On the other hand, an indirect interaction was described by Ong *et al.* where PI3Kp85 was recruited by FRS2/Grb2 complex linking FGFR1 with Akt pathway (Ong, Hadari et al. 2001).

In this study, PPD results indicated an interaction between PI3Kp85 and p110 and FGFR1 peptide (Table 4). Also association between kinase active FGFR1 and PI3K was confirmed by immunoprecipitation (Figure 12). Considering the role of PI3K in signal transduction, it is worth future investigation.

Src family

Another interesting group of signalling molecules is Src family. Src, Fyn and Yes have been identified as binding partners for phospho-Tyr730 of FGFR1 (Table 4) and that result was validated for Src by immunoprecipitation experiment (Figure 12). Src is involved in FGFRs signalling and its activation upon FGF stimulation was previously described (Sandilands, Akbarzadeh et al. 2007). The interaction between FGFR and Src is more complex and often controversial. A direct association between Src/Yes and FGFR1 was described to be essential for cortactin activation (Zhan, Plourde et al. 1994; Zhang, Greendorfer et al. 2006). An indirect interaction between Src and FGFR mediated via FRS2 was also demonstrated (Li, Brunton et al. 2004). The formation of Src/FRS2/FGFR complex triggers inhibition of MAPK pathway and attenuates signal transduction by Sprouty activation

(Li, Brunton et al. 2004). Additionally, FGFR and Src activation is inter-dependent; FGFR activation induces Src phosphorylation whereas, Src controls FGFR activity and regulates its trafficking (Sandilands, Akbarzadeh et al. 2007).

2.2. Endocytic proteins

AP-2 complex, clathrin and epsin-4 were identified as binding partners for Ty677 of FGFR1, whereas Rab5 was found to be specific for Tyr730, Rab1B and Rab10 for Tyr701 and Tyr776, respectively (Table 4). They form a cluster of proteins involved in endocytosis (Figure 11). The fact that several subunits of AP-2 complex were identified with high scores for many peptides (Appendix 1), demonstrated that it was specific hit. However, interaction was not validated by immunoprecipitation and the motif on FGFR1 does not match the sorting signal that promotes endocytosis by the AP2/clathrin complex. AP-2 takes part in the process in which clathrin-coated vesicles bud from the plasma membrane. While clathrin itself is considered to be the driving force for invagination of vesicles during the first steps of endocytosis, the AP-2 adaptor complex attaches clathrin to the membrane (Hirst and Robinson 1998). Adaptor proteins interact with membrane proteins, like GFRs (Hirst and Robinson 1998) and recently, it has been proved that activated EGFR is internalized via clathrin/AP-2 complex (Rappoport and Simon 2009). Further analysis of the interaction between FGFR1 and AP2/clathrin is necessary to establish if FGFR is internalised via clathrin-dependent endocytosis.

Data obtained by PPD revealed Rab5-FGFR1 and Rab1-FGFR1 interactions which were previously described (Vecchione, Cooper et al. 2007). This proteomic study identified Rab5 and Rab1 as novel effectors of FGFR1 signalling. Validation by immunoprecipitation and co-localization between Rab1, Rab5 and activated FGFR1 was shown (Vecchione,

Cooper et al. 2007). Rab1 controls vesicular transport between Golgi and ER, whereas Rab5 is involved in FGFR endocytosis and signal transduction (Vecchione, Cooper et al. 2007).

2.3. Vav-2

Vav-2 was detected as an interacting protein for phospho-Tyr730 of FGFR1 (Table 7) and the association between Vav2 and kinase active FGFR was confirmed by immunoprecipitation (Figure 12). The motif present in the Tyr730 peptide of FGFR1 resembles the binding motif for Vav-2 SH2 domain (Huang, Li et al. 2008).

Table 7. FGFR1 binding motif for SH2 domain of Vav-2.

| Sequence of FGFR1 (pTyr730) | | | | | |
|---------------------------------|----|---------|---------|---------|-------|
| FGFR1 | pY | M | M | M | R |
| Consensus motifs for SH2 domain | | | | | |
| Vav-2 | pY | M/L/V/E | T/M/E/N | P/E/A/D | E/D/M |

Vav-2 is the GTP hydrolase of the Rho/Rac family that takes part in many cellular processes, like formation of cytoskeletal structure, regulation of motility, cell shape, activation of lipids and protein kinase cascades (Bustelo 2000). In the absence of stimulation Vav2 remains in an inactive state in cells, but upon stimulation it gets highly phosphorylated on tyrosine residues by kinases. Activated Vav2 forms complexes with various upstream elements, like kinases and adaptor proteins, for example the association between EGFR and Vav2 via their SH2 domains was reported (Pandey, Podtelejnikov et al. 2000). Also phosphorylation of Vav2 by several kinases including membrane and non-membrane receptors, like EGFR and PDGFR, Jak and Src kinases, has been described (Bustelo 2000). Phosphorylation of Vav2 plays an important role in Vav2 function. Tyrosine phosphorylation

induces final activation of Vav2 protein and triggers activity of its downstream effectors. Biological responses of Vav2 are: GDP-GTP exchange activity towards Rho/Rac proteins, activation of transcription factors, and induction of cytoskeleton responses (Bustelo 2000). Since Vav2 plays a functional role in EGFR and PDGFR signalling it is possible that a FGFR-Vav2 interaction has a potential role in converting extracellular signals into intracellular responses. Recently, Rap1 was identified as a novel binding partner for FGFR1 (Vecchione, Cooper et al. 2007). Since Vav2 is one of Rap1 GEFs (Raaijmakers and Bos 2009), the Vav2-Rap1 interaction might be another way of FGFR-induced MAPK pathway activation.

2.4. IQGAP

IQGAP3 was identified as binding partner for Tyr583/585 of FGFR1 in PPD (Table 4), however further work was continued with IQGAP1 that was identified as a protein interacting with FGFR1 kinase active and kinase dead by previous research (unpublished data A. Vecchione). IQGAP1 was identified as an IQ domain-containing protein with sequence similarity to the Ras GTPase-activating proteins (Briggs and Sacks 2003). There are 3 isoforms of IQGAP1, with IQGAP1 being the best characterised, whereas relatively little is known about IQGAP2 and IQGAP3 (Briggs and Sacks 2003). IQGAP1 is built of a calponin homology domain (CHD), four IQ motifs, a Ras-GAP-related domain and a domain with two conserved tryptophan residues (WW). Due to its multiple protein-binding domains it can interact with numerous proteins simultaneously. IQGAP1 interacting proteins can be divided into several groups: cytoskeleton-associated proteins, small GTPases, receptors, adhesion-associated proteins, phosphatases and kinases (Briggs and Sacks 2003; Brown and Sacks 2006).

IQGAP1 is a scaffolding protein involved in multiple fundamental cellular activities, such as cell migration, adhesion, regulation of the actin cytoskeleton and cell signalling. Recent publications showed that IQGAP1 was involved in signal transduction downstream of several receptors, EGFR, VEGFR and FGFR (Brown and Sacks 2006; Yamaoka-Tojo, Tojo et al. 2006; Bensenor, Kan et al. 2007) where it provides a link between extracellular signals and cytoskeleton responses. It also facilitates the efficiency of Ras/MAPK pathway signalling by coupling its components together (Sacks 2006). It was identified by mass spectroscopy-based screen as a component of EGF-mediated signalling pathway (Brown and Sacks 2006), however, the exact role of IQGAP1 in this pathway is unknown. Results demonstrated by Yamaoka-Tojo *et al.* suggest that IQGAP1 directly binds VEGFR2 in endothelial cells. Stimulation of VEGF promotes association between VEGFR2, Rac1 and IQGAP1 which results in tyrosine phosphorylation of IQGAP1. Increased levels of tyrosine phosphorylated IQGAP1 is important for activation of downstream VEGF signalling in endothelial cells. This mechanism is important for migration and proliferation, as well as maintaining the functional integrity of blood vessels (Yamaoka-Tojo, Tojo et al. 2006).

Furthermore, an interaction between IQGAP1 and FGFR1 has been recently shown (Bensenor, Kan et al. 2007). The results imply that IQGAP1 stimulates branched actin filament assembly and lamellipodial protrusion after FGF2 stimulation. Stimulation of FGF receptor recruits IQGAP1 to the cortically localized cytoplasmic domain of FGFR1. Then, actin filament nucleation is promoted by IQGAP1, WASP and Arp2/3 complex which results in lamellipodia protrusion and cell migration. Additionally, FGFR1, WASP and Arp2/3 fail to be recruited to the cell cortex after FGF2 stimulation in IQGAP1 deficient cells, which suppresses cell motility. The interaction between IQGAP1 and FGFR1 might serve as a

bridge between extracellular signals and cellular responses, however, the mechanism responsible for this is not entirely understood (Bensenor, Kan et al. 2007).

Here, the interaction between IQGAP1 and FGFR1 was investigated using immunoprecipitation. Figure 12 shows the interaction between IQGAP1 and kinase active, kinase dead and truncated version of FGFR1. The presence of IQGAP1 in complex with kinase dead indicated that IQGAP1-FGFR1 interaction was not phospho-dependent. The truncated version of FcFGFR1 has no cytoplasmic region so it should act as a negative control. Binding to truncated receptor might be an artefact caused by much higher expression of truncated FcFGFR1 construct (Figure 12). To further confirm the association of these two proteins, FGFR1 and myc-tagged IQGAP1 were transiently transfected into HEK293T cells and immunoprecipitation of myc-IQGAP1 was performed. Results presented in Figure 13 confirmed an interaction between myc-IQGAP1 and FGFR1. The association is ligand-independent, however, over-expression of FGFR1 WT induces constant phosphorylation of the receptor without FGF stimulation. Thus, the experiment should be repeated with SU5402, FGFR inhibitor, to fully exclude phospho-dependent interaction.

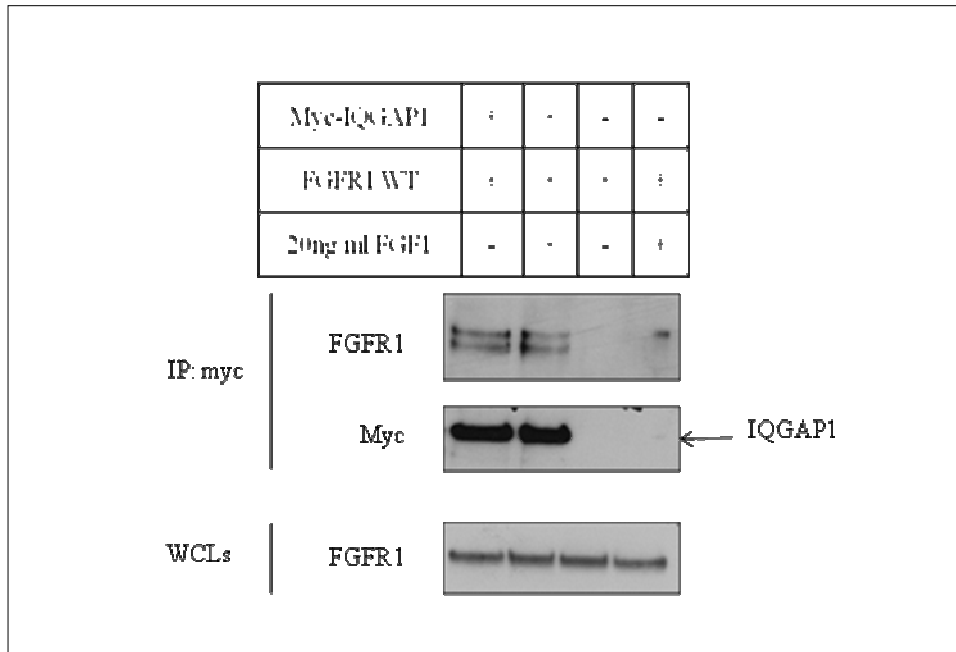


Figure 13. IQGAP1 interacts with FGFR1.

Myc-IQGAP1 and FGFR1 WT were transiently transfected into HEK293T cells. After 48 hours cells were serum-starved and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Equal amounts of proteins were immunoprecipitated using anti-myc antibody and Protein G Sepharose. IP samples and WCLs were resolved by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

Due to the fact that IQGAP1 binds MEK, ERK and B-raf, it is considered as a scaffold protein for mitogen-activated protein kinase signalling (Roy, Li et al. 2004; Roy, Li et al. 2005; Ren, Li et al. 2007). Interestingly, over-expression as well as knockdown of IQGAP1 impaired the EGF-induced activation of MAPK pathway (Roy, Li et al. 2005). These findings suggest that an optimal level of IQGAP1 is required for the activation of B-raf, MEK and ERK. IQGAP1 is considered as a scaffold that links B-raf, MEK and ERK when all components are present in an appropriate stoichiometric ratio. When the concentration of IQGAP1 is too low, functional complexes of IQGAP1 and members of MAPK pathway are unable to form and transmit the signal. However, high overexpression of the scaffold will lead to a separation of the individual components, thus preventing their interaction and signal transmission. When all components of the signalling complex are expressed at equivalent levels, a scaffold will enhance the efficiency and specificity of signalling (Roy, Li et al. 2005; Sacks 2006). IQGAP1 assembles signalling molecules together, Ras, MEK and ERK, and localises these complexes to spatially defined cellular domains. When the ratio between IQGAP1 and the members of Ras/MAPK pathway is disturbed, the signal transduction has been shown to be considerably impaired (Sacks 2006).

The effect of high level of IQGAP1 on ERK activation was examined by transient transfection of myc-IQGAP1 into HEK293T cells (Figure 14). Samples with cells expressing endogenous IQGAP1 and over-expressed myc-IQGAP1 were compared in order to check if altering the level of IQGAP1 modulated the activity of ERK after FGF1 stimulation. Probing with myc antibody showed that over-expression of IQGAP1 was successful. Activation of the MAPK pathway was verified by probing with phospho-ERK antibody, and equal loading was tested by probing with ERK antibody.

Samples where myc-IQGAP1 was over-expressed showed reduced ERK phosphorylation compared to samples with endogenous IQGAP1 (Figure 14) and this is in agreement with previous data (Sacks 2006). Densitometry analysis of ERK and pERK blots was performed from three separate experiments and the level of phospho-ERK was normalized to ERK (Figure 14). Even though the phospho-ERK signal was decreased when myc-IQGAP1 was over-expressed, the difference was not statistically significant ($p=0.09$) (Figure 14). Perhaps, higher expression of myc-IQGAP1 could induce more significant effect. Also, IQGAP1 knock-down could affect ERK activation. Further studies are necessary to examine the exact role of IQGAP1 in FGF-mediated signalling and to verify the details of interaction with FGFR1.

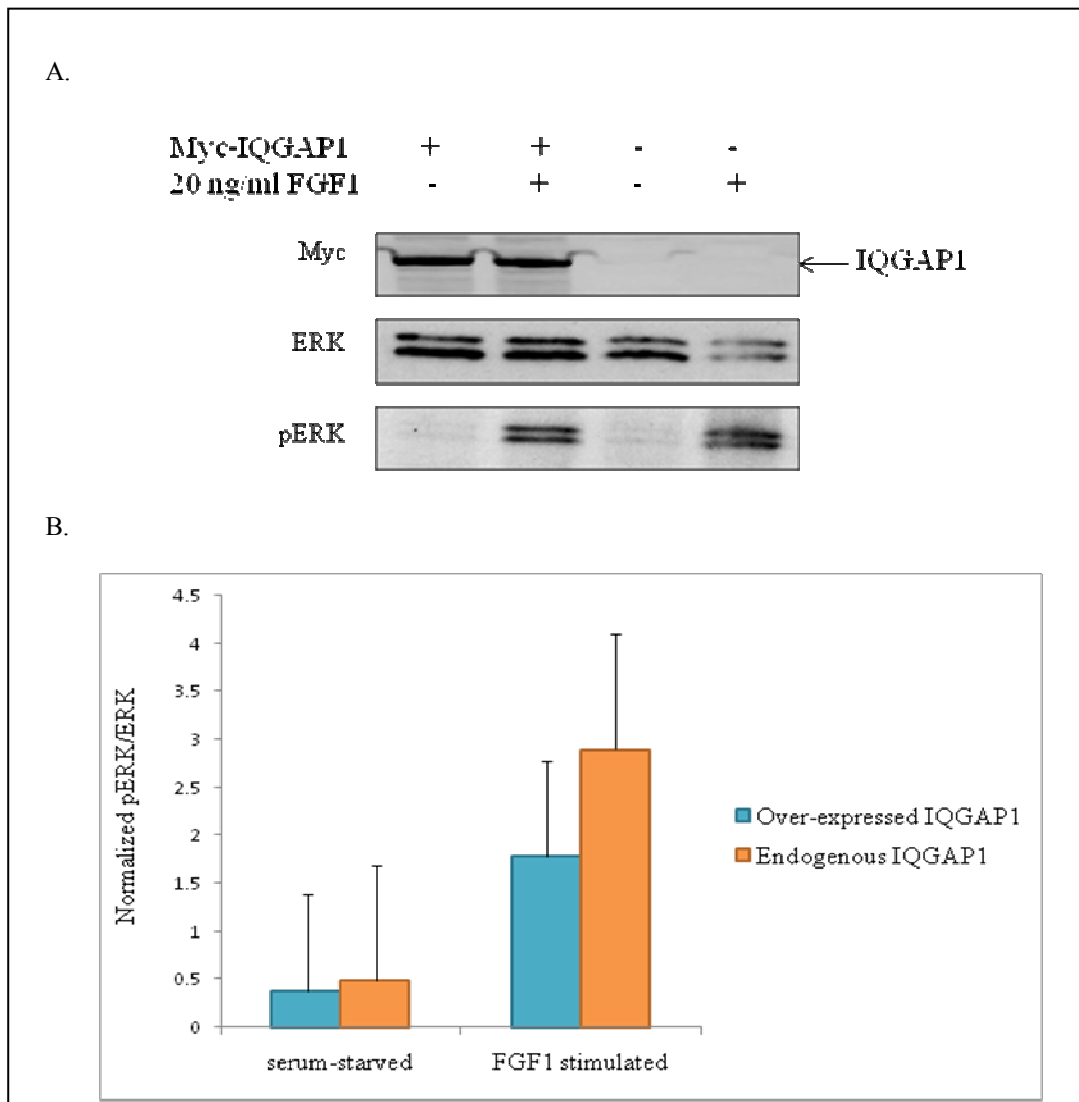


Figure 14. Effect of IQGAP1 over-expression on ERK activation.

- A. Myc-IQGAP1 was transiently transfected into HEK293T cells. After 48 hours cells were serum-starved, stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes and lysed. WCLs were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.
- B. pERK and ERK blots from three separate experiments were analysed by densitometry. T-test was performed ($p=0.09$) for samples over-expressing myc-IQGAP1 versus samples expressing endogenous IQGAP1 (FGF1 stimulated).

Discussion

The significance of protein-protein interactions is highly evaluated and appreciated. Many cellular events are possible thanks to interactions between proteins. Signal transduction is one of the processes that could not be achieved without association of signalling molecules and their co-operation. Signalling is activated by formation of multiprotein complexes to tyrosine-phosphorylated motifs. These motifs, present in cytoplasmic regions of RTKs, mediate interactions with adaptor proteins by binding their SH2 or PTB domains (Pawson 2004).

FGFR signalling, even though it has been studied for years, is not fully understood. For a long time PLC γ 1 and FRS2 were the only known binding partners described for FGFR. Later Shc, PI3K and Src were suggested to interact with FGFRs but the details of these interactions remained elusive. Identification of FGFR binding partners is important for understanding the mechanism of FGF-mediated signal transduction and its disturbances in FGFR-related diseases (Beenken and Mohammadi 2009). An emerging method for such research is proteomics that enables identification of components of signalling pathways. Phospho-proteomics can provide further information regarding regulation of these interactions by specific phosphorylation events. There has been a number of publications describing signalling events accomplished by proteomics (Schulze and Mann 2004; Schulze, Deng et al. 2005; Blagoev and Mann 2006; Zhou, Galan et al. 2007). Furthermore, attempts towards identification of molecules involved in FGFR signalling have also been undertaken (Hinsby, Olsen et al. 2003; Hinsby, Olsen et al. 2004; Vecchione, Cooper et al. 2007).

Peptide pull-down followed by MS/MS has been proven to be an effective screening technique that successfully identified FGFR1 binding partners. PPD is directed towards

finding interactions between motifs present in cytoplasmic regions of FGFR and adaptor proteins. In this study 36 proteins have been shown to bind FGFR1 and many of them are potentially novel, phospho-specific binding partners. Most of identified proteins are composed of SH2 domains which could interact with SH2 consensus binding motifs present on FGFR1. The detection of known binding partners as well as proteins recognizing SH2 binding motifs on FGFR1 proved the specificity of the screen.

The list of identified FGFR binding partners suggests that FGFR signalling is more complex than previously thought. It showed that not only PLC γ 1 interacts with FGFR, but also other signalling proteins, as well as proteins involved in endocytosis of RTKs. This implies a broad role of FGFR signalling that is dynamic and sophisticated process involving many molecules. The choice of binding partner might be cell- and ligand-dependent and the final cellular response is a consequence of combination of up-stream and down-stream effectors.

A significant advantage of PPD is that it detects binding proteins for specific tyrosine residues motifs. Immunoprecipitation with full length FGFR1 isolates all proteins bound to the receptor and it is not suitable to establish the interaction between binding partner and a particular motif present on FGFR1. It also requires the additional step of mutagenesis in order to map the tyrosines responsible for binding particular proteins. PPD allows straightforward identification of binding partners for specific tyrosine-based motifs present on FGFR peptides. Moreover, proteins pulled-down using peptides are likely to be direct binding partners rather than protein complexes, which was previously suggested by other group (Schulze and Mann 2004; Schulze, Deng et al. 2005). However, the possibility that PPD identified indirect binding partners cannot be excluded.

Despite its' benefits, the PPD has some limitations. The obvious weakness of this method is the potential low binding affinity of proteins to the peptides. Because synthetic FGFR1 peptides are outside of the context of the whole protein, the affinity between protein target and peptide might be reduced and therefore, not identified by MS/MS. This might be especially true for low abundance signalling molecules. If the binding between FGFR1 peptide and signalling protein is not strong enough, such an interaction might be too transient to allow detection by MS/MS. This could be the reason why Shb was not detected by mass spectrometry analysis of PPD experiment. Even though the binding between Tyr766 of FGFR1 and Shb was detected by Western blotting during optimisation step, MS/MS failed to identify it. Moreover, some proteins might need two or even three motifs to fully interact with receptor so they fail to bind short fragments of FGFR1.

Additionally, false positives hits might cause a problem during data analysis because, as more abundant proteins, they can mask specific hits. The problem of contaminants binding to affinity Sepharose beads has been addressed before (Trinkle-Mulcahy, Boulon et al. 2008). The detection of false positives was accomplished by comparison between control sample and sample containing FGFR1 peptides. It was assumed that protein hits present in control sample are bound to Dynabeads and subsequently they were deleted as unspecific. However, the number of proteins bound to beads-only was higher than expected which could have masked the detection of specific signalling proteins. Proteins most frequently present in every sample can be divided into two groups – high abundant proteins and proteins recognizing FGFR1 peptides as substrates. The first group are cytoskeletal proteins (actin, tubulin, vimentin, filamin), heat shock proteins, translation factors, and ribosomal proteins. The second group are proteins binding FGFR1 peptides in order to phosphorylate/dephosphorylate them

(kinases, phosphatases), proteins binding basic amino acids (importins, exportins), proteins recognising leucine residues on peptides (ligases, cullins).

Lastly, the PPD experiment is not a quantitative method. PPD leads to identification of binding partners but it does not give the amount of protein bound to sample containing FGFR1 peptide and control sample. In this way the protein hit present in both samples is considered as unspecific. However, the amount of the protein might be significantly different which could suggest specific interaction between FGFR1 peptide and protein. Quantitative proteomics has been successfully used for identification of proteins involved in phospho-events (Hinsby, Olsen et al. 2004; Schulze, Deng et al. 2005; Blagoev and Mann 2006). SILAC eliminates this problem by calculating the differences on protein levels at MS/MS stage. Combining PPD with SILAC would be more efficient method of detection novel binding partners. This was done by Schulze *et al.* where SILAC was used in pull-down assay of EGFR peptides (Schulze and Mann 2004; Schulze, Deng et al. 2005).

All in all, peptide pull-down followed by MS/MS is an effective screening strategy that was employed to obtain qualitative information about FGFR1 binding partners. It can be used as a starting point for detection of novel interactions that later would be confirmed by variety of biochemical experiments.

CHAPTER 4

STAT3 and FGFR1 interaction

Introduction

STAT3 was described in early 90s as a DNA-binding factor which is phosphorylated by IL-6 in the acute-phase inflammatory response (Wegenka, Buschmann et al. 1993; Akira, Nishio et al. 1994; Lutticken, Wegenka et al. 1994). It takes part in migration, differentiation, proliferation, apoptosis and wound healing (Levy and Lee 2002). Just like other members of STAT family, STAT3 is activated by various ligands: cytokines, including interferons and interleukins, growth factors and hormones. Upon ligand binding gp130 receptors dimerize which activates Janus kinases and induces STAT3 recruitment to the receptors. Phosphorylated STAT3 dimerizes through the SH2 domain of one monomer and the phosphotyrosine of the partner. Dimers of STAT3 are translocated to the nucleus where they regulate gene expression (Lim and Cao 2006; Schindler, Levy et al. 2007). STAT3 is also phosphorylated by growth factor receptors, like EGFR and PDGFR, leading to STAT3 activation and DNA binding (David, Wong et al. 1996; Park, Schaefer et al. 1996; Vignais, Sadowski et al. 1996).

1. Identification of STAT3 as a novel binding partner for Tyr677 of FGFR1

Initial peptide pull-down screen identified STAT3 as a novel binding partner for the FGFR1 peptide containing phospho-Tyr677 (Chapter 3). The probability of random match ($p=3.1E-13$) and x-score ($x=188.2$), calculated by Bioworks 3.3.1., indicated high specificity (Table 4). Sixteen STAT3 peptides were identified by MS/MS and overall sequence coverage of STAT3 was 32%. Individual scores for STAT3 peptides are presented in Table 8. The example of the spectrum for one of the STAT3 peptide is presented in Figure 15.

Table 8. Identified STAT3 peptides sequences and their individual scores.

| Peptide | MH+ | DeltaM ppm (parts/million) | z | P (pep) |
|--------------------------------|------------|-------------------------------|---|----------|
| R.LLQTAATAAQGGQANHPTAAVVTEK.Q | 2576.34277 | 0.01325 | 3 | 3.19E-13 |
| R.QFLAPWIESQDWAYAASK.E | 2111.02319 | -0.32441 | 2 | 7.19E-13 |
| K.LLGPVNYSGCQITWAK.F | 1863.94226 | 5.13378 | 2 | 1.03E-12 |
| R.LENWITSLAESQLQTR.Q | 1888.97632 | 4.54877 | 2 | 1.19E-09 |
| K.MQQLEQMLTALDQMR.R | 1835.88123 | 5.81067 | 2 | 1.49E-09 |
| K.VVENLQDDDFNYK.T | 1745.80176 | 1.98508 | 2 | 8.33E-09 |
| K.IMDATNILVSPVLYLPDIPKEEAFGK.Y | 3036.60034 | 14.54342 | 3 | 2.33E-08 |
| K.ESHATLVFHNLLGEIDQQYSR.F | 2457.21582 | 8.43437 | 3 | 2.78E-08 |
| R.SIVSELAGLLSAMEYVQK.T | 1954.02016 | 4.44555 | 3 | 5.00E-08 |
| K.IMDATNILVSPVLYLPDIPKEEAFGK.Y | 3052.59514 | 13.75242 | 3 | 8.63E-08 |
| .SQGDMQDLNGNNQSVTR. | 1880.80391 | -1.96229 | 2 | 6.93E-06 |
| R.SIVSELAGLLSAMEYVQK.T | 1938.02527 | 5.27703 | 3 | 3.83E-05 |
| R.YLEQLHQLYSDSFP MELR.Q | 2269.09570 | 10.96272 | 3 | 7.34E-05 |
| K.TQIQSVEPYTK.Q | 1293.66846 | 1.45218 | 2 | 8.89E-05 |
| K.TLTDEELADWK.R | 1320.63184 | 2.53173 | 2 | 1.47E-04 |
| K.QQLNNMSFAEIMGYK.I | 1886.91394 | 4.29497 | 2 | 2.06E-04 |

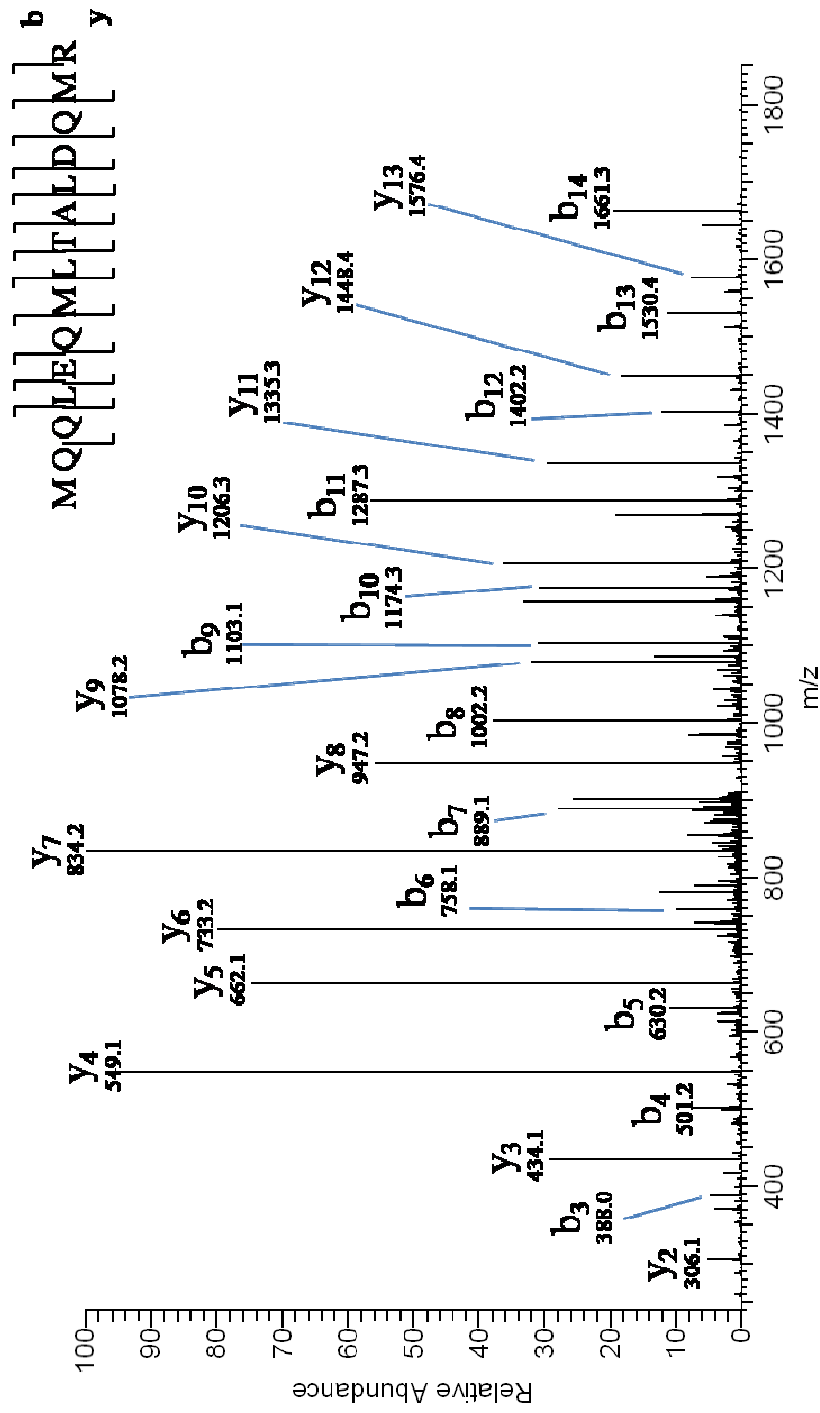


Figure 15. Annotated mass spectrum showing CID fragmentation of STAT3 peptide.

MQQLEQMLTALDQMR, $[M+2H]^{2+}$. One of sixteen STAT3 peptides identified by MS/MS (all with Bioworks probability $<1e-3$).

2. STAT3 SH2 domain is crucial for the interaction with Tyr677 peptide

Tyrosine 677 of FGFR1 is part of a YxxQ motif on FGFR1, which is a consensus binding motif for the STAT3 SH2 domain (Table 9).

Table 9. FGFR1 binding motif for SH2 domain of STAT3

| FGFR1 sequence (Tyr677) | | | | |
|---|----|---|---|---|
| FGFR1 | pY | T | H | Q |
| Consensus binding motif for SH2 domain of STAT3 | | | | |
| STAT3 | pY | X | X | Q |

To confirm the interaction, whole cell lysates from HEK293T cells expressing STAT3 constructs, WT and R609L, were incubated with Tyr677 FGFR1 peptides (Figure 16). The STAT3 R609L, which specifically disrupts the SH2 binding pocket (Zhang, Kee et al. 2000), was generated to test if the SH2 domain of STAT3 was required for association with FGFR1. Figure 16 showed an increased association of STAT3 WT with phospho-Tyr677 FGFR1 peptide, compared to the background levels with non-phosphorylated peptide. R609L mutation in STAT3 abolished peptide-binding ability (Figure 16). Moreover, non-relevant peptide was used to make sure that STAT3 was not bound to FGFR1 peptides in unspecific manner; no association between non-relevant peptides and STAT3 proteins was detected (Figure 16). Western blotting analysis of whole cell lysates showed equal expression of both STAT3 constructs used in this experiment. Altogether, the association between STAT3 WT and FGFR1 peptide was specific and phospho-dependent and the SH2 domain of STAT3 was critical for FGFR1 binding.

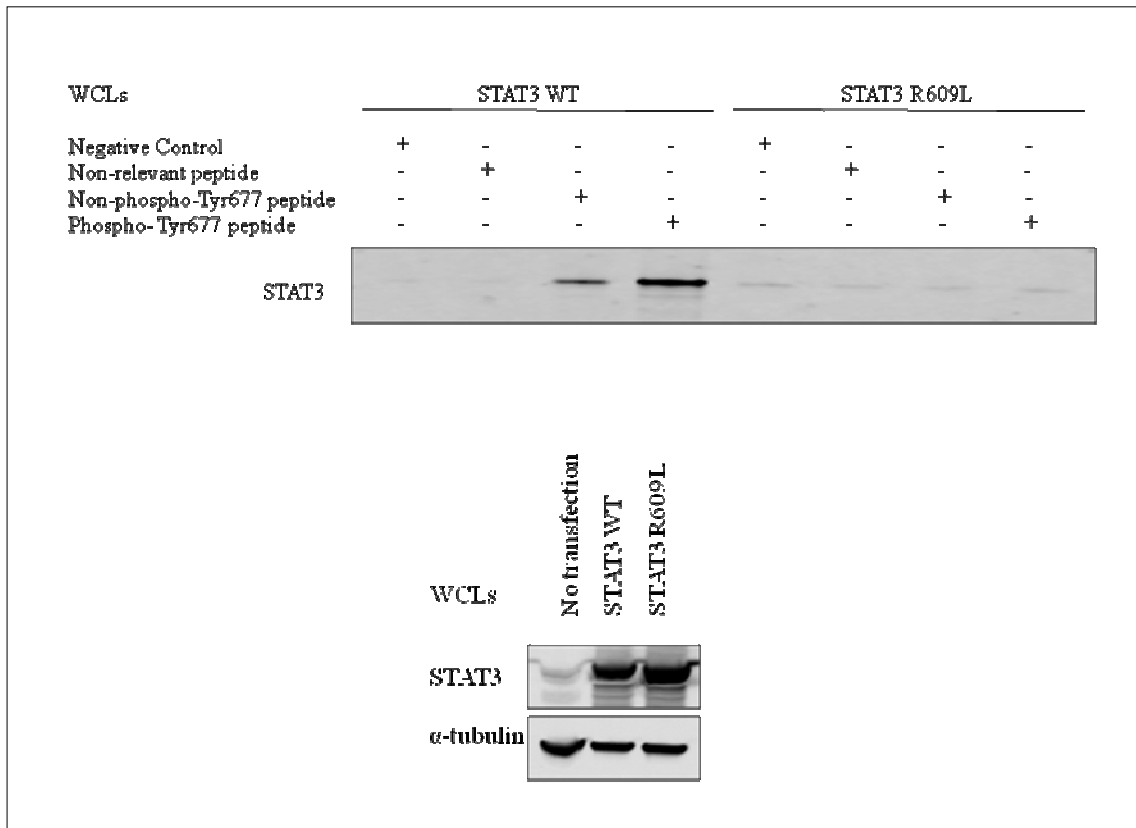


Figure 16. STAT3 SH2 domain is crucial for interaction with FGFR1.

Peptide pull-down was performed using Tyr677 and pTyr677 FGFR1 peptides and non-relevant peptides as control. Peptides were incubated with Dynabeads followed by incubation with whole cell lysates from HEK293T cells over-expressing STAT3 WT or STAT3 R609L. Samples were resolved by SDS-PAGE, transferred to PVDF and probed with STAT3 antibody. Negative control sample did not contain any peptide. Bottom figure presents Western blotting analysis of whole cell lysates used for peptide-pull down experiment.

3. STAT3-FGFR1 interaction depends on Tyr677 of FGFR1

The STAT3-FGFR1 interaction was further investigated by co-immunoprecipitation. FGFR1 construct where tyrosine 677 was substituted with phenylalanine was created. FGFR1 WT and FGFR1 Y677F were expressed in HEK293T cells. Over-expression of FGFR1 in cells is known to induce ligand-independent phosphorylation of the receptor (Hinsby, Olsen et al. 2003). To further clarify phospho-dependent STAT3 binding to FGFR1, an FGFR inhibitor, SU5402, that prevents phosphorylation of the receptor was used. Blots were probed with FGFR1 antibody to check if FGFR1 constructs were equally immunoprecipitated. Activation of FGFRs was checked by probing with pY653/pY654 FGFR antibody. Equal loading and equal expression of STAT3 in WCLs was verified by probing with α -tubulin and STAT3 antibodies, respectively.

The phospho-dependent interaction between FGFR1 WT and STAT3 was confirmed by immunoprecipitation (Figure 17). Decreased association between FGFR1 Y677F and STAT3 proved the importance of Tyr677 of FGFR1 (Figure 17). Taken together, these results indicate that STAT3 and FGFR1 specifically interacted with each other in a phospho-dependent-manner and Tyr677 of FGFR1 played a critical role in STAT3 recruitment.

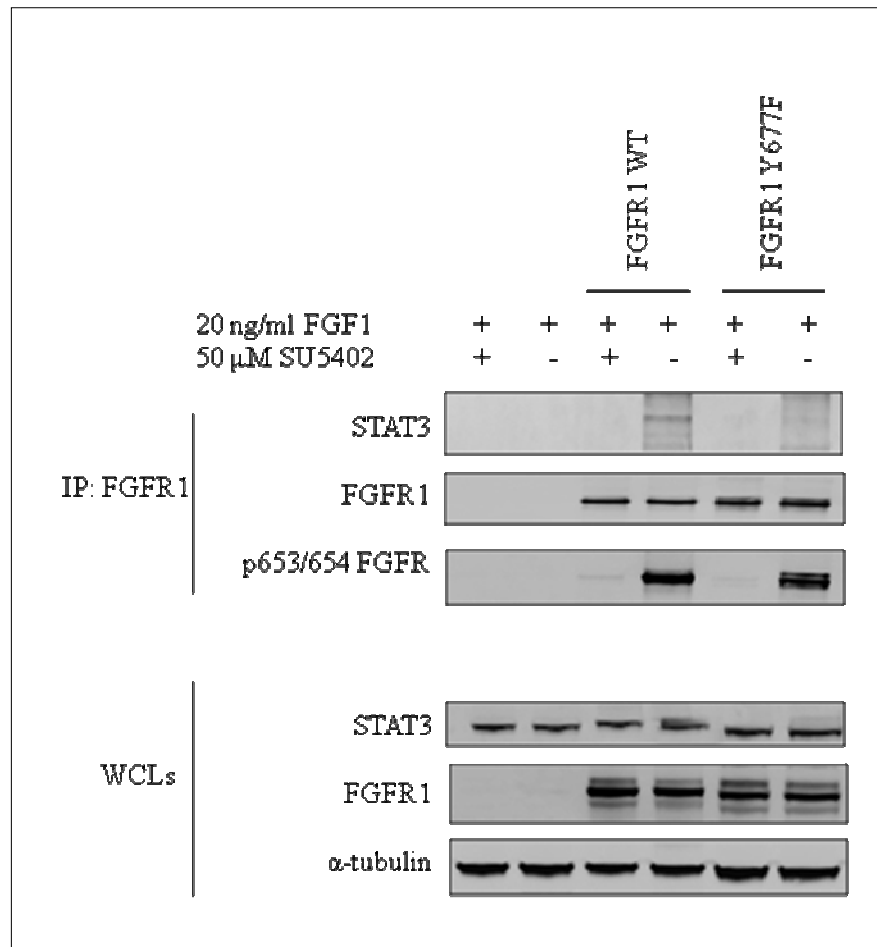


Figure 17. STAT3-FGFR1 interaction depends on Tyr677 of FGFR1.

HEK293T cells were transiently transfected with FGFR1 WT or FGFR1 Y677F, after 48 hours cells were serum-starved, treated with 50 μ M SU5402 or DMSO for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Equal amounts of proteins were immunoprecipitated using FGFR1 antibody conjugated with agarose beads. Immunoprecipitated samples and WCLs were analyzed by Western blotting.

The reverse immunoprecipitation was performed in order to verify the interaction between proteins. The FGFR1 WT and FGFR1 Y677F constructs were transfected into HEK293T cells. One sample was treated with SU5402 in order to check conditions where FGFR1 was not phosphorylated. Endogenous STAT3 was immunoprecipitated using STAT3 antibody and probed with FGFR1 antibody to investigate complex formation between STAT3 and FGFR1. To check equal immunoprecipitation, the blots were re-probed with an STAT3 antibody. The expression level of FGFR1 was verified by probing with FGFR1.

Reverse immunoprecipitation confirmed a phospho-dependent interaction between FGFR1 and STAT3 (Figure 18). STAT3 associated with FGFR1 WT, but not with FGFR1 Y677F, which verified that Tyr677 of FGFR1 was essential to maintain STAT3-FGFR1 association (Figure 18).

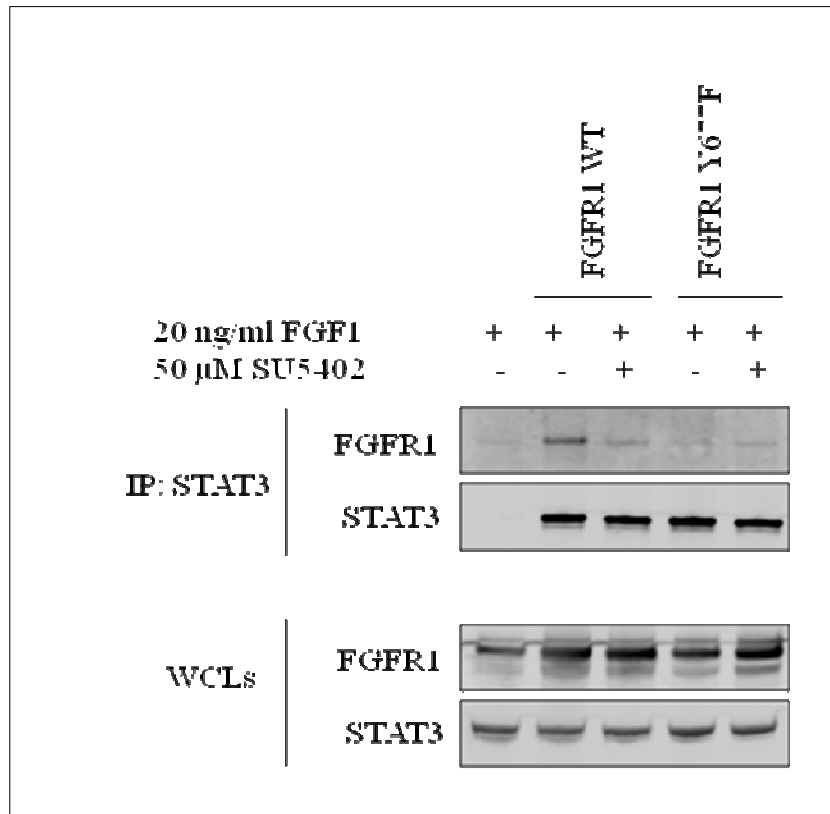


Figure 18. STAT3 associates with FGFR1 WT.

FGFR1 WT and FGFR1 Y677F were transfected into HEK293T cells. After 48 hours cells were serum-starved and stimulated with 20 ng/ml FGF1 and 10 μg/ml heparin for 20 minutes in the absence or presence of 50 μM SU5402. Equal amounts of protein were immunoprecipitated with STAT3 antibody and protein G Sepharose. Samples were resolved by SDS-PAGE, transferred to PVDF membrane and probed for FGFR1 and STAT3.

4. STAT3 binds FGFR1 in an FRS2-independent manner

FRS2 is an adaptor protein which is associated with FGF receptors (Ong, Guy et al. 2000). FRS2 recruits regulators of signalling and plays an important role in Ras/MAPK pathway signal transduction. In order to confirm whether the interaction between FGFR1 and STAT3 is mediated by FRS2, an immunoprecipitation analysis was performed. Four FcFGFR1 constructs were transiently transfected into HEK293T cells. The FcFGFR1 kinase active construct exhibits tyrosine kinase activity due to constitutive dimerization, the kinase dead construct has a mutation (D623A) that results in loss of catalytic activity, the truncated version lacks the C-terminal intracellular domain and the FcFGFR1 VT- construct is kinase active but unable to bind FRS2 (Burgar, Burns et al. 2002). Receptors were immunoprecipitated and probed with STAT3 antibody to investigate the formation of FcFGFR1-STAT3 complex. To check that FGFR1 constructs were equally immunoprecipitated, blots were re-probed with an Fc antibody. The level of STAT3 in all samples was checked by probing with a STAT3 antibody. Phosphorylation of FcFGFR1 constructs was verified by probing the membrane with total phospho-tyrosine antibodies.

A strong association was seen between FcFGFR1 kinase active and STAT3 (Figure 19). The same association existed between FcFGFR1 kinase active VT- and STAT3, demonstrating that formation of the FGFR1-STAT3 complex is FRS2-independent (Figure 19). No interaction was detected between the kinase dead or the truncated version of FcFGFR1 and STAT3 (Figure 19). Densitometric analysis of the STAT3 blot showed an increase in STAT3 binding to kinase active and VT- constructs (Figure 19). Collectively, these data suggest that STAT3 was recruited to phosphorylated FGFR1, independently of FRS2.

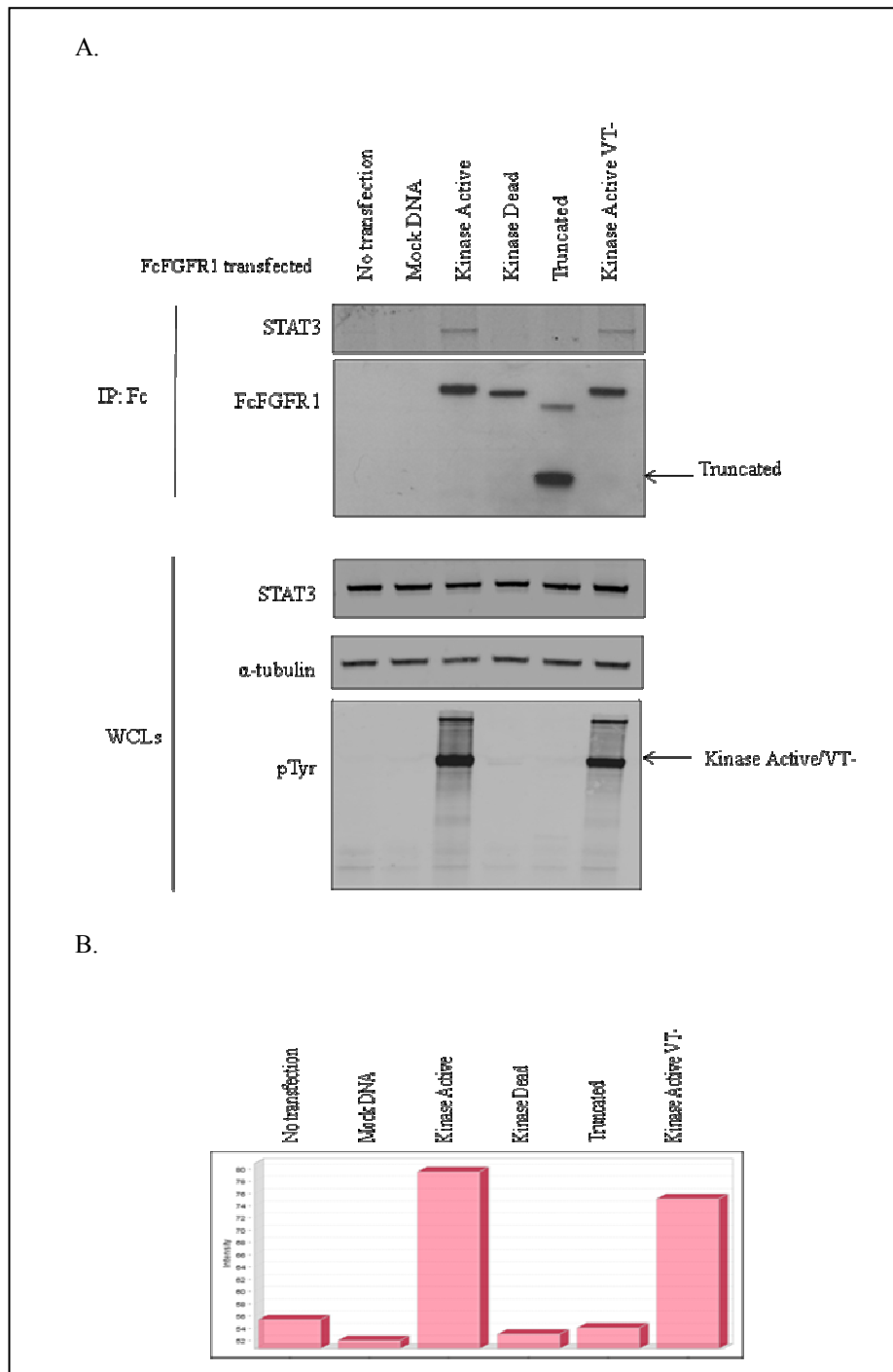


Figure 19. STAT3 binds FGFR1 in an FRS2-independent manner.

A. FcFGFR1 constructs were transfected into HEK293T cells. After 48 hours equal amounts of protein were immunoprecipitated using Sepharose protein-G. Samples were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

B. Intensity analysis of STAT3 blot obtained by Odyssey program.

Discussion

Proteomic analysis identified STAT3 as a FGFR1-associating protein and that interaction was confirmed by a series of biochemical experiments. FGF-mediated activation of STATs was previously described in cells harbouring FGFR mutations (Su, Kitagawa et al. 1997; Hart, Robertson et al. 2000), however the mechanism of the interaction between both proteins remained elusive.

Herein, it was demonstrated for the first time that STAT3-FGFR1 interaction is phospho-dependent and FRS2-independent. Several lines of evidence confirmed this interaction. First, STAT3 was identified as a novel binding partner for Tyr677 of FGFR1 using peptide pull-down followed by mass spectrometry (Chapter 3, Table 4). Second, the phospho-dependent interaction between FGFR1 WT and STAT3 was verified by immunoprecipitation. Reduced association of STAT3 with FGFR1 Y677F mutant indicated the importance of phosphotyrosine 677, in agreement with the proteomics results. Moreover, the interaction was not FRS2-dependent. Lastly, Tyr677 is a part of highly conserved YxxQ SH2-domain binding motif present in the cytoplasmic region of all four FGFRs, supporting the result that FGFR-STAT3 association depends on the STAT3 SH2 domain and FGFR Tyr677.

The YxxQ motif is present in a variety of receptors which recruit STAT3 through its SH2 domain (Stahl, Farruggella et al. 1995; Shao, Xu et al. 2004). The classic interaction between STAT3 and gp130 is maintained by SH2 domain binding to pYxxQ motifs on gp130 receptor (Stahl, Farruggella et al. 1995). Phosphorylated gp130 recruits STAT3 to several pYxxQ motifs where STAT3 is subsequently phosphorylated by Janus family kinases.

Phospho-dependent interactions between growth factor receptors and STAT3 have also been reported previously. EGFR contains two STAT3 SH2-binding motifs at Tyr1068 and Tyr1086 that take part in STAT3 recruitment (Shao, Cheng et al. 2003). Paukku *et al.* demonstrated SH2 domain-dependent recruitment of STAT5 to tyrosine motifs on PDGFR (Paukku, Valgeirsdottir et al. 2000). Similarly, a phospho-dependent association between STAT3 and EphrinB receptor was described (Bong, Lee et al. 2007). On the other hand, phospho-independent association of STAT3 with other RTKs prior to stimulation was also observed. It was found that STAT1, 3, 5a/b and Jak bound ErbB1 even in the absence of EGF, whereas, upon ligand binding Src was recruited to the complex and induced STAT3 activation (Olayioye, Beuvink et al. 1999). Comparable results, phospho-independent STAT3 and Jak association with ErbB-2, were presented by other group (Ren and Schaefer 2002). Thus, association between STATs and GFRs can be receptor type-dependent and cell type-dependent.

CHAPTER 5

FGF-induced STAT3 activation

Introduction

The goal of this chapter was to analyse further FGF-induced mechanism of STAT3 activation. STAT3 activation strictly depends on phosphorylation of tyrosine residue at position 705 (Lim and Cao 2006). Activation of STAT3 mediates differentiation, proliferation, apoptosis and wound healing and is classically induced via the cytokine receptor-associated JAK family of non-receptor tyrosine kinases. JAKs phosphorylate tyrosine residues within the intracellular domain of cytokine receptors, providing SH2 domain mediated docking sites for STAT3. STAT3 is then phosphorylated by JAKs on Tyr705 within the C-terminus yielding STAT dimerisation (Schindler, Levy et al. 2007). Dimeric STAT3 translocates to the nucleus where it induces downstream transcriptional activation of specific target genes (Lim and Cao 2006). The role of serine phosphorylation of STAT3 is more controversial and the transcriptional activity of serine-only-phosphorylated STAT3 is not fully understood. Serine STAT3 phosphorylation was believed to enhance STAT3 transcription activity (Wen, Zhong et al. 1995) however, recently other aspects of SerSTAT3 activity are emerging suggesting more complex role of SerSTAT3 (Decker and Kovarik 2000).

1. FGF1-induced activation of STAT3

1.1. HEK293T, NIH3T3, HeLa and MCF7 cells

The ability of FGF1 to activate STAT3 was examined in HEK293T, 3T3, HeLa and MCF7 cells. All cell lines were stimulated with FGF1 and subsequent tyrosine and serine phosphorylation of STAT3 was investigated by probing with specific antibodies. The

activation of MAPK pathway was checked by probing with pERK antibody. The level of FGFR1 and its activation were examined by FGFR1 and p653/654 FGFR antibodies. Equal loading of samples was verified by probing with STAT3 and ERK antibodies.

TyrSTAT3 phosphorylation was not observed in any of the cell lines even though the level of total STAT3 was detectable (Figure 20). On the contrary, serine STAT3 phosphorylation by FGF1 was observed in all cell types (Figure 20). In HEK293T, NIH3T3 and MCF7 cells SerSTAT3 phosphorylation peaked after 5 minutes and decreased after 60 minutes (Figure 20). HeLa cells showed a high level of phospho-serine STAT3 before stimulation, which did not change after FGF1 stimulation (Figure 20). The FGF1 stimulation induced ERK activation after 2 or 5 minutes, depending on the cell type. Furthermore, the level of FGFR1 was not detectable in any of the cell lines. Phospho-653/654 FGFR was barely detectable in HEK293T and NIH3T3 cells when probed with specific phospho-FGFR antibody which recognises all forms of phosphorylated FGFRs (FGFR1-4) (Figure 20), suggesting that the level of FGFRs in these cell lines was low.

Because the low level of TyrSTAT3 might be the reason why it was not detected by Western blot analysis of WCLs, an immunoprecipitation experiment was performed. Endogenous STAT3 was pulled-out from MCF7 whole cell lysates stimulated with FGF1. Figure 21 A demonstrates that STAT3 was serine but not tyrosine phosphorylated following FGF1 stimulation in MCF7 cells which is in agreement with the previous experiment. The basal level of phospho-serine STAT3 was observed in MCF7 cells prior to stimulation (Figure 21 A).

In order to check if a higher concentration of FGF1 would make a difference in TyrSTAT3 activation, MCF7 cells were stimulated with 50 ng/ml and 100 ng/ml FGF1. Oncostatin M (OSM), used as a positive control, is a cytokine that was described to tyrosine

phosphorylate STAT3 and subsequently induce translocation to the nucleus (Underhill-Day and Heath 2006). An increased concentration of FGF1 did not activate TyrSTAT3 phosphorylation in MCF7 cells, only phospho-serine STAT3 was observed (Figure 21 B). OSM stimulation induced tyrosine and serine STAT3 phosphorylation (Figure 21 B).

In conclusion, tyrosine phosphorylation of STAT3 by FGF1 was not observed, however, serine phosphorylation was detected in all cell types. Thus, further analysis of STAT3 localisation in FGF1-stimulated cells as well as STAT3 phosphorylation in cells over-expressing FGFR1 was undertaken.

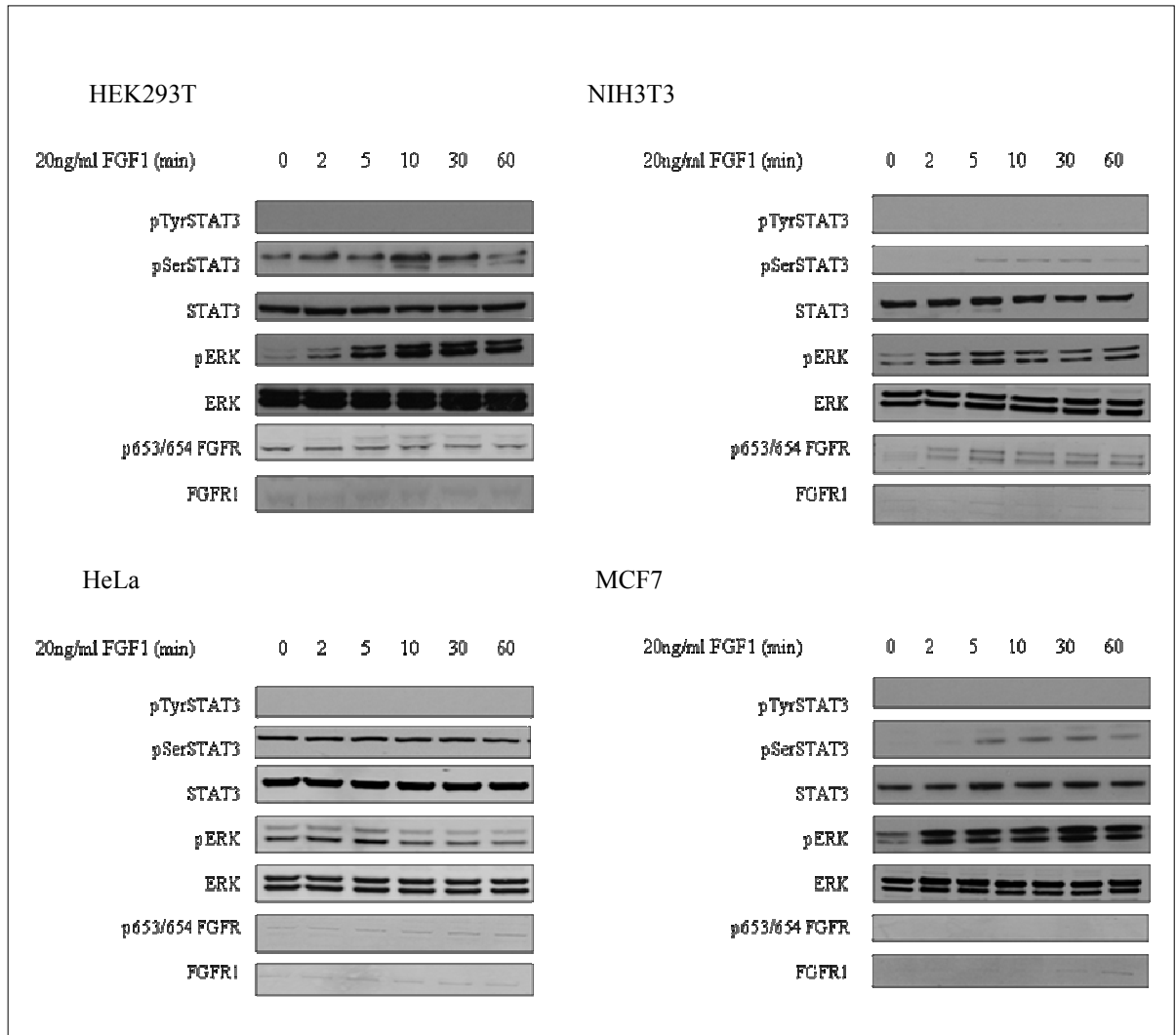


Figure 20. FGF1-induced STAT3 phosphorylation.

Cells HEK293T, NIH3T3, HeLa, MCF7 were serum-starved, stimulated with 20 ng/ml FGF1 and 10 µg/ml heparin for indicated times and lysed. Equal amounts of WCLs were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

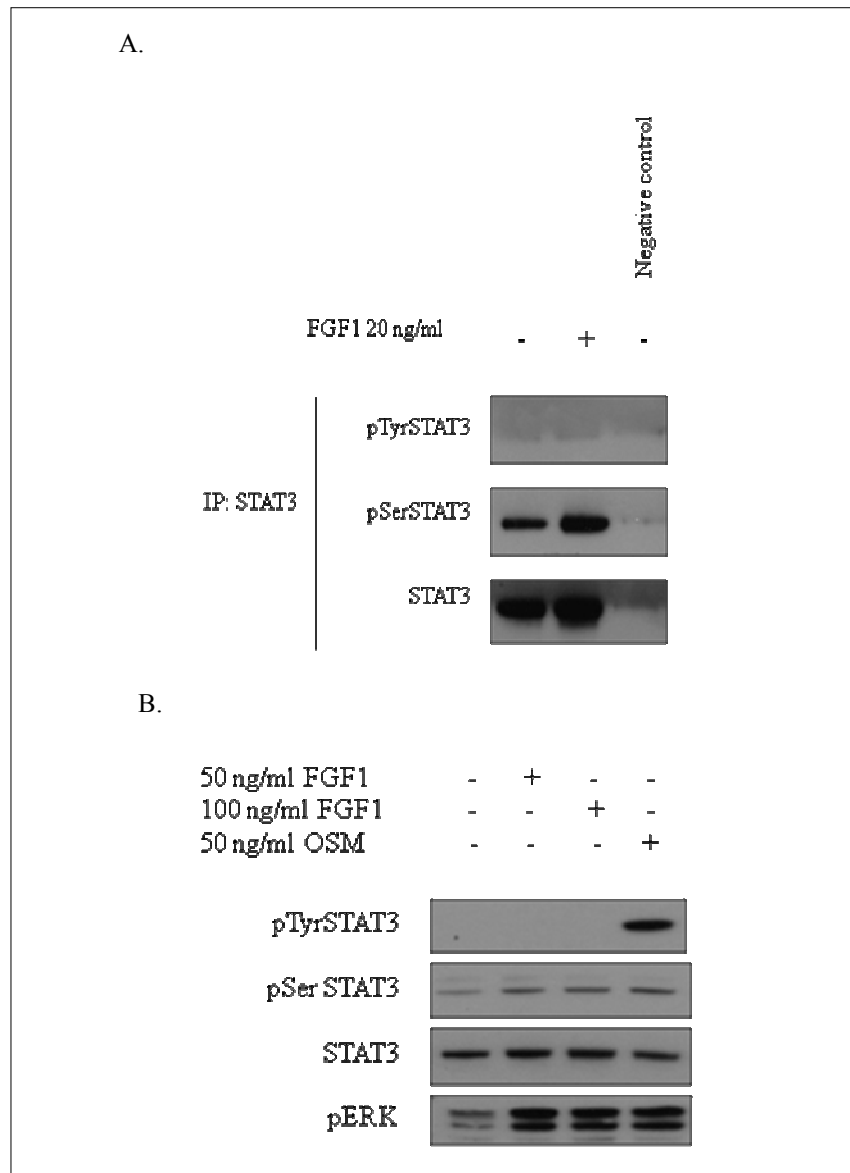


Figure 21. Effect of FGF1 stimulation on STAT3 phosphorylation in MCF7 cells.

- A. MCF7 cells were serum-starved and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 min. Equal amounts of whole cell lysates were immunoprecipitated using STAT3 antibody and protein G Sepharose, negative control had no antibody. Samples were resolved by SDS-PAGE, transferred to PVDF and probed with antibodies.
- B. MCF7 cells were serum-starved and stimulated with 50 ng/ml FGF1 or 100 ng/ml FGF1 and 10 μ g/ml heparin or 50 ng/ml OSM for 20 minutes. Equal amount of proteins were resolved by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

1.2. Localisation of STAT3 in MCF7 cells

The dynamic status of the STAT3/JAK pathway is regulated by two key determinants: STAT3 de-phosphorylation and nuclear export (Guerriero, Dudka et al. 2009), as STAT3 constitutively shuttles between the cytoplasm and nucleus and tyrosine phosphorylation inactivates nuclear export (Pranada, Metz et al. 2004). Upon stimulation, activated TyrSTAT3 accumulates in the nucleus where it drives gene expression. Therefore, STAT3 localisation and activation following FGF1 and OSM stimulation was investigated using immunofluorescence. MCF7 cells were stained using STAT3 and Texas Red conjugated secondary antibody. OSM was used as a positive control.

Prior to stimulation STAT3 was equally distributed throughout the cells (Figure 22). OSM stimulation induced STAT3 accumulation in the nuclei which was a result of tyrosine phosphorylation of STAT3 (Figure 22). The localisation of total STAT3 upon FGF1 stimulation was slightly changed; some cells showed nuclear localisation of STAT3 (Figure 22). In order to check if that effect was due to tyrosine or serine phosphorylation of STAT3, MCF7 cells were stimulated with FGF1 and stained using pTyrSTAT3 and pSerSTAT3 antibodies and Alexa Fluor 594 and Texas Red conjugated secondary antibodies, respectively (Figure 23 and Figure 24). All pictures were taken with the same settings for confocal microscope. OSM stimulation induced clear TyrSTAT3 activation and its nuclear accumulation (Figure 23). TyrSTAT3 in serum-starved cells and FGF1-stimulated was not detectable and the background is likely to be due to the signal from secondary antibody (Figure 23). However, the possibility that there is small amount of TyrSTAT3 in the nucleus in FGF-stimulated cells cannot be excluded.

The phosphorylation of SerSTAT3 was detectable in cells treated with FGF1 and OSM (Figure 24). Even though STAT3 was serine phosphorylated prior to FGF1 treatment,

FGF-induced STAT3 activation

the amount of phosphorylated protein is increased after stimulation (Figure 24). Interestingly, FGF1-activated SerSTAT3 was localised in dot-like structures in the cytosol and in the nuclei of cells, whereas OSM-activated SerSTAT3 was localised mainly in the nuclei (Figure 24). It is possible that FGF1-induced nuclear STAT3 that was observed in Figure 22 could be SerSTAT3. In order to fully validate this matter, cellular fractionation and Western blotting analysis of cytoplasmic and nuclear fractions using total and phospho-specific STAT3 antibodies should be performed.

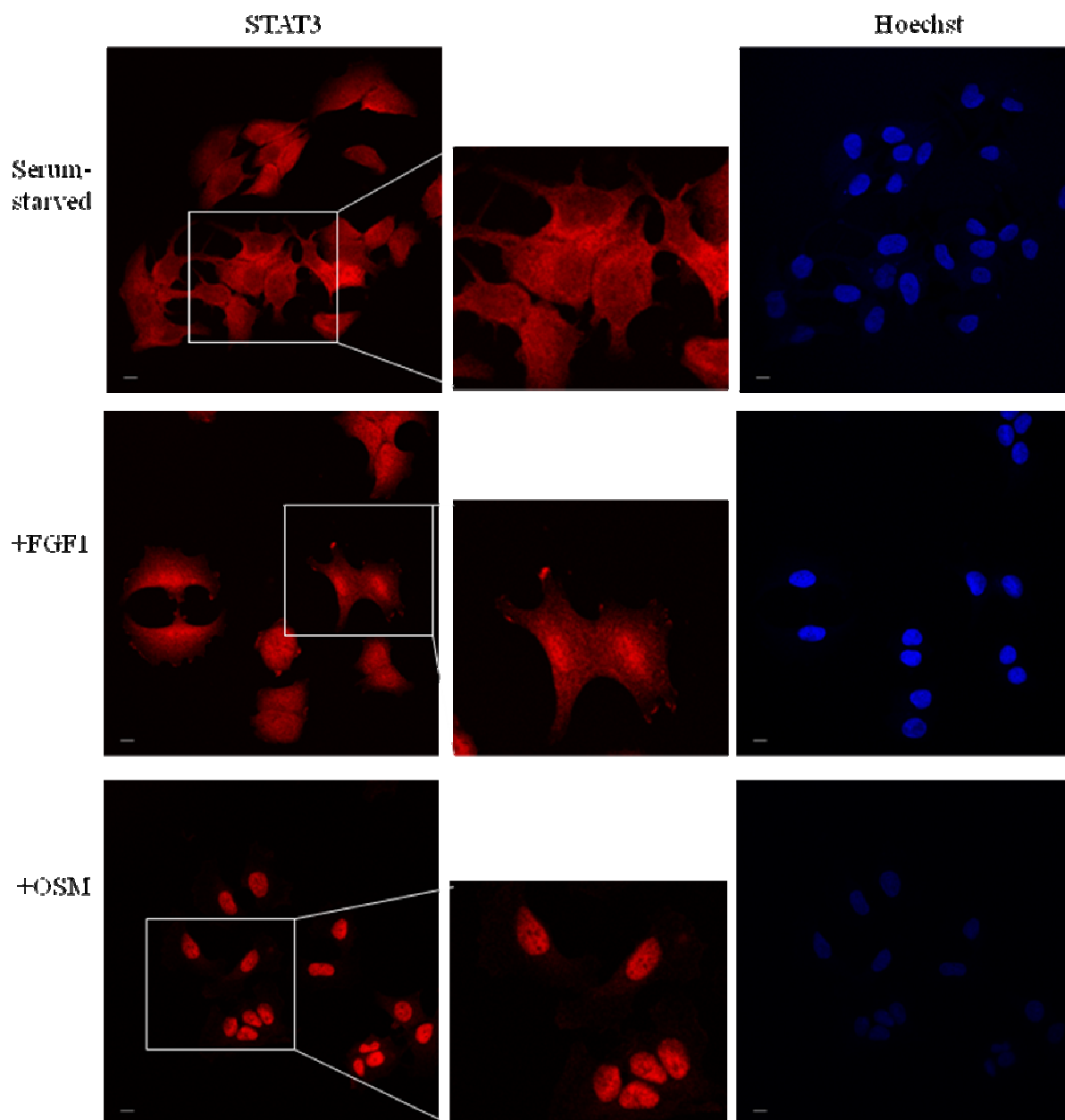


Figure 22. Total STAT3 localisation in MCF7 cells.

MCF7 cells were serum-starved prior to stimulation with 20 ng/ml FGF1 and 10 µg/ml heparin and 50ng/ml OSM for 20 min. Cells were fixed with 4% paraformaldehyde, permeabilized using saponin protocol and stained using STAT3 antibody with a Texas Red conjugated secondary antibody. Nuclei were stained using Hoechst.

Scale bar 10 µm.

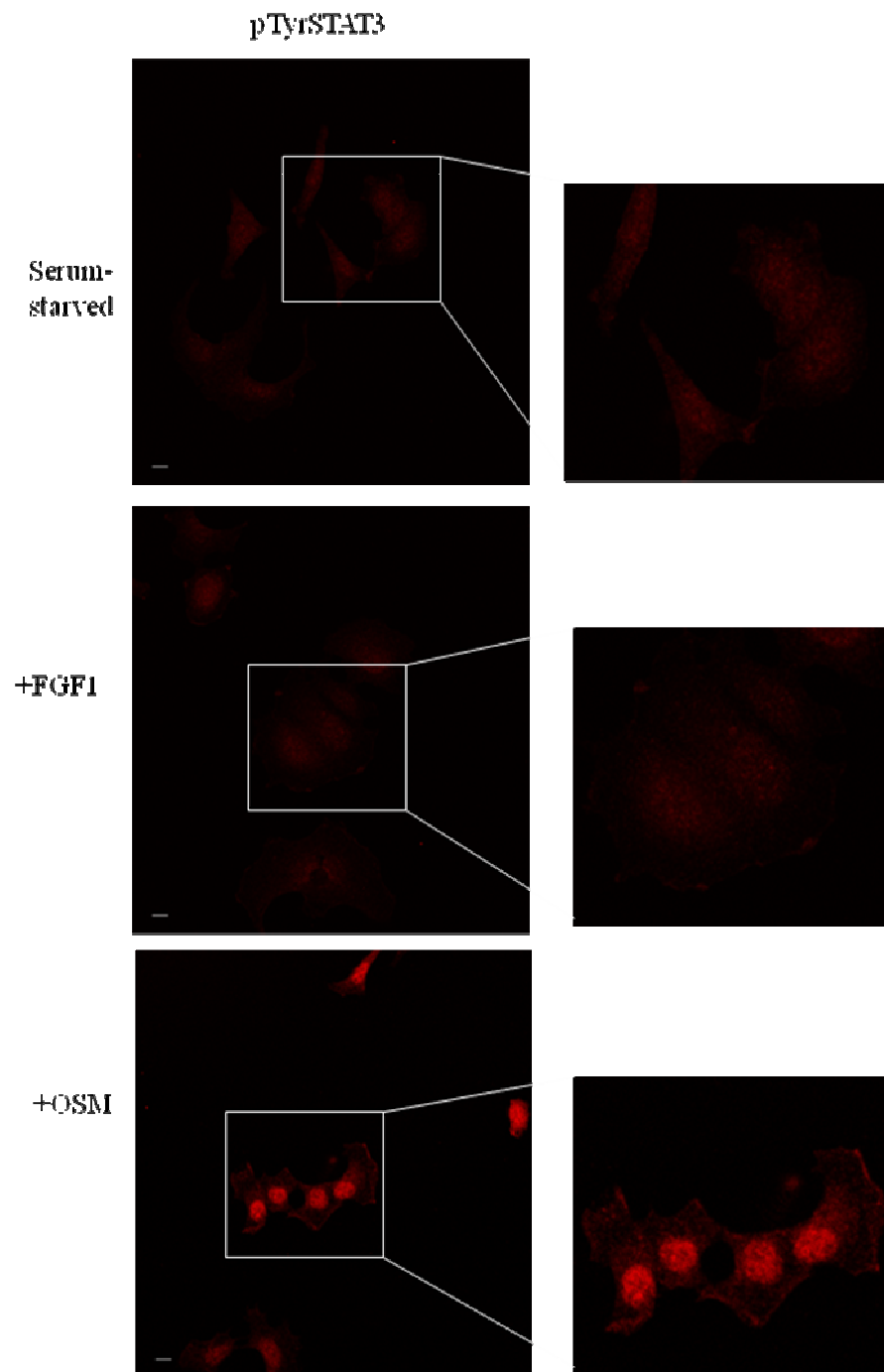


Figure 23. TyrSTAT3 localisation in MCF7 cells.

MCF7 cells were serum-starved prior to stimulation with 20 ng/ml FGF1 and 10 µg/ml heparin and 50 ng/ml OSM for 20 min. Cells were fixed with 4% paraformaldehyde, permeabilized using saponin protocol and stained using pTyrSTAT3 antibody with a Alexa Fluor 594 conjugated secondary antibody. Scale bar 10 µm.

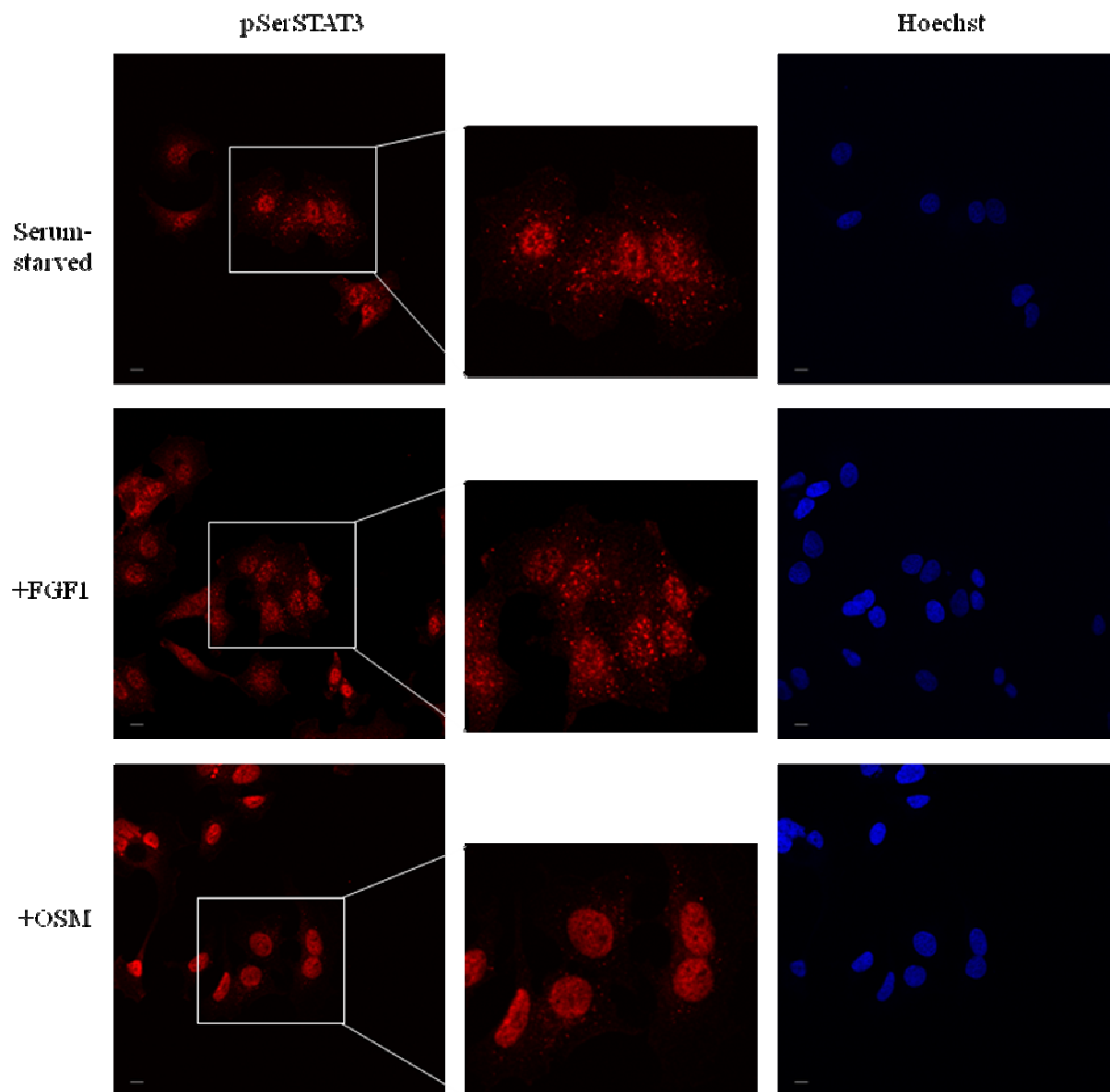


Figure 24. SerSTAT3 localisation in MCF7 cells.

MCF7 cells were serum-starved prior to stimulation with 20 ng/ml FGF1 and 10 μ g/ml heparin and 50 ng/ml OSM for 20 min. Cells were fixed with 4% paraformaldehyde, permeabilized using saponin protocol and stained using pSerSTAT3 antibody with a Texas Red conjugated secondary antibody. Nuclei were stained using Hoechst. Scale bar 10 μ m.

1.3. Localisation of STAT3 in HeLa cells

The localisation of STAT3 in HeLa cells was also studied. Cells were stimulated with FGF1 and stained using STAT3 and FGFR1 antibodies and Texas Red and FITC conjugated secondary antibodies, respectively.

The immunofluorescence analysis showed no nuclear accumulation of STAT3 after FGF1 stimulation in HeLa cells, which suggested no tyrosine phosphorylation (Figure 25). Moreover, the level of FGFR1 in HeLa cells was not high enough to be detected by FGFR1 antibody (Figure 25).

EGF-induced STAT3 activation

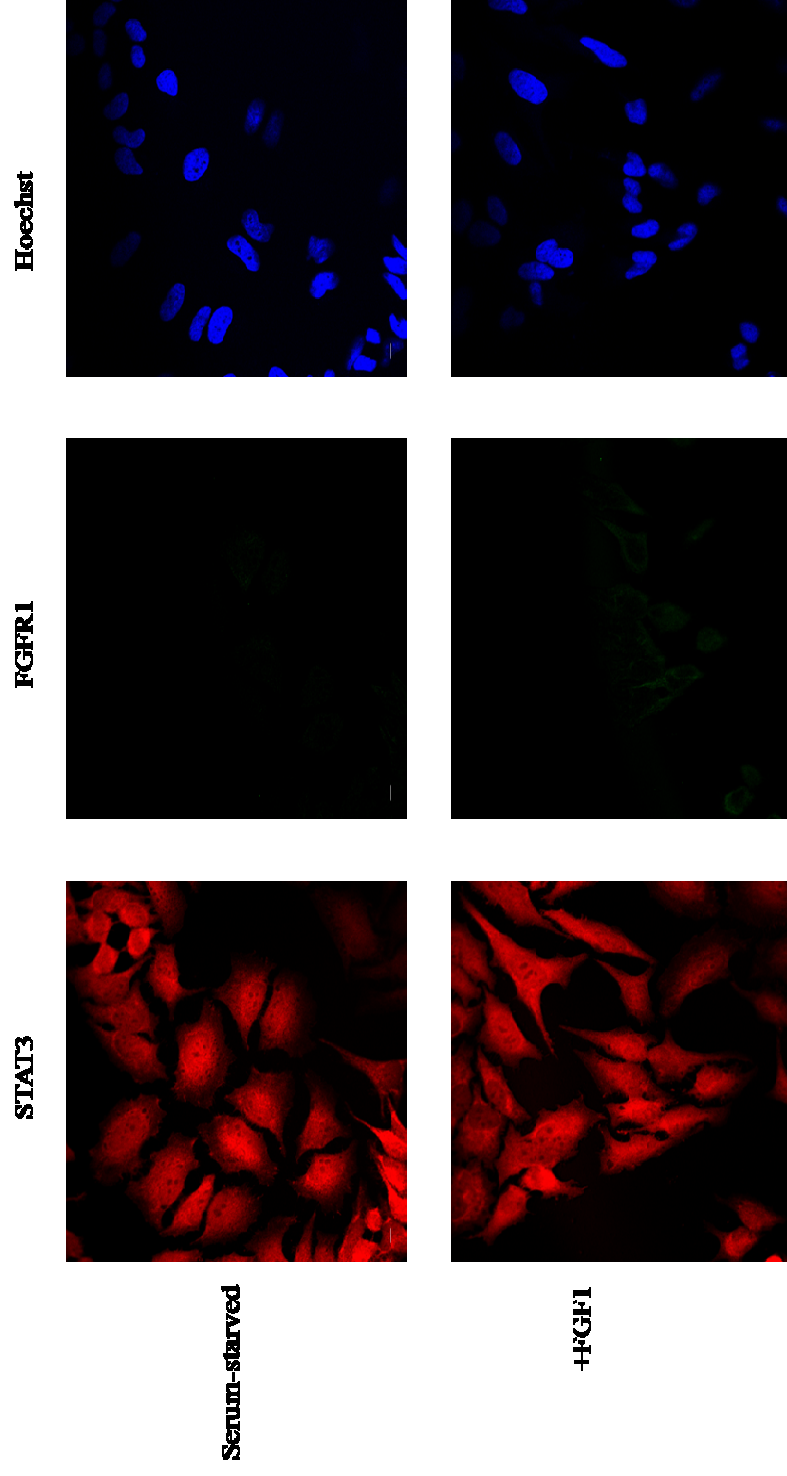


Figure 25. Total STAT3 localisation in HeLa cells.

MCF7 cells were serum-starved overnight prior to stimulation with 20 ng/ml FGF1 and 10 µg/ml heparin and 50 ng/ml OSM for 20 min. Cells were fixed with 4% paraformaldehyde, permeabilized using methanol protocol and stained using STAT3 and FGFR1 antibodies with a Texas Red and FITC conjugated secondary antibodies, respectively. Nuclei were stained using Hoechst. Scale bar 10 µm.

2. STAT3 phosphorylation in cells over-expressing FGFRs

Because no tyrosine phosphorylation of STAT3 was detected in HEK293T, NIH3T3, HeLa and MCF7 cells, it was hypothesized that TyrSTAT3 activation requires elevated levels of FGFRs to become active by FGF. That hypothesis was checked by transient transfection of FGFR1 and FGFR2 into cells and by studying STAT3 activation in cell lines naturally over-expressing FGFRs. FGFR gene amplification has been described in several breast cancer cell lines, which is usually correlated with poor prognosis (Beenken and Mohammadi 2009).

2.1. Over-expression of FGFR induces ligand-independent phosphorylation of the receptor

Before checking TyrSTAT3 activation in cells transiently transfected with FGFRs, the effect of over-expression of FGFR1 and FGFR2 was tested. FGFR1 WT and GFP-FGFR2 were previously described (Hinsby, Olsen et al. 2003; Schuller, Ahmed et al. 2008). Both constructs, FGFR1 and GFP-FGFR2, coding WT human full length proteins, were transfected into HEK293T cells which were serum-starved and stimulated with FGF1 in the presence or absence of SU5402. Expression of the constructs was verified by probing with FGFR1 and FGFR2 antibodies and their phosphorylation was checked by probing with p653/654 FGFR antibody. Activation of MAPK pathway was verified by probing with pERK and equal loading by probing with α -tubulin.

Figure 26 showed equal expression of FGFR1 WT and GFP-FGFR2 constructs. Noticeably, no endogenous FGFRs were detected by antibodies (Figure 26). Furthermore, phosphorylation of over-expressed FGFRs was ligand-independent but activation of phospho-ERK required ligand stimulation (Figure 26). Treatment with SU5402 inhibitor effectively

FGF-induced STAT3 activation

decreased activation of FGFRs. The remainder of the experiments with over-expressed FGFRs were performed with the presence of SU5402 to allow a comparison between phosphorylate and non-phosphorylated FGFRs.

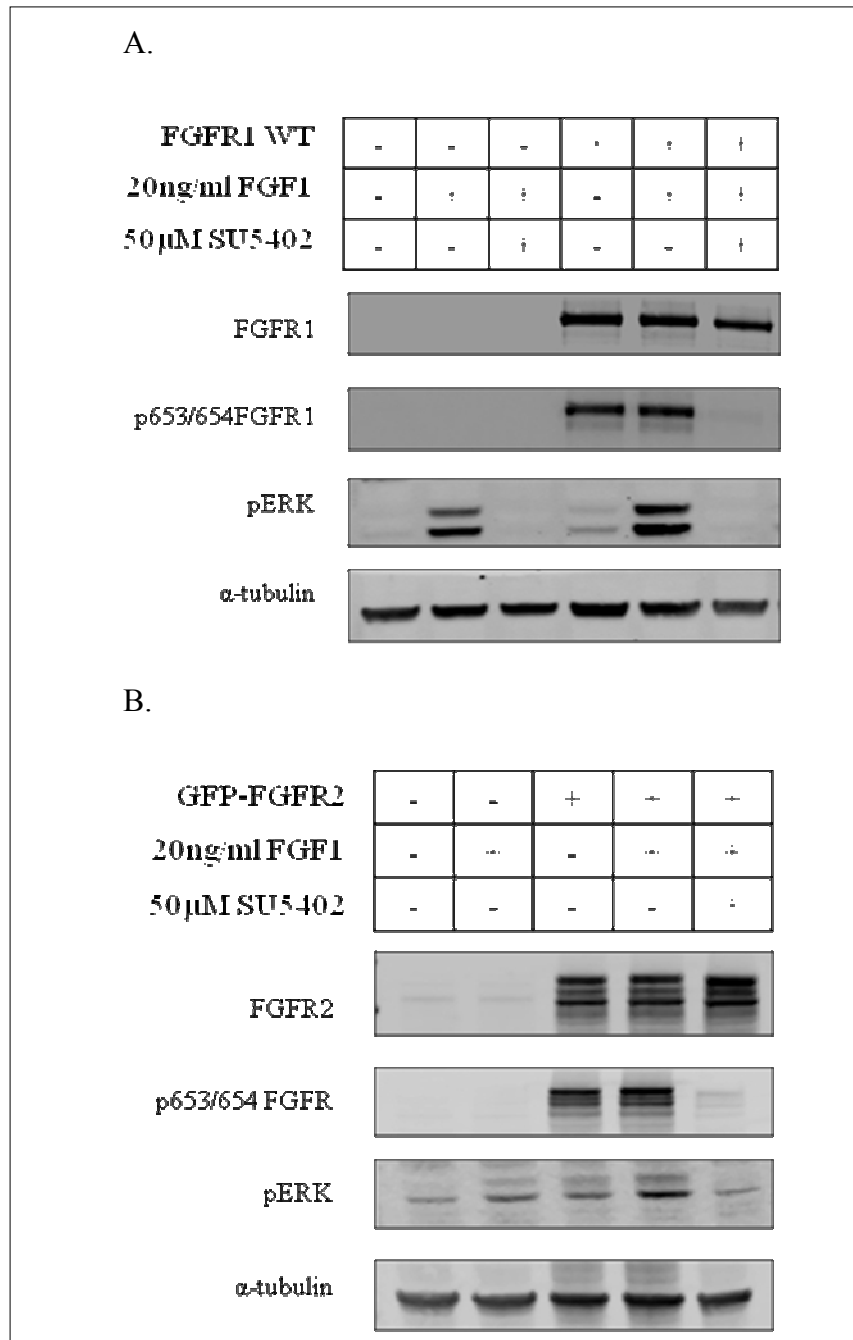


Figure 26. Effect of FGFRs over-expression.

HEK293T were transiently transfected with FGFR1 WT (A) and GFP-FGFR2 (B). After 48 hours cells were serum-starved, treated with 50 μ M SU5402 for 30 minutes where indicated or DMSO and stimulated with 20 ng/ml and 10 μ g/ml heparin for 20 minutes. Equal amounts of WCLs were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

2.2. Tyrosine STAT3 phosphorylation requires over-expression of FGFR1

Four FcFGFR1 (kinase active, kinase dead, truncated form of the receptor and VT-) constructs (Burgar, Burns et al. 2002) were transiently transfected into HEK293T cells in order to study TyrSTAT3 phosphorylation under FGFR1 over-expression. Expression of FcFGFR1 constructs was verified by probing with Fc antibody, phosphorylation of STAT3 was checked by probing with pTyrSTAT3 and pSerSTAT3 antibodies, whereas MAPK pathway activation and equal loading were checked by probing with pERK and α -tubulin antibodies, respectively.

Figure 27 demonstrated STAT3 phosphorylation, tyrosine and serine, by FcFGFR1 kinase active and VT-, the isoform of FGFR1 that does not recruit FRS2. No STAT3 activation was detected in samples over-expressing kinase dead or truncated version of FcFGFR1 (Figure 27). Collectively, these data suggested that both serine and tyrosine of STAT3 were phosphorylated by kinase active FGFR1, independently of FRS2.

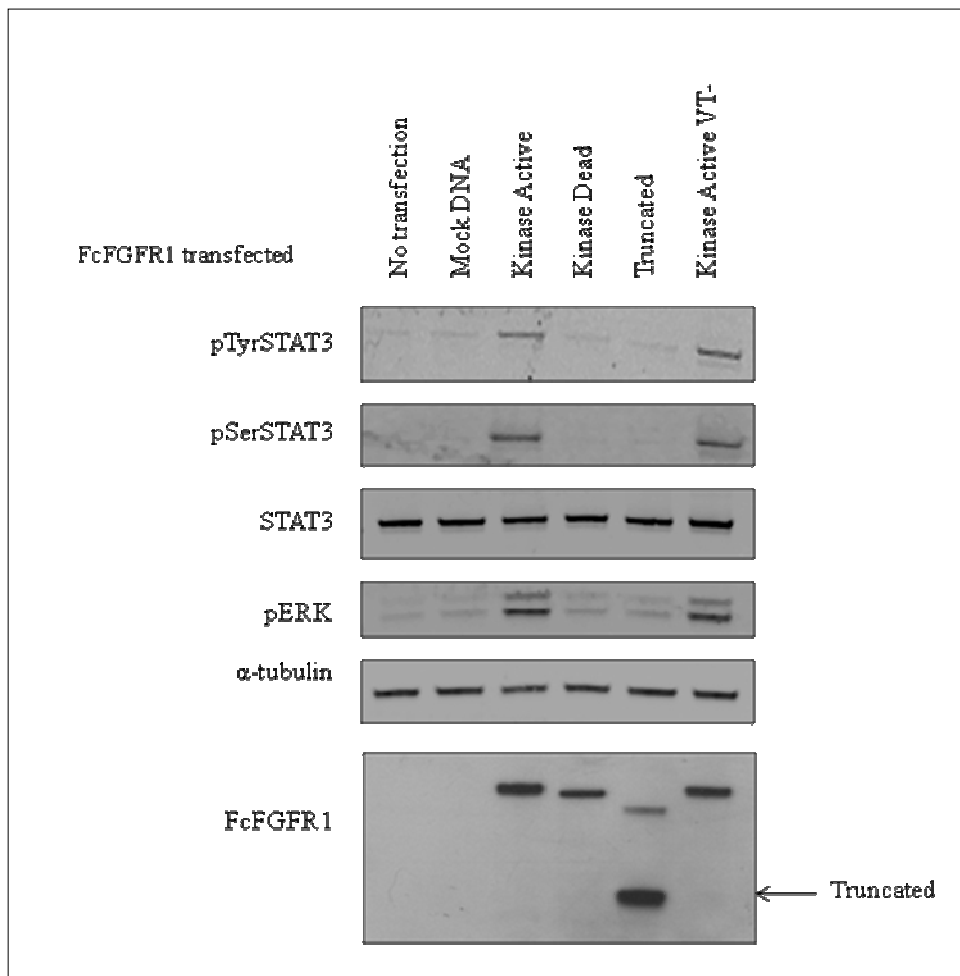


Figure 27. Tyrosine STAT3 phosphorylation requires over-expression of FGFR1.

FcFGFR1 constructs were transiently transfected into HEK293T cells. After 48 hours cells were lysed, equal amount of proteins were separated by SDS-PAGE, transferred on PVDF membrane and probed with antibodies.

2.3. Tyrosine STAT3 phosphorylation depends on Tyr677 of FGFR1

Figure 17 showed the interaction between STAT3 and FGFR1 WT but not FGFR1 Y677F. In order to check phosphorylation of STAT3 by over-expressed FGFR1 WT and FGFR1 Y677F, both constructs were expressed in HEK293T cells. Cells were stimulated with FGF1 in the presence or absence of SU5402 and whole cell lysates were probed with specific antibodies to check STAT3 activation. Equal expression of FGFR1 constructs and their phosphorylation were verified by probing with FGFR1 and p653/654 FGFR antibodies.

Figure 28 confirmed that TyrSTAT3 phosphorylation required over-expression and phosphorylation of FGFR1 WT. Stimulation of endogenous FGFR in HEK293T cells had no effect on STAT3 tyrosine activation which was also shown in previous experiments (Figure 20). SU5402 treatment inhibited FGFR1 phosphorylation and therefore TyrSTAT3 activation (Figure 28). This suggested that kinase activity of FGFR1 WT was necessary to induce activation of tyrosine STAT3. Moreover, tyrosine phosphorylation of STAT3 was decreased with FGFR1 Y677F expression (Figure 28). The difference between TyrSTAT3 activation by FGFR1 WT and FGFR1 Y677F was statistically significant ($p=0.006$).

On the other hand, serine STAT3 activation was not dependent on over-expression of FGFR1. Also the Y677F point mutation in FGFR1 did not affect phosphorylation of SerSTAT3 suggesting that SerSTAT3 was activated downstream of FGFR1 (Figure 28).

Taken together, these results indicated that Tyr677 of FGFR1 played a critical role in STAT3 tyrosine phosphorylation, whereas SerSTAT3 activation occurred as a downstream effect of FGF1-stimulation. Moreover, over-expression of active FGFR1 was necessary to induce TyrSTAT3 phosphorylation.

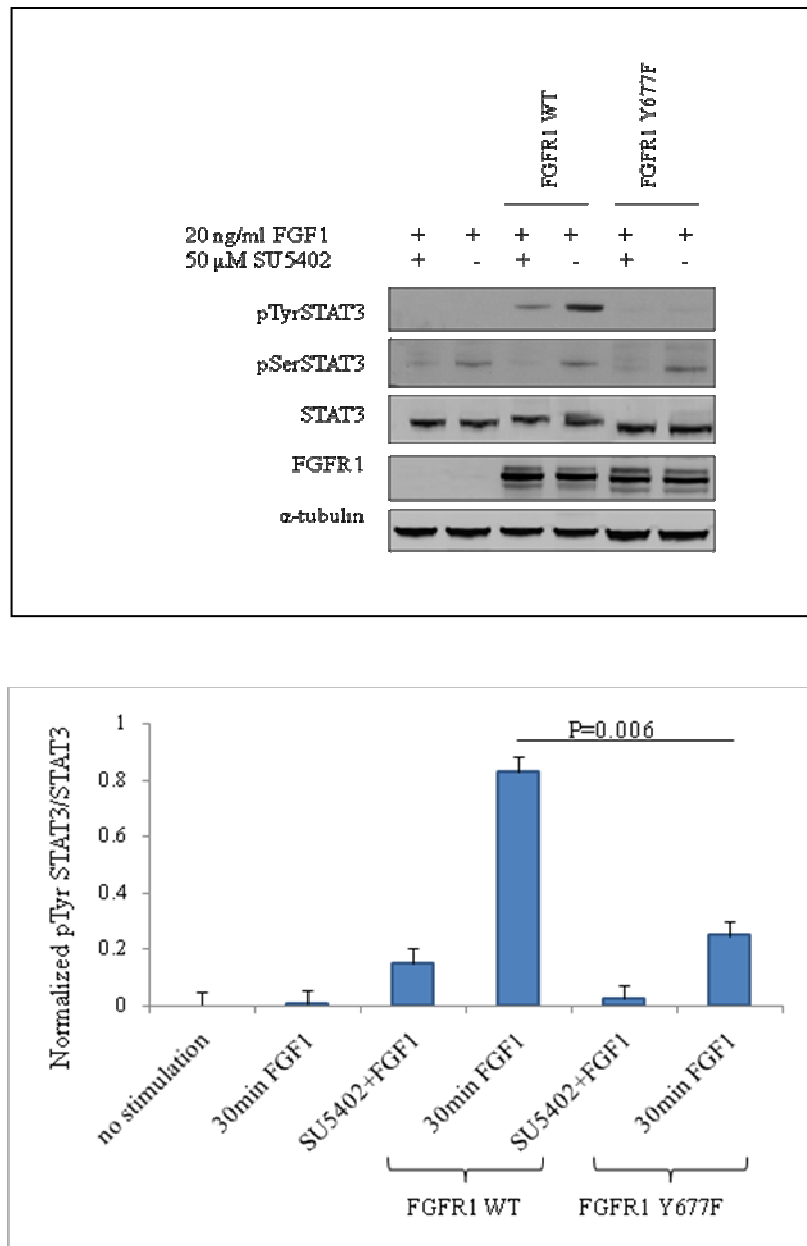


Figure 28. TyrSTAT3 phosphorylation depends on Tyr677 of FGFR1.

HEK293T cells were transiently transfected with FGFR1 WT or FGFR1 Y677F, after 48 hours cells were serum-starved, treated with 50 μM SU5402 or DMSO for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μg/ml heparin for 20 minutes. Equal amounts of proteins were separated by SDS-PAGE, transferred on PVDF membrane and probed with antibodies.

Densitometric analysis of Western blots for pTyrSTAT3 normalized to STAT3. Data are representative of three independent experiments. Error bars, SD.

2.4. Nuclear accumulation of TyrSTAT3 in cells over-expressing FGFR1

STAT3 as a transcription factor translocates to the nucleus where it regulates gene expression (Lim and Cao 2006). Immunofluorescence studies on wild type MCF7 and HeLa cells showed no TyrSTAT3 nuclear accumulation (Figure 22 and Figure 25) therefore, localization of STAT3 in HeLa cells over-expressing FGFR1 WT was examined. Cells were transiently transfected with FGFR1 WT construct and stimulated with FGF1 in the presence or absence of SU5402 inhibitor. Localization of STAT3 and TyrSTAT3 was verified by probing with specific antibodies. FGFR1 antibody was used for detection of cells expressing the FGFR1 WT construct.

Figure 29 demonstrated TyrSTAT3 accumulation in the nuclei of HeLa cells over-expressing FGFR1 WT. Treatment with SU5402 effectively decreased TyrSTAT3 activation and consequently its nuclear accumulation. No TyrSTAT3 was detected in cells that did not express FGFR1 WT construct (Figure 29).

Translocation of total STAT3 to the nucleus was observed in HeLa cells over-expressing FGFR1 WT (Figure 30). It suggests that STAT3 molecules, which were phosphorylated by FGFR1WT, were translocated and accumulated inside the nucleus. However, not every STAT3 protein was phosphorylated as some STAT3 was still observed in the cytoplasm of cells (Figure 30). It might be connected with FGFR1 binding stoichiometry; FGFR1 possesses only one binding motif for STAT3, for comparison, gp130 has got several binding motifs and EGFR two (Stahl, Farruggella et al. 1995; Shao, Cheng et al. 2003).

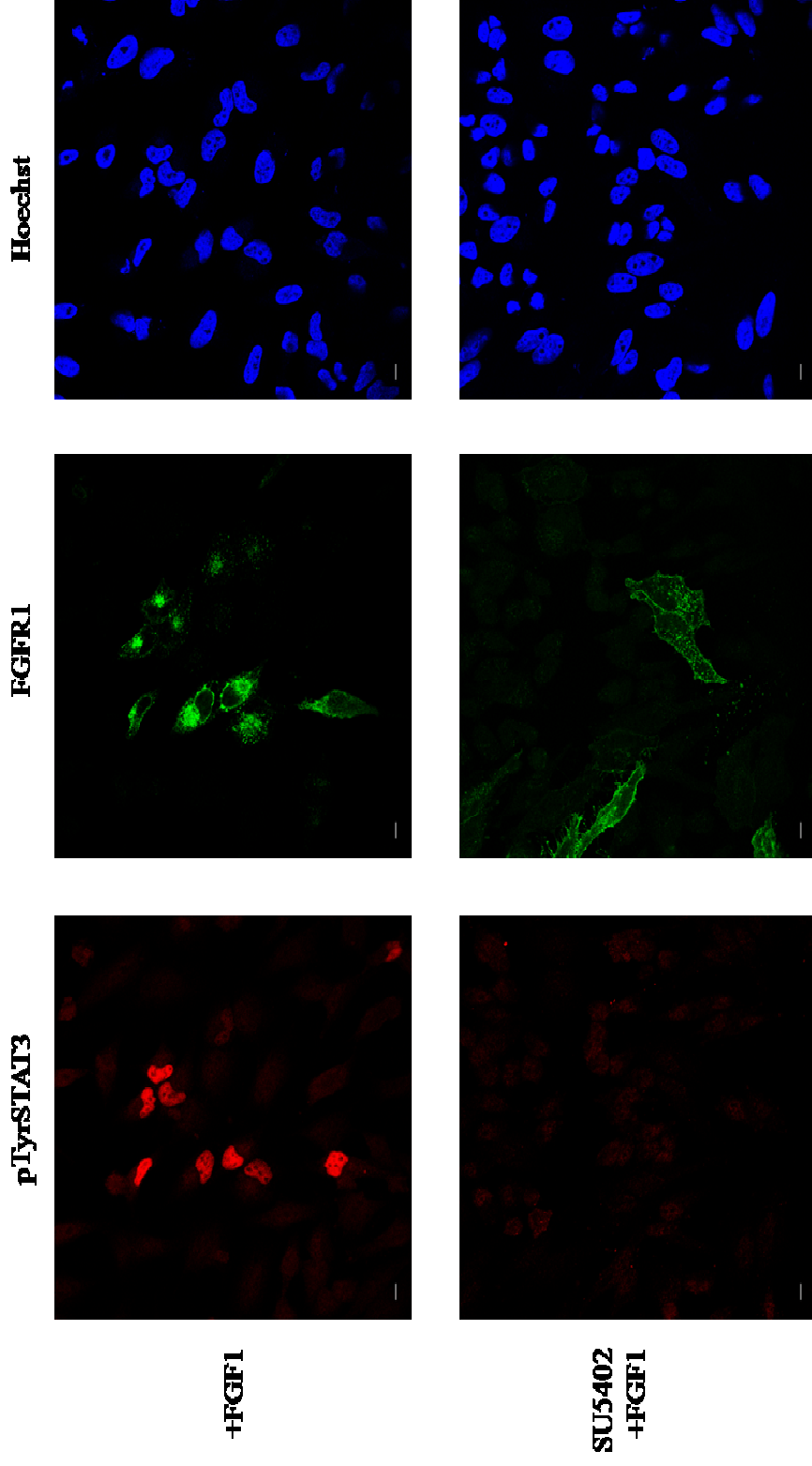


Figure 29. TyrSTAT3 nuclear accumulation in cells over-expressing FGFR1.

HeLa cells were transiently transfected with FGFR1 WT. Cells were serum-starved, treated with 50 μ M SU5402 or DMSO for 30 minutes and stimulated with 20 ng/ml heparin for 20 minutes. Cells were fixed with 4% paraformaldehyde, permeabilized using methanol protocol and stained using pTyrSTAT3 and FGFR1 antibodies and Texas Red or FITC conjugated secondary antibody, respectively. Nuclei were stained with Hoechst. Scale bars 10 μ m.

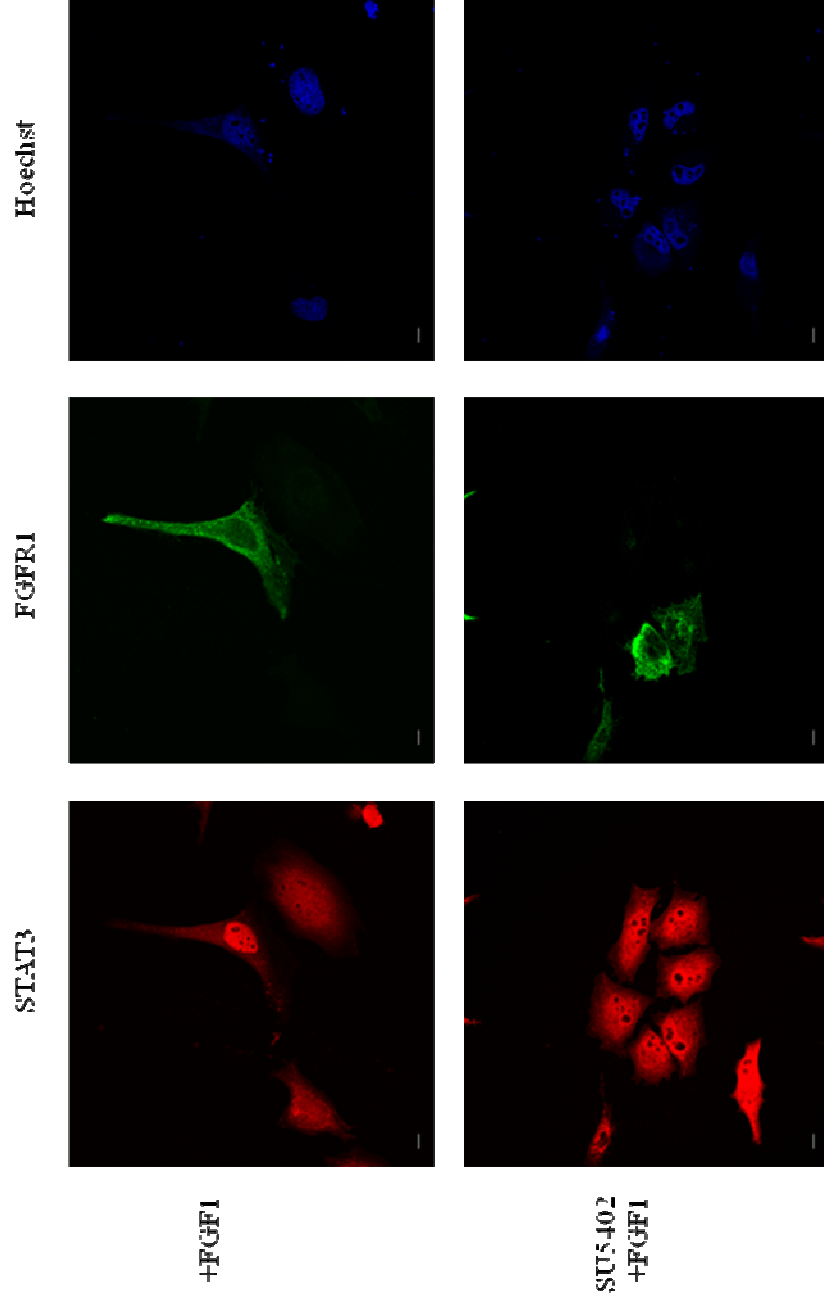


Figure 30. STAT3 nuclear translocation in cells over-expressing FGFR1.

HeLa cells were transiently transfected with FGFR1 WT. Cells were serum-starved, treated with 50 μ M SU5402 or DMSO for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Cells were fixed with 4% paraformaldehyde, permeabilized using methanol protocol and stained using STAT3 and FGFR1 antibodies and Texas Red or FITC conjugated secondary antibody, respectively. Nuclei were stained with Hoechst. Scale bars 10 μ m.

2.5. Over-expression of FGFR2 induces tyrosine STAT3 phosphorylation

Tyr677 of FGFR1 is a part of YxxQ motif present in the cytoplasmic region of the receptor. That motif is SH2 consensus motif for binding STAT3 and is highly conserved in all four human FGF receptors as well as in other vertebrates (Table 10).

Table 10. Comparison of YxxQ motif between FGFRs.

| | Sequence | Position of Tyr |
|--------------------|--------------------------|------------------------|
| human FGFR1 | alfdriy th qsdvws | 677 |
| human FGFR2 | alfdrvy th qsdvws | 680 |
| human FGFR3 | alfdrvy th qsdvws | 671 |
| human FGFR4 | alfdrvy th qsdvws | 666 |
| mouse FGFR1 | alfdriy th qsdvws | 675 |
| rat FGFR1 | alfdriy th qsdvw | 677 |

HEK293T cells were transiently transfected with GFP-FGFR2 in order to check if over-expression of other FGFRs induced TyrSTAT3 activation. Cells were stimulated with FGF1 in the presence or absence of SU5402. Phosphorylation of STAT3 was verified by probing with specific STAT3 antibodies. Expression and activation of GFP-FGFR2 was checked by probing with FGFR2 and p653/654 FGFR antibodies.

Figure 31 showed increased TyrSTAT3 activation in cells over-expressing GFP-FGFR2 that was effectively reduced with SU5402 treatment. This suggested that over-expressed FGFR2 activated tyrosine STAT3 phosphorylation. To check if FGFR2 over-expression induced TyrSTAT3 nuclear accumulation, an immunofluorescence experiment was performed. HeLa cells were transiently transfected with GFP-FGFR2 and stained with

FGF-induced STAT3 activation

TyrSTAT3 antibody and Alexa Fluor 594 conjugated secondary antibody. Figure 32 showed TyrSTAT3 accumulated in the nuclei of cells over-expressing GFP-FGFR2.

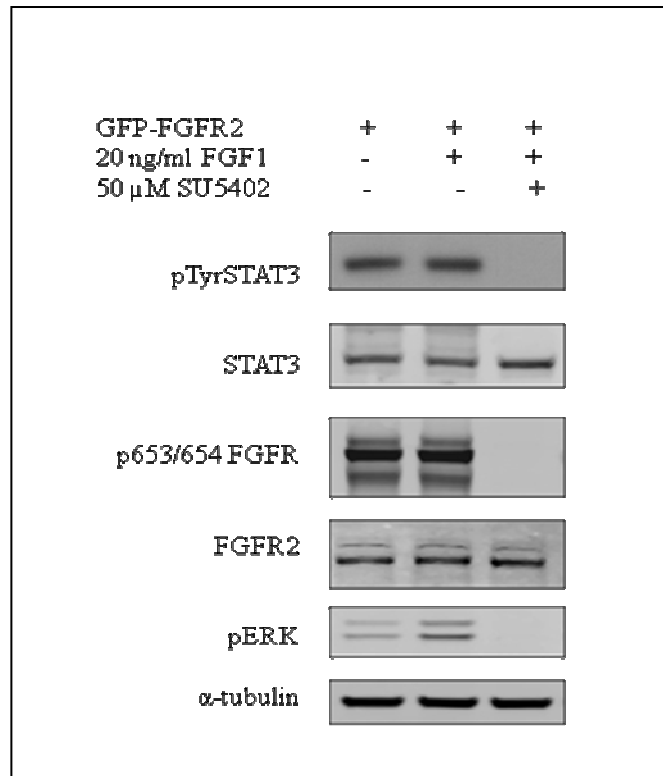


Figure 31. FGFR2 over-expression induces TyrSTAT3 phosphorylation.

HEK293T cells were transiently transfected with GFP-FGFR2. After 48 hours cells were serum-starved, treated with 20 μ M SU5402 for 30 minutes where indicated and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin.

WCLs were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

EGF-induced STAT3 activation

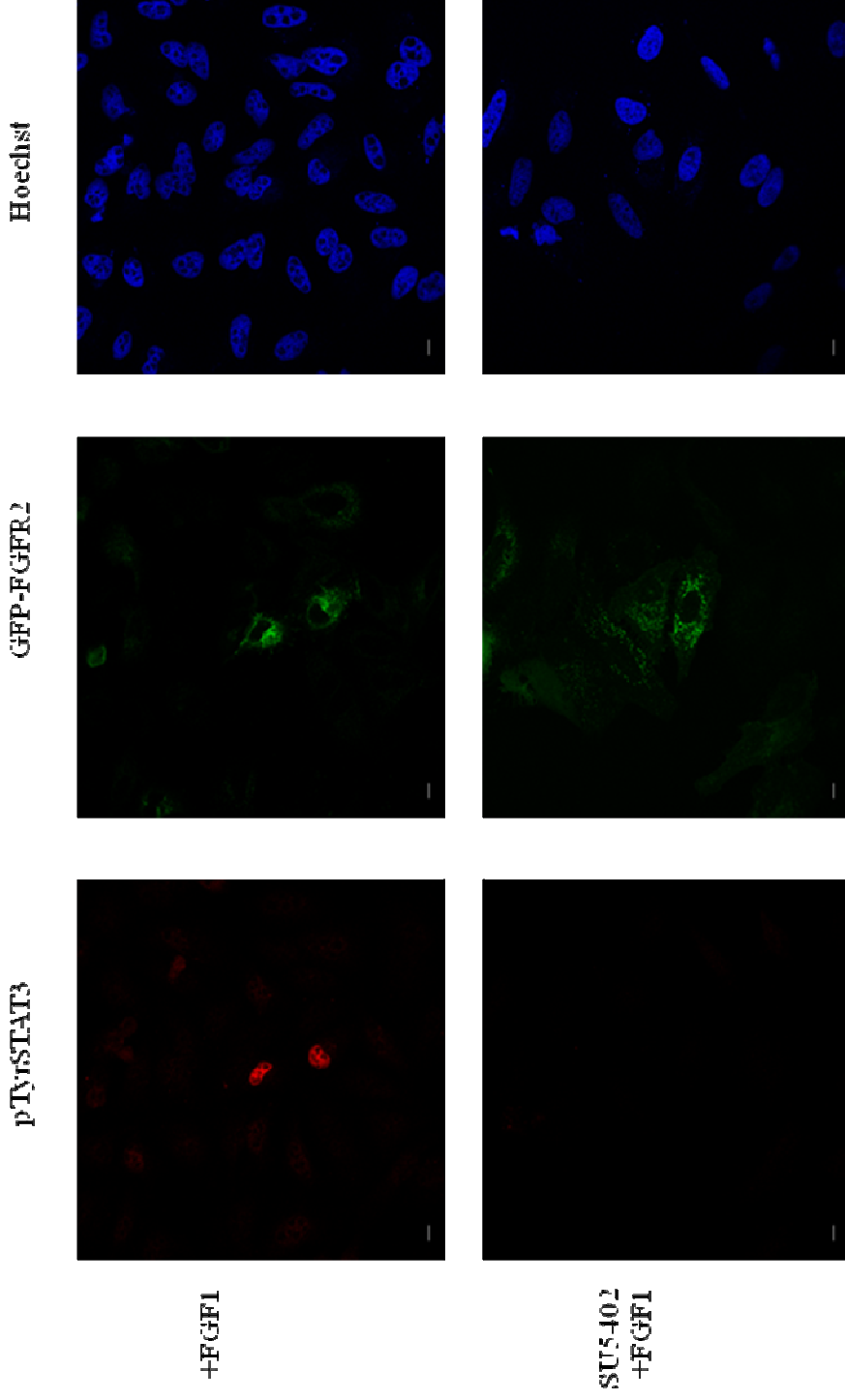


Figure 32. TyrSTAT3 nuclear accumulation in cells over-expressing GFP-FGFR2.

HeLa cells were transiently transfected with GFP-FGFR2. Cells were serum-starved, treated with 50 μ M SU5402 or DMSO for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Cells were fixed with 4% paraformaldehyde, permeabilized using methanol protocol and stained using pTyrSTAT3 antibody and Alexa Fluor 594 conjugated secondary antibody. Nuclei were stained with Hoechst. Scale bars 10 μ m.

2.6. SUM-52PE cell line

As phosphorylation of tyrosine STAT3 required over-expression of FGFRs it was hypothesized that this pathway functions only in cells that express high levels of FGFRs. Several tumour cell lines with FGFR gene amplification (SUM-52PE, MFM-223, MDA-MB-134, MDA-MB-453, ZR-75-1) were tested. One of them was the breast cancer cell line SUM-52PE that was originated from a malignant pleural effusion specimen (Ethier, Langton et al. 1996). SUM-52PE was described as an EGFR-negative cell line expressing erbB-2 protein at single copy levels and very low expression of PTPases (Ethier, Kokeny et al. 1996). Later, it was reported to have 12-fold amplification of the FGFR2 gene and 40-fold over-expression of FGFR2 isoform IIIb (Tannheimer, Rehemtulla et al. 2000). SUM-52PE expresses several variants of FGFR2 and some of them are specific for this cell line, not expressed by normal cells. Different splice variants have different transforming abilities which might be linked to their aggressive potential (Moffa, Tannheimer et al. 2004).

To specifically activate FGFR2 isoform IIIb we used FGF7 (Eswarakumar, Lax et al. 2005). STAT3 was phosphorylated on tyrosine and serine residues after FGF7 stimulation (Figure 33 A). The increased level of TyrSTAT3 and SerSTAT3 are presented graphically (Figure 33 B). To further verify that this effect was due to FGFR2 activity, cells were treated with increasing concentrations of two FGFR inhibitors, SU5402 and PD173074 following stimulation with FGF7 (Figure 34). Phosphorylation of tyrosine STAT3 gradually decreased together with the inhibition of FGFR2 (Figure 34). This reveals a significant role for FGFR2 kinase activity in TyrSTAT3 activation in SUM-52PE cells.

Activation and nuclear accumulation of TyrSTAT3 was confirmed by immunostaining. SUM-52PE cells were stimulated with FGF1 and stained with pTyrSTAT3

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antibody and Alexa Fluor 594 conjugated secondary antibody. FGF-induced TyrSTAT3 phosphorylation was observed in nuclei of stimulated cells, which was effectively reduced with SU5402 treatment (Figure 35).

To confirm an interaction between FGFR2 and STAT3, an immunoprecipitation experiment was performed. Endogenous FGFR2 was pulled down from SUM-52PE whole cell lysates and the presence of STAT3 was verified by probing with anti-STAT3 antibody (Figure 36 A). The reverse immunoprecipitation was also performed, where endogenous STAT3 was pulled down (Figure 36 B). In both experiments the association between STAT3 and FGFR2 was enhanced by FGF1 stimulation, whereas, treatment with SU5402 decreased STAT3 binding to FGFR2, confirming that STAT3 bound preferentially to activated receptor (Figure 36 A and B).

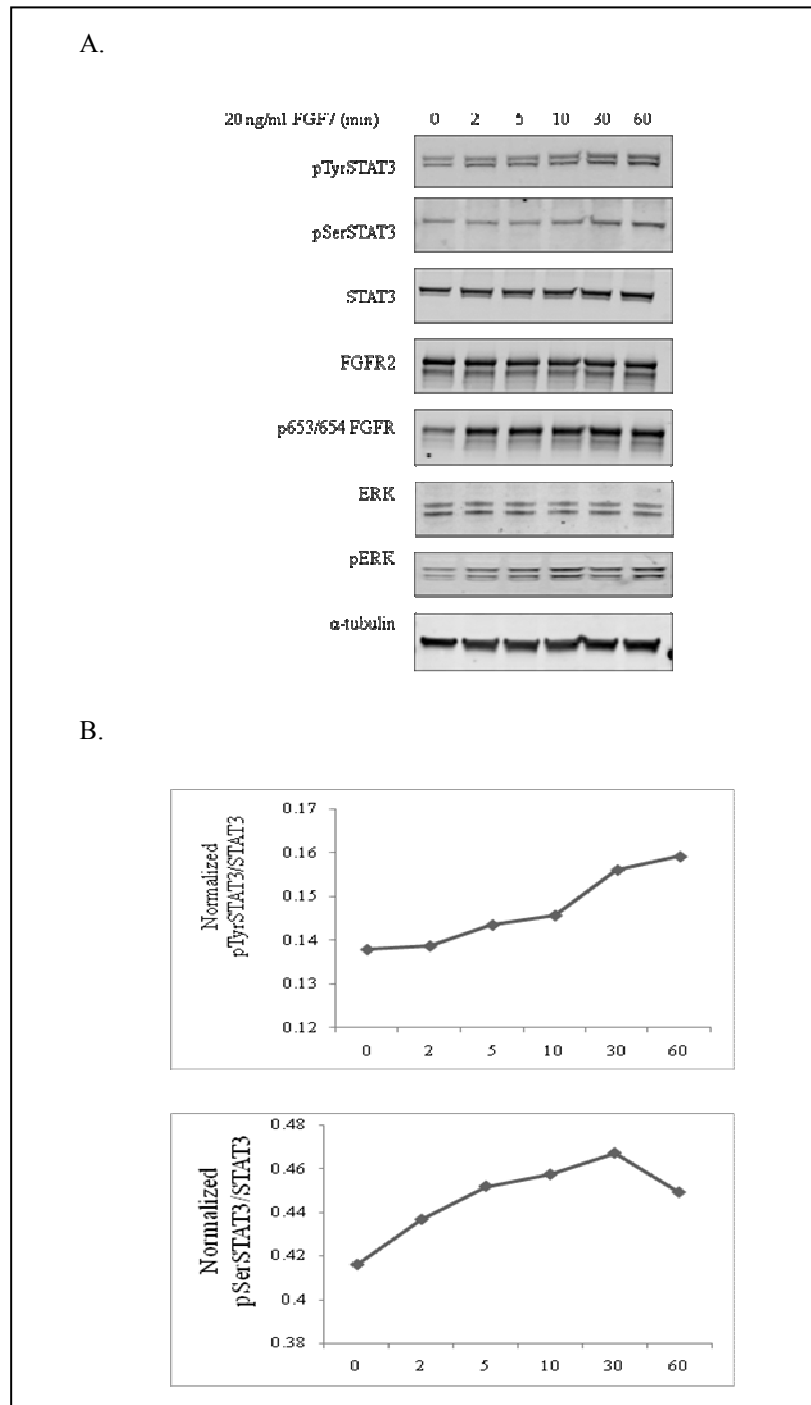


Figure 33. FGF7-induced STAT3 phosphorylation in SUM-52PE cells.

- A. SUM-52PE cells were serum-starved and stimulated with 20 ng/ml FGF7 and 10 μ g/ml heparin for indicated time. WCLs were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.
- B. Densitometric analysis of TyrSTAT3 and SerSTAT3 normalized to STAT3. Data obtained from one experiment.

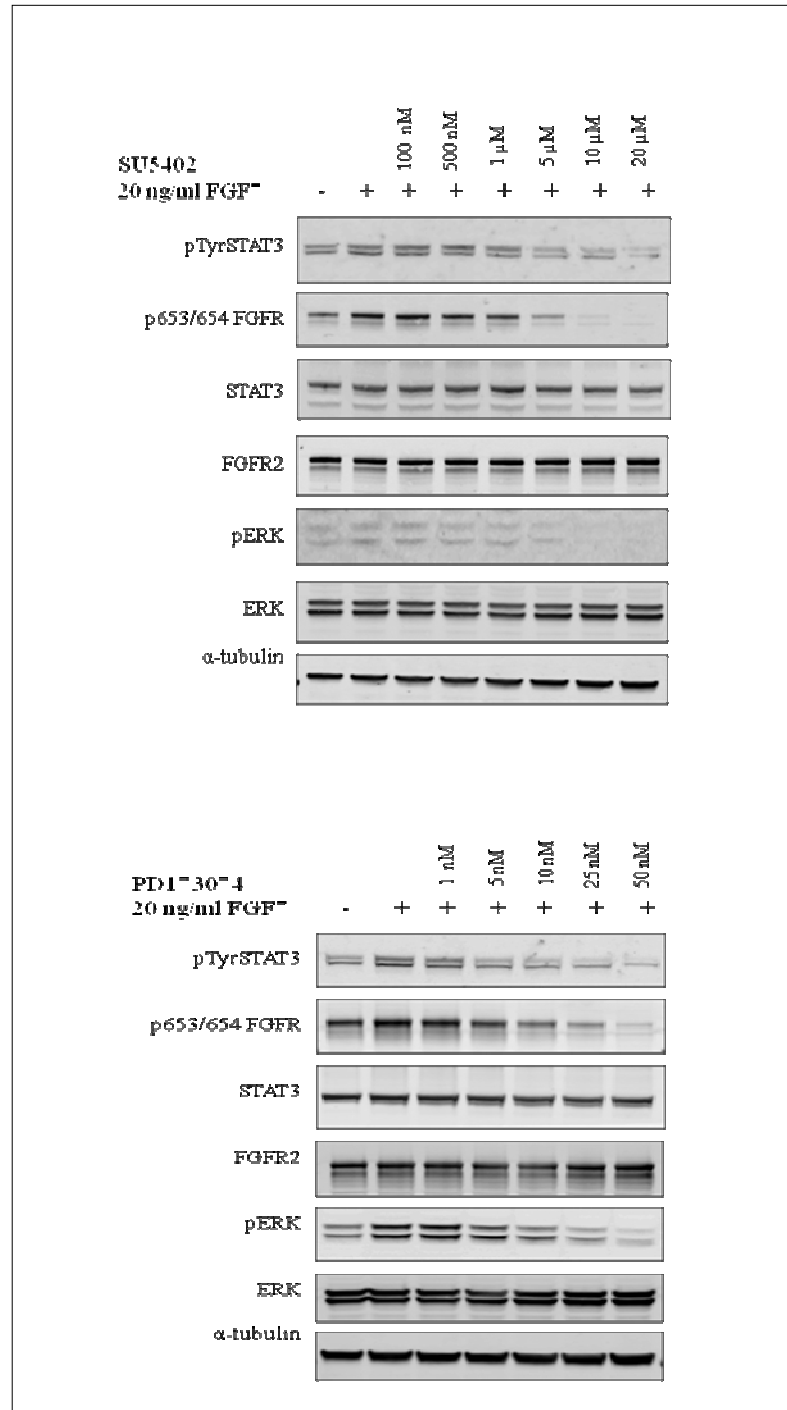


Figure 34. Effect of FGFR2 inhibition on STAT3 phosphorylation in SUM-52PE cells.

SUM-52PE cells were serum-starved, treated with increasing amounts of SU5402, PD173074 or DMSO as control for 30 minutes. Cells were stimulated with 20 ng/ml FGF7 and 10 μg/ml heparin for 20 minutes. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

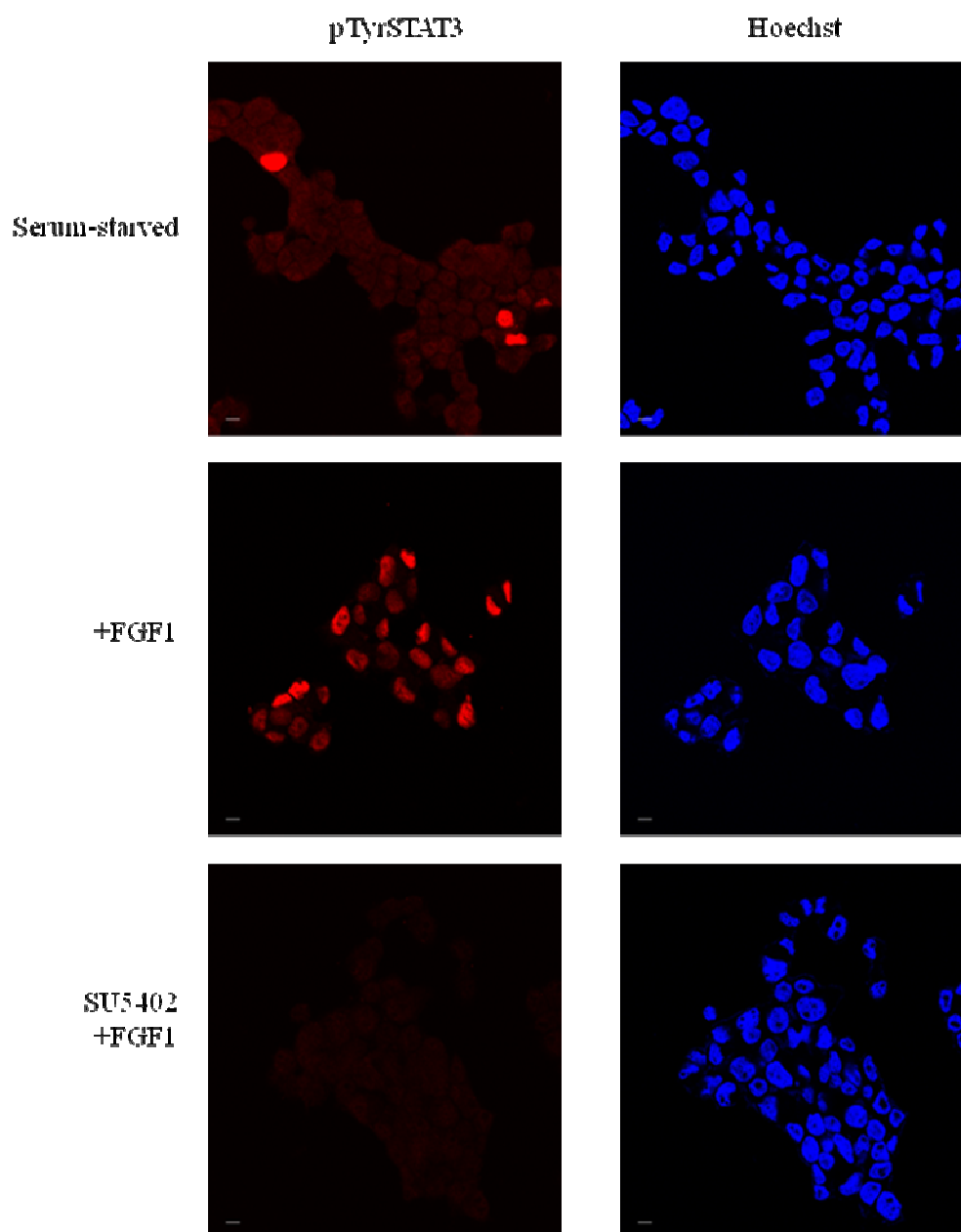


Figure 35. TyrSTAT3 nuclear accumulation in SUM-52PE cells.

SUM-52PE cells were serum-starved, treated with 20 μ M SU5402 or DMSO for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Cells were fixed, permeabilized using methanol protocol and stained using pTyrSTAT3 antibody and Alexa Fluor 594 conjugated secondary antibody. Nuclei were stained using Hoescht. Scale bar 10 μ m.

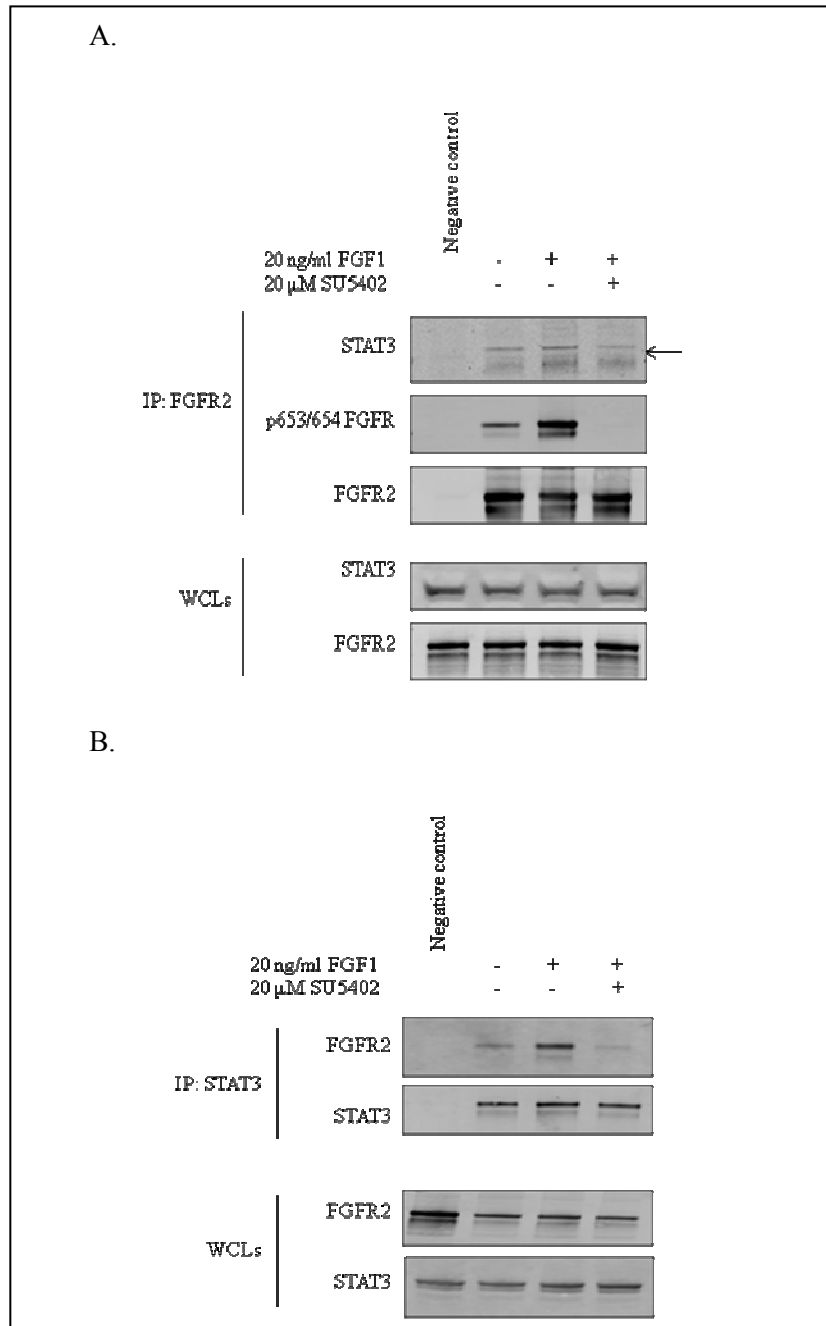


Figure 36. FGFR2 binds STAT3.

- A. SUM-52PE cells were serum-starved, treated with 20 μM SU5402 or DMSO for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μg/ml heparin for 20 minutes. Equal amounts of protein were immunoprecipitated using FGFR2 antibody and protein G-Sepharose. Negative control did not contain antibody. Immunoprecipitation samples and cell lysates were analyzed by Western blotting.
- B. Reverse immunoprecipitation was performed using STAT3 antibody and protein G-Sepharose. Conditions of the experiment were the same as in (A).

2.7. MFM-223 cell line

MFM-223 is a breast cancer cell line described by Hackenberg *et al.* in 1991. They demonstrated that MFM-223 express low levels of androgen receptor but increased estrogen and progesterone receptor levels. MFM-223 became a good model used in studies of androgen receptor (Hackenberg, Lutichens *et al.* 1991; Hackenberg, Hawighorst *et al.* 1993). Recently, amplification of FGFR2 gene was found in MFM-223 cell line (unpublished data by Nick Turner, Institute of Cancer Research, London). Quantitative-PCR showed that MFM-223 has 180 copies of FGFR2 gene, whereas SUM-52PE has 100 copies of FGFR2 gene (personal communication, Nick Turner). In order to check if MFM-223 induced TyrSTAT3 phosphorylation, cells were stimulated with FGF1 and activation of STAT3 was checked by probing whole cell lysates with specific antibodies. Expression of FGFR2 and receptor activation was verified by probing with FGFR2 and p653/654 antibodies, whereas activation of MAPK pathway was verified by probing with pERK antibody.

TyrSTAT3 phosphorylation in MFM-223 cells was observed but only after a long exposure of the blot (Figure 37). The level of FGFR2 was detectable, also phosphorylation of SerSTAT3 and MAPK pathway was observed (Figure 37).

Because the prediction was that FGF stimulation of MFM-223 cells would induce TyrSTAT3 activation on a comparable level to that shown in SUM-52PE cells due to FGFR2 gene amplification, the comparison between FGFR2 protein levels was performed. Three breast cancer cell lines were compared; MCF7 and two cell lines amplifying FGFR2 genes, SUM-52PE and MFM-223. The level of FGFR2 and its phosphorylation was checked by probing cell lysates with FGFR2 and p653/654 antibodies. Equal loading was verified by probing with α -tubulin.

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SUM-52PE cells expressed the highest level of FGFR2 (Figure 38). MFM-223 cells also express FGFR2 but the level of the protein was significantly lower than in SUM-52PE cells (Figure 39). This suggested that even though both cell lines amplify FGFR2 gene, only SUM-52PE expresses the protein on high levels and is able to phosphorylate STAT3. The level of FGFR2 in MCF7 cells was barely detectable (Figure 38 and Figure 39).

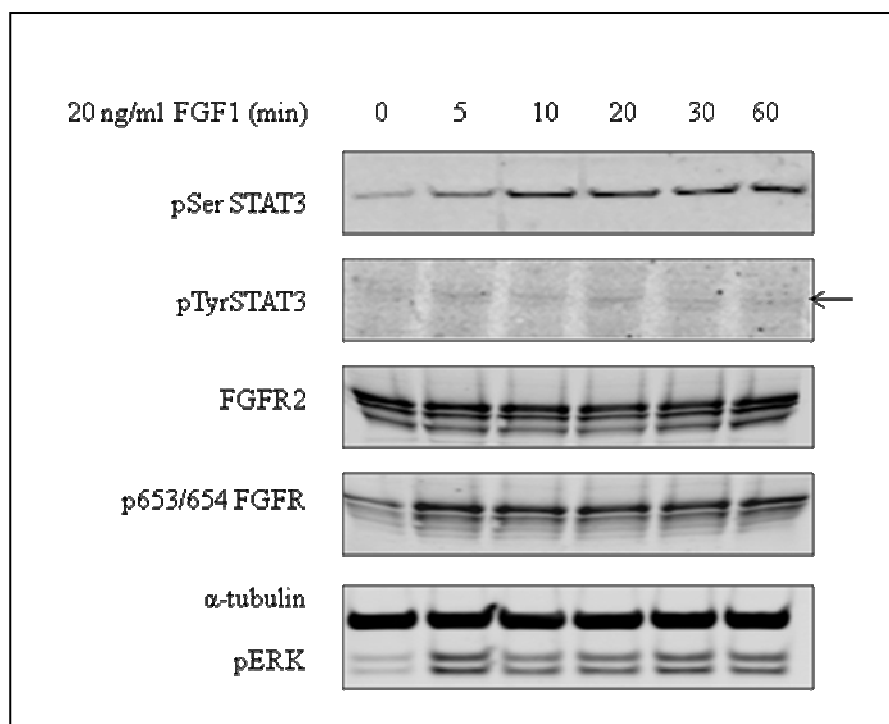


Figure 37. STAT3 phosphorylation in MFM-223 cells.

MFM-223 cells were serum-starved and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for indicated times. Cells were lysed and samples were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

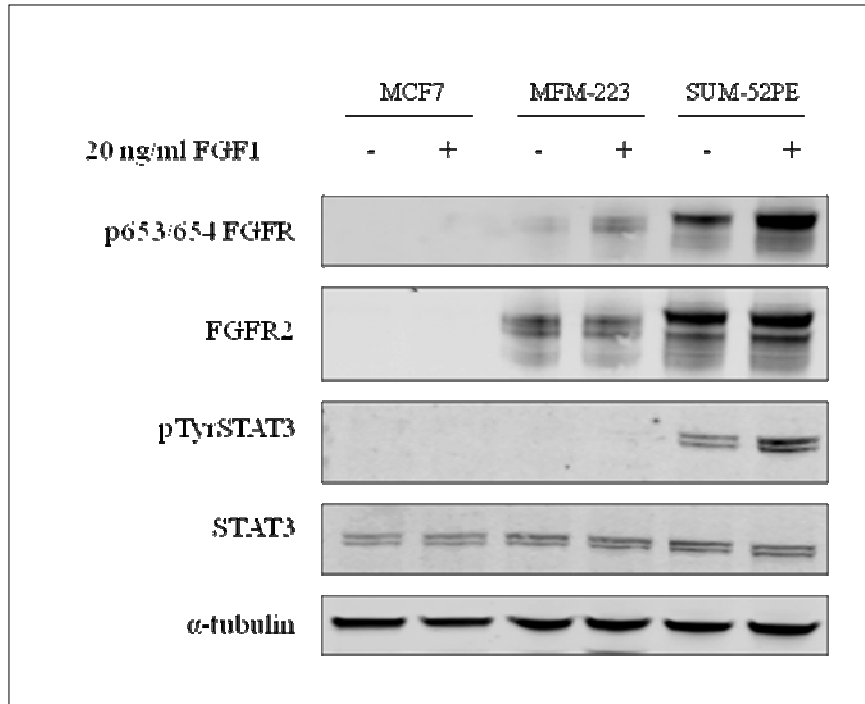


Figure 38. Comparison of FGFR2 levels in MCF7, MFM-223 and SUM-52PE cell lines.

MCF7, MFM-223 and SUM-52PE cells were serum-starved, stimulated with 20 ng/ml and 10 µg/ml heparin for 20 minutes. Cells were lysed, equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

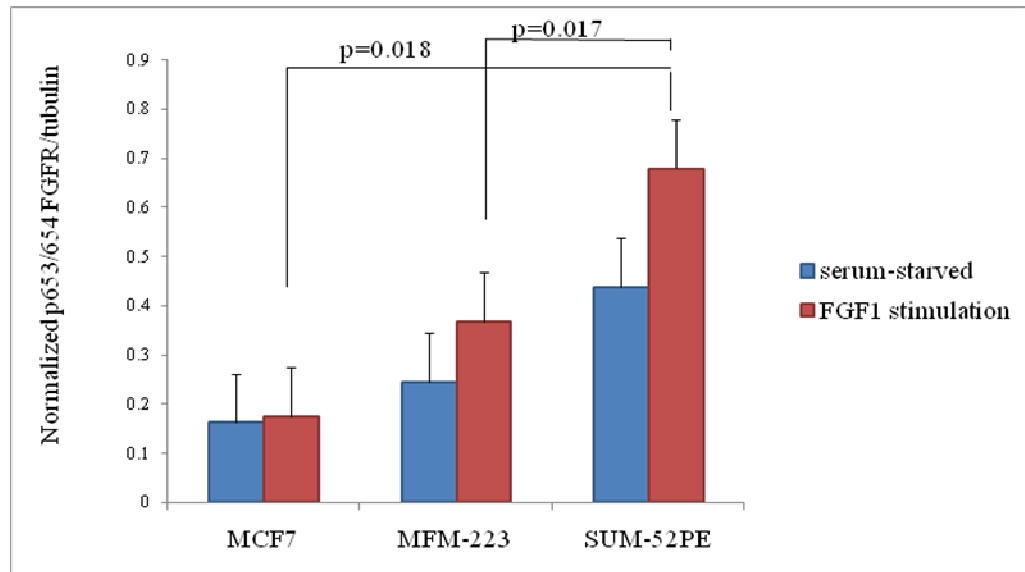
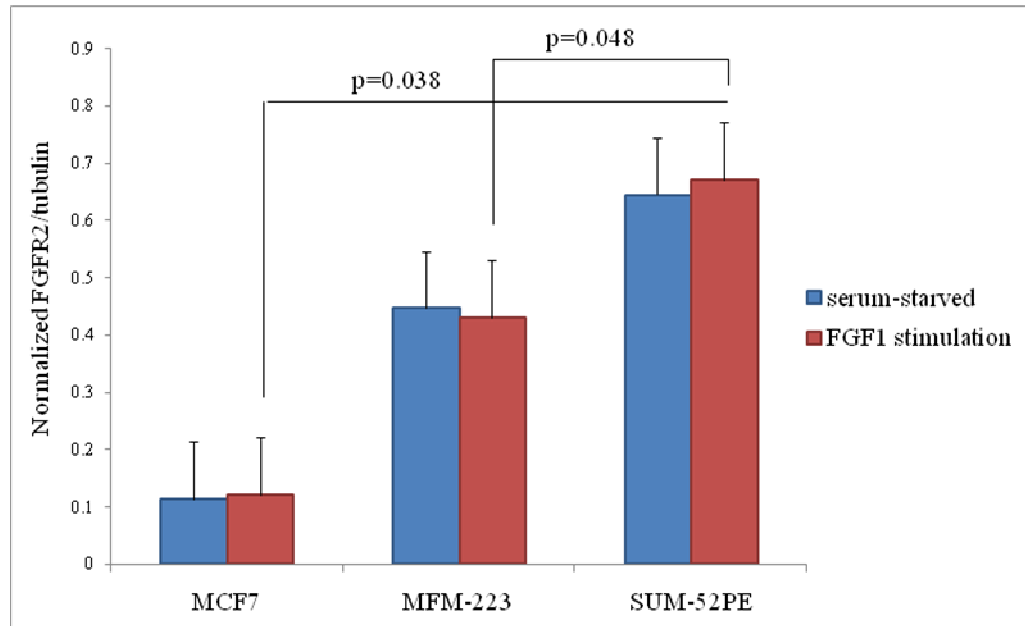


Figure 39. Densitometric analysis of FGFR2 levels.

Densitometric analysis of FGFR2 and p653/654 FGFR blots from Figure 38. FGFR2 and p653/654 FGFR were normalized to α -tubulin. Data obtained from three separate experiments. Error bars, SD.

2.8. MDA-MB-134 and MDA-MB-453 cell lines

MDA-MB-134 and MDA-MB-453 are breast cancer cell lines with amplified FGFRs (McLeskey, Ding et al. 1994). MDA-MB-134 cells are estrogen receptor-positive, they have an amplified FGFR1 gene and express a high level of mRNA for FGFR1 (McLeskey, Ding et al. 1994). MDA-MB-453 was described to express elevated levels of FGFR4 without gene amplification (McLeskey, Ding et al. 1994). Over-expression of FGFR1 has been recently described as common in classic lobular carcinomas (CLC) (Reis-Filho, Simpson et al. 2006; Xian, Pappas et al. 2009). MDA-MB-134 holds distinct gains of 8p12-p11.2 which are suggested to contribute to the survival rate of MDA-MB-134 due to FGFR1 amplification (Reis-Filho, Simpson et al. 2006).

Even though FGFR1 protein expression in MDA-MB-134 cells is higher, no tyrosine STAT3 activation was detected (Figure 40 A). Similarly, FGF1 stimulation of MDA-MB-453 cells did not induce TyrSTAT3 phosphorylation (Figure 40 B). STAT3 was serine phosphorylated in both cell lines (Figure 40 A and B). The signal from p653/654 FGFR antibody was not detectable in MDA-MB-453 cells (data not shown) suggesting low levels of expression of any of FGFR proteins. MDA-MB-134 cells expressed a detectable level of FGFR1 (Figure 40 A). The comparison of p653/654 FGFR activation between MCF7, MFM-223, SUM-52PE and MDA-MB-134 cell lines was also performed (Figure 41). As shown graphically, the phosphorylation level of FGFR1 was much lower in MDA-MB-134 compared with SUM-52PE cells suggesting that the level of FGFR1 protein was not high enough to induce TyrSTAT3 phosphorylation (Figure 41).

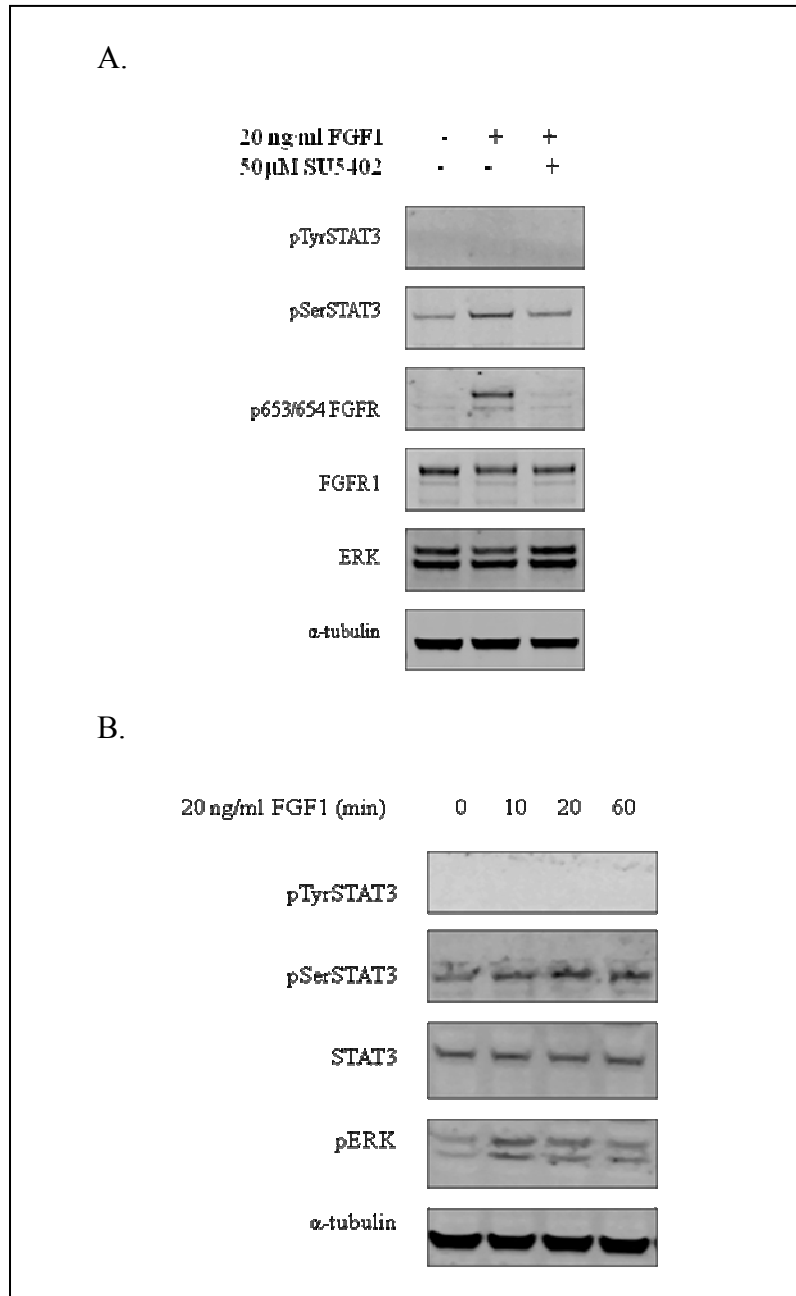


Figure 40. STAT3 phosphorylation in MDA-MB-134 and MDA-MB-453 cells.

- A. MDA-MB-134 cells were serum-starved, treated with 50 μM SU5402 or DMSO as control for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μg/ml heparin for 20 minutes. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.
- B. MDA-MB-453 cells were serum-starved and stimulated with 20 ng/ml FGF1 and 10 μg/ml heparin as indicated. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

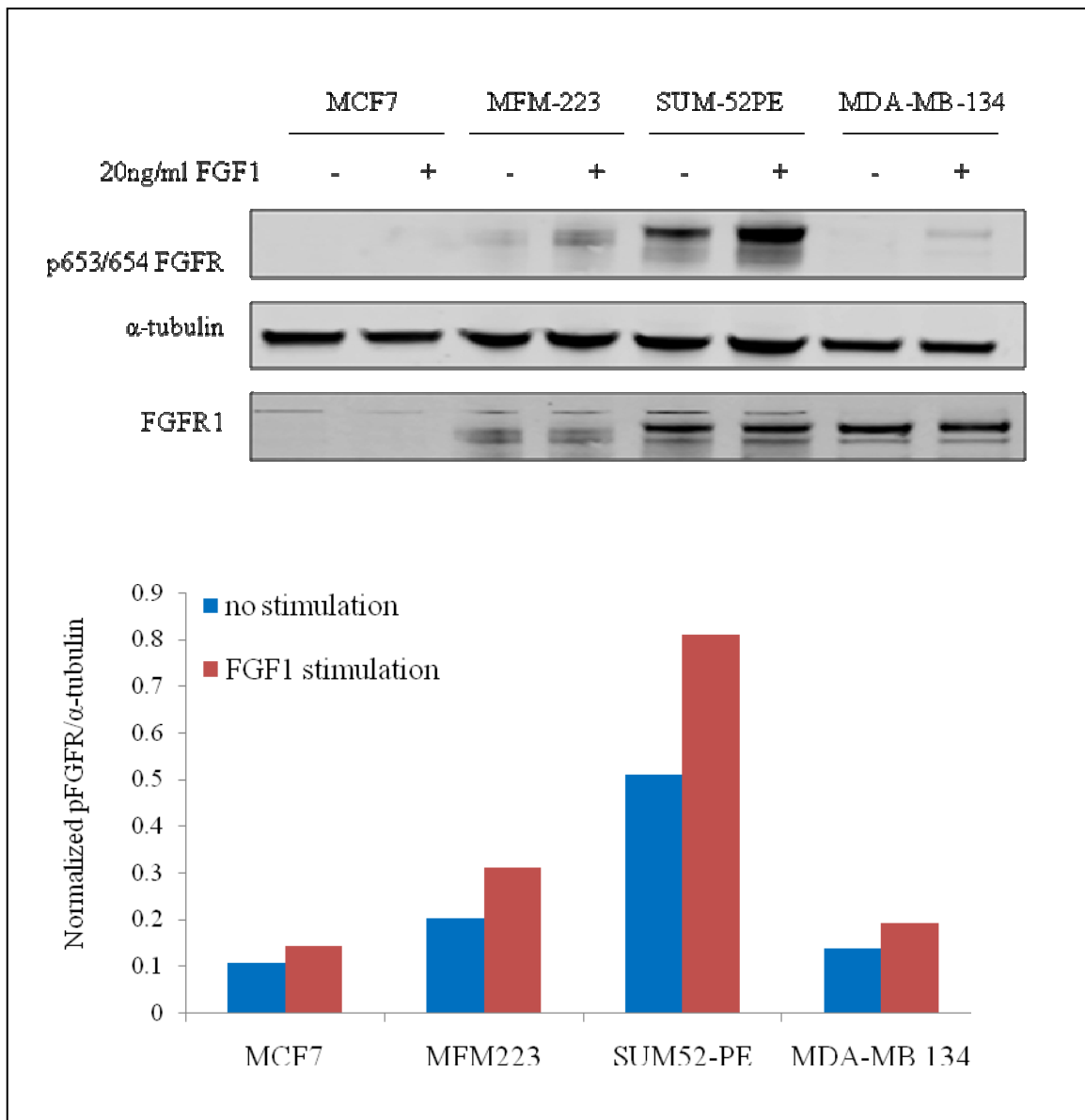


Figure 41. Comparison of p653/654 FGFR levels.

MCF7, MFM-223, SUM-52PE and MDA-MB-134 cells were serum-starved, stimulated with 20 ng/ml and 10 μg/ml heparin for 20 minutes. Cells were lysed, equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

Densitometry analysis of p653/654 FGFR normalized to α-tubulin. Data obtained from one experiment.

2.9. ZR-75-1 cell line

ZR-75-1 cell line has been shown to have FGFR1 amplification (Paterson, Pole et al. 2007) however, no over-expression of the protein (Reis-Filho, Simpson et al. 2006). ZR-75-1 cells were also tested for FGF1-induced STAT3 phosphorylation, but no TyrSTAT3 phosphorylation was observed (Figure 42). The FGFR1 expression in ZR-75-1 cells was compared to HEK293T cells over-expressing FGFR1 WT by probing with FGFR1 antibody (Figure 42). The level of FGFR1 was barely detectable in ZR-75-1 cells suggesting no over-expression of FGFR1. These data are with agreement with results from other group (Reis-Filho, Simpson et al. 2006).

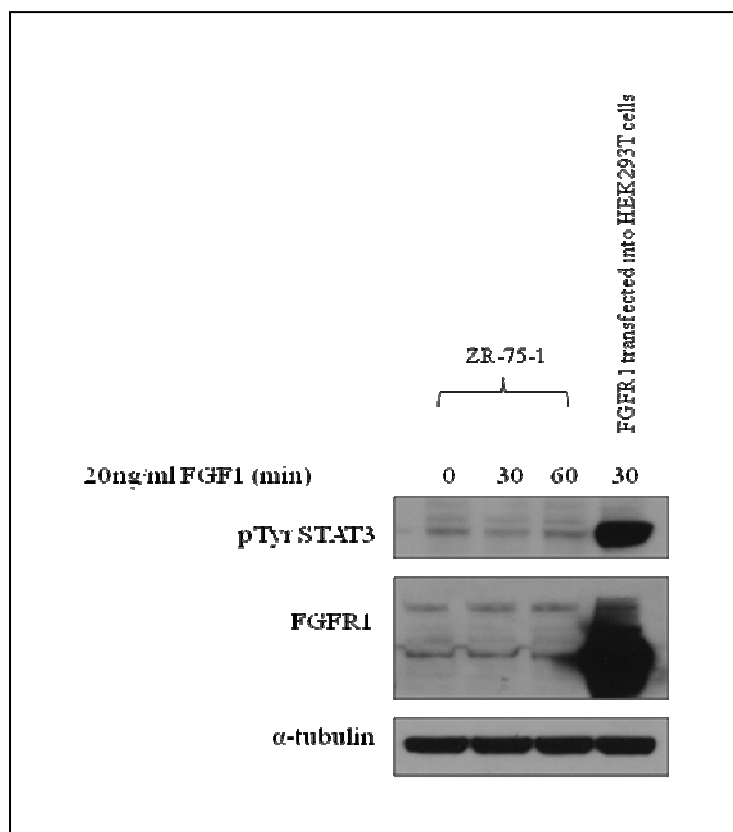


Figure 42. FGFR1 level in ZR-75-1 cells.

ZR-75-1 cells were serum-starved and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for indicated time. Positive control is obtained from HEK293T cells transiently transfected with FGFR1 WT and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin. WCLs were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

2.10. STAT3 activation in cells harbouring FGFR mutations

FGF-mediated activation of STATs was previously described in cells harbouring FGFR3 activating mutations (Su, Kitagawa et al. 1997; Li, Chen et al. 1999; Legeai-Mallet, Benoist-Lasselin et al. 2004). Several cell lines were tested in order to check if FGF1 induced TyrSTAT3 activation in cells harbouring an FGFR mutation.

2.10.1. MEF and Bac16 cells

STAT3 activation was tested in MEF (mouse embryonic fibroblasts) and Bac16 cells. Bac16 cells carry an activating mutation S252P that leads to increased affinity for ligands (Hajihosseini, Lalioti et al. 2004). This mutation is described as a cause of Pfeiffer syndrome (Muenke, Schell et al. 1994). Bac16 cells are mouse fibroblasts that express *Fgfr1* gene that harbours a single mutation corresponding to the human Pfeiffer syndrome (Hajihosseini, Lalioti et al. 2004).

Both cell types, MEF and Bac16, were stimulated with FGF1 and activation of STAT3 and MAPK pathway were checked by probing whole cell lysates with pTyrSTAT3 and pERK antibodies, respectively. Equal loading was verified by probing with ERK. Figure 43 showed no activation of TyrSTAT3 in Bac16 cells and a background level of TyrSTAT3 in MEFs, whereas, OSM stimulation induced strong TyrSTAT3 phosphorylation.

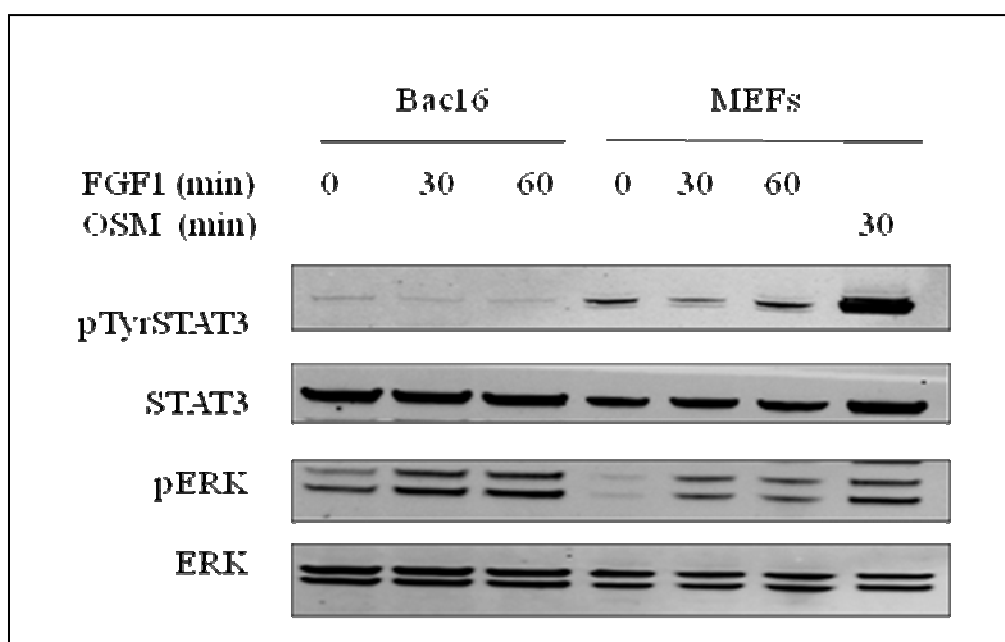


Figure 43. STAT3 phosphorylation in Bac16 and MEF cells.

MEF and Bac16 cells were serum-starved and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin or 50 ng/ml OSM for indicated time. Whole cell lysates were analysed by Western blotting.

2.10.2. Immortalized normal human urothelial cells

Immortalized normal human urothelial cells (TERT-NHUC) (di Martino, L'Hote C et al. 2009) were also used to check tyrosine STAT3 activation. Cells harbouring several different mutations were used. MK5274 IIIB is a wild type FGFR3 3b isoform, MK5275 S249C is a common activating mutation leading to ligand-independent dimerization of the receptors (Adar et al., 2002; d'Avis et al., 1998). MK5276 PLC binding site mutant expresses S249C FGFR3 with a point mutation, Y762F, which prevents PLC γ binding, MK5277 K510A expresses S249C FGFR3 with a point mutation of the kinase loop, K510A, which completely prevents phosphorylation and MK5253 PFB- empty vector (control cells without FGFR3 over-expression). All types of cells were stimulated with FGF1 and TyrSTAT3 phosphorylation was verified by probing with pTyrSTAT3 antibody. No tyrosine phosphorylation of STAT3 was detected (Figure 44). Similarly, STAT1 phosphorylation was not observed in previous studies (di Martino, L'Hote C et al. 2009). Surprisingly, activation of ERK was decreased after stimulation (Figure 44), which disagrees with results obtained by di Martino and colleagues (di Martino, L'Hote C et al. 2009).

Thus, experiments where Bac16 and TERT-NHUC cells were used, suggested that tyrosine STAT3 phosphorylation did not depend on activating mutation in FGF receptors, but rather on elevated level of FGFRs (as shown in SUM-52PE cells).

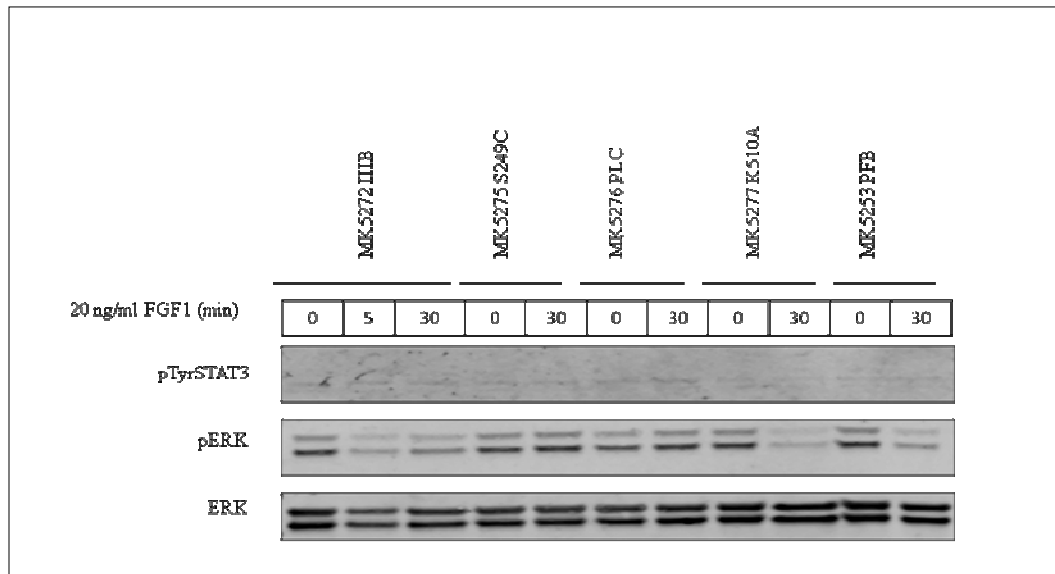


Figure 44. STAT3 phosphorylation in TERT-NHUC cells.

TERT-NHUC were serum-starved and stimulated with 20 ng/ml FGF1 and 10 µg/ml heparin for indicated time. Whole cell lysates were analysed by Western blotting.

MK5274 IIIB (wild type FGFR3 3b isoform)

MK5275 S249C most common activating FGFR3 mutations in bladder tumours

MK5276 PLC binding site mutant expresses S249C FGFR3 with a point mutation, Y762F, which prevents PLCγ binding

MK5277 K510A expresses S249C FGFR3 with a point mutation of the kinase loop, K510A, which prevents phosphorylation

MK5253 PFB- empty vector (control cells without FGFR3 overexpression)

3. Knocking-down FGFR2 in cells over-expressing receptor

In order to validate the data showing that elevated level of FGFR2 causes tyrosine STAT3 activation, the effect of receptor depletion was studied. Several attempts were undertaken to knock-down FGFR2 in SUM-52PE cells. Because SUM-52PE cell line is difficult to transfect, the depletion of FGFR2 was not complete. SUM-52PE cells were transfected with siRNA oligonucleotides against FGFR2 or control siRNA. Cells were stimulated with FGF7 in order to target specifically FGFR2, not other FGF receptors. The efficiency of the knock-down was verified by probing whole cell lysates with FGFR2 antibody, whereas activation of the receptor was tested by probing with p653/654 FGFR antibody. The activation of tyrosine STAT3 and expression of total STAT3 was verified by probing with STAT3 specific antibodies. Equal loading was checked by α -tubulin antibody.

Decreased expression of FGFR2 reduced tyrosine STAT3 phosphorylation, even though the knock-down was not complete (Figure 45 and Figure 46). This result confirmed that FGFR2 activity is essential for FGF-induced tyrosine STAT3 phosphorylation in SUM-52PE cells. The same effect was observed by use of SU5402 and PD173074, where FGFR2 inhibition reduced activation of STAT3 (Figure 34).

Additionally, depletion of the receptor in HEK293T cells over-expressing FGFR2 was tested. HEK293T were transiently transfected with FGFR2-GFP construct and siRNA against FGFR2. The expression of FGFR2 was verified by probing whole cell lysates with FGFR2 antibody, whereas activation of the receptor was tested by probing with p653/654 FGFR antibody. The activation of tyrosine STAT3 and expression of total STAT3 was verified by probing with STAT3 specific antibodies. Equal loading was checked by α -tubulin antibody.

As shown in Figure 47 the level of FGFR2 was decreased in samples where siRNA against FGFR2 were used. Moreover, tyrosine STAT3 activation was reduced in samples expressing lower level of the receptor (Figure 47). Despite the fact that the knock-down of FGFR2 was not complete and the phosphorylation of tyrosine STAT3 was not blocked entirely, the difference between sample over-expressing FGFR2 and samples where FGFR2 was knocked-down, was statistically significant (Figure 47).

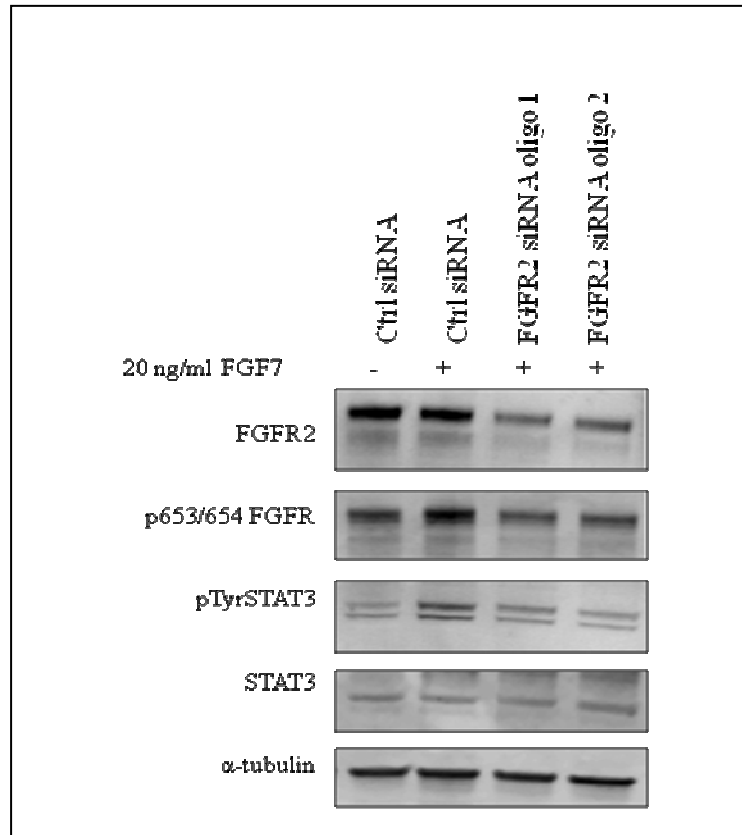


Figure 45. FGFR2 knock-down in SUM-52PE cells.

SUM-52PE cells were transiently transfected using DharmaFECT3 with siRNA against FGFR2 or Ctrl siRNA.

After 48 hours cells were serum-starved and stimulated with 20 ng/ml FGF7 and 10 μ g/ml heparin for 20 minutes. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

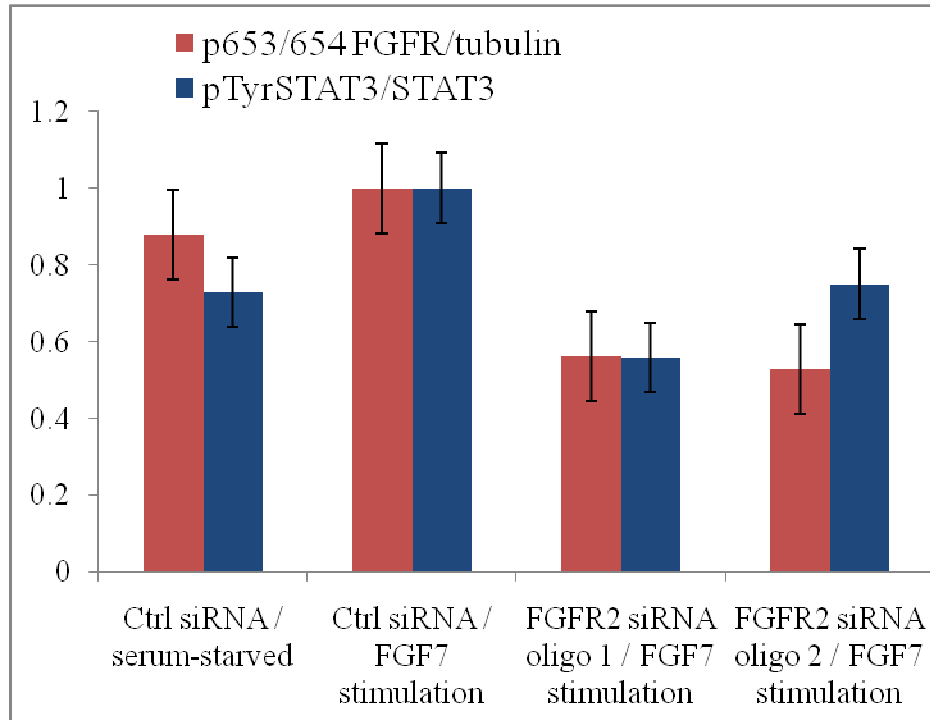


Figure 46. Densitometric analysis of 653/654 FGFR and TyrSTAT3 in SUM-52PE FGFR2-depleted cells.

Densitometric analysis of p653/654 FGFR normalized to α -tubulin and pTyrSTAT3 normalized to STAT3 was performed from three separate experiments.

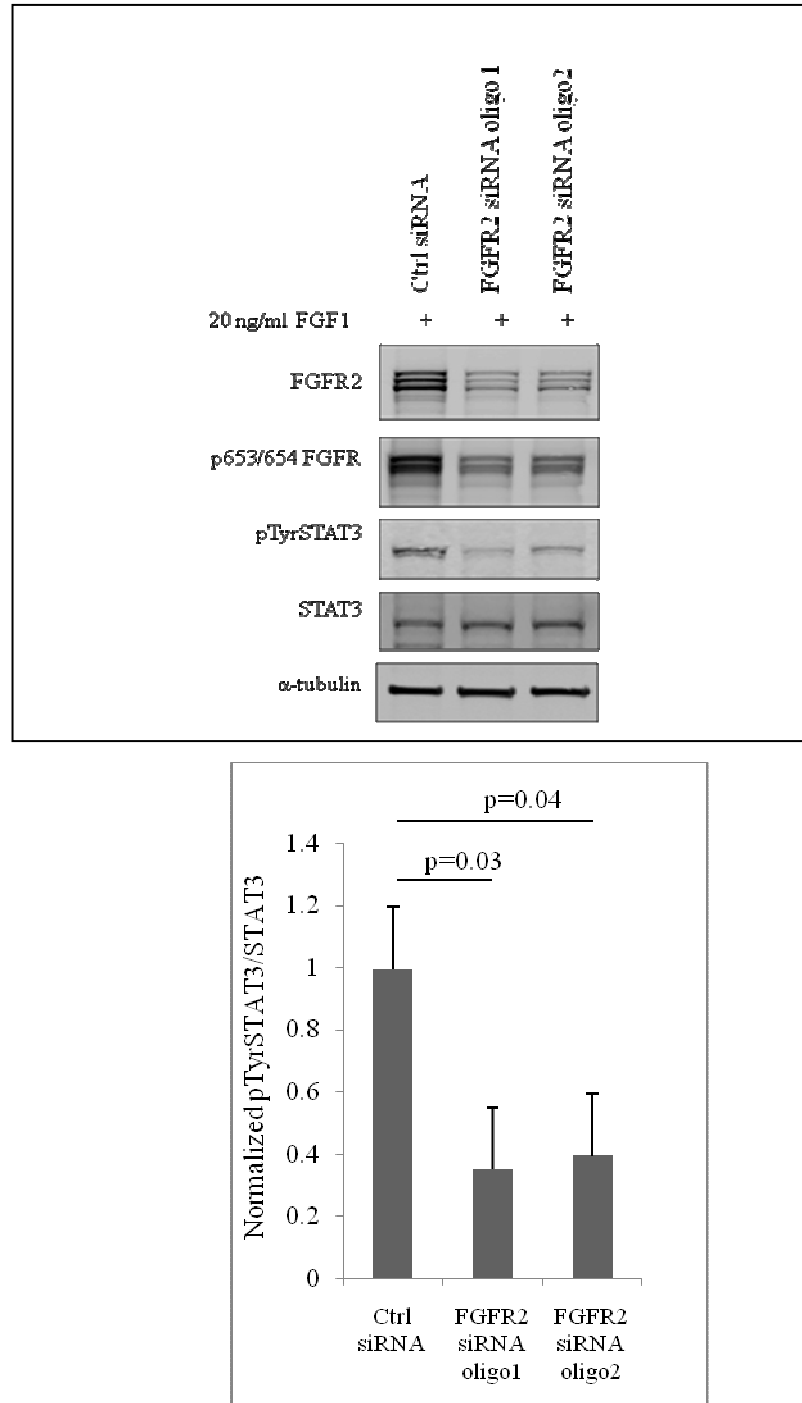


Figure 47. FGFR2 knock-down in HEK293T cells.

HEK293T cells were transiently transfected using Lipofectamine2000 with FGFR2-GFP and siRNA against FGFR2 or Ctrl siRNA. After 48 hours cells were serum-starved and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies. Densitometric analysis for normalized pTyrSTAT3/STAT3 from three separate experiments is presented on the graph.

4. Transcriptional activity of STAT3 in cells over-expressing FGFRs

STAT3 has a combined role as a signal transducer and regulator of transcription. The nuclear activity of STAT3 involves binding to DNA regions and activation of gene transcription. JunB, c-fos and c-myc are examples of the genes regulated by STAT3 (Fujitani, Nakajima et al. 1994; Kiuchi, Nakajima et al. 1999).

Transcriptional activity of STAT3 was investigated by the expression of JunB, c-myc and c-fos. SUM-52PE cells were stimulated for 4 hours with FGF1 and the expression level of STAT3 gene targets was examined. An increased amount of JunB, c-fos and c-myc suggested that STAT3 was transcriptionally active in SUM-52PE cells after FGF1 stimulation (Figure 48). Additionally, SUM-52PE cells were treated with Cucurbitacin I, a STAT3 inhibitor (van Kester, Out-Luiting et al. 2008) to confirm that the observed effect was due to STAT3 activity. The expression of c-fos, JunB and c-myc was verified in cells stimulated with FGF1 for 4 hours and in cells treated with Cucurbitacin I. The efficiency of the inhibitor was tested by probing with pTyrSTAT3 antibody, whereas equal expression was verified by probing with STAT3 and α -tubulin antibodies. It was concluded that reduced expression of STAT3 gene targets in samples treated with inhibitor was due to inhibition of STAT3 activation (Figure 49). The difference between cells treated with Cucurbitacin I and cells stimulated with FGF1 was statistically significant for all STAT3 gene targets.

Furthermore, similar experiment was performed in HEK293T cells over-expressing FGFR1 WT. Cells were stimulated for 4 hours with FGF1 and the level of JunB and c-myc was checked by probing with specific antibodies. Equal expression of FGFR1 constructs was verified by probing with FGFR1 antibody. Before FGF1 stimulation, cells were treated with Cucurbitacin I. Figure 50 showed increased expression of JunB and c-myc which was reduced

FGF-induced STAT3 activation

with the use of Cucurbitacin I. The difference between cells treated with inhibitor and cells stimulated with FGF1 was statistically significant ($p < 0.05$). This suggested that STAT3 activation was required for FGF1-induced expression of JunB and c-myc.

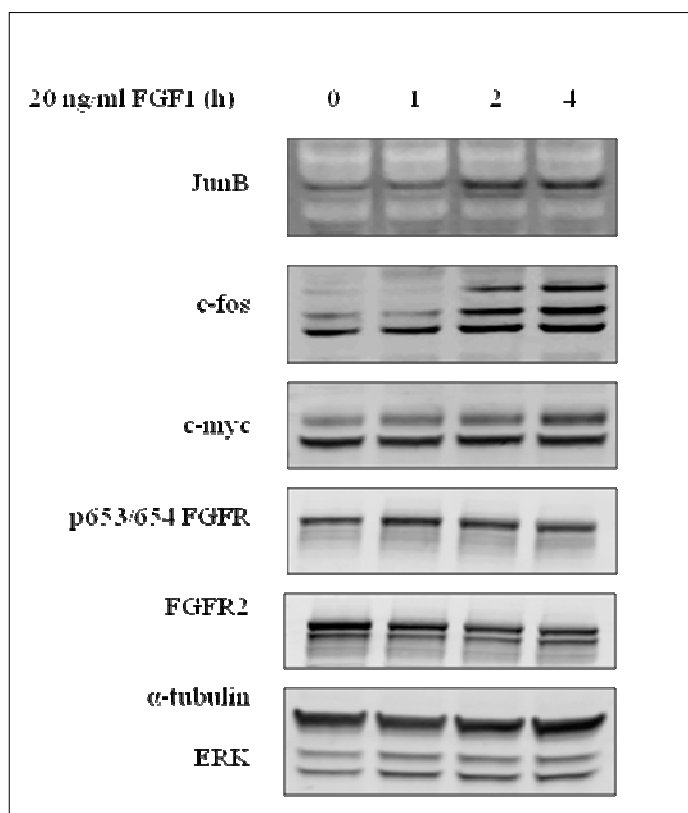


Figure 48. Expression of STAT3 gene targets in SUM-52PE cells.

SUM-52PE cells were serum-starved and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for indicated time. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

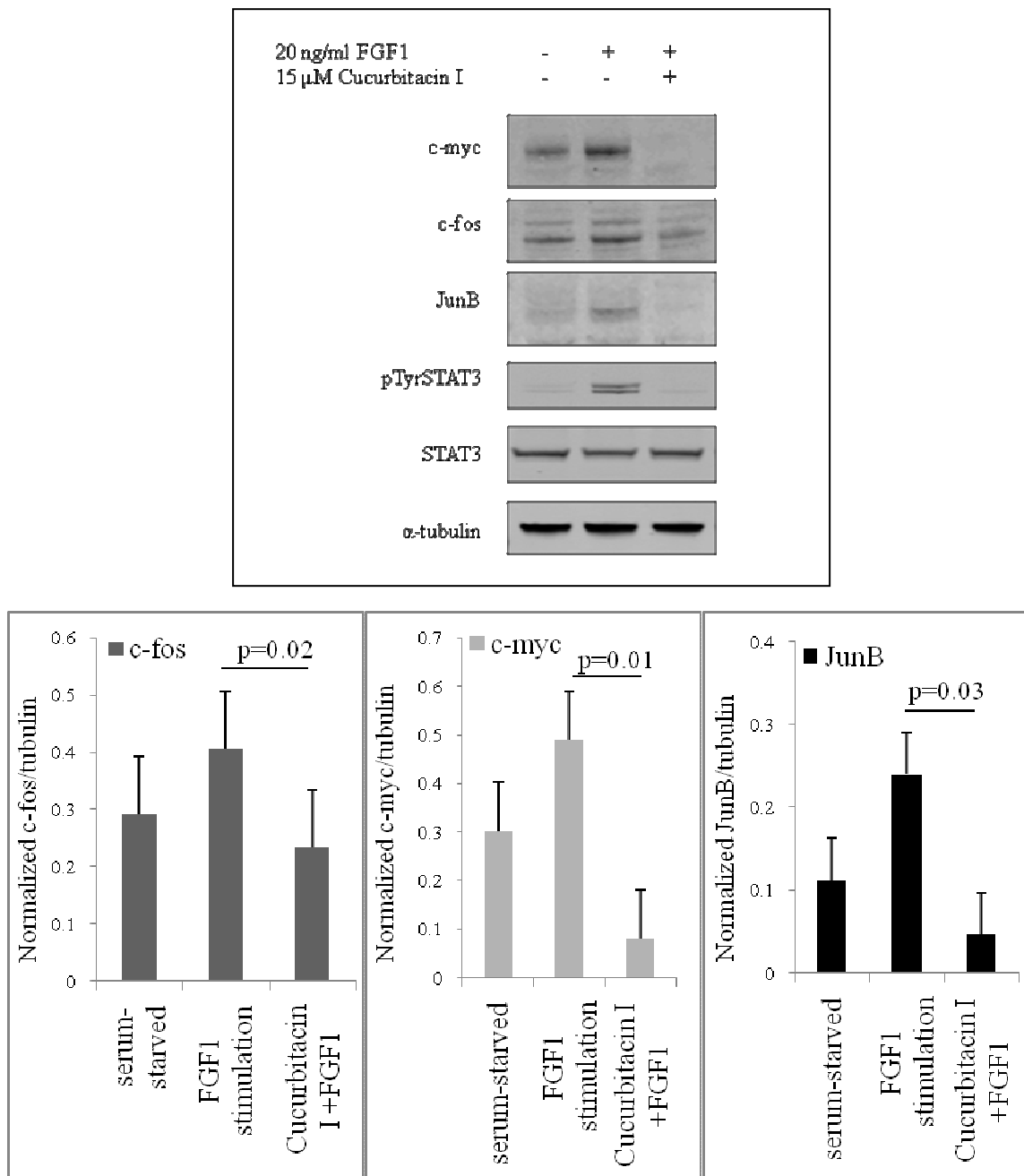


Figure 49. Effect of Cucurbitacin I on c-myc, JunB and c-fos expression in SUM-52PE cells.

SUM-52PE cells were serum-starved, treated with 10 μ M Cucurbitacin I for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 4 hours. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies. Densitometric analysis for c-fos, JunB and c-myc was performed from three separate experiments. T-test for c-fos and c-myc expression in sample treated with inhibitor was calculated versus sample stimulated with FGF1 for 4 hours.

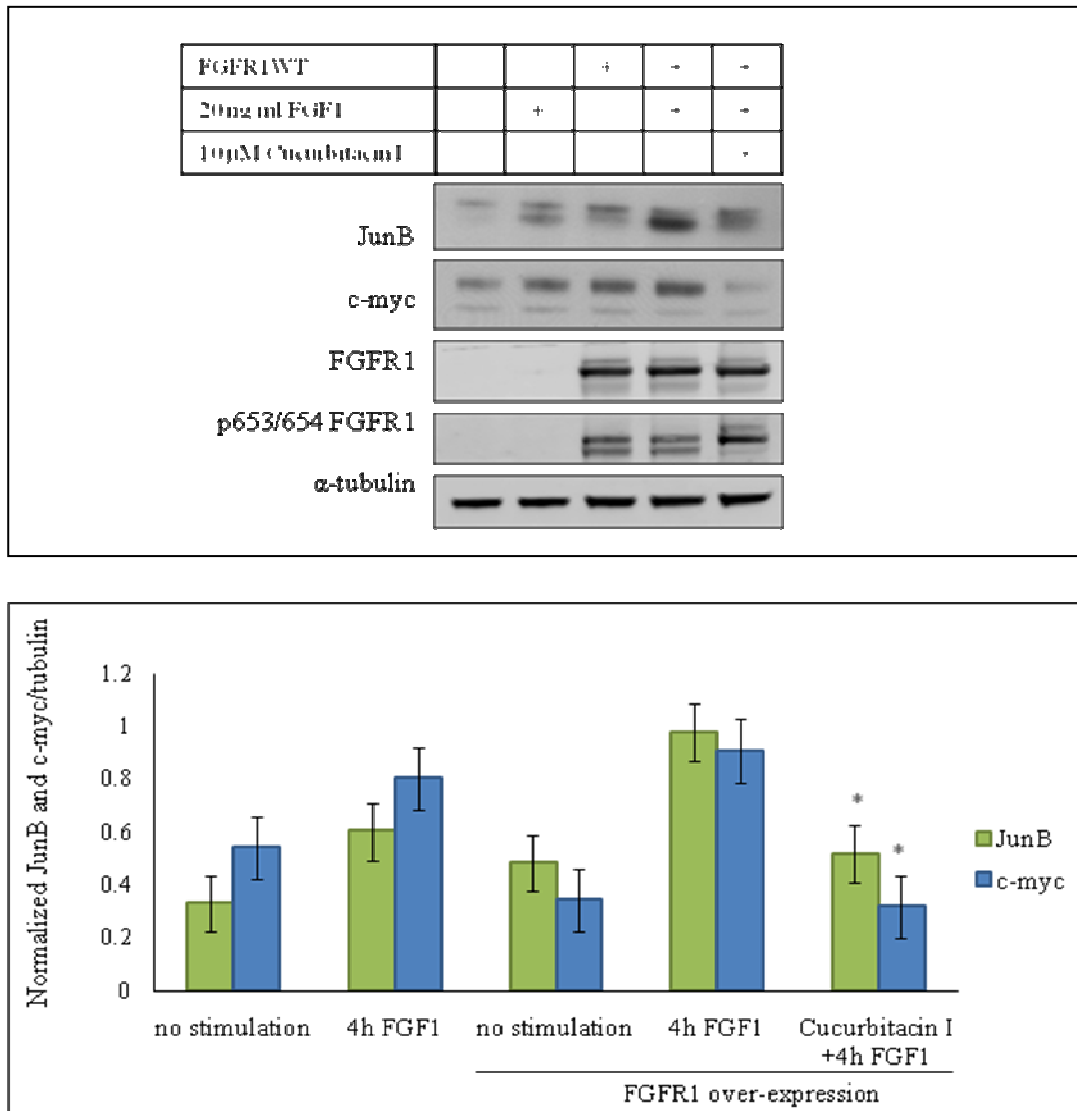


Figure 50. Effect of Cucurbitacin I on expression of JunB and c-myc in HEK293T cells.

HEK 293T cells were transfected with FGFR1. After 48 hours cells were starved, treated with 10 µM Cucurbitacin I for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 µg/ml heparin for 4 hours. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF and probed with antibodies. Densitometric analysis for JunB and c-myc from three separate experiments is presented on the bottom graph. T-test for JunB and c-myc expression in sample treated with inhibitor was calculated versus sample stimulated with FGF1 for 4 hours.

*p<0.05

5. FGF1-induced phosphorylation of serine STAT3

Serine STAT3 phosphorylation plays an important role in regulation of STAT3 activity but the role of SerSTAT3 in FGFR signalling remains unclear. The effect of FGF1 stimulation on tyrosine and serine phosphorylation of STAT3 in several FGF responsive cell lines was examined but stimulation of endogenous FGFR1 had no detectable effect on STAT3 tyrosine activation. On the other hand, FGF-induced SerSTAT3 phosphorylation did not rely on high level of FGFRs (Figure 28). In order to examine which Ser/Thr kinases phosphorylate SerSTAT3 several inhibitors were used: FGFR inhibitor (SU5402), Src inhibitor (SU6656), Jak Inhibitor I, MEK inhibitor (U0126), JNK inhibitor (SP600125), p38 inhibitor (SB203580) and PKC inhibitor (calphostin C). The activation of STAT3 was verified by probing with specific antibodies. MAPK pathway activity was checked by probing with pERK antibody, whereas equal loading was verified by probing with ERK and α -tubulin.

A significant decrease in serine STAT3 phosphorylation with U0126 and SP600125 treatment was observed (Figure 51 A and B). These data suggest that SerSTAT3 was phosphorylated downstream of the JNK and MAPK pathways which were both activated upon FGF1 stimulation. When FGFR1 and Src were inhibited, with SU5402 and SU6656 respectively, the level of phospho-SerSTAT3 was significantly decreased (Figure 51 A and B), presumably due to lack of JNK and MEK activation. Treatment with Jak Inhibitor I, SB203580 and calphostin C did not affect SerSTAT3 activation. No tyrosine STAT3 activation was detected (Figure 51 A). Thus, JNK and MEK kinases were responsible for serine STAT3 phosphorylation which was a downstream effect of FGF1-mediated signalling.

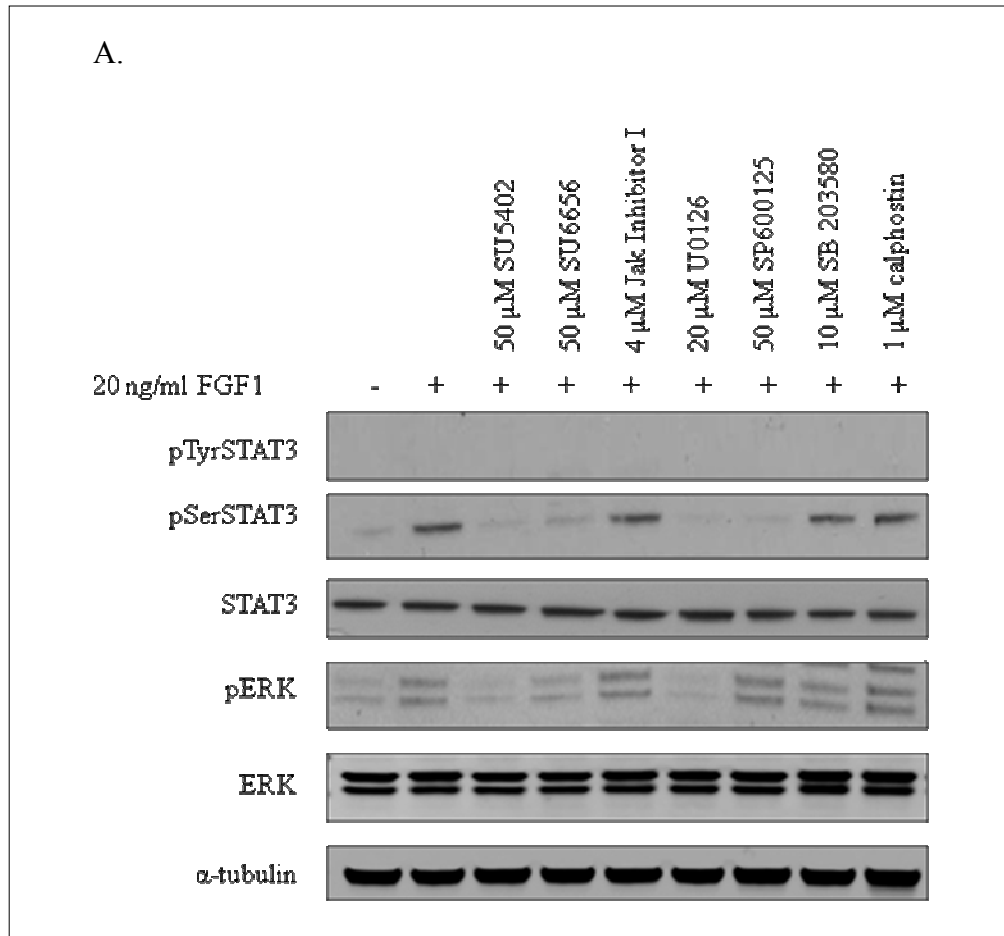


Figure 51. Effect of Tyr and Ser/Thr inhibitors on serine STAT3 phosphorylation.

- A. MCF7 cells were serum-starved, treated with inhibitors, 50 μ M SU5402, 50 μ M SU6656, 4 μ M JAK inhibitor I, 20 μ M U0126, 50 μ M SP600125, 10 μ M SB203580, 1 μ M calphostin C, or DMSO for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

B.

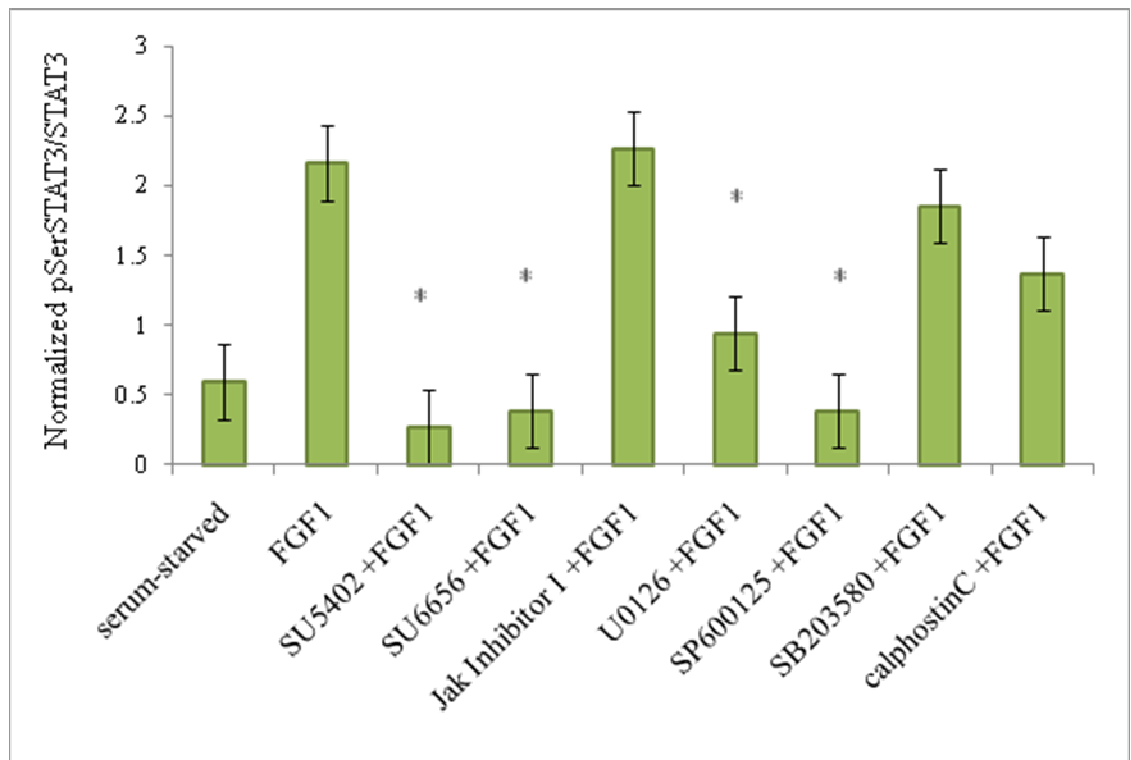


Figure 51. Effect of Tyr and Ser/Thr inhibitors on serine STAT3 phosphorylation.

B. Densitometric analysis of Western blots for pSerSTAT3 normalized to STAT3 was performed from three separate experiments. Error bars, SD. Statistical significance was determined by t-test comparison between each sample treated with inhibitor and sample stimulated with FGF1 (*, $P < 0.05$).

Recently, an involvement of STAT3 in the electron transport chain (ETC) in mitochondria was described (Wegrzyn, Potla et al. 2009). The exact role of STAT3 in ETC is still unknown however, interestingly, only serine phosphorylated STAT3 was necessary for STAT3 activity in regulation of ETC, whereas tyrosine phosphorylation and the DNA binding domain were dispensable (Wegrzyn, Potla et al. 2009). Since FGF1 stimulation induced only SerSTAT3 activation in cells expressing normal levels of FGFRs, it was decided to test if SerSTAT3 localized in the mitochondria after FGF1 stimulation. FGF1-stimulated MCF7 cells were stained with Mitotracker, a specific marker for mitochondria and pSerSTAT3 antibody. The co-localization was verified by merging both pictures however, no significant co-localisation was observed (Figure 52). This suggests that FGF1-induced serine phosphorylated STAT3 was not localized in mitochondria in this case and it may not play a role in regulation of ETC.

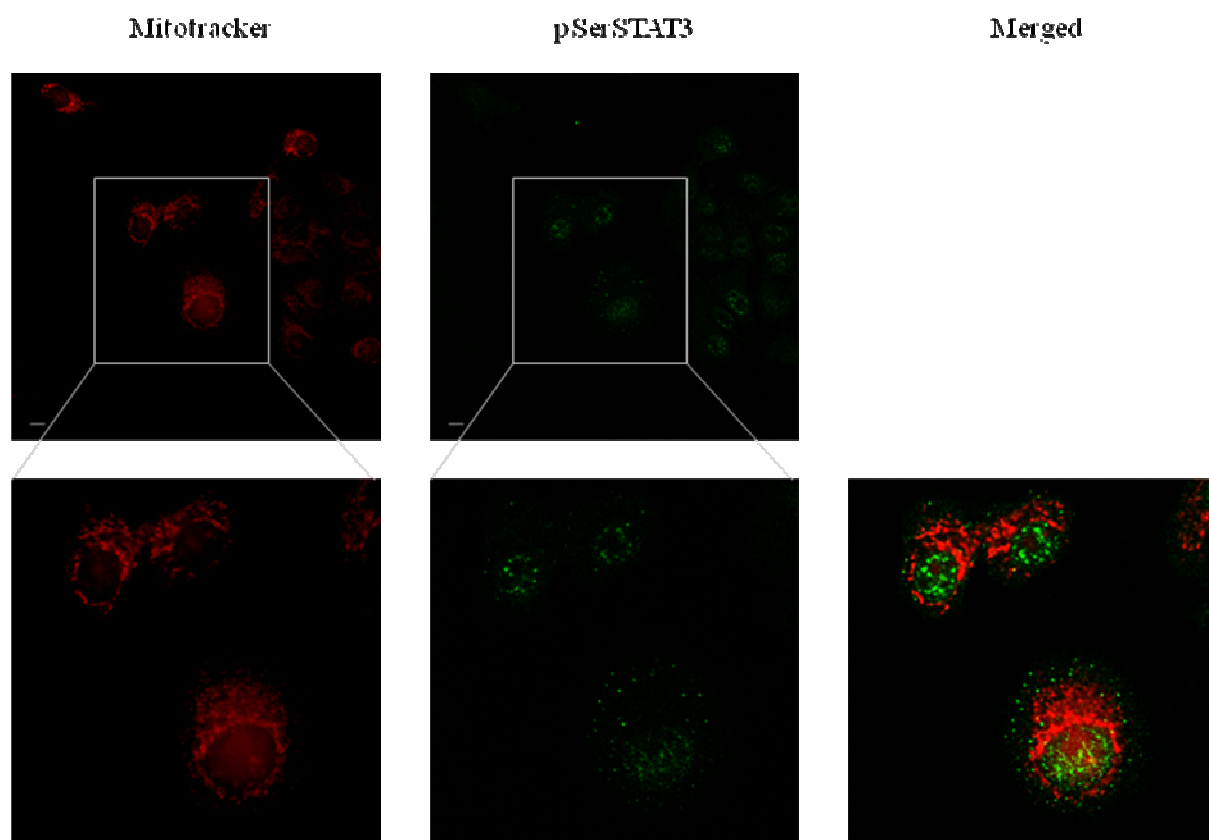


Figure 52. Mitotracker and pSerSTAT3 co-localisation.

MCF7 cells were serum-starved, treated with 100 nM Mitotracker for 15 minutes and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Cells were fixed, permeabilized using methanol protocol and stained with SerSTAT3 and anti-mouse secondary conjugated with FITC. Scale bars 10 μ m.

Discussion

The aim of this chapter was to examine STAT3 phosphorylation upon FGF stimulation. Several cell lines were examined for tyrosine and serine STAT3 activation by a series of biochemical experiments: SerSTAT3 phosphorylation was observed at endogenous level of FGFRs in all cell lines, whereas TyrSTAT3 phosphorylation required over-expression of FGFRs. Apart from over-expression of FGFRs, TyrSTAT3 activation required phosphorylation of the receptor, which indicates that this is a phospho-dependent process. Over-expressed FGFR recruited STAT3 to its cytoplasmic region, pY₆₇₇XXQ, and bound STAT3 via its' SH2 domain. Mutation of tyrosine 677 residue to phenylalanine decreased FGFR1-STAT3 association and STAT3 phosphorylation, indicating the importance of physical interaction between these proteins.

According to recent literature, the common activating mutation in FGFR3, K650E, which causes conformational changes in activation loop (Webster, D'Avis et al. 1996), leads to phosphorylation of STAT1 and STAT3 (Su, Kitagawa et al. 1997; Li, Chen et al. 1999; Legeai-Mallet, Benoist-Lasselin et al. 2004). The same mutation generated in other FGFRs (FGFR1, FGFR2 and FGFR4) results in a similar activation of both STATs (Hart, Robertson et al. 2000). STAT1 was also described as a downstream substrate for FGF signalling that regulates apoptosis and proliferation of chondrocytes (Sahni, Ambrosetti et al. 1999; Sahni, Raz et al. 2001). On the other hand, Krejci *et al.* were unable to detect STAT1 or STAT3 tyrosine activation by FGF in RCS chondrocytes (Krejci, Salazar et al. 2008). However, the activation of STAT1, not STAT3, was observed with over-expression of FGFR3-K650E or kinase active FGFR3, but not cell-borne WT FGFR3 (Krejci, Salazar et al. 2008). The association between STAT1, STAT3 and FGFR3 containing an activating mutation was also

described (Krejci, Salazar et al. 2008). Recently, FGF-induced activation of STAT5 was described as necessary for angiogenesis (Yang, Qiao et al. 2009). STAT5 and STAT1, but not STAT3, can be activated by FGF2 and FGF8b in mouse microvascular endothelial cells and STAT5 activation was detected in samples of human gliomas (Yang, Qiao et al. 2009).

The activation of STATs by other growth factor receptors, like EGFR, VEGFR, was also described (Leaman, Pisharody et al. 1996; Park, Schaefer et al. 1996; Vignais, Sadowski et al. 1996; Olayioye, Beuvink et al. 1999; Wang, Wharton et al. 2000). Often TyrSTATs phosphorylation is induced in cells over-expressing GFRs which is linked with tumourigenesis. Kloth *et al.* claimed that EGF-induced STAT5b activation requires over-expression of EGFR, which was demonstrated in HEK293T cells transiently transfected with EGFR, as well as in MDA-MB-468 breast cancer cell line (Kloth, Catling et al. 2002; Kloth, Laughlin et al. 2003). Furthermore, TyrSTAT3 phosphorylation in MDA-MB-468 cells was described as pro-oncogenic (Garcia, Bowman et al. 2001). Ren *et al.* described that constitutively active mutant of ErbB-2 was capable of TyrSTAT3 activation (Ren and Schaefer 2002). On the contrary, WT ErbB-2 did not induce the same effect (Ren and Schaefer 2002). Similarly, STAT3 activation was described in squamous carcinoma cells (SCC) as downstream effect of EGFR signalling which was limited to malignant keratinocytes (Quadros, Peruzzi et al. 2004). Thus, constitutive activation of STATs induced by various oncogenes, like aberrant tyrosine kinase activity, maintains cell survival, inhibits apoptosis and stimulates proliferation - features typical for cancer development (Bowman, Garcia et al. 2000).

Based on the results obtained in this report, it was hypothesised that the FGFR-STAT3 signalling pathway might be induced in tumour cells with amplified expression of FGFRs. Evidence supporting this theory was derived from two models. In the first model, transient

transfection of FGFR1 WT and FGFR2-GFP in HEK293T or HeLa cells induced TyrSTAT3 activation in contrast to cells with endogenous level of FGFRs. In a second model, FGF stimulation of breast cancer cells SUM-52PE, which naturally over-express FGFR2, also led to TyrSTAT3 phosphorylation. Moreover, translocation of STAT3 into the nuclei and increased expression of JunB, c-myc or c-fos were observed in both models. FGF stimulation of SUM-52PE cells caused an enhanced TyrSTAT3 phosphorylation which was significantly decreased by SU5402 and PD173074 treatment. The knock-down of FGFR2 in SUM52-PE confirmed the result obtained using chemical inhibitors. The high level of FGFR was crucial to induce FGF-mediated STAT3 tyrosine phosphorylation. Therefore, both over-expression and activation of receptor was required for TyrSTAT3 phosphorylation.

Because FGFRs influence on broad range of cellular processes, their expression is highly regulated. Cells express FGFRs on the level that allows sufficient performance of its physiological functions, like MAPK pathway activation. On the other hand, abnormal expression of FGFR genes is often linked with development and progression of a variety of human cancers (Tannheimer, Rehemtulla et al. 2000; Reis-Filho, Simpson et al. 2006; Xian, Pappas et al. 2009). Activation of STAT3 was examined in cell lines which were described to have FGFR gene amplification or FGFR activating mutations. Only SUM-52PE cells were capable of inducing TyrSTAT3 phosphorylation due to very high level of FGFR2 expression compared with other cell lines. MFM-223 cells that also expressed higher level of FGFR2 induced weak phosphorylation of TyrSTAT3 but no tyrosine STAT3 phosphorylation was observed in MDA-MB-134, MDA-MB-453 or ZR-75-1 cells. ZR-75-1 cells, even though they were described to have FGFR1 amplification, do not over-express FGFR1 protein (Reis-Filho, Simpson et al. 2006; Paterson, Pole et al. 2007). Also the levels of FGFRs in MDA-MB-134 and MDA-MB-453 were not sufficient to induce tyrosine phosphorylation of

STAT3. Surprisingly, cells containing an activating mutation, Bac16 and TERT-NHUC, did not induce TyrSTAT3 phosphorylation either. Thus, tyrosine STAT3 phosphorylation by FGF depends strictly on high level of FGFR expression but not on activating mutations.

The role of serine phosphorylated STAT3 is more complex than previously thought and it still needs to be determined. Serine STATs phosphorylation was described as crucial for increased transcriptional activity but has no effect on DNA binding (Wen, Zhong et al. 1995; Wen and Darnell 1997). On the contrary, there is evidence for a negative role of SerSTAT3 which is inhibition of the function of tyrosine phosphorylated STATs (Chung, Uchida et al. 1997; Lim and Cao 1999). Udayakumar *et al.* demonstrated the induction of promatrilysin expression in prostate carcinoma cell line by FGF1-induced SerSTAT3 (Udayakumar, Stratton et al. 2002). Noteworthy, FGF-induced SerSTAT3 activates matrilysin promoter and phosphorylation of TyrSTAT3 is not necessary (Udayakumar, Stratton et al. 2002).

Here, it was shown that STAT3 serine activation was induced by ERK and JNK as a downstream effect of FGFR signalling. Moreover, it did not require over-expression of FGFRs. It is known that the PMSP motif on STAT3 is a substrate for ERK and JNK kinases (Chung, Uchida et al. 1997; Lim and Cao 1999). Likewise, p38 has been shown to play a key role in serine phosphorylation of STAT3 (Turkson, Bowman et al. 1999), however, we presented that p38 had no effect on STAT3 phosphorylation in MCF7 cells. Therefore, the choice of Ser/Thr kinases that phosphorylate SerSTAT3 is probably cell- and ligand-dependent.

Interestingly, serine phosphorylated STAT3 by FGF1 stimulation formed dot-like structures inside cells, especially in the nuclei. These kinds of vesicles were already described as STAT3 nuclear bodies that act as reservoirs of active STAT3 (Herrmann, Sommer et al.

2004). STAT3 is tyrosine and serine phosphorylated in nuclear bodies but it is unknown if SerSTAT3 has similar function as STAT3 activated on both residues. Dot-like structures of SerSTAT3 were also observed in the nucleus in the absence of KAP1 (repressor of transcription). KAP1 was identified as a novel binding partner for STAT3 that negatively regulates SerSTAT3 activity in the nucleus (Tsuruma, Ohbayashi et al. 2008). The correlation between FGF-induced SerSTAT3 and transcription activity needs to be further verified.

Recently, it was suggested that SerSTAT3 takes part in regulation of electron transport chain in mitochondria (Wegrzyn, Potla et al. 2009). Interestingly, SerSTAT3 activity in mitochondria supported Ras-dependent malignant transformation even though Ras does not induce STAT3 activation (Gough, Corlett et al. 2009). It was suggested that SerSTAT3 role in mitochondria of cancer cells involves augmenting electron transport chain activity. The loss of mitochondrial STAT3 activity impaired Ras transformation (Gough, Corlett et al. 2009). Herein, co-localization studies revealed that FGF1-stimulated SerSTAT3 was not translocated into the mitochondria, thus, it is likely that it did not play a role in ETC regulation.

CHAPTER 6

Src and Jak2 involvement in STAT3 activation

Introduction

The aim of this chapter was to investigate the involvement of Src and Jak2 in FGF-induced phosphorylation of STAT3. STATs are activated by a range of cytokines, including interferons and interleukins, growth factors and hormones. STATs activation by cytokines requires the presence of non-receptor kinases, Janus kinases (Jaks) which plays a vital role in STATs phosphorylation (Leaman, Leung et al. 1996). Firstly, Jaks phosphorylate the intracellular domain of the receptor which enables STATs recruitment to the receptor. Secondly, they activate STATs by direct phosphorylation of its tyrosine residue. Moreover, Jaks are necessary not only for cytokine signalling but also for some growth factor receptors (Vignais and Gilman 1999; Garcia, Bowman et al. 2001; Ren and Schaefer 2002). The second family of non-receptor tyrosine kinases involved in STATs pathway is the Src family. Src is a known oncogene and there are several reports describing STAT phosphorylation in cells expressing v-Src (Yu, Meyer et al. 1995; Bromberg, Wrzeszczynska et al. 1999).

1. The role of Src and Jak in STAT3 phosphorylation

In order to study the role of Src and Jak family members in FGFR-mediated activation of STAT3 in SUM-52PE cells, SU6656 and Jak Inhibitor I were used to block their activity. Additionally, SU5402 was used. Activation of STAT3 was verified by probing whole cell lysates with pSerSTAT3 and pTyrSTAT3 antibodies. Equal loading was checked by probing with STAT3 and FGFR2 antibodies, whereas, activation of FGFR2 was tested by probing with p653/654 FGFR antibody.

The result of pharmacological inhibition revealed that both non-receptor kinases were required for full activation of STAT3 by tyrosine phosphorylation (Figure 53). As expected,

tyrosine phosphorylation of STAT3 was significantly decreased with SU5402 treatment suggesting that FGFR2 kinase activity was crucial (Figure 53 A and B). In addition, Src and Jak inhibitors reduced TyrSTAT3 activation indicating that both kinases mediated TyrSTAT3 phosphorylation in FGF1-stimulated SUM-52PE cells (Figure 53 A and B). Results obtained from three separate experiments showed significant difference between samples treated with inhibitors and control samples stimulated with FGF1 (Figure 53 B).

SerSTAT3 phosphorylation was significantly reduced with SU5402 and SU6656 treatment, but not with Jak Inhibitor I (Figure 53 A and B). This suggested a downstream effect on SerSTAT3 activation by FGF and agreed with data obtained for MCF7 cells (Figure 51).

For further validation of the data obtained using SU6656 and Jak Inhibitor I, SUM-52PE cells were treated with Dasatinib and AG490, which are Src family members and Jak2 inhibitors, respectively. The use of inhibitors significantly decreased FGF1-induced tyrosine STAT3 phosphorylation and therefore, confirmed previous results (Figure 54).

Additionally, the observed effect was verified by Src and JAK2 depletion using siRNA oligonucleotides. The level of Src and Jak2 knock-down was checked by probing whole cell lysates with Src and Jak2 antibodies. To check that use of siRNA oligonucleotides did not change the level of STAT3 and FGFR2, whole cell lysates were verified by probing with STAT3 and FGFR2 antibodies. Activation of TyrSTAT3 was tested by probing with specific antibody and statistical analysis was performed from three experiments. Knock-down experiment confirmed that both kinases, Src and Jak2, take part in FGF-induced TyrSTAT3 phosphorylation (Figure 55).

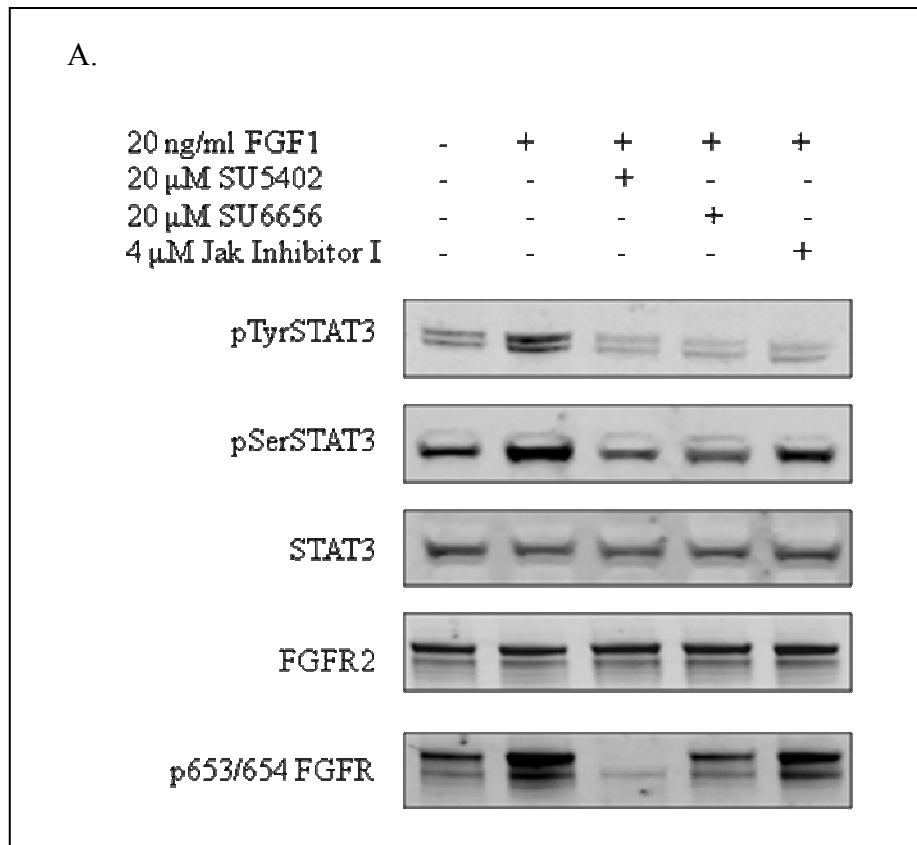


Figure 53. Effect of SU5402, SU6656 and Jak Inhibitor I on STAT3 phosphorylation.

A. SUM-52PE cells were serum-starved, treated with inhibitors, 20 μ M SU5402, 20 μ M SU6656, 4 μ M Jak Inhibitor I or DMSO for 30 minutes, and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

B.

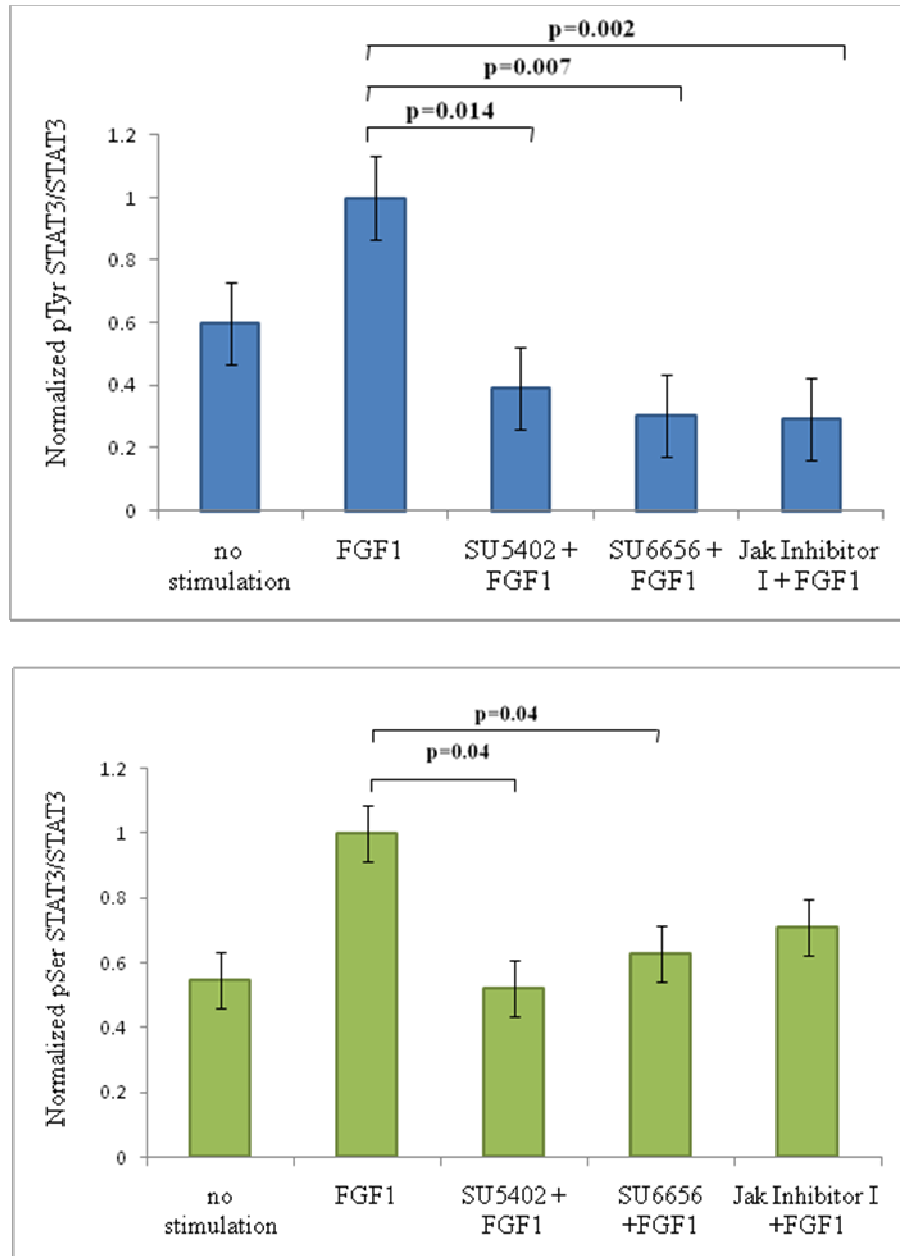


Figure 53. Effect of SU5402, SU6656 and Jak Inhibitor I on STAT3 phosphorylation.

B. Densitometric analysis of Western blots for pTyrSTAT3 and pSerSTAT3 normalized for STAT3 was performed from three separate experiments. Error bars, SD. Statistical significance was determined by t-test.

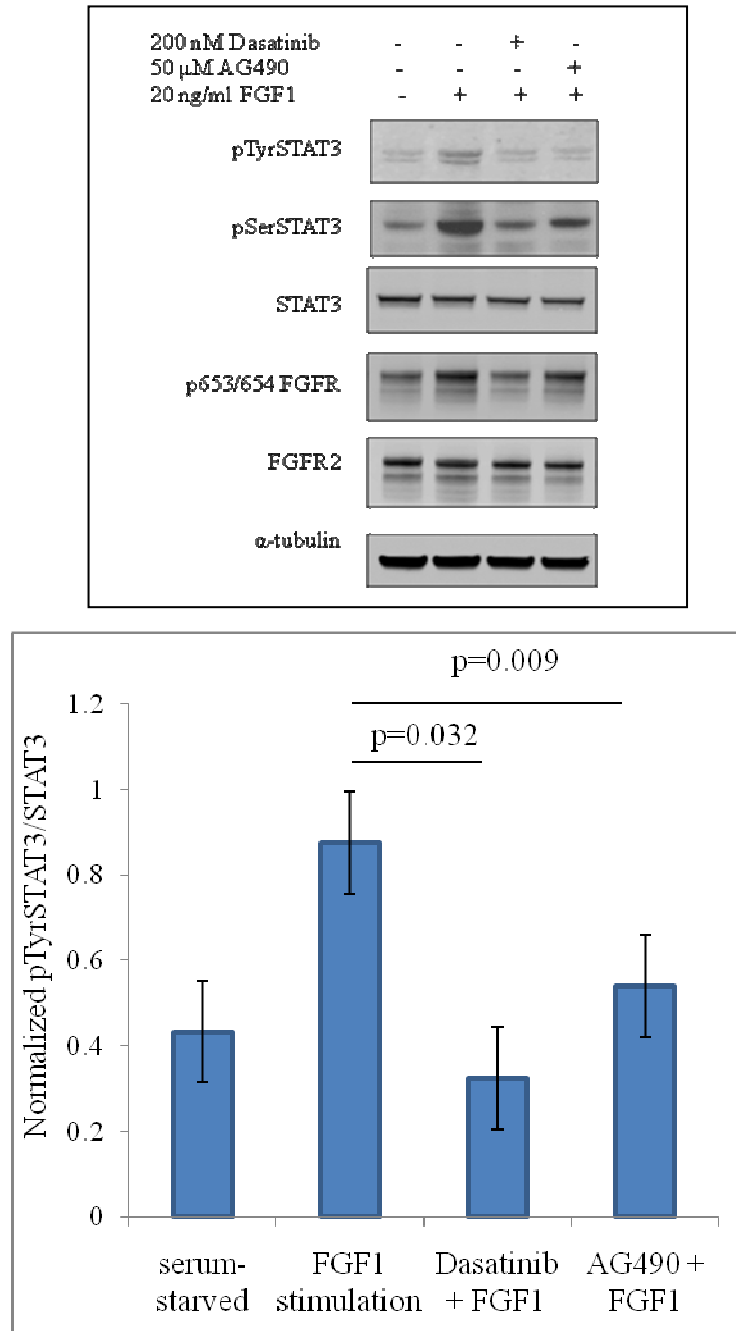


Figure 54. Effect of Dasatinib and AG490 on STAT3 phosphorylation.

SUM-52PE cells were serum-starved, treated with inhibitors, 200 nM Dasatinib and 50 μ M AG490 or DMSO for 30 minutes, and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies. Densitometric analysis of Western blots for pTyrSTAT3 normalized for STAT3 was performed from three separate experiments. Error bars, SD. Statistical significance was determined by t-test.

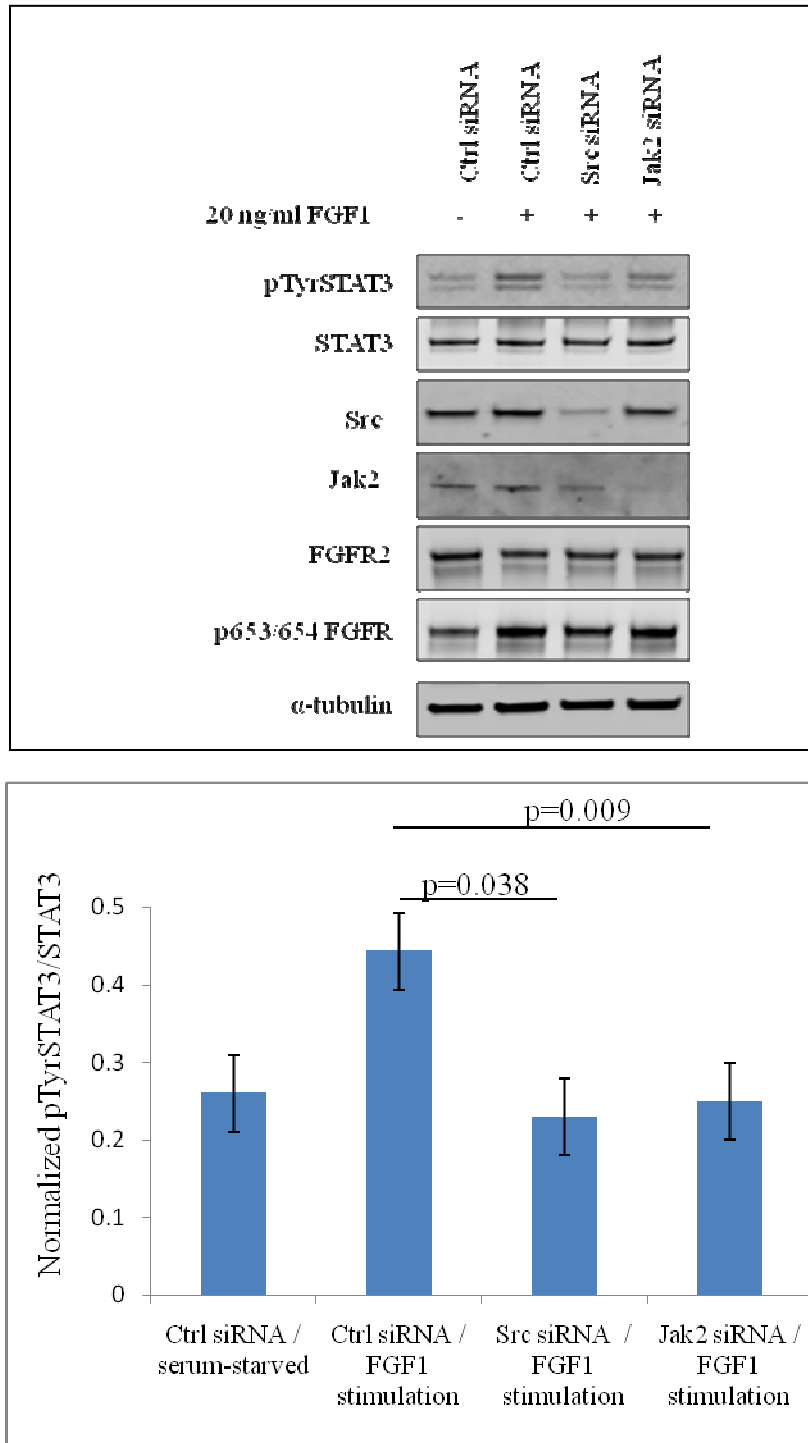


Figure 55. Effect of Src and Jak2 depletion on TyrSTAT3 phosphorylation.

SUM-52PE cells were transfected with Src and Jak2 siRNA oligonucleotides. Cells were serum-starved, stimulated with FGF1 and heparin for 20 minutes. Whole cell lysates were analyzed by Western blotting. Densitometric analysis of Western blots for pTyrSTAT3 normalized to STAT3 was performed from three separate experiments. Error bars, SD. Statistical significance was determined by t-test.

2. The efficiency of Jak inhibitor I

In order to check Jak Inhibitor I efficiency, activation of Jak2 was examined under several conditions. HEK293T cells were transiently transfected with myc-Jak2 and FcFGFR1 constructs. One sample was treated with Jak Inhibitor I. Activation of Jak2 in whole cell lysates was verified by probing with pJak2 antibody. Expression of FcFGFR1 constructs and myc-Jak2 was checked by probing with Fc and myc antibodies, respectively. Figure 56 A shows Jak2 activation with over-expression of FcFGFR1 kinase active that was effectively decreased by treatment with Jak Inhibitor I.

Next, Jak2 activation was tested in HEK293T cells expressing FGFR1 WT. Expression of FGFR1 WT was verified by probing whole cell lysates with FGFR1 antibody and equal loading was checked by probing with α -tubulin. Figure 56 B demonstrated that Jak2 was activated in cells over-expressing FGFR1 WT. This activity was reduced with Jak Inhibitor I treatment (Figure 56 B).

Lastly, MCF7 cells were stimulated with OSM which activates gp130/Jak signalling pathway (Underhill-Day and Heath 2006). One sample was treated with Jak Inhibitor I. Activation of Jak2 was checked by probing with pJak2 antibody. Surprisingly, pJak2 phosphorylation was not very strong, however, it was reduced by Jak Inhibitor I suggesting that both inhibitor and pJak2 antibody were specific (Figure 56 C).

Altogether, three experiments showed that Jak Inhibitor I effectively reduced Jak2 phosphorylation induced by FGFR over-expression or by OSM in MCF7 cells.

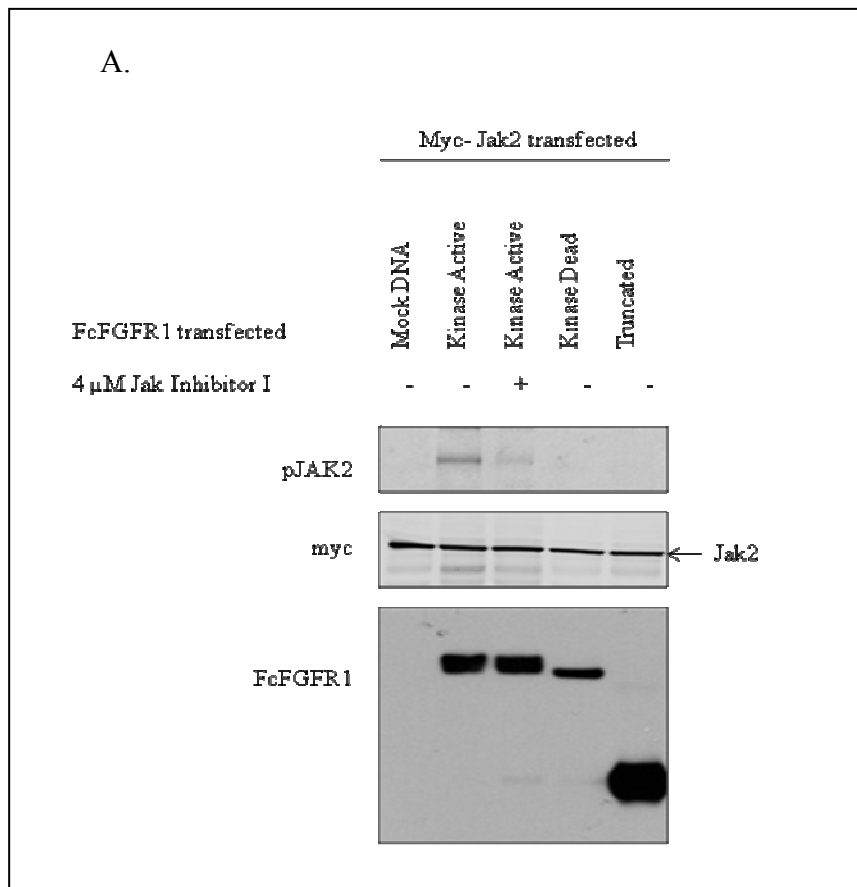


Figure 56. The efficiency of Jak Inhibitor I.

- A. HEK293T cells were transiently transfected with FcFGFR1 constructs. Cells were treated with 4 μ M Jak Inhibitor I for 30 minutes or DMOS. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

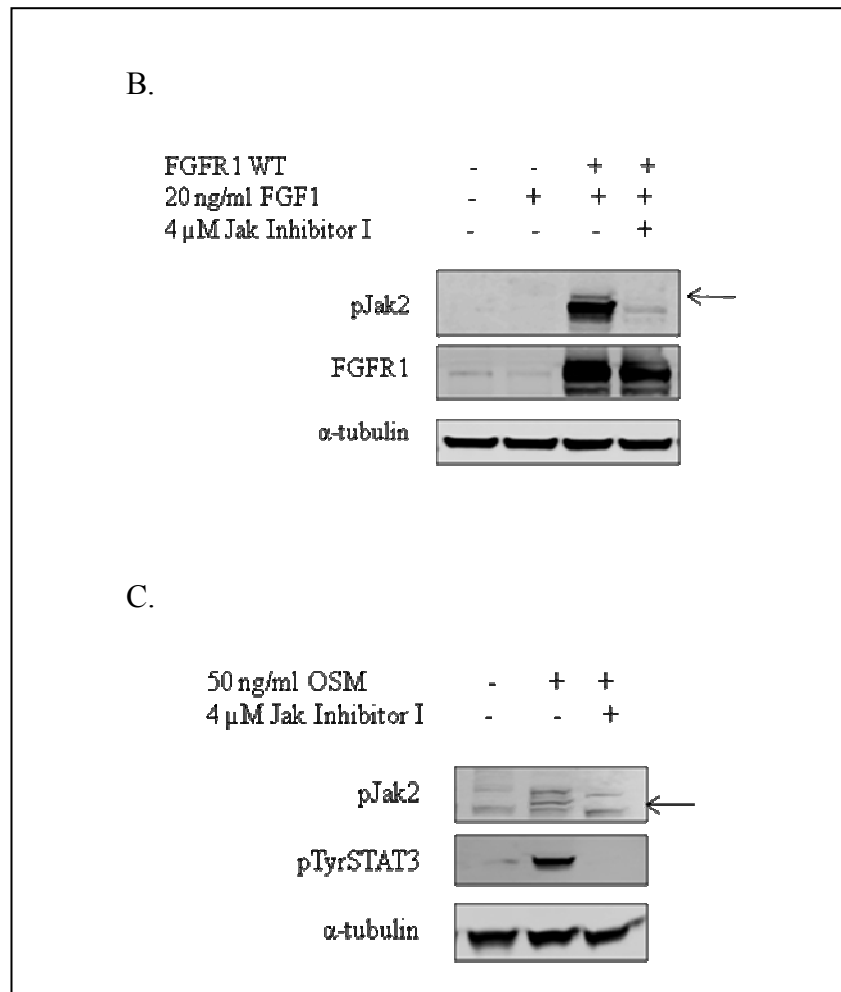


Figure 56. The efficiency of Jak Inhibitor I.

- B. HEK293T cells were transiently transfected with FGFR1 WT construct. Cells were treated with 4 μ M Jak Inhibitor I for 30 minutes or DMSO and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.
- C. MCF7 cells were treated with 4 μ M Jak Inhibitor I for 30 minutes or DMSO and stimulated with 50 ng/ml OSM. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

3. Src and Jak2 form a complex with kinase active FGFR1

To further investigate the involvement of Jak2 and Src kinases in TyrSTAT3 phosphorylation by FGFR, an immunoprecipitation experiment was performed to establish if they associate with the receptor. Src kinase was identified as a binding partner for Tyr730 of FGFR1 in the initial peptide pull-down and the interaction between Src and active FGFR1 was confirmed by immunoprecipitation (Table 4, Figure 12). To verify FRS2 involvement in Src-FGFR interaction, four FcFGFR1 constructs were transiently transfected into HEK293T cells (Burgar, Burns et al. 2002). Their expression was verified by probing with a Fc antibody. Complex formation between Src and FcFGFR1 was tested by probing with Src antibody. Equal expression of Src and its phosphorylation were checked by probing whole cell lysates with Src and p416Src antibodies.

It was found that Src interacted with kinase active as well as with VT- FGFR1 (Figure 57). It suggested FRS2-independent interaction between Src and FGFR1. Increased phosphorylation of Src was demonstrated with kinase active and VT- FGFR1 expression (Figure 57).

Furthermore, the interaction between FGFR1 and myc-Jak2 was demonstrated. Four FcFGFR1 constructs were transiently transfected into HEK293T cells (Burgar, Burns et al. 2002) together with myc-Jak2 construct. The expression of FcFGFR1 constructs and myc-Jak2 was verified by probing with a Fc and a myc antibodies. Complex formation between Jak2 and FcFGFR1 was tested by probing immunoprecipitated samples with myc antibody. Jak2 activation was checked by probing whole cell lysates with pJak2 antibody.

Similarly to Src, Jak2-FGFR1 association was phospho-dependent and FRS2-independent (Figure 58). Phosphorylation of Jak2 was detected with kinase active FGFR1 and

VT- suggesting that Jak2 acts downstream of FGFR1 and FGFR1 kinase activity was necessary for its activation (Figure 58). Altogether, the results showed that FGFR kinase activity was crucial for formation of a complex with Src and Jak2 which were activated downstream of FGFR1.

In order to confirm the data received from previous experiment, reverse immunoprecipitation experiments were performed. FGFR1 WT and myc-Jak2 or Src were co-transfected into HEK293T cells. SU5402 inhibitor was used to test if the interaction was phospho-dependent. Immunoprecipitation was performed using either myc or Src antibody and the formation of the complex between FGFR1 and Jak2 or FGFR1 and Src was verified using FGFR1 antibody. As expected, phospho-specific FGFR1-Jak2 and FGFR1-Src interactions were confirmed by reverse immunoprecipitation (Figure 59 and Figure 60).

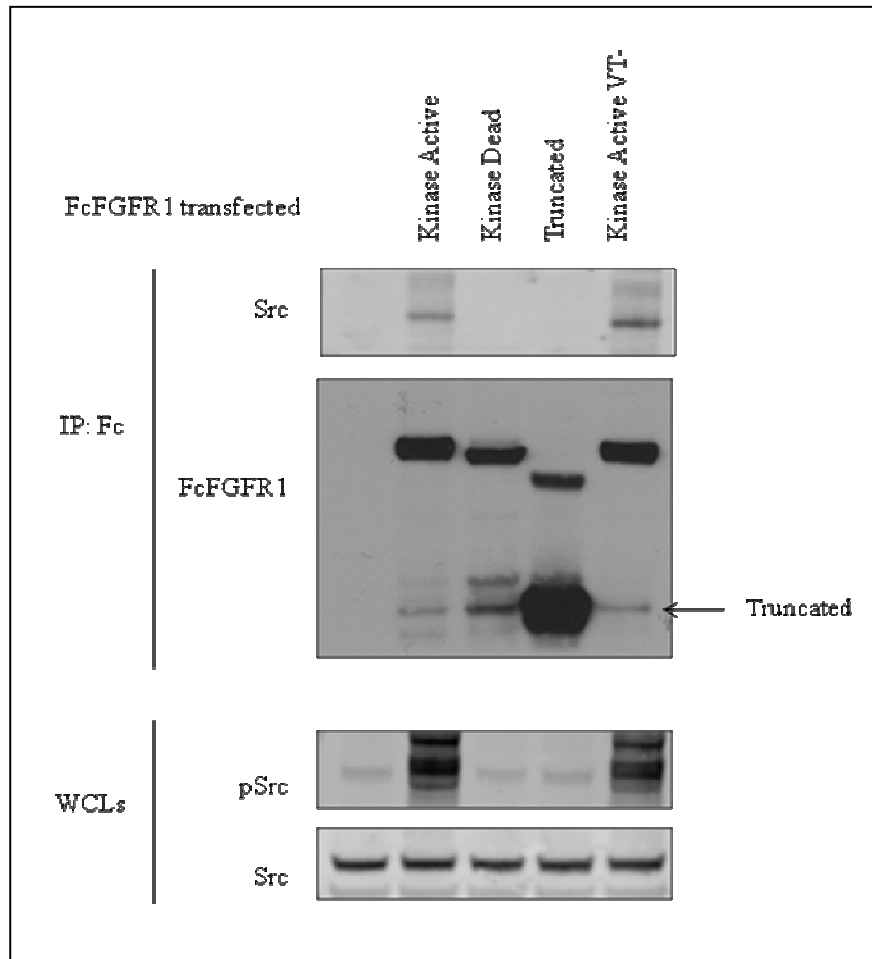


Figure 57. Src forms a complex with FGFR1.

FcFGFR1 constructs were transiently transfected into HEK293T cells. Equal amounts of proteins were immunoprecipitated by Sepharose protein-G. Immunoprecipitated samples and cell lysates were separated on SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

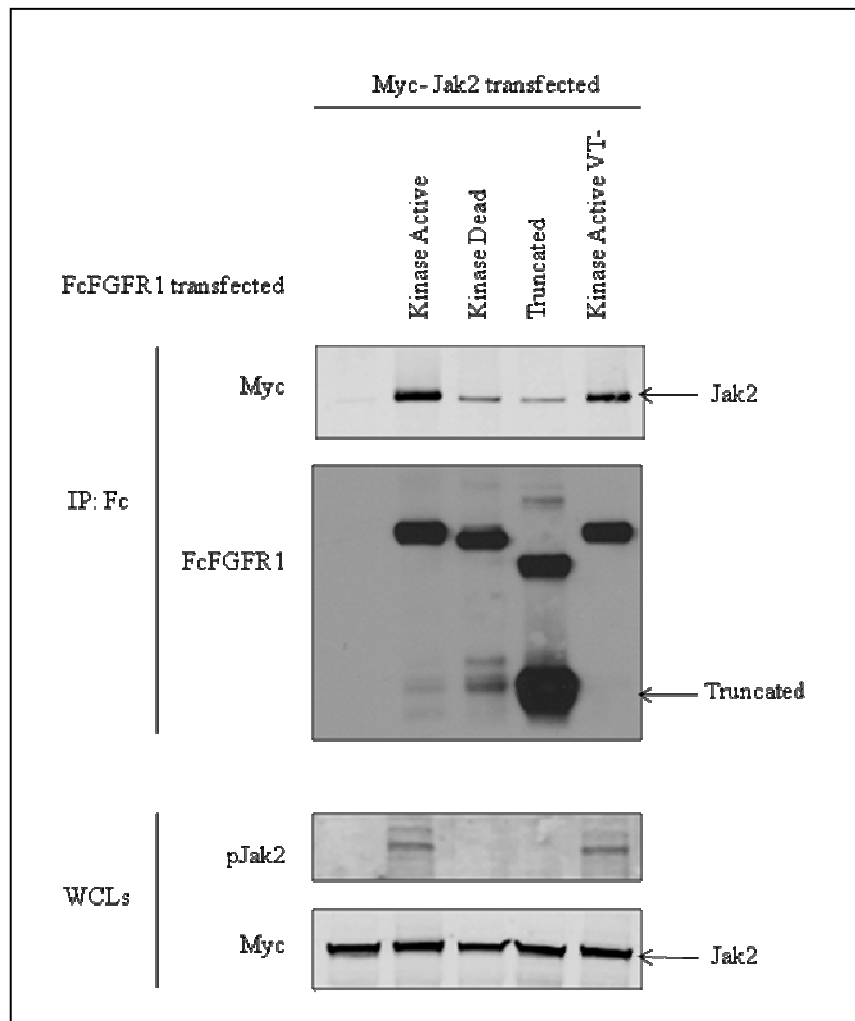


Figure 58. Jak2 forms a complex with FGFR1.

FcFGFR1 and myc-JAK2 constructs were transiently transfected into HEK293T cells. Equal amounts of proteins were immunoprecipitated by Sepharose protein-G. Immunoprecipitated samples and cell lysates were separated on SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

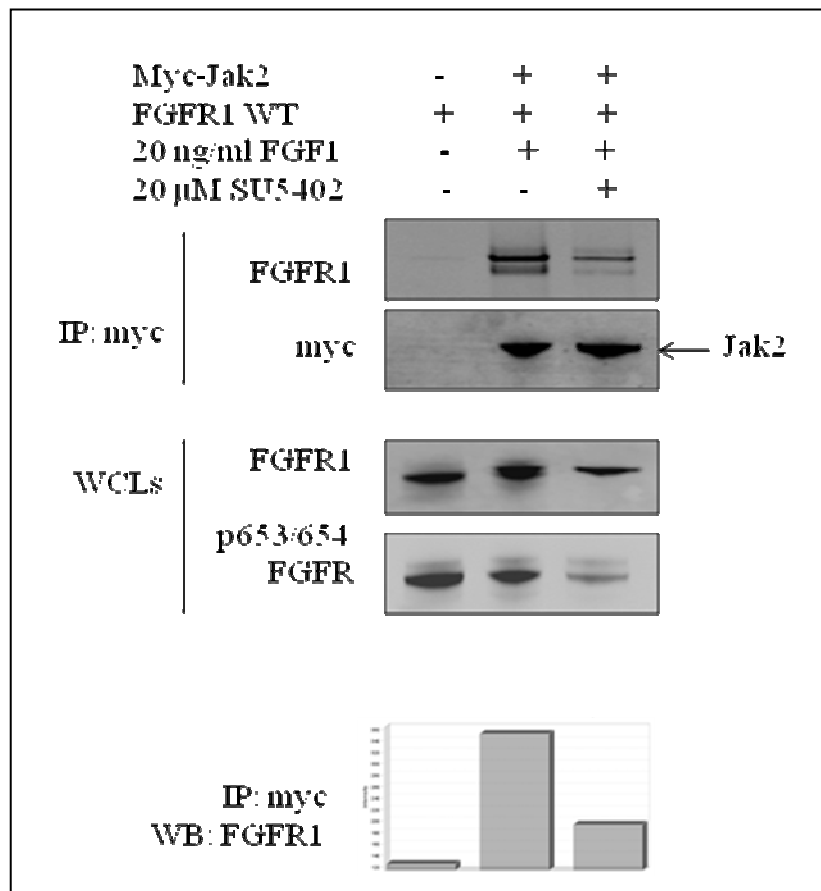


Figure 59. Jak2-FGFR1 interaction.

HEK293T cells were transiently transfected with FGFR1 WT and myc-Jak2. After 48 hours after transfection cells were treated with 20 μ M of SU5402 for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Equal amounts of proteins were immunoprecipitated using myc antibody and protein G Sepharose. Immunoprecipitated samples and whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

Bottom graph represents densitometric analysis of FGFR1 blot (obtained by Odyssey program). Experiment was performed once.

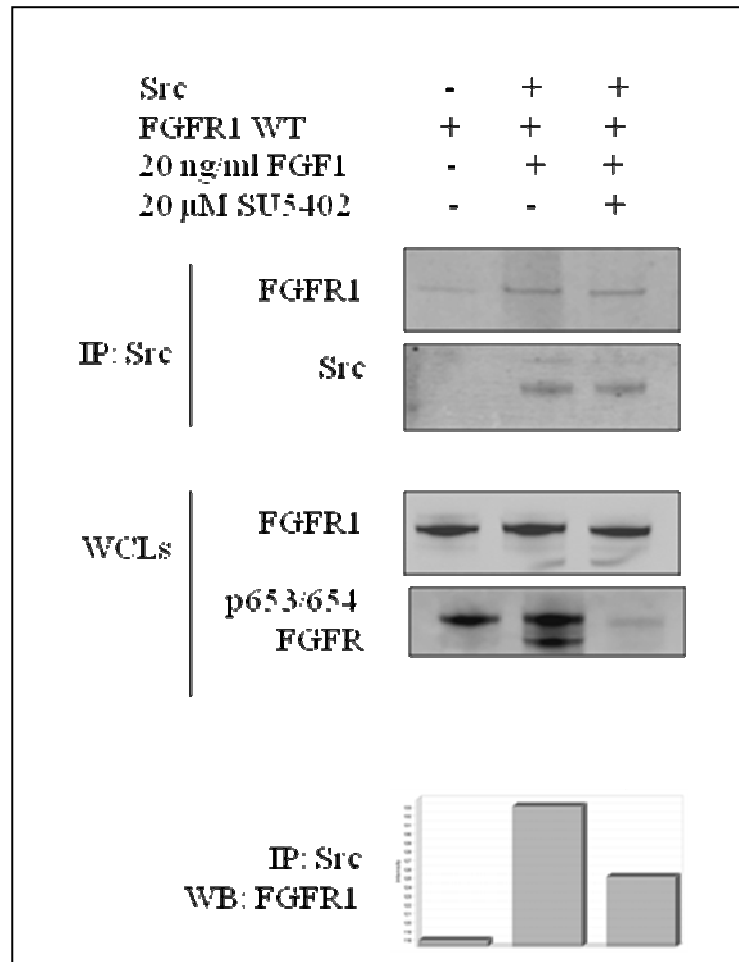


Figure 60. Src-FGFR1 interaction.

HEK293T cells were transiently transfected with FGFR1 WT and Src. After 48 hours after transfection cells were treated with 20 μ M of SU5402 for 30 minutes and stimulated with 20 ng/ml FGF1 and 10 μ g/ml heparin for 20 minutes. Equal amounts of proteins were immunoprecipitated using Src antibody and protein G Sepharose. Immunoprecipitated samples and whole cell lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with antibodies.

Bottom graph represents densitometric analysis of FGFR1 blot (obtained by Odyssey program). Experiment was performed once.

Discussion

The goal of this chapter was to investigate the potential role of the non-receptor tyrosine kinases, Src and Jak, in FGFR-mediated STAT3 activation. Both kinases were shown to act downstream of FGF receptor and their activity was essential for TyrSTAT3 phosphorylation. Moreover, Src and Jak2 associated with FGFR1 kinase active.

In this study the interaction between Src and FGFR1 was demonstrated by peptide pull-down where Src was identified as binding partner for Tyr730 of FGFR1. Immunoprecipitation experiments revealed that complex formation between active FGFR1 and Src was phospho-dependent and FRS2-independent. Therefore, it is possible that Src interacts with FGFRs on many levels, directly or indirectly by FRS2 as shown before (Li, Brunton et al. 2004). It was also proved that Src played a significant role in FGF-mediated STAT3 activation as both Src inhibitors, SU6656 and Dasatinib, inhibited STAT3 phosphorylation on Tyr and Ser residues. The same result was confirmed by Src knock-down. Importantly, Src activation was downstream of FGF and was dependent on receptor tyrosine kinase phosphorylation.

Src family was described as an important kinase in STATs phosphorylation in many cell types under various conditions but its activity is usually dependent on upstream receptor tyrosine kinase activation (Garcia, Bowman et al. 2001). It has been previously reported that EGF stimulation of cells over-expressing Src, but not EGFR, does not induce STATs activation (Garcia, Bowman et al. 2001). Over-expression of both proteins, EGFR and Src, results in STATs enhanced activity, prior to stimulation (Garcia, Yu et al. 1997; Garcia, Bowman et al. 2001), whereas, increased EGFR level (but not Src) leads to ligand-induced STAT3 activation (Garcia, Bowman et al. 2001). Garcia *et al.* proposed a model of STATs activation, prior and after EGF stimulation, in breast cancer cell line over-expressing EGFR

MDA-MB-468. In un-stimulated conditions, STATs are activated by Src and Jak by cooperation with EGFR. After stimulation, EGFR activity leads to STATs phosphorylation by Jaks, independently of Src. This constitutive activation of STATs, induced on many levels, contributes to cancer development by promoting cell survival and inhibition of apoptosis (Garcia, Bowman et al. 2001). Thus, Src can directly phosphorylate STATs but the presence of active upstream receptor tyrosine kinase is crucial.

Moreover, Src is proved to be essential for EGF-induced STAT5a activation in A431 cells by ErbB4, where Jak function is dispensable (Olayioye, Beuvink et al. 1999). Jak and STAT5a pre-associate with ErbB receptor and upon ligand binding Src kinase is recruited to the receptor where it directly phosphorylates STAT5a (Olayioye, Beuvink et al. 1999). Ligand-independent interaction between STAT3, Jak and ErbB-2 receptor was reported by Ren *et al.* This group showed that Src is recruited to the receptor after stimulation, but both kinases, Src and Jak, are required for STAT3 activation (Ren and Schaefer 2002). Similarly, Wang *et al.* described STAT3 association with PDGFR prior to stimulation. Upon ligand binding, Src is recruited and subsequently activates STAT3 (Wang, Wharton et al. 2000). Furthermore, STAT5b phosphorylation, which is induced by over-expressed EGFR, requires Src activity. However, Src function depends on upstream EGFR activation (Kloth, Laughlin et al. 2003). On the other hand, PDGF-induced activation of STAT5 is Src family-independent (Paukku, Valgeirsdottir et al. 2000).

The role of Janus kinases was also investigated and it was found that Jaks2 was necessary for maximal activation of TyrSTAT3 in SUM-52PE cells. Similarly to Src, Jaks activity depended on the concurrent tyrosine kinase activity of FGFR. Furthermore, immunoprecipitation results showed that Jak2 associated with kinase active FGFR1 in FRS2-independent manner.

The association between GFRs and Jak2 was demonstrated before (Olayioye, Beuvink et al. 1999; Ren and Schaefer 2002), the exact mechanism for Jak2 recruitment remains to be further determined. There are several reports explaining the role of Jaks in GFR-induced STATs activation. STATs' signalling differs between growth factor receptors and cytokine receptors. In the case of cytokine receptors, Jaks are necessary to phosphorylate STATs because cytokine receptors lack tyrosine kinase activity. Growth factor receptors have intrinsic tyrosine kinase activity and requirement of Jak is not evident. Thus, STATs activation by various GFRs might be Jak-dependent or Jak-independent. There are several examples in the literature confirming both models of activation. STAT5a phosphorylation by ErbB4 in A413 cells does not rely on Jaks (Olayioye, Beuvink et al. 1999). Similarly, the phosphorylation of STAT1 and STAT3 by EGF is dispensable from Jak2 activation (Leaman, Pisharody et al. 1996). On the contrary, other group demonstrated that ErbB2-mediated STAT3 activation involved Src and Jak2 (Ren and Schaefer 2002). Additionally, MDA-MB-468 breast cancer cell line over-expressing EGFR requires Jaks for TyrSTAT3 activation (Garcia, Bowman et al. 2001). The mechanism by which PDGFR activates STAT3 is unclear and cell-type dependent. It was shown that PDGF-mediated STAT1 and STAT3 phosphorylation is independent of Jaks, suggesting direct mechanism for STATs activation (Vignais, Sadowski et al. 1996). However, later on the same group demonstrated that PDGF-mediated STAT1 and STAT3 activation was distinct, Jak-independent or Jak-dependent, respectively (Vignais and Gilman 1999). Moreover, PDGF-induced STAT5 phosphorylation was Jak-independent (Paukku, Valgeirsdottir et al. 2000). On the contrary, Jak and Src contribute to STAT1 and STAT3 activation by PDGF in human airway smooth muscle cells (HASMC) (Simon, Takahashi et al. 2002) and in pancreatic stellate cells (Masamune, Satoh et al. 2005). Furthermore, the recruitment of STAT3 to ephrinB1 receptor and following

activation is Jak2 dependent (Bong, Lee et al. 2007). Recently, FGF-induced activation of STAT5 via Jak2 and Src was described as necessary for angiogenesis (Yang, Qiao et al. 2009).

Altogether, the role of Src and Jak in GFR-mediated STATs phosphorylation is cell-dependent and usually requires upstream tyrosine kinase activity. The need for Src and Jak also varies between activation of different STATs. Several models presented by many groups describe both, Src/Jak-independent and -dependent STATs activation and the mechanism of non-receptor kinases contribution needs to be determined. In this study STAT3 activation by FGF was detected in breast cancer cell line over-expressing FGFR2. Enhanced activation of FGFR2 in these cells leads to Src- and Jak-dependent STAT3 phosphorylation that can potentially contribute to malignant progression.

CHAPTER 7

Final conclusions

Final conclusions

Cancer progression is a multi-step process that depends on accumulation of a number of genetic changes in a cell (Hanahan and Weinberg 2000). One of the common differences between normal and cancer cells is deregulation of receptor tyrosine kinases signalling (Porter and Vaillancourt 1998; Hynes and MacDonald 2009). RTKs have a broad impact on many processes occurring in the cell thus their activity is highly regulated. Uncontrolled activity of RTKs has been implicated in tumour progression of various cancer types (Zandi, Larsen et al. 2007). Therefore, understanding the molecular mechanisms underlying oncogenic signalling could enable development of therapeutics targeting tumours with aberrant activation of RTKs.

Increased activity of EGFR family members is a characteristic of many human cancers (Wong, Bigner et al. 1987; Umekita, Ohi et al. 2000; Hirsch, Varella-Garcia et al. 2003) and the mechanism for this oncogenic activation is widely studied. Nevertheless, the mechanism of FGFR deregulation in cancer cells is still unidentified. Several events correlated with cancer development were suggested for EGFR family members, including over-expression of the receptor, increased expression of the ligands, activating mutations, loss of negative feedback, attenuation of receptor down-regulation or cross-talk with other receptors (Zandi, Larsen et al. 2007). Increased levels of the receptors in the membrane are often correlated with a more malignant phenotype. Moreover, high expression of EGFRs was described as critical for tumour cell motility and invasion (Pedersen, Tkach et al. 2004). Over-expression of EGFR induces ligand-independent phosphorylation of the receptor caused by spontaneous dimerization, which in turn causes increased activation of down-stream signalling pathways (Pedersen, Tkach et al. 2004; Zandi, Larsen et al. 2007). Receptor over-expression can be a result of gene amplification, increased activity of the promoter or translational deregulation (Zandi, Larsen et al. 2007). Constitutive activity of EGFR can also be induced by mutations in the extra- or intracellular domain of the receptor which generates changes in conformation of

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the receptor and triggers ligand-independent activation (Normanno, De Luca et al. 2006; Zandi, Larsen et al. 2007). Furthermore, the defects in process of EGFR attenuation can also lead to aberrant signalling (Waterman, Katz et al. 2002; Citri and Yarden 2006; Roepstorff, Grovdal et al. 2008). Down-regulation and lysosomal degradation is important to attenuate receptor signalling, thus cells lacking the mechanism to ‘switch-off’ the pathway are more likely to undergo transformation. Sustained activation of down-stream pathways in cells lacking the c-Cbl binding site on EGFR was demonstrated (Waterman, Katz et al. 2002). Moreover, the oncogenic influence of cross-talk between EGFRs and other receptors was demonstrated. The interaction between EGFRs and other molecules, including cytokine receptors, G-protein coupled receptors, ion channels, adhesion molecules and other RTKs, was demonstrated to generate ligand-independent EGFR activation (Zandi, Larsen et al. 2007).

Recently, abnormal expression of FGFRs has been linked with development and progression of a variety of human cancers including breast, prostate and gastric cancer (Giri, Ropiquet et al. 1999; Tannheimer, Rehemtulla et al. 2000; Takeda, Arao et al. 2007). However, despite rapidly advancing knowledge of the prevalence of FGFR amplicons in tumours, the exact impact of enhanced FGFR expression on downstream signalling processes is not known. Thus, it is important to understand how FGFR contributes to the malignant phenotype in order to design tumour-specific treatment.

The role of STAT3 as an oncogene is firmly established (Garcia, Yu et al. 1997; Bromberg, Wrzeszczynska et al. 1999; Bowman, Garcia et al. 2000; Yu, Pardoll et al. 2009). Persistent activation of STAT3 has been observed in many types of cancer, including prostate cancer (Mora, Buettner et al. 2002), breast cancer (Garcia, Bowman et al. 2001), myeloid leukemias (Coffer, Koenderman et al. 2000), head and neck cancer (Song and Grandis 2000).

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STAT3 activation increases cancer cell proliferation, invasion, survival and metastasis (Yu, Pardoll et al. 2009). At the same times STAT3 promotes inflammatory pathways that are pro-oncogenic, including IL-6/Jak/STAT3 pathway (Yu, Pardoll et al. 2009). Thus, targeting STAT3 in tumour cells is a desirable approach in cancer therapy.

In this study, a connection between over-expression of FGFRs and STAT3 activation in cancer cells was demonstrated. STAT3 activation via tyrosine phosphorylation required high level expression of FGFRs and was Src and Jak-dependent. This was demonstrated in the breast cancer cell line SUM-52PE which has an amplified FGFR2 gene and elevated protein expression. Moreover, an association between FGFRs and STAT3 was shown for the first time. The association between FGFR1 and STAT3 was mediated by tyrosine 677 of FGFR1 that is part of a highly conserved YxxQ SH2-domain binding motif for the SH2 domain of STAT3. Mutation of this tyrosine residue significantly reduced the binding of STAT3 to FGFR1. Furthermore, a mutation in the SH2 domain of STAT3 abolished the association with a Tyr677 FGFR1 peptide suggesting that SH2 domain was necessary for the interaction with the receptor. Thus, the physical interaction between phospho-Tyr677 of FGFR1 and SH2 domain of STAT3 was mandatory for STAT3 recruitment and its subsequent phosphorylation.

One of the main goals of this thesis was to establish the mechanism of FGF-induced STAT3 phosphorylation. The activation of STAT3 was tested in several cell lines. Serine STAT3 phosphorylation was observed at an endogenous level of FGFRs in all cells that were tested, whereas TyrSTAT3 phosphorylation required over-expression of FGFRs. FGFR-STAT3 signalling pathway was induced in cells over-expressing FGFRs, either by transient transfection of FGFR1 WT or in SUM-52PE which naturally express FGFR2. FGF stimulation in these cells resulted in enhanced tyrosine STAT3 phosphorylation which was significantly decreased by SU5402 and PD173074 treatment, FGFR inhibitors. Also a knock-

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down experiment in SUM-52PE and HEK293T cells over-expressing FGFR2, confirmed that high levels of FGFRs were necessary to induce FGF-mediated STAT3 phosphorylation.

The question that emerged during the work on this project was why the FGFR-STAT3 pathway was not activated in cells expressing endogenous levels of FGFRs. It is possible that oncogenic over-expression of FGFRs may induce STAT3 activation as an additional signalling pathway leading to an amplified output signal in cancer cells. Thus, the FGFR-STAT3 pathway might not be activated or important in the normal FGF response, but cells highly expressing FGFRs may cross-activate components from other pathways. Also, over-expressed FGFR might recruit low affinity binding partners and this may explain why STAT3 and Jak2 were recruited to over-expressed FGFRs. Moreover, FGFR auto-phosphorylation is an ordered and sequential event that when disturbed, by FGFR over-expression or an activating mutation, might contribute to aberrant assembly of FGFR binding partners (Furdui, Lew et al. 2006; Lew, Furdui et al. 2009). If the order of FGFR phosphorylation changes because of the over-expression, the dynamics of Tyr677 activation could be modified. However, the exact order and dynamics of Tyr677 phosphorylation in cells expressing endogenous and elevated levels of receptors is not known. On the other hand, STAT3 as a substrate might have a weak affinity to FGFRs so it might be difficult to detect TyrSTAT3 activation at endogenous levels of FGFRs.

Another subject that needs to be investigated in the future is amplification of the FGFR gene and over-expression of FGFR at the protein level. The expression level of FGFRs was tested in several cell lines previously described as cell lines with an amplification of FGFR genes (SUM-52PE, MFM-223, MDA-MB-134, MDA-MB-453 and ZR-75-1). Interestingly, FGFR gene amplification was not always correlated to expression of high levels of FGFR proteins. This suggests that over-expression of FGFR proteins is rather an

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uncommon event. FGFR expression might be regulated on many levels. It is possible that over-expressed receptor is quickly degraded or it is down-regulated at the transcription level so it is not expressed even in the presence of gene amplification. Thus, targeting FGFR in cancer cells over-expressing receptor could be an attractive therapeutic target. This topic requires additional studies in order to explain the differences in FGFR protein expression in cancer cells.

Moreover, the role of FGF-induced SerSTAT3 could be investigated further. Serine STAT3 phosphorylation was believed to enhance STAT3 transcription activity (Wen, Zhong et al. 1995) however, recently other aspects of SerSTAT3 activity are emerging (Decker and Kovarik 2000). Here, it was shown that STAT3 serine phosphorylation was induced by ERK and JNK as a downstream effect of FGFR signalling and it did not require over-expression of FGFRs. However, it was not tested if FGF-mediated SerSTAT3 phosphorylation was sufficient to drive gene expression. The physiological function of phospho-SerSTAT3 without tyrosine phosphorylation remains to be determined. Does it act as co-enhancer with other transcription factors or does it regulate expression of different genes than TyrSTAT3? The gene expression pattern between FGF-induced tyrosine phosphorylated STAT3 in cancer cells and serine-only-phosphorylated STAT3 in normal cells could be compared. The emerging evidence about the role of SerSTAT3 in signal-dependent gene expression was suggested (Ng, Cheung et al. 2006). Serine, but not tyrosine, phosphorylated STAT3 was shown to bind DNA and induce gene expression upon stimulation of nerve growth factor (Ng, Cheung et al. 2006). Thus, it is likely that FGF-induced SerSTAT3 could also regulate transcription and this subject needs further studies.

Moreover, involvement of serine STAT3 in oncogenesis was suggested and its activity in cancer cells might be as important as tyrosine phosphorylated STAT3 (Bowman, Garcia et

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al. 2000). Furthermore, the role of serine phosphorylated STAT3 in mitochondria was recently proposed (Gough, Corlett et al. 2009; Wegrzyn, Potla et al. 2009). Serine phosphorylation of STAT3 was shown to induce Ras-dependent malignant transformation by sustaining altered glycolytic and oxidative phosphorylation in cancer cells (Gough, Corlett et al. 2009). Recently, the negative role of SerSTAT3 in suppressing insulin signalling was suggested (Kim, Yoon et al. 2009). High level of amino acids at the plasma membrane that inhibit insulin sensitivity in hepatic cells can signal via SerSTAT3, but not TyrSTAT3. This effect was not observed in cells where STAT3 was knocked-down (Kim, Yoon et al. 2009). Moreover, SerSTAT3 phosphorylation was critical for this effect implicating that STAT3 can be a mediator of excess amino acid signals (Kim, Yoon et al. 2009). Altogether, serine STAT3 plays many more roles than previously thought and it has become an interesting field to explore.

Src and Jak2 were shown to act downstream of FGFR and their activity was also essential for tyrosine STAT3 phosphorylation. However, the exact mechanism for Src and Jak2 recruitment to the receptor and their subsequent STAT3 activation remains to be further determined. Src has been described as an important kinase in STATs phosphorylation in many cell types under various conditions (Olayioye, Beuvink et al. 1999; Ren and Schaefer 2002; Quesnelle, Boehm et al. 2007). Src activation upon FGF stimulation was previously described (Sandilands, Akbarzadeh et al. 2007) and both direct and indirect interaction between Src family members and FGFR was presented (Zhan, Plourde et al. 1994; Li, Brunton et al. 2004). Here, it was shown that complex formation between active FGFR1 and Src was FRS2-independent. It was also demonstrated that Src took part in FGF-mediated tyrosine STAT3 phosphorylation. The contribution of Src in tyrosine STAT3 activation can be explained in two ways. Firstly, Src is a known STAT3 activator and its oncogenic activity was described to

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induce increased STAT3 activation in cancer cells (Yu, Meyer et al. 1995; Bromberg, Wrzeszczynska et al. 1999; Herrmann, Vogt et al. 2007). Thus, Src, which is bound to active FGFR, might directly phosphorylate STAT3. On the other hand, it is known that Src modulates the activity and dynamics of FGFRs and activation of both proteins is inter-dependent (Sandilands, Akbarzadeh et al. 2007). As a result, Src can be indirectly involved in STAT3 activation by maintaining FGFRs activation.

Janus kinases activity was also required for tyrosine phosphorylation of STAT3 in SUM-52PE cells. Additionally, the interaction between Jak2 and FGFR1 was demonstrated as well as FGFR-mediated Jak2 activation. Phospho-dependent Jak2 association with FGFR suggested that Jak2 was recruited only to the activated receptor, directly or indirectly. The direct interaction between Jak2 and FGFR1 could be mediated by the predicted SH2 domain of Jak2, however, the binding motif for the Jak2 SH2 domain is not known (Huang, Li et al. 2008). If the interaction was indirect, but not FRS2-dependent, there must be another adaptor protein recruiting Jak2 to FGFR1, like Src, which is bound to tyrosine residue on FGFR1. Another possibility is that Jak2 binds a motif on FGFR1 that resembles the box1 motif (proline, any amino acid residue, proline, hydrophobic sequence). Jak2 is recruited to gp130 receptor by box1 motif (Tanner, Chen et al. 1995), however, the interaction between Jak2 and the box1 motif is phospho-independent. Therefore, it is rather unlikely that Jak2-FGFR1 association is mediated by FGFR1 box 1-like motif. Another possibility is indirect binding of Jak2 via cytokine receptors by cross-talk between over-expressed FGFR and cytokine receptors. Cross-talk between receptors is a common phenomenon in cancer cells that is often linked with oncogenic signalling (Yu, Kortylewski et al. 2007; Zandi, Larsen et al. 2007). The cross-talk between ErbB2 and ErbB3 and gp130, as well as ErbB2 and OSMR β was described before (Grant, Hammacher et al. 2002). This association was ligand-independent. Moreover,

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EGF stimulation was demonstrated to induce phosphorylation of gp130 (Grant, Hammacher et al. 2002). Simultaneous EGF and OSM stimulation induced cell differentiation as opposed to EGF stimulation that promotes cell proliferation (Grant, Hammacher et al. 2002). Therefore, cross-talk can induce different biological response creating signalling variety. The possibility of cross-talk between FGFR and cytokine receptor needs further investigation by series of co-immunoprecipitation experiments.

Lastly, the influence of FGFR-STAT3 interaction on the behaviour of cancer cells could be examined. In the future, this could be done in the cancer cells expressing high levels of FGFRs, where FGFR2 would be knocked-down. The effect of the knock-down on proliferation or colony forming could be studied. Also activation of other members of STATs family by FGF could be investigated.

Finally, a model for cancer cells that express elevated levels of FGFR proteins is proposed (Figure 61). Over-expression of FGFR leads to ligand-independent phosphorylation which in turn recruits STAT3 to the receptor. At the same time Src and Jak2 form a complex with the receptor resulting in phosphorylation of both kinases. Then, Src and Jak2 facilitate tyrosine STAT3 phosphorylation, which leads to STAT3 dimerisation, translocation in the nucleus and regulation of gene expression (Figure 61 A). Alternatively, over-expressed FGFR may cross-talk with cytokine receptors (Figure 61 B). Thus, it may be that Jak2 recruitment is indirect via cytokine receptor, for example gp130 that dimerises with over-expressed FGFR2 in cancer cells (Figure 60 B).

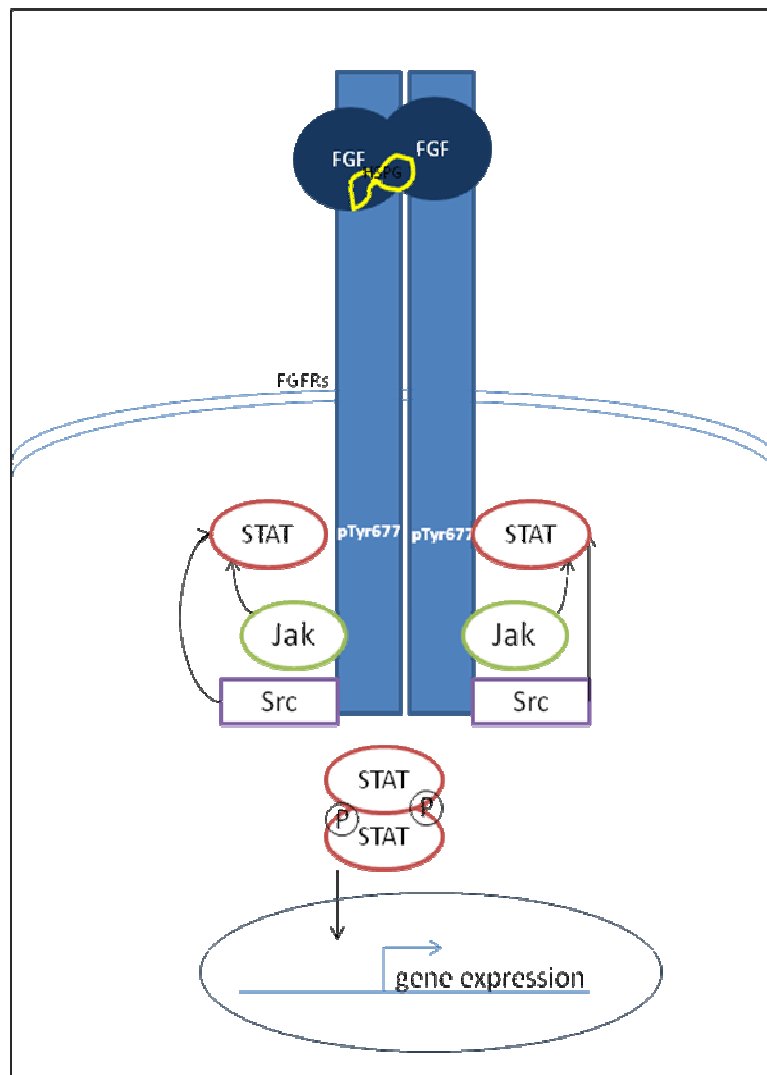
In conclusion, the contribution of STAT3 in FGFRs signalling pathways in cancer cells over-expressing FGFR2, SUM-52PE, was described in this report. It was suggested that STAT3 can be a mediator of amplified FGFR signalling. This study supports a potential role

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for STAT3 in the process of tumorigenesis in cells over-expressing FGFRs, in which case the FGFR-STAT3 pathway might be an attractive therapeutic target.

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A.



B.

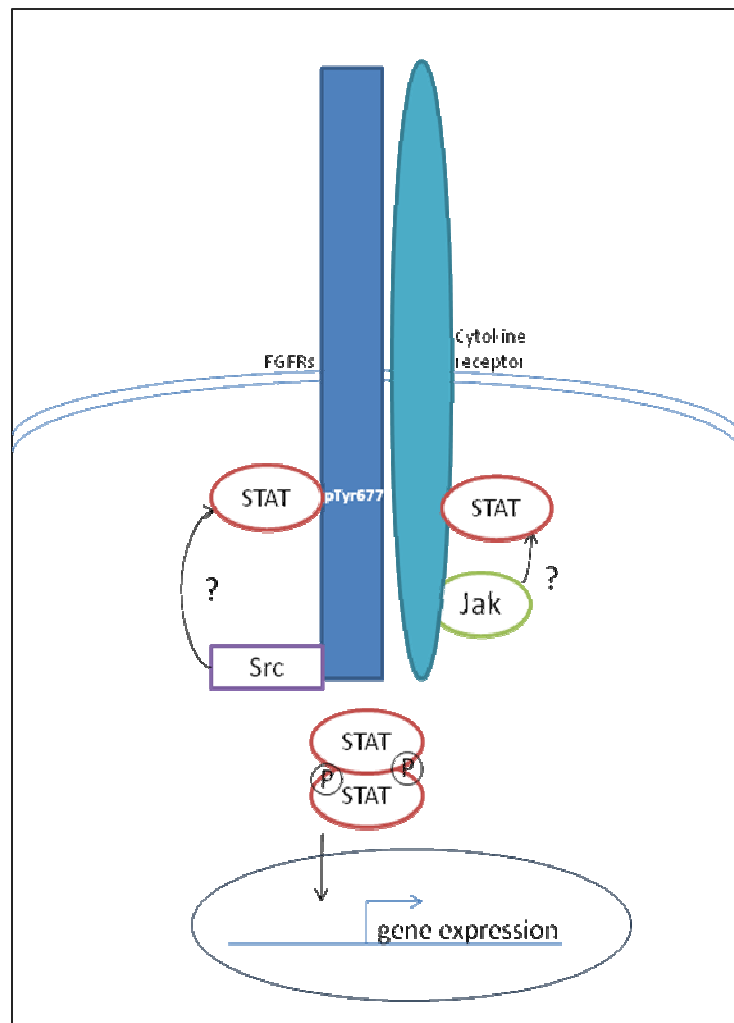


Figure 61. Models of possible FGFR-induced STAT3 activation.

Schematic representation of the proposed models of FGFR-induced STAT3 activation in cell over-expressing FGFRs (description in text). P represents phosphorylation.

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Appendix I

Mass spectrometry results showing the amino acid sequence of peptides identified for the protein hits obtained for FGFR1 peptide in PPD experiment (Chapter 3). The charge and the x-score of each peptide are presented.

| <u>Peptides</u> | <u>Charge</u> | <u>X Score</u> |
|--|---------------|----------------|
| KPCI_HUMAN Protein kinase C iota type | | |
| R.DLKLDNVLLDSEGHK.L | 3.00000 | 3.72E+00 |
| K.ASSSLGLQDFDLLR.V | 2.00000 | 2.50E+00 |
| K.ASSSLGLQDFDLLR.V | 2.00000 | 2.83E+00 |
| TRAF2_HUMAN TNF receptor-associated factor 2 | | |
| K.TATFENIVCVLNR.E | 2.00000 | 1.46E+00 |
| R.IPAIFSPAFYTSR.Y | 2.00000 | 1.68E+00 |
| R.IPAIFSPAFYTSR.Y | 2.00000 | 1.66E+00 |
| R.IPAIFSPAFYTSR.Y | 2.00000 | 1.35E+00 |
| PI4KA_HUMAN Phosphatidylinositol 4-kinase alpha (PI4-kinase alpha) | | |
| R.TLLSLLATEIER.L | 2.00000 | 3.45E+00 |
| K.ASSSAGNLGVLPVIAVLR.R | 2.00000 | 4.94E+00 |
| PERQ2_HUMAN PERQ amino acid-rich with GYF domain-containing protein 2 (Grb10-interacting GYF protein 2) | | |
| R.LQQQQQQQLAQMK.L | 2.00000 | 4.84E+00 |
| R.LQQQQQQQLAQMK.L | 2.00000 | 5.43E+00 |
| R.LQQQQQQQLAQMK.L | 2.00000 | 5.48E+00 |
| K.DVGRPNFEEGGPTSVGR.K | 3.00000 | 4.70E+00 |
| K.SLLEIQEEAR.Q | 2.00000 | 3.04E+00 |
| R.VPFPSPGAPPPHMGELDQER.L | 3.00000 | 5.08E+00 |
| K.MVAYLQDSALDDER.L | 2.00000 | 3.16E+00 |
| R.ALSSGGITSPPLSPALPK.Y | 2.00000 | 3.45E+00 |
| R.ALSSGGITSPPLSPALPK.Y | 2.00000 | 5.09E+00 |
| K.AALSSQQQLALLLQQFQTLK.M | 3.00000 | 4.39E+00 |
| K.AALSSQQQLALLLQQFQTLK.M | 3.00000 | 5.40E+00 |
| K.AALSSQQQLALLLQQFQTLK.M | 3.00000 | 5.43E+00 |
| K.IPSDLLDKEFLPILQEEPLPLALVPFTEEEQR.N | 3.00000 | 4.97E+00 |
| IQA3_HUMAN Ras GTPase-activating-like protein IQGAP3 | | |
| R.HALGAVAQLLQHAAAGK.A | 3.00000 | 5.32E+00 |
| K.IGGILANELSVDEAAVHAAVLAINAEVER.G | 3.00000 | 7.42E+00 |
| NCKP1_HUMAN Nck-associated protein 1 (NAP 1) | | |
| K.AINQIAAALFTIHK.G | 3.00000 | 2.30E+00 |
| R.DVLSYHIPFLVSSIEDFKDHIPR.E | 4.00000 | 4.73E+00 |
| R.DVLSYHIPFLVSSIEDFKDHIPR.E | 4.00000 | 2.77E+00 |
| R.DVLSYHIPFLVSSIEDFKDHIPR.E | 4.00000 | 3.10E+00 |
| PTN1_HUMAN Tyrosine-protein phosphatase non-receptor type 1 | | |
| R.HEASDFPCR.V | 2.00000 | 2.73E+00 |
| R.FSYLAVIEGAK.F | 2.00000 | 3.41E+00 |
| R.FSYLAVIEGAK.F | 2.00000 | 3.42E+00 |
| R.FSYLAVIEGAK.F | 2.00000 | 3.24E+00 |
| DUS3_HUMAN Dual specificity protein phosphatase 3 (EC 3.1.3.48) | | |
| K.LGITHVLNAAEGR.S | 2.00000 | 3.81E+00 |
| R.IYVGNASVAQDIPK.L | 2.00000 | 4.84E+00 |
| R.AADFIDQALAQK.N | 2.00000 | 3.29E+00 |
| -.EIGPNDGFLAQLCQ@LNDR.- | 2.00000 | 4.68E+00 |
| R.EIGPNDGFLAQ@LCQLNDR.L | 2.00000 | 4.64E+00 |
| -.EIGPNDGFLAQLCQLN@DR.- | 2.00000 | 4.18E+00 |
| -.EIGPN@DGFLAQLCQLNDR.- | 2.00000 | 4.12E+00 |
| CRKL_HUMAN Crk-like protein | | |
| K.TALALEVGDIVK.V | 2.00000 | 3.36E+00 |
| K.IGDQEFDHLPALLEYK.I | 2.00000 | 4.76E+00 |
| CSK_HUMAN Tyrosine-protein kinase CSK (EC 2.7.10.2) (C-SRC kinase) | | |
| R.LLYPPETGLFLVR.E | 2.00000 | 1.96E+00 |
| R.HSNLVQLLGVIVEEK.G | 2.00000 | 5.77E+00 |
| RAB1A_HUMAN Ras-related protein Rab-1A | | |
| K.NATNVEQSFMTMAAEIK.K | 2.00000 | 1.82E+00 |
| K.EFADSLGIPFLETSK.N | 2.00000 | 1.62E+00 |
| K.NATNVEQSFMTMAAEIK.K | 2.00000 | 1.63E+00 |
| K.EFADSLGIPFLETSK.N | 2.00000 | 3.28E+00 |

| | | |
|---|---------|----------|
| K.NATNVEQSFMTMAAEIK.K | 2.00000 | 1.32E+00 |
| AP2A2_HUMAN AP-2 complex subunit alpha-2 | | |
| K.KGPSTVTDLEDTKR.D | 2.00000 | 4.88E+00 |
| K.KGPSTVTDLEDTKR.D | 2.00000 | 3.15E+00 |
| K.KGPSTVTDLEDTKR.D | 2.00000 | 3.12E+00 |
| K.TSVSLAVSR.L | 2.00000 | 2.46E+00 |
| R.LLQCYPPDPAVR.G | 2.00000 | 1.33E+00 |
| R.LEPNLQAQMYR.L | 2.00000 | 1.48E+00 |
| R.VIQVINRDDVQGYAAK.T | 2.00000 | 3.85E+00 |
| R.SSPLIQFHLLHSK.F | 2.00000 | 3.21E+00 |
| R.MFIFYGNK.T | 2.00000 | 1.54E+00 |
| R.LTECLETILNK.A | 2.00000 | 2.02E+00 |
| R.TSPDLVPMGDWTSR.V | 2.00000 | 1.14E+00 |
| K.FFQPTEM#ASQDFQQR.W | 2.00000 | 1.75E+00 |
| -.FFQPTEMASQDFQQR.- | 2.00000 | 1.48E+00 |
| K.FVNLFPVEVKPTIQDVL.R.S | 2.00000 | 2.99E+00 |
| K.QLSNPQQEVQNIK.A | 2.00000 | 2.37E+00 |
| R.NPTFM#GLALHCIA SVGSR.E | 2.00000 | 4.31E+00 |
| R.AVDLLYAMCDR.S | 2.00000 | 1.26E+00 |
| -.FFQPTEMASQDFQQR.- | 2.00000 | 1.56E+00 |
| -.FFQPTEMASQDFQQR.- | 2.00000 | 1.86E+00 |
| R.YGGTFQNVSVQLPITL.NK.F | 2.00000 | 3.57E+00 |
| K.NNGVLFENQLLQIGL.K.S | 2.00000 | 3.86E+00 |
| K.FVNLFPVEVKPTIQDVL.R.S | 2.00000 | 2.96E+00 |
| R.NPTFMGLALHCIA SVGSR.E | 2.00000 | 4.01E+00 |
| K.NNGVLFENQLLQIGL.K.S | 2.00000 | 1.79E+00 |
| K.IIGFGSALLEEVDPNPNANFVVGAGIIHTK.T | 2.00000 | 4.18E+00 |
| K.FVNLFPVEVKPTIQDVL.R.S | 2.00000 | 2.89E+00 |
| K.FVNLFPVEVKPTIQDVL.R.S | 2.00000 | 3.29E+00 |
| R.VVHLLNDQHLGVVTAATSLITLAQK.N | 2.00000 | 5.04E+00 |
| R.VVHLLNDQHLGVVTAATSLITLAQK.N | 2.00000 | 4.37E+00 |
| R.NPTFMGLALHCIA SVGSR.E | 2.00000 | 4.11E+00 |
| R.NPTFMGLALHCIA SVGSR.E | 2.00000 | 2.73E+00 |
| K.IIGFGSALLEEVDPNPNANFVVGAGIIHTK.T | 2.00000 | 4.59E+00 |
| K.IIGFGSALLEEVDPNPNANFVVGAGIIHTK.T | 2.00000 | 4.34E+00 |
| R.VVHLLNDQHLGVVTAATSLITLAQK.N | 2.00000 | 5.21E+00 |
| AP2B1_HUMAN AP-2 complex subunit beta-1 | | |
| K.VIAAM#TVGK.D | 2.00000 | 1.77E+00 |
| K.VIAAM#TVGK.D | 2.00000 | 1.93E+00 |
| K.ECHLNADTVSSK.L | 2.00000 | 1.99E+00 |
| K.RPEILKQEIK.V | 2.00000 | 3.18E+00 |
| K.LQNNNVYTIK.R | 2.00000 | 2.47E+00 |
| K.LQNNNVYTIK.R | 2.00000 | 2.70E+00 |
| R.IQPGNPNYTL.SLK.C | 2.00000 | 1.19E+00 |
| -.QGHIYMEMNFTNK.- | 2.00000 | 1.58E+00 |
| K.ALQHM#TDFAIQFNK.N | 2.00000 | 2.02E+00 |
| K.DSDYYNMLLK.L | 2.00000 | 1.77E+00 |
| K.DIPNENELQFOIK.E | 2.00000 | 1.37E+00 |
| K.ALQHMTDFAIQFNK.N | 2.00000 | 2.53E+00 |
| K.DIPNENELQFOIK.E | 2.00000 | 1.79E+00 |
| K.DIPNENELQFOIK.E | 2.00000 | 1.03E+00 |
| K.ALQHMTDFAIQFNK.N | 2.00000 | 2.82E+00 |
| K.LHDINAQM#VEDQGFLDSL.R.D | 2.00000 | 4.78E+00 |
| K.DSDYYNMLLK.K | 2.00000 | 2.09E+00 |
| K.LHDINAQM#VEDQGFLDSL.R.D | 2.00000 | 3.58E+00 |
| K.VNYVVQEIVVIR.D | 2.00000 | 2.55E+00 |
| K.LHDINAQM#VEDQGFLDSL.R.D | 2.00000 | 4.93E+00 |
| K.SQPDMAIM#AVNSFVK.D | 2.00000 | 2.03E+00 |
| -.SQPDM#AIMAVNSFVK.- | 2.00000 | 1.77E+00 |

| | | |
|---|---------|----------|
| K.VNYVVQEAIVVIR.D | 2.00000 | 3.06E+00 |
| K.VNYVVQEAIVVIR.D | 2.00000 | 2.53E+00 |
| K.LHDINAQMVEDQGFLDSL.R.D | 2.00000 | 4.08E+00 |
| K.KPSETQELVQQVLSLATQSDSDNPD.LR.D.R.G | 2.00000 | 2.60E+00 |
| K.KLAPPLVTLSSGEPEVQYVALR.N | 2.00000 | 4.05E+00 |
| K.KPSETQELVQQVLSLATQSDSDNPD.LR.D | 2.00000 | 4.45E+00 |
| K.YPNKYESIATLCENLDSLDEPDAR.A | 2.00000 | 3.69E+00 |
| K.LTN@GIWILAE.LR.I | 2.00000 | 3.68E+00 |
| R.APEVSQYIYQVYDSILKN.- | 2.00000 | 2.08E+00 |
| R.APEVSQYIYQVYDSILKN.- | 2.00000 | 2.70E+00 |
| K.LAPPLVTLSSGEPEVQYVALR.N | 2.00000 | 3.60E+00 |
| K.LAPPLVTLSSGEPEVQYVALR.N | 2.00000 | 4.70E+00 |
| K.LAPPLVTLSSGEPEVQYVALR.N | 2.00000 | 5.21E+00 |
| R.KYPNKYESIATLCENLDSLDEPDAR.A | 2.00000 | 3.54E+00 |
| K.KLAPPLVTLSSGEPEVQYVALR.N | 2.00000 | 4.37E+00 |
| K.KPSETQELVQQVLSLATQSDSDNPD.LR.D.R.G | 2.00000 | 3.22E+00 |
| K.KPSETQELVQQVLSLATQSDSDNPD.LR.D.R.G | 2.00000 | 6.30E+00 |
| K.LTNGIWILAE.LR.I | 2.00000 | 3.39E+00 |
| K.CRAPEVSQYIYQVYDSILKN.- | 2.00000 | 3.82E+00 |
| K.KPSETQELVQQVLSLATQSDSDNPD.LR.D | 2.00000 | 4.56E+00 |
| K.YPNKYESIATLCENLDSLDEPDAR.A | 2.00000 | 3.17E+00 |
| R.APEVSQYIYQVYDSILKN.- | 2.00000 | 2.28E+00 |
| R.APEVSQYIYQVYDSILKN.- | 2.00000 | 1.92E+00 |
| R.APEVSQYIYQVYDSILKN.- | 2.00000 | 2.01E+00 |
| K.LAPPLVTLSSGEPEVQYVALR.N | 2.00000 | 3.93E+00 |
| K.LAPPLVTLSSGEPEVQYVALR.N | 2.00000 | 4.01E+00 |
| CLH1_HUMAN Clathrin heavy chain 1 (CLH-17) | | |
| R.IHEGCEEPATHNALAK.I | 2.00000 | 3.08E+00 |
| K.NNRPSEGPLQTR.L | 2.00000 | 2.41E+00 |
| K.LLLPWLEAR.I | 2.00000 | 1.82E+00 |
| R.NLQNLILITAIK.A | 2.00000 | 3.74E+00 |
| R.KFDVNTSAVQVLIHIGNLDR.A | 2.00000 | 6.29E+00 |
| R.LASTLVHLGEYQAAVDGR.K | 2.00000 | 4.71E+00 |
| K.YHEQLSTQSLIELFESFK.S | 2.00000 | 2.69E+00 |
| K.AFM#TADLPNELIELLEK.I | 2.00000 | 1.07E+00 |
| K.AFMTADLPNELIELLEK.I | 2.00000 | 2.01E+00 |
| AP2A1_HUMAN AP-2 complex subunit alpha-1 | | |
| R.KKGPAGSALDDGR.R | 2.00000 | 4.09E+00 |
| R.KKGPAGSALDDGR.R | 2.00000 | 2.57E+00 |
| R.KKGPAGSALDDGR.R | 2.00000 | 3.59E+00 |
| R.KKGPAGSALDDGR.R | 2.00000 | 2.69E+00 |
| K.ANHMPDAEVTK.A | 2.00000 | 4.05E+00 |
| K.ANHMPDAEVTK.A | 2.00000 | 3.67E+00 |
| K.KGPAGSALDDGR.R | 2.00000 | 2.13E+00 |
| K.GPGAGSALDDGRR.D | 2.00000 | 2.16E+00 |
| R.LEPNAQAQM#YR.L | 2.00000 | 1.44E+00 |
| R.LEPNAQAQM#YR.L | 2.00000 | 1.62E+00 |
| R.INKELANIR.S | 2.00000 | 2.64E+00 |
| R.INKELANIR.S | 2.00000 | 3.27E+00 |
| R.LEPNAQAQM#YR.L | 2.00000 | 1.18E+00 |
| K.QLSLPQQAQK.I | 2.00000 | 1.98E+00 |
| K.TCVSLAVSR.L | 2.00000 | 1.62E+00 |
| K.FHLCSVATR.A | 2.00000 | 2.44E+00 |
| K.FHLCSVATR.A | 2.00000 | 2.75E+00 |
| R.LLQCYPPEDAAVK.G | 2.00000 | 1.10E+00 |
| R.LLQCYPPEDAAVK.G | 2.00000 | 8.91E-01 |
| R.AADLLYAM#CDR.S | 2.00000 | 1.65E+00 |
| R.VLQIVTNRDDVQGYAAK.T | 2.00000 | 4.63E+00 |
| R.YGGAPQALTLK.L | 2.00000 | 2.59E+00 |

| | | |
|--------------------------------|---------|----------|
| R.VLQIVTNRDDVQGYAAK.T | 2.00000 | 3.45E+00 |
| R.VLQIVTNRDDVQGYAAK.T | 2.00000 | 2.88E+00 |
| R.EM#GEAFAADIPR.I | 2.00000 | 2.25E+00 |
| R.EM#GEAFAADIPR.I | 2.00000 | 1.65E+00 |
| R.ACNQLGQFLQHR.E | 2.00000 | 3.28E+00 |
| K.TVFEALQAPACHENM#VK.V | 2.00000 | 2.35E+00 |
| K.TVFEALQAPACHENM#VK.V | 2.00000 | 1.87E+00 |
| R.ACNQLGQFLQHR.E | 2.00000 | 3.04E+00 |
| K.THIDTVINALK.T | 2.00000 | 3.38E+00 |
| K.THIDTVINALK.T | 2.00000 | 2.59E+00 |
| K.ASPDLVPM#GEWTAR.V | 2.00000 | 1.19E+00 |
| K.QSAALCLLR.L | 2.00000 | 2.25E+00 |
| R.ACNQLGQFLQHR.E | 2.00000 | 2.27E+00 |
| R.ACNQLGQFLQHR.E | 2.00000 | 2.54E+00 |
| R.SPPVQFSLHLSK.F | 2.00000 | 1.67E+00 |
| R.SPPVQFSLHLSK.F | 2.00000 | 2.84E+00 |
| K.THIDTVINALK.T | 2.00000 | 2.73E+00 |
| K.ASPDLVPM#GEWTAR.V | 2.00000 | 1.05E+00 |
| K.THIDTVINALKTER.D | 2.00000 | 3.05E+00 |
| K.QSAALCLLR.L | 2.00000 | 2.24E+00 |
| R.SPPVQFSLHLSK.F | 2.00000 | 2.38E+00 |
| K.TVFEALQAPACHENMVK.V | 2.00000 | 1.57E+00 |
| R.LVECLETVLNK.A | 2.00000 | 2.60E+00 |
| R.YLALES#CTLASSEFSHEAVK.T | 2.00000 | 2.34E+00 |
| R.LVECLETVLNK.A | 2.00000 | 2.11E+00 |
| R.NPTFM#CLALHCIANVGSR.E | 2.00000 | 3.86E+00 |
| R.YLETADYAIREEIVLK.V | 2.00000 | 2.85E+00 |
| K.FFQPTEM#AAQDFQ.R.W | 2.00000 | 2.02E+00 |
| K.VGGYILGEFGNLIAGDPR.S | 2.00000 | 2.95E+00 |
| -.GLAVFISDIR.- | 2.00000 | 2.11E+00 |
| R.NPTFM#CLALHCIANVGSR.E | 2.00000 | 2.51E+00 |
| -.GLAVFISDIR.- | 2.00000 | 2.31E+00 |
| R.VAAQVDGGAQVQVNLNIECLR.D | 2.00000 | 3.23E+00 |
| K.FFQPTEM#AAQDFQ.R.W | 2.00000 | 2.16E+00 |
| K.FFQPTEM#AAQDFQ.R.W | 2.00000 | 2.33E+00 |
| K.FFQPTEMAAQDFQ.R.W | 2.00000 | 2.46E+00 |
| R.YLALES#CTLASSEFSHEAVK.T | 2.00000 | 3.45E+00 |
| R.DFLTPPLLSVR.F | 2.00000 | 2.41E+00 |
| K.NNGVLFENQLLQIGVK.S | 2.00000 | 2.80E+00 |
| K.FFQPTEMAAQDFQ.R.W | 2.00000 | 1.03E+00 |
| K.FFQPTEMAAQDFQ.R.W | 2.00000 | 3.74E+00 |
| R.YLALES#CTLASSEFSHEAVK.T | 2.00000 | 3.15E+00 |
| K.NNGVLFENQLLQIGVK.S | 2.00000 | 3.39E+00 |
| K.VGGYILGEFGNLIAGDPR.S | 2.00000 | 3.54E+00 |
| K.N@NGVLFENQLLQIGVK.S | 2.00000 | 3.43E+00 |
| -.NN@GVLFENQLLQIGVK.- | 2.00000 | 3.43E+00 |
| -.NNGVLFEN@QLLQIGVK.- | 2.00000 | 3.03E+00 |
| -.NNGVLFENQ@LLQIGVK.- | 2.00000 | 3.02E+00 |
| -.NNGVLFENQLLQ@IGVK.- | 3.00000 | 2.98E+00 |
| K.LLGFGSALLDNVDPNPNFVAGIIQTK.A | 2.00000 | 3.72E+00 |
| K.VGGYILGEFGNLIAGDPR.S | 2.00000 | 2.09E+00 |
| R.NPTFMCLALHCIANVGSR.E | 2.00000 | 3.18E+00 |
| R.NPTFMCLALHCIANVGSR.E | 2.00000 | 1.90E+00 |
| R.VVHLLNDQHMGVVTAAVSLITCLCK.K | 2.00000 | 2.94E+00 |
| K.VGGYILGEFGNLIAGDPR.S | 2.00000 | 2.21E+00 |
| R.VVHLLNDQHMGVVTAAVSLITCLCK.K | 2.00000 | 3.14E+00 |
| R.VVHLLNDQHMGVVTAAVSLITCLCK.K | 2.00000 | 4.31E+00 |
| R.VVHLLNDQHMGVVTAAVSLITCLCK.K | 2.00000 | 3.32E+00 |

STAT3_HUMAN Signal transducer and activator of transcription 3

| | | |
|---|--------|----------|
| -.SQ@GDM#QDLNGNNQSVTR.- | 3.0000 | 2.02E+00 |
| -.SQGDM#Q@DLNGNNQSVTR.- | 2.0000 | 1.97E+00 |
| K.SQGDM#QDLN@GNNQSVTR.Q | 1.0000 | 1.81E+00 |
| K.TQIQSVEPYTK.Q | 2.0000 | 1.74E+00 |
| R.LLQTAATAAQGGQANHPTAAVVTEK.Q | 2.0000 | 4.41E+00 |
| K.TLTDEELADWK.R | 2.0000 | 1.51E+00 |
| K.LLGPVNYSGCQITWAK.F | 2.0000 | 1.96E+00 |
| R.AILSTKPPGTFLLR.F | 1.0000 | 2.89E+00 |
| K.VVENLQDDDFDFNYK.T | 2.0000 | 1.79E+00 |
| R.YLEQLHQLYSDSFPMELR.Q | 2.0000 | 2.55E+00 |
| K.ESHATLVFHNLGGEIDQQYSR.F | 2.0000 | 4.70E+00 |
| R.YLEQLHQLYSDSFPMELR.Q | 2.0000 | 2.62E+00 |
| K.ESHATLVFHNLGGEIDQQYSR.F | 2.0000 | 3.71E+00 |
| R.LENWITSLAESQLQTR.Q | 2.0000 | 2.46E+00 |
| R.LENWITSLAESQ@LQTR.Q | 2.0000 | 1.99E+00 |
| R.QFLAPWIESQDWAYAASK.E | 2.0000 | 1.75E+00 |
| K.QQLNNMSFAEIMGYK.I | 2.0000 | 1.31E+00 |
| K.MQQLQMLTALDQMR.R | 2.0000 | 2.25E+00 |
| K.IM#DATNILVSPLVYLYPDIPKEEAFGK.Y | 2.0000 | 3.95E+00 |
| K.IMDATNILVSPLVYLYPDIPKEEAFGK.Y | 2.0000 | 3.61E+00 |
| R.SIVSELAGLLSAM#EYVQK.T | 2.0000 | 3.19E+00 |
| R.SIVSELAGLLSAM#EYVQK.T | 2.0000 | 7.70E-01 |
| R.SIVSELAGLLSAMEYVQK.T | 2.0000 | 3.25E+00 |
| AP2M1_HUMAN AP-2 complex subunit mu-1 | | |
| R.SGIYETR.- | 1.0000 | 1.90E+00 |
| R.SPVTNIAR.T | 1.0000 | 2.47E+00 |
| K.SYLSGM#PECK.F | 1.0000 | 1.68E+00 |
| K.SYLSGMPECK.F | 1.0000 | 1.16E+00 |
| K.SNFKPSLLAQK.I | 1.0000 | 2.75E+00 |
| K.SNFKPSLLAQK.I | 1.0000 | 3.54E+00 |
| R.TSFFHVK.R | 1.0000 | 1.70E+00 |
| K.M#CDVM#AAYFGK.I | 1.0000 | 8.22E-01 |
| K.YKASENAIVWK.I | 1.0000 | 2.37E+00 |
| K.SYLSGMPECK.F | 1.0000 | 1.36E+00 |
| K.SNFKPSLLAQK.I | 1.0000 | 3.79E+00 |
| K.ASENAIVWK.I | 1.0000 | 2.84E+00 |
| K.QSIAIDDCTFHQCVR.L | 1.0000 | 2.40E+00 |
| K.EEQSQITSQVTGQIGWR.R | 1.0000 | 2.10E+00 |
| -.MIGGLFIYNHK.G | 1.0000 | 3.31E+00 |
| R.IPTPLNTSGVQVICMK.G | 1.0000 | 2.55E+00 |
| R.SNIWLAAVTK.Q | 1.0000 | 2.99E+00 |
| -.MIGGLFIYNHK.G | 1.0000 | 1.62E+00 |
| R.IPTPLNTSGVQVICMK.G | 1.0000 | 2.83E+00 |
| R.SNIWLAAVTK.Q | 1.0000 | 2.92E+00 |
| K.MCDVMAAYFGK.I | 1.0000 | 1.31E+00 |
| K.WARPPISM#NFEVVPFAPSGLK.V | 1.0000 | 2.94E+00 |
| K.WARPPISM#NFEVVPFAPSGLK.V | 1.0000 | 2.66E+00 |
| K.WARPPISMNFEVVPFAPSGLK.V | 1.0000 | 2.27E+00 |
| K.QNVNAAM#VFEFLYK.M | 1.0000 | 1.33E+00 |
| K.WARPPISMNFEVVPFAPSGLK.V | 1.0000 | 4.06E+00 |
| K.QNVNAAMVFEFLYK.M | 1.0000 | 1.90E+00 |
| K.QNVNAAM#VFEFLYK.M | 1.0000 | 2.03E+00 |
| K.QNVNAAM#VFEFLYK.M | 1.0000 | 1.96E+00 |
| K.QNVNAAMVFEFLYK.M | 1.0000 | 1.81E+00 |
| AP2S1_HUMAN AP-2 complex subunit sigma-1 | | |
| K.HTNFVEFR.N | 1.0000 | 1.71E+00 |
| K.HTNFVEFR.N | 1.0000 | 1.73E+00 |
| K.LIEEVHAVVTVR.D | 1.0000 | 3.85E+00 |

| | | |
|---|---------|----------|
| K.LIEEVHAVVTVR.D | 1.0000 | 2.15E+00 |
| K.LIEEVHAVVTVR.D | 1.0000 | 3.32E+00 |
| K.WYMQFDDEKQK.L | 1.0000 | 1.27E+00 |
| K.LIEEVHAVVTVR.D | 1.0000 | 2.06E+00 |
| K.LIEEVHAVVTVR.D | 1.0000 | 2.72E+00 |
| K.VYTVVDEMFLAGEIR.E | 1.0000 | 2.29E+00 |
| K.VYTVVDEMFLAGEIR.E | 1.0000 | 2.13E+00 |
| K.VYTVVDEMFLAGEIR.E | 1.0000 | 1.98E+00 |
| EPN4_HUMAN Clathrin interactor 1 (Epsin-4) | | |
| K.ASPDQNASTHTPQSSVK.T | 2.0000 | 1.73E+00 |
| K.ASPDQNASTHTPQSSVK.T | 2.0000 | 1.06E+00 |
| K.HIHITQATETTTTR.H | 2.0000 | 2.68E+00 |
| K.HIHITQATETTTTR.H | 2.0000 | 2.31E+00 |
| K.GEFKDEEETVTK.H | 1.0000 | 1.22E+00 |
| K.GEFKDEEETVTK.H | 1.0000 | 1.60E+00 |
| R.SLENYHFVDEHGKQGINIR.Q | 2.0000 | 2.04E+00 |
| K.ATNVVMNYSEIESK.V | 2.0000 | 1.32E+00 |
| K.SLLLLLAYLIR.N | 2.0000 | 2.36E+00 |
| RAB1B_HUMAN Ras-related protein Rab-1B | | |
| K.NATNVEQAFM#TMAAEIK.K | 2.00000 | 4.40E+00 |
| -.NATNVEQAFMTM#AAEIK.- | 2.00000 | 3.97E+00 |
| SRC_HUMAN Proto-oncogene tyrosine-protein kinase Src | | |
| R.LTTVCPTSKPQTQGLAK.D | 2.00000 | 4.56E+00 |
| R.GPSAAFAPAAAEPK.L | 2.00000 | 2.61E+00 |
| R.KLDSGGFYITSR.T | 2.00000 | 4.11E+00 |
| K.LFGGFNSSDVTSPQR.A | 2.00000 | 3.35E+00 |
| K.LFGGFNSSDVTSPQR.A | 2.00000 | 4.05E+00 |
| R.TQFNSLQQLVAYYSK.H | 2.00000 | 4.10E+00 |
| R.AGPLAGGVTTFVALYDYESR.T | 3.00000 | 4.25E+00 |
| R.LPQLVDMAAQIASGMAYVER.M | 3.00000 | 3.85E+00 |
| R.LPQLVDMAAQIASGMAYVER.M | 2.00000 | 5.83E+00 |
| R.LPQLVDMAAQIASGMAYVER.M | 3.00000 | 4.92E+00 |
| K.SDVWSFGILLTELTK.G | 2.00000 | 2.93E+00 |
| SHC1_HUMAN SHC-transforming protein 1 | | |
| R.QAVGGAGPPNPAINGSAPR.D | 2.00000 | 4.52E+00 |
| R.TRVEGGQLGGEWTR.H | 2.00000 | 4.64E+00 |
| R.VPPPPQSVSM*AEQLR.G | 2.00000 | 1.75E+00 |
| R.VEGGQLGGEWTR.H | 2.00000 | 3.86E+00 |
| R.YMGCVEVLQSM*R.A | 2.00000 | 1.74E+00 |
| R.VPPPPQSVSMAEQLR.G | 2.00000 | 2.14E+00 |
| K.VM*GPGVSYLVR.Y | 2.00000 | 2.98E+00 |
| R.PLSSILGR.S | 2.00000 | 2.16E+00 |
| R.VPPPPQSVSMAEQLR.G | 2.00000 | 2.54E+00 |
| K.EPPLGGVDMR.L | 2.00000 | 2.39E+00 |
| R.PTAPNAQTPSHLGATLPVGPVGGDPEVR.K | 3.00000 | 6.17E+00 |
| R.YM*GCVEVLQSMR.A | 2.00000 | 2.31E+00 |
| K.VMGPVSYLVR.Y | 2.00000 | 2.76E+00 |
| R.EAISLVCEAVPGAK.G | 2.00000 | 2.70E+00 |
| K.VMGPVSYLVR.Y | 2.00000 | 2.77E+00 |
| R.ESTTTPGQYVLTGLQSGQPK.H | 2.00000 | 3.65E+00 |
| R.YMGCVEVLQSMR.A | 2.00000 | 2.62E+00 |
| R.ELFDDPSYVNVQNLDK.A | 2.00000 | 3.77E+00 |
| R.ELFDDPSYVNVQNLDK.A | 2.00000 | 3.93E+00 |
| R.EAEALLQLNGDFLVR.E | 2.00000 | 4.09E+00 |
| R.DLFDMPKPFEDALR.V | 3.00000 | 1.85E+00 |
| R.DLFDMPKPFEDALR.V | 2.00000 | 2.95E+00 |
| R.DLFDMPKPFEDALR.V | 2.00000 | 3.22E+00 |
| R.DLFDMPKPFEDALR.V | 3.00000 | 2.11E+00 |
| R.ACHILECEGLAQDVISTIGQAFELR.F | 3.00000 | 6.24E+00 |

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|--|---------|----------|
| R.ACHILECPEGLAQDVISTIGQAFELR.F | 4.00000 | 3.80E+00 |
| P85A_HUMAN Phosphatidylinositol 3-kinase regulatory subunit alpha | | |
| K.IFEEQCQTQER.Y | 2.00000 | 2.30E+00 |
| K.IFEEQCQTQER.Y | 2.00000 | 2.50E+00 |
| K.YQQDQVVKEDNIEAVGKK.L | 3.00000 | 5.06E+00 |
| K.YQQDQVVKEDNIEAVGK.K | 3.00000 | 3.32E+00 |
| R.FSAASSDNTENLIK.V | 2.00000 | 3.81E+00 |
| K.QGCYACSVVDGEVK.H | 2.00000 | 2.43E+00 |
| R.VLSEIFSPMLFR.F | 2.00000 | 3.11E+00 |
| R.VLSEIFSPMLFR.F | 2.00000 | 2.84E+00 |
| K.TEADVEQQALTLPLDLAEQFAPPDIAPPLLIK.L | 3.00000 | 2.84E+00 |
| K.TEADVEQQALTLPLDLAEQFAPPDIAPPLLIK.L | 3.00000 | 2.51E+00 |
| K.YGFSDDLTFSSVVELINHYR.N | 3.00000 | 3.89E+00 |
| K.YGFSDDLTFSSVVELINHYR.N | 3.00000 | 3.83E+00 |
| P85B_HUMAN Phosphatidylinositol 3-kinase regulatory subunit beta | | |
| R.APGPGPPPAAR.- | 2.00000 | 2.70E+00 |
| R.AALQALGVAEGGER.C | 2.00000 | 3.64E+00 |
| R.TAIEAFNETIK.I | 2.00000 | 3.13E+00 |
| R.TATGFGFAEPYNLYGSLK.E | 2.00000 | 4.36E+00 |
| R.ALGATFGPLLR.A | 2.00000 | 3.01E+00 |
| R.ALGATFGPLLR.A | 2.00000 | 2.58E+00 |
| R.GDFPGTYVEFLGPVALAR.P | 2.00000 | 3.57E+00 |
| K.SFLLALPAPLVTPEASAEAR.R | 2.00000 | 4.18E+00 |
| K.SFLLALPAPLVTPEASAEAR.R | 2.00000 | 2.81E+00 |
| RSU1_HUMAN Ras suppressor protein 1 | | |
| R.LTVLPPPELGNLDLTGQK.Q | 2.00000 | 3.20E+00 |
| K.AENNPWVTPIADQFQLGVSHVFEYIR.S | 3.00000 | 2.56E+00 |
| PK3CB_HUMAN Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform | | |
| R.AGLFHGTELLCK.T | 3.00000 | 3.54E+00 |
| K.VFGEDSVGVIFK.N | 2.00000 | 3.30E+00 |
| R.LCDVRPFLPVLK.L | 3.00000 | 2.67E+00 |
| R.VEYVFGDHPLIQFYIR.N | 3.00000 | 3.80E+00 |
| R.EALELLDFNYPDQYVR.E | 2.00000 | 2.15E+00 |
| R.IGQFLFWHLR.S | 3.00000 | 3.60E+00 |
| K.TGQLFHIDFGHILGNFK.S | 4.00000 | 3.49E+00 |
| R.VPFILTYDFIHVIQGGK.T | 3.00000 | 3.83E+00 |
| R.EALSDLQSPNPCVILSELYVEK.C | 3.00000 | 3.60E+00 |
| GRB10_HUMAN Growth factor receptor-bound protein 10 | | |
| R.LLCAEDEQTR.T | 2.00000 | 2.56E+00 |
| R.VIENPAEAQSAALEEGHAWR.K | 3.00000 | 2.50E+00 |
| R.MNILGSQSPLHPSTLSTVIHR.T | 3.00000 | 4.25E+00 |
| K.VVEILADMTAR.D | 2.00000 | 2.94E+00 |
| R.DLCQLLVYK.S | 2.00000 | 2.51E+00 |
| K.QQGLVDGFLLR.D | 2.00000 | 2.41E+00 |
| K.QQGLVDGFLLR.D | 2.00000 | 2.51E+00 |
| K.FSDLIQLVDFYQLNK.G | 2.00000 | 4.08E+00 |
| K.FSDLIQLVDFYQLNK.G | 2.00000 | 4.78E+00 |
| ARAF_HUMAN A-Raf proto-oncogene serine/threonine-protein kinase | | |
| K.VSQPTAEQAQAFK.N | 2.00000 | 2.39E+00 |
| R.EERPLFPQILATIELLQR.S | 3.00000 | 3.69E+00 |
| FYN_HUMAN Proto-oncogene tyrosine-protein kinase Fyn | | |
| R.LIEDNEYTAR.Q | 2.00000 | 2.43E+00 |
| K.WTAPAAALYGR.F | 2.00000 | 2.80E+00 |
| K.TLKPGMTSPESFLEEAQIMK.K | 3.00000 | 4.43E+00 |
| YES_HUMAN Proto-oncogene tyrosine-protein kinase Yes | | |
| K.GRVPYPGM*VNR.E | 3.00000 | 3.33E+00 |
| R.AANILVGENLVCK.I | 2.00000 | 3.27E+00 |
| RAB5A_HUMAN Ras-related protein Rab-5A | | |
| R.QASPNIVIALSGNK.A | 1.00000 | 1.95E+00 |

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|--|---------|----------|
| R.QASPNIVIALSGNK.A | 1.00000 | 1.77E+00 |
| R.GAQAAIVVYDITNEESFAR.A | 1.00000 | 1.60E+00 |
| VAV2_HUMAN Protein vav-2 | | |
| K.EIIELLFHK.M | 2.00000 | 2.71E+00 |
| R.NSELFDPFDLFDVR.D | 1.00000 | 2.21E+00 |
| R.VVWPSAVVFDLAQALR.D | 1.00000 | 3.23E+00 |
| PLCG1_HUMAN 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-1 | | |
| R.LSEPVPQTNAHESK.E | 1.00000 | 3.01E+00 |
| K.NM*LSQVNYR.V | 1.00000 | 2.92E+00 |
| R.GSDASGQLFHGR.A | 2.00000 | 3.16E+00 |
| R.ISQEHLADHFDSR.E | 2.00000 | 3.32E+00 |
| R.ISQEHLADHFDSR.E | 2.00000 | 4.37E+00 |
| -.QITWSR.- | 2.00000 | 1.15E+00 |
| K.EWYHASLTR.A | 2.00000 | 1.73E+00 |
| R.KRNEPNSY AISFR.A | 2.00000 | 3.98E+00 |
| R.GADKIEGAIDIR.E | 2.00000 | 3.81E+00 |
| K.NMLSQVNYR.V | 2.00000 | 2.74E+00 |
| R.GADKIEGAIDIR.E | 1.00000 | 3.26E+00 |
| K.FSDVLHTIK.E | 1.00000 | 2.82E+00 |
| K.LRYPINEEALEK.I | 1.00000 | 4.26E+00 |
| K.IGTAEPDYGALYEGR.N | 1.00000 | 3.33E+00 |
| K.IGTAEPDYGALYEGR.N | 1.00000 | 5.04E+00 |
| R.GVLDVPACQIAIRPEGK.N | 1.00000 | 1.96E+00 |
| R.SGDITYGQFAQLYR.S | 1.00000 | 4.81E+00 |
| R.NPGFYVEANPMPYFK.C | 2.00000 | 4.21E+00 |
| K.VLGDITLLTKPVEISADGLPSPNQLKR.K | 2.00000 | 1.50E+00 |
| R.LLTEYCIETGAPDGSFLVR.E | 2.00000 | 5.24E+00 |
| K.TM*DLPFLEASTLR.A | 2.00000 | 3.16E+00 |
| R.GLEPCAISIEVLGAR.H | 2.00000 | 3.45E+00 |
| K.VLGDITLLTKPVEISADGLPSPNQLK.R | 2.00000 | 3.64E+00 |
| K.TMDLPFLEASTLR.A | 1.00000 | 4.54E+00 |
| K.TMDLPFLEASTLR.A | 1.00000 | 3.70E+00 |
| R.AVPLKNNYSEDELEASLLIK.I | 1.00000 | 2.35E+00 |
| R.ESETFVGDYTLFSWR.N | 2.00000 | 3.57E+00 |
| K.NNYSDELEASLLIK.I | 1.00000 | 4.57E+00 |
| R.SLEVGTVMTLFYSK.K | 1.00000 | 3.89E+00 |
| R.SLEVGTVMTLFYSK.K | 1.00000 | 3.99E+00 |
| R.SLEVGTVMTLFYSK.K | 1.00000 | 3.66E+00 |
| R.LQVQEFMLSFLR.D | 1.00000 | 3.44E+00 |
| R.LQVQEFMLSFLR.D | 1.00000 | 3.70E+00 |
| R.LQVQEFMLSFLR.DPLR.E | 1.00000 | 1.93E+00 |
| R.VQEQGQTVMLGNSEFDSLVDLISYYEK.H | 1.00000 | 3.68E+00 |
| RUFY1_HUMAN RUN and FYVE domain-containing protein 1 (FYVE-finger protein EIP1) | | |
| K.LCPEASDIATSVR.N | 2.00000 | 2.88E+00 |
| R.IGALQLQLSQLHEQCSSEK.E | 3.00000 | 3.74E+00 |
| RAB10_HUMAN Ras-related protein Rab-10 | | |
| -.LQIWDTAGQER.- | 2.00000 | 2.56E+00 |
| -.LQIWDTAGQER.- | 2.00000 | 2.66E+00 |
| K.AFLTLAEDILR.K | 2.00000 | 3.62E+00 |

Appendix II

The sequence of human FGFR1 WT and FGFR1 Y677F.

Human FGFR1 WT

1 atgtggagctggaagtgcctcctcttctgggctgtgctggtcacagccacactctg
> M W S W K C L L F W A V L V T A T L C
57 caccgctagccgctccccgacctgacctgaacaagcccagccctggggagcccctg
> T A R P S P T L P E Q A Q P W G A P
113 tggaagtggagctcctcctgggtccaccccggtgacctgctgcagcttcgctgtcgg
> V E V E S F L V H P G D L L Q L R C R
169 ctgcgggacgatgtgcagagcatcaactggctgcgggacggggtgcagctggcgga
> L R D D V Q S I N W L R D G V Q L A E
225 aagcaaccgcaccgcatcacaggggaggaggtggaggtgcaggactccgtgcccg
> S N R T R I T G E E V E V Q D S V P
281 cagactccggcctctatgcttgctaaccagagcccctcgggacgtgacaccacc
> A D S G L Y A C V T S S P S G S D T T
337 tacttctccgtaaatgtttcagatgctctcccctcctcggaggatgatgatga
> Y F S V N V S D A L P S S E D D D D D
393 tgatgactcctcttcagaggagaagaacagataacaccaaaccacccgtagtc
> D D S S S E E K E T D N T K P N R M
449 ccgtagctccatattggacatccccagaaaagatggaaaagaaattgcatgcagtg
> P W A P Y W A L S P E K M E K K L H A Y
505 ccggctgccaaagacagtgaagttcaaatgcccttcagtgggaccccaaacccac
> P A A K T V K F K C P S S G T P N P T
561 actgcgctggttgaaaaatggcaagaattcaaacctgaccacagaattggaggct
> L R W L K N G K E F K P D H R I G G
617 acaaggtccggtatgccacctggagcatcataatggactctgtggtgcccctctgac
> Y K V R Y A T W S I I M D S V V P S D
673 aaggvcaactaacctgcattgtggagaatgagtacggcagcatcaaccacata
> K G N Y T C I V E N E Y G S I N H T Y
729 ccagctggatgctggtggagcgggtcccctcaccggcccatcctgcaagcagggttgc
> Q L D V V E R S P H R P I L Q A G L
785 ccgccaacaaaacagtggccctgggtagcaacgtggagtccatgtgtaaggtgtac
> P A N K T V A L G S N V E F M C K V Y
841 agtgaccgcagccgcacatccagtggttaagcacatcgaggtgaatgggagcaa
> S D P Q P H I Q W L K H I E V N G S K
897 gattggcccagacaacctgccttatgtccagatcttgaagactgctggagttaata
> I G P D N L P Y V Q I L K T A G V N
953 ccaccgacaaaagatggaggtgcttcacttaagaaatgtctcctttgaggacgca
> T R M E V A L H L R N V S F E D A
1009 ggggagtatacgtgcttggcgggtaactctatcggactctcccatcactctgcatg
> G E Y T C L A G N S I G L S H H S A W
1065 gttgaccgttctggaagccctggaagagaggccggcagtgatgacctgccccctgt
> L T V A L E A L E E R P A V M T S P L
1121 acctggagatcatctattgcacagggccttcctcatctcctgcatggtgggg
> Y L E I I I Y C T G A F L I S C M V Y
1177 tcggtcatcgtctacaagatgaagagtggtagcaagaagagtgactccacagcca
> S V I V Y K M K S G T K K S D F H S Q
1233 gatggctgtgcacaagctggccaagagcatccctctgcgagacaggtaacagtgt
> M A V H K L A K S I P L R R Q V T V
1289 ctgctgactccagtgcatccatgaactctggggttcttctggttcggccatcacgg
> S A D S S A S M N S G V L L V R P S R
1345 ctctcctccagtgggactcccatgctagcaggggtctctgagtatgagcttcccga
> L S S S G T P M L A G V S E Y E L P E
1401 agaccctcgtgggagctgcctcgggacagactgggtcttaggcaaaccctgggag
> D P R W E L P R D R L V L G K P L G
1457 agggctgctttgggcaggtggtggtggcagaggctatcgggctggacaaggacaaa
> E G C F G Q V V L A E A I G L D K D K
1513 cccaaccgtgtgaccaaagtggctgtgaagatggtgaagtgcggacgcaacagagaa
> P N R V T K V A V K M L K S D A T E K
1569 agacttgtcagacctgatctcagaaatggagatgatgaagatgatcggaagcata
> D L S D L I S E M E M M K M I G K H
1625 agaatatcatcaacctgctggggcctgcacgcaggatggtcccttgtatgtcatc
> K N I I N L L G A C T Q D G P L Y V I
1681 gtggagtatgcctccaagggcaacctgcgggagtagctgcaggcccggaggccccc
> V E Y A S K G N L R E Y L Q A R R P P
1737 agggctggaatactgctacaacccagccacaacccagaggagcagctctcctcca
> G L E Y C Y N P S H N P E E Q L S S

1793 aggacctgtgtcctgctgcctaccaggtggcccgaggcatggagtatctggcctcc
>K D L V S C A Y Q V A R G M E Y L A S
1849 aagaagtgcatacaccgagacctggcagccaggaatgtcctgggtgacagaggaaa
>K K C I H R D L A A R N V L V T E D N
1905 tgtgatgaagatagcagactttggcctgcacgggacattcaccacatcgactact
>V M K I A D F G L A R D I H H I D Y
1961 ataaaaagacaaccaacggccgactgcctgtgaagtggatggcaccgaggcatta
>Y K K T T N G R L P V K W M A P E A L
2017 tttgaccggatctacaccaccagagtgatgtgtggtctttcggggtgctcctgtg
>F D R I Y T H Q S D V W S F G V L L W
2073 ggagatcttcaactctgggcggtccccataccccgggtgtgcctgtggaggaacttt
>E I F T L G G S P Y P G V P V E E L
2129 tcaagctgtgaaggagggtcaccgcatggacaagcccagtaactgcaccaacgag
>F K L L K E G H R M D K P S N C T N E
2185 ctgtacatgatgatgctgggactgctggcatgcagtgcctcacagagaccacctt
>L Y M M M R D C W H A V P S Q R P T F
2241 caagcagtggtggaagaccgcatcgtggccttgacctcaaccaggagt
>K Q L V E D L D R I V A L T S N Q E
2297 acctggacctgtccatgcccctggaccagtactccccagctttcccgacaccgg
>Y L D L S M P L D Q Y S P S F P D T R
2353 agctctacgtgctcctcaggggaggattccttctctcatgagccgctgcccga
>S S T C S S G E D S V F S H E P L P E
2409 ggagccctgctgccccgacccagccagcttgccaatggcggactcaaaccg
>E P C L P R H P A Q L A N G G L K R
2465 gctga
>R

Human FGFR1 Y677F

1 atgtggagctggaagtgcctcctcttctgggctgtgctggtcacagccacactctg
>M W S W K C L L F W A V L V T A T L C
57 caccgctaggccgtccccgaccttgctgaacaagcccagccctggggagcccctg
>T A R P S P T L P E Q A Q P W G A P
113 tggagtgaggctccttctggtccacccccgggtgacctgctgcagcttcgctgtcgg
>V E V E S F L V H P G D L L Q L R C R
169 ctgctgggacgatgtgcagagcatcaactggctgctgggacgggggtgcagctggcgga
>L R D D V Q S I N W L R D G V Q L A E
225 aagcaaccgcacccgcatcacaggggaggaggtggaggtgcaggactccgtgcccg
>S N R T R I T G E E V E V Q D S V P
281 cagactccggcctctatgcttgctgaaccagcagcccctcgggacgtgacaccacc
>A D S G L Y A C V T S P S G S D T T
337 tacttctccgtcaatgtttcagatgctctcccctcctcggaggatgatgatgatga
>Y F S V N V S D A L P S S E D D D D D
393 tgatgactcctcttcagaggagaaagaacagataacaccaaaccaaccgatatgc
>D D S S S E E K E T D N T K P N R M
449 ccgtagctccatattggacatccccagaaaagatggaaaagaattgcatgcagtg
>P V A P Y W T S P E K M E K K L H A V
505 ccggctgccaagacagtgaagttcaaatgcccttccagtgggaccccaaacccac
>P A A K T V K F K C P S S G T P N P T
561 actgctgctggtgaaaaatggcaagaattcaaacctgaccacagaattggaggct
>L R W L K N G K E F K P D H R I G G
617 acaaggtccgttatgccacctggagcatcataatggactctgtggtgccctctgac
>Y K V R Y A T W S I I M D S V V P S D
673 aagggcaactacacctgcattgtggagaatgagtacggcagcatcaaccacacata
>K G N Y T C I V E N E Y G S I N H T Y
729 ccagctggatgtcgtggagcgggtcccctcaccggcccatcctgcaagcagggttgc
>Q L D V V E R S P H R P I L Q A G L
785 ccgccaacaaaacagtgccctgggttagcaactggagttcatgtgtaagggtgac
>P A N K T V A L G S N V E F M C K V Y
841 agtgaccgagccgcacatccagtggctaaagcacatcgaggtgaatgggagcaa
>S D P Q P H I Q W L K H I E V N G S K
897 gattggcccagacaacctgccttatgtccagatcttgaagactgctggagttaata
>I G P D N L P Y V Q I L K T A G V N
953 ccaccgacaaagagatggaggtgcttcacttaagaatgtctcctttgaggacgca

>T T D K E M E V L H L R N V S F E D A
 1009 ggggagtatacgtgcttggcgggtaactctatcggactctcccatcactctgcatg
 >G E Y T C L A G N S I G L S H H S A W
 1065 gttgaccgttctggaagccctggaagagaggccggcagtgatgacctcgcccctgt
 >L T V L E A L E E R P A V M T S P L
 1121 acctggagatcatcatctattgcacaggggccttctcatctcctgcatggtgggg
 >Y L E I I I Y C T G A F L I S C M V G
 1177 tcggtcatcgtctacaagatgaagagtgggtaccaagaagagtgacttccacagcca
 >S V I V Y K M K S G T K K S D F H S Q
 1233 gatggctgtgcacaagctggccaagatccctctgcgagacaggtaacagtg
 >M A V H K L A K S I P L R R Q V T V
 1289 ctgctgactccagtgcatccatgaactctggggttcttctggttcggccatcacgg
 >S A D S S A S M N S G V L L V R P S R
 1345 ctctcctccagtgggactcccatgctagcaggggtctctgagatgagcttcccga
 >L S S S G T P M L A G V S E Y E L P E
 1401 agaccctcgctgggagctgcctcgggacagactggctttaggcaaaccctgggag
 >D P R W E L P R D R L V L G K P L G
 1457 agggctgcttgggaggtggtggtggcagaggctatcgggctggacaaggacaaa
 >E G C F G Q V V L A E A I G L D K D K
 1513 cccaaccgtgtgaccaaagtggctgtgaagatggtgaagtcggacgcaacagagaa
 >P N R V T K V A V K M L K S D A T E K
 1569 agacttgcagactgatctcagaatggagatgatgaagatgatcgggaagcata
 >D L S D L I S E M E M M K M I G K H
 1625 agaatatcatcaacctgctggggcctgcacgcaggatggtcccttgtatgtcatc
 >K N I I N L L G A C T Q D G P L Y V I
 1681 gtggagtatgcctccaagggcaacctgcccggagctgacagggcccgaggccccc
 >V E Y A S K G N L R E Y L Q A R R P P
 1737 agggctggaatactgctacaaccccagccacaacccagaggagcagctctcctcca
 >G L E Y C Y N P S H N P E E Q L S S
 1793 aggacctggtgtcctgctgacctaccaggtggcccgaggcatggagtatctggcctcc
 >K D L V S C A Y Q V A R G M E Y L A S
 1849 aagaagtgcatacaccgagacctggcagccaggaatgtcctggtgacagaggacaa
 >K K C I H R D L A A R N V L V T E D N
 1905 tgtgatgaagatgacagacttggcctcgcacgggacattcaccacatcgactact
 >V M K I A D F G L A R D I H H I D Y
 1961 ataaaaagacaaccaacggccgactgcctgtgaagtggatggcaccggaggcatta
 >Y K K T T N G R L P V K W M A P E A L
 2017 tttgaccggatcttccaccaccagagtgatgtgtggtctttcggggtgctcctgtg
 >F D R I F T H Q S D V W S F G V L L W
 2073 ggagatcttactctggggctccccataccccgggtgctgctgtggaggacttt
 >E I F T L G G S P Y P G V P V E E L
 2129 tcaagctgtgaaggagggtcaccgcatggacaagcccagtaactgcaccaacgag
 >F K L L K E G H R M D K P S N C T N E
 2185 ctgtacatgatgatgctgggactgctggcatgcatgcccctcacagagaccacctt
 >L Y M M M R D C W H A V P S Q R P T F
 2241 caagcagctggtggaagacctggaccgcatcgtggccttgacctccaaccaggagt
 >K Q L V E D L D R I V A L T S N Q E
 2297 acctggacctgtccatgccccctggaccagtactccccagctttcccgacaccgg
 >Y L D L S M P L D Q Y S P S F P D T R
 2353 agctctacgtgctcctcaggggaggattccgtcttctctcatgagccgctgcccga
 >S S T C S S G E D S V F S H E P L P E
 2409 ggagccctgctgccccgacaccagcccagcttgccaatggcggactcaaacgcc
 >E P C L P R H P A Q L A N G G L K R
 2465 gctga
 >R

Appendix III

The sequence of mouse STAT3 WT and STAT R609L.

Mouse STAT3 WT

1 atggctcagtggaaccagctgcagcagctggacacacgctacctggagcagctgca
> M A Q W N Q L Q Q L D T R Y L E Q L H
57 ccagctgtacagcgacagcttccccatggagctgcggcagttcctggcaccttgga
> Q L Y S D S F P M E L R Q F L A P W
113 ttgagagtcaagactgggcatatgcagccagcaaagagtcacatgccacgttggtg
> I E S Q D W A Y A A S K E S H A T L V
169 tttcataatctcttgggtgaaattgaccagcaatatagccgattcctgcaagagtc
> F H N L L G E I D Q Q Y S R F L Q E S
225 caatgtcctctatcagcacaaccttcgaagaatcaagcagtttctgcagagcaggt
> N V L Y Q H N L R R I K Q F L Q S R
281 atcttgagaagccaattggaattgcccggatcgtggcccgatgcctgtgggaagag
> Y L E K P M E I A R I V A R C L W E E
337 tctcgcctcctccagacggcagccacggcagcccagcaagggggcaggccaacca
> S R L L Q T A A T A A Q Q G G Q A N H
393 cccaacagccgcccgtagtgcagagagaagcagcagatggtggagcagcatcttcagg
> P T A A V V T E K Q Q M L E Q H L Q
449 atgtccggaagcgagtgaggatctagaacagaaaatgaaggtggtggagaacctc
> D V R K R V Q D L E Q K M K V V E N L
505 caggacgactttgatttcaactacaaaacctcaagagccaaggagacatgcagga
> Q D D F D F N Y K T L K S Q G D M Q D
561 tctgaatggaacaaccagctctgtgaccagacagaagatgcagcagctggaacaga
> L N G N N Q S V T R Q K M Q Q L E Q
617 tgctcagccctggaccagatgcggagaagcattgtgagtgagctggcggggctc
> M L T A L D Q M R R S I V S E L A G L
673 ttgtcagcaatggagtacgtgcagaagacactgactgatgaagagctggctgactg
> L S A M E Y V Q K T L T D E E L A D W
729 gaagagggcggcagcagatcgcgtgcatcggaggccctcccaacatctgcctggacc
> K R R Q Q I A C I G G P P N I C L D
785 gtctggaatactggataacttattagcagaatctcaacttcagaccgccaacaa
> R L E N W I T S L A E S Q L Q T R Q Q
841 attaagaaactggaggagctgcagcagaaagtgtcctacaagggcgaccctatcgt
> I K K L E E L Q Q K V S Y K G D P I V
897 gcagcaccggcccatgctggaggagaggatcgtggagctgttcagaaacttaatga
> Q H R P M L E E R I V E L F R N L M
953 agagtgccttctgtggtggagcggcagccctgcagccatgcaccggaccggccc
> K S A F V V E R Q P C M P M H P D R P
1009 ttagtcatcaagactgggtgtccagtttaccacgaaagtcaggttgctggtcaatt
> L V I K T G V Q F T T K V R L L V K F
1065 tcctgagttgaattatcagcttaaaattaaagtgtgcattgataaagactctgggg
> P E L N Y Q L K I K V C I D K D S G
1121 atgttgctgccctcagaggggtctcgaaatttaacattctgggcacgaacacaaaa
> D V A R G S R K F N I L G T N T K
1177 gtgatgaacatggaggagtctaacaacggcagcctgtctgcagagttcaagcacct
> V M N M E E S N N G S L S A E F K H L
1233 gacccttagggagcagagatgtgggaatggaggccgtgcccaattgtgatgcctcct
> T L R E Q R C G N G G R A N C D A S
1289 tgatcgtgactgaggagctgcacctgatcaccttcgagactgaggtgtaccaccaa
> L I V T E E L H L I T F E T E V Y H Q
1345 ggcctcaagattgacctagagaccctccttgccagttgtggtgatctccaacat
> G L K I D L E T H S L P V V V I S N I
1401 ctgtcagatgccaatgcttgggcatcaatcctgtggtataacatgctgaccaata
> C Q M P N A W A S I L W Y N M L T N
1457 acccaagaacgtgaacttctcactaagccgccaattggaacctgggaccaagtg
> N P K A N V N F F T K P P I G T W D Q V
1513 gccgaggtgctcagctggcagttctcgtccaccaccaagcgggggctgagcatcga
> A E V L S W Q F S S T T K R G L S I E
1569 gcagctgacaacgctggctgagaagctcctagggcctggtgtgaactactcagggt
> Q L T T L A E K L L G P G V N Y S G
1625 gtcagatcacatgggctaattctgcaagaaaacatggctggcaagggcttctcc
> C Q I T W A K F C K E N M A G K G F S
1681 ttctgggtctggctagacaatatcatcgaccttgtgaaaaagtatatcttggcct
> F W V W L D N I I D L V K K Y I L A L
1737 ttggaatgaaggttacatcatgggtttcatcagcaaggagcgggagcgggcatcc
> W N E G Y I M G F I S K E R E R A I
1793 taagcacaagccccgggcaccttctactgcgcttcagcagagcagcaagaa

>L S T K P P G T F L L R F S E S S K E
 1849 ggaggggtcactttcacttgggtggaaaaggacatcagtggcaagaccagatcca
 > G G V T F T W V E K D I S G K T Q I Q
 1905 gtctgtagagccatacacaagcagcagctgaacaacatgtcatttgctgaatca
 > S V E P Y T K Q Q L N N M S F A E I
 1961 tcatgggctataagatcatggatgcgaccaacatcctgggtgtctccacttgtctac
 >I M G Y K I M D A T N I L V S P L V Y
 2017 ctctacccccgacattcccaaggaggaggcatttggaaagtactgtaggccccgagag
 > L Y P D I P K E E A F G K Y C R P E S
 2073 ccaggagcaccgccgaagccgaccaggtagtgtgccccgtacctgaagaccaagt
 > Q E H P E A D P G S A A P Y L K T K
 2129 tcatctgtgtgacaccaacgacctgcagcaataaccattgacctgccgatgtcccc
 >F I C V T P T T C S N T I D L P M S P
 2185 cgcacttttagattcattgatgcagtttggaaataacggtgaaggtgctgagccctc
 > R T L D S L M Q F G N N G E G A E P S
 2241 agcaggaggcagtttgagtcgctcacgtttgacatggatctgacctcgaggtg
 > A G G Q F E S L T F D M D L T S E C
 2297 ctacctccccatgtga
 >A T S P M

Mouse STAT3 R609L

1 atggctcagtggaaccagctgcagcagctggacacacgctacctggagcagctgca
 > M A Q W N Q L Q Q L D T R Y L E Q L H
 57 ccagctgtacagcgcagccttccccatggagctgcggcagttcctggcaccttggga
 > Q L Y S D S F P M E L R Q F L A P W
 113 ttgagagtcaagactgggcatatgcagccagcaagagtcacatgccacgttgggtg
 >I E S Q D W A Y A A S K E S H A T L V
 169 tttcataatctcttgggtgaaattgaccagcaatatagccgattcctgcaagagtc
 > F H N L L G E I D Q Q Y S R F L Q E S
 225 caatgtcctctatcagcacaaccttcgaagaatcaagcagtttctgcagagcaggt
 > N V L Y Q H N L R R I K Q F L Q S R
 281 atcttgagaagccaatggaaaattgccccgatcgtggccccgatgcctgtgggaagag
 >Y L E A K P M E I A R I V A R C L W E E
 337 tctcgcctcctccagcggcagccagcagcccagcaagggggccaggccaacca
 > S R L L Q T A A T A A Q Q G G Q A N H
 393 cccaacagccgccgtagtgacagagaagcagcagatggtggagcagcatcttcagg
 > P T A A V V T E K Q Q M L E Q H L Q
 449 atgtccggaagcagtgaggatctagaacagaaaatgaaggtggtggagaacctc
 >D V R K R V Q D L E Q K M K V V E N L
 505 caggacgactttgatttcaactacaaaacctcaagagccaaggagacatgcagga
 > Q D D F D F N Y K T L K S Q G D M Q D
 561 tctgaatggaaacaaccagtctgtgaccagacagaagatgcagcagctggaacaga
 > L N G N N Q S V T R Q K M Q Q L E Q
 617 tgctcagccctggaccagatgcggagaagcattgtgagtgagctggcggggctc
 >M L T A L D Q M R R S I V S E L A G L
 673 ttgtcagcaatggagtacgtgcagaagacactgactgatgaagagctggctgactg
 > L S A M E Y V Q K T L T D E E L A D W
 729 gaagagggcggcagcagatcgcgtgcatcggaggccctccaacatctgcctggacc
 > K R R Q Q I A C I G G P P N I C L D
 785 gtctggaaaactggataacttcattagcagaatctcaacttcagaccgccaacaa
 >R L E N W I T S L A E S Q L Q T R Q Q
 841 attaagaaaactggaggagctgcagcagaaagtgtcctacaagggcgaccctatcgt
 > I K K L E E L Q Q K V S Y K G D P I V
 897 gcagcaccggccccatgctggaggagaggatcgtggagctgttcagaaacttaatga
 > Q H R P M L E E R I V E L F R N L M
 953 agagtgccttctgtggtggagcggcagccctgcatgccatgcaccggaccggccc
 >K S A F V V E R Q P C M P M H P D R P
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 1065 tcctgagttgaattatcagcttaaaattaaagtgtgcattgataaagactctgggg
 > P E L N Y Q L K I K V C I D K D S G
 1121 atgttgtgcctcagaggggtctcggaatttaacattctgggcacgaacacaaaa
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> V M N M E E S N N G S L S A E F K H L
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 > T L R E Q R C G N G G R A N C D A S
 1289 tgatcgtgactgaggagctgcacctgatcaccttcgagactgagggtaccacaa
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 1345 ggcctcaagattgacctagagaccactccttgccagttgtgggtgatctccaacat
 > G L K I D L E T H S L P V V V I S N I
 1401 ctgtcagatgccaaatgcttgggcatcaatcctgtggtataacatgctgaccaata
 > C Q M P N A W A S I L W Y N M L T N
 1457 accccaagaacgtgaacttcttcaactaagccgccaattggaacctgggaccaagt
 > N P K N V N F F T K P P I G T W D Q V
 1513 gccgaggtgctcagctggcagttctcgtccaccaccaagcgggggctgagcatcga
 > A E V L S W Q F S S T T K R G L S I E
 1569 gcagctgacaacgctggctgagaagctcctagggcctgggtgtaactactcagggt
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 1625 tcagatcacatgggctaaattctgcaagaaaacatggctggcaagggtcttcc
 > C Q I T W A K F C K E N M A G K G F S
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 1961 tcatgggctataagatcatggatgcgaccaacatcctgggtgtctccacttgtctac
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 2017 ctctaccccgacattccaaggaggaggcatttggaaagtactgtaggcccgagag
 > L Y P D I P K E E A F G K Y C R P E S
 2073 ccaggagcaccggcgaagccgacccaggtagtgtgctgccccgtacctgaagaccaagt
 > Q E H P E A D P G S A A P Y L K T K
 2129 tcatctgtgtgacaccaacgacctgcagcaataccattgacctgcccgatgcccc
 > F I C V T P T T C S N T I D L P M S P
 2185 cgcacttttagattcattgatgcagtttggaaataacgggtgaaggtgctgagccctc
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 2241 agcaggagggcagtttgagtcgctcacgtttgacatggatctgacctcggagtggt
 > A G G Q F E S L T F D M D L T S E C
 2297 ctacctccccatgtga
 > A T S P M