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Key words: Shc, FGFR (fibroblast growth factor receptor), FGFR2, Src, GFP (green fluorescent protein), RFP (red fluorescent protein), FLIM (fluorescence lifetime imaging microscopy), FRET (Förster resonance energy transfer), cellular signalling,tyrosine kinase, protein complex

Abbreviations: Shc, Src homology and collagen containing protein; FGF, fibroblast growth factor; FGFR, FGF receptor; c-Src kinase, cellular homologue of Rous sarcoma virus kinase; GFP, green fluorescent protein; RFP, red fluorescent protein; FRET, Förster resonance energy transfer; FLIM, fluorescence lifetime imaging; SH2, Src homology 2 domain; PTB, phosphotyrosine binding domain; CH1, collagen homology 1 domain

Summary:

The adaptor protein Shc plays an important role in the activation of signalling pathways downstream of receptor tyrosine kinases (RTKs) regulating diverse cellular functions such as differentiation, adhesion, migration and mitogenesis. Despite being phosphorylated downstream of members of the fibroblast growth factor receptor (FGFR) family, a direct interaction of Shc with this receptor family has not been described to date. Various studies have suggested potential binding sites for the Shc PTB and/or SH2 domains on the FGFR1, but no interaction of full length Shc with these sites has been reported in vivo. We investigated the importance of the SH2 and PTB domains in recruitment of Shc to the FGFR2(IIIc) to characterise the interaction of these two proteins. Confocal microscopy revealed extensive co-localisation of Shc with the FGFR2. The PTB domain was identified as the critical component of Shc mediating membrane localisation. Data from fluorescent lifetime imaging microscopy (FLIM) revealed that the interaction of Shc and FGFR2 is indirect, suggesting that the adaptor protein forms part of a signalling complex containing the receptor. We identified the non-receptor tyrosine kinase Src as a protein potentially mediating formation of such a ternary complex. Although an interaction between Src and Shc has been described previously, we implicate the Shc SH2 domain as a novel mediator of this association. The recruitment of Shc to the FGFR2 via an indirect mechanism provides new insight into the regulation of protein assembly and activation of various signalling pathways downstream of this RTK.

Introduction:

Fibroblast growth factor receptors (FGFRs) are members of a receptor tyrosine kinase (RTK) family that play an important role in the regulation of a multitude of cellular functions such as apoptosis, proliferation, migration, differentiation and survival (reviewed in [1]). The FGFR family comprises four independent genes (*fgfr1-4*) that can be alternatively spliced to create numerous receptor isoforms. The different FGFRs are highly homologous, with certain regions, such as the kinase domain, demonstrating sequence identity of up to 90% [1, 2]. Differences in the extracellular domain regulate specific activation of individual FGFR isoforms by different FGFs. Upon ligand binding in the presence of auxiliary glycosaminoglycans such as heparan sulphate, autophosphorylation occurs on tyrosine residues in the intracellular region. These phosphorylation events facilitate the recruitment of numerous signalling proteins which subsequently activate various pathways downstream of the FGFR [3, 4]. The FGFR1 has been used primarily as a model system to elucidate signal transduction events from this RTK family. Seven tyrosine residues (largely conserved amongst FGFR2-4) have been shown to become phosphorylated in the FGFR1 intracellular region upon ligand binding [5]. However, despite the sequence homology and the notion that the main difference between FGFR isoforms is the level of phosphorylation but not the type of signalling proteins phosphorylated [6], several studies have shown that subtle differences exist in the signalling events emanating from the different FGFR isoforms [7-9]. These differences may be important in regulating signalling specificity from the different FGFR isoforms, and highlight the importance of understanding the way in which different signalling proteins are recruited to various FGFR isoforms.

Despite various attempts, few proteins that associate directly with the FGFR1 have been identified to date. Of the seven major sites subject to autophosphorylation, only PLCγ binding to Y766 and CrkII binding to Y463 have been described [3, 10]. FRS2 is known to associate with the receptor constitutively and independently of tyrosine phosphorylation in the juxtamembrane region [4]. The proteins Sef and Grb14 have also been shown to bind to FGFR1, but their binding sites have not been described

[11, 12]. Grb2 is able to bind to the EGFR directly via its SH2 domain, but no such interaction could be detected in case of the FGFR1 [13].

In mammals the Shc (Src homology and collagen containing protein) family comprises three different forms of this adaptor protein, namely ShcA, ShcB (also known as Sck) and ShcC, of which the latter two are predominantly expressed in the brain [14-16]. ShcA (most commonly, and from hereon, referred to as Shc) is ubiquitously expressed and itself exists as three isoforms p66Shc, p52Shc and p46Shc, where the numerical values reflect their relative molecular weight. These are encoded by two different transcripts generated from the same gene by use of alternative 5' exons [17]. The p52 and the p46 isoforms are produced from the same transcript by use of alternative start sites. Shc is an adaptor protein involved in signalling downstream of various tyrosine kinase receptors. It contains a collagen homology domain (CH1, a region rich in proline and glycine residues but with no resemblance to the collagen fold) flanked by an SH2 domain on the C-terminus and a PTB domain on the N-terminus (reviewed in [18]). Whereas the SH2 domain mediates association with cell surface receptors such as the T cell and the EGF receptors [19], the PTB domain has been shown to interact with a wider variety of RTKs including the EGF and the insulin receptors, HER2/neu and TrkA [20, 21]. The similarity of the Shc PTB domain fold to that of PH domains means that in addition to mediating binding to tyrosine phosphorylated motifs, it is able to bind to acidic phospholipids (such as phosphatidylinositols) [22]. The CH1 domain contains tyrosine phosphorylation sites, Y239/Y240 and Y317, which are phosphorylated upon growth factor receptor activation and consequently form docking sites for the SH2 domain of the adaptor protein Grb2. The best known role of Shc is the recruitment of the Grb2/Sos complex to activated tyrosine kinase receptors and the subsequent activation of the Ras/Raf/MEK/Erk1/2 pathway [18]. However, it has also been shown to be involved in activation of other downstream signalling pathways. The phosphorylation sites in the CH1 domain have been shown to carry out pleiotropic and non-redundant roles in JNK and p38MAPK activation, cell death and *c-myc* transcription [23].

Numerous studies have shown that Shc is phosphorylated in response to FGF stimulation of cells expressing the FGFR1, FGFR3 or FGFR4 [5, 6, 8, 13, 24, 25]. A

recent report suggested a possible binding site for the Shc PTB domain on FGFR1, since a phosphopeptide based on the sequence surrounding Y730 was able to bind to Shc and block the mitogenic function of FGFR1 [26]. On the other hand, synthetic peptides corresponding to the sequences surrounding phosphorylated Y766, Y730 and Y558 of the FGFR1 can bind to the isolated Shc SH2 domain in vitro [13, 27]. Despite the presence of potential binding sites for the Shc SH2 and/or PTB domains on the FGFR1 (which are conserved amongst other FGFR isoforms), co-precipitation of Shc was only demonstrated in the case of p66 Shc with the FGFR3 and under non-physiological conditions with the FGFR1 in mammalian cells expressing v-Src [28, 29]. The latter finding indicated that direct interaction between the receptor and Shc is possible and that a Shc binding site is present on the FGFR1. Further, the Shc SH2 domain has been shown to block Erk2 activation downstream of the *Pleurodeles* FGFR1 (which is homologous to the human FGFR1) in *Xenopus laevis* oocytes. Although this does not necessarily indicate direct binding between the two proteins, it suggests that such an event would occur via the SH2 domain. Despite such evidence of the involvement of Shc in signal transduction from various FGFR family members and the identification of potential binding sites for Shc on FGFRs, it is clear that the way this adaptor protein is recruited to this receptor family in vivo needs to be investigated further. Identification of the Shc binding site in vivo and the importance of the different Shc domains for recruitment to FGFRs would provide important detail on the activation of effectors such as the different members of the MAPK family downstream of this family of receptor tyrosine kinases.

We chose to focus on the interaction of Shc (p52 and p46 isoforms) with the FGFR2 because signalling from this member of the FGFR family has been poorly investigated to date. Additionally, identification of a Shc binding site on one FGFR isoform may provide important insight into the recruitment of this adaptor to the entire FGFR family. We report here, that Shc co-localises with the FGFR2 at the plasma membrane and in intracellular membrane compartments and that it is associated with the receptor in a manner that allows co-precipitation of the two proteins. We highlight the requirement for the Shc PTB domain in its membrane recruitment and the necessary context of the full length protein (i.e. presence of both the SH2 and PTB domains) for stable association with the receptor. Fluorescence lifetime imaging microscopy revealed that, despite co-localisation and co-precipitation of Shc with the FGFR2,

these two proteins do not interact directly in vivo. Thus Shc must be recruited to a multimeric protein complex upon FGFR2 activation, rather than utilising any of the previously described binding sites on the receptor directly. We identified the tyrosine kinase Src as a binding partner of Shc in cells expressing the FGFR2, which may play an important role in regulating the assembly of the ternary complex involving Shc and the FGFR2. Shc has been shown to interact with various tyrosine kinase receptors, including the EGF, insulin and TrkA receptors. Our observations of a lack of direct association of Shc with the FGFR2 highlight an important alternative mechanism for recruitment of signalling proteins to RTKs to finely control the specific activation of various downstream signalling pathways. The fact that Shc is phosphorylated following FGFR activation, but does not directly associate with them despite the presence of binding sites, highlights the fact that recruitment of adaptor proteins into specific multiprotein signalling assemblies downstream of different receptors plays an important part in regulating signalling specificity.

Methods:

Materials

FGF9 was from R&D systems, heparan sulphate from Sigma-Aldrich. The anti-GFP antibody was purchased from Rockland and the anti-Shc antibodies were from Upstate and BD Transduction Laboratories, the anti-Src antibody was from Cell Signaling Technologies. Secondary horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma-Aldrich.

Constructs

The cDNA encoding the FGFR2(IIIc) was a kind gift from John Heath (University of Birmingham, UK). It was PCR amplified and cloned in frame into the eGFP-N2 vector (Clontech). The vector encoding monomeric RFP (mRFP) for N-terminal or C-terminal RFP-fusion proteins were a kind gift from Tony Ng (King's College London, UK). The cDNA encoding the p52 isoform of human ShcA (amino acids 17-472) was a kind gift from Kodi Ravichandran (University of Virginia, USA). Full length Shc was PCR amplified and cloned into the RFP-C vector to create a C-terminally RFP-tagged protein. The ShcΔPTB construct (refer to Figure 3) was created by PCR amplification with a separate forward primer and cloning into the RFP-C vector. ShcR401A, ShcR175Q and ShcTriple (R112Q/K116A/K139A) were created with the Quikchange mutagenesis kit (Stratagene, applied according to manufacturer's instructions) using the C-terminally RFP-tagged Shc as template. The Shc SH2 domain (as GST fusion in the pGEX-2T vector) was a kind gift from Cosima Baldari (University of Siena, Italy) and was sub-cloned into the RFP-N vector to create an N-terminally tagged fusion protein. The Shc PTB domain was PCR amplified using appropriate primers and cloned in-frame into the RFP-C vector or into the pGEX4T-2 GST-expression vector (Amersham Biosciences).

GST fusion proteins of the SH2 and PTB domains and full length Shc (cloned into the pGEX4T-2 expression vector) were created by expression in the BL21(DE3) strain and purification using GST-Bind Resin (Novagen).

Cell culture

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. Prior to stimulation cells were serum-starved for 18 hours in serum-free DMEM. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable clones expressing the FGFR2-GFP construct were isolated by dilution cloning in 96 well plates and screening for fluorescence.

PC12 cells were cultured in DMEM supplemented with 5% foetal bovine serum and 10% horse serum. Prior to stimulation cells were serum-starved for 18 hours in DMEM with 0.1% horse serum. PC12 cells expressing the FGFR2-GFP were created as described previously [30]. Stable cells expressing the FGFR2-GFP and Shc-RFP were created by additional transfection with cDNA encoding C-terminally RFP-tagged Shc and selecting stable cells by dilution cloning in 96 well plates and screening for fluorescence.

For immunoprecipitation experiments cells were serum starved for 18 hours and stimulated with 10ng/ml FGF9 in the presence of additional 1μg/ml heparan sulphate for 15 minutes and lysed in 20mM TrisHCl pH 7.5, 138mM NaCl, 1mM EGTA, 20mM β-glycerophosphate, 10% (w/v) glycerol, 1mM sodium orthovanadate, 20mM sodium fluoride, supplemented with 1% (v/v) protease inhibitor cocktail III (Calbiochem). Whole cell lysates (2mg total protein) were subjected to pulldown experiments using GST fusion proteins or immunoprecipitation with the appropriate antibodies as indicated. Immunoprecipitants were captured on Protein A or Protein G beads, subjected to four washes with lysis buffer followed by SDS-PAGE and western blotting with the appropriate antibodies as indicated.

Confocal microscopy

Cells were seeded on glass coverslips (coated with poly-D-lysine in the case of PC12 cells) and allowed to adhere overnight. Following serum starvation, cells were stimulated with FGF9 (10ng/ml) in the presence of additional heparan sulphate (1μg/ml) for 15 minutes. Following a wash in phosphate buffered saline (PBS), cells were fixed in 4% paraformaldehyde in PBS pH 8.0. Coverslips were washed in PBS pH 8.0 and mounted on glass slides on a drop of mounting medium (0.1% anti-fade agent 1,4-phenylenediamine (Fluka) in 50% (v/v) glycerol in PBS pH 8.0). Slides

were analysed using a Leica TCS SP2 system with a 63x oil immersion objective. GFP was excited at 488 nm using an argon visible light laser and its emission was detected using a 514/10 nm band selection. Mid-sections of the cells were chosen to avoid interference from cell attachment to the slides. Fluorescence images were collected using a photomultiplier tube interfaced to an Intel Pentium II system running the Leica TCS NT control software. The images presented are representative of 10-15 fields of view analysed per independent experiment. Fluorescent intensity graphs were created using the Leica LCS software. Peaks represent the pixel intensity of GFP (green) and RFP (red) respectively along the points of an arbitrarily drawn line.

Fluorescence lifetime imaging (FLIM)

FLIM analysis was carried out using a Leica TCS SP2 inverted microscope set-up with a 63x water immersion objective, which was adapted for Time-Correlated Single-Photon Counting (TCSPC) FLIM with a Becker & Hickl SPC 830 card using 64 or 256 time channels in a 3 GHz, Pentium IV, 1GB RAM computer. The samples were excited using a femtosecond Titanium Sapphire laser (Coherent Mira, repetition rate 76MHz) that was pumped by a 6.5W solid state laser (Coherent Verdi V6). Images were obtained with a line scan speed of 200Hz. Two-photon excitation was carried out with a wavelength of 900nm and fluorescence was detected through a 525+/-25nm interference filter using a cooled PMC100-01 detector (Becker & Hickl, based on a Hamamatsu H5772P-01 photomultiplier). The fluorescence decays obtained were fitted using a single exponential decay model with Becker & Hickl SPCImage software v2.8.3. GFP fluorescence lifetimes were portrayed in false colour maps. The images shown are representative of at least five independent fields of view analysed. Changes in overall GFP lifetime in the entire field of view were represented graphically in form of lifetime histograms.

Results:

Co-precipitation of Shc and the FGFR2

To assess the co-localisation and interaction of Shc and the FGFR2, we created cell lines expressing a GFP-tagged version of the FGFR2(IIIc). We previously showed that this receptor is active and responds to FGF9 stimulation [30, 31], indicating that GFP-tagging does not interfere with receptor activity. Immunoprecipitation experiments revealed that Shc (p52 and p46 isoforms) and the FGFR2 could be co-precipitated from both HEK 293T and PC12 cells expressing the GFP-tagged version of the FGFR2 (Figure 1). Co-precipitation of Shc with the receptor was somewhat less in PC12 cells compared to HEK 293T cells. This is most likely due to cell-specific differences in protein (particularly of transfected FGFR2-GFP) expression. The observed co-precipitation indicates an interaction between the FGFR2 and Shc.

Co-precipitation of Shc and the FGFR2 did not increase significantly following stimulation with FGF9. An explanation for this observation could be the fact that the FGFR2 is already highly phosphorylated in the unstimulated state, and its level of phosphorylation only increases minimally upon FGF9 stimulation [30, 31]. Association of Shc with this basally phosphorylated receptor may already take place in the absence of FGF stimulation. FGF binding to the receptor would, however, be required to induce activation of downstream signalling pathways, since pathways such as the Erk1/2 pathway are not activated in unstimulated cells [30, 31].

Interestingly, this observation also highlights potential differences in the way proteins are recruited to various FGFR isoforms, since co-precipitation of Shc with the FGFR1 under physiologically relevant conditions had not been observed previously [5, 13]. We hence chose to investigate in more detail the association of Shc with the FGFR2 and its role in intracellular signalling from this receptor.

Co-localisation of Shc with the FGFR2 on the plasma membrane and intracellular membraneous compartments

To confirm the role of Shc in FGFR2 signal transduction suggested by the immunoprecipitation experiments, we chose to investigate the cellular localisation of Shc in relation to the FGFR2. Intracellular co-localisation of two proteins does not indicate their direct interaction, but does highlight the recruitment of two proteins to

the same cellular compartment and the involvement of adaptor proteins in signalling from a certain receptor. In conjunction with co-precipitation experiments, co-localisation may give important insight into the spatial and temporal regulation of protein-protein interactions intracellularly.

HEK 293T cells expressing the FGFR2-GFP were transfected with mRFP-tagged Shc (construct encodes the p52 and p46 isoforms, refer to Figure 3B) and cells were stimulated with FGF9. The mRFP-tagged Shc was phosphorylated in a manner comparable to untagged Shc, which indicates that the mRFP-tag does not interfere with Shc functionality (data not shown). Strong co-localisation of Shc with the FGFR2 at the plasma membrane was observed in HEK 293T cells (Figure 2A, yellow colouring on overlays as well as overlapping RFP (red) and GFP (green) peaks in the fluorescent intensity graphs). Co-localisation on the plasma membrane occurred in both the unstimulated and the FGF9-stimulated state, which is in agreement with the co-immunoprecipitation experiments. In the absence of the FGFR2, Shc was not significantly localised at the plasma membrane (Figure 2B), which confirms that the observed membrane localisation of Shc occurs as a result of an association with the FGFR2.

In PC12 cells, a similar co-localisation pattern was observed (Figure 2A). However, Shc membrane localisation increased more strongly upon FGF9 stimulation compared to HEK 293T cells (increased yellow colouring in the overlay and greater overlap of the red and green peaks in fluorescent intensity graphs). Despite slight cell-specific differences, overall, extensive co-localisation between Shc and the FGFR2 was observed. This further indicates involvement of this adaptor protein in FGFR2 signalling and raises the question whether a direct binding site for Shc on the receptor can be identified and which domain of Shc is involved in mediating the recruitment of Shc to the FGFR2.

The Shc PTB domain is required for its membrane localisation

Shc possesses two domains that could potentially mediate association with phosphorylated tyrosine motifs on the FGFR2: the N-terminal PTB domain and the C-terminal SH2 domain (Figure 3). Both domains have been shown to mediate association of Shc with other receptor tyrosine kinases such as the EGF, PDGF and insulin receptors [20, 32, 33]. Several potential binding sites for either the Shc SH2 domain or the Shc PTB domain on the FGFR1 have been identified in vitro [13, 26,

27]. These sites are conserved between the FGFR1 and FGFR2 and could mediate the recruitment of Shc to the FGFR2 and hence result in the observed co-localisation and co-immunoprecipitation of Shc with this receptor. To assess whether the PTB domain or the SH2 domain or indeed phosphorylation on tyrosine residues in the CH1 domain were required for Shc recruitment to the membrane in response to expression and activation of the FGFR2, we created three Shc constructs that were missing a functional SH2 (ShcR401) or the PTB (ShcΔPTB) domain respectively or in which all three tyrosine residues in the CH1 domain were mutated to phenylalanine (Shc3F) (refer to Figure 3 for a diagrammatic representation of all constructs used and expression of the RFP-tagged constructs in HEK 293T cells). Western blotting revealed the integrity of all RFP-fusion proteins (Figure 3B). All three cDNA constructs were transiently transfected into HEK 293T cells and co-localisation with the FGFR2 was observed in the absence and presence of FGF9 (Figure 4).

Mutation of the arginine residue critical for phosphotyrosine binding by the SH2 domain (ShcR401A) did not affect localisation of Shc to the plasma membrane (Figure 4). The pattern of co-localisation with the receptor was unaltered from that of full length Shc. Similarly, the mutation of residues Y239, Y240 and Y317 to phenylalanine (Shc3F) did not affect co-localisation of Shc with the FGFR2 as highlighted by the fluorescence intensity graphs (Figure 4). These observations indicate that the Shc SH2 domain and tyrosine phosphorylation in the CH1 domain are not required for co-localisation with the FGFR2.

In contrast, the construct lacking the PTB domain (ShcΔPTB) was unable to localise to the plasma membrane (Figure 4). This indicates that the PTB domain plays a major role in mediating the co-localisation of Shc with the FGFR2 at the plasma membrane. Nonetheless, the resolution of confocal microscopically assessed protein co-localisation does not allow assessment of direct protein-protein interactions in vivo. Thus despite the requirement of the PTB domain for Shc co-localisation with the FGFR2, an involvement of the SH2 and CH1 domains in association with the receptor cannot be ruled out by these studies.

The Shc PTB domain can bind to tyrosine phosphorylated motifs on target proteins, but is also able to bind to phospholipids [34, 35]. This dual binding capacity means that removal of the Shc PTB domain as a whole does not discriminate between membrane localisation as a result of phospholipid binding or indeed an interaction with the FGFR2. We therefore chose to individually mutate the binding sites for

phospholipids (ShcTriple) and phosphotyrosine (Shc R175Q) [34] (refer to Figure 3 for diagrammatic representation). As expected, the mutant protein still able to bind phospholipids (ShcR175Q) was membrane localised in a fashion comparable to full length Shc (Figure 4). Previous studies have shown that the ability of the PTB domain to bind both phospholipids and phosphotyrosine is important for its localisation to the membrane [34]. In agreement with this, Shc lacking the ability to bind phospholipids (ShcTriple) was also able to co-localise with the FGFR2 upon FGF9 stimulation (Figure 4). This observation indicates that binding of tyrosine phosphorylated proteins plays a role in Shc recruitment to the membrane in addition to its ability to bind phospholipids via the PTB domain.

Requirement of functional SH2 and PTB domains for recruitment to the FGFR2

Since co-localisation of two proteins does not necessarily infer their direct interaction, we chose to utilise various Shc mutants (refer to Figure 3) to assess which of the Shc domains was important for mediating its co-precipitation with the FGFR2. HEK 293T cells stably expressing the FGFR2-GFP were transiently transfected with equal amounts of DNA encoding Shc, Shc3F, ShcΔPTB or ShcR401A as for co-localisation experiments and stimulated with FGF9. The FGFR2 was immunoprecipitated with an anti-GFP antibody to ensure isolation of only the exogenously expressed FGFR2 (Figure 5). Co-localisation of Shc lacking its three tyrosine phosphorylation sites in the CH1 domain was unaltered from that of full length Shc (Figure 4). Likewise, Shc3F co-precipitated with the FGFR2 to a similar extent as full length Shc (Figure 5, doublets represent the p46 and p52 isoforms), which supports the notion, that phosphorylation in the CH1 domain is not required for Shc to associate with this receptor.

On the contrary, both the ShcR401A and the ShcΔPTB constructs, lacking a functional SH2 and PTB domain respectively, were observed to co-precipitate with the receptor to a significantly lesser extent than full length Shc and could only be observed under prolonged exposure of the western blot. This highlights a requirement for both the SH2 and the PTB domain for effective association with the FGFR2. ShcΔPTB is most likely unable to associate with the FGFR2 because the lack of the PTB domain impairs its recruitment to the plasma membrane and hence the receptor itself. Interestingly, ShcR401A (containing a non-functional SH2 domain) was also unable to associate with the FGFR2 to a great extent, despite its co-localisation being

unaltered from that of full length Shc (Figure 4). Thus despite the ability to mediate efficient co-localisation of Shc with the FGFR2, the presence of the PTB domain alone is not sufficient to result in stable complex formation with the receptor. Taking into account these data, it emerges that the PTB domain is required to bring the SH2 domain into the vicinity of the FGFR2, where the latter then stabilises and/or mediates the association with the FGFR2. In the absence of the PTB domain, the lack of Shc membrane association would be expected to prevent stable association with the FGFR2.

No direct protein-protein interaction takes place between the FGFR2 and Shc

Neither pulldown methods such as immunoprecipitation nor confocal microscopy analysis of protein co-localisation can indicate conclusively whether two proteins interact directly or whether they are found in a ternary complex (i.e. are associated indirectly). Since both the PTB and the SH2 domain seemed to be required for association with the FGFR2, we chose to use fluorescence lifetime imaging (FLIM) to determine unequivocally whether direct association between Shc and the FGFR2 occurs and whether the SH2 or the PTB domain or indeed both mediate this interaction. When an acceptor fluorophore such as RFP is within close enough distance (typically between 1-10nm) to a donor fluorophore (GFP), this will result in Förster resonance energy transfer (FRET) and a subsequent shortening of the donor fluorescence lifetime [36, 37]. By measuring the differences in donor lifetime in the absence or presence of an acceptor fluorophore, direct interaction between two fluorescently tagged proteins can be assessed within a cell. FLIM has previously been used successfully to determine direct protein-protein interactions in vivo and is therefore a suitable technique to characterise the interaction between Shc and the FGFR2 [38].

The average fluorescence lifetime of GFP in the context of the FGFR2-GFP construct was approximately 2ns, as indicated by the peak on the lifetime histogram (Figure 6A). This value is similar to that of GFP alone (Figure 6A). The slightly wider peak in cells expressing FGFR2-GFP compared to GFP only indicates that there is a larger spread of GFP molecules with shorter or longer lifetimes, which may be due to varying conditions in the environment of GFP when located near the plasma membrane [39]. The expression of monomeric RFP in cells also expressing GFP did not result in decreased GFP lifetime (Figure 6A). This illustrates that FRET between

the two fluorophores does not occur unless they are positioned proximal to each other as a result of direct protein-protein interaction between two tagged proteins.

Immunoprecipitation and co-localisation experiments suggested that the PTB domain was necessary to recruit Shc to the membrane, but that the SH2 domain was a major component required for stable complex formation with FGFR2. To determine whether either of these domains is able to mediate direct association of Shc with the receptor, cells were transiently transfected with constructs encoding the RFP-tagged Shc SH2 or PTB domains or full length Shc and stimulated with FGF9 or left unstimulated.

Surprisingly, FLIM analysis revealed, that Shc and the FGFR2 do not associate directly in vivo (Figure 6B). Neither expression of the SH2 or PTB domains on their own, nor of full length Shc induced a significant change in overall GFP lifetime (Figure 6B). The peaks in the lifetime histograms remained centred around 2ns, which indicates that the GFP molecules on the receptor and the RFP molecules on the Shc constructs were not positioned proximal to each other as a result of direct interaction between the tagged proteins. A small left shift of the peak was observed in FGF9 stimulated cells expressing the RFP-tagged SH2 domain. However, since full length Shc did not show a similar pattern this is likely the result of overexpression of the SH2 domain and non-specific binding to tyrosine phosphorylated sites on the FGFR2. The fact that no changes in GFP lifetime could be observed indicates that Shc does not bind directly to the receptor, despite the presence of various potential binding sites. The association of these two proteins must therefore occur indirectly via ternary complex formation.

Src is a candidate protein mediating interaction of Shc with the FGFR2

Since FLIM revealed that no direct interaction takes place between Shc and the FGFR2, we decided to probe for candidate proteins that could mediate ternary complex formation between these two proteins. Shc immunoprecipitation and pulldown experiments using the GST-tagged SH2 and PTB domains were carried out. Although proteins such as Grb2, PLCγ, and IRS4 were observed in Shc immunoprecipitates, we were unable to identify any of these proteins in pulldown experiments using the isolated domains (data not shown). Their presence in Shc immunoprecipitates was therefore likely a result of ternary complex formation. We further tested an interaction of Shc with FRS2, CrkII, SH2-B and c-Cbl, but were unable to show binding of any of these proteins to Shc.

Interestingly, Shc could also be co-immunoprecipitated with the non-receptor tyrosine kinase Src (data not shown). Pulldown experiments using full length Shc also revealed an association between these two proteins (Figure 7B). The role of Src in FGF receptor signalling is somewhat controversial [40], but a direct interaction between the kinase and the FGFR1 has been shown in vitro and a recent study has shown that Src plays an important role in FGFR1 activation and signalling dynamics [41, 42]. The latter study also showed that Src co-localises with the FGFR1. Additionally, we observed co-immunoprecipitation of Src with the FGFR2 (Figure 7C), which further indicates that it may play a role in the recruitment of Shc to the FGFR2 and hence the localisation of Shc to the plasma membrane in cells expressing this receptor. Pulldown experiments using GST-tagged Shc SH2 and PTB domains, respectively, revealed that the association between Shc and Src is mediated via the SH2 domain (Figure 7A). This observation correlates with earlier results highlighting the requirement for a functional Shc SH2 domain for ternary complex formation between Shc and the receptor (Figure 5). Co-precipitation of the FGFR2 with the Shc SH2 domain was observed only in the GST-pulldown assay following ligand stimulation (Figure 7A). This provides further evidence for the role of Src in ternary complex formation. Hence the SH2 domain and the presence of Src play an important role in mediating the association of Shc with the receptor.

Previous studies have shown that Shc is able to bind Src via its PTB domain and result in activation of Src kinase activity [43, 44]. We failed to observe significant association of the Shc PTB domain with Src (Figure 7A). Differences in the cell and receptor systems used in this work compared to other studies may account for the differences observed. An interaction of the Shc SH2 domain with Src has not been described previously. The fact that this interaction could also be observed in unstimulated cells raises the possibility that it is not phosphorylation dependent and is in agreement with the observation that Shc and the FGFR2 co-precipitated in unstimulated cells. Shc has previously been shown to bind to a protein called mPAL via its SH2 domain in a non-phosphorylation-dependent manner [45]. A similar mechanism may be at work in the case of Shc and Src. Alternatively, the Shc SH2 domain could bind to one of the Src autoregulatory tyrosine phosphorylation sites (of which tyrosine 527 would be expected to be phosphorylated in resting cells). Such a binding event may subsequently play a role in regulating kinase activity. Regardless of the precise manner of interaction, our results highlight Src as a candidate protein

mediating the indirect interaction of Shc with the FGFR2. Such a mechanism of recruitment opens up the possibility of interesting new investigations of the precise role of Shc in FGFR2 signalling and its contribution to the activation of different downstream signalling pathways.

Discussion:

Although the adaptor protein Shc has been implicated as a major component in the recruitment of Grb2 to numerous RTKs, its precise involvement in FGF receptor signalling has remained somewhat of a mystery. Our investigation has revealed that Shc co-localises intracellularly with the FGFR2, and that these two proteins can be co-precipitated. This observation contrasts with the fact that co-precipitation of these Shc isoforms could not be observed in the case of FGFR1 or FGFR3 [28, 29]. Additionally, it indicates that despite a high degree of sequence homology between these three FGFR isoforms, differences exist in the way in which Shc is recruited to various members of this growth factor receptor family. Any such differences in the recruitment of Shc to various FGFR isoforms could play an important role in the activation of different pathways downstream of the individual receptors within this family. This is further supported by previous work that highlighted differences in the affinity of FRS2 for FGFR1 and FGFR3 [9]. The differential recruitment of the same signalling proteins to individual members of this receptor tyrosine kinase family would be expected to regulate the generation of specific downstream signals. By expressing different FGFR isoforms at their surface, cells could therefore fine-tune the response to stimulation by FGFs not only by presenting different extracellular domains with varying specificity for FGF isoforms, but also by generating slightly altered signalling pathways intracellularly.

It was somewhat unexpected to find that despite intensive co-localisation and co-precipitation of Shc and the FGFR2 no direct interaction between these two proteins could be observed using a high-resolution FRET technique (Figure 6). It is particularly interesting to note that no interaction was observed despite the presence of various potential binding sites for Shc on the FGFR2 [13, 26, 27]. The indirect association of these two proteins must be mediated via formation of a ternary signalling complex involving at least one other protein. We identified the non-receptor tyrosine kinase Src as a mediator of the indirect interaction between the FGFR2 and Shc. Although a specific binding site for Src on the FGFR2 has not been described, previous work has shown that the Src SH2 domain is able to bind to the FGFR1 in vitro [41]. Further support for the fact that Src plays an important role in

mediating the association of Shc with the FGFR2 comes from a previous study which showed that Shc could be co-precipitated with the FGFR1 in cells overexpressing v-Src [29]. This observation strengthens the proposal that Src plays a role in the recruitment of Shc to a ternary protein complex involving the FGFR2, and further highlights differences in the way proteins are recruited to individual members of the FGFR family.

The Shc PTB domain was required for membrane localisation (Figure 4). However, the addition of the SH2 domain was necessary to obtain stable ternary complex formation with the FGFR2 (Figure 5). Previous studies have shown the lack of localisation of the ShcΔPTB construct to the membranous fraction, whereas full-length Shc was associated with the membrane fractions of unstimulated T cells, BaF cells and COS cells [34]. The data presented here are consistent with these reports. The ability of the PTB domain to bind phospholipids was sufficient to mediate extensive co-localisation of Shc with the FGFR2 at the plasma membrane (Figure 4, R175Q). When considered in conjunction with the fact that the lack of a functional SH2 domain greatly reduced the ability of Shc to co-precipitate with the receptor (Figure 5), a model emerges in which the PTB domain mediates membrane localisation and brings the SH2 domain into the vicinity of the FGFR2. This would then allow the SH2 domain to interact with proteins, such as Src, mediating the formation of the ternary signalling complex between Shc and the FGFR2. We have previously shown, that the Shc SH2 domain is occluded until phosphorylation in the CH1 domain occurs [35]. Recruitment of Shc to the membrane via the PTB domain would ensure its localisation to a site where phosphorylation and consequently interaction with other proteins via the SH2 domain can occur.

In a search for candidate proteins mediating the formation of a ternary complex involving the FGFR2 and Shc, we probed for the interaction of the Shc PTB and/or SH2 domains with numerous proteins known to bind to the FGFR1 or to at least be involved in FGF receptor signalling. We identified the non-receptor tyrosine kinase Src as an interacting partner of the Shc SH2 domain. This observation is in agreement with the fact that the Shc SH2 domain was required for stable association of this adaptor protein with the FGFR2 (Figure 5). Since Src is able to phosphorylate Shc, the interaction of these proteins is likely to play a role in the regulation of Shc

phosphorylation and hence activation of downstream signalling pathways. A previous study showed that binding of phosphatidylinositol-4,5-bisphosphate to the Shc PTB domain stimulates its phosphorylation by Src [46]. This observation supports our model for the FGFR2 system, in which the Shc PTB domain binds to phospholipids in the plasma membrane, whereas the SH2 domain mediates association of Shc with Src. This could subsequently facilitate phosphorylation of Shc by this kinase. Further work would need to be carried out to investigate whether Shc phosphorylation downstream of the FGFR2 does indeed occur via Src and not the receptor itself. Additionally, the fact that Shc co-localisation was not altered upon removing the ability of Shc to bind phospholipids via the PTB domain suggests that the PTB domain could be involved in further stabilising this ternary complex via interactions with other, unidentified proteins. This is supported by the fact that the PTB domain is able to bind phospholipids and phosphoproteins at the same time [35].

Shc has been shown to bind directly to numerous tyrosine kinase receptors. Therefore the observation that the association of Shc with the FGFR2 is indirect despite co-precipitation and the presence of potential Shc binding sites on the receptor was somewhat unexpected. However, the association of Shc with Src and hence indirectly with the FGFR2 may highlight roles for Shc in FGFR2 signalling in addition to Grb2/Sos complex recruitment. Further investigation of the way in which Shc and Src interact and potential effects on the activity of the kinase and/or the recruitment of other proteins involved in signalling from the FGFR2 are required to describe in detail the effects that this novel way of Shc recruitment has on the activation of downstream signalling pathways.

Our results show that despite its ability to bind to numerous tyrosine kinase receptors, Shc does not interact directly with the FGFR2. Taking into consideration the fact that previous studies were unable to show co-precipitation of p52/p46 Shc and the FGFR1 or FGFR3 under physiological conditions, it is likely that in vivo, FGFRs in general do not present a binding site for Shc. In addition to highlighting differences in protein recruitment to different members of the FGFR family, this observation also reveals an important way in which signalling specificity downstream of different RTKs can be achieved. Shc has been shown to bind directly to RTKs such as the EGFR, PDGFR, TrkA and the insulin receptor [20, 21]. The exclusion of full length Shc binding to the

FGFR2 despite the presence of potential binding sites indicates that different RTKs are able to recruit downstream adaptor proteins in a very specific and regulated fashion. Such precise recruitment of proteins to the activated receptor would then be expected to lead to the activation of a specific combination of downstream pathways to relay information from the cell surface to the interior of the cell. Although further investigation is necessary to elucidate the exact mechanism by which Shc is recruited to the FGFR2 via Src, the fact that Shc was recruited to this receptor in an indirect fashion requiring both functional SH2 and PTB domains indicates that various receptors may indeed assemble highly individual signalling complexes to specifically activate a host of downstream signalling pathways.

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Figure legends:

Figure 1: Immunoprecipitation reveals the association of Shc with the FGFR2.
(A) HEK 293T and PC12 cells stably expressing the FGFR2-GFP were stimulated with FGF9 (plus heparan sulphate) or left unstimulated following serum starvation. Whole cell lysates were subjected to immunoprecipitation using an anti-Shc (top panel) or an anti-GFP (bottom panel) antibody. Precipitants were subjected to SDS-PAGE and immunoblotting with appropriate anti-Shc and anti-GFP antibodies as indicated. (B) Immunoprecipitation with an anti-Shc antibody was carried out as in (A) using untransfected HEK 293T cells as a negative control.

Figure 2: Full length Shc co-localises with the FGFR2.
(A) HEK 293T or PC12 cells stably expressing the FGFR2-GFP were transfected with cDNA encoding mRFP-tagged full length Shc (Shc-RFP). The cells were seeded on coverslips, serum starved and stimulated with FGF9 (plus heparan sulphate) or left unstimulated. Paraformaldehyde fixed cells were mounted on slides and analysed using confocal microscopy. Yellow colouring in the overlay indicates co-localisation. The graphical representation indicates the distribution of fluorescence intensity at each point along the arbitrarily drawn line in the overlay. Overlapping peaks of red and green intensity indicate co-localisation. (B) HEK 293T cells were transfected with the Shc-RFP construct in the absence of the FGFR2-GFP and treated as described in (A).

Figure 3: Illustration of the Shc constructs used.
(A) The Shc constructs used are shown diagrammatically including an indication of the amino acids at the domain boundaries. In Shc3F, all three tyrosine phosphorylation sites in the CH1 domain were mutated to phenylalanine (Y239/240F and Y317F). To abolish the phosphotyrosine binding ability of the SH2 domain, arginine 401 was replaced by alanine (ShcR401A). The removal of the PTB domain and insertion of a new start site at the beginning of the CH1 domain (ShcΔPTB) ensured removal of all binding of phospholipids or phosphotyrosine via the PTB domain. A previous study has shown that several point mutations can remove phosphotyrosine and phospholipid recognition of the Shc PTB domain independently

of one another [34]. The substitution of arginine 175 by asparagine (ShcR175Q) prevents the PTB domain from phosphotyrosine binding, and the replacement of arginine 112 by asparagine, lysine 116 by alanine and lysine 139 by alanine (R112Q/K116A/K139A, ShcTriple) removes its ability to recognise phospholipids. (B) All constructs were transiently expressed in HEK 293T cells as RFP-fusion proteins. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with an anti-RFP antibody to assess integrity of the fusion proteins. (FL: full length)

Figure 4: The Shc PTB domain is required for co-localisation of Shc with the FGFR2.

HEK 293T cells stably expressing the FGFR2-GFP were transiently transfected with cDNA constructs encoding ShcR401A, Shc3F, ShcΔPTB, ShcR175Q or ShcTriple as indicated. Cells were seeded on glass cover slips and stimulated with FGF9 (plus heparan sulphate) for 15 minutes following serum starvation or left unstimulated. Cells were fixed using paraformaldehyde and subsequently analysed by confocal microscopy. Overlays of individual images and fluorescent intensity graphs (indicating the intensity of GFP and RFP fluorescent along the arbitrarily drawn line in the overlay) are shown to highlight protein co-localisation.

Figure 5: Requirement for both SH2 and PTB domains for efficient co-precipitation of Shc with FGFR2.

HEK 293T cells stably expressing the FGFR2-GFP were transiently transfected with cDNA constructs encoding RFP, full length Shc, Shc3F, ShcΔPTB or ShcR401A. Cells were stimulated with FGF9 for 15 minutes following serum starvation and lysed. Whole cell lysates were subjected to immunoprecipitation using an anti-GFP antibody, followed by SDS-PAGE and immunoblotting with an anti-Shc antibody and an anti-GFP antibody as loading control (top panel). Whole cell lysates prior to immunoprecipitation were subjected to SDS-PAGE and immunoblotting with an anti-Shc antibody to show expression relative to each other and relative to cellular Shc (bottom panel). (WCL: whole cell lysate)

Figure 6: The interaction between Shc and the FGFR2 is indirect.

(A) HEK 293T cells were transfected with GFP, or GFP and RFP. Alongside HEK 293T cells stably expressing the FGFR2-GFP, these were seeded on coverslips, fixed

with paraformaldehyde and mounted on glass slides. Confocal microscopy and FLIM analysis were carried out as described in "Methods". (B) HEK 293T cells stably expressing the FGFR2 were transiently transfected with cDNA constructs encoding the RFP-tagged SH2 domain, PTB domain or full length Shc as indicated. Cells were seeded on glass coverslips, serum-starved and stimulated with FGF9 (plus heparan sulphate) for 15 minutes or left unstimulated. Cells were fixed in paraformaldehyde and fixed on glass slides. Confocal microscopic and FLIM analysis were carried out as described in "Methods". The lifetime histogram represents the lifetime of all GFP molecules within the field of view. Images are representative of at least five cells.

Figure 7: Shc associates with Src in cells expressing the FGFR2

(A) HEK 293T cells expressing the FGFR2-GFP were left unstimulated or stimulated with FGF9 in the presence of heparan sulphate for 15 minutes. Cell lysates were subjected to the recombinant, GST-tagged SH2 or PTB domains of Shc captured on glutathione resin overnight or GST only as a control. Precipitants were analysed by SDS-PAGE and western blotting with anti-Src (upper panel), anti-GFP (middle panel) and anti-GST (bottom panel) antibodies. (B) Pulldown experiments were carried out as in (A) using full length GST-tagged Shc. Precipitants were analysed by western blotting using an anti-Src antibody and an anti-GST antibody as loading control. (C) Whole cell lysates from untransfected HEK 293T cells or those stably expressing the FGFR2-GFP were immunoprecipitated using an anti-GFP antibody. Immunoprecipitates were analysed by Western blotting with an anti-Src antibody (top panel). In the absence of FGFR2-GFP in untransfected cells, the amount of IgG added to each immunoprecipitation reaction is shown as a control (bottom panel).

Figure 1

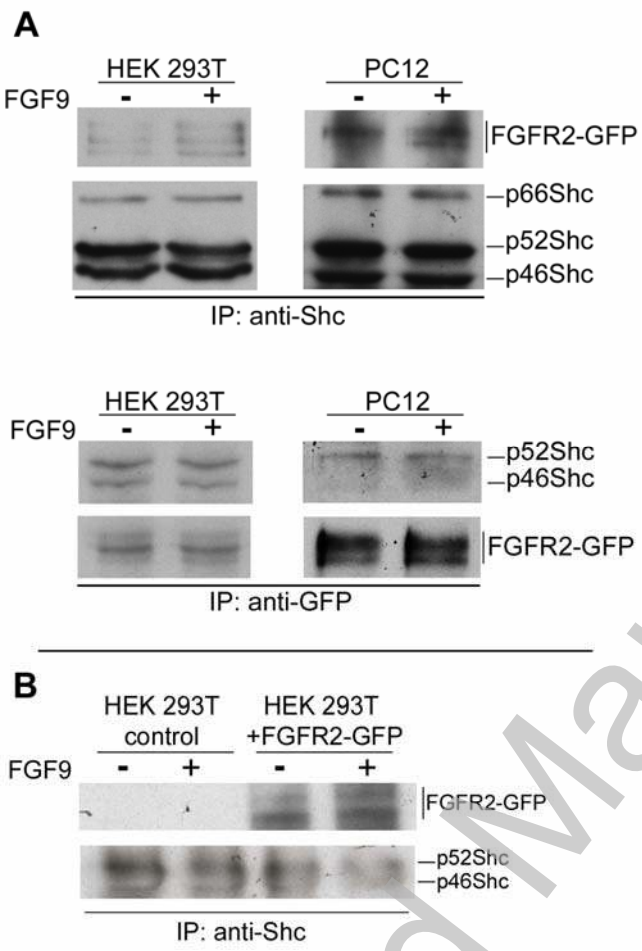

Figure 2

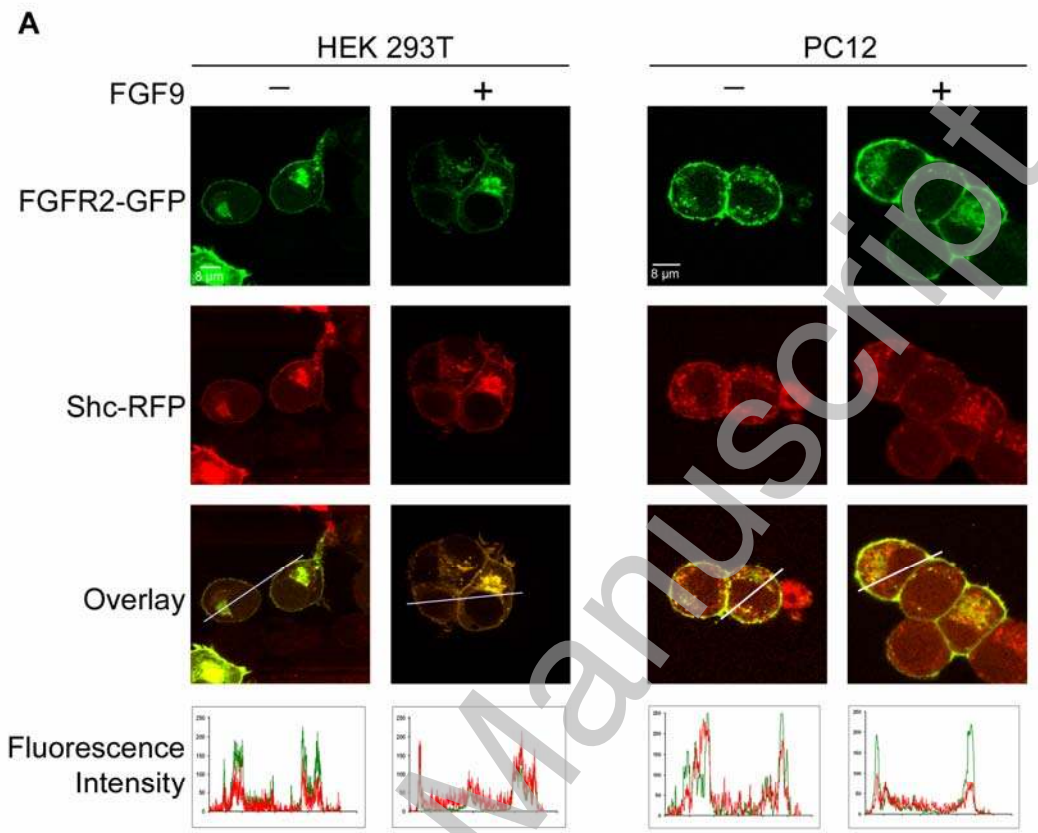

Figure 3

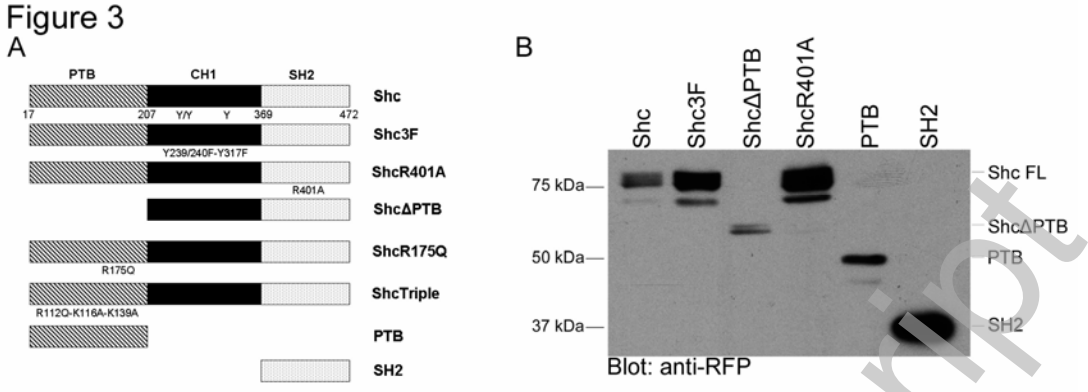

Figure 4

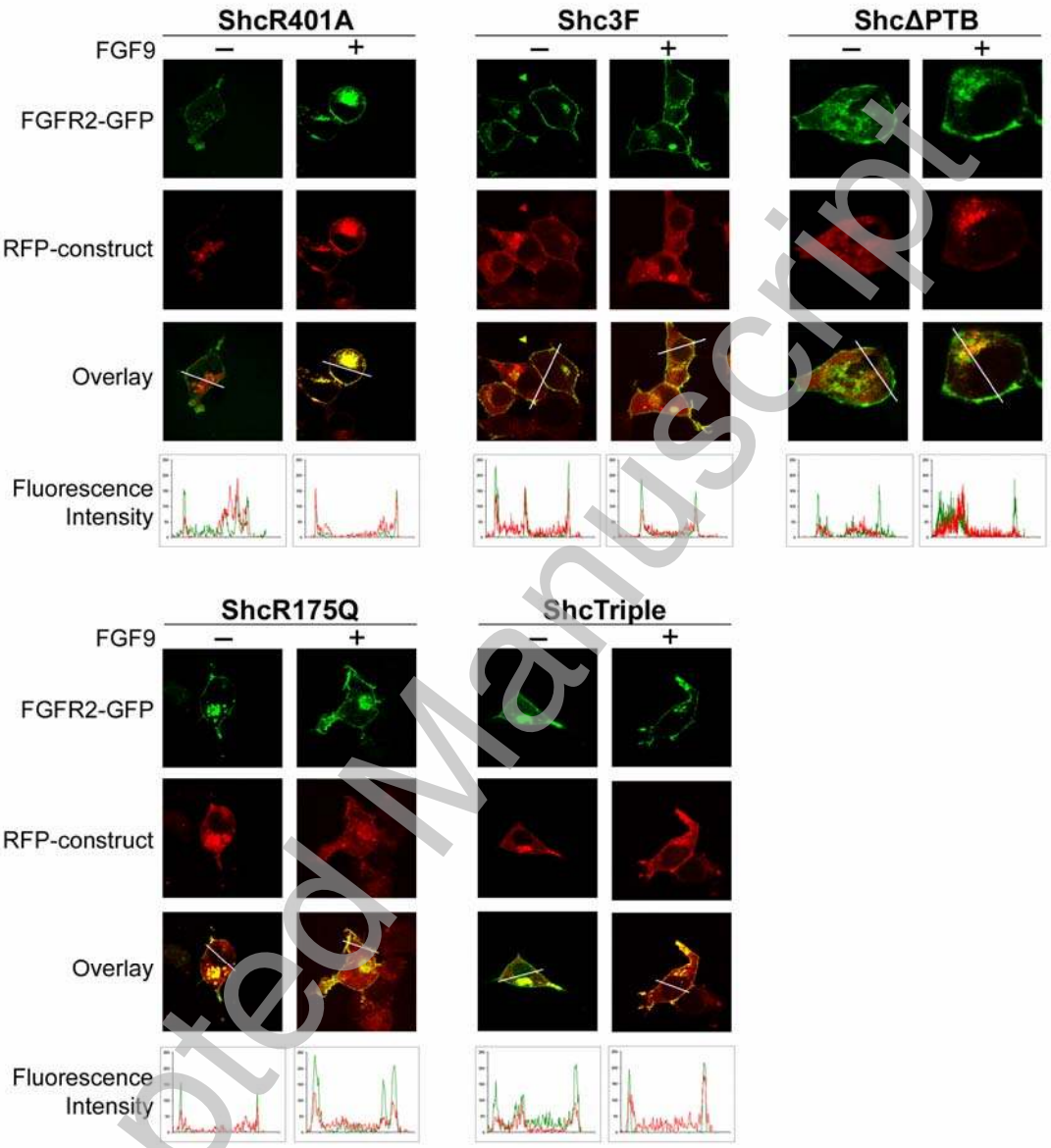

Figure 5

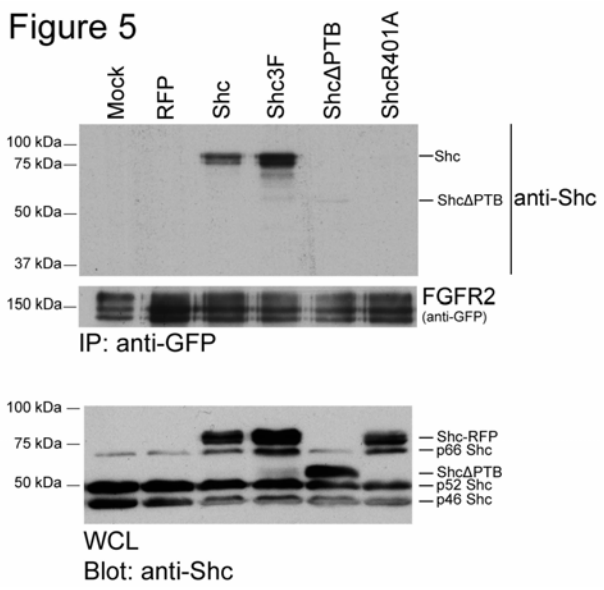

Figure 6

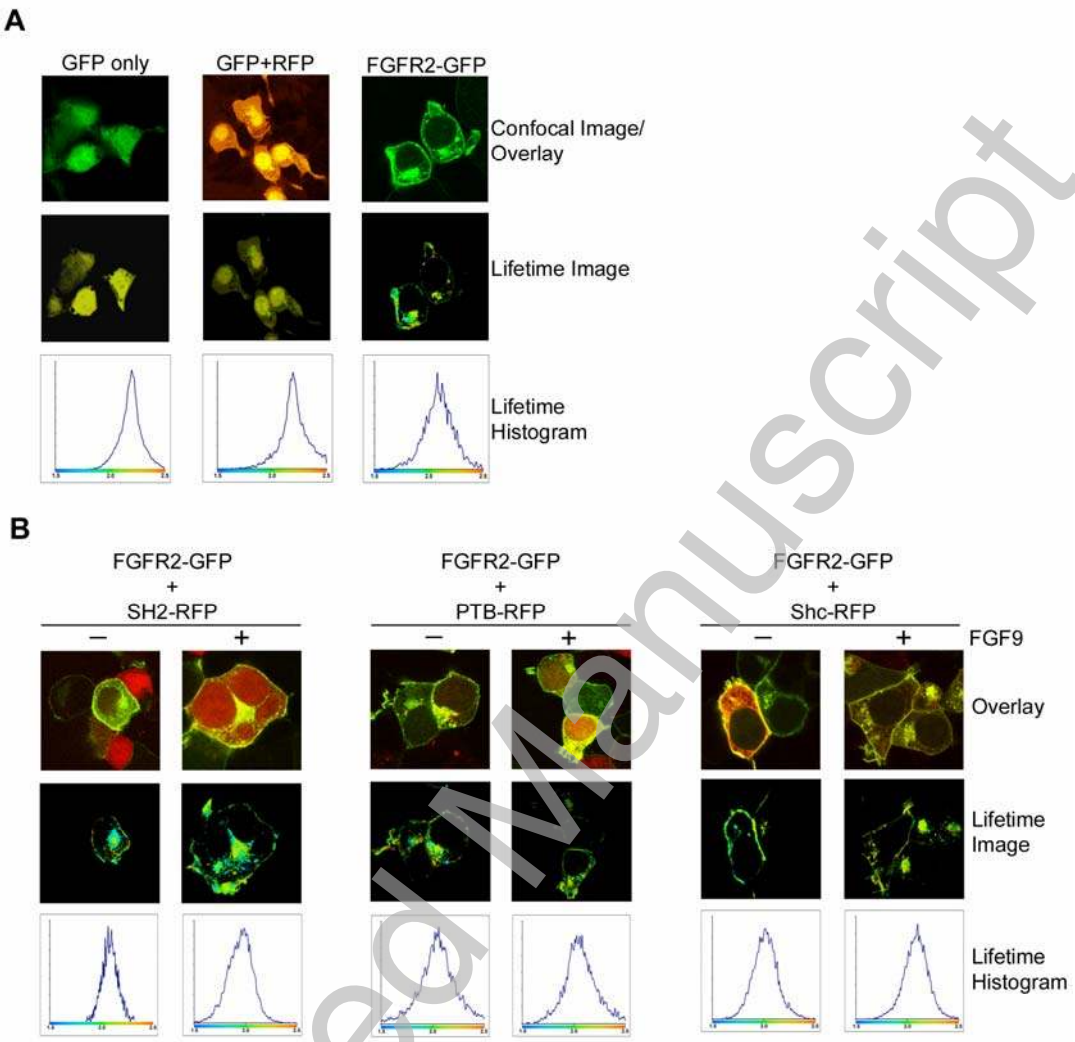

Figure 7