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Extended-Synaptotagmin-2 Mediates FGF Receptor Endocytosis and ERK Activation In Vivo

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SUMMARY

Targeting of activated plasma membrane receptors to endocytic pathways is important in determining the outcome of growth factor signaling. However, the molecular mechanisms are still poorly understood. Here, we show that the synaptotagmin-related membrane protein E-Syt2 is essential for rapid endocytosis of the activated FGF receptor and for functional signal transduction during *Xenopus* development. E-Syt2 depletion prevents an early phase of activated FGF receptor endocytosis that we show is required for ERK activation and the induction of the mesoderm. E-Syt2 interacts selectively with the activated FGF receptor and with Adaptin-2, and is required upstream of Ras activation and of receptor autophosphorylation for ERK activation and the induction of the mesodermal marker Xbra. The data identify E-Syt2 as an endocytic adaptor for the clathrin-mediated pathway whose function is conserved in human and suggest a broader role for the E-Syt subfamily in growth factor signaling.

INTRODUCTION

The fibroblast growth factors (FGFs) are powerful mitogens whose disregulation has been associated with multiple forms of cancers (Eswarakumar et al., 2005), and with cell transformation (Dvorak et al., 2006), angiogenesis (Murakami and Simons, 2008) and metastasis (Chaffer et al., 2007). Their action is also essential for correct germ layer induction and organogenesis during early metazoan development (Bottcher and Niehrs, 2005; Kimelman, 2006; Thisse and Thisse, 2005). FGF signaling occurs via one of four receptor tyrosine kinases (RTKs), the FGF receptors 1 to 4 (FGFR1–4), which transduce the FGF signal to the ERK MAP-kinase (Umbhauer et al., 1995), PI3K, and PLCγ pathways (Sivak et al., 2005).

Endocytosis was originally thought to be a means to extinguish receptor signaling, permitting appropriate responses to sequential signaling events. However, active signaling may continue well after RTK internalization (Baass et al., 1995) and the choice of endocytic pathway can often determine the response to growth factors (Miaczynska et al., 2004). The endocytic pathway has been found to determine signal strength, longevity, and intracellular location, at least in part by directing signaling to appropriate effector pathways (Le Borgne et al., 2005; Le Roy and Wrana, 2005; Polo et al., 2004; Vieira et al., 1996). Thus, the exact manner in which RTKs are internalized will more than often determine the outcome of growth factor signaling.

Despite the wide-ranging biological importance of FGF signaling, the role of receptor endocytosis in determining its physiological outcome is still very poorly understood (Wiedlocha and Sorensen, 2004). It is known that both the catalytic activity and intracellular domains of the FGFRs are necessary for receptor endocytosis (Citores et al., 2001; Sorokin et al., 1994). Recent work has suggested that targeting activated RTKs to the clathrin-dependent endocytic pathway rather than to non-clathrin pathways is necessary if signaling is to be sustained (Sigismund et al., 2008). But, it is still far from clear how activated RTKs in general, and the FGFRs in particular, are selectively recognized and targeted to any endocytic pathway.

We report that the Extended Synaptotagmin-like protein E-Syt2 (Groer et al., 2008; Min et al., 2007) constitutes an early endocytic adaptor for the FGFRs. E-Syt2 is essential for mesoderm induction, and for functional FGFR endocytosis and ERK activation during *Xenopus* embryogenesis. E-Syt2 interacts highly selectively with activated FGFR on the plasma membrane and with the clathrin adaptor complex Adaptin-2 (AP-2), providing a link between receptor activation and endocytosis that is conserved in human.

RESULTS

The Extended Synaptotagmin-like protein E-Syt2 is a membrane protein of unknown function (Groer et al., 2008; Min et al., 2007). However, E-Syt2 orthologs exist in animals as evolutionarily distant as human and worm, suggesting an essential common function (Figure 1A; see Figure S1A available online) (Groer et al., 2008). The *Xenopus* ortholog of human E-Syt2 is predicted to have an N-terminal trans-membrane (TM) domain and a SMP (Synaptotagmin-like, Mitochondrial and lipid binding protein) in silico predicted domain (Lee and Hong, 2006), followed by three C2 domains (Figure 1A). E-Syt2 is maternally expressed and is present throughout early *Xenopus* development, at least as far as stage 40 (Figure S1B). By late gastrula/early neurula (stages 11.5–18) expression is mainly associated with head

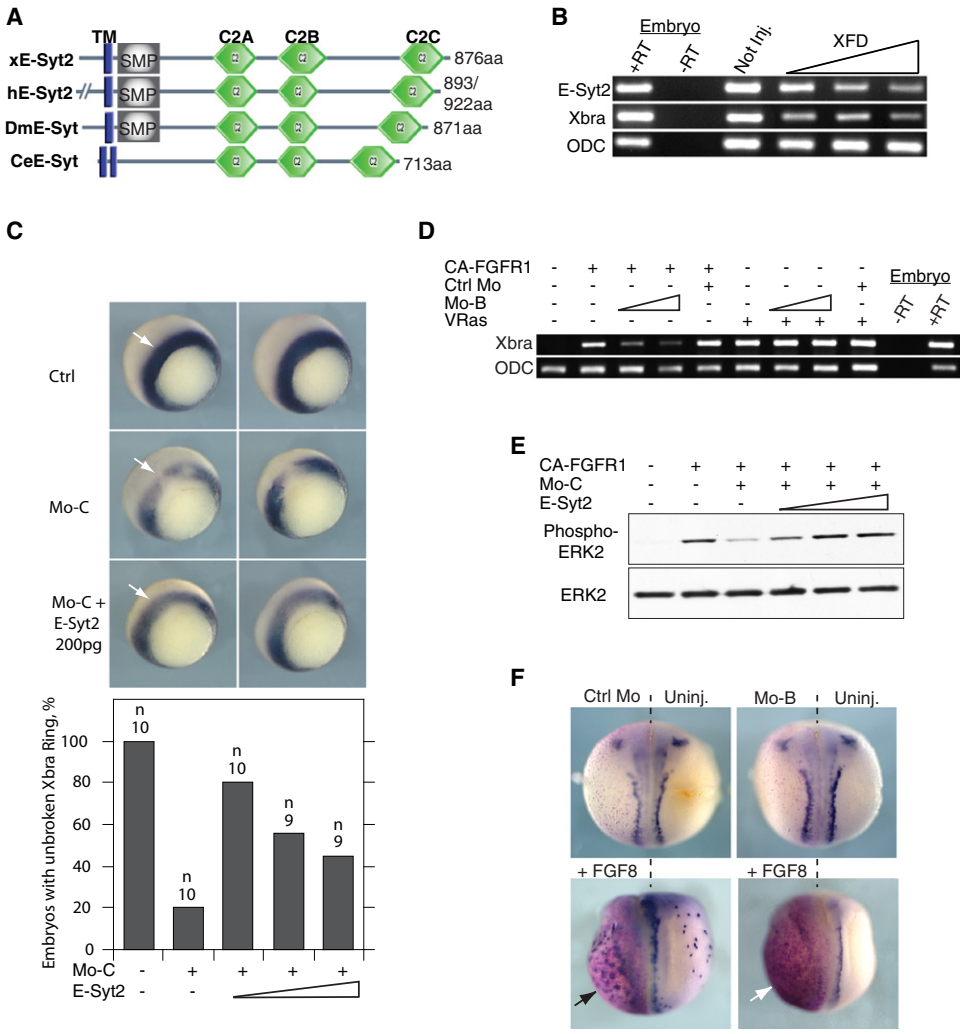

Figure 1. *Xenopus* E-Syt2 Is Required for Induction of the Early Mesoderm Gene Xbra and for FGF Signaling

(A) Predicted structural domains of E-Syt2 orthologs, "x" *Xenopus*, "h" human, "Dm" *Drosophila melanogaster*, "Ce" *Caenorhabditis elegans*.

(B) Zygotic E-Syt2 expression depends on FGF signaling. FGF signaling was inhibited by the expression of the dominant-negative FGF receptor "XFD" (100, 200, and 400 pg) in ventral marginal zone (VMZ) explants.

(C) In vivo Xbra expression is suppressed by E-Syt2 depletion. Dorsal blastomeres of four cell embryos were each injected equatorially with 0.75 pmol of Mo-C and increasing amounts of HA-E-Syt2 mRNA and Xbra expression at stage 10.5 revealed by whole-mount in situ hybridization.

(D) E-Syt2 depletion suppresses ectopic induction of Xbra by activated FGFR1. Embryos were injected with 0.9 or 1.8 pmol Mo-B or Ctrl Mo and with 2.5 pg constitutively active FGFR1 (CA-FGFR1) or 5 pg VRas.

(E) Activated FGFR1 (1.5 pg/cell, four cell embryos) induction of ectopic ERK activation in ACs was also suppressed by depletion of E-Syt2 (3 pmol MoC/cell).

(F) Depletion of E-Syt2 also suppresses FGF8-dependent differentiation. Embryos were unilaterally coinjected with FGF8 (2.5 pg), β-galactosidase (325 pg) mRNAs, and E-Syt2 Mo-B or Ctrl Mo (2 pmol) and subjected to whole-mount in situ to reveal N-tubulin expression (blue). Purple staining (β-galactosidase activity assay) indicates injected side of embryo.

See also Figure S1.

and dorsolateral mesoderm and at later stages (29–33) with eye, cranial neural crest, and somites (Figure S1B).

E-Syt2 Is Required for Mesoderm Formation In Vivo

The expression pattern of E-Syt2 resembled that of FGFR1 (Lea et al., 2009), suggesting that it might form part of the FGF synexpression group (Furthauer et al., 2002; Tsang et al., 2002). Consistent with this, inhibition of FGF signaling in the marginal zone of *Xenopus* embryos by expression of the dominant-negative receptor XFD (Amaya et al., 1991) inhibited the zygotic expression of both E-Syt2 mRNA and the early mesodermal marker Xbra, indicating that *e-syt2* is an FGF-responsive gene (Figure 1B). Specific depletion of E-Syt2 using antisense Morpholinos (Figures S1C and S1D), in turn caused severe trunk shortening, reminiscent of FGF inhibition (Chung et al., 2004; Fletcher and Harland, 2008) and was rescued by the introduction of wild (wt) E-Syt2 but not of a mutant lacking the transmembrane domain (Figure 6A; Figures S1E and S1F). Trunk shortening was also associated with a disruption of somatic muscle formation (Figure S1G). Further, depletion of E-Syt2

from the dorsal medial zone inhibited expression of the early mesodermal marker Xbra, and this expression could be rescued by low-level expression of E-Syt2 (Figure 1C). Together these data suggested that E-Syt2 was required for early mesoderm induction.

E-Syt2 Is Required for FGF Signaling

FGF signaling via the ERK MAP-kinase pathway is essential for the induction and maintenance of mesoderm during *Xenopus* embryogenesis (Amaya et al., 1993; Fletcher and Harland, 2008; Umbhauer et al., 1995). *Xenopus* animal cap (AC) explants do not normally express mesoderm-specific genes. However, activation of the FGF pathway, for example, by ectopic expression of constitutively active FGFR1 (CA-FGFR1), is sufficient to induce mesodermal differentiation and the expression of genes such as Xbra (Figure 1D). Depletion of E-Syt2 strongly inhibited Xbra expression, while expression was fully rescued by the constitutively active VRas (Figure 1D). Further, E-Syt2 depletion also inhibited ERK activation by CA-FGFR1, and this activation was rescued by the reintroduction of E-Syt2 (Figure 1E). Thus, E-Syt2 was required for the induction of Xbra expression and for ERK activation by the FGF pathway, and acted upstream of Ras.

Ectopic FGF8 in *Xenopus* embryos induces the aberrant differentiation of neurons within the embryonic ectoderm and hence provides a further in vivo test for functional FGF signaling (Hardcastle et al., 2000; Figure 1F). Depletion of E-Syt2 from embryos severely attenuated the differentiation of ectopic neurons in embryos unilaterally microinjected with FGF8 mRNA (Figure 1F). This provided further confirmation that E-Syt2 was indeed required for functional FGF signaling.

E-Syt2 Selectively Interacts with the Activated FGF Receptor

Full-length *Xenopus* E-Syt2 localized to the plasma membranes of embryonic blastomeres (Figure 2A) and of HEK293T cells (Figure 5A). This and its requirement upstream of Ras suggested that it might directly interact with the FGF receptor. When E-Syt2 was coexpressed with a range of plasma membrane receptors, it showed a preference for *Xenopus* FGFR1 (Figure 2B). It further displayed a similar level of interaction with the human FGFR1 and indeed with all four receptor homologs FGFR1–4 (Figure 2C). A weak, but reproducible, interaction was also noted with EGFR, but no significant interaction with EphA4 (Winning et al., 1996) or with the Activin/BMP receptor (Chang et al., 1997; Figure 2B). Interaction of E-Syt2 with FGFR1 was found to be dependent on a basal level of receptor activation, since treatment of cells with the FGFR inhibitor SU5402 (EMD/Merck) abrogated the interaction as well as receptor autophosphorylation (Figure 2D). Further, addition of bFGF markedly enhanced the basal level of interaction (Figure 2E). Enhancement of the interaction by bFGF was noted whether FGFR1 was coimmunopreciptated with E-Syt2 or E-Syt2 was coimmunoprecipitated with FGFR1 (upper and lower panels in Figure 2E). The weak interaction of E-Syt2 with the EGFR receptor was also enhanced by receptor activation and suppressed by the EGFR inhibitor tyrphostin (AG1478, Sigma), but activation of the EphA4 receptor (Epp) did not enhance its interaction with E-Syt2, despite strong autophosphorylation (Figure S2). Expression of dominant-negative EGFR in *Xenopus* in fact induces a mild XFD-like phenotype (Amaya et al., 1993; Nie and Chang, 2006). Hence, the interaction of E-Syt2 with activated FGFR and possibly to a lesser degree with activated EGFR provided a potential explanation for its requirement in vivo.

Interaction of E-Syt2 with FGFR1 Is Independent of Receptor Autophosphorylation Required for Signal Transduction

Since E-Syt2 depletion prevented activation of the ERK pathway by CA-FGFR1 but not by VRas, it must act downstream of receptor activation and upstream of Ras (Figure 1D). To more precisely determine the requirement for E-Syt2, we asked whether or not it interacted with the activated receptor independently of signal transduction. FGFR1 activation results in the autophosphorylation of a number of tyrosines within its cytoplasmic domain. Phosphorylation of aa Y463 and Y766 are required for recruitment of Crk, PLCγ, and Shb, and for activation of the ERK pathway (Cross et al., 2002; Lundin et al., 2003; Mohammadi et al., 1992; Ong et al., 2000). However, neither a Y463F nor a Y766F mutation of FGFR1 affected the ability of E-Syt2 to specifically recognize and interact with the activated receptor (Figure 2F). Thus, the interaction of E-Syt2 with activated FGFR1 not only preceded Ras activation but also the recruitment of key downstream effectors.

E-Syt2 Is Required for Endocytosis of Activated FGFR1 and for ERK Activation

The classical Synaptotagmins are believed to direct cargo proteins to the Adaptin-2/clathrin endocytic pathway (Haucke and De Camilli, 1999). Since it is generally accepted that receptor endocytosis is important in growth factor signaling, we hypothesized that E-Syt2 might be required for the endocytic trafficking of FGFR. We, therefore, determined the kinetics of FGFR1 endocytosis in E-Syt2-depleted ACs following bFGF stimulation (Figure 3A; see Experimental Procedures). As expected, receptor endocytosis was arrested at 4°C even after FGF treatment. On the other hand, at 22°C a large proportion of cell surface receptor was transported into endocytic vesicles within 5 min of FGF treatment, and by 15 min receptor internalization was essentially complete. (Internalized FGFR colocalized with markers of early endosomes when assayed in cell culture; see below.) E-Syt2 depletion very strikingly abrogated the rapid phase of receptor endocytosis that occurred within the first 5 min of FGF treatment (Figure 3A). This rapid phase of endocytosis was also efficiently rescued by reintroduction of E-Syt2 (Figure S3). In contrast, after 15 min of FGF treatment, receptor endocytosis in the E-Syt2-depleted ACs approached control levels. A higher level of FGFR, however, remains associated with the plasma membrane than in control ACs (Figure 3A).

ERK activation in control ACs closely correlated with the rapid phase of receptor endocytosis, already being maximal after 5 min of FGF treatment and remaining high for at least 15 min, before returning to the basal level by 30 min (Figure 3B). E-Syt2 depletion abrogated the rapid phase of ERK activation and though some ERK activity was detected at 15 min, it was significantly lower than in control ACs. Thus, E-Syt2 was required for the rapid phase of receptor endocytosis and for the maximal and prolonged activation of ERK.

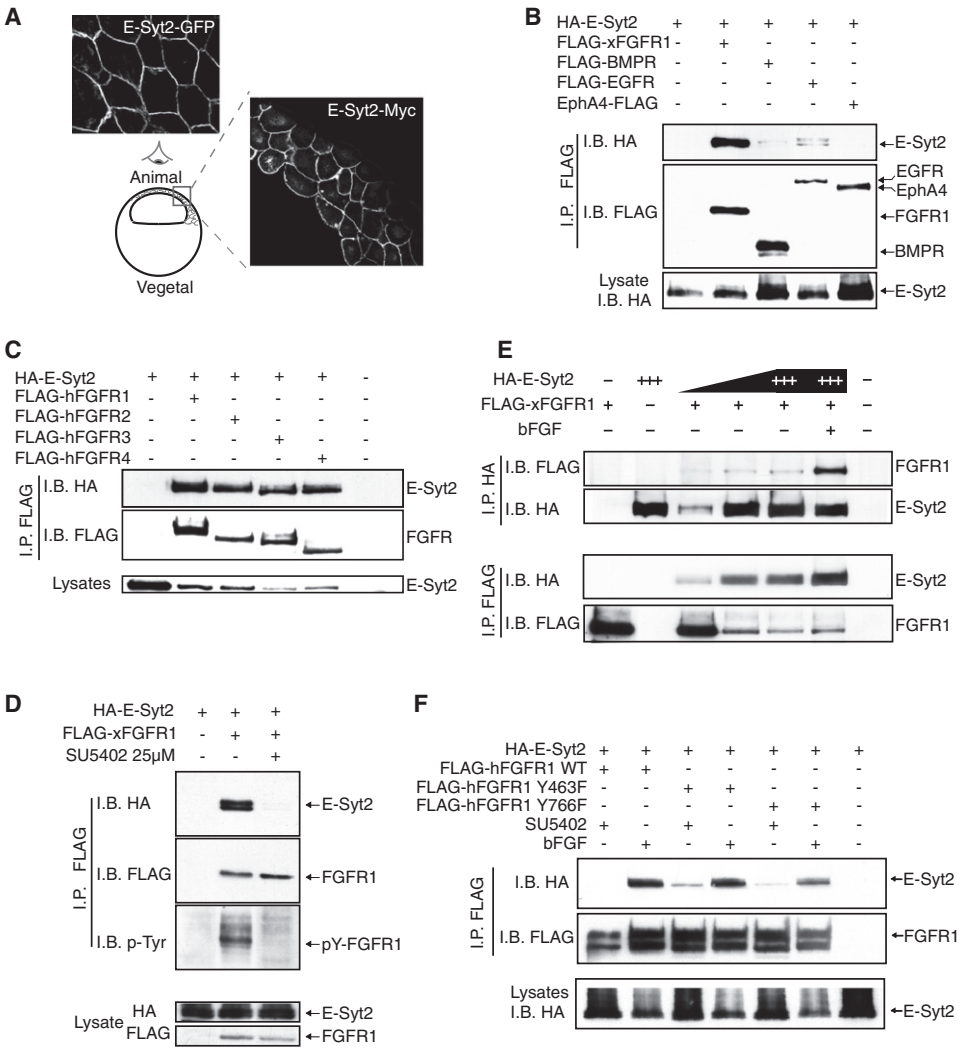

Figure 2. E-Syt2 Is a Membrane Protein that Specifically Interacts with the Activated FGF Receptor

(A) E-Syt2-GFP and E-Syt2-Myc fusions were expressed in *Xenopus* embryos and visualized at blastula either by direct (-GFP) or indirect (-Myc) confocal epifluorescence microscopy.

(B) E-Syt2 interacts selectively with FGFR1. HA-tagged E-Syt2 (HA-E-Syt2) was coexpressed with FLAG-tagged Xenopus FGFR1 (xFLAG-FGFR1), Xenopus BMPR (FLAG-BMPR), human EGFR (FLAG-EGFR), or Xenopus Epp (EphA4) (Epp-FLAG) (Winning et al., 1996) in HEK293T cells and anti-FLAG-coimmunoprecipitated (I.P.) complexes were immunoblotted (I.B.).

(C) HA-tagged E-Syt2 (HA-E-Syt2) was also coexpressed with the FLAG-tagged human FGF receptors 1 to 4 (FLAG-hFGFR1, 2, etc) and analyzed for coimmunoprecipitation as in (B).

(D) E-Syt2 interacts selectively with the activated, tyrosine phosphorylated form of FGFR1. HA-E-Syt2 and FLAG-xFGFR1 were coexpressed in HEK293T cells, FGFR1 inactivated or not with the specific inhibitor SU5402 and proteins immunoprecipitated and blotted as in (B).

(E) bFGF enhances the E-Syt2 interaction with FGFR1. FLAG-xFGFR1 and increasing levels of HA-E-Syt2 were coexpressed as in (B) and where indicated cells were treated with bFGF. Extracts were then immunoprecipitated with either anti-HA or anti-FLAG antibodies and blotted as indicated. "+++" indicates highest level of E-Syt2 transfection.

(F) The E-Syt2/FGFR1 interaction is independent of the recruitment of downstream factors to the receptor. FLAG-hFGFR1 and the point mutants Y463F and Y766F were coexpressed with HA-E-Syt2, cells treated either with SU5402 or with bFGF as in (D) and (E), and anti-FLAG-coimmunoprecipitated (I.P.) complexes immunoblotted (I.B.).

See also Figure S2.

As expected, a dominant-negative (K44E) Dynamin mutant also abrogated FGF-induced receptor endocytosis in ACs (Figure 3C). Coexpression of Dynamin K44E inhibited CA-FGFR1 induction of Xbra to a similar degree as E-Syt2 depletion and, as with depletion of E-Syt2, VRas rescued Xbra expression in K44E-Dynamin expressing ACs (Figures 3D and 1D). Chlorpromazine, a specific inhibitor of the clathrin-mediated endocytic pathway (Wang et al., 1993), suppressed Xbra induction to a similar degree as E-Syt2 depletion and Dynamin-K44E expression while nystatin, a specific inhibitor of the caveolar

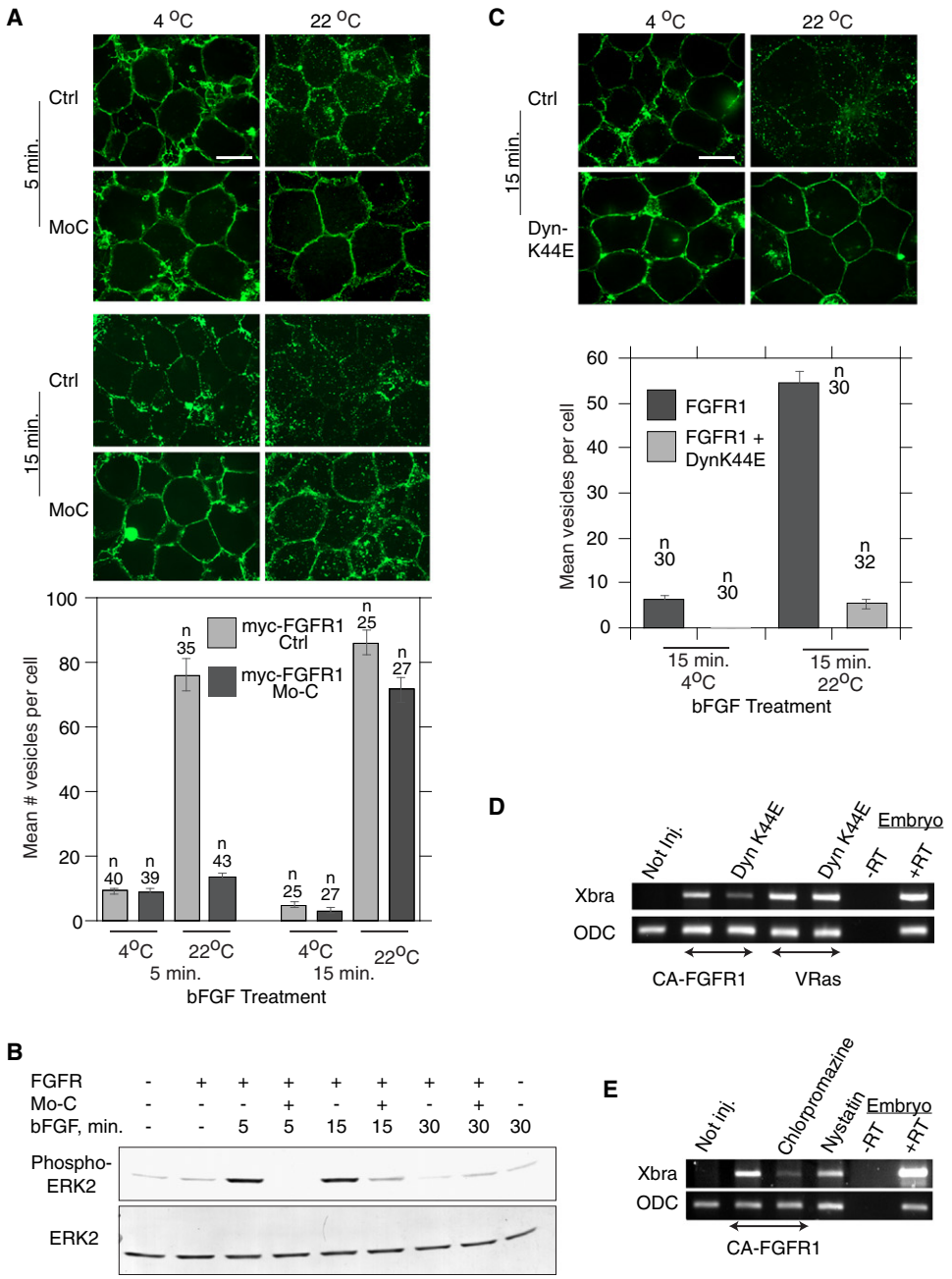

Figure 3. E-Syt2 Is Required In Vivo for FGF Receptor Endocytosis and Signaling via the Clathrin Pathway

(A) E-Syt2 depletion inhibits FGF receptor endocytosis in vivo. Each blastomere of four cell embryos was injected with 6 pmol of Morpholino C (Mo-C) and 20 pg of Xenopus Myc-FGFR1 mRNA. ACs were then isolated and incubated with anti-Myc antibody to label surface receptors. After incubation with bFGF at 4°C or 22°C for the indicated times, ACs were subjected to indirect immunofluorescence labeling to reveal receptor endocytosis. The upper panels show typical images and the lower panel the statistical analysis of receptor internalization.

(B) Embryos were injected and ACs were isolated and treated with bFGF at 22°C as in (A), before analysis of cell extracts for ERK and phospho-ERK levels by immunoblotting.

(C) Dominant-negative Dynamin also inhibits FGFR1 endocytosis in vivo. ACs expressing Myc-FGFR1 alone or with Dynamin K44E (Dyn-K44E) were prepared and treated with bFGF and were analyzed for receptor endocytosis as in (A). In (A) and (C), "n" indicates the number of cells scored, scale bar in micrographs represents 30 μm, and error bars indicate the standard error.

(D) Induction of Xbra in ACs requires FGFR1 endocytosis upstream of VRas. Embryos were injected with either CA-FGFR1 or VRas and DynK44E mRNAs and Xbra and ODC mRNA levels determined by RT-PCR.

(E) ACs expressing CA-FGFR1 were also treated with clathrin or caveolin inhibitors, chlorpromazine and nystatin before RT-PCR analysis as in (D).

In (D) and (E), +RT and –RT refer to control PCR analysis of whole embryo RNAs with and without reverse transcriptase.

See also Figure S3.

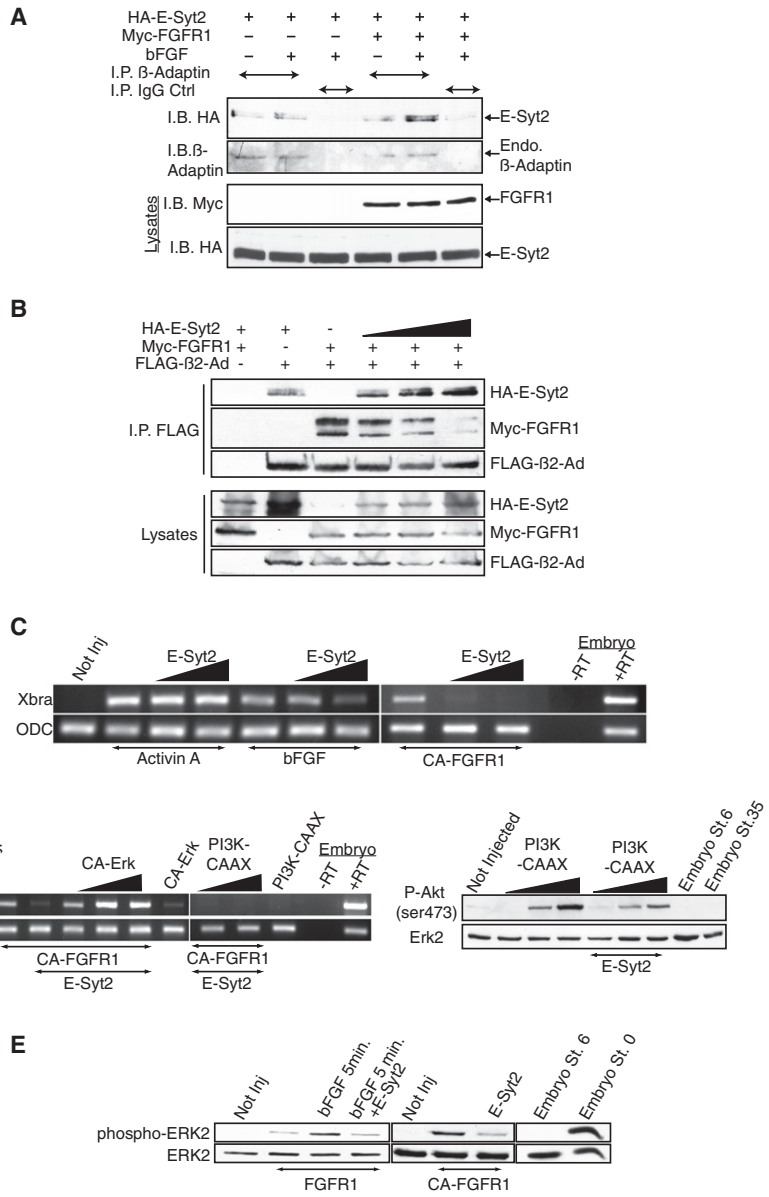

Figure 4. E-Syt2 Interacts with Adaptin-2 to Modulate Receptor Endocytosis, ERK Activation, and Xbra Induction

(A) E-Syt2 interacts with the endogenous AP-2 tetramer. HA-E-Syt2 and Myc-tagged FGFR1 (Myc-FGFR1) were coexpressed in HEK293T cells stimulated or not with bFGF. Endogenous AP-2 complexes were immunoprecipitated with a β_2-Adaptin-specific antibody or a nonspecific control antibody (IgG Ctrl) and I.B.

(B) Both E-Syt2 and FGFR1 interact with the AP-2 complex. β_2-adaptin (FLAG-β2-Ad) was coexpressed with FGFR1 (Myc-FGFR1) and with increasing amounts of E-Syt2 (HA-E-Syt2) and complexes were immunoprecipitated with an anti-FLAG antibody (I.P. FLAG). The upper panels show immunoblots for the coprecipitated proteins and the lower panels show protein levels in the input protein lysate.

(C) E-Syt2 gain of function also inhibits bFGF and activated FGFR1 (CA-FGFR1)-mediated, but not Activin-mediated Xbra induction in ACs.

(D) Left panel, constitutively activated-ERK (CA-ERK) but not constitutively activated PI3K (PI3K-CAAX) expression rescues E-Syt2 inhibition of Xbra induction by CA-FGFR1 in ACs. Right panel, consistent with this PI3K-CAAX activation of Akt in ACs is not strongly affected by E-Syt2 gain of function.

(E) E-Syt2 gain of function inhibits ERK activation by CA-FGFR1 (anti-phospho-Erk immunoblot) in ACs.

See also Figure S4.

pathway (Rothberg et al., 1992), had little effect (cf. Figure 3E with Figures 1D and 3D). Thus, in the *Xenopus* system, functional signaling through FGFR1 occurred predominantly via the clathrin-mediated pathway. Together, the data supported the contention that E-Syt2 depletion inhibited FGF signaling by suppressing the rapid phase of clathrin-dependent receptor endocytosis. E-Syt2 appeared, then, to act as an essential endocytic adaptor in FGF signaling. Since it directly recognized the activated FGF receptor independently of downstream signaling events, the data also suggested that E-Syt2 acted at a very early stage of endocytic trafficking.

E-Syt2 Interacts with the Adaptin-2 Complex

Synaptotagmin 1 (Syt1) catalyzes endocytosis via the clathrin-mediated pathway by targeting cargo proteins to Adaptin-2 (AP-2) via an interaction that involves its C2B domain (Haucke and De Camilli, 1999; Haucke et al., 2000; Poskanzer et al., 2006; von Poser et al., 2000; Zhang et al., 1994). We, therefore, tested whether E-Syt2 interacted with AP-2. In vitro "pull-down" assays showed that the bacterially expressed C2A, -B, and -C domains of E-Syt2 all bound the in vitro translated α subunit of AP-2, while only C2B and -C also bound the μ and σ subunits and none of the C2 domains bound the β subunit (Figure S4A). This was analogous to interactions of Syt-1 with the α and μ subunits of AP-2 (Haucke et al., 2000).

Consistent with the in vitro interactions, immunoprecipitation of the endogenous AP-2 complex from HEK293T cells with an β_2-Adaptin-specific antibody was found to coprecipitate E-Syt2 (Figure 4A). Since the β_2 subunit did not directly interact with E-Syt2, this demonstrated that E-Syt2 indeed interacted with the intact AP-2 complex. The interaction was also detectably enhanced by bFGF stimulation, suggesting that endogenous FGFR might cooperate in the formation of the AP-2/E-Syt2 complex. To investigate this further, FLAG-tagged β_2-Adaptin was coexpressed with E-Syt2 and with FGFR1 in HEK293T cells. Both E-Syt2 and FGFR1 were observed to coimmunoprecipitate with FLAG-β_2-Adaptin, whether expressed separately or together, suggesting the possibility of a tripartite complex (Figure 4B; Figures S4B and S7A). However, stepwise increase in

the expression levels of E-Syt2 competed the interaction between FGFR1 and AP-2 (β_2-Adaptin) (Figure 4B), and this effect was fully reproducible (Figure S4B). These data suggested that either the interactions of E-Syt2 with AP-2 and with FGFR1 were exclusive or that nonstochiometric levels of E-Syt2 independently titrated AP-2 and FGFR1 and in so doing inhibited the formation of the tripartite complex. Such competition is a common but poorly documented occurrence when adaptor proteins are overexpressed (Ruan et al., 1999). Since E-Syt2 was found to interact with FGFR1 and with AP-2 via distinct and distal domains (Figures 6D, 6E, and 7D; Figures S4A and S6A), it seemed unlikely, but not impossible, that AP-2 and FGFR1 simply competed for binding to E-Syt2. Thus, E-Syt2 either interacted with both FGFR1 and AP-2 to form a tripartite complex, or these interactions were exclusive and occurred independently.

E-Syt2 Gain of Function Inhibits Functional FGF Signaling

Since overexpression of E-Syt2 interfered with the interaction between FGFR1 and AP-2, we argued that it should also inhibit functional FGF signaling. Consistent with this, gain of E-Syt2 function in vivo-induced trunk shortening similar to that observed with E-Syt2 depletion (Figure S4C), and suppressed the induction of Xbra in embryo ACs by bFGF and by CA-FGFR1, but not by Activin (Figure 4C). Further, this inhibition could be rescued by activated ERK (CA-ERK), but not by activated PI3K (PI3K-CAAX) despite the latter's ability to activate Akt in ACs (Figure 4D). (Though earlier studies suggested the FGF or Activin pathways were interdependent, more recent work has shown that this is not the case [Tsang et al., 2002; Zhao et al., 2008].) E-Syt2 gain of function also inhibited the activation of ERK by both bFGF and by CA-FGFR1 (Figure 4E). Thus, E-Syt2 gain of function specifically suppressed the induction of Xbra and the activation of ERK by the FGF pathway.

E-Syt2 Gain of Function Inhibits Endocytosis of Activated FGFR1

The data suggested that the dominant-negative gain-of-function effects of E-Syt2 could be due to inhibition of FGFR1 endocytosis. Since E-Syt2 had been observed to compete the FGFR1/AP-2 interaction in HEK293T cells (Figure 4B; Figure S4B), we studied the effects of E-Syt2 gain of function on internalization of FGFR1 in these cells. In FGF-treated control cells, internalization of FGFR1 was rapid and complete, only 7% displaying no internalization 20 min after FGF addition (Figures 5A and B). In contrast, 45% of E-Syt2 expressing cells still showed no receptor endocytosis at all 20 min after FGF addition. FGFR1 positive vesicles were found to colocalize with the early endosome marker EEA1 already 5 min after FGF addition and 15 min later limited colocalization with the late endosome marker Rab7 was also observed (Figure S5A). Inhibition of endocytosis was specific to the FGF receptor, since the parallel uptake of transferrin was unaffected (Figure S5B). Further, FGFR1 endocytosis in the HEK293T cells was clathrin dependent as determined by its sensitivity to chlorpromazine but not to nystatin (Figure S5C). When receptor internalization was blocked by E-Syt2 gain of function, both FGFR1 and E-Syt2 remained fully accessible on the plasma membrane to biotin modification, clearly showing that the receptor did not enter the endocytic pathway (Figure 5C). Thus, E-Syt2 gain of function blocked receptor endocytosis at a step preceding pinching-off of the clathrin-coated pit.

Consistent with the block to receptor internalization, E-Syt2 gain of function in HEK293T cells also significantly suppressed activation of ERK by FGF (Figure 5D). Thus, as for E-Syt2 gain of function and depletion in ACs, the level of ERK activation correlated closely with the degree of internalization of activated FGFR1 in the HEK293T cell system.

Dominant-Negative Effects of E-Syt2 Require Both Putative-TM and C2C Domains

Since E-Syt2 gain of function behaved as a dominant-negative mutation, it was used to map the functional domains of E-Syt2 in FGF signaling. Deletion of either the putative-TM or the C2C domain of E-Syt2 was sufficient to abrogate its ability to inhibit Xbra induction by CA-FGFR1 in *Xenopus* ACs (Figures 6A and 6B). The same mutants were assayed for their dominant-negative effects on endocytosis in HEK293T cells, and here again both the TM and C2C domains were found to be essential, only full-length E-Syt2 displaying an ability to block endocytosis of activated FGFR1 (Figure 6C). Though the C-terminal deletion mutants ΔC2C, ΔC2BC, and ΔC2ABC were partly cytosolic, all mutants displayed a significant degree of association with the plasma membrane. Surprisingly, the ΔTM mutant, lacking a putative transmembrane domain, was almost exclusively associated with the plasma membrane. Thus, the loss of dominant-negative function in E-Syt2 mutants was not simply due to a failure to decorate the plasma membrane and colocalize with FGFR1.

The Putative-TM Domain of E-Syt2 Is Sufficient for Selective Interaction with Activated FGFR1

We argued that one or more of the mutations may have affected the ability of E-Syt2 to interact with the FGF receptor. Each E-Syt2 mutant was therefore assayed for interaction with FGFR1 by coimmunoprecipitation (Figure 6D; Figure S6A). We found that deletion of the N-terminal domain including the putative-TM domain (ΔTM) eliminated the interaction with FGFR1 and that this same domain (aa 1–312, ΔC2ABC) was sufficient for interaction with FGFR1. More importantly, this N-terminal domain encoded the receptor specificity of E-Syt2, its receptor binding being in greater part dependent on receptor activation (Figure 6E). (As will be seen, this was also the case for human E-Syt2 [Figure 7D]). Expression of differentially tagged E-Syt2 mutants revealed that like the Synaptotagmins, E-Syt2 dimerized or oligomerized, and that this required the same N-terminal domain found to interact with FGFR1 (Figures S6B and S6C). Thus, the N-terminal domain of E-Syt2 was responsible for interaction with FGFR1 and for E-Syt2 oligomerization but was not sufficient for the E-Syt2 dominant gain-of-function effects, which also required the C2C domain.

The C2C Domain Constitutes a Phospholipid Binding Domain

C2 domains often display selective phospholipid binding and phospholipids are known to be important in the internalization of receptor complexes (Geppert et al., 1994). In fact, the C2C domain of human E-Syt2a was shown to be sufficient for

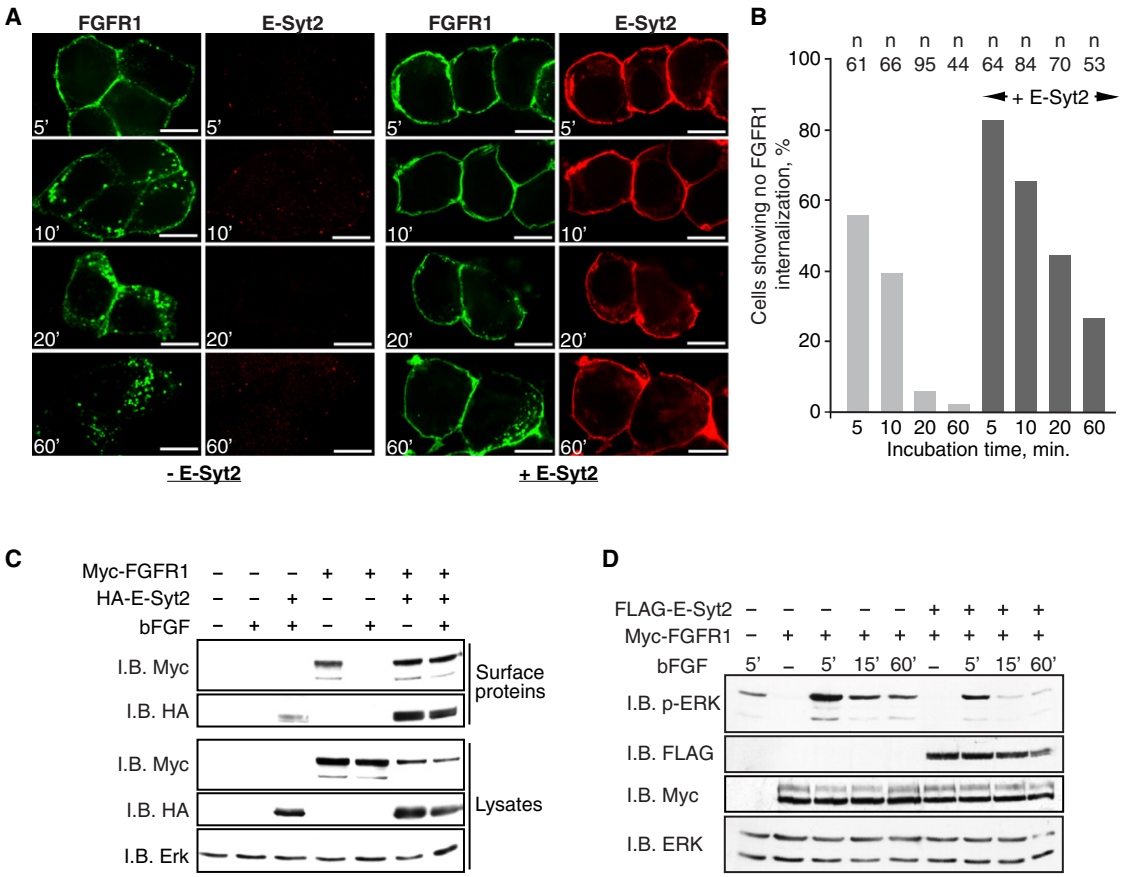

Figure 5. Gain of E-Syt2 Function Inhibits FGFR1 Endocytosis

(A) HEK293T cells were transfected with Myc-FGFR1, with or without HA-E-Syt2, treated with bFGF and internalization of plasma membrane receptor followed. Panels show typical image fields of the localization of the initially plasma membrane fraction of FGFR1 and of total E-Syt2 at various times after bFGF addition. Scale bar, 10 μm.

(B) Statistical analysis of receptor uptake during analyses as in (A). "n" indicates the total number of cells analyzed in repeat experiments.

(C) Myc-FGFR1 and HA-E-Syt2 were expressed as in (A), but surface proteins were biotin labeled before adding bFGF. Biotin-labeled surface proteins were then isolated and analyzed by immunoblotting (I.B.).

(D) Time course of Erk activation following bFGF treatment in the presence or absence of E-Syt2 gain of function. The experiment was performed as in (A), but total protein lysates were recovered at the indicated time following bFGF addition and were analyzed by immunoblotting.

See also Figure S5.

localization to the plasma membrane of HEK293T cells (Min et al., 2007). In agreement with these data, we noted a significant increase in the cytosolic localization of *Xenopus* E-Syt2 when the C2C domain was deleted (Figure 6C) and found that fusion of the C2C domain to a nuclear-targeted GFP was sufficient to displace it to the plasma membrane (Figure S6D). A previous study showed that while the combined C2AB domains of human E-Syt2a displayed Ca^{2+}-dependent binding to synthetic phosphatidyl-serine/-choline liposomes, C2C did not bind these liposomes (Min et al., 2007). To further investigate the activities of the C2C domain, we assayed its ability to bind a broad range of immobilized phospholipid species in comparison with the isolated C2A and C2B domains. In this assay, C2C displayed strong phospholipid binding with a preference for phosphoinositide phosphate (PI-P) species (Figure S6E) and, consistent with the lack of a consensus motif for Ca^{2+} coordination (Cheng et al., 2004; Min et al., 2007), binding was not dependent on the presence of Ca^{2+}. Thus, the ability of the C2C domain to bind phosphoinositol species and to target E-Syt2 to the plasma membrane may in part explain the functional requirements for this domain.

E-Syt2 Function Is Conserved in Human

The closest human ortholog of Xenopus E-Syt2 is translated from a major mRNA splice variant that encodes hE-Syt2b differing in its N-terminal domain from the previously identified hE-Syt2a (Min et al., 2007; Figure S1A).When expressed in HEK293T cells, human E-Syt2b was found to interact with both human and *Xenopus* FGFR1 (Figure 7A). Further, immunoprecipitation of endogenous hE-Syt2 from bFGF stimulated HEK293T cells, using specific anti-E-Syt2 antibodies, coimmunoprecipitated the endogenous FGFR1 receptor, indicating that this interaction occurred naturally within these cells (Figure 7B). In common with its *Xenopus* ortholog, hE-Syt2b displayed a strong and specific interaction with the activated form of human FGFR1, and this interaction required only the N-terminal domain of

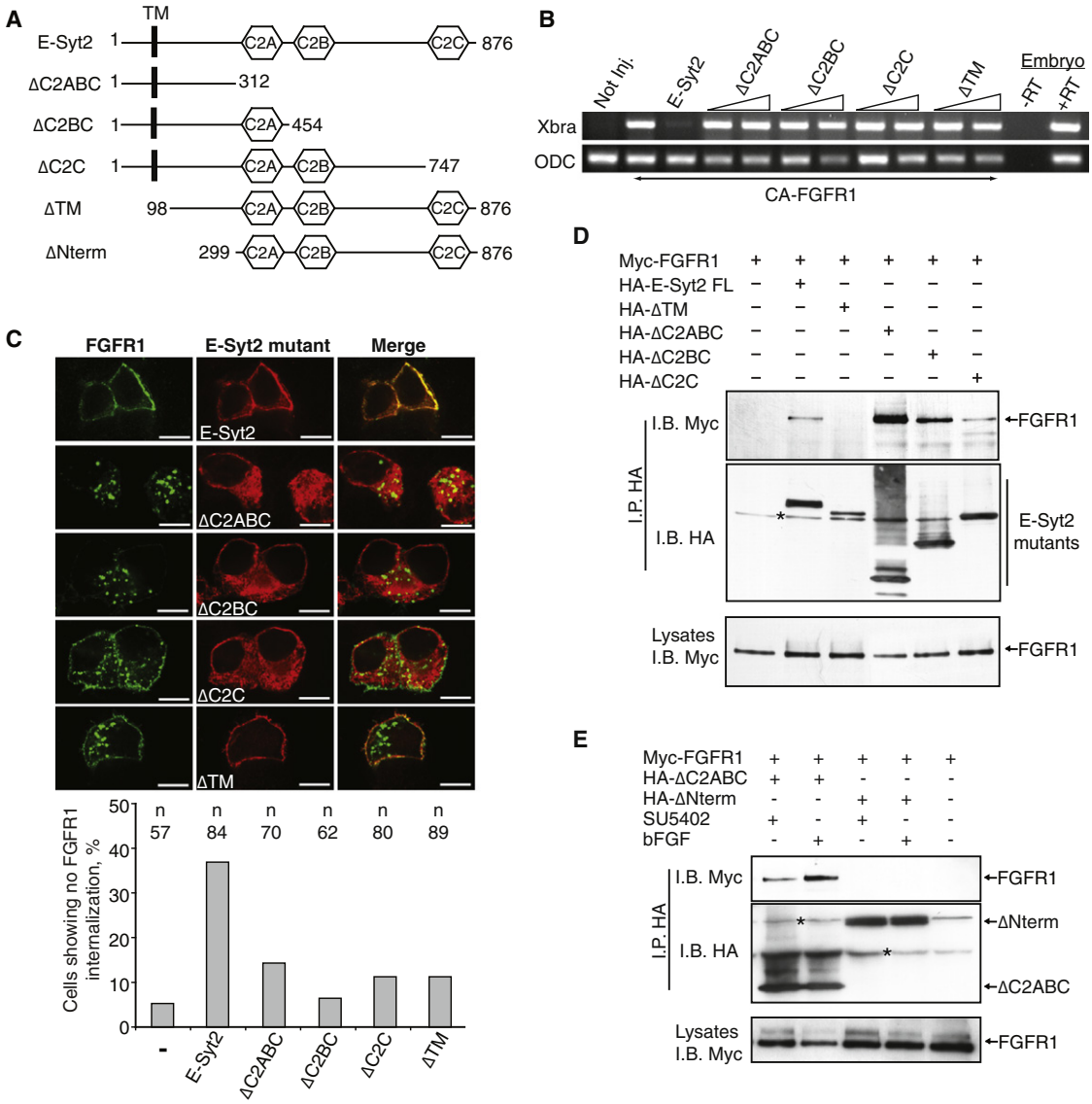

Figure 6. The Transmembrane and the C2C Domains of E-Syt2 Are Both Required for Its Function

(A) Schematic representation of the HA-E-Syt2 deletion mutants used.

(B) Deletion of either the N-terminal or the C2C domain eliminates the dominant-negative effects of E-Syt2 gain of function on Xbra induction. Mutants were expressed and analyzed in AC induced with CA-FGFR1. Four independent experiments gave consistent results.

(C) Only full-length E-Syt2 displays a dominant-negative gain of function effect on FGFR1 internalization. Each E-Syt2 mutant was analyzed in the receptor uptake assay in HEK293T cells as in Figures 5A and 5B. Upper panels show typical immunolocalization of initially membrane-labeled Myc-FGFR1 and of E-Syt2 mutants 20 min after bFGF addition. Scale bar, 10 μm. Lower panel shows statistical analysis of the data.

(D) The N-terminal domain of E-Syt2 is required to mediate its interaction with FGFR1. E-Syt2 mutants were coexpressed with Myc-FGFR1 in HEK293T cells, and HA immunoprecipitates (I.P.) were analyzed by immunoblotting (I.B.).

(E) The HA-ΔC2ABC mutant containing only the first 312 aa of E-Syt2 displays selective binding to activated FGFR1. The analysis in D was repeated for the mutant HA-ΔC2ABC, but cells were treated with either bFGF or with the inhibitor SU5402 before immunoprecipitation and the analysis of Myc-positive complexes.

In (D) and (E), the asterisk indicates the unspecific detection of an endogenous protein by the HA antibody assay.

See also Figure S6.

hE-Syt2b (Figures 7C and 7D). Human E-Syt2b also interacted with AP-2 (again FLAG-tagged on the β subunit) and both it and FGFR1 could be coprecipitated together with AP-2 (Figure S7A). hE-Syt2b localized to the plasma membrane, and hE-Syt2b gain of function blocked FGF-dependent internalization of hFGFR1 (Figure S7B). Thus, human E-Syt2b displayed very similar, if not identical, functionality to its *Xenopus* ortholog, strongly suggesting that E-Syt2 function in the FGF signaling pathway is conserved from amphibia to mammals.

DISCUSSION

Of 16 vertebrate synaptotagmins, functions have as yet been assigned to very few. These functions are mostly related to

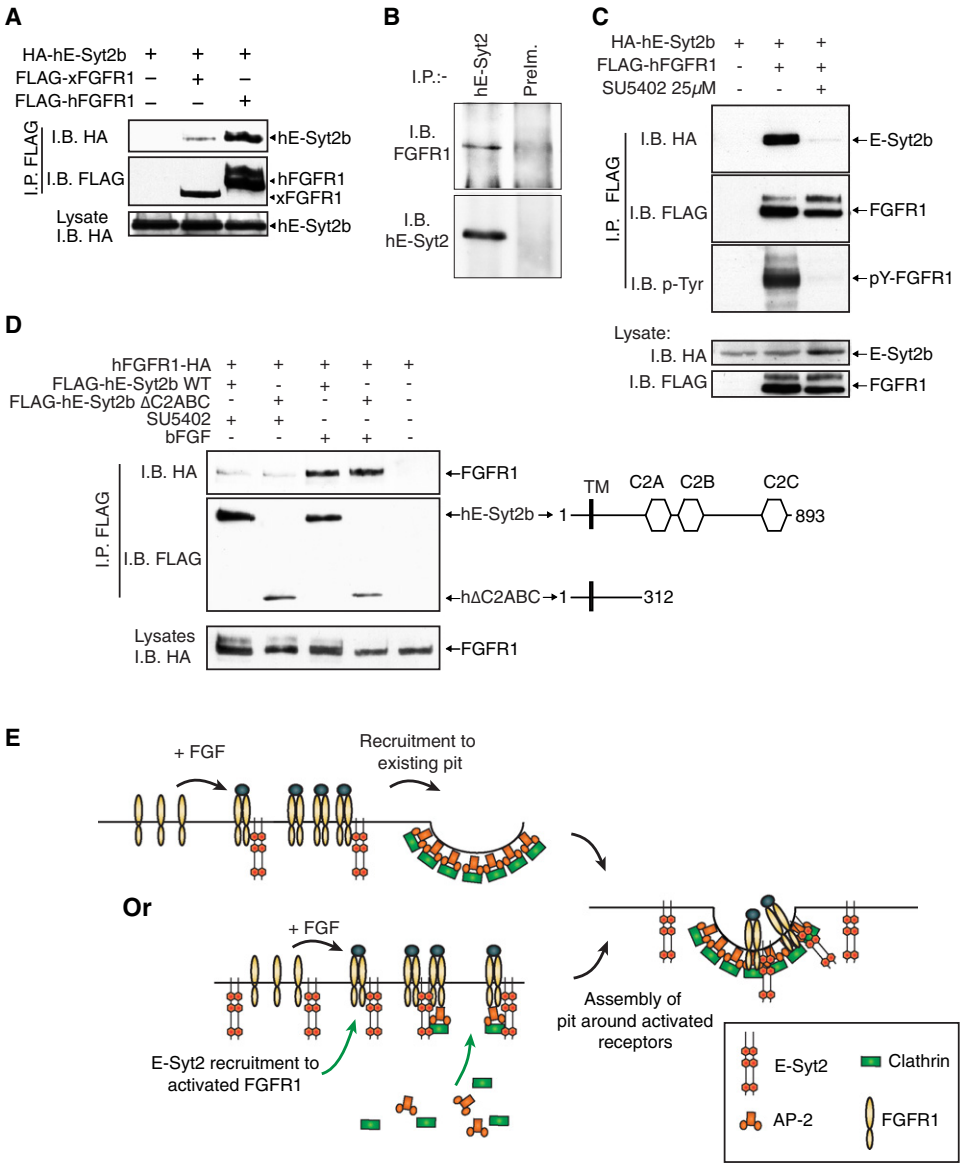

Figure 7. The Interaction between FGFR1 and E-Syt2 Is Conserved in Human

(A) Human E-Syt2b (HA-hE-Syt2b) was coexpressed with *Xenopus* or human FGFR1 (FLAG-xFGFR1 or -hFGFR1) in HEK293T cells, the receptors immunoprecipitated and proteins analyzed by immunoblotting.

(B) Endogenous human E-Syt2 was immunoprecipitated from a whole-cell extract using xE-Syt2 antibody #2 and the precipitate immunoblotted for both hE-Syt2 (hE-Syt2#Pr0863-2932r, see Experimental Procedures) and hFGFR1 (Flg #121, Santa Cruz Biotechnology).

(C) Human E-Syt2b interacts specifically with activated FGFR1. hE-Syt2b (HA-hE-Syt2b) was coexpressed with human FGFR1 (FLAG-hFGFR1) in HEK293T cells and receptor activation inhibited with SU5402. The receptor was then immunoprecipitated and proteins analyzed by immunoblotting.

(D) Specific interaction between hE-Syt2b and activated hFGFR1 requires the N-terminal domain of hE-Syt2b. Full-length hE-Syt2b or the mutant FLAG-hΔC2ABC containing aa 1–312 was coexpressed with hFGFR1 in HEK cells as in (C). Cells were then treated with either bFGF or with the inhibitor SU5402 before immunoprecipitation and analysis of FLAG-positive complexes.

(E) Potential E-Syt2 functions in FGF signaling. Two possible modes of action are suggested; see text for more detail.

See also Figure S7.

neurotransmission, despite broad expression profiles in non-neuronal tissues, e.g., (Andrews and Chakrabarti, 2005). Three synaptotagmin-related protein families, the Ferlins, MCTPs, and the E-Syts, are broadly expressed and each is characterized by a transmembrane domain and multiple C2 domains. Of these, only the E-Syts are like the synaptotagmins in having an N-terminal transmembrane domain and most closely resemble the Tricalbins, which are implicated in receptor endocytosis in yeast (Creutz et al., 2004). We found that E-Syt2 was essential for FGF signaling during early *Xenopus* development. Antisense depletion of E-Syt2 inhibited induction of the early mesodermal marker Xbra by FGF and this inhibition could be rescued by

VRas, suggesting that E-Syt2 was required upstream of Ras. Depletion of E-Syt2 prevented a rapid phase of FGF receptor endocytosis and the temporally linked activation of ERK. E-Syt2 specifically recognized activated FGFR1 on the plasma membrane and also interacted with the Adaptin-2 (AP-2) complex. Though recognition depended on receptor catalytic activity, it was independent of key receptor autophosphorylation events and hence of signal transduction, suggesting the interaction with E-Syt2 was one of the earliest events following receptor activation. E-Syt2, to our knowledge, presents the first example of an endocytic adaptor able to selectively recognize the activated FGF receptor prior to signal transduction and to functionally link it with the AP-2/clathrin pathway.

The data suggest that E-Syt2 either links the activated receptor to AP-2 and clathrin or catalyzes this interaction by presenting and handing-off the receptor to AP-2, a mechanism similar to that suggested to explain Syt-1's ability to catalyze interactions between AP-2 and cargo proteins (Haucke and De Camilli, 1999). E-Syt2 might then stimulate the interaction of the activated receptor with nascent clathrin-coated pits or catalyze the de novo formation of pits around activated receptors (Figure 7E). Given that Syt-1 has been found to both stimulate and regulate the de novo formation of clathrin-coated pits (von Poser et al., 2000), it is tempting to suggest that the latter may in fact be the case.

To our knowledge, E-Syt2 is also the first endocytic adaptor to be identified for the FGF pathway. Several other membrane proteins have been implicated in mesoderm induction by FGF. Sef (Furthauer et al., 2002; Tsang et al., 2002) and xFLRT3 (Bottcher et al., 2004) are both transmembrane proteins shown to interact directly with the FGF receptor and to modulate FGF signaling, see (Bottcher and Niehrs, 2005). Further, in cell culture N-CAM (Cavallaro et al., 2001) and N-cadherin (Suyama et al., 2002) have been shown to modulate FGF signaling. However, unlike E-Syt2 none of these proteins show homologies with potential components of the endocytic machinery nor do they display obvious endocytic activity. Further, their actions have been suggested to be tissue specific. The broad tissue and temporal expression profile of E-Syt2 in *Xenopus* along with its requirement for signaling in vivo suggest it is an essential component of the FGF signaling pathway.

The requirement for receptor endocytosis in FGF signaling has been the subject of some controversy. Some studies argue that membrane localization of activated FGFR1 results in higher MAPK activation (Dammai et al., 2003; Suyama et al., 2002), while others have found that endocytosis or trafficking are required for full FGFR1 signaling (Bryant et al., 2005; Reilly and Maher, 2001; Vecchione et al., 2007). Further, FGF receptor endocytosis is believed to occur via both clathrin and caveolin-dependent pathways (Belleudi et al., 2007; Bryant et al., 2005; Wiedlocha and Sorensen, 2004). Here, we show that inhibition of FGFR1 endocytosis, by loss or gain of E-Syt2 function, by dominant-negative dynamin expression or chlorpromazine inhibition, suppresses mesoderm induction, Xbra expression, and ERK activation. Thus, our data demonstrate the necessity of endocytosis via the clathrin pathway for functional FGF signaling in vivo, and as such parallel the findings for the EGF receptor (Vieira et al., 1996). Further, we demonstrate that rapid FGFR endocytosis is essential for functional signaling and ERK activation during *Xenopus* embryogenesis.

Since the synaptotagmins function both in endo- and exocytosis (Sudhof, 2004), it is tempting to speculate that E-Syt2 could also be implicated in the exocytosis and/or the recycling of the FGFR1, a possibility we are currently pursuing. In this context, it is worth noting that other unrelated C2 domain proteins have been identified as important in cell trafficking events (Gallagher and Knoblich, 2006; Jaekel and Klein, 2006) and for cell adhesion (Pilot et al., 2006).

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Full-length E-Syt2 cDNA was amplified from an I.M.A.G.E. clone 5543078 (GenBank NM_001087117). All the mutants or epitope-tagged constructs were created by PCR from the original I.M.A.G.E. clone and subcloned in pT7Ts-HA (P. Krieg, modified) and pCDNA3. A short cDNA encoding human E-Syt2b was obtained from Kazusa DNA Research Institute (KIAA1228) and the missing 5′ sequences obtained by PCR amplification from HEK293T cells. Sequencing showed the product to be identical to GenBank NM_020728. It was then subcloned into pT7Ts-HA and pCDNA3. *Xenopus* FGFR1 and CA-FGFR1 (K562E) were from R. E. Friesel and C. Niehrs, and FLAG-β_2-adaptin from S. Laporte. Wherever the source of E-Syt2 and FGFR1 is not specified the *Xenopus* forms were used. The position of epitope tags is indicated as N- or C-terminal by prefixing or suffixing the epitope indicator.

Morpholinos and Antibodies

Five Morpholino sequences against the *Xenopus* E-Syt2 cDNAs were made, two complementary to the sequence downstream from the ATG start codon referred to as A and A-like and two complementary to the adjacent 5′ UTR sequence referred to as B and B-like, and one complementary to a 5′ UTR sequence further 5′ referred to as C: A; TCTCTGCGCTGCTCTCGGAAGACAT, A-like; 5′-TCTCAGCGCCGCTCTCGGAAGCCAT, B; 5′-GTGAATTAACTGCG ACAGAGAGAGA, B-like; 5′-GTGAATTGACTGCGCCTGAGAGAGA, C; 5′-CT CTTCCAGCCCTGCCTCAGCCCAA. A 5 mismatch Morpholino was used as control; 5′-GTCAATTAAGTGCCACACAGACAGA. Of these five Morpholinos only the B and C Morpholinos downregulated E-Syt2 protein levels and had significant biological effects. The rabbit polyclonal antibody xE-Syt2#2 was generated from a bacterially expressed protein containing aa 97–180 of *Xenopus* E-Syt2. The rabbit polyclonal antibody hE-Syt2#Pr0863-2932r was generated and affinity-purified against aa 802–822 of hE-Syt2b (NM_020728) (21st Century Biochemicals).

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed on albino or pigmented *Xenopus laevis* (Nasco) as described (Harland, 1991). The probe used for E-Syt2 was to nucleotides 2506–2765 (NM_001087117).

Embryo Manipulation, Injection, and Explants

Embryos were staged using the Nieuwkoop and Faber tables (Nieuwkoop and Faber, 1967). *Xenopus* embryos in 2% Ficoll (GE HealthCare) in 0.5× MMR were injected with either mRNA or antisense Morpholino oligonucleotides at the two or four cell stage (AC explants). mRNA was produced using the mMessage kit (Ambion). The amount of Morpholino or mRNA injected was as indicated. Animal Cap (AC) explants were removed at stage 8–9 using forceps on agarose coated dishes in 1 × Barth's medium and cultured in 0.5 × Barth's medium in parallel with control. Ventral marginal zone (VMZ) explants were isolated at stage 10.5 using an eyebrow knife on agarose coated dishes in 1 × Barth's medium. bFGF was purchased from Sigma and Activin A from R&D.

Gene Expression Analysis by RT-PCR

Total RNA was prepared using Trizol (Invitrogen). RT-PCR was performed using the primers, RT-PCR protocol, and PCR cycles standardize by the

Xenopus research community to be within the linear range of amplification, see Xenbase (http://www.xenbase.org).

Pull-Down and Phospholipid Binding Assays

Pull-downs were carried out essentially as in Vadlamudi et al. (2002). Phospholipid binding assays were performed on PIP Strips (Echelon) essentially as in Schulz and Creutz (2004).

Cell Culture, Transfection, and Inhibitors

HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Wisent). 1.25 × 10^6 293T cells were seeded on poly-L-lysine (1 mg/ml) (Sigma) treated 60 mm Petri dishes 24 hr prior to transfection. Transfections were performed by calcium phosphate precipitation as described (Gingras et al., 2002), for 7 hr in the presence of 25 μM chloroquine (Sigma) using 8 μg of DNA. For cotransfections the ratio of DNA was 1:1 unless otherwise stated. Where indicated, cells were treated for 20 min with 20 ng/ml of bFGF (Invitrogen) or EGF (Invitrogen), for 16 hr with 25 μM SU5402 (EMD/Merck) or 70 min with 30 nM tyrphostin (AG 1478, Sigma), or for 1 hr either with chlorpromazine (Sigma), 10 μg/ml, or with nystatin (Sigma), 10 μg/ml. For AC experiments, the inhibitors were added immediately after AC isolation.

Coimmunoprecipitation

HEK293T cells were processed for coimmunoprecipitation 24 hr posttransfection. Cells were washed twice with ice-cold PBS and lysed in 300 μl (per 60 mm dishes) of coimmunoprecipitation buffer, essentially as (Bokoch et al., 1996). For Western blotting, antibodies were used at 1/5000 (HA), 1/1000 (Myc, Cell Signaling; E-Syt2), 1/400 (FLAG, Sigma), 1/5000 (Phospho-Erk, Sigma; β_2-Adaptin, BD Biosciences) and 1/3000 (GST, Sigma) and 1/10,000 (anti-phosphotyrosine PY99, Santa Cruz Biotechnology).

Imaging of Proteins in *Xenopus*

Embryos expressing fluorescent proteins were dissected as necessary and fixed (3.7% formaldehyde, 1 mM $MgCl_2$ in PBS) for 20 min at room temperature. Explants were washed four times in PBS before mounting in 1:1 glycine buffer: glycerol (glycine buffer; 0.2 M glycine, 0.3 M NaCl, 0.175 M NaOH, 15 mM NaN_3). For immunofluorescence in ACs, embryos were fixed in MEMFA for 1 hr, dehydrated in methanol, and permeabilized for 2 hr in Dent's fixative. ACs were rehydrated in PBS and blocked with 10% goat serum and 2% BSA (Sigma) for 2 hr at room temperature. Both primary and secondary antibody incubations were performed overnight at 4°C on a Nutator. Five washes of 1 hr each were performed at room temperature after each antibody incubation. Images were obtained on an UltraView spinning disk microscope (Perkin-Elmer).

Biotin Labeling, Receptor Uptake Assays, and Immunofluorescence

For receptor uptake assays in ACs, four cell stage embryos were injected with mRNA encoding Myc-xFGFR1 with E-Syt2 or control Morpholino. ACs were removed at stage 9 and incubated for 1 hr with Myc antibody in 0.3 × Barth's, washed twice in 0.3 × Barth's, and incubated for different times in 0.3 × Barth's plus 20 ng/ml bFGF (Sigma) and 5 μg/ml of heparin (Sigma). They were then washed twice in PBS plus $MgCl_2$ (1 mM) and fixed in 4% paraformaldehyde for 20 min at 37°C. ACs were permeabilized in 0.2% Triton X-100 (Sigma), blocked with 10% goat serum (Wisent) in PBS-$MgCl_2$ for 1 hr at room temperature , and incubated with α-rabbit Alexa-488 (1/250, Invitrogen) for 1 hr before mounting (1:1 glycine buffer: glycerol). 3D images stacks were obtained on an UltraView spinning disk microscope (Perkin-Elmer) and analyzed using Volocity (Perkin-Elmer). FGFR1 positive vesicles at least 2 μm from the plasma membrane were counted for each cell throughout 15 optical sections (7.5 μm).

Biotin labeling was performed as in Lee et al. (2006), and in-cell receptor uptake assay as in Wernick et al. (2005) with the difference that a Myc antibody (Abcam, ab9106) was used at a concentration of 5 μg/ml. 293T cells were exposed to 20 ng/ml of bFGF and 5 μg/ml of heparin (Sigma) at 37°C for different times. Receptor colocalization assays with endosomal markers were performed essentially as for uptake assays. However, the second antibody labeling after cell fixation was performed in two steps. Mouse anti-FLAG antibody bound plasma membrane (external) FGFR1 was visualized (and saturated) before cell permeabilization by incubation with an Alexa405-conjugated goat anti-mouse antibody. After, permeabilization with Triton X-100, mouse anti-FLAG antibody bound to endocytosed (internalized) FGFR1 was detected with an Alexa488-conjugated goat anti-mouse antibody. EEA1 and Rab7 were labeled with first antibody (respectively C45B10 and D95F2, Cell Signaling) immediately after cell permeabilization and revealed with Alexa568 conjugated goat anti-rabbit antibody in parallel with detection of endocytosed FGFR1. 3D colocalization of endocytosed FGFR1 with EEA1 or Rab7 was determined on the three-color confocal image stacks using Volocity software (Perkin-Elmer).

For transferrin uptake assays 293T cells were processed as for an antibody uptake assay. Transferrin-Alexa-568 20 μg/ml (Invitrogen) was added with the bFGF and heparin and cells were incubated at 37°C for 20 min. Immunofluorescences were carried out as described above with the difference that an α-mouse Alexa-405 (Invitrogen) was used as secondary antibody and imaging was performed on a FV1000 confocal microscope (Olympus).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at doi:10.1016/j.devcel.2010.08.007.

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