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FGF stimulation leads to tyrosine phosphorylation of the protein SNT-1, which binds to activated FGF receptors. The objective of this work was to determine if SNT-1 protein had a central role in transducing the signals that lead to FGF-mediated antiestrogen resistant growth. We expressed an SNT-1 PTB domain in MCF-7 cells using a modified tetracycline inducible expression system. Upon induction of the PTB domain, we observed a significant decrease of FGF-dependent tyrosine phosphorylation of wild type SNT-1, strong inhibition of complex formation between SNT-1, Gab-1 and Sos-1, blocked activation of Ras as well as phosphorylation of MAP kinase indicating that the SNT-1 PTB domain was functioning in a dominant negative manner. Under the same conditions we did not observe a decrease in phosphorylation of AKT and Thr 389 of p70S6K, components of PI-3 kinase pathway. FGF dependent colony formation of MCF-7 cells in media containing the antiestrogen ICI 182,780 was inhibited upon induction of SNT-1 PTB domain. We conclude that SNT-1 plays a major role in the FGF-dependent activation of Ras/MAPK pathway and proliferation of MCF-7 cells and that overexpression of SNT-1 PTB domain can block these responses. We also suggest some SNT-1 independent mechanisms of PI-3K activation by FGF.

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Introduction

Various molecular mechanisms are responsible for tumor cells acquiring the ability to grow in the presence of tamoxifen, an antiestrogen effectively used as adjuvant therapy for women with estrogen receptor positive (ER+) breast cancer. One such mechanism is the ability of growth factor signaling to confer estrogen independent and antiestrogen resistant phenotypes to ER+ breast cancer cell lines. Evidence for a possible role of FGF signaling in estrogen-independent growth of breast tumors is attained through the study of clonal and polyclonal FGF-transfected MCF-7 cell lines, which are capable of forming large, progressively growing tumors in ovariectomized or tamoxifen-treated nude mice. Moreover, FGF-transfected MCF-7 cells are metastatic, forming micrometastases in lymph nodes, lungs and other organs [1 2 3]. In addition to being resistant to tamoxifen, the FGF-transfected cell lines are cross-resistant to the antiestrogen ICI 182,780 (Faslodex) and two aromatase inhibitors [4]. These results suggest FGF signaling may represent a general mechanism allowing ER+ breast cancer cells to bypass a requirement of ER activation for growth.

The RAS/MAPK pathway plays an important role in signaling via FGF receptors. Published work from other laboratories has indicated that a lipid-anchored docking protein called SNT-1 links FGFR molecules with the Ras/MAP kinase signaling pathway [5]. It was demonstrated that FRS2 protein, a mouse analog of SNT-1 protein, also plays a role in FGF-induced phosphatidylinositol-3(PI-3) kinase activation [6]. Targeted disruption of the FRS2 gene caused severe impairment in mouse development resulting in embryonic lethality. Mouse embryo fibroblasts deficient in FRS2α demonstrate impaired MAPK stimulation, PI-3 kinase activity, cell proliferation and cell migration. These results demonstrate the critical role of the FRS2/SNT-1 protein in FGF receptor-mediated signal transduction pathways [6]. SNT-1 protein contains a phosphotyrosine binding (PTB) domain at its amino terminus. This PTB domain binds to a juxtamembrane region of FGFR1 [7]. Several proteins, including Grb2, Sos, Shp2, Crk, Gab1 bind to SNTs following FGF induced SNT phosphorylation [8 9]. Some cellular fractions of atypical protein kinase C are able to bind SNT-1 regardless of its phosphorylation [10]. Because FGF is a growth factor that may have paracrine as well as autocrine effects within a tumor, including effects on angiogenesis, interference with this binding may also block the growth promotion that occurs in endothelial cells or other stromal elements.

Blocking FGF signaling at the level of the adaptor protein SNT-1, which is important for the recruitment of a multiprotein complex to the activated FGF receptor, could inhibit growth of antiestrogen resistant breast cancer cells. SNT-1 appears to be the critical link in multiple signaling pathways, many of which may be implicated in the variety of cellular responses seen in cancer cells. In this proposal, we intended to validate SNT-1 as a useful target for drug development (objective 1), determine if all four FGF receptors can interact with SNT-1 through homologous regions (objective 2), and determine a crystal structure of the SNT-1 PTB domain (objective 3).

BODY

Chemicals, growth factors and immunological reagents. $p13^{Suc1}$ agarose, anti-FRS2/SNT-1, anti-Gab1, anti-Sos1, anti-phospho-Shc(Y317), anti-Shc, anti-Grb2 (clone 3F2), anti-phosphotyrosine (clone 4G10) antibodies and Ras activation kit were obtained from Upstate Biotechnology, anti-FRS2 from Santa Cruz Biotechnology, c-myc antibodies used for immunoblotting–from Oncogene Research Products, c-myc antibodies used for fluorescent microscopy–from Invitrogen, Phospho-Raf (Ser338) antobody, PhosphoPlus AKT (Ser473) antibody kit, PhosphoPlus Map kinase (Thr202/Tyr204) antobidy kit from Cell Signaling, PhosphoPlus p70S6 kinase antobody kits (Thr 389, Ser411, Thr421/Ser424) from New England BioLabs, Texas Red dye-conjugated AffiniPure Donkey Anti-Mouse IgG and normal goat serum from Jackson ImmunoResearch Laboratories, Protein A Sepharose from Pharmacia Biotech. Recombinant human FGF-1 was obtained from Promega, heregulin (HRG-β1) - from R&D Systems. Cell lysis buffer was obtained from New England BioLabs. ICI 182780 (Faslodex®) was a gift from AstraZeneca or was obtained from Tocris and was dissolved in 70% EtOH prior to use. 17β–estradiol and heparin were from Sigma.

Plasmids and expression constructs.
GFP-MAPK plasmid [11] was obtained from Dr. Eisuke Nishida (Kyoto University, Japan). Vector pEF6/MycHisA was purchased from Invitrogen. Methods of isolation RNA, cells. PTB domains of SNT-1 (Accession number AF036717) and SNT-2 (Accession number AF036718 were obtained from ML20 cells by RT PCR. Total RNA was isolated from one 100 mm dish using the phenol-chloroform [12]. The PCR fragment of the PTB domain was amplified using the following primers:
sense primer 5'-AAGCTTGCCATGGGTAGCTGTTGTAGC-3',
antisence primer 5'- CTCGAGTCCTGGAGTTGTAGGTGT-3'.
Cloning sites HindIII and XhoI are underlined in the primers.
Primers for full length SNT-1: sense primer was the same as used for PTB domain RT PCR, antisense primer: 5'- GAATTCTCACATGGGCAGATCAGTACTA-3'. The following primers were used to amplify SNT-2 PTB domain using the total RNA isolated from ML20 cells and placenta cells:
sense primer 5'-GGATCCGACACCATGGGGAGCTGCTGCAGC-3',
antisence primer 5'-GAATTCCTACAGAGGCAGGTCGGTGCT-3'.
Primers were engineered with the sites BamHI and EcoRI. PR PCR was performed using access RT-PCR System from Promega.

Cell lines and cell culture. The breast cancer cell line ML-20 is an MCF-7-derived cell line that is stably transfected with a LacZ expression vector [13]. Cells were maintained in Phenol Red Free (PRF) and phenol red containing Improved Modified Eagle's Medium (IMEM) (Mediatech Cellgro) supplemented with 5-10% fetal bovine serum (FBS, GibcoBRL was obtained from Invitrogen. In some experiments charcoal-stripped calf serum (CCS) was used to strip cells of endogenous estrogens. Calf serum was treated with dextran-coated activated charcoal [1,4] to obtain CCS.

Transient and stable transfection strategies: Transient transfection assays were utilized to test whether SNT-1 PTB domain can block the phosphorylation of GFP-ERK2 fusion protein. 2.5 X

10^5 ML20 cells were plated in 60-mm tissue culture dishes in 5% FBS IMEM. They were transfected the next day using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Cells were cotransfected with 0.5 μg of GFP-ERK2 plasmid and 2 μg of pEF6/PTB plasmid or the control pEF6 vector. Transfected cells were incubated for 48 hours in 5% FBS IMEM to allow for gene expression. The transfected cells were then stripped of estrogen and incubated in the presence of growth factors for 30 minutes. Lysates (30 μg) were then analysed by Western Blotting with PhosphoPlus MAP kinase antibodies.

To obtain clonal and polyclonal populations of ML20 cells constitutively expressing SNT-1 PTB domain, ML20 cells were seeded in 100-mm tissue culture dishes in 5% FBS IMEM. Cells were transfected the next day in serum-free IMEM medium with 5 μg of pEF6/PTB plasmid or control vector pEF6. Lipofectamine Plus reagent was used according to the manufacturer's instructions. Cells expressing PTB domain were selected in the presence of 3 μg/ml of blasticidin (Invitrogen). Separate colonies and total pools were further expanded in the presence of blasticidin and clones expressing high levels of SNT-1 PTB domain were selected by c-myc immunofluorescence. Stable transfection experiments designed to obtain doxycycline-regulatable expression of SNT-1 PTB domain will be described in the Results section.

Growth factor stimulation. ML20 cells were stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM over 8 hours and incubated overnight in serum free CCS-PRF-IMEM. Cells transfected with doxycycline inducible PTB domain were induced with 200 ng/ml of doxycycline (Dox) for 24-48 hours. During process of stripping Dox remained present in the media. Cells were stimulated with 20 ng/ml of FGF-1 in the presence of 50 μg/ml of heparin or with 10 μg/ml of Heregulin β-1 for 15-30 minutes prior to lysis. Cells were washed once in ice-cold PBS and lysed using lysis buffer (Cell Signaling) supplemented with 1 mM phenylmethylsulfonyl fluoride. The protein concentrations in the cellular lysates were determined by the bicinchoninic acid method (Pierce).

$p13^{Sucl}$ agarose precipitaton. $p13^{Sucl}$ agarose was used to precipitate endogenous SNT-1 protein from cellular lysates. 30 μl of 50% gel slurry was incubated with 1-2 mg of protein from total cellular lysate for 18 hours at 4°C. The precipitates were washed three times in lysis buffer, boiled for 5 minutes in 2X Laemmli sample buffer before SDS-polyacrylamide electrophoresis in 7% gels then subjected to immunoblotting with different antibodies.

Western immunoblotting. Western immunoblotting was performed on cellular lysates. A portion of cellular lysate was run on Criterion® pre-cast 10% SDS-PAGE gels (Bio-Rad). Proteins were transferred onto Protran® filters (Bio-Rad) by electroblotting, probed with antibodies. Enhanced chemiluminescence detection was performed using the Supersignal kit (Pierce).

Active Ras pulldown assays were performed using Ras Activation Assay Kit (Upstate Biotechnology). ML 20 cells expressing SNT-1 PTB domain in Dox-regulatable manner were stripped of estrogen, induced by 200 ng/ml of Dox and stimulated by FGF-1 and Heregulin β1 for 5 minutes. 500 μg of cellular lysate proteins were incubated with 10 μg of Raf-1 RBD agarose at 4°C for 30 min , washed 3 times with lysis buffer, boiled 5 minutes in 2X Laemmli

sample buffer before SDS-polyacrylamide electrophoresis in 12% gels followed by immunoblotting with anti-Ras antibodies.

Colony forming tests. Cells were plated in 5% PRF-FBS-IMEM and were allowed to attach overnight. The expression of PTB domain was induced by 100 ng/ml doxycycline in 5% PRF-FBS-IMEM with heparin at 50 μg and the following additions: ICI 182,780 at 10^{-7} M, FGF-1 at 20 ng/ml, heregulin β-1 at 10 μg/ml, 17β–estradiol at 10^{-7M}. Media were changed every 48 hours for a period of 3 weeks. Colonies were fixed with formalin and stained with 0.05% crystal violet and counted.

Task 1. Validate SNT-1 as a target for drug development by demonstrating that a truncated protein consisting of an N terminal myristylation signal and PTB domain can function as a dominant negative inhibitor of FGF signaling in ER+ breast cancer cells.

Cloning SNT-1 PTB domain. 140 N-terminal aminoacids of SNT-1, containing a myristilation domain responsible for membrane targeting and a PTB domain involved in the interaction with juxtamembrane domain of FGF receptor, are deleted of the docking sites that are tyrosine phosphorylated in response to FGF. We expected that this fragment of SNT-1 would function as a dominant negative molecule preventing endogenous SNT-1 from the binding FGF receptor and from subsequent phosphorylation of SNT-1 multiple docking sites, and thus inhibit FGF signaling. In the first year of the project we encountered difficulties involving the use of the vector pCMVTag form Stratagene for the expression of the PTB domain of SNT-1. Therefore, we employed a different expression system. We have used RT PCR with RNA from MCF-7-derived ML20 breast cancer cell line to place the N-terminal 140 aminoacids of SNT-1 in vector pEF6 under the control of EF1α promoter and in frame with c-myc epitope that is upstream of the termination codon (pEF6/PTB).

Constitutive expression of SNT-1 PTB domain in MCF-7 breast cancer cell line. The breast cancer cell line ML20 was stably transfected with the plasmid pEF6/PTB or with the empty pEF6 vector, which confers resistance to blasticidin. Stably transfected clonal and polyclonal populations were selected and expanded in the presence of 3 μg/ml of blasticidin. Selected clones and polyclonal population of the transfected cells were assayed for expression of SNT-1 PTB domain by immunofluorescence. Several clones expressing high constitutive levels of c-myc tagged SNT-1 PTB domain upon FGF induction were subjected to Western blot with c-myc antibody to detect the levels of protein expression (Fig. 1A). Clones 2/1 and 5 were selected for future studies.

Expression of SNT-1 PTB domain in the vector pEF6 significantly inhibits tyrosine phosphorylation of endogenous SNT-1 in FGF dependent conditions. We expected that abrogation of the binding of endogenous SNT-1 to the juxtamembrane domain of FGF receptor would reduce tyrosine phosphorylation of wild type SNT-1 protein in response to FGF stimulation. To examine this possibility, we precipitated endogenous SNT-1 with p13suc1 agarose from total lysates of cells expressing the PTB domain of SNT-1. Cells were stripped in CCS containing medium and serum-starved overnight for estrogen deprivation. The media then was supplemented by FGF-1. As it is shown at Fig. 1B, FGF stimulation caused tyrosine

phosphorylation of endogenous SNT-1 in cells transfected with the vector pEF6 alone. Tyrosine phosphorylation of wild-type SNT-1 was significantly reduced in clones 2/1 and 5, which express high levels of PTB domain compared to untransfected cells or to the cells containing only vector pEF6. Since polyclonal populations transfected by pEF6/PTB plasmid did not show as high and homogenous c-myc staining in immunofluorescence experiments compared to individual clones, we did not observe a significant inhibition of SNT-1 tyrosine phosphorylation in the polyclonal population upon FGF treatment.

Effect of SNT-1 PTB domain constitutive expression in the vector pEF6 on ERK and AKT phosphorylation upon FGF induction. The MAPK cascade transduces signal from FGF receptor tyrosine kinase through Ras to sequential activation of Raf serine/threonine kinases, MEK, and the MAP kinases ERK1 and ERK2. To determine if abrogation of SNT-1 tyrosine phosphorylation would unlink FGFR activation from activation of MAP kinase, we determined the levels of MAPK phosphorylation in total cellular lysates of cells stably transfected with pEF6/PTB plasmid (Fig.1). Surprisingly, we did not observe a significant change of MAPK phosphorylation in the total lysates of the cells expressing SNT-1 PTB domain upon FGF induction in estrogen depleted conditions. Next, we examined the possibility of abrogation of another pathway important for FGF signaling, the PI3-kinase dependent pathway. For this purpose, we determined if SNT-1 PTB domain overexpression would cause inhibited phosphorylation of AKT (PKB), a component of PI3 kinase pathway (Fig.1). AKT phosphorylation remained intact upon FGF stimulation in the estrogen depleted conditions in the cells expressing SNT-1 PTB domain as compared to untransfected cells or to the cells transfected with vector alone, although it should be pointed out that FGF exposure did not cause significant levels of AKT phosphorylation in the control cells. To confirm the absence of a physiological effect of SNT-1 PTB domain in these cells, we utilized a colony forming test. The increase in blasticidin resistant colony formation observed with FGF treatment in media containing ICI 182,780 was not altered by PTB domain expression (data not shown). To determine whether cells, constitutively expressing SNT-1 PTB domain were utilizing some compensatory mechanisms which allowed them to bypass a requirement for SNT-1 protein were selected during expansion of the clonal and polyclonal populations stably expressing SNT-1 PTB domain, we studied the ability of the SNT-1 PTB domain to inhibit MAP kinase activation in MCF-7 cells transiently cotransfected with GFP-ERK reporter and the pEF6/PTB plasmid. Differences in size between GFP-ERK fusion protein (80 kD) and endogenous ERK1 and ERK2 (42 and 44 kD) allowed observation of the phosphorylation of GPF-ERK transiently expressed in these cells. 48 hours after transfection cells were depleted of estrogen and MAPK phosphorylation was induced by incubation with FGF-1 for 30 minutes. The levels of MAPK were greatly reduced in the cells transiently expressing PTB domain compared to the control transfected with the vector alone (Fig. 2). These data confirmed that expression of SNT-1 PTB domain in breast cancer cell line MCF-7 leads to abrogation of wild type SNT-1 phosphorylation in response to FGF treatment, followed by significant diminution of MAPK activation. Based on these data we conclude that SNT-1 PTB domain can function as a dominant negative molecule in breast cancer cells in FGF dependent conditions and that evidently there are some SNT-1 independent mechanisms involved in FGF-1 signaling.

Doxycycline(Dox)-inducible expression of SNT-1 PTB domain. To overcome the selection for cells with an increased utilization of compensatory mechanisms that may bypass a

requirement for a functional SNT-1 protein when the PTB domain is constitutively overexpressed, we utilized a tet-on inducible expression system that allowed us to achieve high levels of SNT-1 PTB domain protein expression in the presence of doxycycline with no detectable basal expression in its absence. We used a recently described tetracycline-inducible system that uses the synthetic reverse tet transactivator rtT2S-M2 [14]. A stable ML20 cell line transfected with CMV-driven rtT2S-M2, called C9, was first isolated. This line was retransfected with a TRE-reporter vector into which SNT-1 PTB domain was subcloned as a c-myc-tagged molecule. The TRE-PTB module was flanked by 2 double copies of a 250 bp 'insulator' from the chicken β-globin 5' hypersensitive site (manuscript in preparation). This insulator has been shown to protect transgenes from being silenced and also to protect these genes from nearby enhancers. The polyclonal population and several clones were isolated, and a line called C9 TRE-ins PTB-c-myc was established (TRE-Ins/PTB). First, we demonstrated by immunofluorescent staining with an anti c-myc antibody that PTB domain was induced with 48 hours Dox treatment (200 ng/ml) in clones 29 and 13 (data not shown), with almost undetectable expression in uninduced cells. Polyclonal populations showed mosaic expression of this protein. High levels of PTB domain expression in clones 29 and 13 were confirmed by Western blot with c-myc antibodies (Fig 3).

Dox-induced SNT-1 PTB domain expression abrogates phosphorylation of endogenous SNT-1 and its interaction with Gab1 and Sos1. To determine the dominant negative function of Dox inducible PTB domain expressed in the stably transfected ML 20 cell line, we studied levels of wild type SNT-1 tyrosine phosphorylation in $p13^{Suc1}$ captured fraction of the cellular lysate of Clone 29. Cells were stripped for estrogen deprivation in CCS containing medium and serum-starved overnight in the presence or in the absence of Dox. FGF supplementation of these media caused tyrosine phosphorylation of endogenous SNT-1 in cells transfected with vector pTRE-Ins alone. Induction of PTB domain expression with Dox led to significant decrease of SNT-1 phosphorylation in Clone 29 cells (Fig. 4).

In response to FGF stimulation, several proteins including Grb1/Sos and Gab1/Shp2 are recruited to the C-terminal region of the docking protein SNT-1 following its phosphorylation at multiple tyrosine residues leading to activation of the Ras/MAPK, PI-3 kinase and other signaling pathways. To further explore the dominant negative effect of SNT-1 PTB domain in FGF signaling pathways, we determined the ability of endogenous SNT-1 to form a complex with Gab1 and Sos1 proteins upon FGF treatment in cells where expression of PTB domain is induced with Dox treatment. Endogenous SNT-1 was precipitated by $p13^{Suc1}$ agarose from estrogen depleted and FGF-1 stimulated cellular lysates in the presence and in the absence of Dox. The presence of Gab1 and Sos1 proteins in the $p13^{Suc1}$ captured material was determined by Western blot. As shows in Fig. 4, the involvement of Gab1 and Sos1 in the multiprotein complex with SNT-1 in FGF dependent conditions was significantly decreased upon Dox induction of PTB domain expression. Abrogation of the recruitment of multiple proteins to the C-terminus of wild type SNT-1 confirmed the dominant negative action of PTB domain overexpression in MCF-7 cells.

SNT-1 PTB domain inhibits activation of Ras and MAP kinase by FGF-1. To determine whether abrogation of engagement of the multiple proteins in the complex with SNT-1 would subsequently abrogate the transfer of FGF-generated signal to its downstream targets, MAP

kinase and AKT, we further examined activation of these major signaling pathways involved in FGF signaling.

Formation of the complex of SNT-1 with proteins Grb2 and Sos1 is considered an initial step in the activation of the Ras/Raf/MAPK signaling pathway [15 5]. Based SNT-1 PTB domain inhibition of Sos1 protein involvement in the complex with endogenous SNT-1 upon FGF induction, we expected that activation of Ras by FGF-1 will also be prevented when the PTB domain is overexpressed. Indeed, in the lysates of cells stably transfected with pTRE Ins/PTB plasmid (Clone 13 and Clone 29) we observed a significant inhibition of Ras binding to the Ras-binding domain of Raf-1 upon induction of PTB domain synthesis by Dox and stimulation of cellular proliferation by FGF-1 (Fig. 5). Since SNT-1 was not shown to be able to interact with erb2-erb3 receptor(s), it was not expected to be involved in regulation of the heregulin β-1 induced signaling pathway [16,17]. In the same conditions in cells transfected with vector pTRE Ins alone Ras activation remained uninfluenced by Dox treatment. Specificity of Ras inhibition for FGF-dependent conditions in these two clones was confirmed by the absence of a Ras inhibitory effect by Dox upon heregulin β-1 stimulation.

Next we determined MAPK phosphorylation by Western blotting of total lysates of cells stimulated with FGF, heregulin β-1 and 5% FBS upon induction of PTB domain expression with 200 ng/ml of Dox (Fig.6). Cells deprived of estrogen by repeated changes of CCS-PRF-IMEM and starved overnight in serum free PRF-IMEM were then exposed to growth factor-supplemented conditions in PRF-IMEM. Dox remained in the media upon estrogen deprivation. We found that Dox treatment at this concentration did not cause significant changes in the levels of MAPK activation in untransfected cells or in the cells transfected with vector alone. On the other hand, when Dox was added to Clone 29 and Clone 13 cells, phosphorylation of MAPK decreased to the levels observed in the absence of FGF-1. Exposure to heregulin β-1 caused much greater levels of MAPK phosphorylation than FGF treatment. These levels remained unchanged upon induction of PTB domain expression with Dox. Since MAPK activation represents a major step in FGF dependent proliferation of breast cancer cells, its significant inhibition in Clone 29 and Clone 13 cells in a FGF-dependent but not heregulin β-1-dependent conditions confirms FGF-specific dominant negative effect of SNT-1 PTB domain in MCF-7 cells.

Influence of SNT-1 PTB domain expression on the phosphorylation of AKT and p70S6K.

To further investigate potential dominant negative properties of SNT-1 PTB domain expression in the regulation of FGF-mediated proliferation of breast cancer cells, we studied the activity of different components of the PI3 kinase pathway. This signaling cascade is activated by multiple growth factors and results primarily in cell survival. In response to FGF stimulation of Swiss 3T3 cells, activation of PI3 kinase/AKT signaling pathway is mediated by binding of the SH2 domain of Grb2 to tyrosine phosphorylated SNT-1/FRS2a and by binding the SH3 domain of Grb2 to a proline-rich region of Met-binding domain of Gab1[18]. Since we demonstrated a significant inhibition of the involvement of Gab1 in SNT-1 associated multiprotein complex by SNT-1 PTB domain expression in MCF-7 cells, it would be reasonable to expect a dominant negative effect of this molecule in the PI3 kinase pathway. We studied the potential of the SNT-1 PTB domain to inhibit phophorylation of the PI3 kinase downstream effector protein, AKT (PKB). Our experiments did not show a significant induction of AKT

Ser/Thr phosphorylation upon stimulation with FGF-1 in estrogen depleted conditions over the basal levels in MCF-7 cells transfected with rtT2S-M2 (C9) or empty TRE-reporter vector (Fig. 7). We also did not observe any inhibition of AKT phosphorylation in cells stably expressing SNT-1 PTB domain upon Dox induction. Control treatment of the same cells in the same conditions with heregulin β-1 instead of FGF-1 resulted in a large increase in AKT phosphorylation levels which, as expected, were insensitive to the PTB domain expression. Most likely, our results reflect the differences in levels of FGF-dependent induction of AKT phosphorylation in different cells lines.

Another important mediator of the PI3 kinase pathway is the mitogen-stimulated serine/threonine kinase p70S6K, which modulates the translation of an mRNA subset which includes those that encode ribosomal proteins and translation elongation factors [19 20 21 22]. It signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD [23]. Activity of the cytoplasmic isoform p70S6K is regulated by phosphorylation of eight different residues distributed throughout the protein [24 25 26]. To further study the effect of SNT-1 PTB domain expression on different signaling pathways in MCF-7 cells, we investigated the influence of the PTB domain on phosphorylation patterns of p70S6 kinase in lysates of cells treated with FGF-1 or heregulin β–1 upon Dox induction of PTB domain synthesis. We studied the effects of SNT-1 PTB domain overexpression on phosphorylation of sites Thr 421/Ser 424 and Ser 411, located in the pseudosubstrate domain of p70S6K, and the site Thr389, which is located in the linker domain of p70S6K and is also critical for activity of the protein.

Results of Western blot analysis showed that phosphorylation of all these sites was induced by FGF-1 as well as heregulin β-1. In cells transfected with the vector pTREIns alone, this phosphorylation was not influenced by Dox (Fig.8). FGF-induced phosphorylation of sites Thr 421/Ser 424 and Ser 411 in cells stably expressing Dox-inducible SNT-1 PTB domain (Clone 13 and Clone 29) was decreased to background levels upon Dox induction, while heregulin β-1 induced phosphorylation of these sites was unchanged. Since the sites are targets of MAPK, the Dox dependent inhibition of their phosporylation is likely a direct effect of the reduced MAPK activity under these conditions. On the other hand, Thr 389, which is phosphorylated in response to PI-3 kinase activation, remained phosphorylated in these clones in the presence of FGF-1 and Dox as well as in the presence of heregulin β-1 and Dox. These data show that FGF-1 and heregulin β-1 can induce phosphorylation of the sites located both in the pseudosubstrate domain and in the linker site Thr 389. Expression of SNT-1 PTB domain in FGF-treated conditions selectively inhibited phosphorylation of Thr421/Thr424 and Ser 411 located in the pseudosubstrate domain of p70S6K leaving phosphorylation of the linker site Thr389 intact. All these sites remained phosphorylated in the control when the same cell lines were incubated in the presence of heregulin β-1 and Dox, which confirmed the specificity of the observed effects for the FGF signaling pathway. Mitogen induced activation of p70S6K requires multiple input including PI3-kinase- and mTOR-dependent activation which results in phosphorylation of the hydrophobic motif site Thr 389 as well as the activation loop site Thr229. We show that the PI3-kinase inhibitor LY294002, as well as Rapamycin, an inhibitor of mTOR, completely block the FGF-1 induced phsophorylation of Thr389 while this site still remained phosphorylated upon induction of the dominant negative SNT-1 mutant (Fig. 9). This suggests an alternative mechanism of activation in FGF stimulated cells that is independent of the activation of Gab1.

A possible mechanism for compensation of an inactive SNT-1 protein in FGF signaling could involve increased signaling through the ShcA adaptor protein [27], [28]. If this hypothesis is correct, the levels of protein ShcA phosphorylation would be expected to be increased in cells expressing the SNT-1 PTB domain to compensate for the absence of SNT-1 protein function in these cells. We induced these cells with 20 ng of FGF-1 in the serum free PRF-IMEM for 30, 60, and 180 min, and determined the levels of Y317 tyrosine phosphorylation using anti-phospho-SHC (Y317) antibodies. This experiment did show increased levels of ShcA phosphorylation in the clones stably transfected with the PTB domain of SNT-1 (Fig.10). To confirm involvement of protein ShcA in the FGF pathway, we intend to use dominant negative mutants to abrogate Shc signaling. We have recently received a HA tagged Shc Y239F, Y240F,Y317F triple mutant from Dr. K. Ravichandran that has a dominant negative function [29]. To determine its role in the FGF signaling pathway, we plan to cotransfect cells expressing the PTB domain of SNT-1 with this mutant and GFP-MAPK and check the possibility of further decreasing the residual MAPK phosphorylation that occurs in these cells upon FGF induction.

SNT-1 PTB domain exhibits a dominant negative effect on the anchorage-dependent growth of ML20 cells in FGF-dependent conditions. Previous studies showed that supplementation of the ICI 182,780 containing media with fibroblast growth factor can restore cell growth at different levels. These studies indicated that estrogen independence and antiestrogen resistant growh of ER+ breast cancer cells may be achieved through FGF signaling pathways in a manner that is likely to be independent of ER pathways. Here we employed a colony forming assay to study if the Dox inducible expression of SNT-1 PTB domain would be able to prevent FGF-dependent antiestrogen resistant growth of these cells (Fig. 11). We found that colony number and size were inhibited by Dox treatment under all growth conditions in both the TRE vector control and the TRE Ins/PTB transfected cells. However, the increase in the colony number and size that was observed in the pTRE Ins/PTB Clone 29 cells when FGF-1 was added to ICI 182,780 containing media was reduced by Dox treatment to a greater extent than that observed in either FGF treated vector control cells with Dox or the heregulin-treated pTRE Ins Clone 29 cells with Dox.

Overexpression of SNT-1 in breast cancer cells leads to increased FGF-dependent phosphorylation of MAP kinase. To investigate a potential role of SNT-1 in proliferation of ER+ breast cancer cells, we cloned a c-myc tagged full size SNT-1 in the vector pTRE-Ins to express it in a Dox-regulatable manner. We stably transfected ML20 Clone 18 cells, which express and secrete FGF-1 [3], with this construct. The polyclonal populations and several clones were isolated, and a cell line called pTRE-Ins/SNT-1 was established. We determined by immunoblotting with anti c-myc antibodies that SNT-1 was induced with 48 h Dox treatment (200 ng/ml) with undetectable expression in uninduced cells (Fig.12). To study the levels of MAPK phosphorylation in estrogen-independent conditions, cells were stripped of estrogen with three changes of 5% CCS-PRF-IMEM over 36 hours and incubated overnight in serum free PRF-IMEM medium. 50 μg of total cellular protein was loaded in each well for immunoblotting with phospho-MAPK and MAPK antibodies. We did not observe any changes in MAPK phosphorylation levels upon DOX-induction of the cells transfected with vector alone, while it was significantly increased in the clonal and polyclonal populations expressing SNT-1(Fig.13). Since proliferation of these cells in the absence of estrogen and in the presence of anriestrogen is

mediated mainly by induction of FGF-dependent pathway by overproduced FGF-1, these experiments suggest a potential role of SNT-1 in tumor progression.

Expression of SNT-1 shRNA inhibits activation of MAP kinase. To investigate if abrogation of SNT-1 expression would be critical for the FGF pathway in breast cancer cells and to study mechanisms of its action in the FGF signaling pathway, we employed the siRNA mediated gene silencing approach [30]. We have designed 21-nucleotide siRNA duplexes using Dharmacon siDesign Center and transiently transfected the ML20 cell linewith these oligonucleotides using Oligofectamine reagent (Invitrogen). Being aware of technical issues of the siRNA approach, including unspecific targeting of some proteins [31], we carefully examined the possibility of unspecific matches when designing the siRNA molecules. We also designed several siRNA molecules targeting the same gene. Levels of mRNA degradation were examined by real-time PCR. Sequences of the most efficient oligonucleotides #575 and #1463 (degrading 80% of SNT-1 mRNA), were used to design small hairpin (sh) oligonucleotides. These oligonucleotides were cloned into the shRNA expression vector pSuper provided by Dr. R. Agami [32]. These constructs were used to study FGF-induced phosphorylation of MAP kinase by transient cotransfection of ML20 cells with GFP-Erk1 plasmid obtained from Dr. E. Nishida (Kyoto University, Japan). Expression of both SNT-1 shRNAs caused significant inhibition of MAPK phosphorylation mediated by FGF-1 but not by heregulin beta-1 in estrogen depleted conditions (Fig.14). We will consider stable expression of shRNA in the analysis of the mechanism of Sprouty and Sef action in growth signaling pathways.

Task 2. Study of SNT-1-FGFR1 interaction in vivo: mammalian two-hybrid system.

To determine the minimal interacting region within the FGFR juxtamembrane region that is capable of interacting with the SNT-1 PTB domain and to create a system for screening of drugs inhibiting the interaction between SNT-1 and FGF receptors, we utilized a mammalian two-hybrid assay (Clontech). The PTB domain and juxtamembrane domain of FGFR1 each were cloned separately in the pBIND vector as fusion proteins with GAL4 DNA binding domain and the vector pACT as fusion proteins with the herpes simplex virus VP16 activation domain. ML20, Cos7 and HIN3T3 cells were transiently cotransfected with the newly constructed plasmids and the reporter plasmid pG5Luc which contains five GAL4 binding sites upstream of a minimal TATA box. As a result of these experiments we found that the PTB domain of SNT-1 and the juxtamembrane domein of FGFR1 can interact with each other but this interaction is very weak compared to two positive control interacting fusion proteins, Gal4-Id and MyoD-VP16 provided in the kit. Consequently, we have abandoned this approach. Furthermore, the need to define minimal interacting domain is now less pressing in light of the recent publication of Ong S.H. [33].

Task 3. Determine the crystal structure of the SNT-1 PTB domain alone and comlexed to peptides from the juxtamembrane regions of the four FGF receptors.

As we reported at the end of the second and third yeas, SNT-1 PTB domain (residues 11-140) was overexpressed in Escherichia coli, and recombinant protein was purified using an N-terminal 6His

Tag. Although the protein ran as a single band in SDS PAGE, the native gel electrophoresis showed smeared bands. The phosphotyrosine binding (PTB) domain of human SNT-1 contains 130 amino acids (11-140). Determination of the solution structure of the PTB domain indicated that residues in the C-terminus (117-140) could form a flexible loop structure. This may cause the protein to be less stable in solution and allow the protein to take multiple confirmations that prevent the PTB domain from crystallization. Residues from 110 to 116 form a β-strand (β8) that interact with polypeptides derived from FGFRs (Fig.15). Based on this hypothesis and in consideration of retaining the correct fold and possible interactions with polypeptide, we made two new DNA constructs by removing the flexible loop: one coding for protein from 11 to 116 (PTB1), and the other from 11 to 119 (PTB2). Removal of the loop may increase the protein stability and chances of protein crystallization. The two proteins were over expressed in *E. coli* cells and purified in large scale. The proteins have been tested for crystallization in this research project.

DNA cloning and Protein expression. DNA fragments of SNT1, PTB1 and PTB2 were amplified by PCR with one 5' primer and two 3' primers for PTB1 and PTB2:

5' NdeI (+): ACT TGA TTC CAT ATG GAC ACT GTC CCA GAT AAC CAT

3' BamHI (-) from PTB1: CTC CTC GGA TCC TTA TGG CTC TTC CAC CAC ATT TAT

3' BamHI (-) from PTB2: CTC CTC GGA TCC TTA TTC TAC AAC TGG CTC TTC CAC

These DNA fragments were then cloned into the expression vector pET28 with NdeI and BamHI restriction enzymes. pET28 contains a His_6 affinity tag that can be used for protein purification by coupling with Ni^{2+} ions of metal cheating agarose.

E. coli cells were transfected with the plasmid containing one of the DNA fragments and the cell clones were selected for insertions by antibiotics. Subsequent DNA sequencing was performed to confirm the correct DNA seqences. Large-scale cell cultures were then prepared to overexpress PTB1 or PTB2 protein. It was found that at 37° C most of the protein formed insoluble aggregates, known as inclusion bodies. The soluble portion of protein was increased significantly through optimization of expression conditions by lowering induction temperature and inducer concentration.

Protein purification and characterization. PTB proteins were purified by two different approaches. For protein expressed as inclusion bodies, the insoluble protein was separated from the cell crude extract by centrifugation and then denatured by either adding 4-6 M guanidine chloride or increasing the pH value of the protein solution. The protein was further purified by Ni^{2+} affinity chromatography with imidazole as the elutent. For the soluble protein, cell extract was directly loaded onto the Ni^{2+} affinity column and purified through a designed washing scheme and eluted by an imidazole gradient.

The purified protein samples were characterized by protein electrophoresis in native or denatured conditions. Under denatured conditions, protein migrated as a major band with a molecular weight of about 13 kDa (Fig.16) and several minor bands. However, the native protein gel showed smeared bands of both PTB1 and PTB2, either purified from soluble protein or from the denaturing-refolding process, (Fig.17), indicating that protein molecules were unstable. Many conditions were tested for protein stabilization, such as addition of DTT, glycerol, detergents and other reagents. No improvement in protein stability was observed after extensive effort. This result is similar to that of the original PTB

domain protein. It may be concluded that removal of the C-terminal flexible loop does not improve the protein stability.

To stabilize the protein in solution and to facilitate crystallization, four polypeptides derived from FGFRs have been synthesized. SNT1 incubated with one of these polypeptides showed some degree of increased stability. However, the native protein gel still showed smeared bands (Fig.17), indicating the conformation of protein molecule is heterogeneous.

Peptide	*Sequence*	*Receptor*
PS65	(411-430)-QMAVHKLAKSIPLRRQVTVS	FGFR-1
PS66	(412-431)-QPAVHKLTKRIPLRRQVTVS	FGFR-2
PS68	(408-427)-SPTVHKISRFPLKRQVSLES	FGFR-3
PS67	(403-422)-PATVQKLSRFPLARQFSLES	FGFR-4

Crystallization screening. We have screened several thousand conditions for crystallization of the PTB domain by the vapor diffusion sitting-drop method, both with and without the presence of the synthetic peptides. Currently, no crystals other than some micro-crystals have been observed under various conditions. The best lead condition was with PS66 peptide present in a solution containing 0.1 M tri-sodium citrate (pH 5.6), 35%(v/v) tert-butanol. However, expanded optimization screening experiments did not yield better crystals.

Key research accomplishments.

- The c-myc tagged PTB domain of SNT-1 protein was overexpressed in the MCF-7 breast cancer cell line in either a constitutive or inducible manner using vectors pEF6 and pTRE Ins, respectively. The vector pTRE Ins allowed inducible overexpression of the protein at very high levels and avoidance of the potential problems of adaptation to constitutive overexpression of SNT-1 PTB with compensatory mechanisms of FGF signaling.

- Dominant negative properties of the SNT-1 PTB domain were confirmed by several observations: an inhibition of the phosphorylation of wild-type SNT-1, MAPK and MAPK-dependent Thr421/Thr424 and Ser411 in p70-S6K; significant decrease of Gab1 and Sos1 recruitment to the FGFR-SNT-1 complex; significant inhibition of Ras binding to the Ras-binding domain of Raf-1 upon FGF stimulation; inhibition of FGF-dependent growth phenotypes in a colony forming assay in a doxycycline regulatable manner.

- Although inducible SNT-1 PTB overexpression resulted in specific inhibition of FGF-dependent MAPK there was no effect on phosphorylation of AKT or PI3Kinase-dependent site Thr389 in p70 S6K. However, phosphorylation of Thr389 was completely abrogated by the PI3-kinase inhibitor LY294002, as well as Rapamycin, an inhibitor of mTOR. This suggests an alternative mechanism of activation in FGF stimulated cells that is independent of the activation of Gab1.

- Abrogation of SNT-1 synthesis by specific shRNA inhibits the FGF mediated but not heregulin β-1 mediated activation of MAP kinase. This confirms the critical role of SNT-1 in the FGF-dependent Ras-MAPK signaling pathway.

- Our results suggest a compensatory mechanism involving increased signaling through Shc may substitute for SNT-1 function in the FGF pathway in MCF-7 cells.

- Stable doxycycline-inducible expression of the full size SNT-1 in MCF-7 Clone 18 cells producing and secreting FGF-1 leads to the increase in MAP kinase phosphorylation in estrogen depleted conditions. This suggests the role of SNT-1 in the developing an antiestrogen resistance in breast cancer cells.

- Lead conditions for crystallization of purified SNT-1 PTB domain have been determined.

Reportable outcomes.

Marina Manuvakhova, Susan Hays, Jaideep Thottassery, Norman R. Estes, Zhican Qu, Francis G Kern. The role of SNT-1/FRS2 in growth factor mediated abtiestrogen resistance of breast cancer cells. Abstracts of 95th Annual Meeting of the American Association for Cancer Research. March 27-31, Orlando, FL, #2425.

Marina Manuvakhova, Jaideep Thottassery, Susan Hays, Zhican Qu, Sarah S. Rentz, Louise Westbrook, and Francis G. Kern. Doxycycline-regulated overexpression of the FRS2/SNT-1 PTB domain has a dominant negative effect on major components of FGF signaling in MCF-7 breast cancer cells. Abstracts of 94th Annual Meeting of the American Association for Cancer Research. July 11-14, 2003, Washington, D.C., # 4968.

Zhican Qu, Jaideep Thottassery, Sabrina van Ginkel, Marina Manuvakhova, Louise Westbrook, Carrie Roland-Lazenby, and Francis G. Kern. Improved performance of a doxycycline-regulated gene expression system in a breast carcinoma cell line using a modified tet-on transactivator and an insulator-flanked TRE vector. Abstracts of 94th Annual Meeting of the American Association for Cancer Research. July 11-14, 2003, Washington, D.C., #2290.

Marina Manuvakhova, Jaideep Thottassery, Susan Hays, and Francis G. Kern. Evidence for involvement of SNT-1 independent mechanisms in FGF signaling in breast cancer cells. Abstracts of the 93rd Annual Meeting of the American Association for Cancer Research. April 6-10, 2002, San-Francisco, California, p.728.

M. Manuvakhova, J. Thottassery, S. Hays, Z. Qu., F.G. Kern. SNT-1 PTB domain overexpression has a dominant negative effect on FGF signaling in an ER+ breast cancer cell line. 2002. Era of Hope. Department of Defense Breast Cancer Research Program Meeting. P3-19.

Conclusions.

At the end of the third year of our study, we have completed the major goals listed in Tasks 1,2,3. It was shown that the truncated protein consisting of an N-terminal myristylation signal and the PTB domain with a deleted carboxy terminal region containing phosphotyrosine docking sites for Grb2 and SHP-2 signaling molecules can be overexpressed in breast cancer cells at very high levels in either a constitutive or regulatable manner. The truncated protein exhibits dominant negative properties by abrogating the major pathways involved in FGF signaling resulting in the inhibition of FGF-dependent growth phenotypes in a colony forming assay in a doxycycline regulatable manner. The use of other growth factors such as heregulin β-1 that also permit growth in antiestrogen containing media allowed us to determine the specificity of the dominant negative properties of the PTB domain for FGF signaling. These results allowed us to validate SNT-1 as a useful target for design of the molecules that will be able to inhibit its binding to activated FGF receptors.

We also showed that in the cells expressing SNT-1 PTB domain in an inducible manner, some components of PI-3 kinase signaling pathway are not affected suggesting that a separate mechanism of their activation exists in these cells. It would be important to explore the activation state of the PI3 kinase pathway in the cells expressing the dominant negative mutant of SNT-1 protein. We would consider an involvement of a potential signal adapter, Shc, as alternative way of linking FGF receptor activation to the activation of PI-3 kinase in breast cancer cells. If Shc-dependent signal transduction is indeed involved in cellular adaptation to the effects of SNT-1 dominant negative mutants, this fact will be taken into consideration in consequent drug design approaches.

Although expression of four fibroblast growth factors was detected in human breast tumors or cells lines [34 35 36 37], the role of SNT-1/FRS2 is mostly defined in FGFR1-induced pathways. Sequence alignment of the potential SNT-1 binding site in the juxtamembrane region of FGFR-1 with the juxtamembrane domains of the FGFR-2, -3,and -4 receptors demonstrated the binding potential of SNT-1 for all four receptors. Indeed, the direct interaction of SNT-1 with FGFR3 receptor has already been demonstrated [38]. It has also been shown that the quantity and quality of SNT-1 phosphorylation is both FGFR isotype and cell type-specific in prostate epithelial cells at different stages of malignancy [39]. It is possible that in breast cancer cells the efficiency of SNT-1 PTB domain interaction with different FGF receptor isotypes can be different, leading to different levels of abrogation of FGF-dependent pathways. This question needs further elucidation.

The coordinates for the NMR derived structure published by another group (13) should be available in the near future. This structure can be used for drug design. The NMR structure in essence validated our initial homology model by demonstrating an interaction of the basic region of the FGFR-1 peptide with an acidic groove that constitutes a second binding site distinct from the phosphotyrosine binding site. Consequently we are now focusing our efforts on the specificity of the interaction of SNT-1 protein with different FGF receptors. We plan to first use a Biacore apparatus to determine if a high affinity interaction of the PTB domain occurs with FGFR1, 2, 3, and 4 juxtamembrane peptides.

We experienced difficulties in obtaining diffractable crystals of the PTB domain of human SNT-1. It is possible that truncation of SNT-1 exposes a hydrophobic protein surface or flexible loops that cause protein aggregation or heterogeneous conformation distribution in solution. To overcome this problem, we would pursue in the future the full-length SNT-1 protein expression and crystallization. Structure of the full length SNT-1 should provide more information for targeting SNT-1 signaling. Expression of SNT-1 in bacterial cells may produce insoluble protein. Therefore, a eukaryotic cell expression system may be used to produce the SNT-1 protein, such as the Baculovirus/insect cell system or yeast cells.

Acronyms and abbreviations.

FGF – fibroblast growth factor
FGFR – fibroblast growth factor receptor
PRF – phenol red free
ER – estrogen receptor
PTB – phosphotyrosine binding domain
CCS – charcoal stripped serum
GFP – green fluorescent protein
MAPK – mitogen-activated protein kinase
Dox – doxycycline
E2 – 17β estradiol

References

1. McLeskey, S. W., Kurebayashi, J., Honig, S. F., Zwiebel, J., Lippman, M. E., Dickson, R. B., and Kern, F. G. Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. Cancer Res., *53*: 2168-2177, 1993.

2. McLeskey, S. W., Zhang, L., Kharbanda, S., Kurebayashi, J., Lippman, M. E., Dickson, R. B., and Kern, F. G. Fibroblast growth factor overexpressing breast carcinoma cells as models of angiogenesis and metastasis. Breast Cancer Res Treat, *39*: 103-117, 1996.

3. Zhang, L., Kharbanda, S., Chen, D., Bullocks, J., Miller, D. L., Ding, I. Y. F., Hanfelt, J., McLeskey, S. W., and Kern, F. G. MCF-7 breast carcinoma cells overexpressing FGF-1 form vascularized, metastatic tumors in ovariectomized or tamoxifen-treated nude mice. Oncogene, *15*: 2093-2108, 1997.

4. McLeskey, S. W., Zhang, L., El-Ashry, D., Trock, B. J., Lopez, C. A., Kharbanda, S., Tobias, C. A., Lorant, L. A., Hannum, R. S., Dickson, R. B., and Kern, F. G. Tamoxifen-

resistant FGF-transfected MCF-7 cells are cross-resistant *in vivo* to the antiestrogen, ICI 182,780 and two aromatase inhibitors. Clin.Cancer Res., *4*: 697-711, 1998.

5. Kouhara, H., Hadari, Y. R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., and Schlessinger, J. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell, *89*: 693-702, 1997.

6. Hadari, Y. R., Gotoh, N., Kouhara, H., Lax, I., and Schlessinger, J. Critical role for the docking-protein FRS2alpha in FGF receptor- mediated signal transduction pathways. Proc.Natl.Acad.Sci U.S.A, *98*: 8578-8583, 2001.

7. Xu, H., Lee, K. W., and Goldfarb, M. Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins. J.Biol.Chem., *273*: 17987-17990, 1998.

8. Hadari, Y. R., Kouhara, H., Lax, I., and Schlessinger, J. Binding of Shp2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. Mol.Cell Biol., *18*: 3966-3973, 1998.

9. Larsson, H., Klint, P., Landgren, E., and Claesson-Welsh, L. Fibroblast Growth Factor Receptor-1-mediated Endothelial Cell Proliferation Is Dependent on the Src Homology (SH) 2/SH3 Domain- containing Adaptor Protein Crk. J.Biol.Chem., *274*: 25726-25734, 1999.

10. Lim, Y. P., Low, B. C., Lim, J., Wong, E. S., and Guy, G. R. Association of atypical protein kinase C isotypes with the docker protein FRS2 in fibroblast growth factor signaling. J.Biol.Chem., *274*: 19025-19034, 1999.

11. Sasaki, A., Taketomi, T., Wakioka, T., Kato, R., and Yoshimura, A. Identification of a Dominant Negative Mutant of Sprouty That Potentiates Fibroblast Growth Factor- but Not Epidermal Growth Factor- induced ERK Activation. J Biol.Chem, *276*: 36804-36808, 2001.

12. Xie, W. Q. and Rothblum, L. I. Rapid, small-scale RNA isolation from tissue culture cells. Biotechniques, *11*: 324, 326-324, 327, 1991.

13. Kurebayashi, J., McLeskey, S. W., Johnson, M. D., Lippman, M. E., Dickson, R. B., and Kern, F. G. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and lacZ. Cancer Res., *53*: 2178-2187, 1993.

14. Urlinger, S., Baron, U., Thellmann, M., Hasan, M. T., Bujard, H., and Hillen, W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc.Natl.Acad.Sci.U.S.A, *97*: 7963-7968, 2000.

15. Ong, S. H., Goh, K. C., Lim, Y. P., Low, B. C., Klint, P., Claesson-Welsh, L., Cao, X., Tan, Y. H., and Guy, G. R. Suc1-associated neurotrophic factor target (SNT) protein is a

major FGF-stimulated tyrosine phosphorylated 90-kDa protein which binds to the SH2 domain of GRB2. Biochem.Biophys.Res.Commun., *225*: 1021-1026, 1996.

16. Rabin, S. J., Cleghon, V., and Kaplan, D. R. SNT, a differentiation-specific target of neurotrophic factor-induced tyrosine kinase activity in neurons and PC12 cells. Mol.Cell Biol., *13*: 2203-2213, 1993.

17. Kimpinski, K. and Mearow, K. Neurite growth promotion by nerve growth factor and insulin-like growth factor-1 in cultured adult sensory neurons: role of phosphoinositide 3-kinase and mitogen activated protein kinase. J.Neurosci.Res., *63*: 486-499, 2001.

18. Ong, S. H., Hadari, Y. R., Gotoh, N., Guy, G. R., Schlessinger, J., and Lax, I. Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. Proc.Natl.Acad.Sci.U.S.A, *98*: 6074-6079, 2001.

19. Cheatham, L., Monfar, M., Chou, M. M., and Blenis, J. Structural and functional analysis of pp70S6k. Proc.Natl.Acad.Sci.U.S.A, *92*: 11696-11700, 1995.

20. Romanelli, A., Martin, K. A., Toker, A., and Blenis, J. p70 S6 kinase is regulated by protein kinase Czeta and participates in a phosphoinositide 3-kinase-regulated signalling complex. Mol.Cell Biol., *19*: 2921-2928, 1999.

21. Romanelli, A., Dreisbach, V. C., and Blenis, J. Characterization of phosphatidylinositol 3-kinase-dependent phosphorylation of the hydrophobic motif site Thr(389) in p70 S6 kinase 1. J.Biol.Chem., *277*: 40281-40289, 2002.

22. Gonzalez-Garcia, A., Garrido, E., Hernandez, C., Alvarez, B., Jimenez, C., Cantrell, D. A., Pullen, N., and Carrera, A. C. A new role for the p85-phosphatidylinositol 3-kinase regulatory subunit linking FRAP to p70 S6 kinase activation. J Biol.Chem, *277*: 1500-1508, 2002.

23. Harada, H., Andersen, J. S., Mann, M., Terada, N., and Korsmeyer, S. J. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc.Natl.Acad.Sci U.S.A, *98*: 9666-9670, 2001.

24. Ferrari, S., Bannwarth, W., Morley, S. J., Totty, N. F., and Thomas, G. Activation of p70s6k is associated with phosphorylation of four clustered sites displaying Ser/Thr-Pro motifs. Proc.Natl.Acad.Sci.U.S.A, *89*: 7282-7286, 1992.

25. Han, J. W., Pearson, R. B., Dennis, P. B., and Thomas, G. Rapamycin, wortmannin, and the methylxanthine SQ20006 inactivate p70s6k by inducing dephosphorylation of the same subset of sites. J.Biol.Chem., *270*: 21396-21403, 1995.

26. Dennis, P. B., Pullen, N., Pearson, R. B., Kozma, S. C., and Thomas, G. Phosphorylation sites in the autoinhibitory domain participate in p70(s6k) activation loop phosphorylation. J.Biol.Chem., *273*: 14845-14852, 1998.

27. Klint, P., Kanda, S., and Claesson-Welsh, L. Shc and a novel 89-kda component couple to the Grb2-Sos complex in fibroblast growth factor-2-stimulated cells. J.Biol.Chem., *270*: 23337-23344, 1995.

28. Meakin, S. O., MacDonald, J. I., Gryz, E. A., Kubu, C. J., and Verdi, J. M. The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation. J.Biol.Chem., *274*: 9861-9870, 1999.

29. Walk, S. F., March, M. E., and Ravichandran, K. S. Roles of Lck, Syk and ZAP-70 tyrosine kinases in TCR-mediated phosphorylation of the adapter protein Shc. Eur.J.Immunol., *28*: 2265-2275, 1998.

30. Elbashir, S. M., Lendeckel, W., and Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev., *15*: 188-200, 2001.

31. Scacheri, P. C., Rozenblatt-Rosen, O., Caplen, N. J., Wolfsberg, T. G., Umayam, L., Lee, J. C., Hughes, C. M., Shanmugam, K. S., Bhattacharjee, A., Meyerson, M., and Collins, F. S. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. Proc.Natl.Acad.Sci.U.S.A, *101*: 1892-1897, 2004.

32. Brummelkamp, T. R., Bernards, R., and Agami, R. A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. Science, *296*: 550-553, 2002.

33. Ong, S. H., Guy, G. R., Hadari, Y. R., Laks, S., Gotoh, N., Schlessinger, J., and Lax, I. FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors [In Process Citation]. Mol.Cell Biol.2000.Feb.;20.(3.):979.-89., *20*: 979-989.

34. Smith, J., Yelland, A., Baillie, R., and Coombes, R. C. Acidic and basic fibroblast growth factors in human breast tissue. Eur.J.Cancer, *30A*: 496-503, 1994.

35. Renaud, F., El Yazidi, I., Boilly-Marer, Y., Courtois, Y., and Laurent, M. Expression and regulation by serum of multiple FGF1 mRNA in normal transformed, and malignant human mammary epithelial cells. Biochem.Biophys.Res.Commun., *219*: 679-685, 1996.

36. Yoshimura, N., Sano, H., Hashiramoto, A., Yamada, R., Nakajima, H., Kondo, M., and Oka, T. The expression and localization of fibroblast growth factor-1 (FGF-1) and FGF receptor-1 (FGFR-1) in human breast cancer. Clin.Immunol.Immunopathol., *89*: 28-34, 1998.

37. Tanaka, A., Furuya, A., Yamasaki, M., Hanai, N., Kuriki, K., Kamiakito, T., Kobayashi, Y., Yoshida, H., Koike, M., and Fukayama, M. High frequency of fibroblast growth factor (FGF) 8 expression in clinical prostate cancers and breast tissues, immunohistochemically demonstrated by a newly established neutralizing monoclonal antibody against FGF 8. Cancer Res, *58*: 2053-2056, 1998.

38. Ehrhard, K. N., Jacoby, J. J., Fu, X. Y., Jahn, R., and Dohlman, H. G. Use of G-protein fusions to monitor integral membrane protein-protein interactions in yeast. Nat.Biotechnol., *18*: 1075-1079, 2000.

39. Wang, F. In Vitro Cell Dev.Biol.Anim, *38*: 178-183, 2002.

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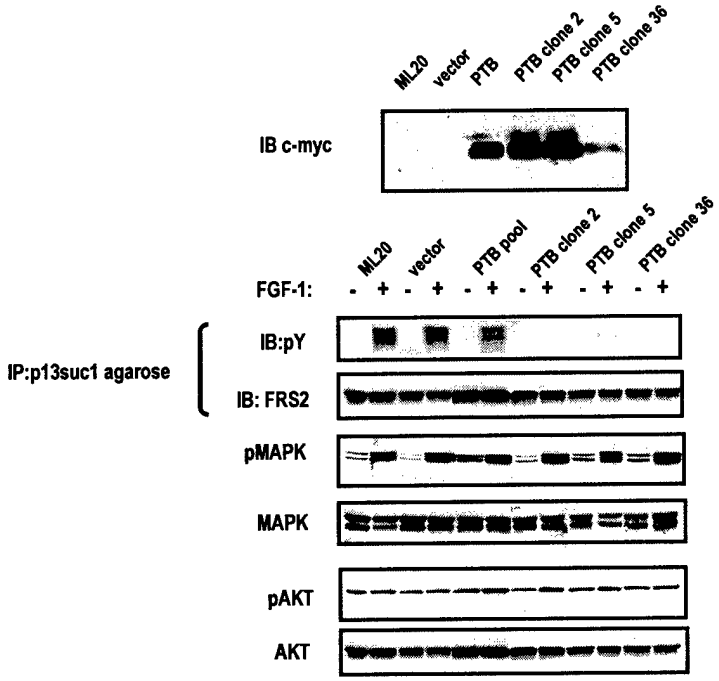
Appendix

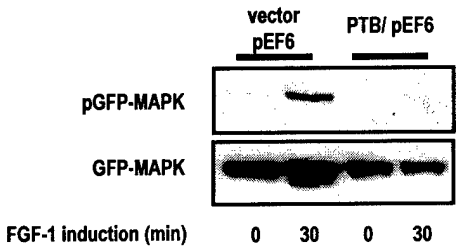
Figures and Legends

Fig.1. Constitutive expression of the SNT-1 PTB domain in MCF-7 cells blocks FGF-1 induced tyrosine phophorylation of the endogenous SNT-1 protein but fails to reduce FGF induced MAPK or AKT phosphorylation



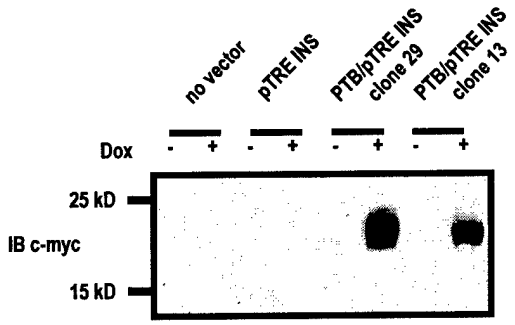
ML20 cells, stably transfected with PTB/pEF6 plasmid, pEF6 vector, or untransfected, were stripped of estrogen in 5% CCS-PRF-IMEM, incubated overnight in serum free PRF-IMEM, and induced with 20 ng/ml FGF-1 and 50 μg/ml heparin in serum free PRF-IMEM for 15 minutes. Total cellular lysates were loaded on 8-16% SDS-PAGE for immunoblotting. (A). Constitutive expression of SNT-1 PTB domain in ML20 cells. Immunoblot with monoclonal mouse c-myc antibody 9E10 (Oncogene) (100μg of protein/well). (B). Phosphorylation of endogenous SNT-1, MAPK and AKT in the clones and polyclonal populations constitutively expressing SNT-1 PTB domain. Immunoblots with phospho-MAPK and total MAPK antibodies (30μg of protein/well) and phospho-AKT and total AKT antibodies (100μg of protein/well). Tyrosine phosphorylation of the endogenous wild-type SNT-1 is shown in p13Suc1-agarose (Upstate Biotechnology) captured fraction of 1mg of total cellular lysates electrophoresed in 7.5% SDS-PAGE and immunoblotted with 4G10 antiphosphotyrosine antibodies.

Fig.2. Influence of the SNT-1 PTB domain on the phosphorylation of transiently transfected GFP-MAPK in ML20 cells



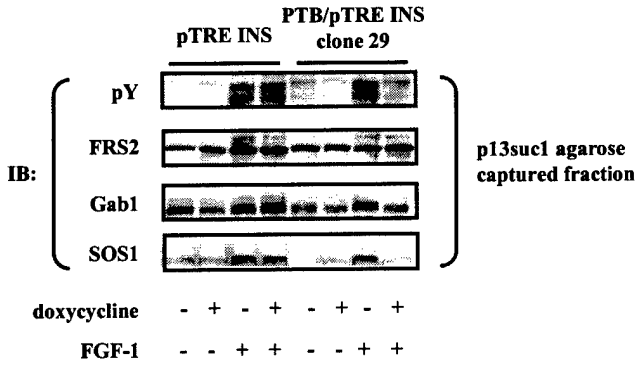
$2x10^5$ ML20 cells in a 60 mm dish were transiently cotransfected with 0.5 μg GFP-MAPK and 2 μg PTB/pEF6 or pEF6 vector. Cells were stripped of estrogen by 3 changes of 5% CCS-PRF-IMEM over 8 hours, incubated overnight in serum-free PRF-IMEM, and induced with 20 ng/ml FGF-1 and 50 μg/ml heparin in serum free-PRF-IMEM for 30 minutes. 30 μg of total cellular lysate was loaded in each well of 12% SDS-PAGE for immunoblotting with phospho-MAPK and MAPK antibodies.

Fig.3. Doxycycline regulated expression of the SNT-1 PTB domain in clones of MCF-7 cells expressing a modified tet-on transactivator



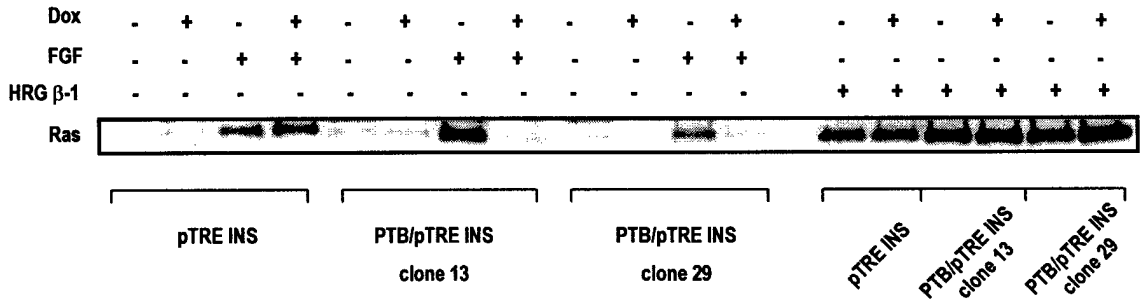
C9 ML20 cells expressing the modified tet on transactivator rtTA2 SM2 were stably transfected with the empty pTRE-INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain. The PTB domain expression was induced with 200 ng/ml doxycycline for 48 hours. 5 μg of total cellular lysates were loaded on an 8-16% gradient polyacrylamide gel and used for immunoblotting with the 9E10 mouse monoclonal antibody against the c-myc epitope.

Fig.4. Doxycycline-inducible expression of SNT-1 PTB domain in MCF-7 cells block FGF-1 induced tyrosine phosphorylation of the endogenous SNT-1 protein and inhibits its association with Gab1 and SOS1



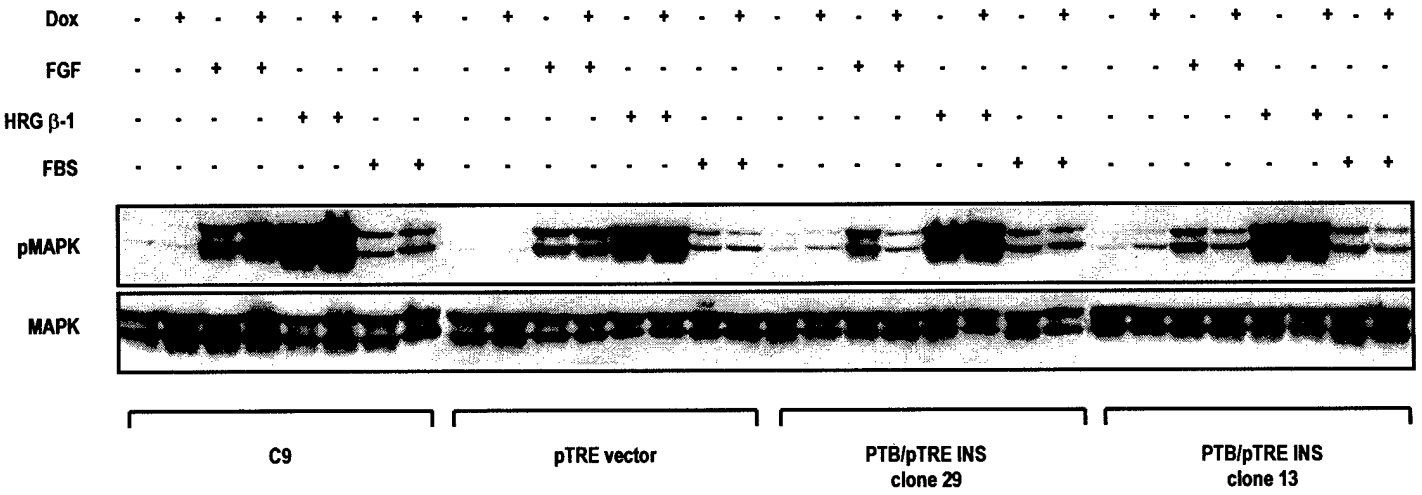
C9 ML20 cells expressing the modified tet on transactivator rtTA2 SM2 were stably transfected with the empty pTRE-INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain. The PTB domain expression was induced with 200 ng/ml doxycycline for 24 hours. The cells were then stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM medium over 8 hours and incubated overnight in serum free PRF-IMEM medium. During this process doxycycline remained present at 200 ng/ml where indicated. Tyrosine phosphorylation of endogenous wild-type SNT-1 and complex formation with Gab1 and SOS1 are shown in p13suc1 agarose captured fraction of 2mg of total cell lysates electrophoreses in 7.5% SDS-PAGE and immunoblotted with 4G10 antiphosphotyrosine antibodies, Gab1 and Sos1.

Fig.5 Doxycycline-inducible expression of SNT-1 PTB domain in MCF-7 cells significantly reduces activation of Ras



C9 ML20 cells expressing the modified tet on transactivator rtTA2 SM2 were stably transfected with the empty pTRE-INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain. The PTB domain expression was induced with 200 ng/ml doxycycline for 24 hours. The cells were then stripped of estrogen with 4 changes of 5% CCS-PRF-IMEM medium over 32 hours and incubated overnight in serum free PRF-IMEM medium. During this process doxycycline remained present at 200 ng/ml where indicated. Cells were then treated with 20 ng/ml FGF-1 or 10 μg/ml Heregulin β-1 for 5 min. Cells were lysed and 500 μg of cellular lysate was incubated with 10 μl Raf-1 RBD agarose from the Ras activation kit at 4°C for 30 min. The agarose pull-downs were washed three times with lysis buffer, boiled with 2X Laemmli sample buffer and fractionated on SDS-PAGE gels, followed by Western immunoblotting with anti-Ras antibodies.

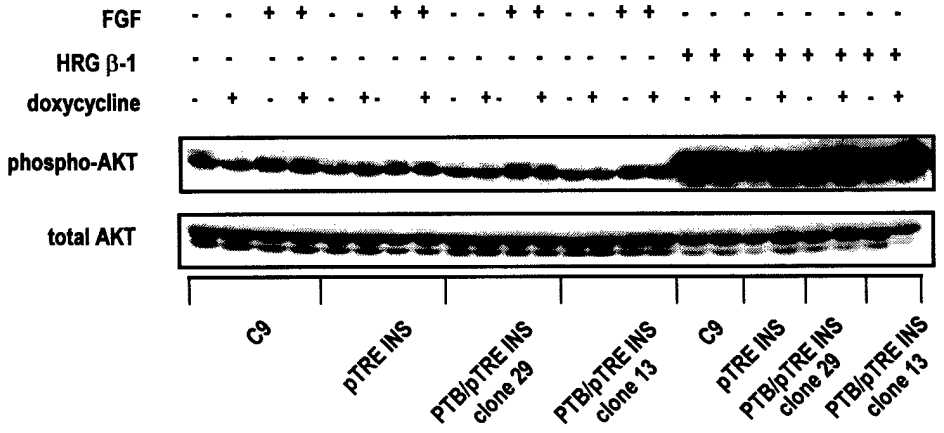
Fig.6. SNT-1 PTB domain specifically reduces FGF-stimulated MAPK phosphorylation when overepressed in a doxycycline regulatable manner



C9 ML20 cells expressing the modified tet on transactivator rtTA2 SM2 were stably transfected with the empty pTRE-INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain. The PTB domain expression was induced with 200 ng/ml doxycycline for 24 hours. The cells were then stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM medium over 8 hours and incubated overnight in serum free PRF-IMEM medium. During this process doxycycline remained present at 200 ng/ml where indicated. MAPK phosphorylation was induced by a 30 minute incubation with 20 ng/ml FGF-1, 10 μg/ml Heregulin β-1, or 5% FBS. 30 μg of the total cellular protein was

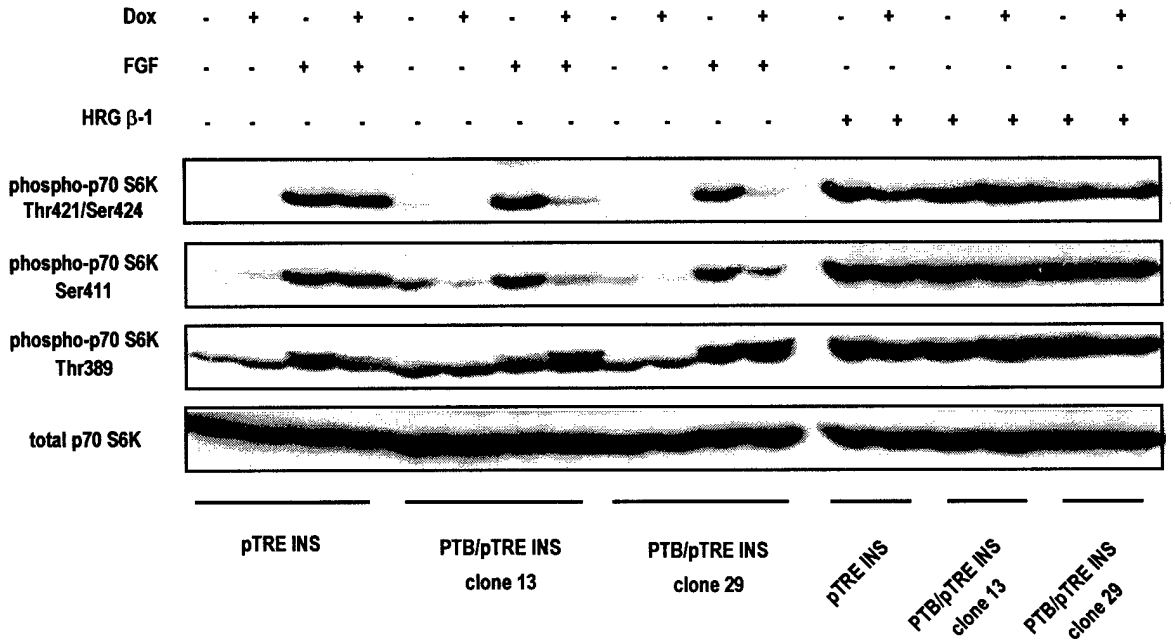
loaded in each well for immunoblotting with phospho-MAPK and MAPK antibodies.

Fig.7. SNT-1 PTB domain does not reduce FGF-stimulated AKT phosphorylation when overexpressed in a doxycycline regulatable manner



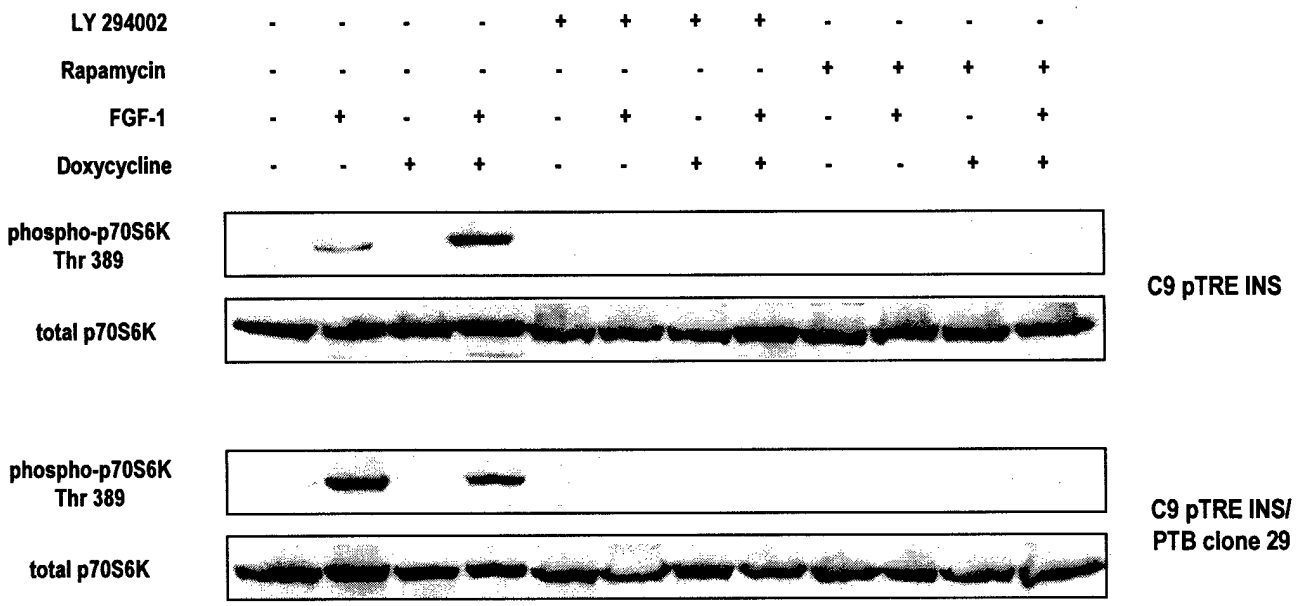
C9 ML20 cells transfected with the empty pTRE-INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain were incubated with 200 ng/ml of Dox for 24 hours to induce the PTB domain expression. The cells were then stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM medium over 8 hours and incubated overnight in serum free PRF-IMEM medium. During this process doxycycline remained present at 200 ng/ml where indicated. AKT phosphorylation was induced by a 30 minute incubation with 20 ng/ml FGF-1 or 10 mg/ml Heregulin β-1. 80 μg of the total cellular protein was loaded in each well for immunoblotting with phospho-AKT and AKT.

Fig.8. Effect of doxycycline-regulatable overexpression SNT-1 PTB domain on phosphorylation of the sites Thr 421/Ser 424, Ser 411 and Thr389 of p70S6K



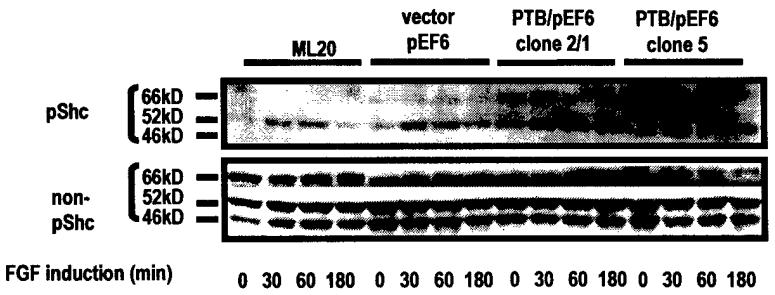
C9 ML20 cells transfected with the empty pTRE-INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain were incubated with 200 ng/ml of Dox for 24 hours to induce the PTB domain expression. The cells were then stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM medium over 8 hours and incubated overnight in serum free PRF-IMEM medium. During this process doxycycline remained present at 200 ng/ml where indicated. P70S6K phosphorylation was induced by a 30 minute incubation with 20 ng/ml FGF-1 or 10 μg/ml Heregulin β-1. 80 μg of the total cellular protein was loaded in each well for immunoblotting with antibodies against phospho-Thr 421/Ser 424, phospho-Ser 411 and phospho-Thr389 of p70S6K and p70S6K.

Fig.9. Effect of LY 294002 and Rapamycin on FGF-1 induced phosphorylation of Thr389 of p70S6K in C9 cells stably transfected with the vector pTRE INS and pTRE INS expressing the PTB domain of SNT-1



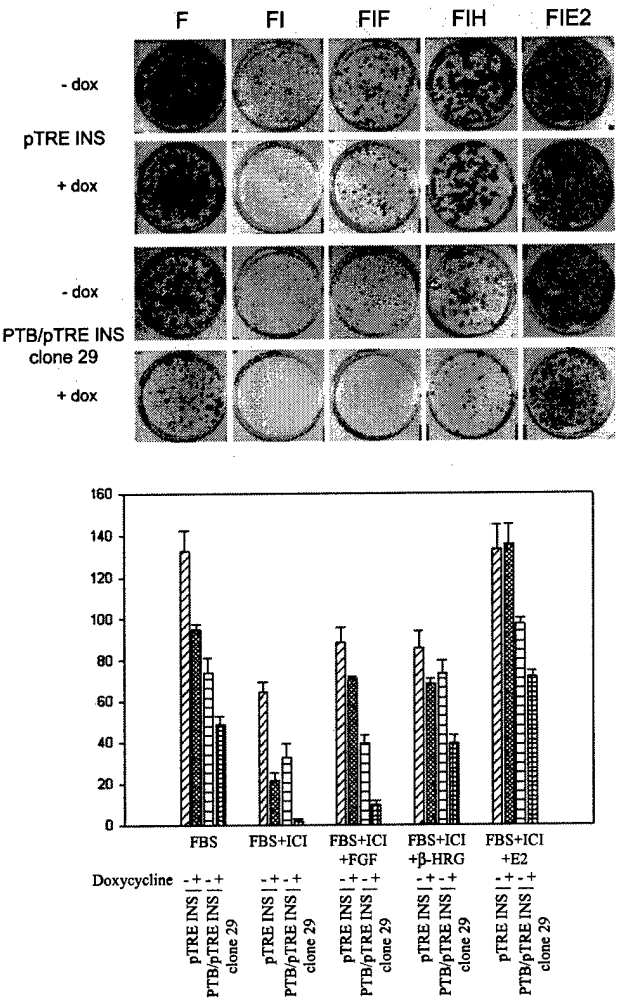
C9 ML20 cells transfected with the empty pTRE INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain were incubated with 200 ng/ml of Dox for 24 hours to induce the PTB domain expression. The cells were then stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM medium over 8 hours and incubated overnight in serum free PRF-IMEM medium. During this process doxycycline remained present at 200 ng/ml where indicated. Cells were pretreated for 30 minutes with 3μM LY 294002 or with 3nM Rapamycin. p70S6K phosphorylation was induced by a 20 minute incubation with 20 ng/ml FGF-1 while LY 294002 and Rapamycin remained present in the medium. 55 μg of the total cellular protein was loaded in each well for immunoblotting with antibodies against phospho-Thr389 of p70S6K and p70S6K.

Fig.10. Increased FGF-induced phosphorylation of ShcA isoforms in ML20 cells stably transfected with the PTB domain of SNT-1 cloned in the pEF6 vector



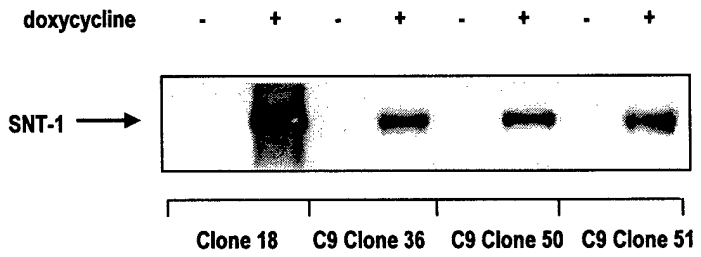
ML20 cells stably transfected with PTB/pEF6 plasmid, pEF6 vector, or untransfected, were treated as described in the legend to Figure 5 and induced with 20 ng/ml FGF-1 and 50 μg/ml heparin in serum- free PRF-IMEM for 30, 60, and 180 minutes. 30 μg of total cellular lysate was loaded in each well of 12% SDS-PAGE for immunoblotting with phospho-Shc and total Shc antibodies (Upstate Biotechnology).

Fig.11 Effect of SNT-1 PTB domain overexpression on anchorage-dependent growth of ML20 cells (colony forming test)



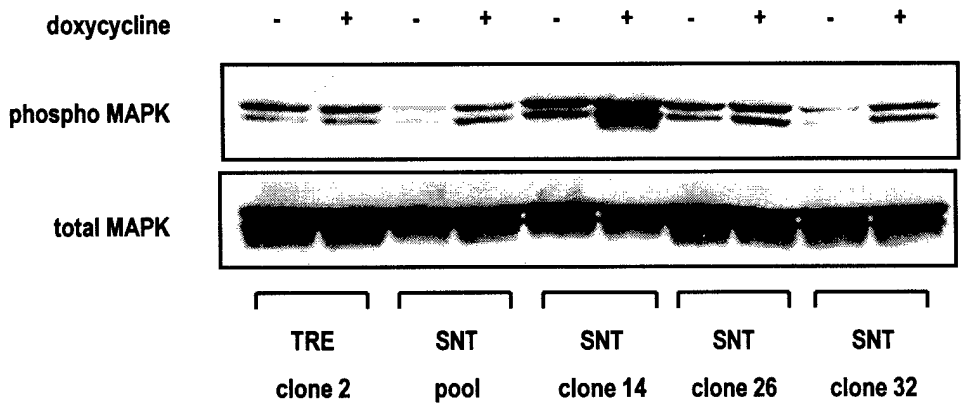
(A). C9 ML20 cells expressing the modified tet on transactivator rtTA2 SM2 were stably transfected with the empty pTRE-INS vector or with the same vector with an inserted SNT-1 myc-tagged PTB domain. Cells were plated in 5% PRF-FBS-IMEM. 24 hours later PTB domain expression was induced with 100 ng/ml doxycycline in 5% PRF-FBS-IMEM with heparin at 50 μg/ml and the following additions: ICI at 10^{-7}M, FGF-1 at 20 ng/ml, HRG-β1 at 10 μg/ml and β-estradiol at 10^{-9}M. Media was changed every 47 hours for a period of 3 weeks. Colonies were stained with 0.05% crystal violet and counted. (B). Graphic presentation of the results of colony counts.

Fig.12. Doxycycline-regulated expression of SNT-1 in C9 ML20 cells and MCF-7 cells stably expressing FGF-1 (Clone 18)



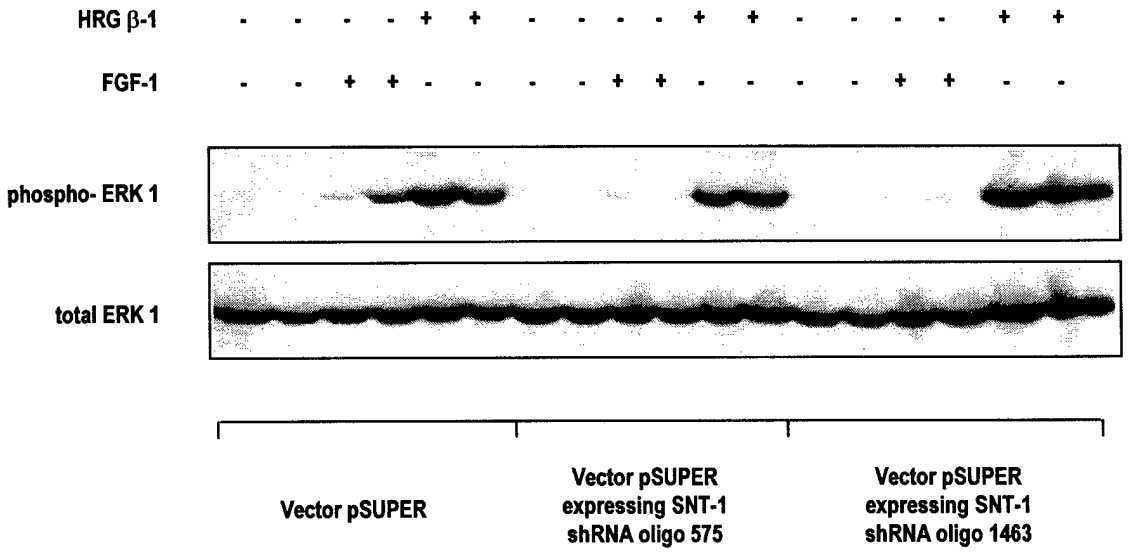
C9 ML20 cells and Clone 18 cells expressing the modified tet on transactivator rtTA2 SM2 were stably transfected with the vector pTRE INS with and inserted c-myc tagged SNT-1 gene. The SNT-1 expression was induced with 200 ng/ml of doxycycline for 24 hours. 2 μg of total cellular protein was loaded in each well for immunoblotting with c-myc antibodies.

Fig.13. Doxycycline regulated expression of SNT-1 in ML20 clone 18 cells leads to increased phophorylation of MAP-kinase



MCF-7 Clone 18 cells expressing the modified tet on transactivator rtTA2 SM2 were stable transfected with the empty vector pTRE-INS or with the same vector with an inserted SNT-1 gene. The SNT-1 expression was induced with 200 ng/ml doxycycline for 24 hours. The cells were then stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM medium over a 24 hour period and incubated overnight in serum free PRF-IMEM medium. During this process doxycycline remained present where indicated. 50 μg of the total cellular protein was loaded in each well for immunoblotting with phospho-MAPK and MAPK abtibodies.

Fig.14. Effect of the SNT-1 short hairpin RNA (shRNA) on FGF-dependent phophorylation of GFP-ERK 1



ML20 cells were transiently cotransfected with either the vector pSUPER or the same vector expressing short hairpin SNT-1 (sh) RNAs and the vector expressing GFP-ERK 1. Cells were incubated for 24 hours in 10% FBS IMEM, stripped of estrogen with 3 changes of 5% CCS-PRF-IMEM medium over 8 hours and incubated overnight in serum free PRF-IMEM medium. ERK 1 phosphorylation was induced by a 30 minute incubation with 20 ng/ml FGF-1. 130 μg of total cellular protein was loaded in each well for immunoblotting with phospho-MAPK and MAPK antibodies.

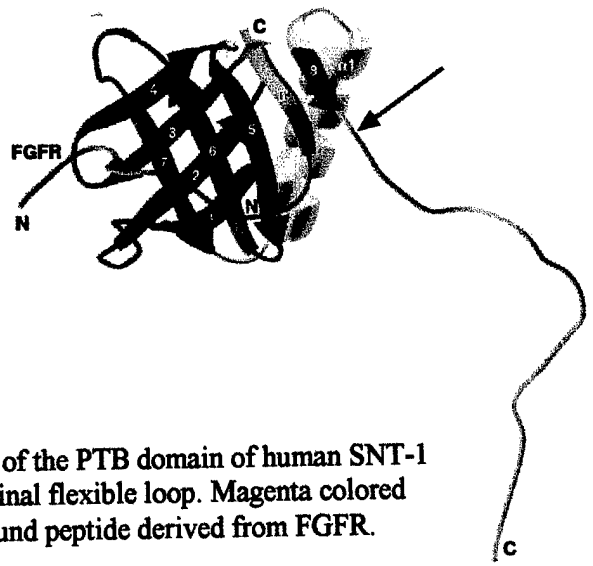
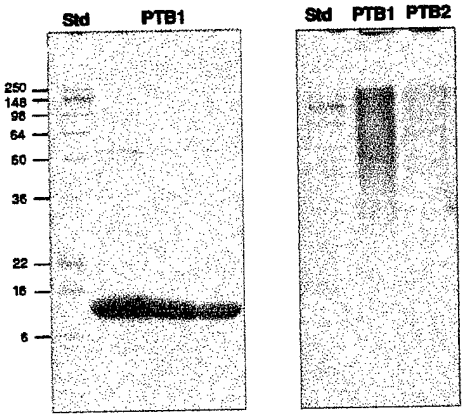


Fig. 15. Model of the PTB domain of human SNT-1 with the C-terminal flexible loop. Magenta colored segment is a bound peptide derived from FGFR.



(a) SDS PAGE (b) Native gel

Fig. 16. Electrophoresis of purified PTB domains: (a) SDS-polyacrylamide gel (15%) analysis of purified PTB1 protein. Protein samples were denatured by heating at 90° C for 5 min. (b) Native gel analysis of PTB1 and PTB2. The gels were stained with Coomassie brilliant blue to visualize the bands.

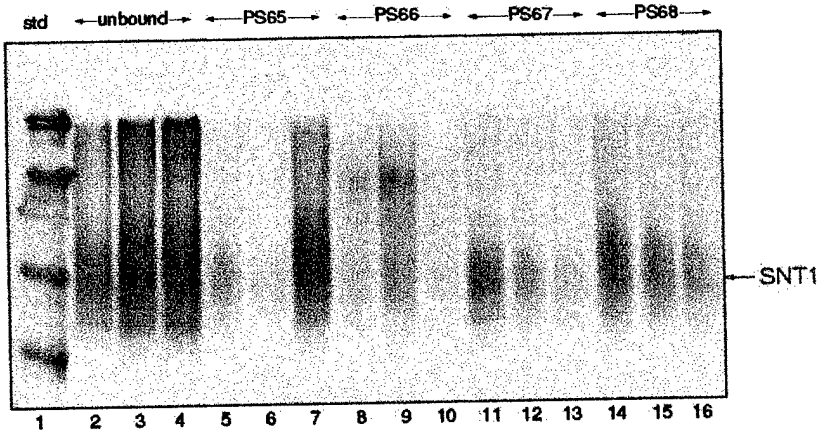


Fig. 17. Electrophoresis analysis of peptide bound PTB domain under native condition.