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(54) Title: METHODS OF PREVENTING TUMOR METASTASIS, TREATING AND PROGNOSING CANCER AND IDENTIFYING AGENTS WHICH ARE PUTATIVE METASTASIS INHIBITORS

(57) Abstract: A method of preventing tumor metastasis with the proviso that the tumor is not glioma is provided. The method comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2), thereby preventing tumor metastasis. Also, provided is a method of treating cancer. The method comprising, administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer, thereby treating cancer.

METHODS OF PREVENTING TUMOR METASTASIS, TREATING AND PROGNOSING CANCER AND IDENTIFYING AGENTS WHICH ARE PUTATIVE METASTASIS INHIBITORS

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of preventing tumor metastasis, treating and prognosing cancer and identifying agents which are putative metastasis inhibitors.

Cell motility supports a variety of physiological and pathological processes, including tumor metastasis (Ridley, 2011). The onset of migration is driven by actin polymerization and Rho-family GTPases, which instigate formation of lamellipodia and filopodia. Burgeoning evidence implicates another type of actin-driven protrusions, called invadopodia, in matrix degradation (Murphy and Courtneidge, 2011). To seed metastases, migratory breast cancer cells form invadopodia and infiltrate into nearby vessels. Studies aiming to characterize gene expression signatures associated with metastasis of breast cancer cells to lungs (Minn et al., 2005) and brain (Bos et al., 2009) identified sets of genes that underlie site-specific metastasis. Interestingly, both sets include members of the epidermal growth factor (EGF) family, suggesting that signaling by the shared receptor, EGFR, supports metastatic dissemination.

Intracellular trafficking emerges as a key feature of cell migration and tumor progression (Mosesson et al., 2008). For example, it has been shown that mutant p53 promotes metastasis through enhanced integrin and EGFR trafficking, which depend on the Rab-coupling protein (RCP) (Muller et al., 2010). Along with Rab proteins, phosphoinositides play pivotal roles in cellular compartmentalization by determining vesicles identity (Yuan and Cantley, 2008). For example, phosphorylation at the D3 position of $PI(4,5)P_2$ (phosphatidyl-inositol 4,5-bisphosphate) by phosphatidylinositol 3-kinase (PI3K) generates $PI(3,4,5)P_3$, which is necessary for invadopodia formation (Yamaguchi et al., 2011). Similarly, $PI(4,5)P_2$ regulates multiple proteins controlling endocytosis and actin dynamics (Saarikangas et al., 2010), but its levels are stringently controlled by two additional types of enzymes: phospholipase C ($PLC\gamma$) promotes $PI(4,5)P_2$ hydrolysis, which activates Cofilin (an actin-severing protein) and drives mammary cell migration (van Rheenen et al., 2007). Likewise, inositol polyphosphate 5-

phosphatases, such as Synaptojanin 2 (SYNJ2), dephosphorylate the D5 position of the inositol ring and control glioma cell migration (Chuang et al., 2004; Malecz et al., 2000). In addition homozygous mutations were identified in certain prostate cancer samples Rossi et al. Cancer Genet Cytogenet. 2005 Sep;161(2):97-103.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of preventing tumor metastasis with the proviso that the tumor is not glioma, the method comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2), thereby preventing tumor metastasis.

According to an aspect of some embodiments of the present invention there is provided a method of treating cancer the method comprising, administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer, thereby treating cancer.

According to an aspect of some embodiments of the present invention there is provided an inhibitor of synaptojanin 2 (SYNJ2) for preventing tumor metastasis with the proviso that said tumor is not glioma.

According to an aspect of some embodiments of the present invention there is provided an inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer for treating cancer.

According to some embodiments of the invention, the cell surface receptor associated with the onset or progression of cancer is a receptor tyrosine kinase.

According to some embodiments of the invention, the receptor tyrosine kinase is an ErbB receptor.

According to some embodiments of the invention, the ErbB receptor is Epidermal Growth Factor Receptor (EGFR).

According to an aspect of some embodiments of the present invention there is provided a method of identifying a putative inhibitor of tumor metastasis, the method comprising analyzing SYNJ2-mediated processing of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ in the presence of a test agent, wherein a decreased processing of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ in the

presence of the test agent as compared to same in an absence thereof is indicative of a putative inhibitor of tumor metastasis.

According to some embodiments of the invention, the analyzing SYNJ2-mediated processing of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ is performed by a competition assay.

According to some embodiments of the invention, the competition assay assays displacement of a $PI(3,4)P_2$ binding domain from a complex comprising the $PI(3,4)P_2$ binding domain bound to $PI(3,4)P_2$.

According to some embodiments of the invention, the competition assay is a fluorescence polarization competitive assay.

According to an aspect of some embodiments of the present invention there is provided a method of prognosing cancer in a subject in need thereof, the method comprising determining a level or activity of SYNJ2 in a cancer cell of the subject, wherein an upregulation in the level of activity of the SYNJ2 in the cancer cell of the subject compared to same in a cell of an unaffected control sample, is indicative of a poor prognosis.

According to some embodiments of the invention, the method further comprises augmenting the prognosis using a Gold standard method.

According to some embodiments of the invention, the Gold standard method comprises detection of a marker.

According to some embodiments of the invention, the marker is selected from the group consisting of HER-2 and estrogen receptor (ER).

According to some embodiments of the invention, the metastasis is EGF dependent.

According to some embodiments of the invention, the cancer is breast cancer.

According to some embodiments of the invention, the inhibitor of SYNJ2 is selected from the group consisting of a small molecule, an antibody, a peptide and a nucleic acid silencing agent.

According to some embodiments of the invention, the small molecule is selected from the molecules listed in Table 2.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture for the treatment of cancer or prevention of cancer metastasis,

comprising a packaging material packaging an inhibitor of SYNJ2 and an inhibitor of a cell surface receptor associated with an onset or progression of cancer.

According to some embodiments of the invention, the inhibitor of the cell surface receptor associated with the onset or progression of cancer is an antibody.

According to some embodiments of the invention, the inhibitor of the cell surface receptor associated with the onset or progression of cancer is a small molecule inhibitor.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

Figures 1A-I show that EGF promotes invasive growth of mammary cells and induces a specific set of genes. Figure 1A - MCF10A cells were plated in the absence of growth factors and allowed to form clusters. Seventy-two hours later, cells were treated with the indicated growth factors (each at 10 ng/mL) and phase contrast images were taken 24 hours later (scale bar, 50μm). Figure 1B - MCF10A cells were plated in migration or invasion chambers, as indicated, in the presence of the indicated ligands (10 ng/mL), and 18 hours later cells that migrated to the lower compartment were stained with crystal violet (left panel). Shown are quantifications of migration and invasion signals, normalized to the effect of EGF treatment. Data represent mean ± S.D. of biological triplicates from a representative experiment that was repeated twice (right

panel). Figure 1C - MCF10A cells were plated in transwell inserts in EGF-containing medium, without or with the inhibitors AG-1478 (1 μM), U0126 (5 μM), or Wortmannin (200 nM), and allowed to migrate for 18 hours. Data represent mean ± S.D. of triplicates. The experiment was repeated twice. Figure 1D - A list of 425 genes specifically induced in human mammary MCF10A cells by EGF (and not by serum (Amit et al., 2007)), was intersected with genes that were up-regulated in the context of metastasis of MDA-MB-231 cells (1,597 genes) (Minn et al., 2005). One of the 23 overlapping genes encodes the 5'-phosphatidylinositol lipid phosphatase Synaptojanin-2 (SYNJ2). Figure 1E - MCF10A cells were infected with lentiviral particles encoding LacZ (Ctrl) or SYNJ2-GFP (SYNJ2-OX). Expression levels of the endogenous SYNJ2 and the SYNJ2-GFP fusion protein were determined by immunoblotting, and equal protein loading was confirmed by probing for tubulin. Figure 1F - The Ctrl and SYNJ2-OX clones of MCF10A cells were plated in migration chambers (5 x 10^4 cells/well) in the absence (NT) or presence of EGF (10 ng/mL) and allowed to migrate for 22 hours. Migrating cells that reached the other side of the filter were stained with crystal violet and images were taken. Figure 1G - MCF10A cells were transfected with siRNA control (siCtrl) or siRNA directed to SYNJ2 (siSYNJ2), and protein levels of SYNJ2 were determined 36 hours later by immunoblotting. Equal protein loading was confirmed by immunoblotting for Ras-GAP. Figure 1H - The cells presented in G were plated in migration chambers (5 x 10^4 cells/well) in the absence (NT) or presence of EGF (10 ng/mL) and allowed to migrate for 22 hours. Migrating cells that reached the lower face of the filter were stained with crystal violet and images were captured. Figure 1I - Confluent cultures of MCF10A cells were treated with the indicated siRNAs. Once monolayers formed, they were subjected to an automated scratching system that monitors the rate of scratch closure.

Figures 2A-E show that transcriptional induction of SYNJ2 by EGF promotes invasive growth. Figure 2A - Serum-starved MCF10A cells were stimulated with EGF (20 ng/mL) or serum (5 %), and SYNJ2 mRNA expression was assayed by using microarrays or RT-qPCR. Figure 2B - MCF10A cells were stimulated with EGF, extracted and immunoblotted as indicated. Figure 2C - MCF10A cells, infected with viruses encoding GFP-SYNJ2 (SYNJ2-OX) or LacZ as control (Ctrl), were cultured for 4 days in the absence or presence of EGF. Phase contrast (top, bar: 100 μm) and

confocal images (bottom, bar: 20 μm) using phalloidin and DAPI were obtained. Figures 2D-E - MCF10A cells were cultured for 22 hours in migration or invasion chambers (5-6 x 10^4 cells/well) in the absence (NT) or presence of EGF (10 ng/mL). Cells that reached the filter's bottom were stained and filter's coverage quantified (mean ± S.D.).

Figures 3A-G show inducible translocation of SYNJ2 to the leading edge accompanies mammary cell migration and invasion. Figure 3A - MDA-MB-231 cells were infected with lentiviral particles encoding LacZ (Ctrl) or a V5-tagged SYNJ2 (SYNJ2-V5), along with control shRNA (shCtrl) or an shRNA directed against SYNJ2 (shSYNJ2). Protein levels of V5-SYNJ2 and endogenous SYNJ2 were determined by immunoblotting. Equal protein loading was confirmed by immunoblotting for AKT. Figure 3B - Phase images (left panels) and invasion images (right panels) of MDA-MB-231 cells stably over-expressing SYNJ2, or LacZ as control. The invasive capacities were determined in triplicates using an invasion assay, and invading cells were quantified and normalized to control (Ctrl). Scale bar, 50μm. Figure 3C - MDA-MB-231 cells were transfected with siRNA oligonucleotides directed to SYNJ2 (or siCtrl). Following 36 hours, protein levels of SYNJ2 were determined by immunoblotting. Equal protein loading was confirmed by immunoblotting for Ras-GAP. Figure 3D - Cells from C were plated in migration or invasion chambers and incubated for 18 hours. The migration and invasion signals were quantified and normalized to EGF-treated siCtrl cells. Data shown are means ± S.D. of triplicates. Figure 3E - MDA-MB-231 cells transiently expressing GFP-SYNJ2 were plated on glass coverslips and stimulated with TGFα (10 ng/mL). Time-lapse microscopy photos were taken (every 10 seconds). The images shown are inverted, with black spots representing SYNJ2 and its assembly at the base of lamellipodia. Scale bar, 10μm. Figure 3F - MDA-MB-231 cells were immunostained for endogenous SYNJ2 and F-actin using TRITC-phalloidin. The squared area is magnified. Scale bar, 10μm. Figure 3G - MCF10A cells were stimulated with EGF for 18 hours, and then immunostained for endogenous SYNJ2 and counter-stained for F-actin using TRITC-phalloidin. Scale bar, 10 μm.

Figures 4A-F show that the catalytic activity of SYNJ2 is essential for invasive growth. Figures 4A-B - MDA-MB-231 cells expressing SYNJ2 (SYNJ2-OX) or shRNA to SYNJ2 (shSYNJ2), as well as control cells, were seeded in 5 % Matrigel.

Images were captured after six days, and invasive spheroids quantified (mean ± S.D.). Scale bars, 50 μm. Figures 4C-D - shSYNJ2-expressing MDA-MB-231 cells were infected with WT SYNJ2 (shSYNJ2+$SYNJ2^{WT}$) or with a catalytically disabled mutant (shSYNJ2+$SYNJ2^{CD}$). Cells were either extracted and immunoblotted as indicated, or they were allowed to invade for 18 hours in invasion chambers. Images of the invaded cells and their normalized quantification are shown (mean ± S.D). Figure 4E – show scanning electron micrographs of shCtrl and shSYNJ2 cells grown on fibronectin. Scale bar, 2 μm. Figure 4F - Images of F-actin in the indicated MDA-MB-231 cells stained with phalloidin and DAPI. Z-axis sections (lines) and magnified areas are shown. Arrowheads mark swollen structures. Scale bar, 10 μm.

Figures 5A-H show the subcellular localization of SYNJ2. Figure 5A - MDA-MB-231 cells expressing GFP-SYNJ2 were transfected with an RFP-Clathrin and plated on fibronectin-coated plates. Using spinning-disc microscopy, cells were imaged every five seconds. Arrowheads mark a newly formed leading edge. Scale bar, 5 μm. Figure 5B - Representative time frames depicting assembly and disassembly of SYNJ2 at the leading edge (upper two rows) and underneath the cell body. For the lower rows, cells were transfected with a mCherry-lifeACT plasmid and plated on collagen. Thereafter, cells were imaged at 1 minute intervals. Arrowheads were inserted for reference. Note the difference in time scales. Scale bar, 1 μm. Figure 5C - Cells were simultaneously imaged by TIRF and epifluorescence microscopy and signals converted into kymographs (X-axis). Arrowheads mark signal initiation. Scale bar, 5 μm. Figure 5D - Cells were imaged using spinning disc confocal microscopy 5 minutes before and 5 minutes after treatment with Dyngo-4a (30 μM; a Dynamin-2 inhibitor). Scale bar, 5 μm. Figure 5E - MDA-MB-231 cells stably expressing GFP-SYNJ2 were pre-incubated with Dyngo-4a (30 μM; 30 min), or with solvent (DMSO). Cell lysates were subjected to immunoprecipitation with anti-GFP antibodies (or with no antibody; *-Ab*), and then immunoblotted, along with a sample (5%) of the cell lysate, with the indicated antibodies. Figure 5F - Cells were plated on fibronectin, fixed and immunostained for endogenous Rac1. Scale bar, 10 μm. Figure 5G - Cells were imaged using confocal microscopy 5 minutes prior to and 5 minutes after a 30min-long treatment with NSC-23766 (5μM). Scale bar, 5 μm. Figure 5H - MDA-MB-231 cells were treated with the

indicated siRNA oligonucleotides. Cell extracts were blotted for SYNJ2 and Ras-GAP. GTP-Rac1 levels were determined using an ELISA-based assay (Cytoskeleton).

Figures 6A-D show SYNJ2 localization to the leading edge is distinct from caveolins distribution and depends on F-actin, cholesterol and PI3K. Figure 6A - MDA-MB-231 cells expressing GFP-SYNJ2 and co-expressing RFP-Cav1 were simultaneously imaged over time, and signals converted into kymographs (X and Y axis). Note the transient nature of SYNJ2 assemblies and stable appearance of Caveolin 1. Scale bar, 5μm. Figure 6B - The left panel depicts the distribution (% of pits versus lifetime) of 150 randomly selected SYNJ2 assemblies, imaged as in Figure 5A (5 second intervals, single plane, spinning disk confocal). The right panel depicts the average (± SEM) relative intensity of assemblies that showed a 55 seconds lifetime. Figure 6C - MDA-MB-231 cells stably-expressing GFP-SYNJ2 were treated with MβCD (10 mM, 15 minutes) or with Wortmannin (500 nM, 15 minutes). Images of the same selected cells were captured every 6 seconds, either prior to or following treatment, and signals were converted into kymographs (representing the squared insets in the left panels). Scale bar, 20μm. Figure 6D - MDA-MB-231 cells stably co-expressing GFP-SYNJ2 and lifeACT-mCherry were treated with LatrunculinB (1 μM, 15 minutes). Images were acquired either prior to or following treatment. Scale bar, 5 μm.

Figures 7A-E show SYNJ2 depletion arrests EGFR in intracellular vesicles Figure 7A - MCF10A cells stably expressing shRNA control (shCtrl) or shRNA specific to SYNJ2 (shSYNJ2) were extracted three days after plating in EGF-containing medium. Immunoblots were probed for SYNJ2, EGFR, phosphorylated tyrosine 1068 of EGFR (pEGFR), phosphorylated ERK (pERK), and Ras-GAP, as a loading control. Figure 7B - MCF10A cells were transfected with siRNA control, or siRNA directed against SYNJ2, in the presence of EGF. Confocal immunofluorescence analysis was performed using EGFR and SYNJ2 antibodies. Note that only the SYNJ2-depleted cell (asterisk) displays EGFR trafficking defects. Scale bar, 10 μm. Figure 7C - Three derivatives of MDA-MB-231 cells were immunostained for EGFR and counterstained for DAPI and F-actin: (i) cells in which SYNJ2 was knocked-down (shSYNJ2; left column), (ii) the same cells infected by lentiviral gene transfer corresponding to the catalytically-dead form (shSYNJ2+SYNJ2^{CD}; middle column), and (iii) cells in which

SYNJ2 was knocked-down and the wild type form was introduced by infection (shSYNJ2 +SYNJ2^{WT}; right column). Scale bar, 20 μm. Figure 7D - Ubiquitinated EGFR levels (densitometry). Figure 7E - MDA-MB-231 derivatives were stimulated with 488-Tfn (5 minutes, 10 μg/mL). Cells were fixed on ice, acid-washed and analysed for signal intensity.

Figures 8A-I show that SYNJ2 regulates EGFR trafficking and chemotaxis. Figure 8A - Whole extracts of MDA-MB-231 cells transfected with the indicated siRNAs were immunoblotted as indicated. Figure 8B - FACS (left) and ^{125}I-EGF binding (right; in triplicates) analyses of surface EGFR in the indicated MDA-MB-231 subclones. Figure 8C - shCtrl and shSYNJ2 cells were grown on fibronectin and immunostained for EGFR and F-actin. Bar, 20 μm. Figure 8D - Rose plots of tracks of shCtrl and shSYNJ2 MDA-MB-231 cells, which migrated in chemotaxis chambers upon exposure to an EGF gradient. The red tracks indicate cells migrating toward EGF. Figure 8E - Starved MDA-MB-231 derivatives were treated with EGF (10 ng/mL) and cell lysates were subjected to immunoprecipitation and immunoblotting as indicated. Figure 8F - Cells were cultured as in C and immunostained for active EGFR (pY1045) and F-actin. Bar, 10 μm. Figure 8G - The indicated MDA-MB-231 derivatives were treated with EGF (10 ng/ml) for 5 hours and extracts immunoblotted as indicated. Figure 8H - The indicated MDA-MB-231 derivatives were exposed to Alexa Fluor 488-Tfn (25 μg/ml; 5 min), acid-washed to remove surface-bound ligands, and images taken at the indicated intervals. Normalized fluorescence signals are shown. Bar, 10 μm. Figure 8I - MDA-MB-231 cells, pre-treated with siCtrl or siSYNJ2, were stimulated with Alexa Fluor 488-EGF (20 μg/ml; 10 min), acid-washed, incubated at 37°C for the indicated intervals and analysed by FACS.

Figures 9A-D show that SYNJ2 is necessary for both vesicular trafficking and focal adhesion formation. Figure 9A - MDA-MB-231 derivatives (shCtrl and shSYNJ2) were fixed and stained for EEA1, F-actin and nuclei (DAPI). Scale bar, 10 μm. Figure 9B - MDA-MB-231 derivatives, namely shCtrl and shSYNJ2 cells, were probed for integrin beta-1, F-actin and DAPI (scale bar, 20 μm). Figure 9C - MDA-MB-231 cells were treated with siCtrl and siSYNJ2 for 48 hours and then immunostained for integrin beta-1 and phosphorylated EGFR. Figure 9D - Immunofluorescence analysis of MDA-MB-231 derivatives for paxillin, nuclei (DAPI), and F-actin (using TRITC-phalloidin).

The paxillin signal was quantified in cytoplasmic regions relative to focal adhesions, and the numbers of focal adhesions per cell were also quantified. In addition, the shapes of focal adhesions were quantified by determining deviations from a perfect circle (eccentricity). Scale bar, 10 μm.

Figures 10A-F show that SYNJ2 depletion perturbs phosphoinositide homeostasis, inflates early endosomes and disassembles focal adhesions. Figure 10A - MDA-MB-231 cells, expressing shCtrl or shSYNJ2, were transfected with a GFP-Rab4 plasmid and 48 hours later cells were fixed and counterstained for F-actin using TRITC-phalloidin. Figure 10B - MDA-MB-231 derivatives were immunostained for Rab5, F-actin and nuclei (DAPI). Images were quantified for the size and number of Rab5-positive vesicle, as well as for the average cell area. Scale bars, 10 μm. Figure 10C - Phosphoinositides extracted from ^{3}H-phosphoinositol labeled derivatives of MDA-MB-231 cells, were separated by chromatography and their levels determined in three different experiments (signals normalized to shCtrl cells). Figure 10D - shCtrl and shSYNJ2 MDA-MB-231 cells were probed for pY1068-EGFR, Paxillin and F-actin (co-localization signal are white). Scale bar, 10 μm). Figure 10E - shCtrl and shSYNJ2 MDA-MB-231 cells were seeded. Unattached cells were removed 20 min later and attached cells were imaged and quantified for surface area. Figure 10F - MDA-MB-231 cells, stably expressing shCtrl or shSYNJ2, were plated on RTCA E-plates and real-time impedance measurements were recorded in 5 sec intervals for 80 min, and then in 10 min intervals for additional 80 min. Means of 2 replicates (± S.D.) are shown.

Figures 11A-G show that SYNJ2 regulates protease secretion and invadopodium assembly. Figure 11A - shCtrl and shSYNJ2 MDA-MB-231 cells were cultured in Matrigel for 5 days, fixed and immunostained for MMP-9. Signal intensities were converted into heat-maps and plotted against distance from colony cores. Arrowheads mark spheroid boundaries. Bar, 50 μm. Figure 11B - Supernatants from control MDA-MB-231 cells and cells stably overexpressing SYNJ2 were analyzed in triplicates for MMP-2 and MMP-9 activity using gelatin zymography. Figure 11C - MDA-MB-231 cells stably expressing GFP-SYNJ2 were plated onto coverslips pre-coated with cross-linked fluorescent gelatin. Three hours later, cells were probed for GFP and F-actin, and invadopodial structures detected (arrowheads). Bar, 10 μm. Figure 11D - MDA-MB-231 cells overexpressing SYNJ2 (SYNJ2-OX), as well as cells pre-treated with siCtrl or

siSYNJ2 oligonucleotides, were plated on coverslips pre-coated with cross-linked fluorescent gelatin and invadopodial structures were quantified in three independent experiments. Figure 11E - Invadopodial structures of MDA-MB-231 cells treated with the indicated siRNAs were detected by gelatin degradation, as well as by staining for F-actin or TKS5. Arrowheads (Z-axis images) mark invadopodia. Bar, 10 μm. Figure 11F - MDA-MB-231 cells expressing siCtrl or siSYNJ2 were plated on gelatin-coated coverslips and processed as in C using phalloidin and antibodies to the phosphorylated form of EGFR (tyrosine 1068). Scale bar, 10 μm. Figure 11G - Media conditioned over 3 days by the indicated MDA-MB-231 derivatives were examined using an ELISA-based assay for EGF-like ligands.

Figures 12A-G show that SYJN2 regulates matrix degradation and invadopodia assembly. Figure 12A - The indicated siRNA-treated MDA-MB-231 cells were plated in triplicates, cultured for 3 days and their conditioned media were separated electrophoretically using a gelatin (0.1%) embedded gel, followed by protein staining to quantify MMP-2 and MMP-9 proteolytic activity. Figure 12B - Co-immunoprecipitation analysis using GFP-conjugated beads and cleared extracts of MDA-MB-231 cells stably expressing GFP-SYNJ2. Figure 12C - MDA-MB-231 cells stably expressing GFP-SYNJ2 were transfected with a RFP-Cortactin plasmid and plated on collagen plates. Live-cell image analysis was performed forty-eight hours later, and representative snapshot images of both peripheral and central cell areas were captured. Scale bar 5 μm. Figure 12D - The indicated derivatives of MDA-MB-231 cells were transfected with a plasmid encoding a Myc-tagged PH domain of Tapp1 (a $PI(3,4)P_2$ binder) and 48 hours later they were plated on gelatin-coated surfaces. The co-distribution of F-actin, aggregated TKS5 and $PI(3,4)P_2$ (Tapp1) was visualized and quantified using confocal microscopy. Scale bar, 10 μm.

Figure 12E - MDA-MB-231 cells expressing siCtrl or siSYNJ2 were plated onto FITC-gelatin coated glass coverslips and incubated for 3 hours. Cells were then fixed and immunostained for CD44, and counter stained for F-actin with TRITC-phalloidin. Cells were visualized using fluorescence microscopy, and invadopodia were detected by observing holes in the FITC-gelatin matrix. The framed areas are enlarged. Scale bar, 10 μm. Figure 12F - An antibody to CD44 was used for FACS analysis of surface expression by shCtrl and shSYNJ2 cells. Indicated are the fractions of cells

corresponding to the framed regions. Figure 12G - MDA-MB-231 cells pre-treated with siCtrl or siSYNJ2 were plated onto FITC-gelatin coated glass coverslips and incubated for 3 hours. Cells were then fixed and immunostained for MT1-MMP, and counter stained for F-actin with TRITC-phalloidin. Scale bar, 10 μm.

Figures 13A-H show that the enzymatic activity of SYNJ2 propels metastatic spread of mammary tumor cells. Figure 13A - The indicated derivatives of RFP-expressing MDA-MB-231 cells ($2X10^6$/mouse) were implanted in the fat pad of female SCID mice (10-11 per group). Tumor size (mean ± S.D.) was measured 2 and 6 weeks post implantation. Figures 13B-C - Metastases that appeared six weeks post-implantation in axillary and distant lymph nodes (Figure 13B), or lungs (Figure 13C), are shown. Asterisks mark *p* values: * < 0.05, ** < 0.01 and *** < 0.001. Figures 13D-F - Control (LacZ) and SYNJ2-overexpresseing (SYNJ2-OX) RFP-labelled MDA-MB-231 cells were implanted in animals as in A and tumor size (Figure 13D), as well as metastases to lymph nodes (Figure 13E) and lungs (Figure 13F) were quantified 6 and 8 weeks post implantation. Figures 13G-H - The indicated MDA-MB-231-RFP derivatives were injected either intravenously ($1.5X10^5$ per mouse; tail vein), or in the mammary fat pad ($2.5X10^6$ per mouse) of 5-week old female SCID mice. Four weeks later, lungs from mice injected into the vein were examined for RFP signals (left and middle panels). Peripheral blood was collected from the fat pad-treated group four weeks later. Samples were purified on a gradient of ficol and the numbers of RFP-positive circulating tumor cells (CTC) were scored per $1x10^6$ FACS readings and normalized to tumor weight.

Figure 14 is an in-vivo imaging of local and distant lymph node metastases. Representative images of local (ipsilateral) and distant (contralateral) lymph node metastases in mice that were inoculated with MDA-MB-231-RFP cells and analysed 6 weeks later (see Figure 13B). Prior to imaging, mice were anaesthetized and their fur was removed for visualization and quantification of metastases in lymph nodes.

Figure 15 is a working model depicting the integrated action of SYNJ2 in cell migration and invasion. EGFR-loaded recycling endosomes position active receptors at the ventral membrane, and this is followed by local activation of PI3K. Phosphorylation of membranal $PI(4,5)P_2$ by PI3K generates $PI(3,4,5)P_3$, which is dephosphorylated by SYNJ2 to $PI(3,4)P_2$. The latter recruits TKS5, which anchors Cortactin and nucleates

actin polymerization. In parallel, SYNJ2 controls delivery of adhesion molecules like CD44, and proteases like MT1-MMP, to degrade the extracellular matrix (ECM) and establish new invasive structures, the invadopodia. In a similar way, EGFR delivery to the cell periphery leads to breakdown of $PI(4,5)P_2$ by SYNJ2 (and phospholipase C), which locally activates Dynamin and actin severing enzymes like Cofilin to dissolve cortical actin fibres and initiate actin-filled, integrin-rich protrusions called lamellipodia. The horizontal arrow marks the direction of cell migration. Color-coded segments of the plasma membrane denote specific PI phospholipids.

Figures 16A-C show that SYNJ2 is highly expressed in aggressive breast tumors. Figure 16A - Immunohistochemistry and tissue microarrays were used to stratify 331 invasive breast carcinomas according to SYNJ2 abundance (high, medium and low). The relative fraction of tumors is presented according to clinical subtypes. Figure 16B - Representative images of SYNJ2 staining demonstrating intensities and patterns (magnified in the right column) observed in a luminal case (an asterisk marks expression by endothelial cells as control), and both basal-like and HER2-overexpressing breast tumors. Figure 16C - Kaplan-Meier curves stratified according to SYNJ2 mRNA expression in cohorts of 286 (left; GSE2034) or 99 (right; GSE19783) breast cancer patients.

Figures 17A-B show the principles of the fluorescence polarization assay utilized for measuring the 5'-phosphatase activity of SYNJ2. Figure 17A is a scheme demonstrating the general principle that an unbound PI(3,4)P2 fluorescent probe gives rise to low polarization readings, while the bound PI(3,4)P2 fluorescent probe increases the polarization readings. Figure 17B is a representative bar graph showing SYNJ2 5'-phosphatase activity detection as measured by polarization values (mP).

FIG. 18 depicts the amino acid and nucleic acid sequences (SEQ ID NO: 13 and 14, respectively) of the Flag-TAPP1 PH domain-His that was cloned into pET28 plasmid and expressed in E.coli. The TAPP1-PH domain is marked in yellow.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of preventing tumor metastasis, treating and prognosing cancer and identifying agents which are putative metastasis inhibitors.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Growth factors propel cell migration and metastasis, but underlying mechanisms are incompletely understood.

The present inventors have now identified synaptojanin-2 (SYNJ2) as a master module in regulating invadopodia and lamellipodia in vitro and cancer metastasis in vivo.

As is illustrated hereinbelow and in the Examples section which follows, the present inventors substantiated their finding in vitro, in animals and in patient specimens. Specifically, employing EGF-stimulated mammary cells the present inventors link the lipid phosphatase synaptojanin 2 (SYNJ2) to an invasive phenotype, and relate high SYNJ2, to short survival rates of cancer patients. Knockdown of SYNJ2 robustly impaired metastasis of mammary tumor cells in an animal model. In vitro, SYNJ2-depleted cells exhibited derailed trafficking of EGFR and integrins, resulting in deformed focal adhesions, arrested lamellipodia and disappearance of invadopodia. Without being bound to theory it is suggested that recycling of active EGFRs focally promotes SYNJ2-mediated dephosphorylation of specific phosphoinositol lipids, thereby instigating formation of both invadopodia and lamellipodia and facilitates tumor progression (see Figure 15).

Thus, according to an aspect of the invention there is provided a method of preventing tumor metastasis with the proviso that said tumor is not glioma, the method comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2), thereby preventing tumor metastasis.

As used herein the phrase "tumor metastasis" refers to a malignant tumor which spreads out of its primary location to other parts of the body e.g., breast cancer which metastasizes to the lungs.

As used herein the terms "cancer" and "tumor" are interchangeably used. The term refers to a malignant growth or tumor caused by abnormal and uncontrolled cell division.

As used herein the term "preventing" refers to arresting, halting, inhibiting the metastatic process or progression and subsequent metastasis.

According to yet another aspect, there is provided a method of treating cancer the method comprising, administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer, thereby treating cancer.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

Non-limiting examples of cancers which can be treated (or prognosed) according to some embodiments of the invention include any solid or non-solid cancer and/or cancer metastasis, including, but is not limiting to, tumors of the gastrointestinal tract (colon carcinoma, rectal carcinoma, colorectal carcinoma, colorectal cancer, colorectal adenoma, hereditary nonpolyposis type 1, hereditary nonpolyposis type 2, hereditary nonpolyposis type 3, hereditary nonpolyposis type 6; colorectal cancer, hereditary nonpolyposis type 7, small and/or large bowel carcinoma, esophageal carcinoma, tylosis with esophageal cancer, stomach carcinoma, pancreatic carcinoma, pancreatic endocrine tumors), endometrial carcinoma, dermatofibrosarcoma protuberans, gallbladder carcinoma, Biliary tract tumors, prostate cancer, prostate adenocarcinoma, renal cancer (e.g., Wilms' tumor type 2 or type 1), liver cancer (e.g., hepatoblastoma, hepatocellular carcinoma, hepatocellular cancer), bladder cancer, embryonal rhabdomyosarcoma, germ cell tumor, trophoblastic tumor, testicular germ cells tumor, immature teratoma of ovary, uterine, epithelial ovarian, sacrococcygeal tumor, choriocarcinoma, placental site trophoblastic tumor, epithelial adult tumor, ovarian carcinoma, serous ovarian cancer, ovarian sex cord tumors, cervical carcinoma, uterine cervix carcinoma, small-cell and non-small cell lung carcinoma, nasopharyngeal, breast carcinoma (e.g., ductal breast cancer, invasive intraductal breast cancer, sporadic ; breast cancer, susceptibility to breast cancer, type 4 breast cancer, breast cancer-1, breast cancer-3; breast-ovarian cancer), squamous cell carcinoma (e.g., in head and neck), neurogenic tumor, astrocytoma, ganglioblastoma, neuroblastoma, lymphomas (e.g., Hodgkin's disease, non-Hodgkin's lymphoma, B cell, Burkitt,

cutaneous T cell, histiocytic, lymphoblastic, T cell, thymic), gliomas, adenocarcinoma, adrenal tumor, hereditary adrenocortical carcinoma, brain malignancy (tumor), various other carcinomas (e.g., bronchogenic large cell, ductal, Ehrlich-Lettre ascites, epidermoid, large cell, Lewis lung, medullary, mucoepidermoid, oat cell, small cell, spindle cell, spinocellular, transitional cell, undifferentiated, carcinosarcoma, choriocarcinoma, cystadenocarcinoma), ependimoblastoma, epithelioma, erythroleukemia (e.g., Friend, lymphoblast), fibrosarcoma, giant cell tumor, glial tumor, glioblastoma (e.g., multiforme, astrocytoma), glioma hepatoma, heterohybridoma, heteromyeloma, histiocytoma, hybridoma (e.g., B cell), hypernephroma, insulinoma, islet tumor, keratoma, leiomyoblastoma, leiomyosarcoma, leukemia (e.g., acute lymphatic, acute lymphoblastic, acute lymphoblastic pre-B cell, acute lymphoblastic T cell leukemia, acute - megakaryoblastic, monocytic, acute myelogenous, acute myeloid, acute myeloid with eosinophilia, B cell, basophilic, chronic myeloid, chronic, B cell, eosinophilic, Friend, granulocytic or myelocytic, hairy cell, lymphocytic, megakaryoblastic, monocytic, monocytic-macrophage, myeloblastic, myeloid, myelomonocytic, plasma cell, pre-B cell, promyelocytic, subacute, T cell, lymphoid neoplasm, predisposition to myeloid malignancy, acute nonlymphocytic leukemia), lymphosarcoma, melanoma, mammary tumor, mastocytoma, medulloblastoma, mesothelioma, metastatic tumor, monocyte tumor, multiple myeloma, myelodysplastic syndrome, myeloma, nephroblastoma, nervous tissue glial tumor, nervous tissue neuronal tumor, neurinoma, neuroblastoma, oligodendroglioma, osteochondroma, osteomyeloma, osteosarcoma (e.g., Ewing's), papilloma, transitional cell, pheochromocytoma, pituitary tumor (invasive), plasmacytoma, retinoblastoma, rhabdomyosarcoma, sarcoma (e.g., Ewing's, histiocytic cell, Jensen, osteogenic, reticulum cell), schwannoma, subcutaneous tumor, teratocarcinoma (e.g., pluripotent), teratoma, testicular tumor, thymoma and trichoepithelioma, gastric cancer, fibrosarcoma, glioblastoma multiforme; multiple glomus tumors, Li-Fraumeni syndrome, liposarcoma, lynch cancer family syndrome II, male germ cell tumor, mast cell leukemia, medullary thyroid, multiple meningioma, endocrine neoplasia myxosarcoma, paraganglioma, familial nonchromaffin, pilomatricoma, papillary, familial and sporadic, rhabdoid predisposition syndrome, familial, rhabdoid tumors, soft tissue sarcoma, and Turcot syndrome with glioblastoma.

According to a specific embodiment, the cancer is breast cancer.

According to a specific embodiment, the cancer (or the cancer metastasis) is EGF-regulated.

According to another preferred embodiment, the cancer is characterized by over-expression or up-regulation of an ErbB receptor molecule such as EGFR or HER2.

Mutations that lead to EGFR overexpression (known as upregulation) or overactivity have been associated with a number of cancers, including lung cancer, anal cancers and glioblastoma multiforme. In this latter case a more or less specific mutation of EGFR, called EGFRvIII is often observed. Mutations, amplifications or misregulations of EGFR or family members are implicated in about 30 % of all epithelial cancers.

Mutations involving EGFR could lead to its constant activation, which could result in uncontrolled cell division – a predisposition for cancer. Consequently, mutations of EGFR have been identified in several types of cancer, and it is the target of an expanding class of anticancer therapies [Zhang 2007 *J. Clin. Invest.* **117** (8): 2051–8].

Amplification or over-expression of the *ERBB2* gene occurs in approximately 30 % of breast cancers. It is strongly associated with increased disease recurrence and a worse prognosis. Over-expression is also known to occur in ovarian, stomach, and aggressive forms of uterine cancer, such as uterine serous endometrial carcinoma.

Following is a list of cancers in which members of the ErbB family of receptor tyrosine kinases are implicated.

ErbB-1 - adrenocortical cancer, biliary cancer, cervical cancer, colorectal cancer, esophageal cancer, gallbladder cancer, gastric cancer, glioblastoma, head and neck cancer, lung cancer (non-small cell, squamous cell carcinoma, adenocarcinoma, and large cell lung cancer), pancreatic cancer, salivary gland cancer, diarrhea benign neoplasm, invasive carcinoma, skin disease, ductal carcinoma in situ, paronychia.

ErbB-2 – biliary cancer, bladder cancer, breast cancer, cholangiocarcinoma, esophageal cancer, gallbladder cancer, gastric cancer, glioblastoma, ovarian cancer, pancreatic cancer, salivary gland cancer. According to a specific embodiment the cancer is breast or gastric cancer.

ErbB-3 – breast cancer, lung cancer and viral leukemia.

ErbB-4 – breast cancer, viral leukemia, medulloblastoma, lung cancer and mammary tumor.

As used herein the term "subject" refers to a mammal (e.g., human) who has been diagnosed with cancer.

As used herein synaptojanin-2 or SYNJ2 refers to Synaptic inositol-1,4,5-trisphosphate 5-phosphatase 2, EC 3.1.3.36. Synaptojanin-2 is a ubiquitously expressed inositol polyphosphate 5-phosphatase (SEQ ID NO: 1 and 2, referring to polynucleotide and encoded polypeptide, respectively).

As used herein the phrase "inhibitor of synaptojanin 2 (SYNJ2)" refers to a molecule which decreases or downregulates the expression or activity of SYNJ2.

Downregulating can be by more than 10 %, 20 %, 30%, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or a complete inhibition (100 % loss of activity or expression as determined by a given assay such as described hereinbelow).

Downregulating the expression of SYNJ2 can be effected at the DNA, RNA or protein level as described hereinbelow. A SYNJ2 activity refers to its catalytic activity [as a phosphatase, converting $PI(3,4,5)P_3$ into $PI(3,4)P_2$], its signaling activity (interacts with dynamin, cotractin, see Figures 5E-H) or cellular localization. In the latter case the inhibitor of SYNJ2 will alter the protein's cellular localization.

Thus, downregulation of SYNJ2 can be effected on the genomic and/or the transcript level using a variety of molecules which knock-in the gene or interfere with its transcription and/or translation [e.g., nucleic acid silencing agents e.g., nucleic acid (RNA) silencing agents e.g., antisense, siRNA, shRNA, micro-RNA, Ribozyme and DNAzyme], or on the protein level using e.g., antagonists, enzymes that cleave the polypeptide and the like.

Following is a list of agents capable of downregulating expression level and/or activity of SYNJ2.

One example, of an agent capable of downregulating SYNJ2 is an antibody or antibody fragment capable of specifically binding SYNJ2. Preferably, the antibody specifically binds at least one epitope of SYNJ2. As SYNJ2 is a cellular protein, measures are taken to introduce the antibody into the cells. As used herein, the term "epitope" refers to any antigenic determinant on an antigen to which the paratope of an antibody binds.

Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')2, and Fv that are capable of binding to macrophages. These functional antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')2, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')2 is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Antibody fragments according to some embodiments of the invention can be prepared by proteolytic hydrolysis of the antibody or by expression in E. coli or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')2. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting

from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [Biochem. J. 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar et al. [Proc. Nat'l Acad. Sci. USA 69:2659-62 (19720]. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as E. coli. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by [Whitlow and Filpula, Methods 2: 97-105 (1991); Bird et al., Science 242:423-426 (1988); Pack et al., Bio/Technology 11:1271-77 (1993); and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [Methods, 2: 106-10 (1991)].

Anti SYNJ2 are commercially available. Examples of suppliers of anti human SYNJ2 monoclonal antibodies include, but are not limited to, Amsbio, Atlas

Antibodies, AbD Serotec, United States Biological, antibodies-online.com, Genway, Proteintech Group and more. Antibodies of the invention are rendered non-immunogenic for therapeutic applications.

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been

substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10,: 779-783 (1992); Lonberg et al., Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

Downregulation of SYNJ2 can be also achieved by RNA silencing. As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression] mediated by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of specifically inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g, the full translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include noncoding

RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include dsRNAs such as siRNAs, miRNAs and shRNAs. In one embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression.

According to an embodiment of the invention, the RNA silencing agent is specific to the target RNA (e.g., SYNJ2) and does not cross inhibit or silence a gene or a splice variant which exhibits 99% or less global homology to the target gene, e.g., less than 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81% global homology to the target gene.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla. Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

Accordingly, some embodiments of the invention contemplate use of dsRNA to downregulate protein expression from mRNA.

According to one embodiment, the dsRNA is greater than 30 bp. The use of long dsRNAs (i.e. dsRNA greater than 30 bp) has been very limited owing to the belief that these longer regions of double stranded RNA will result in the induction of the interferon and PKR response. However, the use of long dsRNAs can provide numerous advantages in that the cell can select the optimal silencing sequence alleviating the need to test numerous siRNAs; long dsRNAs will allow for silencing libraries to have less complexity than would be necessary for siRNAs; and, perhaps most importantly, long dsRNA could prevent viral escape mutations when used as therapeutics.

Various studies demonstrate that long dsRNAs can be used to silence gene expression without inducing the stress response or causing significant off-target effects - see for example [Strat et al., Nucleic Acids Research, 2006, Vol. 34, No. 13 3803–3810; Bhargava A et al. Brain Res. Protoc. 2004;13:115–125; Diallo M., et al., Oligonucleotides. 2003;13:381–392; Paddison P.J., et al., Proc. Natl Acad. Sci. USA. 2002;99:1443–1448; Tran N., et al., FEBS Lett. 2004;573:127–134].

In particular, the invention according to some embodiments thereof contemplates introduction of long dsRNA (over 30 base transcripts) for gene silencing in cells where the interferon pathway is not activated (e.g. embryonic cells and oocytes) see for example Billy et al., PNAS 2001, Vol 98, pages 14428-14433 and Diallo et al, Oligonucleotides, October 1, 2003, 13(5): 381-392. doi:10.1089/154545703322617069.

The invention according to some embodiments thereof also contemplates introduction of long dsRNA specifically designed not to induce the interferon and PKR pathways for down-regulating gene expression. For example, Shinagwa and Ishii [*Genes & Dev.* 17 (11): 1340-1345, 2003] have developed a vector, named pDECAP, to express long double-strand RNA from an RNA polymerase II (Pol II) promoter. Because the transcripts from pDECAP lack both the 5'-cap structure and the 3'-poly(A) tail that facilitate ds-RNA export to the cytoplasm, long ds-RNA from pDECAP does not induce the interferon response.

Another method of evading the interferon and PKR pathways in mammalian systems is by introduction of small inhibitory RNAs (siRNAs) either via transfection or endogenous expression.

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 basepairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is theorized to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

It has been found that position of the 3'-overhang influences potency of a siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

The strands of a double-stranded interfering RNA (e.g., an siRNA) may be connected to form a hairpin or stem-loop structure (e.g., an shRNA). Thus, as mentioned the RNA silencing agent of some embodiments of the invention may also be a short hairpin RNA (shRNA).

Examples of small interfering RNA molecules can be found in Chuang et al. (supra) SJ2–1 (coding region 1612–1633; 5′AACGTGAACGGAGGAAAGCAG, SEQ ID NO: 3), SJ2–2 (region 5419–5440 in the 3′ untranslated region; 5′CTCTTGCTGATACGCGATATT, SEQ ID NO: 4); or Rusk et al. [Curr Biol. 2003 Apr 15;13(8):659-63. Erratum in: Curr Biol. 2003 Sep 30;13(19):1746], teaching siRNA to the coding regions 1612-1633 or 4925-4946 of SYNJ2.

Other examples of siRNA sequences that successfully downregulate SYNJ2 mRNA levels include, but are not limited to GAAGAAACAUCCCUUUGAU (SEQ ID NO: 5) and GGACAGCACUGCAGGUGUU (SEQ ID NO: 6).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide

analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-UUCAAGAGA-3' (Brummelkamp, T. R. et al. (2002) Science 296: 550) and 5'-UUUGUGUAG-3' (Castanotto, D. et al. (2002) RNA 8:1454). It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

Examples of shRNA sequences that successfully downregulate SYNJ2 mRNA levels include, but are not limited to, CCGGCCTACGATACAAGCGACAAATCTCGAAGATTTGTCGCTTGTATCGTAG GTTTTTG (SEQ ID NO: 7); CCGGCGAGAGGAGATCATTCGGAAACTCGAGTTTCCGAATGATCTCCTCTC GTTTTTG (SEQ ID NO: 8); CCGGCCGGAAGAACAGTTTGAGCAACTCGAGTTGCTCAAACTGTTCTTCCG GTTTTTG (SEQ ID NO: 9).

Synthesis of RNA silencing agents suitable for use with some embodiments of the invention can be effected as follows. First, the SYNJ2 mRNA sequence is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level (wwwdotambiondotcom/techlib/tn/91/912dothtml).

Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat etc.) using any sequence alignment software, such as the BLAST software available from the NCBI server (wwwdotncbidotnlmdotnihdotgov/BL

AST/). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

It will be appreciated that the RNA silencing agent of some embodiments of the invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

In some embodiments, the RNA silencing agent provided herein can be functionally associated with a cell-penetrating peptide." As used herein, a "cell-penetrating peptide" is a peptide that comprises a short (about 12-30 residues) amino acid sequence or functional motif that confers the energy-independent (i.e., non-endocytotic) translocation properties associated with transport of the membrane-permeable complex across the plasma and/or nuclear membranes of a cell. The cell-penetrating peptide used in the membrane-permeable complex of some embodiments of the invention preferably comprises at least one non-functional cysteine residue, which is either free or derivatized to form a disulfide link with a double-stranded ribonucleic acid that has been modified for such linkage. Representative amino acid motifs conferring such properties are listed in U.S. Pat. No. 6,348,185, the contents of which are expressly incorporated herein by reference. The cell-penetrating peptides of some embodiments of the invention preferably include, but are not limited to, penetratin, transportan, pIsl, TAT(48-60), pVEC, MTS, and MAP.

mRNAs to be targeted using RNA silencing agents include, but are not limited to, those whose expression is correlated with an undesired phenotypic trait. Exemplary mRNAs that may be targeted are those that encode truncated proteins i.e. comprise deletions. Accordingly the RNA silencing agent of some embodiments of the invention

may be targeted to a bridging region on either side of the deletion. Introduction of such RNA silencing agents into a cell would cause a down-regulation of the mutated protein while leaving the non-mutated protein unaffected.

According to another embodiment the RNA silencing agent may be a miRNA.

The term "microRNA", "miRNA", and "miR" are synonymous and refer to a collection of non-coding single-stranded RNA molecules of about 19-28 nucleotides in length, which regulate gene expression. miRNAs are found in a wide range of organisms (virusesdotfwdarwdothumans) and have been shown to play a role in development, homeostasis, and disease etiology.

Below is a brief description of the mechanism of miRNA activity.

Genes coding for miRNAs are transcribed leading to production of an miRNA precursor known as the pri-miRNA. The pri-miRNA is typically part of a polycistronic RNA comprising multiple pri-miRNAs. The pri-miRNA may form a hairpin with a stem and loop. The stem may comprise mismatched bases.

The hairpin structure of the pri-miRNA is recognized by Drosha, which is an RNase III endonuclease. Drosha typically recognizes terminal loops in the pri-miRNA and cleaves approximately two helical turns into the stem to produce a 60–70 nucleotide precursor known as the pre-miRNA. Drosha cleaves the pri-miRNA with a staggered cut typical of RNase III endonucleases yielding a pre-miRNA stem loop with a 5' phosphate and ~2 nucleotide 3' overhang. It is estimated that approximately one helical turn of stem (~10 nucleotides) extending beyond the Drosha cleavage site is essential for efficient processing. The pre-miRNA is then actively transported from the nucleus to the cytoplasm by Ran-GTP and the export receptor Ex-portin-5.

The double-stranded stem of the pre-miRNA is then recognized by Dicer, which is also an RNase III endonuclease. Dicer may also recognize the 5' phosphate and 3' overhang at the base of the stem loop. Dicer then cleaves off the terminal loop two helical turns away from the base of the stem loop leaving an additional 5' phosphate and ~2 nucleotide 3' overhang. The resulting siRNA-like duplex, which may comprise mismatches, comprises the mature miRNA and a similar-sized fragment known as the miRNA*. The miRNA and miRNA* may be derived from opposing arms of the pri-miRNA and pre-miRNA. MiRNA* sequences may be found in libraries of cloned miRNAs but typically at lower frequency than the miRNAs.

Although initially present as a double-stranded species with miRNA*, the miRNA eventually become incorporated as a single-stranded RNA into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). Various proteins can form the RISC, which can lead to variability in specificity for miRNA/miRNA* duplexes, binding site of the target gene, activity of miRNA (repress or activate), and which strand of the miRNA/miRNA* duplex is loaded in to the RISC.

When the miRNA strand of the miRNA:miRNA* duplex is loaded into the RISC, the miRNA* is removed and degraded. The strand of the miRNA:miRNA* duplex that is loaded into the RISC is the strand whose 5' end is less tightly paired. In cases where both ends of the miRNA:miRNA* have roughly equivalent 5' pairing, both miRNA and miRNA* may have gene silencing activity.

The RISC identifies target nucleic acids based on high levels of complementarity between the miRNA and the mRNA, especially by nucleotides 2-7 of the miRNA.

A number of studies have looked at the base-pairing requirement between miRNA and its mRNA target for achieving efficient inhibition of translation (reviewed by Bartel 2004, Cell 116-281). In mammalian cells, the first 8 nucleotides of the miRNA may be important (Doench & Sharp 2004 GenesDev 2004-504). However, other parts of the microRNA may also participate in mRNA binding. Moreover, sufficient base pairing at the 3' can compensate for insufficient pairing at the 5' (Brennecke et al, 2005 PLoS 3-e85). Computation studies, analyzing miRNA binding on whole genomes have suggested a specific role for bases 2-7 at the 5' of the miRNA in target binding but the role of the first nucleotide, found usually to be "A" was also recognized (Lewis et at 2005 Cell 120-15). Similarly, nucleotides 1-7 or 2-8 were used to identify and validate targets by Krek et al (2005, Nat Genet 37-495).

The target sites in the mRNA may be in the 5' UTR, the 3' UTR or in the coding region. Interestingly, multiple miRNAs may regulate the same mRNA target by recognizing the same or multiple sites. The presence of multiple miRNA binding sites in most genetically identified targets may indicate that the cooperative action of multiple RISCs provides the most efficient translational inhibition.

MiRNAs may direct the RISC to downregulate gene expression by either of two mechanisms: mRNA cleavage or translational repression. The miRNA may specify

cleavage of the mRNA if the mRNA has a certain degree of complementarity to the miRNA. When a miRNA guides cleavage, the cut is typically between the nucleotides pairing to residues 10 and 11 of the miRNA. Alternatively, the miRNA may repress translation if the miRNA does not have the requisite degree of complementarity to the miRNA. Translational repression may be more prevalent in animals since animals may have a lower degree of complementarity between the miRNA and binding site.

It should be noted that there may be variability in the 5' and 3' ends of any pair of miRNA and miRNA*. This variability may be due to variability in the enzymatic processing of Drosha and Dicer with respect to the site of cleavage. Variability at the 5' and 3' ends of miRNA and miRNA* may also be due to mismatches in the stem structures of the pri-miRNA and pre-miRNA. The mismatches of the stem strands may lead to a population of different hairpin structures. Variability in the stem structures may also lead to variability in the products of cleavage by Drosha and Dicer.

The term "microRNA mimic" refers to synthetic non-coding RNAs that are capable of entering the RNAi pathway and regulating gene expression. miRNA mimics imitate the function of endogenous microRNAs (miRNAs) and can be designed as mature, double stranded molecules or mimic precursors (e.g., or pre-miRNAs). miRNA mimics can be comprised of modified or unmodified RNA, DNA, RNA-DNA hybrids, or alternative nucleic acid chemistries (e.g., LNAs or 2'-O,4'-C-ethylene-bridged nucleic acids (ENA)). For mature, double stranded miRNA mimics, the length of the duplex region can vary between 13-33, 18-24 or 21-23 nucleotides. The miRNA may also comprise a total of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 nucleotides. The sequence of the miRNA may be the first 13-33 nucleotides of the pre-miRNA. The sequence of the miRNA may also be the last 13-33 nucleotides of the pre-miRNA.

It will be appreciated from the description provided herein above, that contacting cancer cells with a miRNA may be affected in a number of ways:

1. Transiently transfecting the cancer cells with the mature double stranded miRNA.
2. Stably, or transiently transfecting the cancer cells with an expression vector which encodes the mature miRNA.

3. Stably, or transiently transfecting the cancer cells with an expression vector which encodes the pre-miRNA. The pre-miRNA sequence may comprise from 45-90, 60-80 or 60-70 nucleotides. The sequence of the pre-miRNA may comprise a miRNA and a miRNA* as set forth herein. The sequence of the pre-miRNA may also be that of a pri-miRNA excluding from 0-160 nucleotides from the 5' and 3' ends of the pri-miRNA.

4. Stably, or transiently transfecting the cancer cells with an expression vector which encodes the pri-miRNA. The pri-miRNA sequence may comprise from 45-30,000, 50-25,000, 100-20,000, 1,000-1,500 or 80-100 nucleotides. The sequence of the pri-miRNA may comprise a pre-miRNA, miRNA and miRNA*, as set forth herein, and variants thereof.

Another agent capable of downregulating a SYNJ2 is a DNAzyme molecule capable of specifically cleaving an mRNA transcript or DNA sequence of the SYNJ2. DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 1997;943:4262) A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 199; for rev of DNAzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNAzymes recognizing single and double-stranded target cleavage sites have been disclosed in U.S. Pat. No. 6,326,174 to Joyce et al. DNAzymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis in vivo (Itoh et al, 20002, Abstract 409, Ann Meeting Am Soc Gen Ther wwwdotasgtdotorg). In another application, DNAzymes complementary to bcr-ab1 oncogenes were successful in inhibiting the oncogenes expression in leukemia cells, and lessening relapse rates in autologous bone marrow transplant in cases of CML and ALL.

Downregulation of a SYNJ2 can also be effected by using an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the SYNJ2.

Design of antisense molecules which can be used to efficiently downregulate a SYNJ2 must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which specifically binds the designated mRNA within cells in a way which inhibits translation thereof.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types [see, for example, Luft J Mol Med 76: 75-6 (1998); Kronenwett et al. Blood 91: 852-62 (1998); Rajur et al. Bioconjug Chem 8: 935-40 (1997); Lavigne et al. Biochem Biophys Res Commun 237: 566-71 (1997) and Aoki et al. (1997) Biochem Biophys Res Commun 231: 540-5 (1997)].

In addition, algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton et al. Biotechnol Bioeng 65: 1-9 (1999)].

Such algorithms have been successfully used to implement an antisense approach in cells. For example, the algorithm developed by Walton et al. enabled scientists to successfully design antisense oligonucleotides for rabbit beta-globin (RBG) and mouse tumor necrosis factor-alpha (TNF alpha) transcripts. The same research group has more recently reported that the antisense activity of rationally selected oligonucleotides against three model target mRNAs (human lactate dehydrogenase A and B and rat gp130) in cell culture as evaluated by a kinetic PCR technique proved effective in almost all cases, including tests against three different targets in two cell types with phosphodiester and phosphorothioate oligonucleotide chemistries.

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an in vitro system were also published (Matveeva et al., Nature Biotechnology 16: 1374 - 1375 (1998)].

For example, a suitable antisense oligonucleotides targeted against the SYNJ2 mRNA (which is coding for the SYNJ2 protein) would be of the following sequences: CCCTTTGTCTGCCACCTCCT (SEQ ID NO: 10), ACCCATCTTGCTCTCTCCC (SEQ ID NO: 11) and TCTTCCTCCACCACAGCACC (SEQ ID NO: 12).

Several clinical trials have demonstrated safety, feasibility and activity of antisense oligonucleotides. For example, antisense oligonucleotides suitable for the treatment of cancer have been successfully used [Holmund et al., Curr Opin Mol Ther 1:372-85 (1999)], while treatment of hematological malignancies via antisense oligonucleotides targeting c-myb gene, p53 and Bcl-2 had entered clinical trials and had been shown to be tolerated by patients [Gerwitz Curr Opin Mol Ther 1:297-306 (1999)].

More recently, antisense-mediated suppression of human heparanase gene expression has been reported to inhibit pleural dissemination of human cancer cells in a mouse model [Uno et al., Cancer Res 61:7855-60 (2001)].

Thus, the current consensus is that recent developments in the field of antisense technology which, as described above, have led to the generation of highly accurate antisense design algorithms and a wide variety of oligonucleotide delivery systems, enable an ordinarily skilled artisan to design and implement antisense approaches suitable for downregulating expression of known sequences without having to resort to undue trial and error experimentation.

Another agent capable of downregulating a SYNJ2 is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding a SYNJ2. Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch et al., Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch et al., Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized

ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

Another agent capable of downregulating SYNJ2 would be any molecule which binds to and/or cleaves SYNJ2.

The present teachings unveiled a general mechanism underlying the roles of SYNJ2 in cellular motility.

The principles are outlined in Figure 15. Accordingly, a key event entails EGF-induced up-regulation of SYNJ2, and consequent depletion of three phosphoinositides: $PI(4,5)P_2$, $PI(3,4,5)P_3$ and $PI(3,5)P_2$. SYNJ2-mediated $PI(4,5)P_2$ dephosphorylation is paralleled by degradation of $PI(4,5)P_2$ by phospholipase C-gamma, and phosphorylation by PI3K, which generates $PI(3,4,5)P_3$. Collectively, stimulation of the three enzymes by EGF dissociates a group of $PI(4,5)P_2$ binders from the plasma membrane, and also generates $PI(4,5)P_2$-devoid endocytic vesicles. Concurrently, SYNJ2 converts $PI(3,4,5)P_3$ into $PI(3,4)P_2$, which is essential for invadopodia formation. Once in place, $PI(3,4)P_2$ binds TKS5 and nucleates a Dynamin and Cortactin-centered complex that enables Cofilin to generate actin barbed ends within invadopodia. According to the present results, SYNJ2 is involved also in the next invadopodia maturation steps, namely secretion of MMPs and delivery of MT1-MMP and other surface molecules, such as CD44. In a similar way, SYNJ2 controls delivery of EGFRs and integrin to the leading edge, and likely activates Cofillin, a pivotal event dictating formation of lamellipodial protrusions.

These findings can be harnessed towards identifying SYNJ2 inhibitors, which are putative inhibitors of tumor metastasis.

Thus, according to an aspect of the invention there is provided a method of identifying a putative inhibitor of tumor metastasis, the method comprising analyzing SYNJ2-mediated processing of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ in the presence of a test agent, wherein a decreased processing of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ in said presence of said test

agent as compared to same in an absence thereof is indicative of a putative inhibitor of tumor metastasis.

The test agent may be a biomolecule (protein e.g., a peptide or an antibody, nucleic acid molecule e.g., silencing agent, a carbohydrate, a lipid or a combination of same) or a small molecule (e.g., chemical).

The method can be effected in vivo or in vitro. The latter may be implemented in a cellular system or using a cell-free system.

An exemplary assay, involves analyzing SYNJ2-mediated processing of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ by a competition assay.

Accordingly, the competition assay tests displacement of a $PI(3,4)P_2$ binding domain from a complex comprising the $PI(3,4)P_2$ binding domain bound to $PI(3,4)P_2$.

According to an exemplary embodiment, a fluorescence polarization competitive assay is employed. The assay relies on the principle that once molecules bind a sequestering bigger element (e.g., a protein) their movement in space is significantly decreased. This phenomenon can be detected and measured using a fluorescent probe, which allows assaying fluorescent polarization following measurements from the parallel and perpendicular planes of the sample. Accordingly, unbound fluorescent molecules in solution give rise to very low polarization readings, but when a detector (e.g., a binding protein) that binds (sequesters) these molecules is added to the solution, the fluorescent molecules are stabilized in a confined composition that increases the polarization readings in the solution.

For example, the assay may comprise a $PI(3,4)P_2$ binding domain (e.g.; PH-domain e.g.; Tapp1 PH domain, SEQ ID NOs: 15-16) and a fluorescent $PI(3,4)P_2$, along with a recombinant SYNJ2 and its non-fluorescent substrate, $PI(3,4,5)P_3$. The product of SYNJ2'S catalytic activity displaces the fluorescent $PI(3,4)P_2$, thus decreasing fluorescence polarization.

According to specific embodiments a commercial 5' PI(3,4,5)P3 Phosphatase Activity Fluorescence Polarization Assay is used (e.g.; Echelon Bioscience, cat. no. K-1400).

According to specific embodiments, a reaction mix comprising SYNJ2 and PI(3,4,5)P3, as a substrate, is incubated under conditions which allow catalytic activity (dephosphorylation) of SYNJ2, with or without a test agent is prepared. The test agent

may be, for example, a small molecule, a nucleic acid molecule, a peptide, an antibody, a carbohydrate or a combination of same. Following incubation, the solution containing the PI(3,4)P2 products is mixed with a mixture of PI(3,4)P2 binding protein (e.g.; PH-domain of Tapp1, SEQ ID NO: 15) and a fluorescent PI(3,4)P2 and fluorescence polarization is measured. The polarization values measured in this assay decreases as the bound fluorescent PI(3,4)P2 molecules are being displaced by un-labeled PI(3,4)P2 produced by the enzymatic activity of SYNJ2 and the amount of unbound fluorescent PI(3,4)P2 molecules in the solution increases. In the case where the value of fluorescence polarization in the presence of the test agent increases in comparison to the value in the absence of the test agent, the test agent is a putative SYNJ2 inhibitor.

Once identified, the functionality of the test agent as an anti metastatic drug is further substantiated using relevant assays, such as the gelatin-zymography assay, transwell assay and test animals as exemplified further below.

Using this methodology the present inventors have identified a number of small molecules which can be used as SYNJ2 inhibitors according to some embodiments of the present invention. These molecules are depicted in Figure 19 and shown in Table 2 of Example 10, hereinbelow.

As mentioned, the inhibitor of SYNJ2 is administered in addition to an inhibitor of a cell surface receptor associated with an onset or progression of cancer. According to an embodiment of the invention, the receptor is an oncogene.

Examples of receptors which may be targeted according to the present teachings are receptor tyrosine kinases such as those EGFR, PDGFR, VEGFR, FGFR and ErbB-2.

Other surface molecules which can be targeted include integrins matrix metallo proteinases (MMPs), dynamin, TKS5 and CD44.

Inhibitors of cell surface molecules are well known in the art. A non-limiting list of such inhibitors is provided infra.

Thus for example, the identification of EGFR as an oncogene has led to the development of anticancer therapeutics directed against EGFR.

Cetuximab and panitumumab are examples of monoclonal antibody inhibitors. Other monoclonals in clinical development are zalutumumab, nimotuzumab, and matuzumab. The monoclonal antibodies block the extracellular ligand binding domain.

With the binding site blocked, signal molecules can no longer attach there and activate the tyrosine kinase.

Another method is using small molecules to inhibit the EGFR tyrosine kinase, which is on the cytoplasmic side of the receptor. Without kinase activity, EGFR is unable to activate itself, which is a prerequisite for binding of downstream adaptor proteins. Ostensibly by halting the signaling cascade in cells that rely on this pathway for growth, tumor proliferation and migration is diminished. Gefitinib, erlotinib, and lapatinib (mixed EGFR and ERBB2 inhibitor) are examples of small molecule kinase inhibitors. Other examples include, Iressa and Tarceva directly target the EGFR.

HER2 is the target of the monoclonal antibody trastuzumab (marketed as Herceptin). Trastuzumab is effective only in cancers where HER2 is over-expressed. Another monoclonal antibody, Pertuzumab, which inhibits dimerization of HER2 and HER3 receptors, was approved by the FDA for use in combination with trastuzumab in June 2012.

Additionally, NeuVax (Galena Biopharma) is a peptide-based immunotherapy that directs "killer" T cells to target and destroy cancer cells that express HER2.

The expression of HER2 is regulated by signaling through estrogen receptors. Estradiol and tamoxifen acting through the estrogen receptor down-regulate the expression of HER2.

Examples of antibodies which can be used according to the present teachings are listed below and are by no way meant to be limiting.

Table 1

Antibody	Brand name	Approval date	Type	Target	Approved treatment(s)
Alemtuzumab	Campath	2001	humanized	CD52	Chronic lymphocytic leukemia
Bevacizumab	Avastin	2004	humanized	vascular endothelial growth factor	colorectal cancer

Brentuximab vedotin	Adcetris	2011	chimeric	CD30	Hodgkin lymphoma, Anaplastic large-cell lymphoma
Cetuximab	Erbitux	2004	chimeric	epidermal growth factor receptor	colorectal cancer
Gemtuzumab ozogamicin	Mylotarg	2000	humanized	CD33	acute myelogenous leukemia (with calicheamicin)
Ibritumomab tiuxetan	Zevalin	2002	murine	CD20	non-Hodgkin lymphoma (with yttrium-90 or indium-111)
Panitumumab	Vectibix	2006	human	epidermal growth factor receptor	colorectal cancer
Rituximab	Rituxan, Mabthera	1997	chimeric	CD20	non-Hodgkin lymphoma
Trastuzumab	Herceptin	1998	humanized	ErbB2	breast cancer

The inhibitors of the SYNJ2 and optionally the inhibitor of the cell surface receptor as described herein can be administered to the subject *per se* or in a pharmaceutical composition where it is mixed with suitable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the inhibitor of the SYNJ2 (and optionally the inhibitor of the cell surface receptor) accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, intraperitoneal, intranasal, or intraocular injections.

Conventional approaches for drug delivery to the central nervous system (CNS) include: neurosurgical strategies (e.g., intracerebral injection or intracerebroventricular infusion); molecular manipulation of the agent (e.g., production of a chimeric fusion protein that comprises a transport peptide that has an affinity for an endothelial cell surface molecule in combination with an agent that is itself incapable of crossing the BBB) in an attempt to exploit one of the endogenous transport pathways of the BBB; pharmacological strategies designed to increase the lipid solubility of an agent (e.g., conjugation of water-soluble agents to lipid or cholesterol carriers); and the transitory disruption of the integrity of the BBB by hyperosmotic disruption (resulting from the infusion of a mannitol solution into the carotid artery or the use of a biologically active agent such as an angiotensin peptide). However, each of these strategies has limitations, such as the inherent risks associated with an invasive surgical procedure, a size limitation imposed by a limitation inherent in the endogenous transport systems,

potentially undesirable biological side effects associated with the systemic administration of a chimeric molecule comprised of a carrier motif that could be active outside of the CNS, and the possible risk of brain damage within regions of the brain where the BBB is disrupted, which renders it a suboptimal delivery method.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

The term "tissue" refers to part of an organism consisting of cells designed to perform a function or functions. Examples include, but are not limited to, brain tissue, retina, skin tissue, hepatic tissue, pancreatic tissue, bone, cartilage, connective tissue, blood tissue, muscle tissue, cardiac tissue brain tissue, vascular tissue, renal tissue, pulmonary tissue, gonadal tissue, hematopoietic tissue.

Pharmaceutical compositions of some embodiments of the invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with some embodiments of the invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use

can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to some embodiments of the invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules

and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of some embodiments of the invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of some embodiments of the invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (SYNJ2 inhibitor) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., cancer or metastatic cancer) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide SYNJ2 inhibitor levels of the active ingredient are sufficient to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of some embodiments of the invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a

form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

In line with the contribution of SYNJ2 to cell migration, the present inventors observed a significant up-regulation of SYNJ2 mRNA and protein levels in aggressive subtypes of cancer, suggesting that SYNJ2 may be used as a prognostic marker.

Thus, according to an aspect of the invention there is provided a method of prognosing cancer in a subject in need thereof, the method comprising determining a level or activity of SYNJ2 in a cancer cell of the subject, wherein an upregulation in said level of activity of said SYNJ2 in said cancer cell of the subject compared to same in a cell of an unaffected control sample, is indicative of a poor prognosis.

As used herein the term "prognosing" refers to determining the outcome of the disease (cancer).

As used herein "poor prognosis" refers to increased risk of recurrence of the disease and/or increased risk of death due to the disease.

As used herein the term "level" refers to expression level at the DNA (gene amplification), RNA or protein.

As used herein "SYNJ2 activity" refers to primarily to its phosphatase activity i.e., converting $PI(3,4,5)P_3$ to $PI(3,4)P_2$.

According to a specific embodiment, the activity is assayed using an in vitro activity assay.

In vitro activity assays: In these methods the activity of a particular enzyme (in this case phosphatase) is measured in a protein mixture extracted from the cells. The activity can be measured in a spectrophotometer well using colorimetric methods or can be measured in a non-denaturing acrylamide gel (*i.e.*, activity gel). Following electrophoresis the gel is soaked in a solution containing a substrate and colorimetric reagents. The resulting stained band corresponds to the enzymatic activity of the

protein of interest. If well calibrated and within the linear range of response, the amount of enzyme present in the sample is proportional to the amount of color produced. An enzyme standard is generally employed to improve quantitative accuracy.

A specific assay for SYNJ2 is described above, where the $PI(3,4,5)P_3$ to $PI(3,4)P_2$ conversion activity is tested.

Methods of detecting expression and/or activity of proteins

Protein expression of SYNJ2 can be determined using methods known in the arts.

Enzyme linked immunosorbent assay (ELISA): This method involves fixation of a sample (e.g., fixed cells or a proteinaceous solution) containing a protein substrate to a surface such as a well of a microtiter plate. A substrate specific antibody coupled to an enzyme is applied and allowed to bind to the substrate. Presence of the antibody is then detected and quantitated by a colorimetric reaction employing the enzyme coupled to the antibody. Enzymes commonly employed in this method include horseradish peroxidase and alkaline phosphatase. If well calibrated and within the linear range of response, the amount of substrate present in the sample is proportional to the amount of color produced. A substrate standard is generally employed to improve quantitative accuracy.

Western blot: This method involves separation of a substrate from other protein by means of an acrylamide gel followed by transfer of the substrate to a membrane (e.g., nylon or PVDF). Presence of the substrate is then detected by antibodies specific to the substrate, which are in turn detected by antibody binding reagents. Antibody binding reagents may be, for example, protein A, or other antibodies. Antibody binding reagents may be radiolabeled or enzyme linked as described hereinabove. Detection may be by autoradiography, colorimetric reaction or chemiluminescence. This method allows both quantitation of an amount of substrate and determination of its identity by a relative position on the membrane which is indicative of a migration distance in the acrylamide gel during electrophoresis.

Radio-immunoassay (RIA): In one version, this method involves precipitation of the desired protein (*i.e.*, the substrate) with a specific antibody and radiolabeled antibody binding protein (e.g., protein A labeled with I^{125}) immobilized on a

precipitable carrier such as agarose beads. The number of counts in the precipitated pellet is proportional to the amount of substrate.

In an alternate version of the RIA, a labeled substrate and an unlabelled antibody binding protein are employed. A sample containing an unknown amount of substrate is added in varying amounts. The decrease in precipitated counts from the labeled substrate is proportional to the amount of substrate in the added sample.

Fluorescence activated cell sorting (FACS): This method involves detection of a substrate *in situ* in cells by substrate specific antibodies. The substrate specific antibodies are linked to fluorophores. Detection is by means of a cell sorting machine which reads the wavelength of light emitted from each cell as it passes through a light beam. This method may employ two or more antibodies simultaneously.

Immunohistochemical analysis: This method involves detection of a substrate *in situ* in fixed cells by substrate specific antibodies. The substrate specific antibodies may be enzyme linked or linked to fluorophores. Detection is by microscopy and subjective or automatic evaluation. If enzyme linked antibodies are employed, a colorimetric reaction may be required. It will be appreciated that immunohistochemistry is often followed by counterstaining of the cell nuclei using for example Hematoxyline or Giemsa stain.

In situ activity assay: According to this method, a chromogenic substrate is applied on the cells containing an active enzyme and the enzyme catalyzes a reaction in which the substrate is decomposed to produce a chromogenic product visible by a light or a fluorescent microscope.

Alternatively or additionally, the level of SYNJ2 is detected at the RNA level using methods which are well known in the arts and some are described infra.

Methods of detecting the expression level of RNA

The expression level of the RNA in the cells of some embodiments of the invention can be determined using methods known in the arts.

Northern Blot analysis: This method involves the detection of a particular RNA in a mixture of RNAs. An RNA sample is denatured by treatment with an agent (e.g., formaldehyde) that prevents hydrogen bonding between base pairs, ensuring that all the RNA molecules have an unfolded, linear conformation. The individual RNA molecules are then separated according to size by gel electrophoresis and transferred to a

nitrocellulose or a nylon-based membrane to which the denatured RNAs adhere. The membrane is then exposed to labeled DNA probes. Probes may be labeled using radio-isotopes or enzyme linked nucleotides. Detection may be using autoradiography, colorimetric reaction or chemiluminescence. This method allows both quantitation of an amount of particular RNA molecules and determination of its identity by a relative position on the membrane which is indicative of a migration distance in the gel during electrophoresis.

RT-PCR analysis: This method uses PCR amplification of relatively rare RNAs molecules. First, RNA molecules are purified from the cells and converted into complementary DNA (cDNA) using a reverse transcriptase enzyme (such as an MMLV-RT) and primers such as, oligo dT, random hexamers or gene specific primers. Then by applying gene specific primers and Taq DNA polymerase, a PCR amplification reaction is carried out in a PCR machine. Those of skills in the art are capable of selecting the length and sequence of the gene specific primers and the PCR conditions (*i.e.*, annealing temperatures, number of cycles and the like) which are suitable for detecting specific RNA molecules. It will be appreciated that a semi-quantitative RT-PCR reaction can be employed by adjusting the number of PCR cycles and comparing the amplification product to known controls.

RNA in situ hybridization stain: In this method DNA or RNA probes are attached to the RNA molecules present in the cells. Generally, the cells are first fixed to microscopic slides to preserve the cellular structure and to prevent the RNA molecules from being degraded and then are subjected to hybridization buffer containing the labeled probe. The hybridization buffer includes reagents such as formamide and salts (e.g., sodium chloride and sodium citrate) which enable specific hybridization of the DNA or RNA probes with their target mRNA molecules *in situ* while avoiding non-specific binding of probe. Those of skills in the art are capable of adjusting the hybridization conditions (*i.e.*, temperature, concentration of salts and formamide and the like) to specific probes and types of cells. Following hybridization, any unbound probe is washed off and the bound probe is detected using known methods. For example, if a radio-labeled probe is used, then the slide is subjected to a photographic emulsion which reveals signals generated using radio-labeled probes; if the probe was labeled with an enzyme then the enzyme-specific substrate is added for the formation of a

colorimetric reaction; if the probe is labeled using a fluorescent label, then the bound probe is revealed using a fluorescent microscope; if the probe is labeled using a tag (e.g., digoxigenin, biotin, and the like) then the bound probe can be detected following interaction with a tag-specific antibody which can be detected using known methods.

In situ RT-PCR stain: This method is described in Nuovo GJ, et al. [Intracellular localization of polymerase chain reaction (PCR)-amplified hepatitis C cDNA. Am J Surg Pathol. 1993, 17: 683-90] and Komminoth P, et al. [Evaluation of methods for hepatitis C virus detection in archival liver biopsies. Comparison of histology, immunohistochemistry, *in situ* hybridization, reverse transcriptase polymerase chain reaction (RT-PCR) and *in situ* RT-PCR. Pathol Res Pract. 1994, 190: 1017-25]. Briefly, the RT-PCR reaction is performed on fixed cells by incorporating labeled nucleotides to the PCR reaction. The reaction is carried on using a specific *in situ* RT-PCR apparatus such as the laser-capture microdissection PixCell I LCM system available from Arcturus Engineering (Mountainview, CA).

DNA microarrays/DNA chips:

The expression of thousands of genes may be analyzed simultaneously using DNA microarrays, allowing analysis of the complete transcriptional program of an organism during specific developmental processes or physiological responses. DNA microarrays consist of thousands of individual gene sequences attached to closely packed areas on the surface of a support such as a glass microscope slide. Various methods have been developed for preparing DNA microarrays. In one method, an approximately 1 kilobase segment of the coding region of each gene for analysis is individually PCR amplified. A robotic apparatus is employed to apply each amplified DNA sample to closely spaced zones on the surface of a glass microscope slide, which is subsequently processed by thermal and chemical treatment to bind the DNA sequences to the surface of the support and denature them. Typically, such arrays are about 2 x 2 cm and contain about individual nucleic acids 6000 spots. In a variant of the technique, multiple DNA oligonucleotides, usually 20 nucleotides in length, are synthesized from an initial nucleotide that is covalently bound to the surface of a support, such that tens of thousands of identical oligonucleotides are synthesized in a small square zone on the surface of the support. Multiple oligonucleotide sequences from a single gene are synthesized in neighboring regions of the slide for analysis of

expression of that gene. Hence, thousands of genes can be represented on one glass slide. Such arrays of synthetic oligonucleotides may be referred to in the art as "DNA chips", as opposed to "DNA microarrays", as described above [Lodish et al. (eds.). Chapter 7.8: DNA Microarrays: Analyzing Genome-Wide Expression. In: Molecular Cell Biology, 4th ed., W. H. Freeman, New York. (2000)].

The prognosis can be substantiated by using Gold standard methods e.g., imaging methods, biopsy sampling, marker expression, immunohistochemistry and the like.

The following is a specific example for breast cancer but is by no way meant to be limiting. The prognosis of breast cancer is usually determined by the disease stage (TNM stage) after surgery that assesses the size of tumor (T), the status of metastasis to adjacent lymph nodes (N), and the presence or absence of distant metastasis to other organs (M). The prognosis of patients classified according to TNM stage is different even in the same stage. In other words, in the same stage of breast cancer, the prognosis can be determined by the expression of estrogen or progesterone receptor (ER or PR) and the over-expression of HER2 protein or the amplification of the gene.

The agents of some embodiments of the invention which are described hereinabove for detecting the SYNJ2 may be included in a diagnostic kit/article of manufacture preferably along with appropriate instructions for use and labels indicating FDA approval for use in diagnosing and/or assessing cancer stage and/or prognosis.

Such a kit can include, for example, at least one container including at least one of the above described diagnostic agents (e.g., anti SYNJ2 antibody e.g., along with anti-HER2 and/or anti ER or oligonucleotide probes/primers for these targets) and an imaging reagent packed in another container (e.g., enzymes, secondary antibodies, buffers, chromogenic substrates, fluorogenic material). The kit may also include appropriate buffers and preservatives for improving the shelf-life of the kit.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the

additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof. Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases “ranging/ranges between” a first indicate number and a second indicate number and “ranging/ranges from” a first indicate number “to” a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term “treating” includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination

in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); “Nucleic

Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

MATERIALS AND METHODS

Cell migration, invasion and chemotaxis assays

Cells were plated in triplicates in the upper compartment of a Transwell tray (BD Bioscience), and allowed to migrate through the intervening membrane for 18 hours. Thereafter, cells were fixed in paraformaldehyde (3%), permeabilized in Triton X-100 (0.05%) and stained with methyl violet (0.02%). Non-migrating cells, growing on the upper side of the filter, were removed and migrated cells photographed. Invasion assays were performed using BioCoat Matrigel Chambers. For chemotaxis chambers from ibidi (München, Germany) and time-lapse imaging were used. The positions of cell nuclei were tracked using ImageJ.

Phosphoinositide analyses

Cells were incubated for 30 minutes in inositol-free medium, which was changed to medium supplemented with both [^{3}H]-inositol and dialyzed serum (10%). Cells were cultured for three days, rinsed and extracted in 1M HCl followed by 1M Methanol. The cells were then scraped and extracted in chloroform, and then in methanol:0.1M EDTA pH8.0, and the organic phase evaporated. Thereafter, extracts were de-acetylated, separated by anionic-exchange HPLC (Agilent 1200) using two partisphere SAX columns (Whatman) in tandem, and a four-step gradient of ammonium

phosphate pH 6.0. The radiolabelled eluate was detected by an online flow scintillation analyzer and quantified using ProFSA software (Perkin-Elmer).

Gelatin zymography

To detect MMP-2 activity, biological samples were separated electrophoretically on 10% polyacrylamide/0.1% gelatin-embedded gels. The gels were then washed in 2.5% Triton X-100, and incubated at 37°C for 36 hours in 50 mM Tris-HCl (pH 7.5), containing 0.2 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% Brij 35, and 1 mM p-aminophenylmercuric acetate.

Metastasis tests in animals

Female CB-17 SCID mice (Harlan Laboratories, Haslett, MI; 15 per group) were implanted in the fat pad with MDA-MB-231 cells (1.4 $x10^6$ cells/mouse). Two and six weeks post implantation, mice were anesthetized, tumor sizes were measured and metastases in lymph nodes were visualized using a fluorescent binocular. For lung metastases, mice were sacrificed, lungs were removed, washed, and images were acquired using a fluorescent binocular. Two-sided Fischer's exact test was used for analysis of lymph node metastasis. Tumor growth measurements used the Exact-sig [2x1-tailed]) Mann-Whitney test.

Reagents

Unless indicated, Human recombinant growth factors and other materials were purchased from Sigma (St. Louis, MO, USA). Radioactive materials and a chemiluminescence kit for immunoblotting were obtained from Amersham (Buckinghamshire, UK). The EGFR-kinase inhibitor AG1478, MEK inhibitor U0126 and the PI3K inhibitor Wortmannin were from Calbiochem (San Diego, CA). Plates for wound-healing assays were from ibidi (Munich, Germany). 35-mm glass-bottom dishes for time-lapse imaging were purchased from MaTek (Ashland, MA). Murine monoclonal antibody (mAb) 111.6 to the EGF-receptor was generated in our laboratory. Anti-EGFR for western blot analysis was from Alexis (Lausen, Switzerland). Anti Ras-GAP and anti-AKT antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-EEA1, anti-Rab5, anti-Rab4, and anti Rac1 were from BD Transduction Laboratories (Franklin Lakes, NJ). Anti-SYNJ2 mAb was from Abnova (Taipei, Taiwan). The following secondary antibodies were used: goat anti-mouse IgG and goat anti-rabbit IgG antibodies conjugated to Horseradish peroxidase (HRP) were purchased

from Jackson ImmunoResearch Laboratories (Bar Harbor, Maine). Texas-red transferrin, goat anti mouse Alexa-488, Alexa-555 and Alexa-647 secondary antibodies were from Invitrogen (Carlsbad, CA).

- siRNA control was from "Thermo scientific Dharmacon" cat. D-001810-10-05; siRNA sequence against SYNJ2 is as set forth in SEQ ID NO: 6 - GGACAGCACUGCAGGUGUU; all shRNA were from SIGMA Israel: shRNA control- cat. SHC002; shRNA sequences against SYNJ2 used is CCGGCCGGAAGAACAGTTTGAGCAACTCGAGTTGCTCAAACTGTTCTTCCG GTTTTTG (SEQ ID NO: 9).

Cell lines and transfections

MCF10A cells were grown in DMEM:F12 (1:1) medium supplemented with antibiotics, insulin (10 μg/mL), cholera toxin (0.1 μg/mL), hydrocortisone (0.5 μg/mL), heat-inactivated horse serum (5% vol/vol), and EGF (10 ng/mL). Human mammary MDA-MB-231 cells were grown in RPMI-1640 (Gibco BRL; Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 1mM sodium pyruvate and a penicillin-streptomycin mixture (100 unit/ml; 0.1 mg/ml; Beit Haemek, Israel). The MDA-MB-231-RFP stable cell-line was a kind gift from Prof. Hadasa Degani (The Weizmann Institute of Science, Israel). Plasmid transfections were performed using Fugen-HD according to the manufacture's guidelines (Roche, Mannheim, Germany). Alternatively, for transient mRNA knockdown experiments using siRNA oligonucleotides, cells were transfected with Oligofectamine (Invitrogen).

Lentiviral vectors and virus production

Non-targeted shRNA hairpins (control) and hairpins directed against human SYNJ2 were produced in HEK-293T cells following the manufacture's guidelines (Sigma). Target cells were infected with shRNA-encoding lentiviruses supplemented with polybrene (8 μg/mL), and cultured in the presence of puromycin (2 μg/mL) for 4 days. Stable gene-specific delivery of human SYNJ2 was performed using the ViraPower lentiviral expression system (Invitrogen), following the manufacture's guidelines.

Immunofluorescence and image processing

Cells were grown on fibronectin-coated cover slips for 48 hours. Following treatments, cells were washed, permeabilized using 0.02% Triton X- 100 and 3%

paraformaldehyde, and fixed for 20 minutes. Confocal microscopy was performed using either a Zeiss LSM-710 microscope, or a spinning disk microscope (Zeiss 100×, NA 1.45; Yokogawa CSU-22; Zeiss fully automated, inverted 200 M; Photometrics HQ-CCD camera) and solid state lasers (473, 561 and 660 nm, exposure times: 0.25-1 sec), under the command of Slidebook™. 3D image stacks were acquired every 70–300 ms along the Z-axis by varying the position of the piezo electrically controlled stage (step size: 0.1-0.4 μm). Alternatively, live cell fluorescence microscopy was carried out using the DeltaVision system (Applied Precision, Issaqua, WA) and images were processed using the priism software.

Radiolabeling of EGF

Human recombinant EGF was labeled with IODOGEN as follows: EGF (5 μg) was mixed in an Iodogen-coated tube (1 mg of reagent) with $Na^{125}I$ (1mCi). Following 15 minutes of incubation at 23^0C, albumin was added to a final concentration of 0.1 mg/ml, and the mixture was separated on an Excellulose GF-5 column.

Receptor downregulation assay

MDA-MB-231 cells were seeded in triplicates for each time point in 24-well plates, with an additional well plated for control. 48 hours later, cells were starved for 4 hours and stimulated with EGF (2 ng/ml) at 37°C for the indicated time intervals. Subsequently, they were placed on ice, rinsed once with binding buffer (DME medium, albumin 1 %, Hepes 20 mM, pH 7.5), and subjected to mild acid/salt wash (0.2 M Na Acetate buffer pH 4.5, 0.5 M NaCl) to remove surface-bound EGF. Thereafter, cells were incubated with a radiolabelled EGF for 1.5 hours at 4°C and rinsed with binding buffer. The control well was incubated with a radiolabelled EGF and an excess of unlabelled EGF. Finally, cells were lysed with 1M NaOH, and radioactivity was determined using a γ–counter. Data represent the percentage of receptors on the cell surface relative to time 0.

Determination of surface EGF-receptor

Cells (2 x 10^4/well) were seeded in triplicates in 24-well plates, with an additional well plated for control. Thereafter, cells were incubated with a radiolabelled EGF for 1.5 hours at 4°C and rinsed with binding buffer. The control well was incubated with a radiolabelled EGF and an excess of unlabelled EGF. Finally, cells

were lysed in 1M NaOH solution and radioactivity was determined. Data represent the percentage of receptors on the cell surface relative to control cells.

Immunoblotting analysis

Cells were washed briefly with ice-cold saline, and scraped in a buffered detergent solution (25 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Na-deoxycholate, 1% NP-40, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4 and a protease inhibitor cocktail diluted at 1:1000). For equal gel loading, protein concentrations were determined by using the BCA (Pierce) reagent. Following gel electrophoresis, proteins were transferred to a nitrocellulose membrane. Membranes were blocked in TBST buffer (0.02 M Tris-Hcl (pH 7.5), 0.15 M NaCl and 0.05% Tween 20) containing 10% low-fat milk, blotted with a primary antibody for 1 hour, washed with TBST and incubated for 30 minutes with a secondary antibody conjugated to HRP.

Wound healing (scratch) assays

Wound healing assays were performed according to manufacturer's protocol (iBidi, Germany). Briefly, MCF10A cells were trypsinized, re-suspended in EGF-deprived medium (7.0×10^5 cells/mL) and 70 µl plated into each well, resulting in a confluent layer within 24 hours. Thereafter, Culture–Inserts were removed by using sterile tweezers and cells were allowed to migrate for 2 hours.

Scanning and transmission electron microscopy

Cells were fixed in saline supplemented with 4% paraformaldehyde and 2% sucrose. Samples were washed and subjected to a second fixative (3% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer supplemented with 1% sucrose and 5 mM $CaCl_2$, pH 7.4). Cells were washed in 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide in cacodylate buffer for 1 hour. For scanning electron microscopy (SEM), the post-fixed samples were washed twice and treated with 1% tannic acid for 5 minutes followed by another wash and treatment with 1% uranyl acetate for 30 minutes. Samples were dehydrated in graded ethanol, and made conductive by sputtering with a gold-palladium film. The samples were photographed using a scanning electron microscope (Leo Supra 55/Vp Zeiss, Thornwood, NY).

Receptor recycling assay

MDA-MB-231 cells were pre-incubated for 30 minutes at 37ºC with Alexa Fluor 488-transferrin (25 µg/ml in serum-free medium) or for 10 minutes with Alexa Fluor 488-EGF (40 ng/mL). Surface-bound ligands were detached by incubation for 30 minute at 4°C in an acidic buffer (150mM NaCl, 1mM $MgCl_2$, 0.125mM $CaCl_2$, 0.1M glycine), prior to transfer to 37ºC for the indicated time intervals, to allow for recycling of the internalized ligands. Cells were analyzed either by imaging or by FACS.

Real-time cell impedance analysis

Measurements of cell spreading and adhesion were recorded by using the RTCA-Xcelligence System (Roche Diagnostics, Mannheim, Germany). Gold microelectrode E-plates-16 were washed once in saline. Cells (2,500 per well) were first seeded and then impedance data (cell index; derived as a relative change in measured electrical impedance) was recorded in the indicated intervals. The data was analyzed using software package 1.2 provided by the manufacturer.

TAPP1-PH domain expression and purification

A construct encoding TAPP1-PH domain and an N-terminal Flag tag and C-terminal 6xHis tag (SEQ ID NO.: 13, Figure 18) was cloned into pET28 plasmid and expressed in *E. coli* BL21(DE3) following induction with 200 µM IPTG. The bacteria were grown at 15 °C and then lysed with a cell disrupter. Cell debris was removed by centrifugation and the protein was captured on a Ni column (HisPrep FF 16/10, GE Healthcare) equilibrated with 50 mM Tris pH 8, 0.5 M NaCl and 20 mM imidazole. The protein was eluted in the same buffer containing 0.5 M Imidazole. Fractions containing the TAPP1-PH domain were injected into a size exclusion column (Hiload_26/60_Superdex 75, GE Healthcare) equilibrated with buffer containing 50 mM Tris pH 8 and 100 mM NaCl. The pooled peak containing TAPP1-PH domain was diluted three fold with 20 mM sodium phosphate buffer pH 7.2 and loaded onto a cation exchange column (HiTrap_SP_FF_5ml, GE Healthcare) equilibrated with the same phosphate buffer. The pure protein was eluted from the column with a linear gradient of the phosphate buffer containing 1 M NaCl (TAPP1-PH domain elutes at 200 mM NaCl). The fractions containing the pure TAPP1-PH domain as evaluated by SDS-PAGE were pooled together and protein concentration was determined by Bradford

reagent and OD_{280} (extinction coefficient of 20,520) quantization. The protein was divided into aliquots, flash frozen with liquid nitrogen and stored at -80 °C.

5' phosphatase activity of SYNJ2

Measurements of the ability of SYNJ2 to hydrolyze the 5-phosphate from PI(3,4,5)P3 to generate PI(3,4)P2 were recorded by a competitive assay, based on fluorescence polarization as a read out. Stabilizing SOP lipid mix (x50) was prepared in a glass tube by adding 100 µl of SOPS (Avanti Inc., 50 mg / ml in chloroform) and 50 µl Cholesterol (Sigma Aldrich, 10 mg / ml in chloroform). The mix was air-dried using gentle nitrogen steam to evaporate the chloroform. The evaporated lipid mix was then re-suspended in 10 ml of 0.25 mg / ml $C_{12}E_8$ (Avanti Inc.) by 1 minute vortex at room temperature. A reaction mix comprising PBS, DTT, MgCl2 (all from Sigma Aldrich), SOP lipid mix (x50), full length purified SYNJ2 (OriGene, cat no. TP315160) and PI(3,4,5)P3 (Echelon Bioscience, cat no. P-3908), with or without a tested compound. Once PI(3,4,5)P3 was added, the reaction mix was incubated in 33 °C for 8 minutes to allow production of PI(3,4)P2 by SYNJ2 5'-phosphatase activity. Following incubation the of reaction was stopped by adding a detection mix comprising PBS, DTT, detector proteins (PH domain of TAPP1), SOP lipid mix (x50), fluorescently-labeled PI(3,4)P2 (Echelon Bioscience, cat no. C34M6) and EDTA (Sigma Aldrich). Fluorescence polarization was measured using an appropriate plate reader and filter set compatible with BODIPY® TMR dye (550 nm excitation / 580 nm polarizing emission filters). Unlabeled PI(3,4)P2 control was purchased from Echelon Bioscience (Cat no. P-3408).

EXAMPLE 2

EGF-INDUCED ELEVATED EXPRESSION OF SYNJ2 PROMOTES MAMMARY CELL INVASION

Human mammary epithelial cells (MCF10A) exhibit strong migratory and invasive phenotypes when cultured with EGF family ligands (Figures 1A and 1B), but treatment with serum is insufficient to propel cell motility. Co-incubation of EGF along with inhibitors of EGFR (AG1478), MEK (U0126) or PI3K (Wortmannin) reduced motility (Figure 1C), suggesting that both MEK/ERK and PI3K activities are essential for EGF-induced migration. Importantly, the EGFR-induced motile phenotype associates with transcriptional upregulation of 425 genes (Amit et al., 2007). To identify

genes that propel metastasis, this gene-set was intersected with a larger set of genes that undergo up-regulation during *in vivo* selection of metastatic sub-clones of breast cancer cells (Minn et al., 2005). The group of 23 overlapping genes (Figure 1D) included the gene encoding Synaptojanin-2 (SYNJ2), a lipid phosphatase implicated in glioma cell invasion (Chuang et al., 2004). EGF-induced up-regulation of SYNJ2 was validated by PCR and immunoblotting (Figures 2A and 2B).

Next, MCF10A cells were transformed and sub-cloned to stably overexpress SYNJ2 (as a GFP fusion; SYNJ2-OX, Figure 1E). When plated in EGF-deprived medium, SYNJ2-OX cells displayed a pro-migratory phenotype characterized by membrane ruffling (Figure 2C), along with enhanced basal and EGF-induced migratory and invasive capacities (Figures 2D and 2C). Conversely, knockdown of SYNJ2 using small interfering RNAs (siRNAs; Figure 1G) significantly reduced cell invasion, as well as individual and collective migration (Figures 2E, 1H and 1J). In conclusion, EGF-induced up-regulation of SYNJ2 drives a robust invasive phenotype of mammary cells.

EXAMPLE 3

THE PHOSPHATASE ACTIVITY OF SYNJ2 IS ESSENTIAL FOR INVASIVENESS OF MAMMARY CELLS

To enable in vivo experiments, the highly metastatic MDA-MB-231 breast cancer Red fluorescent protein (RFP) expressing cells were used to generate subclones overexpressing either SYNJ2 or LacZ (control), as well as sub-clones expressing shControl or SYNJ2-specific hairpins (shSYNJ2; Figure 3A). Enhanced expression of SYNJ2 conferred an elongated morphology in 2D cultures (Figure 3B) and extensive invasive arms, when cells were grown in 3D cultures (Figure 4A). Conversely, SYNJ2 knockdown abrogated invasive patterns (Figure 4B). Similarly, over-expression enhanced invasive capacities by ~3.2 fold (Figure 3B), and knockdown (Figure 3C) inhibited migration and invasion (Figure 3D). To examine roles for the catalytic phosphatase activity, shSYNJ2 cells with lentiviral particles encoding either a WT SYNJ2 or a catalytically-dead form (D388A and D726A; Figure 4C) harboring point mutations in each of the conserved WXGDXN(F/Y)R motifs (Jefferson and Majerus, 1996) within the phosphatase/nuclease domain (Pfam: PF03372). Unlike WT SYNJ2, re-expression of the mutant failed to restore the invasive capacity (Figure 4D),

indicating that the phosphatase activity of SYNJ2 is essential for the invasive phenotype.

The failure of shSYNJ2 cells to migrate was further supported by both scanning electron microscopy (Figure 4E) and F-actin staining, which revealed severe actin organization defects and an increase in cell height (Figure 4F). Importantly, also noted were actin patches clustered around circular moieties (Figure 4F; arrowheads). Accordingly, time-lapse microscopy analyses of shSYNJ2 cells confirmed the existence of abnormal intracellular vesicles, suggesting that SYNJ2 knockdown derailed vesicular trafficking. Next, the sub-cellular localization of SYNJ2 was examined. Time-lapse images of MDA-MB-231 cells expressing GFP-SYNJ2 (Figure 3E), as well as immunofluorescence using anti-SYNJ2 antibodies (Figure 3F), reflected two major patterns of SYNJ2 distribution: small peripheral assemblies, which localized to the leading edge (black arrowheads in Figure 3E), and a second population of larger assemblies, which were located closer to the cell centre (blue arrowheads). Notably, shortly after stimulation of MDA-MB-231 cells with an EGFR ligand (TGF-alpha), SYNJ2 rapidly assembled at the base of emerging lamellipodia, underneath the forming leading edge (Figures 3E, 3F). Interestingly, similar analyses performed with MCF10A cells indicated that SYNJ2 initially co-localizes with F-actin at cell-to-cell junctions, but translocates to the leading edge, typically to the base of lamellipodia, upon stimulation with EGF (Figure 3G). In conclusion, these observations indicate that growth factors regulate not only the levels of SYNJ2 expression, but also its dynamic recruitment to the leading edge.

EXAMPLE 4

RECRUITMENT OF SYNJ2 TO THE VENTRAL MEMBRANE DEPENDS ON DYNAMIN AND Rac1

To investigate the dynamics of SYNJ2's sites of localization, a stably expressing GFP-SYNJ2 MDA-MB-231 subclone (GFP-SYNJ2 cells) was generated and analyzed for the formation and consumption of GFP-SYNJ2 puncta. These were classified into kinetically distinct sub-populations: dynamic puncta that localized to ruffling membranes and puncta localized to discrete regions proximal to the cell centre (Figure 5A). Notably, GFP-SYNJ2 puncta showed minimal overlap with assemblies marked by

RFP-Clathrin light chain A (Figure 5A) or RFP-Caveolin 1 (Figure 6A), suggesting minor localization to Clathrin-coated pits or to caveolae. Importantly, newly formed peripheral puncta heralded nascent lamellipodia, as their appearance preceded local formation of lamellipodia. In contrast, the more central and stable clusters of puncta, which co-localized with actin, persisted for ~30 minutes (Figure 5B). Accordingly, tracking of individual assemblies (Figure 6B; left) revealed remarkably wide distribution of lifetimes: short-lived (~20-40s, 60% of assemblies), intermediate lifetimes, and long-lived assemblies (~10% of assemblies). Initiation of the intermediate group was followed by a continuous increase in fluorescence intensity, while the assembly remained static in terms of movement (Figure 6B; right). This dynamic pattern resembles that of Clathrin-coated pits (Ehrlich et al., 2004) and suggests the formation and consumption of trafficking intermediates.

The mostly bimodal compartmentalization of GFP-SYNJ2 at the ventral membrane was reinforced by the synchronous appearance and disappearance of fluorescence signals in experiments employing both epifluorescence (red; relatively insensitive to changes in the Z dimension) and total internal reflection microscopy (TIRF, green; limited to ~ 200 nm depth). Because puncta appeared yellow throughout their lifetime (Figure 5C), the present inventors concluded that SYNJ2 assembles within the plane of the ventral plasma membrane. By employing a panel of inhibitors it was found that the assembly was dramatically inhibited by cholesterol depletion (Figure 6C; left), suggesting that cholesterol-rich membrane microdomains are needed for SYNJ2 recruitment to the ventral membrane. A similar inhibitory effect was induced by Wortmannin (Figure 6C; right), suggesting a role for PI3K. Another requirement was revealed by employing Dyngo-4a, an inhibitor of Dynamin, the large GTPase that mediates the scission step of clathrin-dependent and clathrin-independent carriers, and whose inhibition leads to accumulation of U-shape invagination intermediates (Macia et al., 2006). Because Dyngo-4a strongly arrested the dynamic assemblies of SYNJ2 at the plasma membrane (Figure 5D), the present inventors concluded that SYNJ2 is recruited to nascent trafficking intermediates regulated by Dynamin. Because Dynamin has been implicated as a facilitator of cell migration and invasion (Kruchten and McNiven, 2006), its physical interactions with SYNJ2 was tested. This experiment confirmed

complex formation between active Dynamin and SYNJ2 (Figure 5E), in line with an extended role for Dynamin in both endocytosis and actin-based migration.

SYNJ2 can physically interact with GTP-loaded Rac1 (Malecz et al., 2000), and inducible activation of Rac1 requires internalization and subsequent recycling (Palamidessi et al., 2008). Hence, the coincidence of the peripheral puncta of SYNJ2 coincide with Rac1 was tested. Indeed, immunostaining of endogenous Rac1 revealed co-localization with peripheral puncta of GFP-SYNJ2 (Figure 5F). Moreover, inhibition of GTP loading onto Rac1 (using NSC-23766) dramatically reduced the number of GFP-SYNJ2 puncta (Figure 5G). Complementarily, SYNJ2 knockdown reduced the levels of GTP-loaded Rac1 in MDA-MB-231 cells (Figure 5H). In accord with a regulatory role for Rac1 and the actin cytoskeleton in recruiting SYNJ2 to the membrane, inhibition of actin dynamics with Latrunculin abrogated GFP-SYNJ2 dynamics (Figure 6D). Taken together, these results associate the peripheral SYNJ2 assemblies, with a dynamin-mediated endocytic pathway that depends on cholesterol, 3'-phosphoinositides, actin and active Rac1. Notably, this pathway shares several attributes with clathrin-independent carriers that enable rapid membrane and adhesion turnover at the leading edge of migrating fibroblasts (Howes et al., 2010).

EXAMPLE 5

SYNJ2 CONTROLS VESICULAR TRAFFICKING OF CELL SURFACE RECEPTORS

Although EGF-treated shSYNJ2-MCF10A cells displayed higher levels of total and phosphorylated EGFR relative to control cells, this translated to lower, rather than higher activation of ERK (Figure 7A). Along this line, it was noted that SYNJ2 knockdown trapped EGFRs in enlarged intracellular vesicles (Figure 7B). Consistent with trapping, immunoblotting of MDA-MB-231 cells similarly revealed that EGFR levels were stabilized in siSYNJ2 cells (Figure 8A), but quantification of surface EGFR by using two methods indicated significantly lower surface levels (Figure 8B). Intracellular trapping of EGFR bears functional consequences: in line with their well-characterized chemotactic function (Mouneimne et al., 2006; van Rheenen et al., 2007), EGFRs localized to the leading edge of mammary cells, but EGFRs of shSYNJ2 cells lost their polarized distribution and accumulated in large, actin-decorated vesicles

(Figure 8C). Notably, EGFR trafficking defects observed in shSYNJ2 cells could be rescued by WT SYNJ2, but not by a catalytically-dead form (Figure 7C), indicating that the phosphatase activity of SYNJ2 is essential for vesicular trafficking of EGFRs to and from the leading edge, where it mediates the chemotactic response to gradients of EGF. Consistent with this model, shSYNJ2 cells severely lost the ability to migrate along a gradient of EGF (Figure 8D).

The abnormal accumulation of EGFR in SYNJ2-depleted cells could reflect defects in EGFR delivery, arrested recycling, or impaired sorting for degradation, a process regulated by ubiquitination (Goh et al., 2010). Consistent with impaired sorting, SYNJ2-depleted cells exhibited significantly higher basal EGFR ubiquitination, which was only weakly altered in response to EGF (Figures 8E and 7D). Furthermore, despite being tagged for degradation by phosphorylation of tyrosine 1045 (a docking site for the ubiquitin ligase c-Cbl; Figure 8F), an EGF stimulation experiment confirmed normal activation (tyrosine 1068 phosphorylation) but defective degradation in shSYNJ2 cells (Figure 8G). To address a recycling defect, fluorescent ligands were employed to follow the extensive recycling of the transferrin receptor (TfR), as well as the weaker recycling of EGFR. Although TfR internalization was not affected, recycling was markedly decreased in shSYNJ2 cells and, conversely, markedly accelerated in SYNJ2-OX cells (Figures 8H and 7E). Likewise, flow cytometry analyses indicated defective recycling of fluorescent-EGF (Figure 8I), and live cell imaging confirmed ligand accumulation within the large vesicles of SYNJ2-depleted cells. In conclusion, these results indicate that SYNJ2 is essential for proper recycling of both EGFR and TfR.

EXAMPLE 6

SYNJ2 KNOCKDOWN PERTURBS HOMEOSTASIS OF PHOSPHOINOSITOL LIPIDS AND ALTERS BOTH ENDOCYTOSIS AND ADHESION

The endocytic system maintains several distinct compartments, which are defined by specific phosphoinositides (PI) (Gruenberg and Stenmark, 2004), and the present analyses uncovered strong dependency on SYNJ2. For example, by probing early endosomes for EEA1, a PI(3)P-binder, it was found that its spatial organization was markedly altered in SYNJ2-depleted cells (Figure 9A). Similarly, probing the recycling compartment using GFP-tagged Rab4, uncovered strong associations with the

circular actin patches of shSYNJ2 cells (Figure 10A). The distribution of another marker of early endosome, Rab5, also reflected dependence on SYNJ2 (Figure 10B). Whereas the number of Rab5-positive vesicles was significantly lower in shSYNJ2-depleted cells, their average size increased and they partly localized to circular actin patches (Figure 9A). To uncover underlying alterations in phosphoinositides, shCtrl and shSYNJ2 MDA-MB-231 cells that were biosynthetically labeled were compared, and thereafter their phospholipids were extracted (Figure 10C). The results showed that mainly PI(3)P, but also $PI(4,5)P_2$ and $PI(3,5)P_2$ were present at higher levels in shSYNJ2 cells, whereas PI(4)P levels remained unaltered and levels of both $PI(3,4)P_2$ and $PI(3,4,5)P_3$ were hardly detectable by this method. While these results confirm the notion that SYNJ2 targets primarily the D5 position of PIs, the present inventors assume that the rather limited global effects observed represent larger local differences. In conclusion, these observations reaffirm that SYNJ2 controls cargo sorting at the early endosome, as well as in the subsequent recycling step.

Along with recycling of RTKs like EGFR, vesicular trafficking of integrins and their interactions with downstream partners, such as Paxillin, play major roles in cell migration and focal adhesion (FA) maturation (Guo and Giancotti, 2004). Accordingly, beta-1 integrin and phosphorylated-EGFR (pEGFR) localized to FAs of MDA-MB-231 cells. By contrast, due to abnormal accumulation in large vesicles, both proteins failed to localize to the periphery of SYNJ2-depleted cells (Figures 10D, S5B and S5C). Moreover, using Paxillin as a marker of mature FAs, it was found that FAs assumed a round and relatively short appearance in shSYNJ2 cells (Figure 9D). Taken together, these observations imply that SYNJ2 is required for substrate adhesion, a scenario examined by measuring cell spreading using two methods (Figures 10E and 10F). The results demonstrated attenuated adhesion of shSYNJ2 cells, which is attributed to defective delivery of both integrins and RTKs to FAs.

EXAMPLE 7

SYNJ2 REGULATES THE ASSEMBLY OF INVADOPODIA

Matrix-based 3D cultures of MDA-MB-231 cells normally display wedge-shaped protrusions, but shSYNJ2 cells displayed roundish extensions (Figure 11A), suggesting defective matrix degradation. To test this, confocal immunofluorescence

images of MMP-9 were obtained, and it was noted that shSYNJ2 spheroids displayed a relatively sharp decrease of MMP-9 abundance at their borders (Figure 11A), likely due to impaired secretion. Indeed, zymography assays performed on conditioned media confirmed defective MMP-9 secretion by cells that were treated with siSYNJ2 oligonucleotides, but MMP-2 secretion remained unaltered (Figure 12A). Conversely, media conditioned by cells overexpressing SYNJ2 displayed a substantial increase in MMP-9 activity (Figure 11B), in line involvement of SYNJ2 in MMP secretion.

To visualize focal proteolysis, cells were plated on cross-linked fluorescent gelatin and probed for the actin-centered, matrix-degrading organelles called invadopodia (Murphy and Courtneidge, 2011). In line with previous reports, active matrix proteolysis corresponded to actin dots localized underneath the cell body. Importantly, SYNJ2-GFP puncta co-localized with these structures (Figure 11C, arrowheads), which resembled the actin-associated long-lived puncta presented in Figure 5B. Expression levels of SYNJ2 are clearly correlated with invadopodia occurrence; whereas SYNJ2 overexpression almost doubled the fraction of invadopodia-containing cells, siSYNJ2 significantly reduced the incidence of invadopodia (Figure 11D), implying causal relationships. Next, potential physical associations between SYNJ2 and Cortactin, a well-characterized marker of invadopodia, was examined and found that SYNJ2 and Cortactin co-immunoprecipitate (Figure 12B), as well as co-localize to both invadopodia and leading edges (Figure 12C). To firmly establish a driving role for SYNJ2, TKS5 was observed, a $PI(3,4)P_2$ and a binder of Cortactin that serves as a signpost of invadopodia (Courtneidge et al., 2005). As expected, endogenous TKS5 localized to multiple ventral sites of matrix degradation in control MDA-MB-231 cells, but almost no active sites were found in siSYNJ2 cells, and TKS5 lost its ventral location (Figure 6E; X-Y and Z panels). Furthermore, because invadopodial TKS5 anchors at $PI(3,4)P_2$ (Oikawa et al., 2008), a $PI(3,4)P_2$-binding domain, namely the PH domain of Tapp1 was used as a probe. Consistent with previous reports, ectopic expression of the PH domain reduced the number of invadopodia, but nevertheless the remaining signal co-localized with TKS5 and actin cores (Figure 12D). In conclusion, SYNJ2 appears necessary at a step preceding TKS5 engagement, consistent with sequential action of PI3K (Yamaguchi et al., 2011) and SYNJ2, which

respectively generate $PI(3,4,5)P_3$ and then $PI(3,4)P_2$, to anchor TKS5 at sites of EGFR-induced activation of PI3K.

In line with an EGFR-PI3K-SYNJ2 scenario, the active form of EGFR (pEGFR) was detected in proteolytically active invadopodia, but EGFRs of SYNJ2-depleted cells localized to swollen vesicles (Figure 11F). The mechanism responsible for local receptor activation remains unknown. According to one model, cleavage of pro-ligands, such as the heparin-binding EGF (HB-EGF), by a complex comprising MMP-7 and CD44, might locally stimulate EGFR (Yu et al., 2002). In line with this model, SYNJ2 abundance was correlated with secretion of EGFR ligands (Figure 11G), and detected co-localization of CD44 with the actin cores of invadopodia (Figure 12E). Likewise, using flow cytometry, it was found that surface expression of CD44 was strongly suppressed in shSYNJ2 cells relative to control cells (Figure 12F). Yet another critical step in the maturation of invadopodia is the recruitment of the membrane type-1 matrix metalloproteinase (MT1-MMP), which activates soluble MMPs (Wang and McNiven, 2012). Accordingly, it was found that in control cells MT1-MMP corresponded to sites of invadopodial protrusions, but MT1-MMP molecules of siSYNJ2 cells formed large aggregates, which were not associated with matrix degradation (Figure 9E). Taken together, these observations imply that SYNJ2 is essential for invadopodia priming, as well as for targeting to this organelle both proteases and two previously unrecognized residents, CD44 and an active EGFR.

EXAMPLE 8

SYNJ2 PROMOTES TUMOR GROWTH AND METASTATIC SPREAD IN A MAMMARY ANIMAL MODEL

To assess the effect of SYNJ2 on metastatic dissemination *in vivo* MDA-MB-231-RFP cells (and derivatives) were implanted into the mammary fat pad of female mice, and two or six weeks later measured both tumor size (Figure 13A) and metastases (Figure 13B). Primary tumor growth was significantly faster in the shCtrl and shSYNJ2+$SYNJ2^{WT}$ ('active rescue') groups, relative to the shSYNJ2 and the 'inactive rescue' (shSYNJ2+$SYNJ2^{CD}$) groups. The metastatic behavior similarly correlated with SYNJ2: the shSYNJ2 and the 'inactive rescue' group displayed significant reduction in metastatic spread to local and distant lymph nodes (Figures 13B and 14). In order to

examine distant metastases, mice were sacrificed and their lungs evaluated. Lungs of animals implanted with shSYNJ2 cells, or the 'inactive rescue' cells, showed a dramatic reduction in the number and size of metastases, compared to animals inoculated with the shCtrl or the 'active rescue' cells (Figure 13C). Taken together, these results implicate SYNJ2 in metastasis promotion.

Similarly, xenografts overexpressing SYNJ2 were monitored. As expected, SYNJ2-OX cells gave rise to faster growing tumors (Figure 13D), and they also displayed earlier onset of nodal metastases (Figure 13E). Consistent with robust lymphatic invasion, the lungs of animals implanted with SYNJ2-OX cells showed an increase in the number of metastases (Figure 13F). Next, the effect of SYNJ2 on intravasation or extravasation was tested. Hence, sub-clones of MDA-MB-231-RFP cells were either directly injected into the circulation (tail vein) of female mice and scored for lung colonization (extravasation), or they were implanted in the fat pad and scored in blood as circulating tumor cells (CTCs; intravasation). Note that these experiments took into account the size differences between the respective primary tumors. The normalized results indicated that SYNJ2 is necessary for both intravasation ($p=0.0031$) and extravasation ($p=0.0082$; Figure 13G). This conclusion was further tested by using GFP-SYNJ2 overexpressing cells (Figure 13H). Notably, the intravasation results obtained in this experiment displayed statistical significance, but the ability of SYNJ2-OX cells to better extravasate and colonize a distant organ did not reach significance, suggesting that the observed strong effects of SYNJ2 on local and distant metastasis are primarily due to enhanced intravasation into lymph and blood vessels.

EXAMPLE 9

SYNJ2 IS ASSOCIATED WITH AGGRESSIVE HUMAN BREAST TUMORS

To address SYNJ2's relevance to human cancer, the transcript levels of SYNJ2 were analyzed in the NCI-60 panel of 60 human cancer lines. In line with contribution to motile phenotypes, it was found that high transcript levels of SYNJ2 associate with mesenchymal phenotypes. Next, a set of 331 paraffin-embedded samples of breast carcinomas NJ2 were immunostained (Figure 16A). Importantly, expression intensity of SYNJ2 was positively associated with prognostically unfavorable subtypes defined by

HER2 overexpression ($p<0.001$) and/or lack of estrogen receptor ($p<0.001$). However, no significant association was found between SYNJ2 abundance and age, histological subtype, axillary lymph node status, and differentiation grade. Interestingly, staining patterns for SYNJ2 also varied; whereas HER2+ tumors exhibited mostly membranal staining, luminal and triple negative tumors displayed cytoplasmic staining (Figure 16B). To support the findings, SYNJ2 mRNA levels were analyzed in two cohorts of breast cancer specimens and an association was found with shorter patient survival rates (Figure 16C). Altogether, these observations support involvement of SYNJ2 in progression of breast cancer, but they leave open the mechanism behind transcript up-regulation.

In summary, the observations made in animals, along with the clinical data and the *in vitro* experiments, clearly indicate that dephosphorylation of inositol lipids by SYNJ2 is critical for the metastatic process, primarily because of the cardinal roles played by phosphoinositides in trafficking of cell surface molecules to and from invadopodia and the leading edge. Below is presented a working model (Figure 15) and discuss the multiple functions of SYNJ2 in the broad context of tumor progression.

EXAMPLE 10

SELECTIVE INHIBITORS OF THE 5' PHOSPHATASE ACTIVITY OF SYNJ2

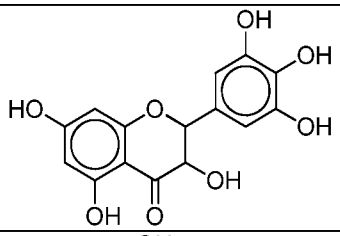
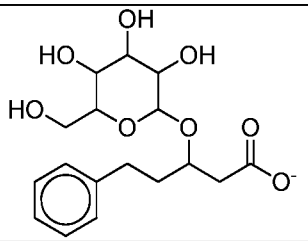
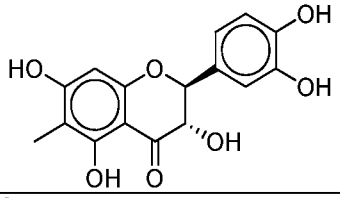
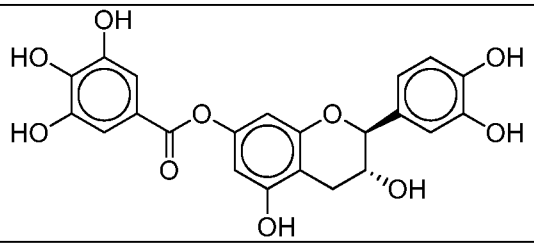
In order to identify selective inhibitors of SYNJ2 phosphatase activity, the present inventors utilized a fluorescence polarization competitive assay relying on the principle that molecules are constantly rotating and moving in space but once bound to another bigger element (e.g., a protein) their movement is dramatically limited. These changes in movement can be detected and measured using fluorescent molecules (i.e., probes) that in unbound state give rise to very low polarization readings, but when a detector (e.g., a binding protein) that binds these molecules is added to the solution, the fluorescent molecules are stabilized in a confined composition that increases the polarization readings in the solution (see Figure 17A).

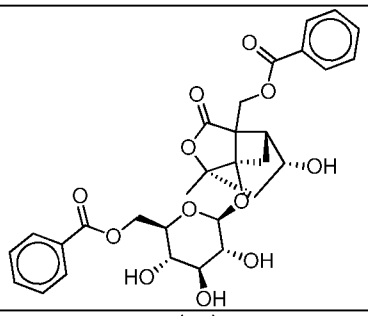
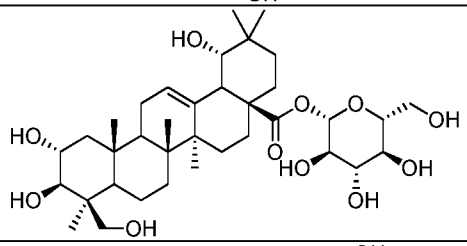
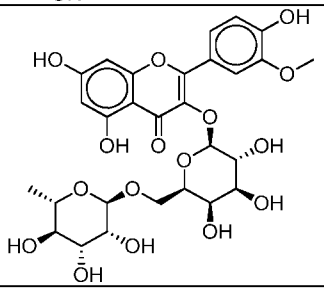
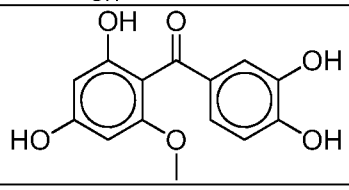
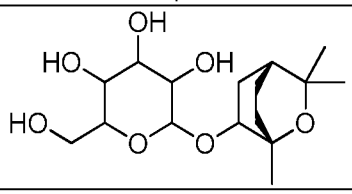
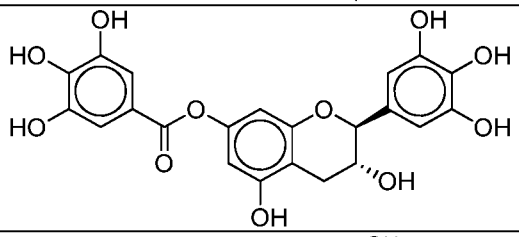
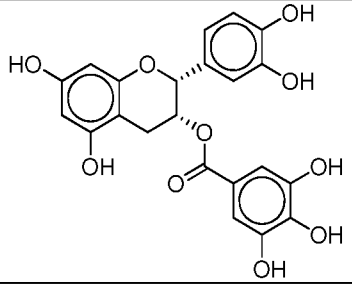
In the screen preformed, the present inventors measured the enzymatic activity of SYNJ2 to de-phosphorylate the 5' position of PI(3,4,5,)P3 to produce PI(3,4)P2, in the presence of different compounds. Once the enzymatic reaction was completed/stopped, the solution containing the PI(3,4)P2 products was mixed with a

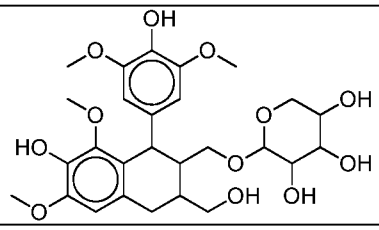
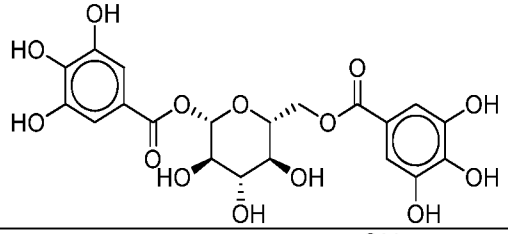
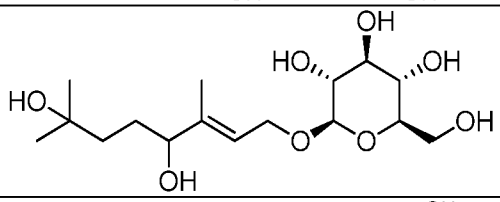
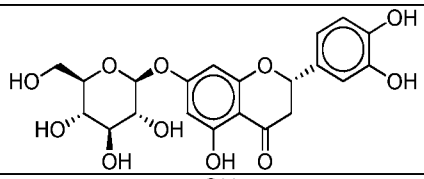
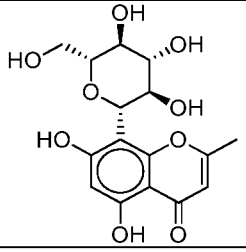
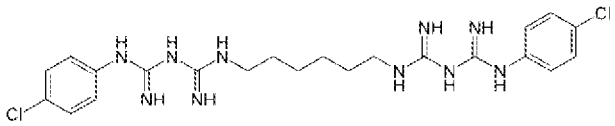
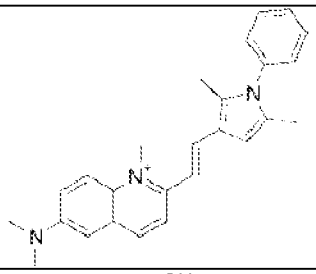
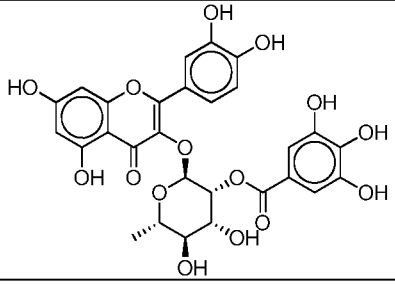
mixture of PI(3,4)P2 binding protein (detector) and a fluorescent PI(3,4)P2 (probe). The detector protein used was the purified PH-domain of Tapp1 that selectively binds PI(3,4)P2 (SEQ ID NO.: 15). As demonstrated in Figure 17B, the polarization values measured in this assay decreased as the bound PI(3,4)P2 fluorescent probes were being displaced by un-labeled PI(3,4)P2 produced by the enzymatic activity of SYNJ2 and the amount of unbound fluorescent probe in the solution increased.

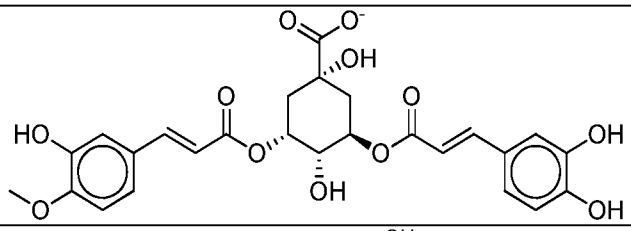
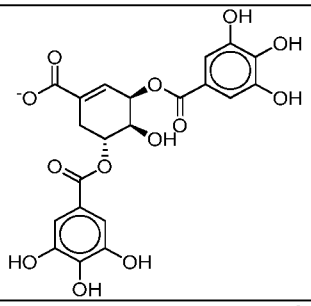
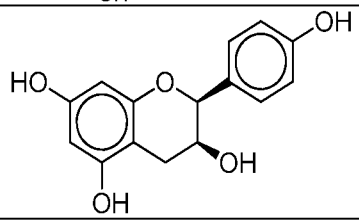
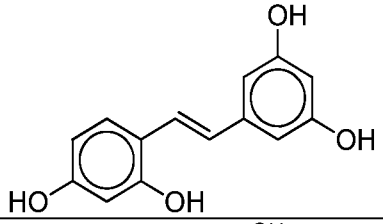
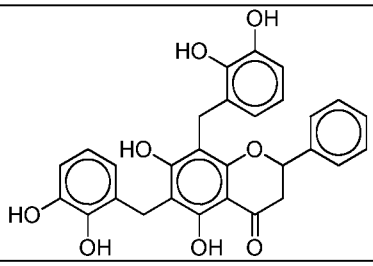
Table 2 below depicts the various compounds indentified using this method that were able to inhibit the production of PI(3,4)P2 by SYNJ2.

Table 2: Identified selective inhibitors of SYNJ2

No	AnalytiCon Cat. No. [CAS No.]	Compound name	IC50 (μM)	Structure
1	NP-001872	Ampelopsin	3.17	
2	NP-003195	3-Hydroxy-5-phenylpentanoic acid 3-D-glucopyranoside	1.87	
3	NP-012228	Cedeodarin	2.19	
4	NP-014109	(-)-Catechin-7-gallate	3.44	

5	NP-015304	Paeonivayin	6.02	
6	NP-003491	Sericoside	3.52	
7	NP-000303	Keioside	3.4	
8	NP-005201	2,4,3',4'-tetrahydroxy-6-methoxy-benzophenone	2.84	
9	NP-001585	2-Hydroxy-1,8-cineole-glucopyranoside	1.98	
10	NP-014110	(-)-Gallocatechin-7-gallate	1.075	
11	NP-002326	(-)-Epicatechin-3-gallate	2.88	

12	NP-000360		1.67	
13	NP-002973	1,6-Digalloylglucose	7.53	
14	NP-015222	3,7-dimethyloct-2-ene-1,4,7-triol 1-β-D-glucopyranoside	1.24	
15	NP-000181	Pyracanthoside	0.874	
16	NP-008708	Isobiflorin	2.71	
17	[CAS: 55-56-1]	Chlorhexidine	10	
18	[CAS: 3546-41-6]	Pyrvinium pamoate	1.25	
19	NP-003299	Quercitrin gallate	2.94	

20	NP-013254	4'-O-Methyl-3,5-di-O-caffeoylquinic acid	3.17	
21	NP-012429	3,5-Di-O-galloylshikimic acid	3.75	
22	NP-002325	Epiafzelechin	15.9	
23	NP-003143	Oxyresveratrol	6.65	
24	NP-012649	6,8-di-(2,3-dihydroxybenzyl)-pinocembrin	10.3	

DISCUSSION

The functions of SYNJ2 as an integrative master regulator of cell migration and tumor metastasis are demonstrated, likely due to its ability to control the levels of PI phospholipids acting as both second messengers and signposts determining the identity of specific membrane sub-domains. Another reflection of the multiplicity of SYNJ2's action is the mostly bimodal ventral localization to invadopodia and lamellipodia. Accordingly, SYNJ2 forms physical complexes with prominent regulators of actin dynamics (e.g., Dynamin, Cortactin and Rac1). One key to understanding SYNJ2's action is the ability to control endocytic trafficking. Frequent packaging of portions of the plasma membrane in vesicles, which constantly feed actin-based protrusions propels

cell migration (Ridley, 2011). SYNJ2 of lamellipodia exhibits remarkable dynamism (Figure 3B), and live-cell imaging implies that SYNJ2 recruitment marks sites of new lamellipodia formation. Because SYNJ2 molecules located at the leading edge depend on Dynamin, Rac1, actin polymerization and cholesterol, but its distribution is distinct from that of Caveolin-1 and Clathrin, the present inventors assume that it represents a Dynamin-dependent variant of the Clathrin-independent carriers (CLICs), which sustain membrane turnover at the leading edge (Howes et al., 2010).

A series of elegant studies implicated SYNJ1 in synaptic vesicle recycling in neurons (Cremona et al., 1999). In mice, deletion of SYNJ1 caused elevation of steady-state $PI(4,5)P_2$, accumulation of Clathrin-coated vesicles, and a delay in post-endocytic vesicle reavailability (Mani et al., 2007). These observations suggest that dephosphorylation of $PI(4,5)P_2$, which would allow vesicle coat shedding, underlies the phenotype. In analogy, the present SYNJ2-depleted mammary cells displayed intracellular accumulation of active EGFRs. Receptor ubiquitination status and markers of the endocytic pathway indicated that trafficking is arrested at sorting endosomes, where internalized receptors are normally shunted to either recycling or degradation. Conceivably, the defect is due to an inability to disassemble $PI(4,5)P_2$-binding proteins associated with the vesicle's coat or with its actin comet tails (Kaksonen et al., 2003). Thus, in similarity to the defects in synaptic transmission observed upon SYNJ1 ablation, loss of SYNJ2 severely impairs cell migration and invasion due to arrested trafficking of surface molecules essential for motility.

The observed elevation of PI(3)P and $PI(3,5)P_2$ (Figure 5C), regulators of early and late endosomes, respectively, in SYNJ2-depleted cells proposes additional trafficking mechanisms. A regulatory role for $PI(3,5)P_2$ has been reinforced by the identification of multiple binders, such as integrins and several Rab proteins (Catimel et al., 2008). PI(3)P is phosphorylated by PIKfyve, a 5-kinase implicated in cycling between endosomes and the trans-Golgi network, the route delivering MT1-MMP to invadopodia (Poincloux et al., 2009). Hence, in addition to dephosphorylation of $PI(4,5)P_2$, SYNJ2 likely processes $PI(3,5)P_2$ to fine tune the PI(3)P pool of early endosomes and coordinate both exocytosis of MT1-MMP and recycling of integrins, as well as EGFR.

When introduced into animals, shSYNJ2 MDA-MB-231 cells severely lost

metastatic potential, due to reduced ability to reach lymph nodes and blood vessels (Figures 13A-H). In an attempt to integrate these results and the in vitro phenotypes, outlined in Figure 15 are mechanisms underlying the roles of SYNJ2 in cellular motility. Accordingly, a key event entails EGF-induced up-regulation of SYNJ2, and consequent depletion of three phosphoinositides: $PI(4,5)P_2$, $PI(3,4,5)P_3$ and $PI(3,5)P_2$. SYNJ2-mediated $PI(4,5)P_2$ dephosphorylation is paralleled by degradation of $PI(4,5)P_2$ by phospholipase C-gamma, and phosphorylation by PI3K, which generates $PI(3,4,5)P_3$. Collectively, stimulation of the three enzymes by EGF dissociates a group of $PI(4,5)P_2$ binders from the plasma membrane, and also generates $PI(4,5)P_2$-devoid endocytic vesicles. Concurrently, SYNJ2 converts $PI(3,4,5)P_3$ into $PI(3,4)P_2$, which is essential for invadopodia formation. In line with this model, it has been reported that PI3K is needed for invadopodia formation. Once in place, $PI(3,4)P_2$ binds TKS5 and nucleates a Dynamin and Cortactin-centered complex that enables Cofilin to generate actin barbed ends within invadopodia. According to the present results, SYNJ2 is involved also in the next invadopodia maturation steps, namely secretion of MMPs and delivery of MT1-MMP and other surface molecules, such as CD44. In a similar way, SYNJ2 controls delivery of EGFRs and integrin to the leading edge, and likely activates Cofillin, a pivotal event dictating formation of lamellipodial protrusions.

In line with SYNJ2's contribution to cell migration in vitro and metastasis in animals, the present survey of breast cancer specimens observed significant up-regulation of SYNJ2 mRNA and protein levels in aggressive subtypes of the disease. In addition, using data from two cohorts, an association between high SYNJ2 mRNA expression and shorter survival of breast cancer patients was observed.

In summary, the present study attributes essential metastasis-initiating events to EGF-induced local activation of PI3K and global up-regulation of SYNJ2, whose sequential action upon $PI(4,5)P_2$ regulates actin dynamics at the leading edge, as well as generates $PI(3,4)P_2$, the signpost of invadopodia. Furthermore, the present study identified various compounds that selectively inhibited generation of PI(3,4)P2 by SYNJ2.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations

will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

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WHAT IS CLAIMED IS:

1. A method of preventing tumor metastasis with the proviso that said tumor is not glioma, the method comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2), thereby preventing tumor metastasis.

2. An inhibitor of synaptojanin 2 (SYNJ2) for preventing tumor metastasis with the proviso that said tumor is not glioma.

3. A method of treating cancer the method comprising, administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer, thereby treating cancer.

4. An inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer for treating cancer.

5. The method of claim 3, wherein said cell surface receptor associated with said onset or progression of cancer is a receptor tyrosine kinase.

6. The method of claim 5, wherein said receptor tyrosine kinase is an ErbB receptor.

7. The method of claim 6, wherein said ErbB receptor is Epidermal Growth Factor Receptor (EGFR).

8. A method of identifying a putative inhibitor of tumor metastasis, the method comprising analyzing SYNJ2-mediated processing of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ in the presence of a test agent, wherein a decreased processing of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ in said presence of said test agent as compared to same in an absence thereof is indicative of a putative inhibitor of tumor metastasis.

9. The method of claim 8, wherein said analyzing SYNJ2-mediated processing of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ is performed by a competition assay.

10. The method of claim 9, wherein said competition assay assays displacement of a $PI(3,4)P_2$ binding domain from a complex comprising said $PI(3,4)P_2$ binding domain bound to $PI(3,4)P_2$.

11. The method of any one of claims 9-10, wherein said competition assay is a fluorescence polarization competitive assay.

12. A method of prognosing cancer in a subject in need thereof, the method comprising determining a level or activity of SYNJ2 in a cancer cell of the subject, wherein an upregulation in said level of activity of said SYNJ2 in said cancer cell of the subject compared to same in a cell of an unaffected control sample, is indicative of a poor prognosis.

13. The method of claim 12, further comprising augmenting said prognosis using a Gold standard method.

14. The method of claim 13, wherein said Gold standard method comprises detection of a marker.

15. The method of claim 14, wherein said marker is selected from the group consisting of HER-2 and estrogen receptor (ER).

16. The method of claim 1 or 12, wherein said metastasis is EGF dependent.

17. The method of claim 3 or 12, wherein said cancer is breast cancer.

18. The method of claim 1 or 3, wherein said inhibitor of SYNJ2 is selected from the group consisting of a small molecule, an antibody, a peptide and a nucleic acid silencing agent.

19. The method of claim 18, wherein said small molecule is selected from the molecules listed in Table 2.

20. An article of manufacture for the treatment of cancer or prevention of cancer metastasis, comprising a packaging material packaging an inhibitor of SYNJ2 and an inhibitor of a cell surface receptor associated with an onset or progression of cancer.

21. The method of claim 3 or the article of manufacture of claim 20, wherein said inhibitor of said cell surface receptor associated with said onset or progression of cancer is an antibody.

22. The method of claim 3 or the article of manufacture of claim 20, wherein said inhibitor of said cell surface receptor associated with said onset or progression of cancer is a small molecule inhibitor.

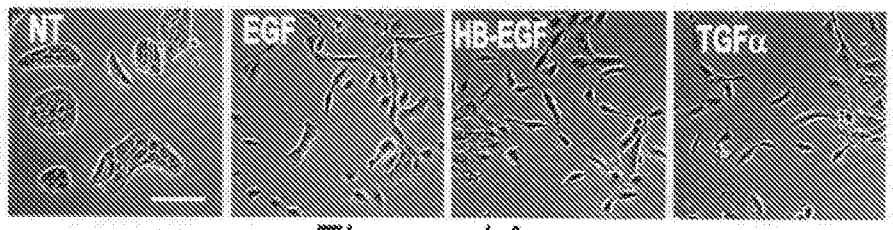


Figure 1A

Figure 1B

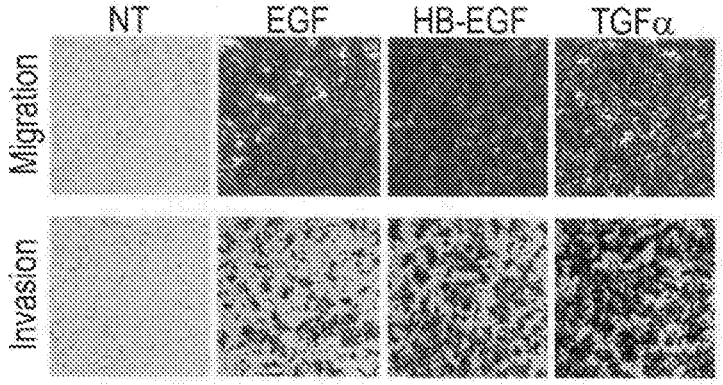
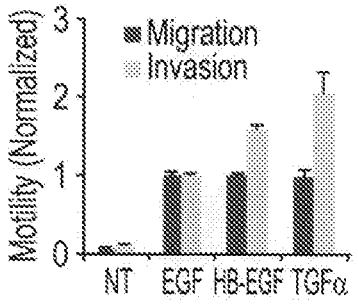
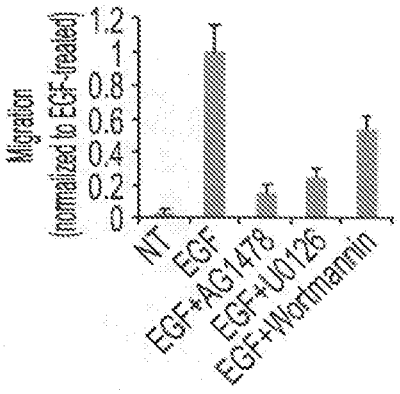


Figure 1C





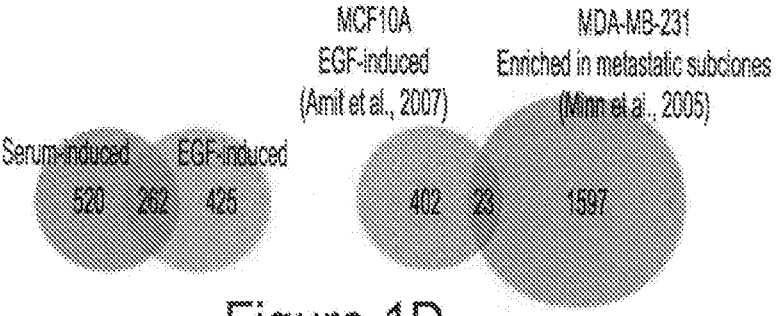
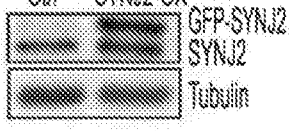


Figure 1D

Figure 1I

Figure 1E



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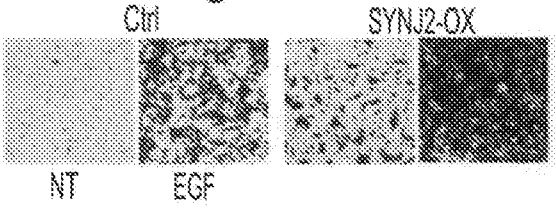


Figure 1G

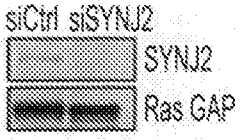
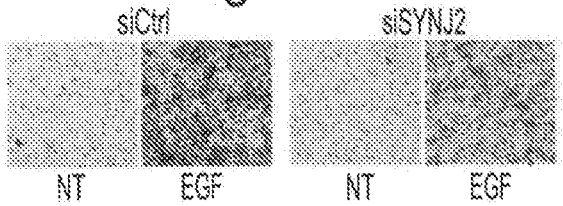


Figure 1H



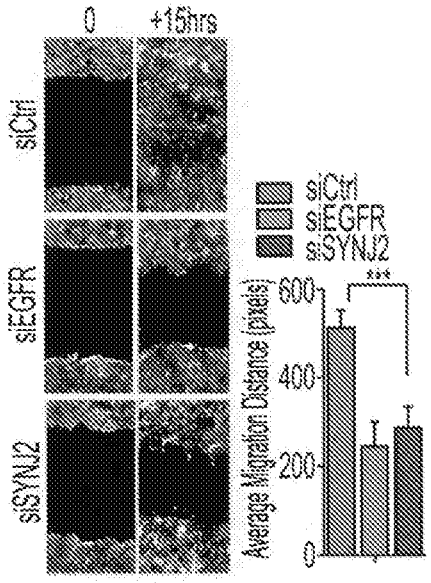
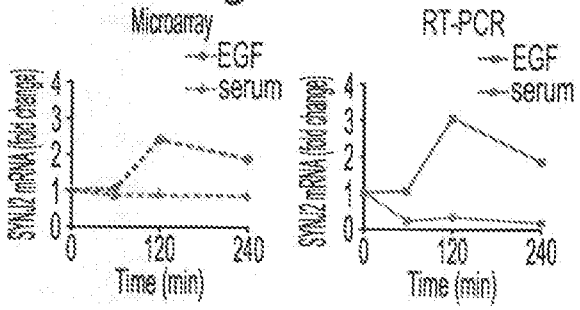


Figure 2A



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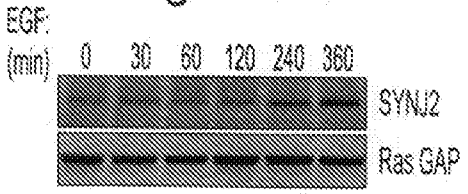

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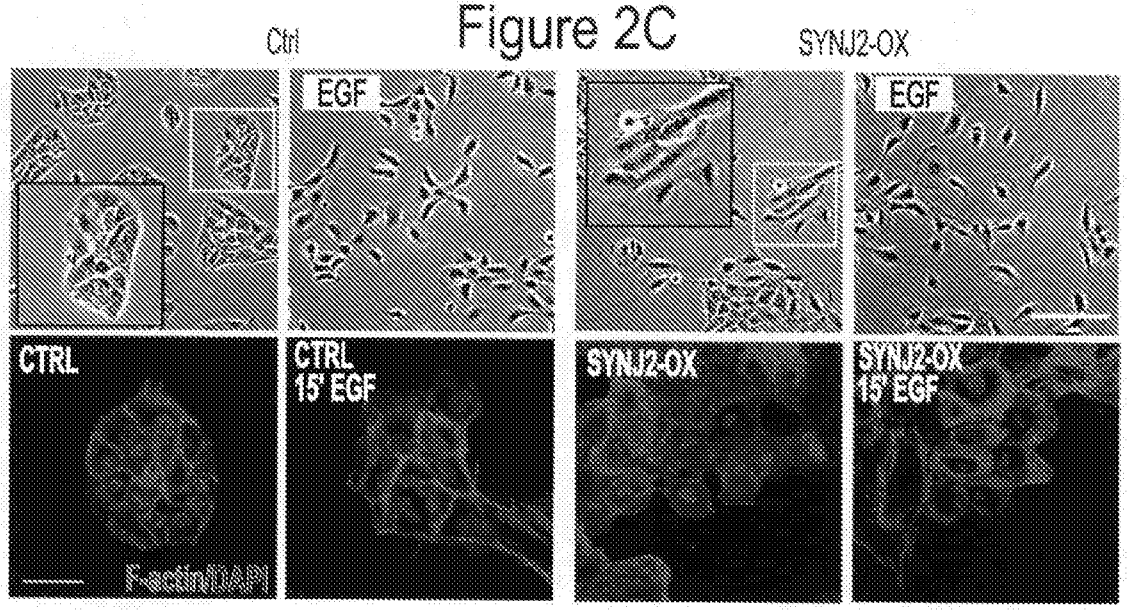

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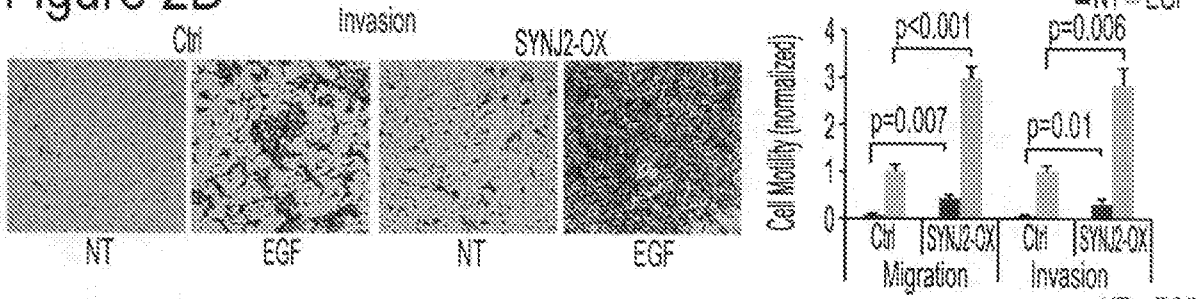

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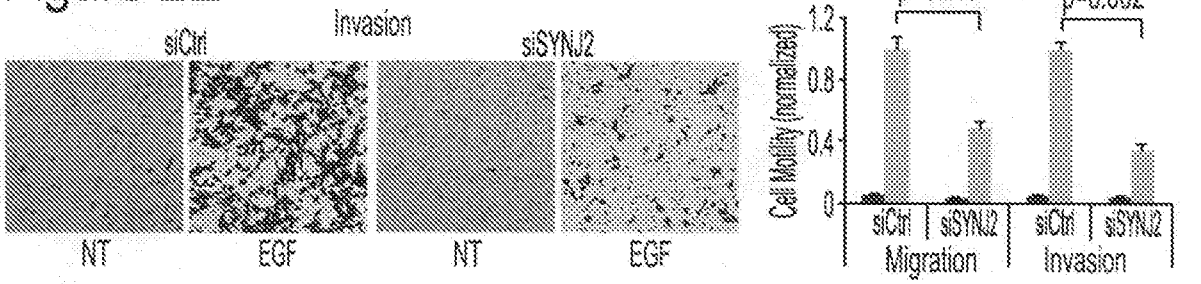
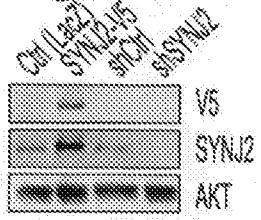


Figure 3A



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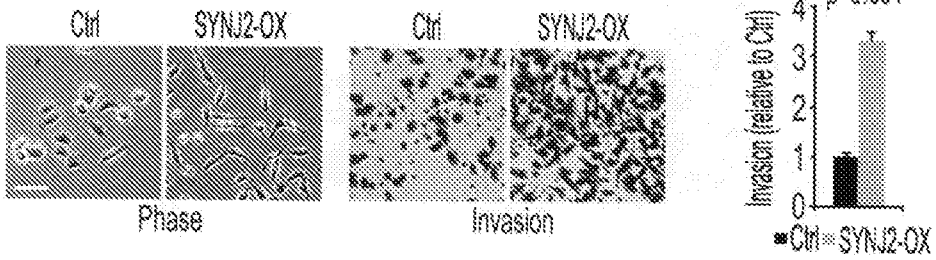

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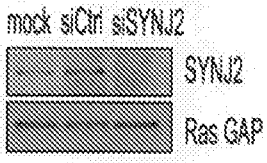

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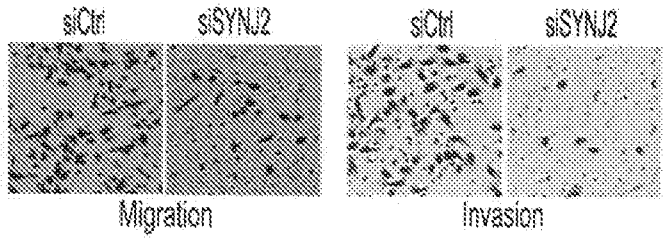

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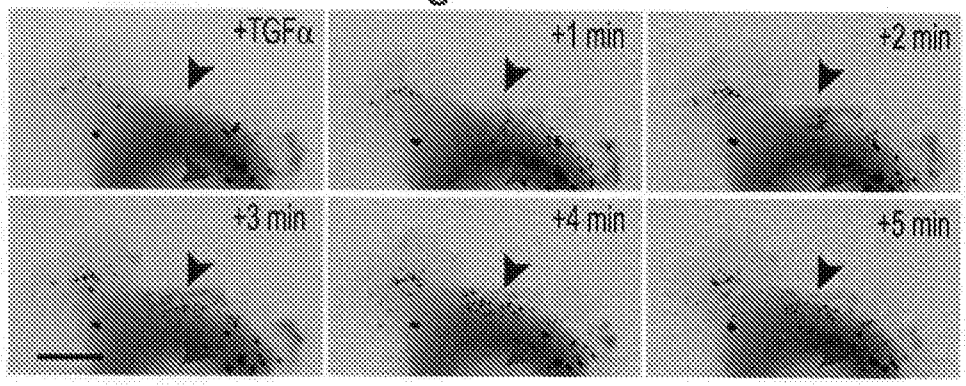
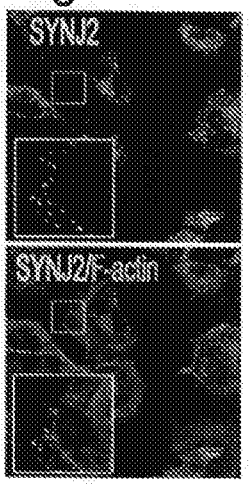


Figure 3F



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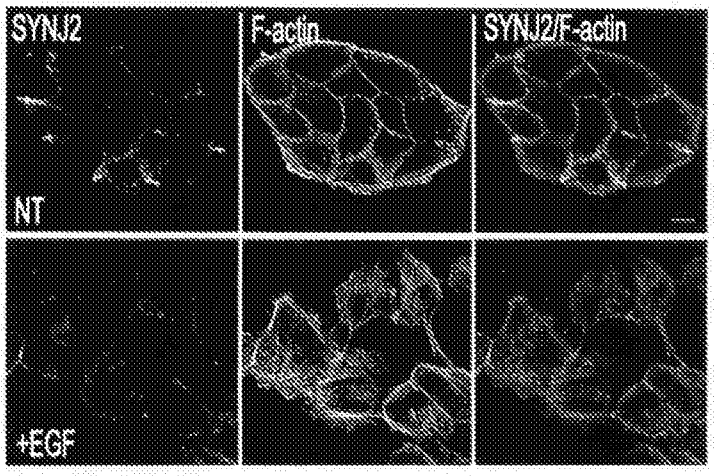
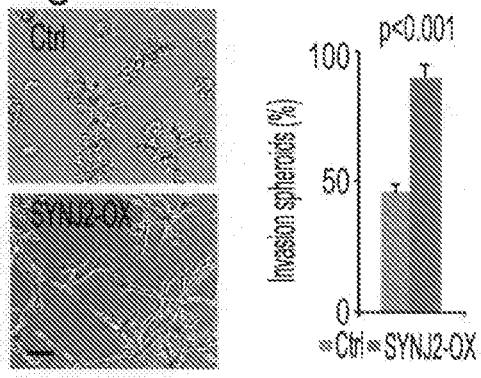


Figure 4A



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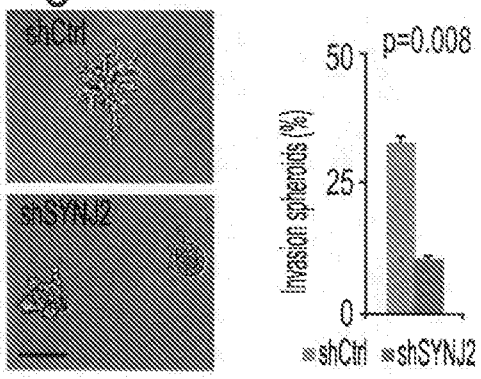

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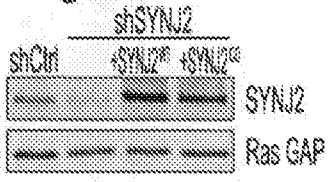

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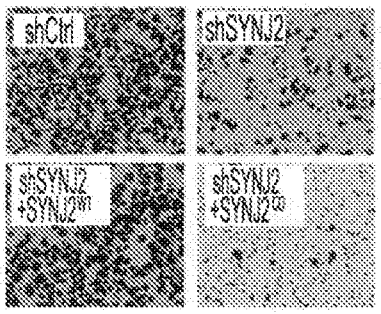

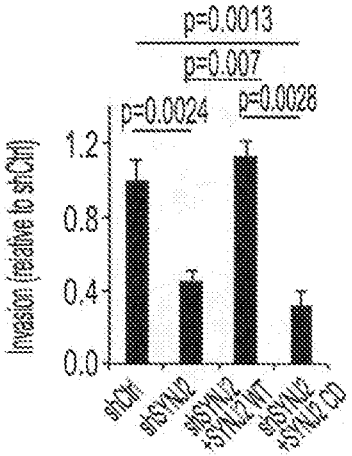

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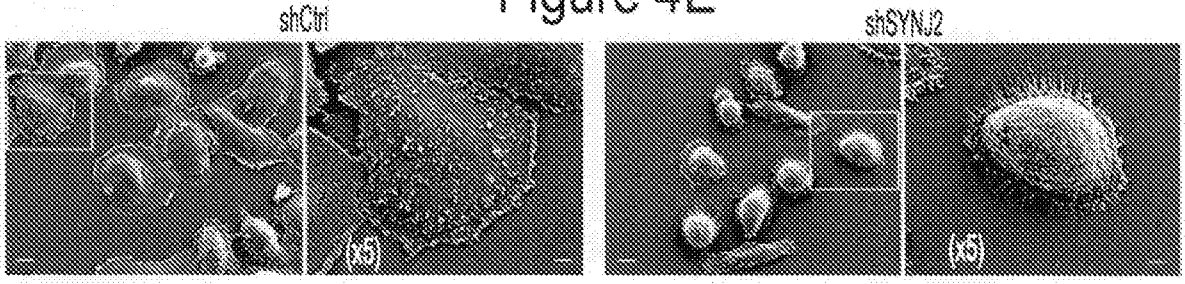


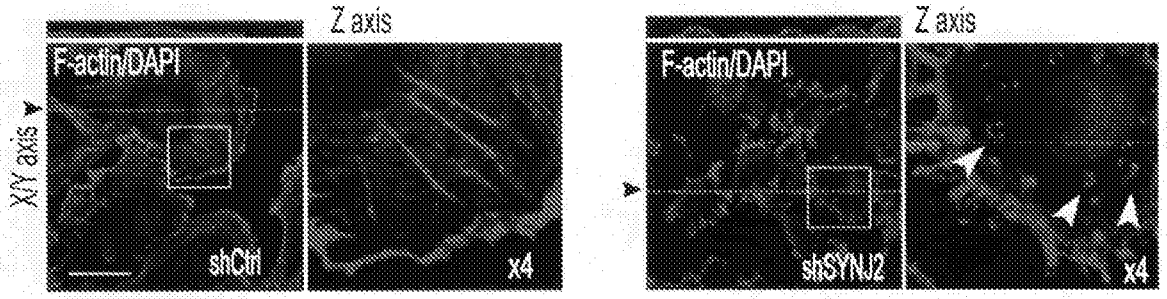
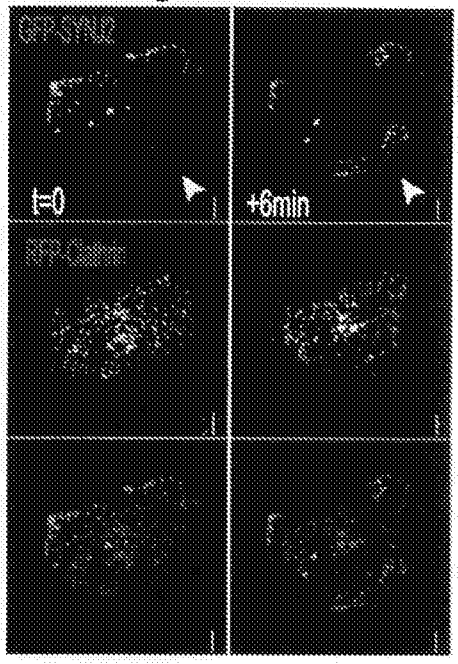


Figure 4F

Figure 5A



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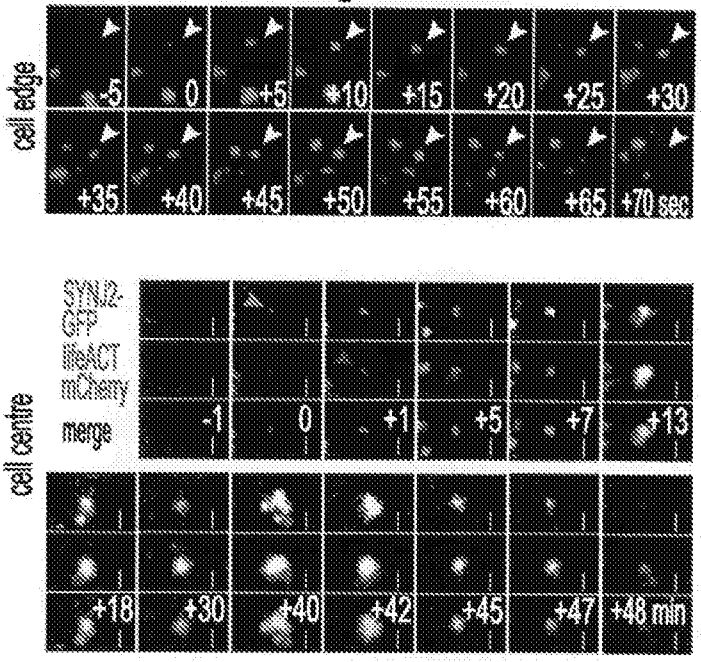

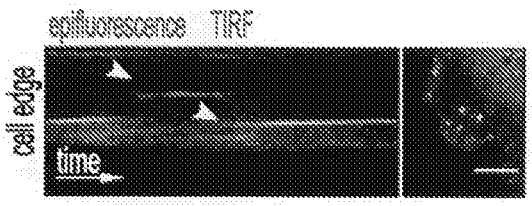

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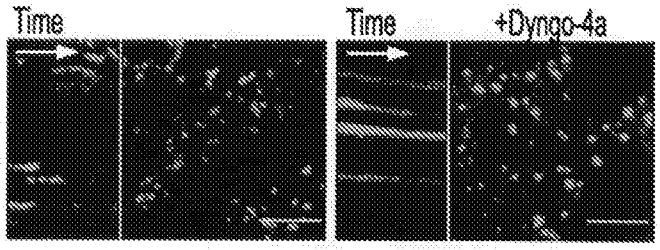

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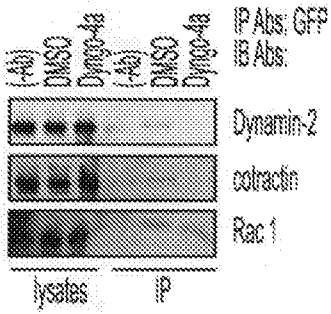

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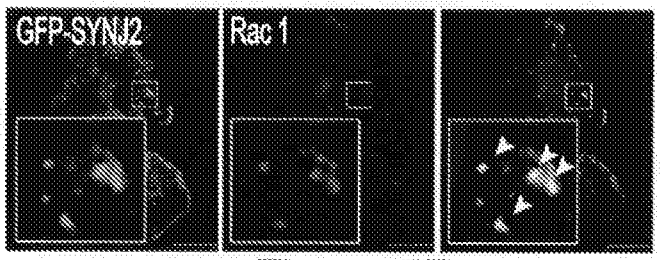


Figure 5F

Figure 5G

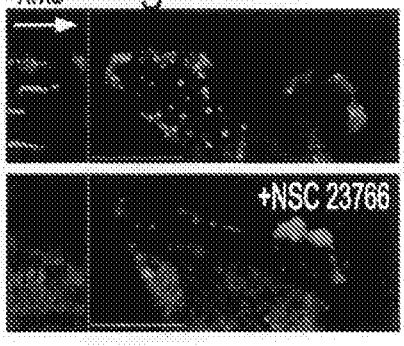


Figure 5H

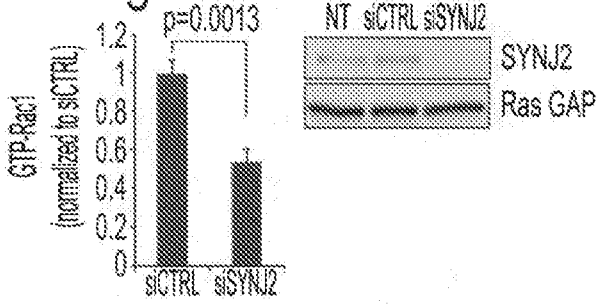
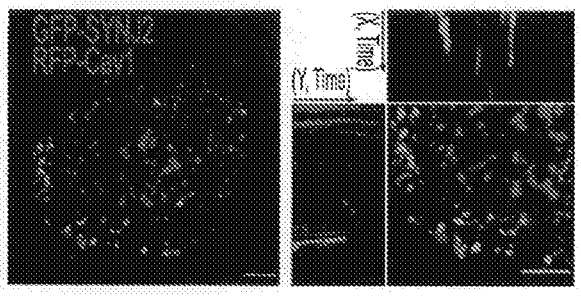
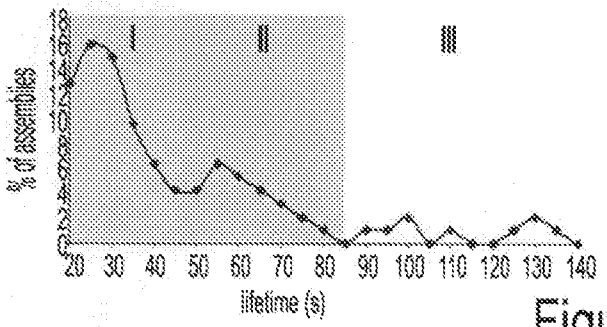


Figure 6A





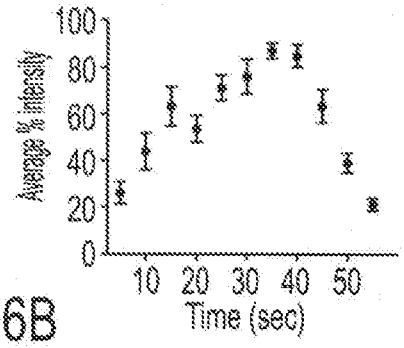
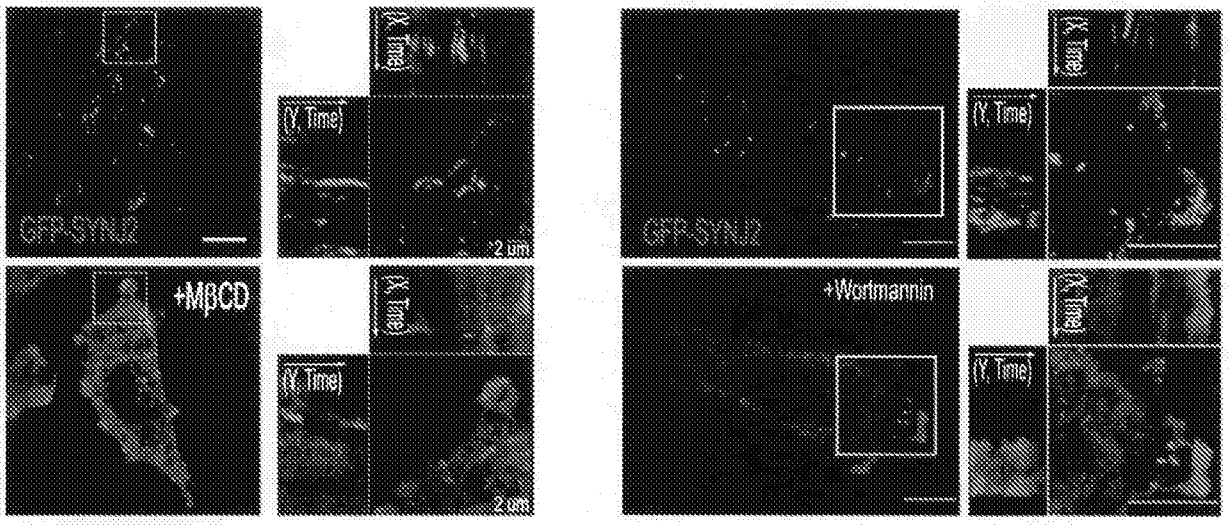


Figure 6B

Figure 6C



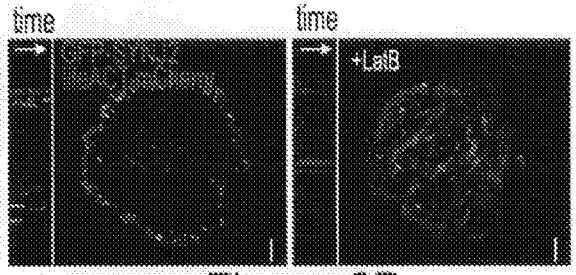


Figure 6D

Figure 7A

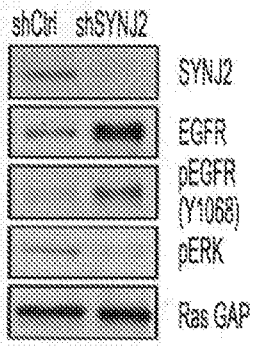


Figure 7B

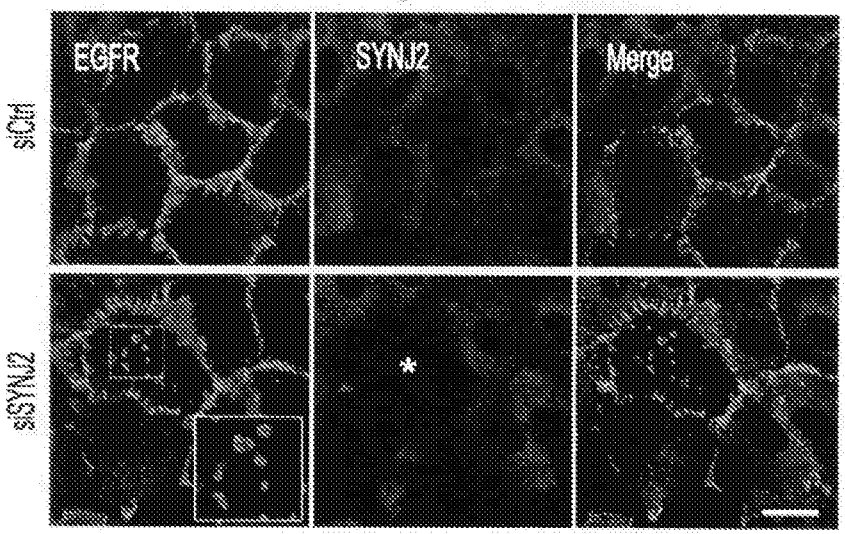


Figure 7C

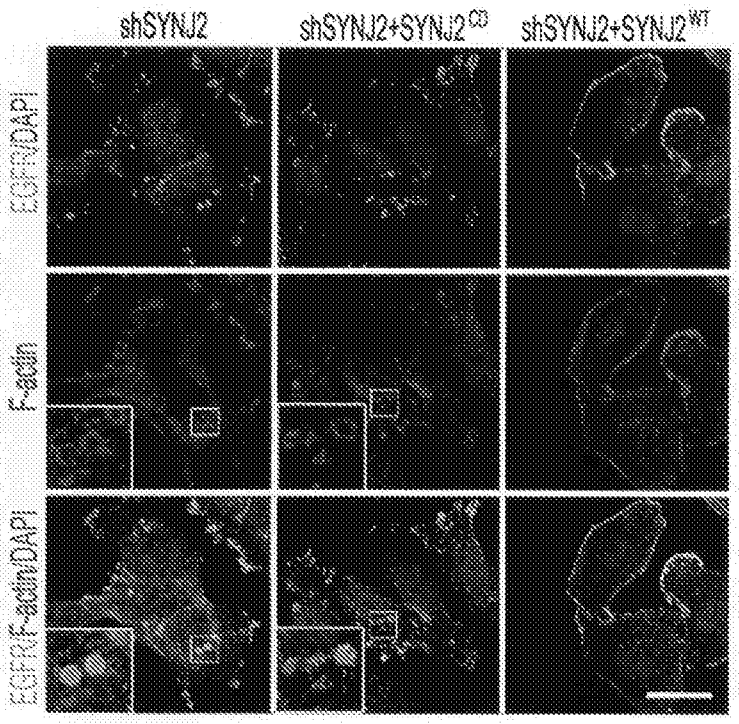
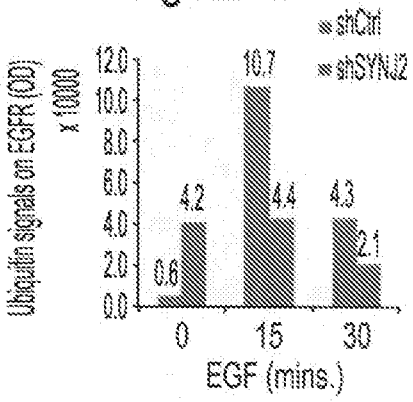


Figure 7D



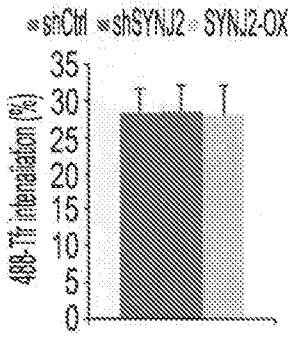
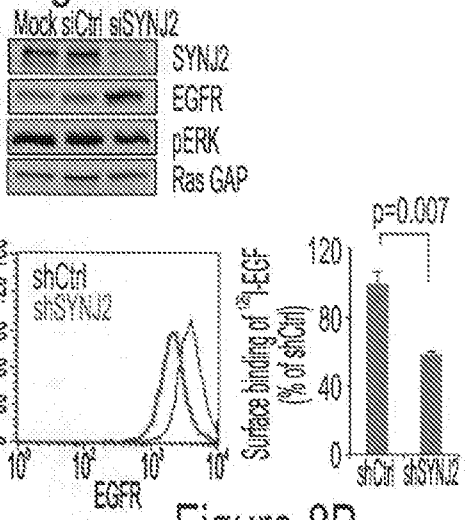


Figure 7E

Figure 8A



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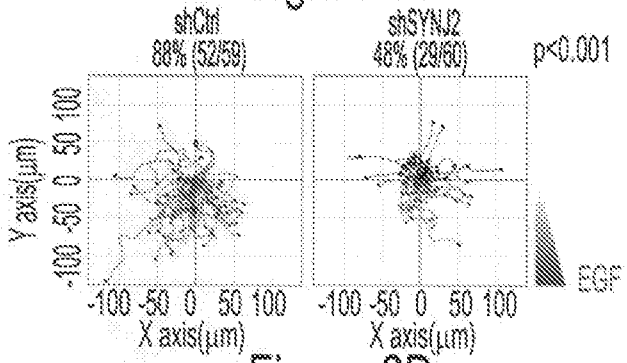
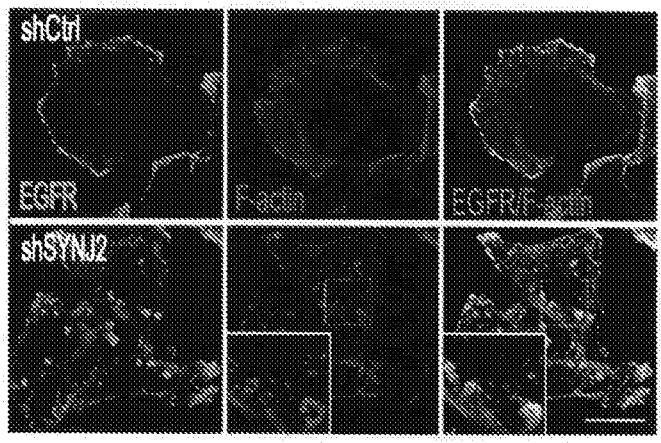


Figure 8D

Figure 8C



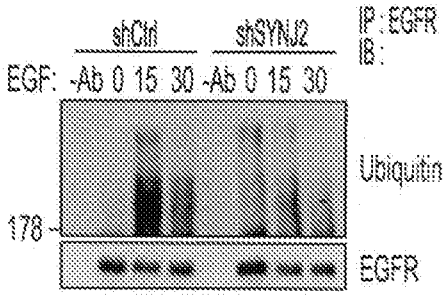


Figure 8E

Figure 8F

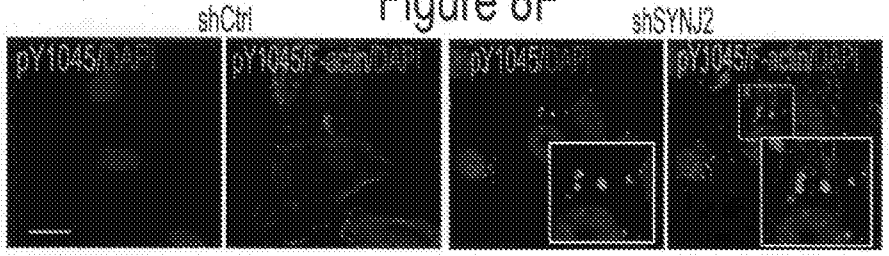
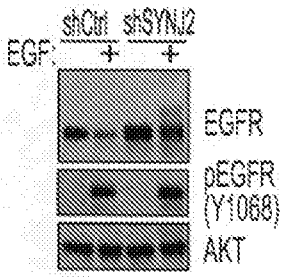


Figure 8G



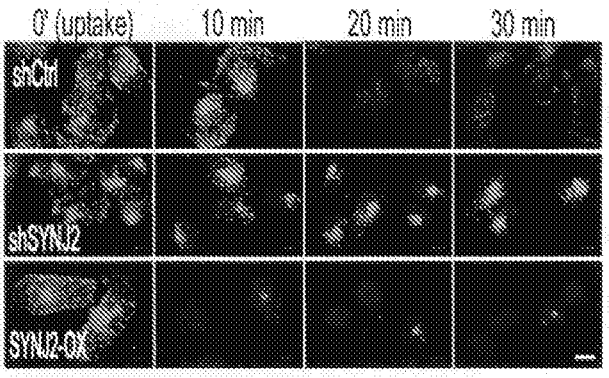
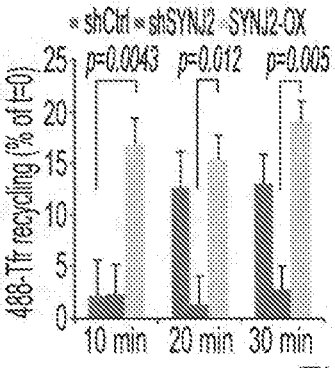


Figure 8H



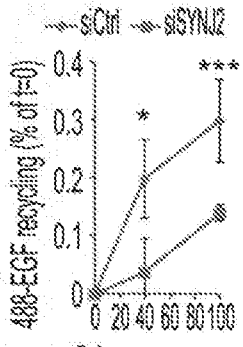


Figure 8I

Figure 9A

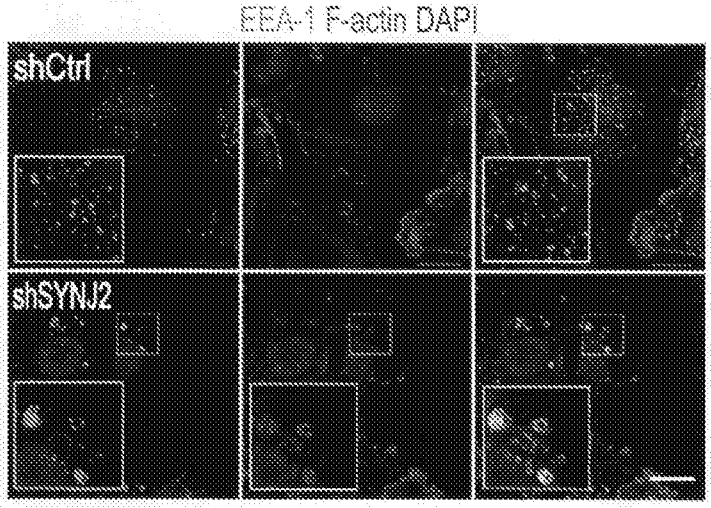


Figure 9B

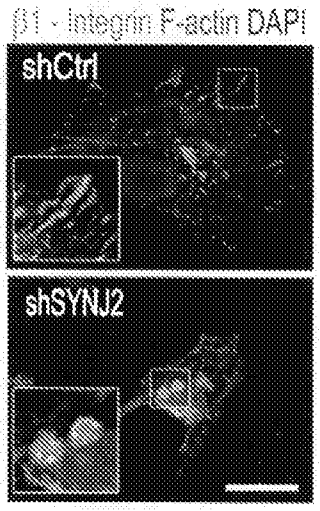


Figure 9C

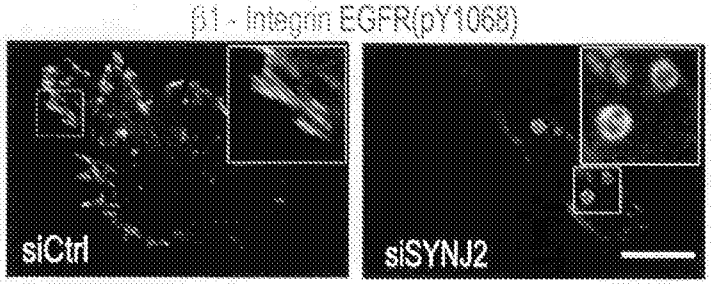
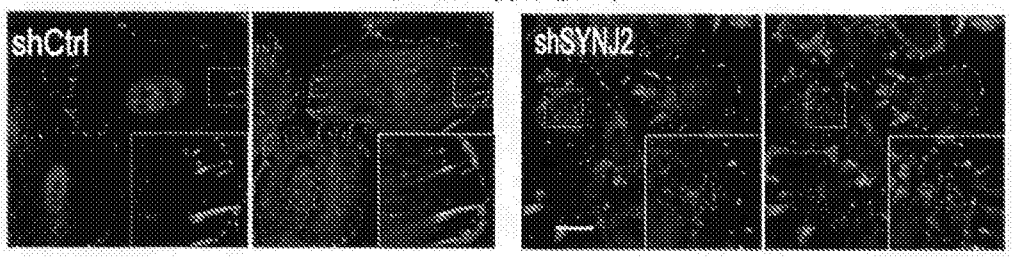


Figure 9D



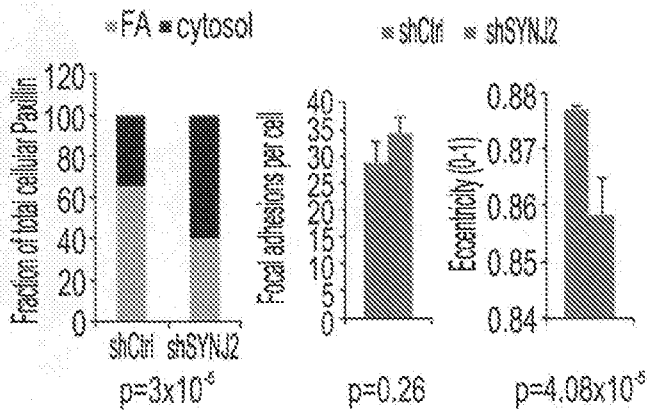
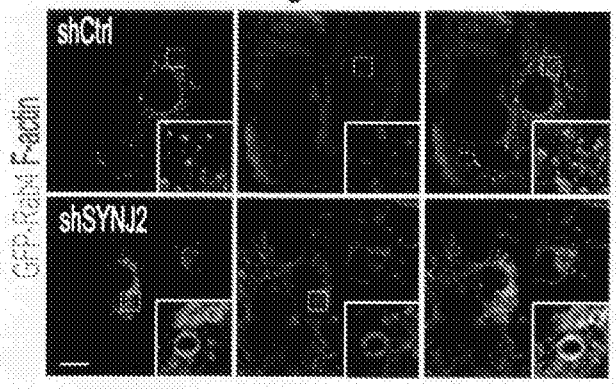


Figure 10A



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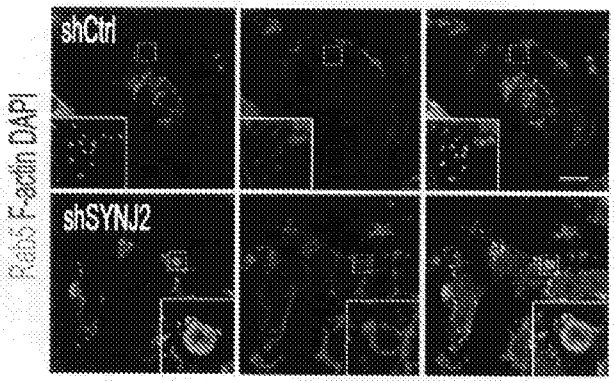


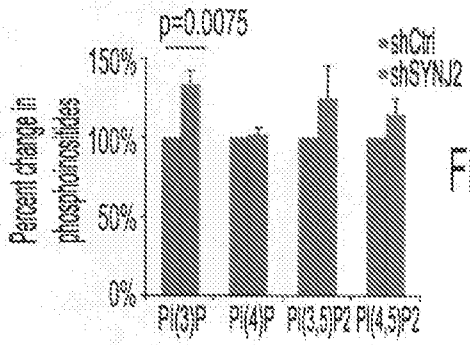
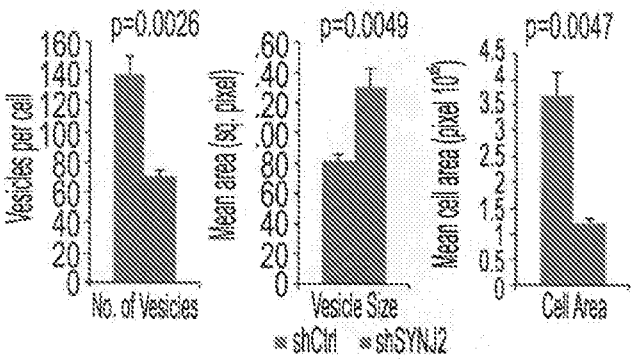


Figure 10C



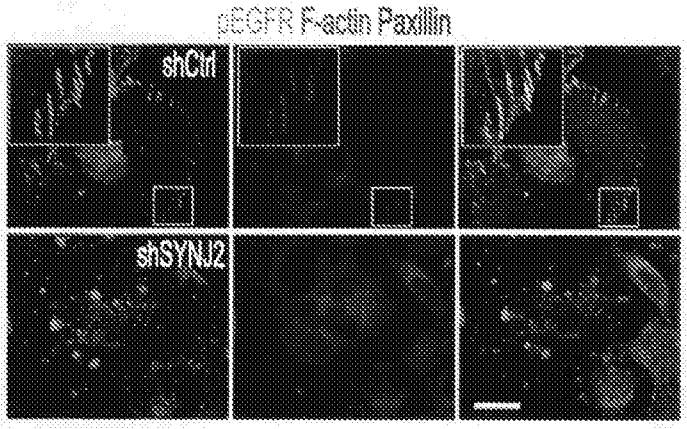


Figure 10D

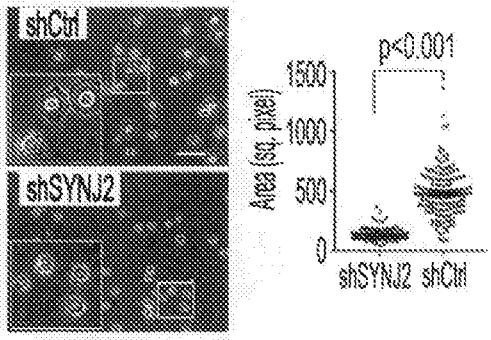


Figure 10E

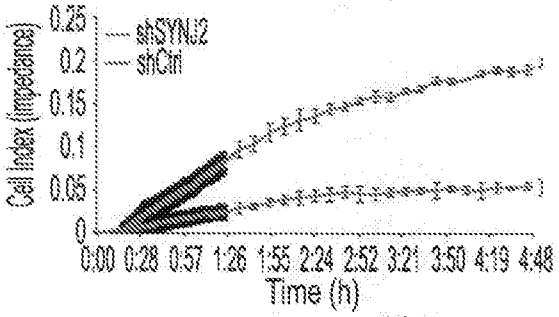
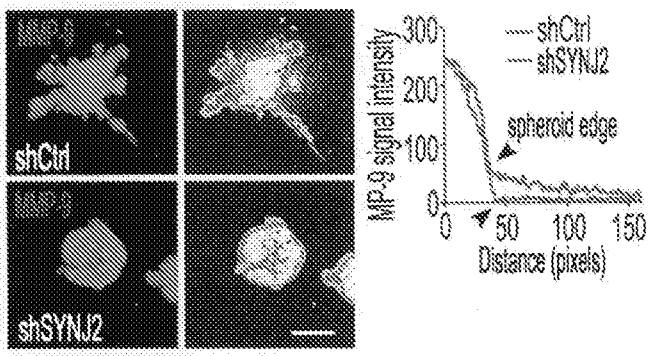


Figure 10F

Figure 11A



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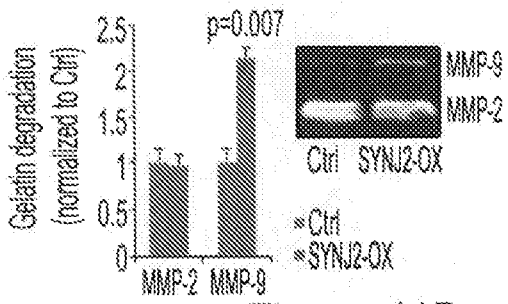
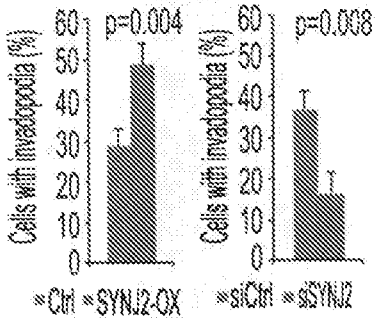


Figure 11C

Figure 11D



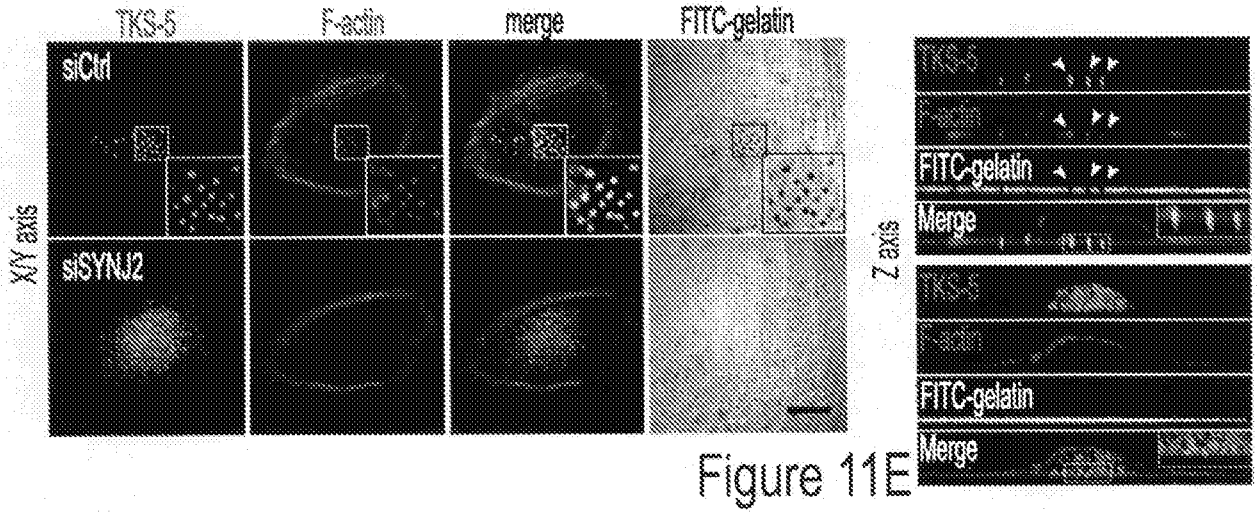


Figure 11E

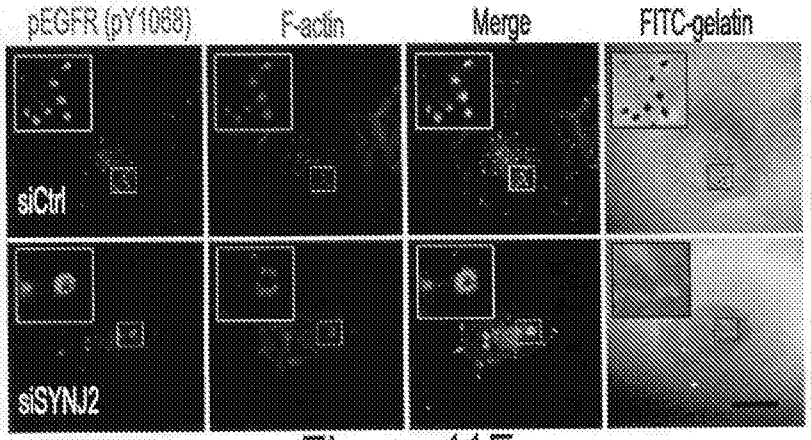


Figure 11F

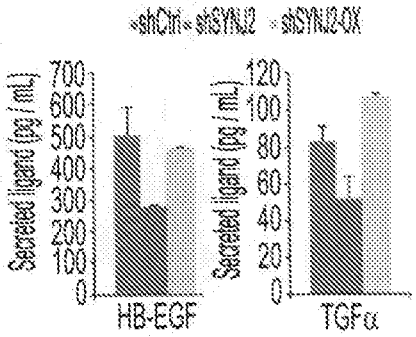
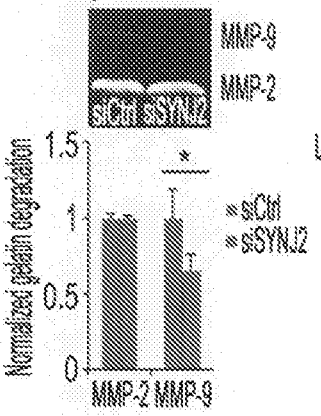


Figure 11G

Figure 12A



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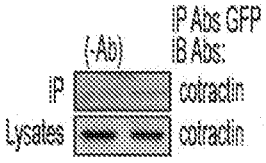


Figure 12C

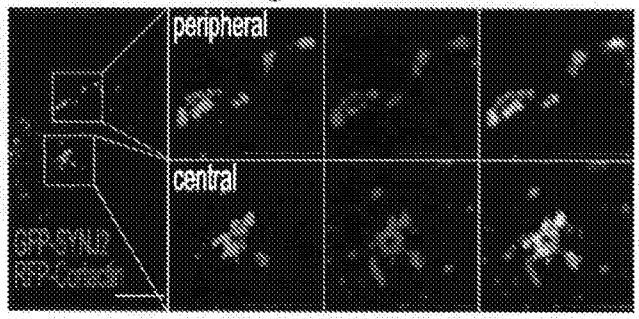
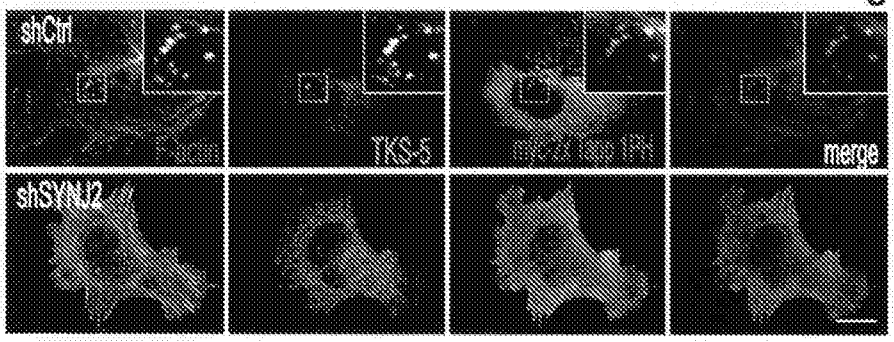
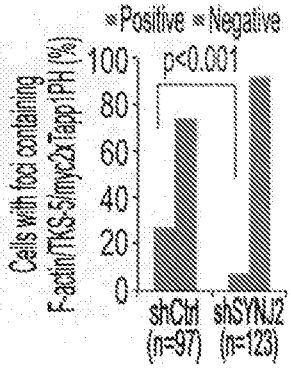


Figure 12D





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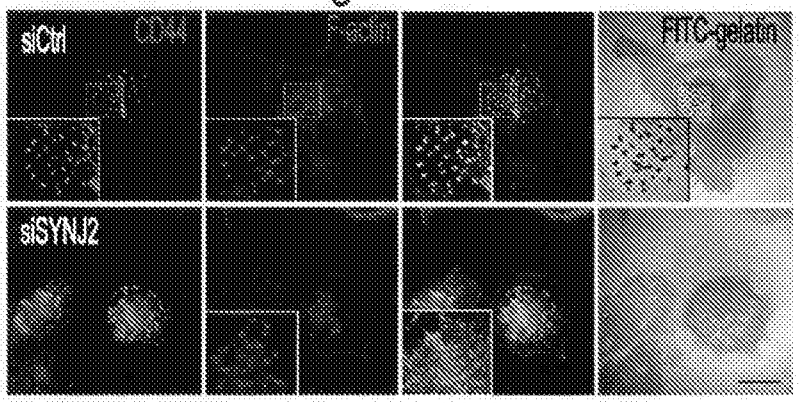


Figure 12F

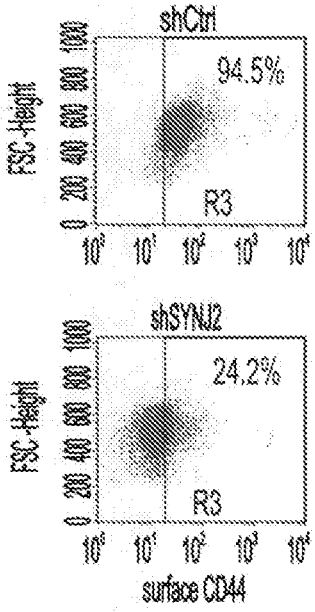
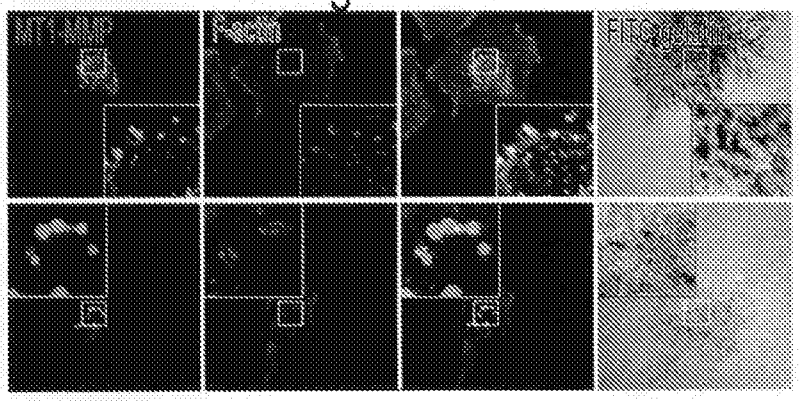
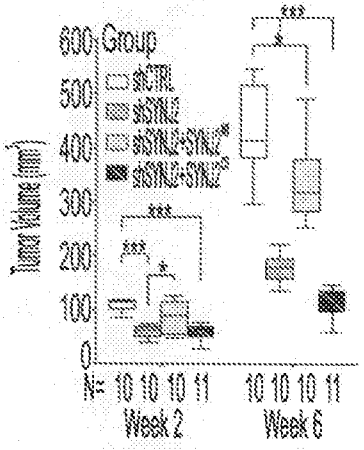


Figure 12G





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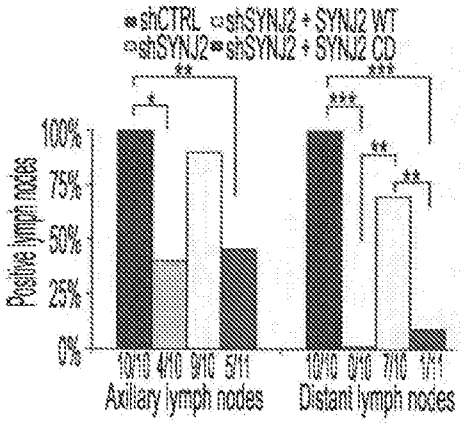


Figure 13B

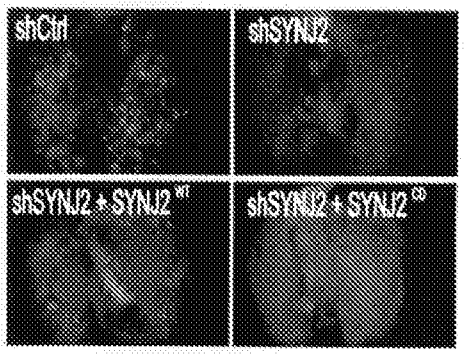
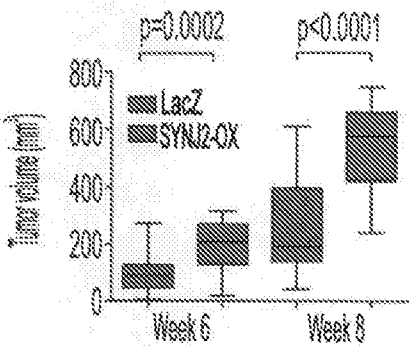


Figure 13C



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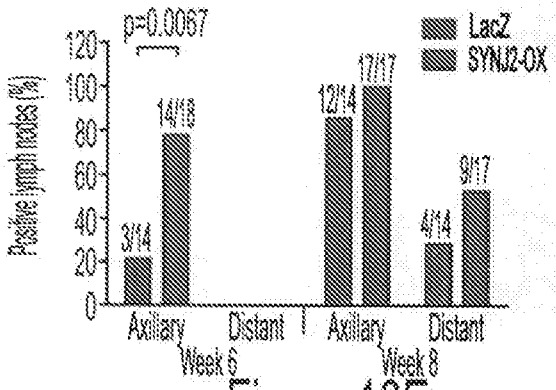

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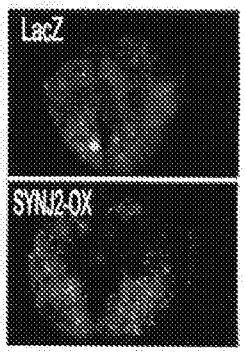
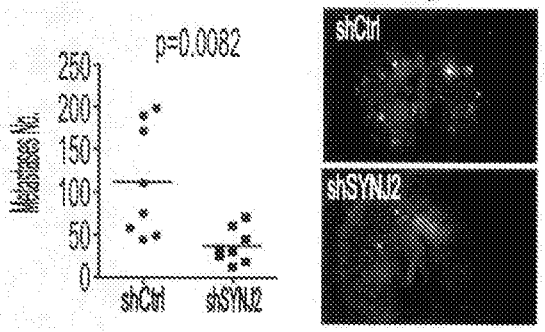


Figure 13F

Figure 13G



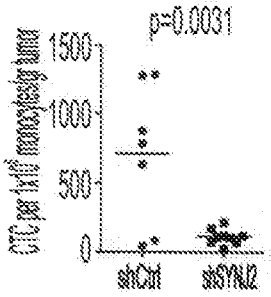
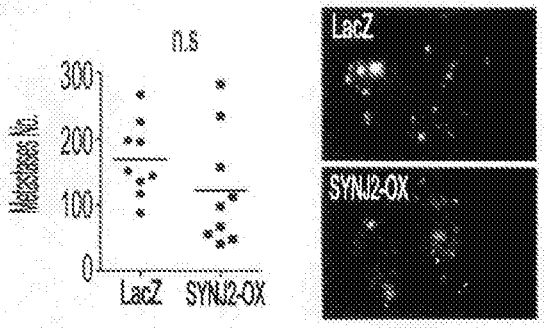
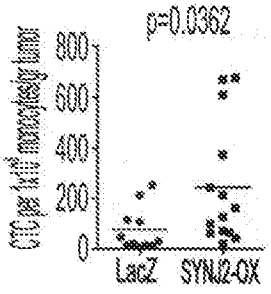
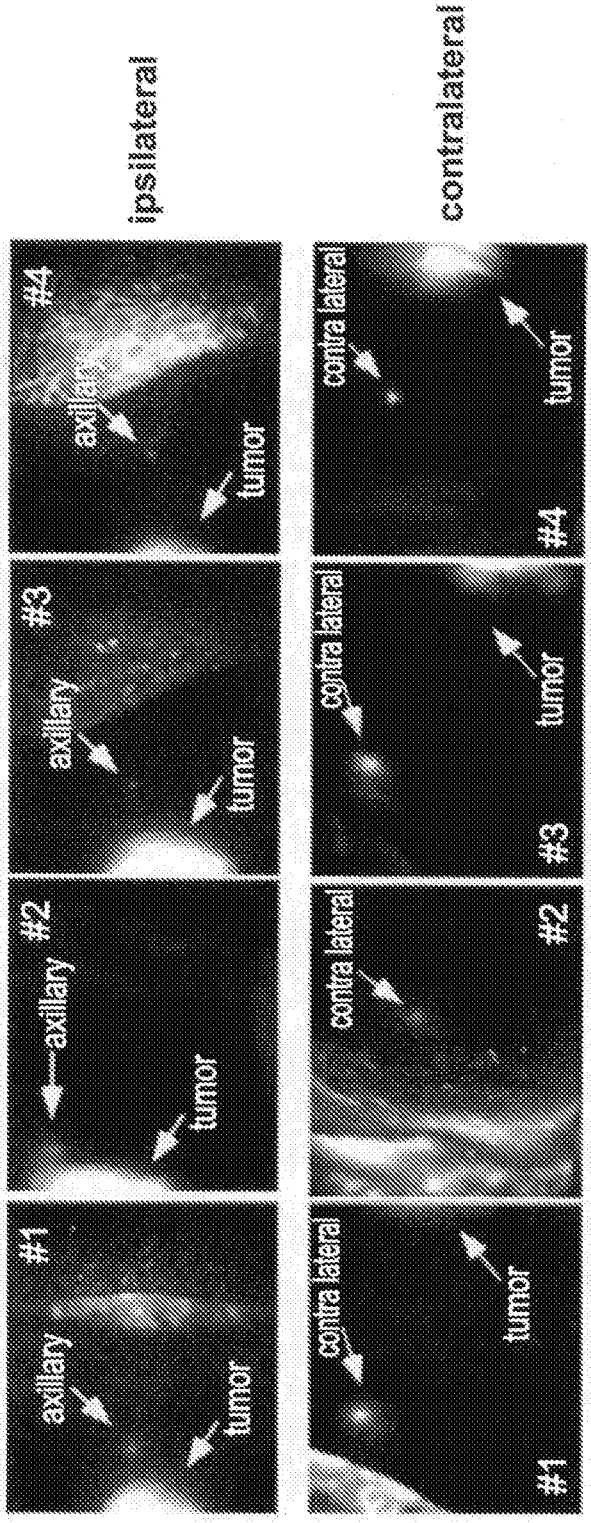


Figure 13H



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axillary
tumor
#2
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tumor
#3
axillary
tumor
#4
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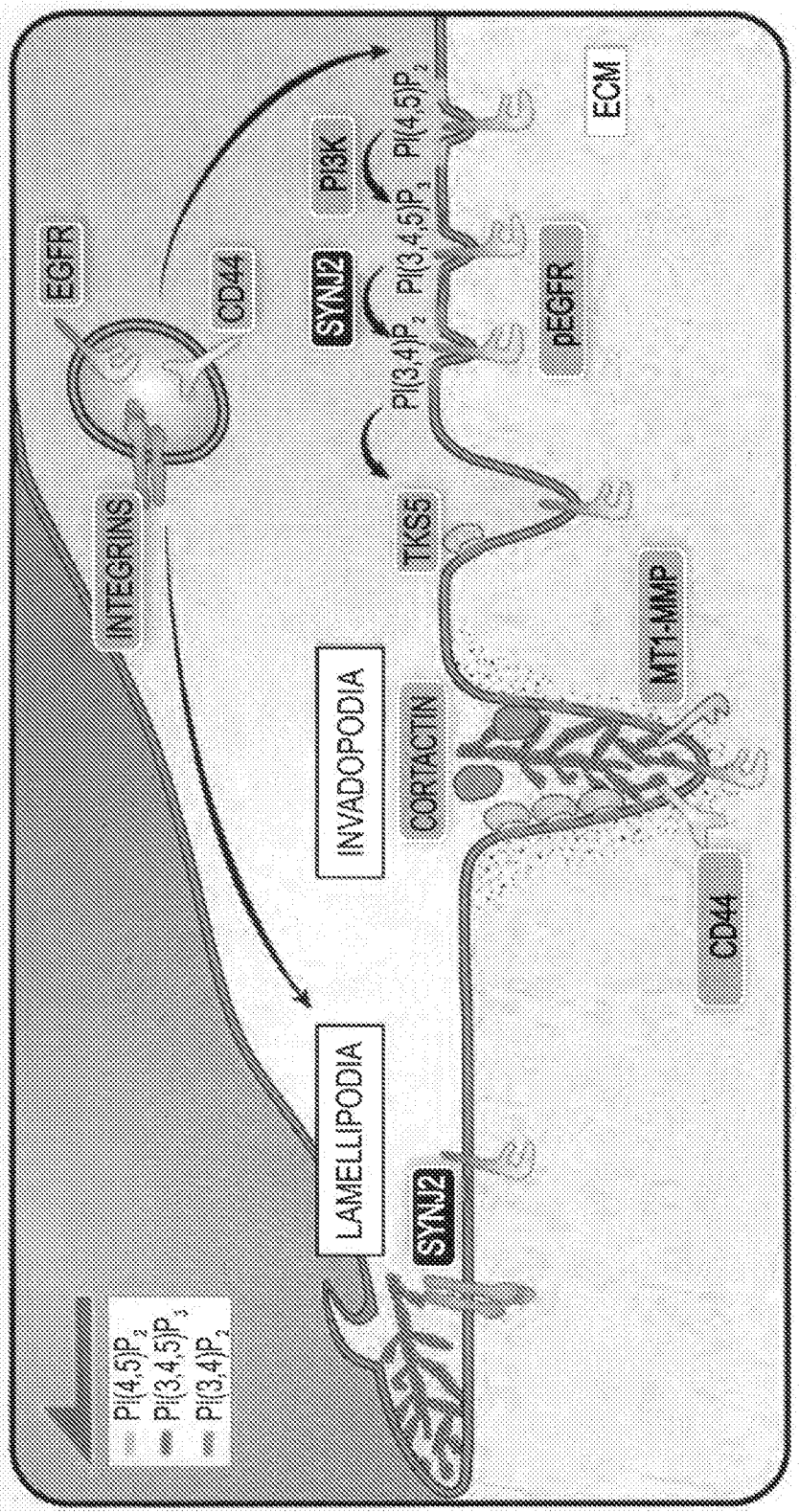
contra lateral
#1
tumor
contra lateral
#2
contra lateral
#3
tumor
contra lateral
#4
tumor

Figure 14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INTEGRINS
SYN2
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PI(4,5)P2
PI(3,4,5)P3
PI(3,4)P2
PI(4,5)P2
PI(3,4)P2
PI(3,4,5)P3
ECM
pEGFR
TKS5
INVADOPODIA
CORTACTIN
MT1-MMP
LAMELLIPODIA
SYN2
CD44

Figure 15

Figure 16A

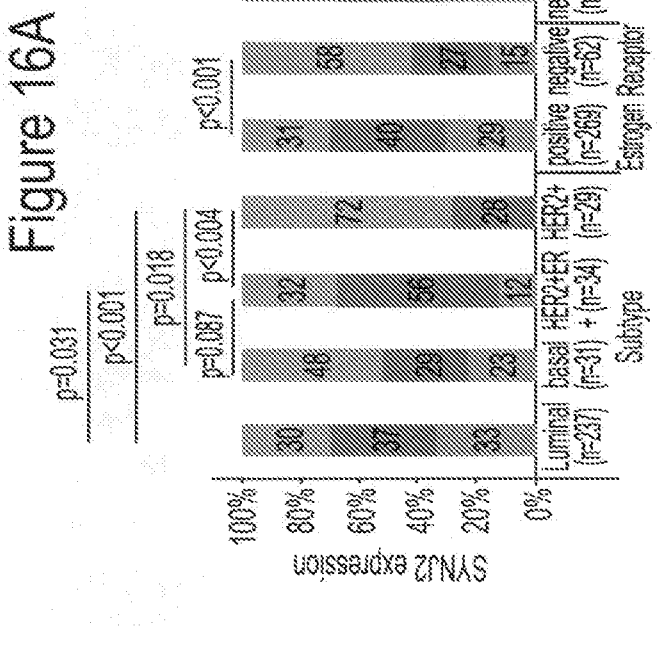


Figure 16B

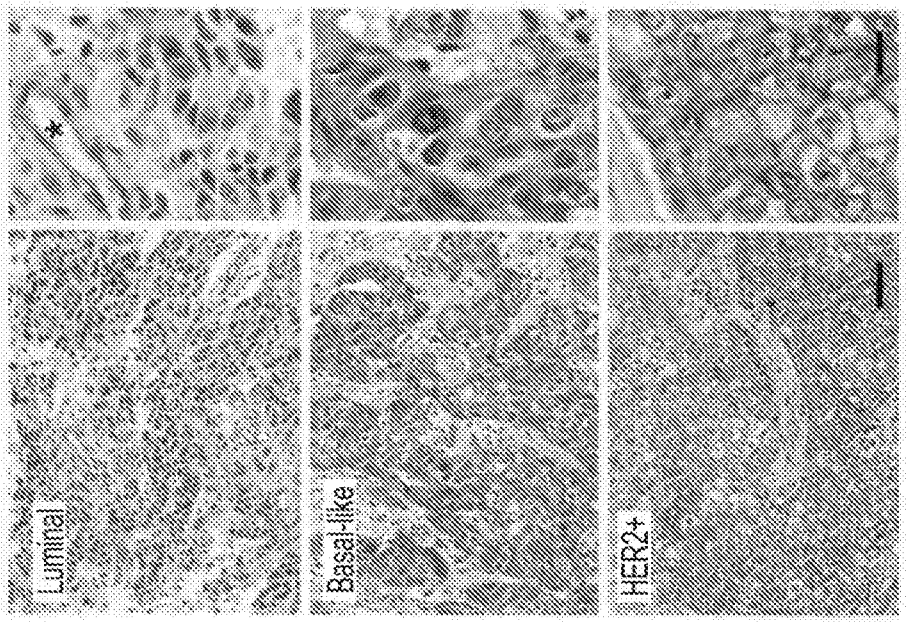
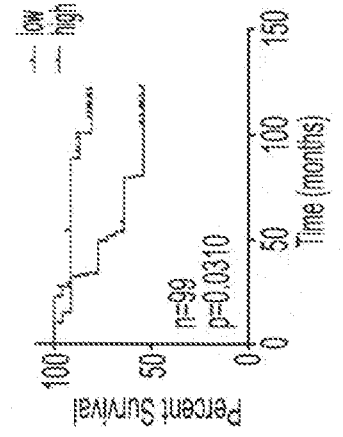


Figure 16C



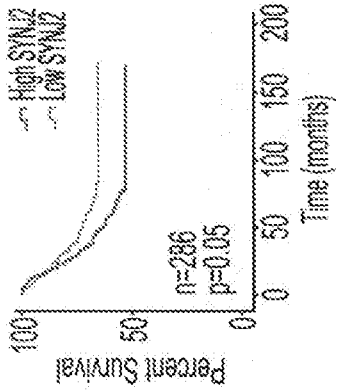


Figure 17A

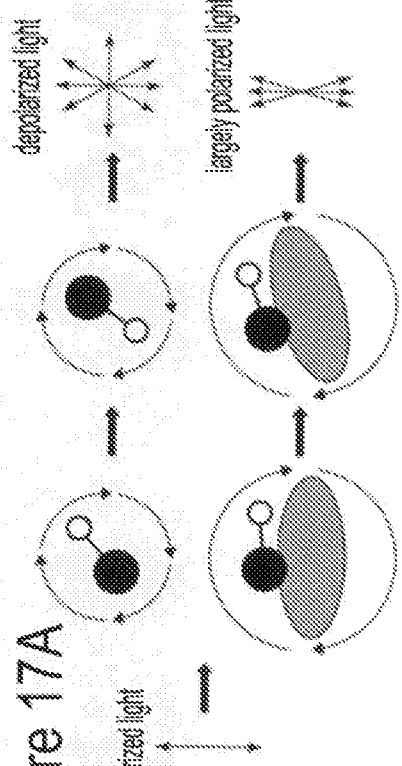


Figure 17B

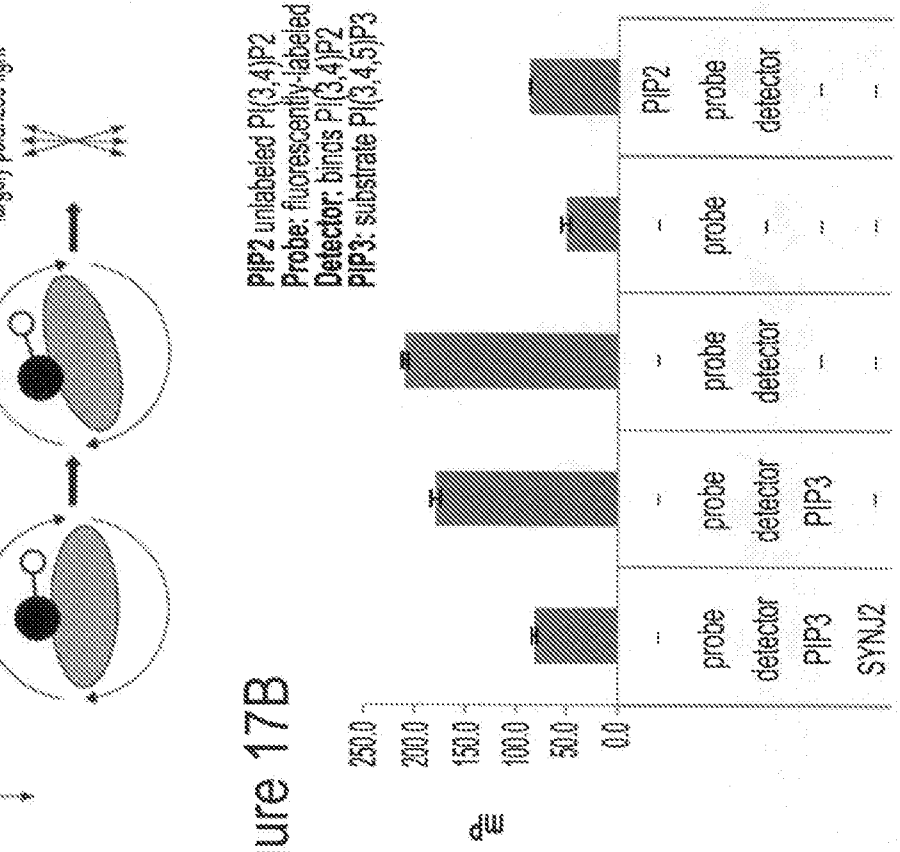


Figure 18

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M D Y K D D D D K G G S P Y F T P K P P Q D S T V I K A G Y

91/31 121/41 151/51
tgt gta aaa caa gga gca gtg atg aaa aac tgg aag aga aga tat ttt caa ttg gat gaa aac aca ata ggc tac ttc aaa tct gaa ctg
C V K Q G A V M K N W K R R Y F Q L D E N T I G Y F K S E L

181/61 211/71 241/81
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E K E P L R V I P L K E V H K V Q E C K Q S D I M M R D N L

271/91 301/101 331/111
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L A K V K P G N F K V Q T V S P R E P A S K V T E Q A L L R

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721/241
cac cac cac cac cac tga
H H H H H *

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

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— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the international search report:
24 July 2014

(54) Title: METHODS OF PREVENTING TUMOR METASTASIS, TREATING AND PROGNOSING CANCER AND IDENTIFYING AGENTS WHICH ARE PUTATIVE METASTASIS INHIBITORS

(57) Abstract: A method of preventing tumor metastasis with the proviso that the tumor is not glioma is provided. The method comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2), thereby preventing tumor metastasis. Also, provided is a method of treating cancer. The method comprising, administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer, thereby treating cancer.

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2013/050986

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 A61K45/06 A61K31/353 A61K31/7048 A61P35/00
A61P35/04
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FENG NI ET AL: "Flavonoid Ampelopsin Inhibits the Growth and Metastasis of Prostate Cancer In Vitro and in Mice", PLOS ONE, vol. 7, no. 6, 5 June 2012 (2012-06-05), pages 1-8, XP055098872, ISSN: 1932-6203, DOI: 10.1371/journal.pone.0038802 page 1, right-hand column, last paragraph - page 2, left-hand column, paragraph 2 page 4, left-hand column, paragraph 1 ----- -/--	1,2,16, 18,19

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 January 2014	26/05/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Haider, Ursula

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2013/050986

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7, 18-22(completely); 16, 17(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2013/050986

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Ya-Yu Chuang ET AL: "Role of Synaptojanin 2 in Glioma Cell Migration and Invasion", Cancer Research, 15 November 2004 (2004-11-15), pages 8271-8275, XP055098695, Retrieved from the Internet: URL:http://cancerres.aacrjournals.org/content/64/22/8271.full.pdf [retrieved on 2014-01-27] the whole document	1,16,18, 19
X	WO 03/057239 A1 (RES DEV FOUNDATION [US]) 17 July 2003 (2003-07-17) claims 2, 3 page 17, lines 19-24 page 30, lines 14-17	1,2,18, 19
X	MEPUR H. RAVINDRANATH ET AL: "Differential Growth Suppression of Human Melanoma Cells by Tea (Camellia sinensis) Epicatechins (ECG, EGC and EGCG)", CANCER EPIDEMIOLOGY, BIOMARKERS & PREVENTION, vol. 158, no. 1, 22 October 2007 (2007-10-22), pages 523-530, XP055098877, ISSN: 1055-9965, DOI: 10.1158/1055-9965.EPI-03-0040 the whole document page 525, right-hand column, paragraph 3	3-7, 18-22
X	MIN-HSIUNG PAN ET AL: "Multistage carcinogenesis process as molecular targets in cancer chemoprevention by epicatechin-3-gallate", FOOD & FUNCTION, vol. 2, no. 2, 1 January 2011 (2011-01-01) , page 101, XP055098881, ISSN: 2042-6496, DOI: 10.1039/c0fo00174k the whole document page 105	1,2,18, 19
X	WO 2008/156644 A2 (FRANK DAVID A [US]) 24 December 2008 (2008-12-24) claim 1 page 12, line 20 page 25, lines 24-25 page 33, lines 18-21 page 41, lines 13-21	1,2,18, 19
X	WO 00/57875 A1 (PURDUE RESEARCH FOUNDATION [US]) 5 October 2000 (2000-10-05) page 7, lines 19-27 page 36 - page 37	3-7, 17-22

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2013/050986

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STYLLI S S ET AL: "Invadopodia: At the cutting edge of tumour invasion", JOURNAL OF CLINICAL NEUROSCIENCE, vol. 15, no. 7, 1 July 2008 (2008-07-01), pages 725-737, XP022689319, CHURCHILL LIVINGSTONE, GB ISSN: 0967-5868, DOI: 10.1016/J.JOCN.2008.03.003 [retrieved on 2008-05-12] abstract page 731, right-hand column, paragraph 4 page 726, left-hand column, paragraph 1 page 726, right-hand column, paragraph 3 -----	1,2,16, 18
A	MALECZ NICOLE ET AL: "Synaptojanin 2, a novel Rac1 effector that regulates clathrin-mediated endocytosis", CURRENT BIOLOGY, vol. 10, no. 21, 2 November 2000 (2000-11-02), pages 1383-1386, XP002373482, the whole document -----	3-7, 17-19, 21,22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IL2013/050986

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03057239 A1	17-07-2003	AU 2002357399 A1	24-07-2003
		CA 2471750 A1	17-07-2003
		EP 1458404 A1	22-09-2004
		US 2003175834 A1	18-09-2003
		WO 03057239 A1	17-07-2003
WO 2008156644 A2	24-12-2008	NONE	
WO 0057875 A1	05-10-2000	AU 4043900 A	16-10-2000
		US 6410061 B1	25-06-2002
		US 2002176897 A1	28-11-2002
		WO 0057875 A1	05-10-2000

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-7, 18-22(completely); 16, 17(partially)

A method of preventing tumor metastasis with the proviso that said tumour is not glioma, the method comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2), thereby preventing tumor metastasis.
A method of treating cancer the method comprising, administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer, thereby treating cancer.
An article of manufacture for the treatment of cancer or prevention of cancer metastasis, comprising a packaging material packaging an inhibitor of SYNJ2 and an inhibitor of a cell surface receptor associated with an onset or progression of cancer.

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2. claims: 8-11

A method of identifying a putative inhibitor of tumor metastasis, the method comprising analyzing SYNJ2-mediated processing of PI(3,4,5)P3 to PI(3,4)P2 in the presence of a test agent, wherein a decreased processing of PI(3,4,5)P3 to PI(3,4)P2 in said presence of said test agent as compared to same in an absence thereof is indicative of a putative inhibitor of tumor metastasis.

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3. claims: 12-15(completely); 16, 17(partially)

A method of prognosing cancer in a subject in need thereof, the method comprising determining a level or activity of SYNJ2 in a cancer cell of the subject, wherein an upregulation in said level of activity of said SYNJ2 in said cancer cell of the subject compared to same in a cell of an unaffected control sample, is indicative of a poor prognosis.

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(72) Inventors: **YARDEN, Yosef**; 4 Ktalav Street, Neve Willner, The Weizmann Institute of Science, 7610002 Rehovot (IL). **BEN-CHETRIT, Nir**; c/o Yeda Research and Development Co. Ltd., at the Weizmann Institute of Science., P.O. Box 95, 7610002 Rehovot (IL).

(74) Agents: **EHRLICH, Gal** et al.; G. E. Ehrlich (1995) Ltd., 11 Menachem Begin Road, 5268104 Ramat Gan (IL).

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(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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(54) Title: METHODS OF PREVENTING TUMOR METASTASIS, TREATING AND PROGNOSING CANCER AND IDENTIFYING AGENTS WHICH ARE PUTATIVE METASTASIS INHIBITORS

(57) Abstract: A method of preventing tumor metastasis with the proviso that the tumor is not glioma is provided. The method comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2), thereby preventing tumor metastasis. Also, provided is a method of treating cancer. The method comprising, administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer, thereby treating cancer.

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代理人 齐晓静

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A61K 45/06(2006.01)
A61K 31/353(2006.01)
A61K 31/7048(2006.01)
A61P 35/00(2006.01)
A61P 35/04(2006.01)

权利要求书2页 说明书45页
序列表18页 附图18页

(54)发明名称

预防肿瘤转移、癌症治疗和预后及鉴定为推定转移抑制剂的试剂的方法

(57)摘要

提供了一种预防肿瘤转移的方法，条件是所述肿瘤不是神经胶质瘤。所述方法包括向有需要的受试者施用治疗有效量的突触囊泡磷酸酶2(SYNJ2)抑制剂，从而预防肿瘤转移。同样，提供了一种治疗癌症的方法。所述方法包括向有需要的受试者施用治疗有效量的突触囊泡磷酸酶2(SYNJ2)抑制剂和与癌症发作或进展相关的细胞表面受体的抑制剂，从而治疗癌症。

CN 104822390 A

1. 一种预防肿瘤转移的方法，条件是所述肿瘤不是神经胶质瘤，所述方法包括向有需要的受试者施用治疗有效量的突触囊泡磷酸酶 2(SYNJ2) 抑制剂，从而预防肿瘤转移。

2. 一种用于预防肿瘤转移的突触囊泡磷酸酶 2(SYNJ2) 抑制剂，条件是所述肿瘤不是神经胶质瘤。

3. 一种治疗癌症的方法，所述方法包括向有需要的受试者施用治疗有效量的突触囊泡磷酸酶 2(SYNJ2) 抑制剂和与癌症发作或进展相关的细胞表面受体的抑制剂，从而治疗癌症。

4. 一种突触囊泡磷酸酶 2(SYNJ2) 抑制剂和一种与癌症发作或进展相关的细胞表面受体的抑制剂，用于治疗癌症。

5. 根据权利要求 3 所述的方法，其中所述的与癌症发作或进展相关的细胞表面受体为受体酪氨酸激酶。

6. 根据权利要求 5 所述的方法，其中所述受体酪氨酸激酶为 ErbB 受体。

7. 根据权利要求 6 所述的方法，其中所述 ErbB 受体为表皮生长因子受体 (EGFR)。

8. 一种肿瘤转移的推定抑制剂的鉴定方法，所述方法包括在试验试剂的存在下分析 SYNJ2 介导的 PI(3,4,5)P_3向 PI(3,4)P_2的变化，其中在所述试验试剂的所述存在下 PI(3,4,5)P_3向 PI(3,4)P_2的变化与所述试验试剂不存在时相比的减少是肿瘤转移的推定抑制剂的指示。

9. 根据权利要求 8 所述的方法，其中所述分析 SYNJ2 介导的 PI(3,4,5)P_3向 PI(3,4)P_2的变化通过竞争测定法进行。

10. 根据权利要求 9 所述的方法，其中所述竞争测定法由包含与 PI(3,4)P_2结合的所述 PI(3,4)P_2结合结构域的复合物来测定 PI(3,4)P_2结合结构域的位移。

11. 根据权利要求 9-10 中任一项所述的方法，其中所述竞争测定法为荧光偏振竞争测定法。

12. 一种在有需要的受试者中癌症预后的方法，所述方法包括测定受试者癌细胞中 SYNJ2 的水平或活性，其中，与在未受影响的对照样品的细胞中相比，在所述受试者癌细胞中所述 SYNJ2 的所述活性水平的上调是预后不良的指示。

13. 根据权利要求 12 所述的方法，还包括使用金标法增进所述预后。

14. 根据权利要求 13 所述的方法，其中所述金标法包括标记的检测。

15. 根据权利要求 14 所述的方法，其中所述标记选自由 HER-2 和雌激素受体 (ER) 所组成的组。

16. 根据权利要求 1 或 12 所述的方法，其中所述转移为 EGF 依赖型。

17. 根据权利要求 3 或 12 所述的方法，其中所述癌症为乳腺癌。

18. 根据权利要求 1 或 3 所述的方法，其中所述 SYNJ2 抑制剂选自由小分子、抗体、肽和核酸沉默剂所组成的组。

19. 根据权利要求 18 所述的方法，其中所述小分子选自表 2 中所列的分子。

20. 一种用于治疗癌症或预防癌转移的制品，其包括包装材料，所述包装材料包装有 SYNJ2 抑制剂和与癌症发作或进展相关的细胞表面受体的抑制剂。

21. 根据权利要求 3 所述的方法或根据权利要求 20 所述的制品，其中所述的与癌症发作或进展相关的细胞表面受体的抑制剂为抗体。

22. 根据权利要求 3 所述的方法或根据权利要求 20 所述的制品，其中所述的与癌症发作或进展相关的细胞表面受体的抑制剂为小分子抑制剂。

预防肿瘤转移、癌症治疗和预后及鉴定为推定转移抑制剂的试剂的方法

[0001] 技术领域和发明背景

[0002] 本发明，在其一些实施方案中，涉及预防肿瘤转移、癌症治疗和预后及鉴定为推定转移抑制剂的试剂的方法。

[0003] 细胞运动性支持包括肿瘤转移在内的各种生理和病理过程 (Ridley,2011)。迁移的开始由肌动蛋白聚合和促使板状伪足和丝状伪足形成的 Rho 家族 GTP 酶驱动。越来越多的证据使得另一类肌动蛋白驱动的突出，称为侵袭伪足牵连于基质降解中 (Murphy 和 Courtneidge,2011)。为进行转移，乳腺癌游走细胞形成侵袭伪足并渗入附近血管中。旨在表征与乳腺癌细胞向肺部 (Minn 等,2005) 和脑部 (Bos 等,2009) 转移相关的基因表达特征的研究鉴定了成为定点转移基础的多组基因。有趣的是，两组均包括表皮生长因子 (EGF) 家族的成员，表明通过共用受体 EGFR 发信号支持转移性传播。

[0004] 胞内运输作为细胞迁移和肿瘤进展的关键特征出现 (Mosesson 等,2008)。例如，已经证实突变体 p53 通过增强型整联蛋白和取决于 Rab 偶联蛋白 (RCP) 的 EGFR 运输促进转移 (Muller 等,2010)。随同 Rab 蛋白一起，磷酸肌醇通过确定囊泡同一性在细胞区域化中起关键作用 (Yuan 和 Cantley,2008)。例如，在 PI(4,5)P_2(磷脂酰肌醇 -4,5 二磷酸) 的 D3 位置通过磷脂酰肌醇 3- 激酶 (PI3K) 的磷酸化生成侵袭伪足形成所必需的 PI(3,4,5)P_3(Yamaguchi 等,2011)。类似地，PI(4,5)P_2调节控制胞吞作用和肌动蛋白动力学特性的多种蛋白质 (Saarikangas 等,2010)，但是其水平受另外两类酶严格控制 ：磷脂酶 C(PLCγ) 促进 PI(4,5)P_2水解，这样激活丝切蛋白 (Cofilin)(一种肌动蛋白切割蛋白) 并且驱动乳腺细胞迁移 (van Rheenen 等,2007)。同样，肌醇多磷酸 5- 磷酸酶，例如突触囊泡磷酸酶 2(SYNJ2)，为肌醇环的 D5 位置脱去磷酸并且控制神经胶质瘤细胞迁移 (Chuang 等,2004 ；Malecz 等,2000)。另外在某些前列腺癌样品中鉴定出纯合突变，Rossi 等 Cancer Genet Cytogenet.2005 年 9 月 ;161(2):97-103。

发明内容

[0005] 根据本发明一些实施方案的一方面，提供了一种预防肿瘤转移的方法，条件是所述肿瘤不是神经胶质瘤，所述方法包括向有需要的受试者施用治疗有效量的突触囊泡磷酸酶 2(SYNJ2) 抑制剂，从而预防肿瘤转移。

[0006] 根据本发明一些实施方案的一方面，提供了一种治疗癌症的方法，所述方法包括向有需要的受试者施用治疗有效量的突触囊泡磷酸酶 2(SYNJ2) 抑制剂和与癌症发作或进展相关的细胞表面受体的抑制剂，从而治疗癌症。

[0007] 根据本发明一些实施方案的一方面，提供了一种用于预防肿瘤转移的突触囊泡磷酸酶 2(SYNJ2) 抑制剂，条件是所述肿瘤不是神经胶质瘤。

[0008] 根据本发明一些实施方案的一方面，提供了一种用于治疗癌症的突触囊泡磷酸酶 2(SYNJ2) 抑制剂和一种用于治疗癌症与癌症发作或进展相关的细胞表面受体的抑制剂。

[0009] 根据本发明的一些实施方案，与癌症发作或进展相关的细胞表面受体为受体酪氨

酸激酶。

[0010] 根据本发明的一些实施方案，所述受体酪氨酸激酶为 ErbB 受体。

[0011] 根据本发明的一些实施方案，所述 ErbB 受体为表皮生长因子受体 (EGFR)。

[0012] 根据本发明一些实施方案的一方面，提供了一种鉴定肿瘤转移的推定抑制剂的方法，所述方法包括在试验试剂的存在下，分析 SYNJ2 介导的 PI(3,4,5)P_3向 PI(3,4)P_2的加工，其中在试验试剂的存在下 PI(3,4,5)P_3向 PI(3,4)P_2的加工与试验试剂不存在时相比减少，表明为肿瘤转移的推定抑制剂。

[0013] 根据本发明的一些实施方案，所述分析 SYNJ2 介导的 PI(3,4,5)P_3向 PI(3,4)P_2的加工通过竞争测定法进行。

[0014] 根据本发明的一些实施方案，所述竞争测定法测定 PI(3,4)P_2结合结构域从包含与 PI(3,4)P_2结合的 PI(3,4)P_2结合结构域的复合物的位移。

[0015] 根据本发明的一些实施方案，所述竞争测定法为荧光偏振竞争测定法。

[0016] 根据本发明一些实施方案的一方面，提供了一种在有需要的受试者中癌症预后的方法，所述方法包括测定受试者癌细胞中 SYNJ2 的水平或活性，其中在受试者癌细胞中所述 SYNJ2 的活性水平与在未受影响的对照样品的细胞中相比上调，表明预后不良。

[0017] 根据本发明的一些实施方案，所述方法还包括使用金标法增进预后。

[0018] 根据本发明的一些实施方案，所述金标法包括标记的检测。

[0019] 根据本发明的一些实施方案，所述标记选自 HER-2 和雌激素受体 (ER)。

[0020] 根据本发明的一些实施方案，所述转移为 EGF 依赖型。

[0021] 根据本发明的一些实施方案，所述癌症为乳腺癌。

[0022] 根据本发明的一些实施方案，所述 SYNJ2 抑制剂选自小分子、抗体、肽和核酸沉默剂。

[0023] 根据本发明的一些实施方案，所述小分子选自表 2 中所列的分子。

[0024] 根据本发明一些实施方案的一方面，提供了一种用于治疗癌症或预防癌转移的制品，其包括包装 SYNJ2 抑制剂和与癌症发作或进展相关的细胞表面受体的抑制剂的包装材料。

[0025] 根据本发明的一些实施方案，与所述癌症发作或进展相关的细胞表面受体的抑制剂为抗体。

[0026] 根据本发明的一些实施方案，与所述癌症发作或进展相关的所述细胞表面受体的所述抑制剂为小分子抑制剂。

[0027] 除非另有定义，本文使用的所有技术和 / 或科学术语具有与本发明所属领域中普通技术人员通常所理解的相同含义。虽然与本文所述相似或等效的方法和材料可用于本发明实施方案的实践或试验中，但下面对示例性方法和 / 或材料进行了描述。如有冲突，以包括定义在内的专利说明书为准。另外，材料、方法和实例仅为说明性而非旨在必定限制。

[0028] 附图简述

[0029] 本文仅以举例的方式，参考附图描述本发明的一些实施方案。现详细地具体参考附图，应强调的是所示细节是举例而言并且是为了说明性讨论本发明的实施方案。在这点上，以附图做出的描述使得本发明的实施方案可如何实践对本领域的技术人员显而易见。

[0030] 图中：

[0031] 图 1A-I 显示 EGF 促进乳腺细胞的侵袭性生长并诱导了一组特定的基因。图 1A- 在生长因子不存在时接种 MCF10A 细胞并使其形成细胞团。72h 后，用指定生长因子（各 10ng/mL）处理细胞并在 24h 后拍摄相衬图像（比例尺，50μm）。图 1B- 按指示，在指定配体（10ng/mL）的存在下，在迁移或侵袭小室内接种 MCF10A 细胞，并且在 18h 后，迁移到下部隔室的细胞经结晶紫染色（左图）。示出了迁移和侵袭信号的量化，归一化为 EGF 处理的效应。数据表示三份生物样品来自重复两次的代表性实验的平均值 ±S.D.（右图）。图 1C- 在 transwell 插件中将 MCF10A 细胞接种于含 EFG，没有或有抑制剂 AG-1478(1μM)、U0126(5μM) 或渥曼青霉素 (Wortmannin)(200nM) 的培养基中，并使其迁移 18h。数据表示三份样品的平均值 ±S.D.。实验重复两次。图 1D- 人乳腺 MCF10A 细胞中受 EGF（而不是受血清 (Amit 等，2007)）特异性诱导的一系列 425 个基因与在 MDA-MB-231 细胞转移的情况下上调的基因 (1,597 个基因）交叉 (Minn 等，2005)。23 个重叠基因中的一个编码 5’- 磷脂酰肌醇脂质磷酸酶突触囊泡磷酸酶 -2(SYNJ2)。图 1E- 用编码 LacZ(Ctrl) 或 SYNJ2-GFP(SYNJ2-OX) 的慢病毒粒子感染 MCF10A 细胞。通过免疫印迹法测定内源 SYNJ2 和 SYNJ2-GFP 融合蛋白的表达水平，并通过对微管蛋白的探测确认蛋白质负荷相等。图 1F- 在 EGF 不存在 (NT) 或存在 (10ng/mL) 时在迁移小室内接种 MCF10A 细胞的 Ctrl 和 SYNJ2-OX 克隆 (5×10^4个细胞 / 孔）并使其迁移 22h。到达滤器另一侧的迁移细胞经结晶紫染色并拍摄图像。图 1G- 用 siRNA 对照 (siCtrl) 或针对 SYNJ2 的 siRNA(siSYNJ2) 转染 MCF10A 细胞，并且在 36h 后通过免疫印迹法测定 SYNJ2 的蛋白质水平。通过对 Ras-GAP 的免疫印迹确认蛋白质负荷相等。图 1H- 在 EGF 不存在 (NT) 或存在 (10ng/mL) 时在迁移小室内接种 G 中提供的细胞 (5×10^4个细胞 / 孔）并使其迁移 22h。到达滤器下表面的迁移细胞经结晶紫染色并捕捉图像。图 1I- 用指定 siRNA 处理 MCF10A 细胞的铺满培养物。一旦形成单层，就使其进入监测擦伤闭合速率的自动化擦伤系统。

[0032] 图 2A-E 显示 EGF 对 SYNJ2 的转录诱导促进侵袭性生长。图 2A- 用 EGF(20ng/mL) 或血清 (5％）刺激血清饥饿 MCF10A 细胞，并且使用微阵列或 RT-qPCR 测定 SYNJ2 mRNA 表达。图 2B- 按指示用 EGF 刺激 MCF10A 细胞，提取并做免疫印迹。图 2C- 在 EGF 不存在或存在时，培养感染了编码 GFP-SYNJ2(SYNJ2-OX) 或作为对照 (Ctrl) 的 LacZ 的病毒的 MCF10A 细胞 4 天。使用鬼笔环肽和 DAPI 获得相衬图像（上，比例尺 :100μm) 和共聚焦图像（下，比例尺 :20μm)。图 2D-E- 在 EGF 不存在 (NT) 或存在 (10ng/mL) 时在迁移或侵袭小室内培养 MCF10A 细胞 (5-6$\times10^4$个细胞 / 孔)22h。到达滤器底部的细胞染色并且量化滤器的覆盖范围（平均值 ±S.D.)。

[0033] 图 3A-G 显示乳腺细胞迁移和侵袭伴有 SYNJ2 向前缘的诱导型易位。图 3A- 用编码 LacZ(Ctrl) 或 V5 标记的 SYNJ2(SYNJ2-V5) 的慢病毒粒子，连同对照 shRNA(shCtrl) 或针对 SYNJ2 的 shRNA(shSYNJ2) 一起，感染 MDA-MB-231 细胞。通过免疫印迹法测定 V5-SYNJ2 和内源 SYNJ2 的蛋白质水平。通过对 AKT 的免疫印迹确认蛋白质负荷相等。图 3B- 稳定过表达 SYNJ2 或作为对照的 LacZ 的 MDA-MB-231 细胞的相位图像（左图）和侵袭图像（右图）。使用侵袭测定法测定侵袭能力，一式三份，并且量化侵袭细胞并归一化为对照 (Ctrl)。比例尺，50μm。图 3C- 用针对 SYNJ2 的 siRNA 寡聚核苷酸（或 siCtrl) 转染 MDA-MB-231 细胞。36h 后，通过免疫印迹法测定 SYNJ2 的蛋白质水平。通过对 Ras-GAP 的免疫印迹确认蛋白质负荷相等。图 3D- 在迁移或侵袭小室内接种来自 C 的细胞并培养 18h。量化迁移和侵

袭信号并归一化为经 EGF 处理的 siCtrl 细胞。所示数据为三份样品的平均值 ±S.D.。图 3E- 将瞬时表达 GFP-SYNJ2 的 MDA-MB-231 细胞涂到盖玻片上并用 TGFα (10ng/mL) 刺激。(每 10s) 拍摄延时显微照片。倒置所示图像，黑斑表示 SYNJ2 及其在板状伪足基部的组件。比例尺，10μm。图 3F- 使用 TRITC- 鬼笔环肽对 MDA-MB-231 细胞的内源 SYNJ2 和 F- 肌动蛋白免疫染色。正方形区域放大。比例尺，10μm。图 3G- 用 EGF 刺激 MCF10A 细胞 18h，然后使用 TRITC- 鬼笔环肽对内源 SYNJ2 免疫染色而 F- 肌动蛋白复染。比例尺，10μm。

[0034] 图 4A-F 显示 SYNJ2 的催化活性对于侵袭性生长必不可少。图 4A-B- 在 5％ Matrigel 中接种表达 SYNJ2(SYNJ2-OX) 或针对 SYNJ2 的 shRNA(shSYNJ2) 的 MDA-MB-231 细胞以及对照细胞。6 天后捕捉图像，并量化侵袭性球体（平均值 ±S.D.）。比例尺，50μm。图 4C-D- 用野生型 SYNJ2(shSYNJ2+SYNJ2^{WT}) 或用催化失能的突变体 (shSYNJ2+SYNJ2^{CD}) 感染表达 shSYNJ2 的 MDA-MB-231 细胞。按指示提取细胞并做免疫印迹，或使其在侵袭小室内侵袭 18h。示出了侵袭细胞及其标准量化的图像（平均值 ±S.D.）。图 4E- 示出了在纤连蛋白上生长的 shCtrl 和 shSYNJ2 细胞的扫描电子显微照片。比例尺，2μm。图 4F- 指定 MDA-MB-231 细胞中经鬼笔环肽和 DAPI 染色的 F- 肌动蛋白的图像。示出了 Z 轴部分（线）和放大区域。箭头标记溶胀结构。比例尺，10μm。

[0035] 图 5A-H 示出了 SYNJ2 的亚细胞定位。图 5A- 用 RFP- 网格蛋白转染表达 GFP-SYNJ2 的 MDA-MB-231 细胞并接种于涂有纤连蛋白的板上。使用转盘式显微镜术，每 5s 为细胞成像。箭头标记新形成的前缘。比例尺，5μm。图 5B- 描绘 SYNJ2 在前缘（上面两行）和细胞体下面装配和分解的代表时帧。对于下面的行而言，细胞经 mCherry-lifeACT 质粒转染并接种于胶原上。之后，间隔 1min 为细胞成像。插入箭头以便参考。注意时间尺度的差异。比例尺，1μm。图 5C- 同时通过 TIRF 和落射荧光显微镜术为细胞成像并将信号转换成波动曲线 (X 轴)。箭头标记信号起始。比例尺，5μm。图 5D- 在用 Dyngo-4a(30μM；一种发动蛋白 -2 抑制剂）处理前 5min 和处理后 5min，使用转盘式共聚焦显微镜术为细胞成像。比例尺，5μm。图 5E- 用 Dyngo-4a(30μM；30min) 或用溶剂 (DMSO) 预培养稳定表达 GFP-SYNJ2 的 MDA-MB-231 细胞。用抗 GFP 抗体（或不用抗体；-Ab) 使细胞裂解物进行免疫沉淀，然后连同细胞裂解物样品 (5％）一起，用指定抗体做免疫印迹。图 5F- 将细胞接种于纤连蛋白上，固定并免疫染色内源 Rac1。比例尺，10μm。图 5G- 在用 NSC-23766(5μM) 进行 30min 长的处理前 5min 和处理后 5min，使用共聚焦显微镜术为细胞成像。图 5H- 用指定 siRNA 寡聚核苷酸处理 MDA-MB-231 细胞。为细胞提取物的 SYNJ2 和 Ras-GAP 做印迹。使用基于 ELISA 的测定法测定 GTP-Rac1 水平（细胞骨架）。

[0036] 图 6A-D 显示 SYNJ2 向前缘的定位与小窝蛋白分布不同并且取决于 F- 肌动蛋白、胆固醇和 PI3K。图 6A- 随时间推移同时为表达 GFP-SYNJ2 和共表达 RFP-Cav1 的 MDA-MB-231 细胞成像，并将信号转换成波动曲线 (X 和 Y 轴）。注意 SYNJ2 组件的瞬时性质和小窝蛋白 1 的稳定外观。比例尺，5μm。图 6B- 左图描绘了如图 5A 中成像（间隔 5s，单平面、转盘式共聚焦）的 150 种随机选择的 SYNJ2 组件的分布（相对于寿命的小窝％）。右图描绘了显示 55s 寿命的组件的平均 (±SEM) 相对强度。图 6C- 用 MβCD(10mM,15min) 或渥曼青霉素 (500nM,15min) 处理稳定表达 GFP-SYNJ2 的 MDA-MB-231 细胞。在处理之前或之后每 6s 捕捉所选相同细胞的图像，并将信号转换成波动曲线（表示左图中的正方形插图）。比例尺，20μm。图 6D- 用拉春库林 B(Latrunculin B)(1μM,15min) 处理稳定共表达 GFP-SYNJ2 和

lifeACT-mCherry 的 MDA-MB-231 细胞。在处理之前或之后获取图像。比例尺,5μm。

[0037] 图 7A-E 显示 SYNJ2 损耗使 EGFR 留在细胞内囊泡中。图 7A- 接种于含 EGF 的培养基中 3 天后,提取稳定表达 shRNA 对照 (shCtrl) 或对 SYNJ2 有特异性的 shRNA(shSYNJ2) 的 MCF10A 细胞。探测 SYNJ2、EGFR、EGFR 磷酸化酪氨酸 1068(pEGFR)、磷酸化 ERK(pERK) 和作为上样对照的 Ras-GAP 的免疫印迹。图 7B- 在 EGF 的存在下,用 siRNA 对照或针对 SYNJ2 的 siRNA 转染 MCF10A 细胞。使用 EGFR 和 SYNJ2 抗体进行共聚焦免疫荧光分析。注意仅 SYNJ2 缺乏型细胞 (星号) 显示出 EGFR 运输缺陷。比例尺,10μm。图 7C- 为 MDA-MB-231 细胞的三种衍生细胞的 EGFR 免疫染色而 DAPI 和 F- 肌动蛋白复染 :(i) 其中 SYNJ2 敲减的细胞 (shSYNJ2 ;左边一列),(ii) 通过与催化非活性形式相对应的慢病毒基因转移感染的相同细胞 (shSYNJ2+SYNJ2^{CD};中间一列),和 (iii) 其中 SYNJ2 敲减并通过感染引入野生型形式的细胞 (shSYNJ2+SYNJ2^{WT};右边一列)。比例尺,20μm。图 7D- 泛素化 EGFR 水平 (密度测定法)。图 7E- 用 488-Tfn 刺激 MDA-MB-231 衍生细胞 (5min,10μg/mL)。将细胞于冰上固定,经酸洗并分析信号强度。

[0038] 图 8A-I 显示 SYNJ2 调节 EGFR 运输和趋化性。图 8A- 按指示,为用指定 siRNA 转染的 MDA-MB-231 细胞的全部提取物做免疫印迹。图 8B- 指定 MDA-MB-231 亚克隆的表面 EGFR 的 FACS(左) 和 ^{125}I-EGF 结合 (右 ;一式三份) 分析。图 8C- 使 shCtrl 和 shSYNJ2 细胞在纤连蛋白上生长并对 EGFR 和 F- 肌动蛋白免疫染色。比例尺,20μm。图 8D- 暴露于 EGF 梯度后,在趋化小室内迁移的 shCtrl 和 shSYNJ2 MDA-MB-231 细胞的轨迹玫瑰图。红色轨迹指细胞向 EGF 迁移。图 8E- 用 EGF(10ng/mL) 处理饥饿 MDA-MB-231 衍生细胞并且按指示将细胞裂解物进行免疫沉淀和免疫印迹。图 8F- 与 C 中一样,培养细胞并对活性 EGFR(pY1045) 和 F- 肌动蛋白免疫染色。比例尺,10μm。图 8G- 用 EGF(10ng/ml) 处理指定 MDA-MB-231 衍生细胞 5h 并且按指示为提取物做免疫印迹。图 8H- 将指定 MDA-MB-231 衍生细胞暴露于 Alexa Fluor 488-Tfn(25μg/ml ;5min),酸洗以去除表面结合的配体,并以指定时间间隔拍摄图像。示出了归一化荧光信号。比例尺,10μm。图 8I- 用 Alexa Fluor 488-EGF(20μg/ml ;10min) 刺激经 siCtrl 或 siSYNJ2 预处理的 MDA-MB-231 细胞,酸洗,在 37℃下培养指定时间间隔并通过 FACS 分析。

[0039] 图 9A-D 显示 SYNJ2 对于囊泡运输和粘着斑形成是必需的。图 9A- 固定 MDA-MB-231 衍生细胞 (shCtrl 和 shSYNJ2) 并对 EEA1、F- 肌动蛋白和细胞核 (DAPI) 染色。比例尺,10μm。图 9B- 为 MDA-MB-231 衍生细胞,即 shCtrl 和 shSYNJ2 细胞探测整联蛋白 β-1、F- 肌动蛋白和 DAPI(比例尺,20μm)。图 9C- 用 siCtrl 和 siSYNJ2 处理 MDA-MB-231 细胞 48h,然后对整联蛋白 β-1 和磷酸化 EGFR 免疫染色。图 9D-MDA-MB-231 衍生细胞的桩蛋白、细胞核 (DAPI) 和 F- 肌动蛋白的免疫荧光分析 (使用 TRITC- 鬼笔环肽)。在胞质区内相对于粘着斑量化桩蛋白信号,并且还量化了每个细胞粘着斑的数量。另外,通过测定与完整圆的偏差 (偏心率) 量化粘着斑的形状。

[0040] 图 10A-F 显示 SYNJ2 损耗扰乱了磷酸肌醇体内平衡,使初级内体充气并分解粘着斑。图 10A- 用 GFP-Rab4 质粒转染表达 shCtrl 或 shSYNJ2 的 MDA-MB-231 细胞并且在 48h 后固定细胞并使用 TRITC- 鬼笔环肽对 F- 肌动蛋白复染。图 10B- 对 MDA-MB-231 衍生细胞的 Rab5、F- 肌动蛋白和细胞核 (DAPI) 免疫染色。量化图像中 Rab5 阳性囊泡的尺寸和数量以及平均细胞面积。比例尺,10μm。图 10C- 通过色谱法分离从 ^{3}H- 磷酯酰肌醇标记的

MDA-MB-231 细胞衍生细胞中提取的磷酸肌醇并且在 3 次不同实验中测定其水平（信号归一化为 shCtrl 细胞）。图 10D- 探测 shCtrl 和 shSYNJ2 MDA-MB-231 细胞的 pY1068-EGFR、桩蛋白和 - 肌动蛋白（共定位信号为白色）。比例尺，10μm。图 10E- 接种 shCtrl 和 shSYNJ2 MDA-MB-231 细胞。20min 后去除独立细胞并且为贴壁细胞成像并量化其表面积。图 10F- 在 RTCA E 板上接种稳定表达 shCtrl 或 shSYNJ2 的 MDA-MB-231 细胞并且间隔 5s 记录实时阻抗测量 80min，然后间隔 10min 再记录 80min。示出了 2 次重复的平均值（±S.D.）。

[0041] 图 11A-G 显示 SYNJ2 调节蛋白酶分泌和侵袭伪足装配。图 11A- 在 Matrigel 中培养 shCtrl 和 shSYNJ2 MDA-MB-231 细胞 5 天，固定并对 MMP-9 免疫染色。将信号强度转换成热图并根据与菌落核心的距离绘图。箭头标记球体边界。比例尺，50μm。图 11B- 使用明胶酶谱法分析来自对照 MDA-MB-231 细胞和稳定表达 SYNJ2 的细胞的上清液的 MMP-2 和 MMP-9 活性，一式三份。图 11C- 将稳定表达 GFP-SYNJ2 的 MDA-MB-231 细胞涂到预先涂有交联荧光明胶的盖玻片上。3h 后，探测细胞的 GFP 和 F- 肌动蛋白，并检测侵袭伪足结构（箭头）。比例尺，10μm。图 11D- 将过表达 SYNJ2(SYNJ2-OX) 的 MDA-MB-231 细胞以及经 siCtrl 或 siSYNJ2 寡聚核苷酸预处理的细胞涂到预先涂有交联荧光明胶的盖玻片上并且在 3 次独立实验中量化侵袭伪足结构。图 11E- 通过明胶降解，以及通过对 F- 肌动蛋白或 TKS5 染色检测经指定 siRNA 处理的 MDA-MB-231 细胞的侵袭伪足结构。箭头 (Z 轴图像）标记侵袭伪足。比例尺，10μm。图 11F- 将表达 siCtrl 或 siSYNJ2 的 MDA-MB-231 细胞涂到涂有明胶的盖玻片上并且与 C 中一样使用鬼笔环肽和 EGFR 磷酸化形式（酪氨酸 1068) 的抗体加工。比例尺，10μm。图 11G- 使用基于 ELISA 的测定法检查用指定 MDA-MB-231 衍生细胞调节 3 天的培养基的 EGF 样配体。

[0042] 图 12A-G 显示 SYJN2 调节基质降解和侵袭伪足装配。图 12A- 接种经指定 siRNA 处理的 MDA-MB-231 细胞，培养 3 天并且使用明胶 (0.1％）包埋凝胶经电泳分离其条件培养基，接着蛋白质染色以量化 MMP-2 和 MMP-9 蛋白水解活性。图 12B- 使用稳定表达 GFP-SYNJ2 的 MDA-MB-231 细胞的 GFP 偶联珠粒和澄清提取物进行的免疫共沉淀分析。图 12C- 用 RFP- 皮动蛋白质粒转染稳定表达 GFP-SYNJ2 的 MDA-MB-231 细胞并接种于胶原板上。48h 后进行活细胞图像分析，并且捕捉外围和中央细胞区域的代表性快照图像。比例尺，5μm。图 12D- 用编码 Tapp1（一种 PI(3,4)P_2结合剂）的经 Myc 标记的 PH 结构域的质粒转染 MDA-MB-231 细胞的指定衍生细胞并且在 48h 后，将其接种在涂有明胶的表面。使用共聚焦显微镜术目测 F- 肌动蛋白、聚集的 TKS5 和 PI(3,4)P_2(Tapp1) 的共同分布并且量化。比例尺，10μm。

[0043] 图 12E- 将表达 siCtrl 或 siSYNJ2 的 MDA-MB-231 细胞接种到涂有 FITC- 明胶的盖玻片上并培养 3h。然后固定细胞并对 CD44 免疫染色，并且用 TRITC- 鬼笔环肽对 F- 肌动蛋白复染。使用荧光显微镜术目测细胞，并且在 FITC- 明胶基质中通过观察孔检测侵袭伪足。加框区域放大。比例尺，10μm。图 12F-CD44 的抗体用于 shCtrl 和 shSYNJ2 细胞表面表达的 FACS 分析。指出了与加框区域相对应的细胞的分数。图 12G- 将用 siCtrl 或 siSYNJ2 预处理的 MDA-MB-231 细胞接种到涂有 FITC- 明胶的盖玻片上并培养 3h。然后固定细胞并对 MT1-MMP 免疫染色，并且用 TRITC- 鬼笔环肽对 F- 肌动蛋白复染。比例尺，10μm。

[0044] 图 13A-H 显示 SYNJ2 的酶活性驱使乳腺肿瘤细胞转移扩散。图 13A- 在雌性 SCID 小鼠（每组 10-11 只）的脂肪垫上植入表达 RFP 的 MDA-MB-231 细胞的指定衍生细胞 (2×10^6

个 / 只小鼠)。移植 2 和 6 周后测量肿瘤大小 (平均值 ±S.D.)。图 13B-C- 示出了移植 6 周后在腋窝和远侧淋巴结 (图 13B) 或肺部 (图 13C) 中出现的转移。星号标记 p 值 : *<0.05,**<0.01 和 ***<0.001。图 13D-F- 与 A 中一样,在动物体内植入过表达对照 (LacZ) 和 SYNJ2(SYNJ2-OX)、经 RFP 标记的 MDA-MB-231 细胞并且在移植 6 和 8 周后量化肿瘤大小 (图 13D) 以及向淋巴结 (图 13E) 和肺部 (图 13F) 的转移。图 G-H- 经静脉 (1.5×10^5个 / 只小鼠 ;尾静脉) 或在 5 周龄雌性 SCID 小鼠的乳腺脂肪垫内 (2.5×10^6个 / 只小鼠),注射指定 MDA-MB-231-RFP 衍生细胞。4 周后,检查来自经静脉注射的小鼠的肺部的 RFP 信号 (左侧和中间的图)。4 周后从脂肪垫处理组收集外周血。按 ficol 梯度纯化样品并且每 $1x10^6$个 FACS 读数为 RFP 阳性循环肿瘤细胞的数量评分并归一化为肿瘤重量。

[0045] 图 14 为局部和远侧淋巴结转移的活体成像。接种了 MDA-MB-231-RFP 细胞并于 6 周后分析的小鼠体内局部 (同侧) 和远侧 (对侧) 淋巴结转移的代表性图像 (见图 13B)。成像之前,麻醉小鼠并去除其毛皮以便目测和量化淋巴结内的转移。

[0046] 图 15 是描绘 SYNJ2 在细胞迁移和侵袭中的综合作用的工作模型。载 EGFR 的再循环核内体将活性受体定位在腹侧膜处,并且在这之后局部激活 PI3K。PI3K 对膜 PI(4,5)P_2 的磷酸化生成 PI(3,4,5)P_3,PI(3,4,5)P_3 经 SYNJ2 脱去磷酸成为 PI(3,4)P_2。后者募集 TKS5,这样锚定皮动蛋白并对肌动蛋白聚合起核心作用。同时,SYNJ2 控制粘着分子如 CD44 和蛋白酶如 MT1-MMP 的递送,以降解细胞外基质 (ECM) 并建立新的侵袭结构,侵袭伪足。以类似方式,EGFR 向细胞外周的递送导致 PI(4,5)P_2 受 SYNJ2(和磷脂酶 C) 分解,这样局部激活了发动蛋白和肌动蛋白切割酶如丝切蛋白以溶解皮层肌动蛋白纤维并引发肌动蛋白填充的、富含整联蛋白的突出,称为板状伪足。水平箭头标记细胞迁移的方向。质膜的彩色编码部分指特定 PI 磷脂。

[0047] 图 16A-C- 显示 SYNJ2 在侵蚀性乳腺肿瘤中高度表达。图 16A- 根据 SYNJ2 丰度 (高、中和低),使用免疫组织化学和组织微阵列为 331 例侵袭性乳腺癌分层。根据临床亚型提出肿瘤的相对分数。图 16B- 展示在细胞腔情况下 (星号标记作为对照的内皮细胞的表达) 及在基底样和 HER2 过表达乳腺肿瘤中观察到的强度和模式 (右侧一列放大) 的 SYNJ2 染色的代表性图像。图 16C- 根据 286 名 (左侧 ;GSE2034) 或 99 名 (右侧 ;GSE19783) 乳腺癌患者组群中的 SYNJ2 mRNA 表达分层的 Kaplan-Meier 曲线。

[0048] 图 17A-B 示出了用于测量 SYNJ2 的 5'- 磷酸酶活性的荧光偏振测定法的原理。图 17A 是展示未结合 PI(3,4)P2 荧光探针产生低偏振读数,而结合 PI(3,4)P2 荧光探针使偏振读数增大的一般原理的示意图。图 17B 是显示通过偏振度 (mP) 测量的 SYNJ2 5'- 磷酸酶活性检测的代表性柱状图。

[0049] 图 18 描绘了克隆到 pET28 质粒中并且在大肠杆菌 (E.coli) 中表达的 Flag-TAPP1 PH 结构域 -His 的氨基酸和核酸序列 (分别为 SEQ ID NO:13 和 14)。TAPP1-PH 结构域标记为黄色。

具体实施方式

[0050] 本发明,在其一些实施方案中,涉及预防肿瘤转移、癌症治疗和预后及鉴定为推定转移抑制剂的试剂的方法。

[0051] 详细解释本发明的至少一个实施方案之前,应理解本发明在其应用上不一定限于

以下描述中提出或实施例中举例说明的详情。本发明能够有其它实施方案或能够以各种方式实践或实施。

[0052] 生长因子驱使细胞迁移和转移，但根本机制未完全了解。

[0053] 本发明现已鉴定突触囊泡磷酸酶 -2(SYNJ2) 为调节体外侵袭伪足和板状伪足和体内癌转移中的主要模块。

[0054] 如下文和之后的实施例部分所述，本发明人在动物和患者样本中证实了其在体外的发现。具体地，采用经 EGF 刺激的乳腺细胞，本发明人将脂质磷酸酶突触囊泡磷酸酶 2(SYNJ2) 与侵袭性表现型联系起来，并且将高 SYNJ2 于癌症患者的短期存活率关联起来。SYNJ2 的敲减稳健地削弱了动物模型中乳腺肿瘤细胞的转移。在体外，SYNJ2 缺乏型细胞表现出 EGFR 和整联蛋白脱轨运输，导致粘着斑变形、板状伪足受阻和侵袭伪足消失。不受理论约束，表明活性 EGFR 的再循环局部促进 SYNJ2 介导的特定磷酸肌醇脂质脱磷酸，从而促使侵袭伪足和板状伪足形成并促进肿瘤进展 (见图 15)。

[0055] 因此，根据本发明的一方面，提供了一种预防肿瘤转移的方法，条件是所述肿瘤不是神经胶质瘤，所述方法包括向有需要的受试者施用治疗有效量的突触囊泡磷酸酶 2(SYNJ2) 抑制剂，从而预防肿瘤转移。

[0056] 如本文所使用，术语“肿瘤转移”指从其首发位置扩散到身体其它部位的恶性肿瘤，例如转移到肺部的乳腺癌。

[0057] 如本文所使用，术语“癌症”和“肿瘤”可交换使用。该术语指异常且不受控制的细胞分裂引起的恶性生长或肿瘤。

[0058] 如本文所使用，术语“预防”指阻止、中断、抑制转移过程或进展和后续转移。

[0059] 根据另一方面，提供了一种治疗癌症的方法，所述方法包括向有需要的受试者施用治疗有效量的突触囊泡磷酸酶 2(SYNJ2) 抑制剂和与癌症发作或进展相关的细胞表面受体的抑制剂，从而治疗癌症。

[0060] 如本文所使用，术语“治疗”包括消除、大体上抑制、减缓或逆转病状的进展，大体上改善病状的临床或审美症状，或大体上预防病状的临床或审美症状出现。

[0061] 可根据本发明的一些实施方案治疗 (或预后) 的癌症的非限制性实例包括任何实体或非实体癌和 / 或癌转移，包括但不限于胃肠道肿瘤 (结肠癌、直肠癌、结直肠癌瘤、结直肠癌、结直肠腺瘤、1 型遗传性非息肉病、2 型遗传性非息肉病、3 型遗传性非息肉病、6 型遗传性非息肉病、结直肠癌、7 型遗传性非息肉病、小肠和 / 或大肠癌瘤、食道癌、伴有食道癌的胼胝症、胃癌、胰腺癌、胰腺内分泌肿瘤)、子宫内膜癌、隆凸性皮肤纤维肉瘤、胆囊癌、胆道肿瘤、前列腺癌、前列腺腺癌、肾癌 (例如，2 型或 1 型维尔姆斯氏瘤 (Wilms' tumor))、肝癌 (例如，肝胚细胞瘤、肝细胞癌瘤、肝细胞癌)、膀胱癌、胚胎性横纹肌肉瘤、胚细胞瘤、滋养细胞瘤、睾丸生殖细胞瘤、卵巢未成熟畸胎瘤、子宫、卵巢上皮性、骶尾瘤、绒毛膜癌、胎盘部位滋养细胞瘤、成人上皮细胞瘤、卵巢癌、浆液性卵巢癌、卵巢性索肿瘤、宫颈癌、子宫颈癌、小细胞和非小细胞肺癌、鼻咽、乳腺癌 (例如，乳腺导管癌、乳腺浸润性导管癌、散发性乳腺癌、乳腺癌敏感性、4 型乳腺癌、乳腺癌 -1、乳腺癌 -3、乳腺 - 卵巢癌)、鳞状细胞癌 (例如，在头颈部)、神经源性肿瘤、星形细胞瘤、多形性胶质母细胞瘤、成神经细胞瘤、淋巴瘤 (例如，霍奇金病 (Hodgkin's disease)、非霍奇金淋巴瘤 (non-Hodgkin's lymphoma)、B 细胞瘤、伯基特淋巴瘤 (Burkitt)、皮肤 T 细胞瘤、组织细胞瘤、淋巴细胞瘤、T 细胞瘤、胸

腺肿瘤）、神经胶质瘤、腺癌、肾上腺瘤、遗传性肾上腺皮质癌、脑部恶性（肿瘤）、各种其它癌（例如，支气管原大细胞癌、导管癌、Ehrlich-Lettre 腹水、表皮样癌、大细胞癌、Lewis 肺癌、髓样癌、粘膜表皮样瘤、燕麦细胞癌、小细胞癌、梭形细胞癌、棘细胞癌、移行细胞癌、未分化癌、癌肉瘤、绒毛膜癌、囊腺癌）、室管膜母细胞瘤、上皮瘤、红白血病（例如，弗兰德红白血病 (Friend)、淋巴母细胞红白血病）、纤维肉瘤、巨细胞瘤、胶质瘤、成胶质细胞瘤（例如，多形性、星形细胞瘤）、神经胶质瘤、肝细胞瘤、异种杂交瘤、杂交骨髓瘤、组织细胞瘤、杂交瘤（例如，B 细胞）、肾上腺样瘤、胰岛瘤、岛细胞瘤、角质瘤、成平滑肌瘤、平滑肌肉瘤、白血病（例如，急性淋巴细胞白血病、急性淋巴母细胞白血病、急性淋巴母细胞性前 B 细胞白血病、急性淋巴母细胞性 T 细胞白血病、急性成巨核细胞白血病、单核细胞白血病、急性髓细胞白血病、急性髓性白血病、伴有嗜酸粒细胞增多的急性髓性白血病、B 细胞性白血病、嗜碱细胞性白血病、慢性髓性白血病、慢性白血病、B 细胞性白血病、嗜酸细胞性白血病、弗兰德白血病 (Friend)、粒细胞或髓细胞性白血病、毛细胞白血病、淋巴细胞性白血病、巨核细胞白血病、单核细胞白血病、单核 - 巨噬细胞白血病、成髓细胞性白血病、骨髓性白血病、慢性骨髓单核细胞性白血病、浆细胞白血病、前 B 细胞白血病、前髓细胞性白血病、亚急性、T 细胞、淋巴肿瘤、髓系恶性肿瘤素因、急性非淋巴细胞性白血病）、淋巴肉瘤、黑素瘤、乳腺肿瘤、肥大细胞瘤、成神经管细胞瘤、间皮瘤、转移瘤、单核细胞瘤、多发性骨髓瘤、骨髓发育异常综合征、骨髓瘤、肾母细胞瘤、神经组织胶质瘤、神经组织神经元肿瘤、神经鞘瘤、成神经细胞瘤、少突神经胶质瘤、骨软骨瘤、骨骨髓瘤、骨肉瘤（例如，尤文氏肉瘤 (Ewing's)）、乳头状瘤、移行细胞瘤、嗜铬细胞瘤、垂体瘤（侵袭型）、浆细胞瘤、成视网膜细胞瘤、横纹肌肉瘤、肉瘤（例如，尤文氏肉瘤、组织细胞肉瘤、延森氏肉瘤 (Jense)、骨原性肉瘤、网状细胞肉瘤）、神经鞘瘤、皮下瘤、畸胎癌（例如，多能畸胎癌）、畸胎瘤、睾丸瘤、胸腺瘤和毛发上皮瘤、胃癌、纤维肉瘤、多形性成胶质细胞瘤；多发性血管球瘤、李 - 佛美尼综合征 (Li-Fraumeni syndrome)、脂肪肉瘤、Lynch 癌家族综合征 II、男性生殖细胞肿瘤、肥大细胞白血病、甲状腺髓样癌、多发性脑膜瘤、内分泌瘤病粘液肉瘤、副神经节瘤、家族性非嗜铬瘤、毛母质瘤、乳头瘤、家族性和分散性、横纹肌样瘤易感综合征、家族性、横纹肌样瘤、软组织肉瘤和伴有成胶质细胞瘤的 Turcot 综合征。

[0062] 根据一特定实施方案，所述癌症为乳腺癌。

[0063] 根据一特定实施方案，所述癌症（或癌转移）为受 EGF 调节。

[0064] 根据另一优选实施方案，所述癌症的特征在于 ErbB 受体分子例如 EGFR 或 HER2 过表达或上调。

[0065] 已经将导致 EGFR 过表达（称为上调）或过度活性的突变与许多癌症，包括肺癌、肛门癌和多形性成胶质细胞瘤相联系起来。在后一种情况下，常常观察到 EGFR 或多或少的特异性突变，称为 EGFRvIII。在所有上皮癌的约 30％中牵涉到 EGFR 或家族成员的突变、扩增或误调节。

[0066] 牵涉 EGFR 的突变可导致其不断活化，这样可导致细胞分裂不受控制 - 癌症素因。相反，已经在几个类型的癌症中鉴定出 EGFR 的突变，并且是一类不断扩大的抗癌疗法的目标 [Zhang 2007 J.Clin.Invest.117(8):2051 - 8]。

[0067] 在约 30％的乳腺癌中出现 ERBB2 基因扩增或过表达。与疾病复发增加和预后较差有极大关联。还了解到在卵巢癌、胃癌和侵蚀形式的子宫癌，例如浆液性子宫内膜癌中出现

过表达。

[0068] 下面是癌症列表，其中涉及了受体酪氨酸激酶 ErbB 家族的成员。

[0069] ErbB-1- 肾上腺皮质癌、胆管癌、宫颈癌、结直肠癌、食道癌、胆囊癌、胃癌、成胶质细胞瘤、头颈癌、肺癌（非小细胞、鳞状细胞癌、腺癌和大细胞肺癌）、胰腺癌、唾液腺癌、腹泻、良性赘生物、浸润性癌、皮肤病、导管原位癌、甲沟炎。

[0070] ErbB-2- 胆管癌、膀胱癌、乳腺癌、胆管细胞癌、食道癌、胆囊癌、胃癌、成胶质细胞瘤、卵巢癌、胰腺癌、唾液腺癌。根据一特定实施方案，所述癌症为乳腺癌或胃癌。

[0071] ErbB-3- 乳腺癌、肺癌和病毒性白血病。

[0072] ErbB-4- 乳腺癌、病毒性白血病、髓母细胞瘤、肺癌和乳腺肿瘤。

[0073] 如本文所使用，术语“受试者”指已经诊断患有癌症的哺乳动物（例如，人）。

[0074] 如本文所使用，突触囊泡磷酸酶 -2 或 SYNJ2 指突触肌醇 -1,4,5- 三磷酸 5- 磷酸酶 2，EC 3.1.3.36。突触囊泡磷酸酶 -2 为泛表达磷酸肌醇 5- 磷酸酶 (SEQ ID NO:1 和 2 分别指多聚核苷酸和编码的多肽）。

[0075] 如本文所使用，短语“突触囊泡磷酸酶 2(SYNJ2) 抑制剂”指降低或下调 SYNJ2 的表达或活性的分子。

[0076] 下调可超过 10％、20％、30％、40％、50％、60％、70％、80％、90％或完全抑制（通过给定测定法，例如下文所述测定法测定活性或表达 100％损失）。

[0077] 下调 SYNJ2 的表达可如下文所述在 DNA、RNA 或蛋白质水平上实现。SYNJ2 活性指其催化活性 [作为磷酸酶，将 PI(3,4,5)P_3转化为 PI(3,4)P_2]、其信号活性（与发动蛋白、皮动蛋白相互作用，见图 5E-H) 或细胞定位。在后一种情况下，SYNJ2 抑制剂将改变蛋白质的细胞定位。

[0078] 因此，可在基因组和 / 或转录产物水平上使用敲入所述基因或干扰其转录和 / 或翻译的各种分子 [例如，核酸沉默剂例如核酸 (RNA) 沉默剂（例如反义、siRNA、shRNA、微 RNA、核酶和 DNA 酶）]，或在蛋白质水平上使用例如拮抗剂、裂解多肽的酶等实现 SYNJ2 的下调。

[0079] 以下是一系列能够下调 SYNJ2 的表达水平和 / 或活性的试剂。

[0080] 能够下调 SYNJ2 的试剂的一个实例为能够特异性结合 SYNJ2 的抗体或抗体片段。优选地，所述抗体特异性结合 SYNJ2 的至少一个表位。当 SYNJ2 为细胞蛋白时，采取措施将抗体引入细胞内。如本文所使用，术语“表位”指抗体结合部位在抗原上结合的任何抗原决定簇。

[0081] 表位决定簇通常由分子化学活性表面分组，例如氨基酸或碳水化合物侧链组成并且通常具有特定三维结构特征以及特定电荷特征。

[0082] 如本发明中所使用，术语“抗体”包括完整分子及其能够与巨噬细胞结合的功能片段，例如 Fab、F(ab')2 和 Fv。这些功能性抗体片段定义如下 :(1)Fab，可通过用木瓜蛋白酶消化全抗体得到完整轻链和一条重链的一部分生成的含抗体分子的单价抗原结合片段的片段 ;(2)Fab'，可通过用胃蛋白酶处理全抗体，接着还原得到完整轻链和重链的一部分获得的抗体分子片段 ;每个抗体分子获得两个 Fab' 片段 ;(3)(Fab')2，可通过用胃蛋白酶处理全抗体，无需后续还原得到的抗体片段 ;F(ab')2 是通过两个二硫键结合在一起的两个 Fab' 片段的二聚体 ;(4)Fv，定义为含表示成两条链的轻链可变区和重链可变区的基因工

程化片段；和 (5) 单链抗体（“SCA”），含有轻链可变区和重链可变区，通过适合多肽接头连接成基因融合单链分子的基因工程化分子。

[0083] 生产多克隆和单克隆抗体及其片段的方法在本领域众所周知（见例如，Harlow 和 Lane，Antibodies:A Laboratory Manual,Cold Spring Harbor Laboratory,New York,1988，其以引用的方式并入本文）。

[0084] 根据本发明一些实施方案所述的抗体片段可通过抗体的蛋白水解或通过在大肠杆菌或哺乳动物细胞（例如中国仓鼠卵巢细胞培养物或其它蛋白表达系统）中表达编码所述片段的 DNA 制备。可用传统方法通过胃蛋白酶或木瓜蛋白酶消化全抗体获得抗体片段。例如，可通过用胃蛋白酶酶促裂解抗体生成抗体片段以提供 5S 片段表示的 F(ab')2。可使用硫醇还原剂，和任选地由二硫键裂解产生的巯基的封端基团进一步裂解该片段，以生成 3.5S Fab' 单价片段。可选地，使用胃蛋白酶酶促裂解直接生成两个单价 Fab' 片段和一个 Fc 片段。例如，Goldenberg，美国专利第 4,036,945 号和第 4,331,647 号及其中所含参考文献中描述了这些方法，所述专利特此以引用的方式整体并入。同样见 Porter,R. R.[Biochem.J.73:119-126(1959)]。也可使用其它裂解抗体的方法，例如分离重链形成单价轻链 - 重链片段，进一步裂解片段或其它酶促、化学或基因技术，只要所述片段与完整抗体所识别的抗原结合。

[0085] Fv 片段包含 VH 和 VL 链的缔合。这种缔合可为非共价，如 Inbar 等 [Proc.Nat'l Acad.Sci.USA 69:2659-62(19720] 所述。可选地，可变链可通过分子间二硫键连接或通过化学物质例如戊二醛交联。优选地，Fv 片段包含通过肽接头连接的 VH 和 VL 链。通过构建包含通过寡聚核苷酸连接的编码 VH 和 VL 结构域的 DNA 序列的结构基因制备这些单链抗原结合蛋白 (sFv)。将结构基因插入表达载体中，随后将表达载体引入宿主细胞例如大肠杆菌。重组宿主细胞合成带有桥接两个 V 结构域的接头肽的多肽链。例如，[Whitlow 和 Filpula,Methods 2:97-105(1991)；Bird 等，Science 242:423-426(1988)；Pack 等，Bio/Technology 11:1271-77(1993)；和美国专利第 4,946,778 号描述了生成 sFv 的方法，其特此以引用的方式整体并入。

[0086] 抗体的另一种形式是编码单个互补决定区 (CDR) 的肽。可通过构建编码目标抗体的 CDR 的基因获得 CDR 肽（“最小识别单位”）。例如，通过使用聚合酶链式反应制备此类基因，以由抗体生成细胞的 RNA 合成可变区。见，例如，Larrick 和 Fry[Methods,2:106-10(1991)]。

[0087] 抗 SYNJ2 市场上可买到。抗人 SYNJ2 单克隆抗体的供应商实例包括但不限于 Amsbio、Atlas Antibodies、AbD Serotec、United States Biological、antibodies-online.com、Genway、Proteintech Group 等。致使本发明的抗体为非免疫原性，以供治疗应用。

[0088] 非人（例如，鼠）抗体的人源化形式为含有源自非人类免疫球蛋白的最小序列的免疫球蛋白、免疫球蛋白链或其片段（例如 Fv、Fab、Fab'、F(ab').sub.2 或抗体的其它抗原结合子序列）的嵌合分子。人源化抗体包括人免疫球蛋白（受者抗体），其中形成受者互补决定区 (CDR) 的残基由形成非人类物种（供体抗体），例如小鼠、大鼠或兔的具有所需特异性、亲和力和容量的 CDR 的残基置换。在一些情况下，人免疫球蛋白的 Fv 骨架残基由相应的非人类残基置换。人源化抗体可能还包含在受者抗体或导入的 CDR 或骨

架序列中都不存在的残基。一般而言，人源化抗体将大体上包含至少一个并且通常两个可变结构域的全部，其中所有或大体上所有的 CDR 区与非人类免疫球蛋白的 CDR 区相对应并且所有或大体上所有的 FR 区为人免疫球蛋白共有序列的 FR 区。人源化抗体最好还将包含免疫球蛋白恒定区 (Fc)，通常为人免疫球蛋白恒定区的至少一部分 [Jones 等，Nature,321:522-525(1986) ;Riechmann 等，Nature,332:323-329(1988) ;和 Presta，Curr. Op.Struct.Biol.,2:593-596(1992)]。

[0089] 人源化非人抗体的方法在本领域众所周知。通常，人源化抗体具有一个或多个从非人类来源引入其中的氨基酸残基。这些非人类氨基酸残基常常称为导入残基，其通常从导入可变结构域获得。基本上可按照 Winter 和同事的方法 [Jones 等，Nature,321:522-525(1986) ;Riechmann 等，Nature 332:323-327(1988) ;Verhoeyen 等，Science,239:1534-1536(1988)]，通过用啮齿动物 CDR 或 CDR 序列取代人抗体的相应序列进行人源化。相应地，此类人源化抗体为嵌合抗体 (美国专利第 4,816,567 号)，其中已经用来自非人类物种的相应序列取代了大体上不大完整的人可变结构域。在实践中，人源化抗体通常是其中一些 CDR 残基和可能一些 FR 残基由来自啮齿动物抗体中类似位点的残基取代的人抗体。

[0090] 也可使用本领域已知的各种技术，包括噬菌体展示库生成人抗体 [Hoogenboom 和 Winter，J.Mol.Biol.,227:381(1991) ;Marks 等，J.Mol.Biol.,222:581(1991)]。Cole 等和 Boerner 等的技术也可用于制备人单克隆抗体 (Cole 等，Monoclonal Antibodies and Cancer Therapy,Alan R.Liss， 第 77 页 (1985) 和 Boerner 等，J.Immunol.,147(1):86-95(1991)]。类似地，可通过将人免疫球蛋白基因座引入转基因动物，例如内源免疫球蛋白基因已经部分或完全灭活的小鼠体内，制成人抗体。激发后，观察到人抗体生成，这在各个方面，包括基因重排、装配和抗体谱，这都非常类似于在人类中所见。例如，在美国专利第 5,545,807、5,545,806、5,569,825、5,625,126、5,633,425、5,661,016 号和下列科技出版物 :Marks 等，Bio/Technology 10,:779-783(1992) ;Lonberg 等，Nature 368:856-859(1994) ;Morrison，Nature 368 812-13(1994) ;Fishwild 等，Nature Biotechnology 14,845-51(1996) ;Neuberger，Nature Biotechnology 14:826(1996) ;和 Lonberg 和 Huszar，Intern.Rev.Immunol.13,65-93(1995). 中描述了这种方法。

[0091] 也可通过 RNA 沉默实现 SYNJ2 的下调。如本文所使用，短语“RNA 沉默”指由导致相应的蛋白质编码基因的表达抑制或“沉默”的 RNA 分子介导的一组调控机制 [例如，RNA 干扰 (RNAi)、转录基因沉默 (TGS)、转录后基因沉默 (PTGS)、压制、共抑制和翻译阻遏]。已经在许多类型的生物，包括植物、动物和真菌中观察到 RNA 沉默。

[0092] 如本文所使用，术语“RNA 沉默剂”指能够特异性抑制或“沉默”靶基因的表达的 RNA。在某些实施方案中，RNA 沉默剂能够通过转录后沉默机制防止 mRNA 分子完全加工 (例如，全部翻译和 / 或表达)。RNA 沉默剂包括非编码 RNA 分子，例如包含配对链的 RNA 双链体，以及可由其生成如此小的非编码 RNA 的前体 RNA。示例性 RNA 沉默剂包括 dsRNA 例如 siRNA、miRNA 和 shRNA。在一个实施方案中，RNA 沉默剂能够诱导 RNA 干扰。在另一实施方案中，RNA 沉默剂能够介导翻译阻遏。

[0093] 根据本发明的一个实施方案，RNA 沉默剂对靶 RNA(例如，SYNJ2) 有特异性并不

交叉抑制或沉默与靶基因表现出 99％或更低总体同源性，例如与靶基因表现出低于 98％、97％、96％、95％、94％、93％、92％、91％、90％、89％、88％、87％、86％、85％、84％、83％、82％、81％总体同源性的基因或剪接变体。

[0094] RNA 干扰指动物中由短干扰 RNA(siRNA) 介导的序列特异性转录后基因沉默过程。植物中的相应过程通常称为转录后基因沉默或 RNA 沉默并且在真菌中也称为压制。转录后基因沉默的过程被认为是用于防止外源基因表达的进化上保守的细胞防御机制并且通常由不同区系和门共用。可能已经响应于经由特异性破坏同源单链 RNA 或病毒基因组 RNA 的细胞反应，源自病毒感染或转座子元件向宿主基因组的随机整合的双链 RNA(dsRNA) 生成，进化出这种对外源基因表达的防护。

[0095] 细胞中长 dsRNA 的存在刺激称为 dicer 的核糖核酸酶 III 的活性。在将 dsRNA 加工成称为短干扰 RNA(siRNA) 的 dsRNA 短片段中涉及到 dicer。源自 dicer 活性的短干扰 RNA 通常长度为约 21 至约 23 个核苷酸并且包含约 19 个碱基对的双链体。RNAi 反应也是通常称为 RNA 诱导沉默复合物 (RISC) 的核酸内切酶复合物的特征，所述复合物介导具有与 siRNA 双链体的反义链互补的序列的单链 RNA 裂解。在与 siRNA 双链体的反义链互补的区域中间发生靶 RNA 裂解。

[0096] 相应地，本发明的一些实施方案考虑到 dsRNA 下调由 mRNA 表达蛋白质的用途。

[0097] 根据一个实施方案，dsRNA 大于 30bp。由于相信双链 RNA 的这些较长区域将导致诱导干扰素和 PKR 反应，所以长 dsRNA（即大于 30bp 的 dsRNA) 的使用已经非常受限。然而，使用长 dsRNA 可以提供许多优势，细胞可以选择减少试验许多 siRNA 的需要的最佳沉默序列；长 dsRNA 将允许沉默库具有比对于 siRNA 而言所需更低的复杂性；并且，可能最重要的是，长 dsRNA 在用作治疗剂时可防止病毒逃避突变。

[0098] 各种研究证明长 dsRNA 可用于沉默基因表达，不会诱导应激反应或产生显著的脱靶效应－见例如 [Strat 等，Nucleic Acids Research，2006 年，第 34 卷，No.13 3803－3810；Bhargava A 等，Brain Res.Protoc.2004；13:115－125；Diallo M. 等，Oligonucleotides.2003；13:381－392；Paddison P.J. 等，Proc.Natl Acad.Sci. USA.2002；99:1443－1448；Tran N. 等，FEBS Lett.2004；573:127－134]。

[0099] 具体而言，本发明根据其一些实施方式考虑到在干扰素途径未激活的细胞（例如胚细胞和卵母细胞）中引入长 dsRNA（超过 30 个碱基的转录产物）用于基因沉默，见例如 Billy 等，PNAS 2001，第 98 卷，第 14428-14433 页和 Diallo 等，Oligonucleotides，2003 年 10 月 1 日，13(5):381-392.doi:10.1089/154545703322617069。

[0100] 本发明根据其一些实施方式还考虑到引入特别设计为不诱导干扰素和 PKR 途径的长 dsRNA 用于下调基因表达。例如，Shinagwa 和 Ishii[Genes&Dev.17(11):1340-1345,2003] 已经研发出称为 pDECAP 的载体，以从 RNA 聚合酶 II(Pol II) 启动子表达长双链 RNA。因为来自 pDECAP 的转录产物缺乏促进 ds-RNA 输出到细胞质的 5'－帽结构和 3'－聚 (A) 尾部，所以来自 pDECAP 的长 ds-RNA 不诱导干扰素反应。

[0101] 在哺乳动物体系中避开干扰素和 PKR 途径的另一种方法是经由转染或内源性表达引入小抑制性 RNA(siRNA)。

[0102] 术语“siRNA”指诱导 RNA 干扰 (RNAi) 途径的小抑制性 RNA 双链体（通常介于 18-30 个碱基对之间）。虽然最近已经描述 25-30 个碱基长度的化学合成 RNA 双链体与相

同位置的21mer相比，效力增强可高达100倍，但是通常siRNA经化学合成为21mer，具有中间19bp双链体区和末端的对称2-碱基3'-突出。观察到在引发RNAi中使用较长RNA获得的更强效力经推理是由于为Dicer提供底物(27mer)而非产物(21mer)产生并且这提高了siRNA双链体进入RISC的速率或效率。

[0103] 已经发现3'-突出的位置影响siRNA的效力并且在反义链上具有3'-突出的非对称双链体通常比在有义链上具有3'-突出的非对称双链体更有效(Rose等，2005)。因为在靶向反义转录产物时观察到相反的功效模式，所以这可归因于非对称链载入RISC中。

[0104] 双链干扰RNA(例如，siRNA)的链可连接形成发夹或茎-环结构(例如，shRNA)。因此，正如提到的那样，本发明一些实施方案的RNA沉默剂也可为短发夹RNA(shRNA)。

[0105] 可在Chuang等(同上)SJ2-1(编码区1612-1633；5′ AACGTGAACGGAGGAAAGCAG，SEQ ID NO:3)、SJ2-2(3′非翻译区内的区域5419-5440；5′ CTCTTGCTGATACGCGATATT，SEQ ID NO:4)；或传授SYNJ2的编码区1612-1633或4925-4946的siRNA的Rusk等[Curr Biol.2003年4月15日；13(8):659-63.Erratum in:Curr Biol.2003年9月30日；13(19):1746]中找到小干扰RNA分子的实例。

[0106] 成功下调SYNJ2 mRNA水平的siRNA序列的其它实例包括但不限于GAAGAAACAUCCCUUUGAU(SEQ ID NO:5)和GGACAGCACUGCAGGUGUU(SEQ ID NO:6)。

[0107] 术语“shRNA”，如本文所使用，指具有茎-环结构，包含互补序列的第一和第二区域，所述区域的互补程度和定向足够使得在所述区域之间发生碱基配对，第一和第二区域通过环区连接，由于环区内核苷酸(或核苷酸类似物)之间缺乏碱基配对而产生环的RNA剂。环内核苷酸的数量是介于并包括3-23或5-15或7-13或4-9或9-11的数量。环内一些核苷酸可涉及与环内其它核苷酸的碱基-对相互作用。可用于形成环的寡核苷酸的实例包括5'-UUCAAGAGA-3'(Brummelkamp,T.R.等(2002)Science 296:550)和5'-UUUGUGUAG-3'(Castanotto,D.等(2002)RNA 8:1454)。本领域的技术人员将认识到所生成的单链寡核苷酸形成包含能够与RNAi机制相互作用的双链区的茎-环或发夹结构。

[0108] 成功下调SYNJ2 mRNA水平的shRNA序列的实例包括但不限于CCGGCCTACGATACAAGCGACAAATCTCGAAGATTTGTCGCTTGTATCGTAGGTTTTTG(SEQ ID NO:7)；CCGGCGAGAGGAGATCATTCGGAAACTCGAGTTTCCGAATGATCTCCTCTCGTTTTTG(SEQ ID NO:8)；CCGGCCGGAAGAACAGTTTGAGCAACTCGAGTTGCTCAAACTGTTCTTCCGGTTTTTG(SEQ ID NO:9)。

[0109] 适合与本发明的一些实施方案一起使用的RNA沉默剂的合成可如下实现。第一，在AUG起始密码子下游扫描SYNJ2 mRNA序列的AA二核苷酸序列。将每个AA和3'相邻的19个核苷酸的出现记录为潜在siRNA靶位点。优选地，因为非翻译区(UTR)更富含调节蛋白结合位点，所以siRNA靶位点选自开放阅读框。UTR结合蛋白和/或翻译起始复合物可能干扰siRNA内切核酸酶复合物的结合[Tuschl ChemBiochem.2:239-245]。不过应认识到，正如对于GAPDH所证明那样，其中对准5'UTR的siRNA介导细胞GAPDH mRNA减少约90％并且完全去除了蛋白质含量(wwwdotambiondotcom/techlib/tn/91/912dothtml)，对准非翻译区的siRNA也可能有效。

[0110] 第二，使用任何序列比对软件，例如可从NCBI服务器(wwwdotncbidotnlmdotnihdotgov/BLAST/)获得的BLAST软件，将潜在靶位点与适当的基因组数据库(例如，人、小鼠、大鼠等)作比较。筛选出表现出与其它编码序列的显著同源

性的推定靶位点。

[0111] 选择合格靶序列作为 siRNA 合成的模板。优选的序列是包括低 G/C 含量的序列，因为已经证实与 G/C 含量高于 55％的序列相比，其介导基因沉默更有效。沿着靶基因的长度优选几个靶位点做评价。为了更好地评价所选 siRNA，优选一起使用阴性对照。阴性对照 siRNA 优选包括与 siRNA 相同的核苷酸组成，但是与基因组没有显著同源性。因此，优选使用 siRNA 的乱序核苷酸序列，条件是其未显示出与其它任何基因的任何显著同源性。

[0112] 应认识到，本发明一些实施方案的 RNA 沉默剂无需限制为仅含 RNA 的那些分子，但是进一步涵盖经化学修饰的核苷酸和非核苷酸。

[0113] 在一些实施方案中，本文提供的 RNA 沉默剂可与细胞穿透肽在功能上相关。如本文所使用，“细胞穿透肽”是包含赋予与跨细胞质和 / 或细胞核膜运输膜渗透性复合物相关的非能量依赖性（即，非胞吞）易位性质的短（约 12-30 个残基）氨基酸序列或功能基序。在本发明一些实施方案的膜渗透性复合物中使用的细胞穿透肽优选包含至少一个非功能性半胱氨酸残基，其游离或经衍生化与为这样连接已经修饰的双链核糖核酸形成二硫键。在美国专利第 6,348,185 号中列出了赋予此类性质的代表性氨基酸基序，其内容明确地以引用的方式并入本文。本发明一些实施方案的细胞穿透肽优选包括但不限于 penetratin、transportan、pIsl、TAT(48-60)、pVEC、MTS 和 MAP。

[0114] 要使用 RNA 沉默剂靶向的 mRNA 包括但不限于其表达与不良表型性状相关的 mRNA。可能靶向的示例性 mRNA 是编码截短蛋白，即包含缺失的 mRNA。相应地，本发明一些实施方案的 RNA 沉默剂可靶向所述缺失任一侧的桥接区。向细胞中引入此类 RNA 沉默剂会引起突变蛋白下调，同时使非突变蛋白不受影响。

[0115] 根据另一实施方案，RNA 沉默剂可为 miRNA。

[0116] 术语“微 RNA”、“miRNA”和“miR”同义并且指长度约 19-28 个核苷酸，调节基因表达的非编码单链 RNA 分子的总称。在各种生物 (virusesdotfwdarwdothumans) 中发现 miRNA 并且已经证实在发育、体内稳态和疾病病因学上起作用。

[0117] 下面是对 miRNA 活性机制的简单描述。

[0118] 编码 miRNA 的基因转录，导致称为 pri-miRNA 的 miRNA 前体生成。pri-miRNA 通常是包含多个 pri-miRNA 的多顺反子 RNA 的一部分。pri-miRNA 可与茎和环形成发夹。茎可能包含错配碱基。

[0119] pri-miRNA 的发夹结构由为 RNA 酶 III 核酸内切酶的 Drosha 识别。Drosha 通常识别 pri-miRNA 中的末端环并且将约两个螺旋转角裂解成茎以生成称为 pre-miRNA 的 60-70 个核苷酸的前体。Drosha 以 RNA 酶 III 核酸内切酶典型的交错切口裂解 pre-miRNA，产生带 5’磷酸和～ 2 个核苷酸的 3’突出的 pre-miRNA 茎环。据估计茎（～ 10 个核苷酸）伸出 Drosha 裂解位点的约一个螺旋转角对有效加工必不可少。然后 pre-miRNA 通过 Ran-GTP 和输出受体输出蛋白 -5 从细胞核主动运输到细胞质。

[0120] 然后 pre-miRNA 的双链茎由也是 RNA 酶 III 核酸内切酶的 Dicer 识别。Dicer 也可识别在茎环基部的 5’磷酸和 3’突出。然后 Dicer 裂解掉末端环，两个螺旋转角离开茎环基部，留下另外的 5’磷酸和～ 2 个核苷酸的 3’突出。所生成的可能包含错配的 siRNA 样双链体包含成熟 miRNA 和称为 miRNA* 的相似大小的片段。miRNA 和 miRNA* 可能源自 pri-miRNA 和 pre-miRNA 的相对臂。可在克隆 miRNA 库中找到 miRNA* 序列，但是通常频率

比 miRNA 低。

[0121] 虽然最初与 miRNA* 一起作为双链物种存在，但是 miRNA 最终作为单链 RNA 并入称为 RNA 诱导沉默复合物 (RISC) 的核糖核蛋白复合物中。各种蛋白质均可形成 RISC，这样可在对 miRNA/miRNA* 双链体的特异性、靶基因结合位点、miRNA 活性（阻遏或激活）和 miRNA/miRNA* 双链体的哪一条链载入 RISC 的方面导致可变性。

[0122] 当 miRNA:miRNA* 双链体的 miRNA 链载入 RISC 时，去除并降解 miRNA*。miRNA:miRNA* 双链体中载入 RISC 的链是其 5’ 末端配对不太牢固的链。在 miRNA:miRNA* 的两端具有大致相当的 5’ 配对的情况下，miRNA 和 miRNA* 可能都具有基因沉默活性。

[0123] RISC 基于 miRNA 和 mRNA 之间的高互补性水平，特别是通过 miRNA 的核苷酸 2-7 识别靶核酸。

[0124] 许多研究已着眼于为实现翻译的有效抑制，miRNA 及其 mRNA 靶标之间的碱基配对要求（经 Bartel 2004 年审核，Cell 116-281）。在哺乳动物细胞中，miRNA 的前 8 个核苷酸可能很重要 (Doench&Sharp 2004GenesDev 2004-504)。然而，微 RNA 的其它部分也可能参与 mRNA 结合。而且，3’ 处足够的碱基配对可补偿 5’ 处的配对不足 (Brennecke 等，2005 PLoS 3-e85)。分析全基因组上 miRNA 结合的计算研究已经表明在靶标结合中对 miRNA 的 5’ 处的碱基 2-7 的特定作用，但是也公认了通常发现为“A”的第一个核苷酸的作用 (Lewis 等 2005 Cell 120-15)。类似地，Krek 等使用核苷酸 1-7 或 2-8 鉴定和验证靶标 (2005, Nat Genet 37-495)。

[0125] mRNA 中的靶位点可能在 5’UTR、3’UTR 或编码区内。有趣的是，多个 miRNA 可通过识别相同或多个位点调节相同 mRNA 靶标。大多数基因识别靶标中多个 miRNA 结合位点的存在可能表明多个 RISC 的协同作用提供了最有效的翻译抑制。

[0126] miRNA 可指示 RISC 通过两种机制中的任一种下调基因表达 :mRNA 裂解或翻译阻遏。如果 mRNA 与 miRNA 有一定程度的互补性，则 miRNA 可指定 mRNA 的裂解。当 miRNA 指导裂解时，切口通常介于与 miRNA 的残基 10 和 11 的核苷酸配对之间。可选地，如果 miRNA 与 miRNA 没有必要程度的互补性，则 miRNA 可阻遏翻译。翻译阻遏可能在动物中更普遍，因为动物可能在 miRNA 和结合位点之间具有更低程度的互补性。

[0127] 应该注意的是，在任一对 miRNA 和 miRNA* 的 5’ 和 3’ 末端可能存在可变性。这种可变性可能是由于 Drosha 和 Dicer 对于裂解位点酶促加工的可变性。miRNA 和 miRNA* 的 5’ 和 3’ 末端的可变性也可能是由于 pri-miRNA 和 pre-miRNA 茎结构中的错配。茎链的错配可能产生一群不同的发夹结构。茎结构的可变性也可能导致 Drosha 和 Dicer 裂解产物的可变性。

[0128] 术语“微 RNA 模拟物”指能够进入 RNAi 途径并调节基因表达的合成非编码 RNA。miRNA 模拟物模仿内源微 RNA(miRNA) 的功能并且可设计成成熟、双链分子或模拟物前体（例如，pre-miRNA）。miRNA 模拟物可由经修饰或未经修饰的 RNA、DNA、RNA-DNA 杂交体或替代核酸化学物质（例如，LNA 或 2’-O,4’-C- 乙烯 - 桥接核酸 (ENA)）构成。对于成熟、双链 miRNA 模拟物而言，双链体区的长度可在 13-33、18-24 或 21-23 个核苷酸之间变化。miRNA 也可包含总计至少 5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39 或 40 个核苷酸。miRNA 的序列也可能是 pre-miRNA 的前 13-33 个核苷酸。miRNA 的序列也可能是 pre-miRNA 的后 13-33 个核苷

酸。

[0129] 从上文提供的描述应认识到,可按许多方式实现癌细胞与 miRNA 接触 :

[0130] 1. 用成熟双链 miRNA 瞬时转染癌细胞。

[0131] 2. 用编码成熟 miRNA 的表达载体稳定或瞬时转染癌细胞。

[0132] 3. 用编码 pre-miRNA 的表达载体稳定或瞬时转染癌细胞。pre-miRNA 序列可能包含 45-90、60-80 或 60-70 个核苷酸。pre-miRNA 序列可能包含如本文所述的 miRNA 和 miRNA*。pre-miRNA 序列也可能是从 pri-miRNA 的 5’和 3’末端排除 0-160 个核苷酸的 pri-miRNA 序列。

[0133] 4. 用编码 pri-miRNA 的表达载体稳定或瞬时转染癌细胞。pri-miRNA 序列可能包含 45-30,000、50-25,000、100-20,000、1,000-1,500 或 80-100 个核苷酸。pri-miRNA 序列可能包含如本文所述的 pre-miRNA、miRNA 和 miRNA* 及其变体。

[0134] 能够下调 SYNJ2 另一种试剂是能够特异性裂解 SYNJ2 的 mRNA 转录产物或 DNA 序列的 DNA 酶分子。DNA 酶是能够裂解单链和双链靶序列的单链多聚核苷酸 (Breaker,R. R. 和 Joyce,G.Chemistry and Biology 1995 ;2:655 ;Santoro,S.W.&Joyce,G.F.Proc. Natl,Acad.Sci.USA 1997 ;943:4262)。已经提出了 DNA 酶的一般模型 (“10-23”模型)。“10-23”DNA 酶具有一个 15 个脱氧核糖核苷酸的催化结构域,其侧面是两个各 7-9 个脱氧核糖核苷酸的底物识别结构域。这类 DNA 酶可在嘌呤 : 嘧啶接点处有效裂解其底物 (Santoro,S.W.&Joyce,G.F.Proc.Natl,Acad.Sci.USA 199 ;对 DNA 酶的评论,见 Khachigian,LM[Curr Opin Mol Ther 4:119-21(2002)]。

[0135] 在 Joyce 等的美国专利第 6,326,174 号中已经公开了构建和扩增识别单链和双链靶裂解位点的合成、工程化 DNA 酶的实例。最近观察到针对人尿激酶受体的类似设计的 DNA 酶抑制尿激酶受体表达,并且成功抑制体内结肠癌细胞转移 (Itoh 等,20002,Abstract 409,Ann Meeting Am Soc Gen Ther wwwdotasgtdotorg)。在另一种应用中,与 bcr-ab1 致癌基因互补的 DNA 酶在白血病细胞中成功抑制致癌基因表达,并且在 CML 和 ALL 的情况下减小了在自身骨髓移植中的复发率。

[0136] 也可使用能够与编码 SYNJ2 的 mRNA 转录产物特异性杂交的反义多聚核苷酸实现 SYNJ2 的下调。

[0137] 必须在考虑对反义方法重要的两个方面的同时实现可用于有效下调 SYNJ2 的反义分子的设计。第一方面是寡核苷酸向适当细胞的细胞质的递送,而第二方面是以抑制其翻译的方式特异性结合细胞内指定 mRNA 的寡核苷酸的设计。

[0138] 现有技术传授了许多可用于有效地将寡核苷酸递送到各种细胞类型的递送策略 [见,例如,Luft J Mol Med 76:75-6(1998) ;Kronenwett 等 Blood 91:852-62(1998) ; Rajur 等 Bioconjug Chem 8:935-40(1997) ;Lavigne 等 Biochem Biophys Res Commun 237:566-71(1997) 和 Aoki 等 (1997)Biochem Biophys Res Commun 231:540-5(1997)]。

[0139] 另外,根据说明靶 mRNA 和寡核苷酸中结构改变的能量学的热力学循环,鉴定对其靶 mRNA 的预测结合亲和力最高的序列的算法也可用 [见,例如,Walton 等 Biotechnol Bioeng 65:1-9(1999)]。

[0140] 此类算法已经成功用于在细胞中实现反义方法。例如,Walton 等研发的算法使得科学家们能够成功设计兔 β- 珠蛋白 (RBG) 和小鼠肿瘤坏死因子 -α (TNFα) 转录产物的

反义寡核苷酸。同一研究小组新近已报道，通过动力学 PCR 技术评估，在细胞培养物中对三种模型靶 mRNA（人乳酸脱氢酶 A 和 B 和兔 gp130）合理选择的寡核苷酸的反义活性证明在几乎所有情况下，包括在具有磷酸二酯和硫代磷酸酯寡核苷酸化学性质的两种细胞类型中对三种不同靶标的试验中有效。

[0141] 另外，还公开了使用体外系统设计和预测特定寡核苷酸的效率的几种方法(Matveeva 等，Nature Biotechnology 16:1374-1375(1998)]。

[0142] 例如，靶向 SYNJ2 mRNA（其编码 SYNJ2 蛋白）的适合反义寡核苷酸将具有以下序列 :CCCTTTGTCTGCCACCTCCT(SEQ ID NO:10)、ACCCATCTTGCTCTCTCCC(SEQ ID NO:11) 和 TCTTCCTCCACCACAGCACC(SEQ ID NO:12)。

[0143] 几项临床试验已经证明反义寡核苷酸的安全性、可行性和活性。例如，已经成功使用了适合治疗癌症的反义寡核苷酸 [Holmund 等，Curr Opin Mol Ther1:372-85(1999)]，而经由靶向 c-myb 基因、p53 和 Bcl-2 的反义寡核苷酸治疗血液恶性肿瘤已进入临床试验并且已经证实患者耐受 [Gerwitz Curr Opin Mol Ther1:297-306(1999)]。

[0144] 最近，已经报道在小鼠模型中反义介导的人乙酰肝素酶基因表达抑制，抑制了人癌细胞的胸膜种植转移 [Uno 等，Cancer Res 61:7855-60(2001)]。

[0145] 因此，目前的共识是在如上所述，已导致产生高度精确的反义设计算法和各种寡核苷酸递送系统的反义技术领域的最新发展，使得普通技术人员能够设计并实现适合下调已知序列的表达，而无需采取不当试验和错误实验的反义方法。

[0146] 能够下调 SYNJ2 的另一种试剂是能够特异性裂解编码 SYNJ2 的 mRNA 转录产物的核酶分子。核酶正越来越多地用于通过裂解编码目标蛋白的 mRNA，序列特异性抑制基因表达 [Welch 等，Curr Opin Biotechnol.9:486-96(1998)]。将核酶设计为裂解任何特定靶 RNA 的可能性已使其在基础研究和治疗应用中成为宝贵的工具。在治疗领域，核酶已开发用于靶向传染病中的病毒 RNA、癌症中的显性致癌基因和遗传病中的特定体细胞突变 [Welch 等，Clin Diagn Virol.10:163-71(1998)]。最为显著的是，HIV 患者的几种核酶基因疗法已经处于 1 期试验。最近，核酶已经用于转基因动物研究、基因靶标验证和途径说明。几种核酶处于临床试验的不同阶段。ANGIOZYME 是要在人类临床试验中研究的首个经化学合成的核酶。ANGIOZYME 特异性抑制 VEGF-r（血管内皮生长因子受体），血管生成途径中的一种关键组分的形成。Ribozyme Pharmaceuticals,Inc. 以及其它公司已经证明了抗血管生成疗法在动物模型中的重要性。在细胞培养测定法中发现 HEPTAZYME，设计为选择性破坏丙型肝炎病毒 (HCV)RNA 的一种核酶，减少丙型肝炎病毒 RNA 有效 (Ribozyme Pharmaceuticals,Incorporated-WEB 首页）。

[0147] 能够下调 SYNJ2 的另一种试剂将为结合和 / 或裂解 SYNJ2 的任何分子。

[0148] 本发明揭开了成为 SYNJ2 在细胞运动中的作用基础的一般机理。

[0149] 图 15 中列出了原理。相应地，关键事件需要 EGF 诱导的 SYNJ2 上调及由此引起的 3 种磷酸肌醇损耗 :PI(4,5)P_2、PI(3,4,5)P_3和 PI(3,5)P_2。SYNJ2 介导的 PI(4,5)P_2脱磷酸与 PI(4,5)P_2受磷脂酶 C-γ 降解和受 PI3K 磷酸化并行，这样生成 PI(3,4,5)P_3。总的来说，3 种酶受 EGF 的刺激使一组 PI(4,5)P_2结合剂从质膜离解，并且还生成无 PI(4,5)P_2的内吞囊泡。同时，SYNJ2 将 PI(3,4,5)P_3转化成侵袭伪足形成所必需的 PI(3,4)P_2。一旦到位，PI(3,4)P_2就结合 TKS5 并且使以发动蛋白和皮动蛋白为中心的复合物成核，该复合物使得

丝切蛋白能够在侵袭伪足内产生肌动蛋白钩端。根据本结果，SYNJ2 也涉及下一侵袭伪足突变步骤，即 MMP 分泌及 MT1-MMP 和其它表面分子例如 CD44 的递送。以类似方式，SYNJ2 控制 EGFR 和整联蛋白向前缘的递送，并且很可能激活丝切蛋白，这是指示板状伪足突出形成的关键事件。

[0150] 这些发现可用于鉴定为肿瘤转移推定抑制剂的 SYNJ2 抑制剂。

[0151] 因此，根据本发明的一方面，提供了一种鉴定肿瘤转移的推定抑制剂的方法，所述方法包括在试验试剂的存在下，分析 SYNJ2 介导的 PI(3,4,5)P_3向 PI(3,4)P_2的加工，其中在所述试验试剂的所述存在下 PI(3,4,5)P_3向 PI(3,4)P_2的加工，与其不存在时 PI(3,4,5)P_3向 PI(3,4)P_2的加工相比减少，表明为肿瘤转移的推定抑制剂。

[0152] 试验试剂可为生物分子（蛋白质（例如肽或抗体）、核酸分子（例如沉默剂）、碳水化合物、脂质或其组合）或小分子（例如，化学品）。

[0153] 所述方法可在体内或体外实现。后者可在细胞体系中或使用无细胞体系执行。

[0154] 示例性测定法牵涉通过竞争测定法分析 SYNJ2 介导的 PI(3,4,5)P_3向 PI(3,4)P_2的加工。

[0155] 相应地，竞争测定法测试 PI(3,4)P_2结合结构域从包含与 PI(3,4)P_2结合的 PI(3,4)P_2结合结构域的复合物的位移。

[0156] 根据一示例性实施方案，采用荧光偏振竞争测定法。所述测定法依赖一旦分子结合更大的隔绝元件（例如，蛋白质），其在空间上的运动就显著降低的原理。可使用允许在从样品的平行和垂直平面测量后，测定荧光偏振的荧光探针检测和测量这种现象。相应地，溶液中未结合的荧光分子产生极低偏振读数，但是向溶液添加结合（隔绝）这些分子的检测剂（例如，结合蛋白）时，荧光分子在增大溶液中的偏振读数的受限组合物中稳定。

[0157] 例如，所述测定法可包括 PI(3,4)P_2结合结构域（例如，PH- 结构域例如 Tapp1PH 结构域，SEQ ID NO:15-16）和荧光 PI(3,4)P_2，连同重组 SYNJ2 及其非荧光底物，PI(3,4,5)P_3。SYNJ2 催化活性的产物置换荧光 PI(3,4)P_2，从而降低荧光偏振。

[0158] 根据特定实施方案，使用商业 5'PI(3,4,5)P3 磷酸酶活性荧光偏振测定法（例如，Echelon Bioscience，产品目录号 K-1400）。

[0159] 根据特定实施方案，在准备或不准备试验试剂，允许 SYNJ2 催化活性（脱磷酸）的条件下温育包含 SYNJ2 和作为底物的 PI(3,4,5)P3 的反应混合物。例如，试验试剂可为小分子、核酸分子、肽、抗体、碳水化合物或其组合。温育后，将含 PI(3,4)P2 产物的溶液与 PI(3,4)P2 结合蛋白（例如，Tapp1 的 PH- 结构域，SEQ ID NO:15）和荧光 PI(3,4)P2 的混合物混合并测量荧光偏振。该测定法中测得的偏振值在由通过 SYNJ2 酶活性生成的未标记 PI(3,4)P2 置换结合的荧光 PI(3,4)P2 分子时减小并且未结合荧光 PI(3,4)P2 分子的量增加。在试验试剂的存在下荧光偏振值与试验试剂不存在时的值相比增大的情况下，试验试剂为推定 SYNJ2 抑制剂。

[0160] 一经鉴定，就使用如以下进一步举例说明的相关测定法，例如明胶酶谱测定法、transwell 测定法和试验动物进一步证实试验试剂作为抗转移药物的功能性。

[0161] 使用这种方法，本发明人已经根据本发明的一些实施方案鉴定了许多可用作 SYNJ2 抑制剂的小分子。图 19 中描绘了这些分子并于下文，实施例 10 的表 2 中示出。

[0162] 正如提到的那样，除与癌症发作或进展相关的细胞表面受体的抑制剂外，还施用

SYNJ2 抑制剂。根据本发明的一个实施方案，受体为致癌基因。

[0163] 可根据本发明靶向的受体的实例为受体酪氨酸激酶，例如 EGFR、PDGFR、VEGFR、FGFR 和 ErbB-2。

[0164] 可靶向的其它表面分子包括整联蛋白基质金属蛋白酶 (MMP)、发动蛋白、TKS5 和 CD44。

[0165] 细胞表面分子的抑制剂在本领域众所周知。下文提供了此类抑制剂的非限制性列表。

[0166] 因此例如，鉴定 EGFR 为致癌基因已导致针对 EGFR 的抗癌疗法的发展。

[0167] 西妥昔单抗 (cetuximab) 和帕尼单抗 (panitumumab) 是单克隆抗体抑制剂的实例。临床研究的其它单克隆抗体为扎鲁木单抗 (zalutumumab)、尼妥珠单抗 (nimotuzumab) 和马妥珠单抗 (matuzumab)。单克隆抗体阻滞细胞外配体结合结构域。阻滞结合位点后，信号分子再也无法附着于此和激活酪氨酸激酶。

[0168] 另一种方法是使用小分子抑制在受体胞质一侧的 EGFR 酪氨酸激酶。没有激酶活性，EGFR 自身无法活化，这是下游衔接蛋白结合的先决条件。表面上通过中断依赖于该途径生长的细胞内的信号级联，减少肿瘤增殖和迁移。吉非替尼 (gefitinib)、埃罗替尼 (erlotinib) 和拉帕替尼 (lapatinib)(混合的 EGFR 和 ERBB2 抑制剂) 是小分子激酶抑制剂的实例。其它实例包括直接靶向 EGFR 的易瑞沙 (Iressa) 和特罗凯 (Tarceva)。

[0169] HER2 是单克隆抗体曲妥珠单抗 (trastuzumab)(作为 Herceptin 在市场上出售) 的靶标。曲妥珠单抗仅在 HER2 过表达的癌症中有效。另一种抑制 HER2 和 HER3 受体二聚化的单克隆抗体，帕妥株单抗 (Pertuzumab)，于 2012 年 6 月经 FDA 批准与曲妥珠单抗组合使用。

[0170] 另外，NeuVax(Galena Biopharma) 是指示“杀伤”T 细胞靶向并破坏表达 HER2 的癌细胞的肽基免疫疗法。

[0171] 通过雌激素受体发信号调节 HER2 的表达。通过雌激素受体作用的雌二醇 (estradiol) 和它莫西芬 (tamoxifen) 下调 HER2 的表达。

[0172] 下面列出了可根据本发明使用的抗体的实例而决非意在为限制性。

[0173] 表 1

[0174]

抗体	商标名称	批准日期	类型	靶标	批准治疗
阿仑单抗(Alemtuzumab)	Campath	2001 年	人源化	CD52	慢性淋巴细胞性白血病
贝伐单抗(Bevacizumab)	Avastin	2004 年	人源化	血管内皮生长因子	结直肠癌
贝伦妥单抗维多汀(Brentuximab vedotin)	Adcetris	2011 年	嵌合	CD30	霍奇金淋巴瘤、间变性大细胞淋巴瘤
西妥昔单抗(Cetuximab)	Erbitux	2004 年	嵌合	表皮生长因子受体	结直肠癌
吉妥珠单抗奥唑米星(Gemtuzumab ozogamicin)	Mylotarg	2000 年	人源化	CD33	急性髓性白血病(连同卡里奇霉素(calicheamicin))
替伊莫单抗(Ibritumomab tiuxetan)	Zevalin	2002 年	鼠	CD20	非霍奇金淋巴瘤(连同钇-90 或铟-111)
帕尼单抗(Panitumumab)	Vectibix	2006 年	人	表皮生长因子受体	结直肠癌
利妥昔单抗(Rituximab)	Rituxan、Mabthera	1997 年	嵌合	CD20	非霍奇金淋巴瘤
曲妥珠单抗(Trastuzumab)	Herceptin	1998 年	人源化	ErbB2	乳腺癌

[0175] SYNJ2 的抑制剂和任选如本文所述的细胞表面受体的抑制剂可本身或呈与适合载体或赋形剂混合的药物组合物施用给受试者。

[0176] 如本文所使用，“药物组合物”指本文所述一种或多种活性成分与其它化学组分例如生理上适合的载体和赋形剂的制剂。药物组合物的目的是利于向生物施用化合物。

[0177] 本文中术语“活性成分”指可解释生物效应的 SYNJ2 抑制剂（和任选细胞表面受体的抑制剂）。

[0178] 在下文中，可交换使用的短语“生理上可接受的载体”和“药学上可接受的载体”指不会对生物产生显著刺激并且不会消除施用化合物的生物活性和特性的载体或稀释剂。佐剂包括在这些短语下。

[0179] 本文中术语“赋形剂”指添加到药物组合物中以更利于施用活性成分的惰性物质。赋形剂的实例包括但不限于碳酸钙、磷酸钙、各种糖和各类淀粉、纤维素衍生物、明胶、植物油和聚乙二醇。

[0180] 在“Remington's Pharmaceutical Sciences,”Mack Publishing Co.,Easton,PA 最新版中可找到配制和施用药物的技术，其以引用的方式并入本文。

[0181] 适合的施用途径可能包括（例如）口服、直肠、透粘膜，尤其是经鼻、肠内或肠胃外递送，包括肌肉、皮下和髓内注射以及胸内、直接心室内、心脏内递送到（例如）右或左心室腔、常见冠状动脉内，经静脉、腹膜内、鼻内或眼内注射。

[0182] 向中枢神经系统 (CNS) 递送药物的传统方法包括：神经外科策略（例如，脑内注射或脑室灌注）；试剂的分子操纵（例如，生成包含对内皮细胞表面分子有亲和力的转运肽结合本身不能够跨过 BBB 的试剂的嵌合融合蛋白）以试图利用 BBB 的其中一个内源运输途径；设计用于增加试剂的脂溶性的药理学策略（例如，水溶性试剂与脂质或胆固醇载体偶联）；和通过高渗破坏（由向颈动脉注入甘露糖醇溶液或使用生物活性剂例如血管紧张素

肽引起）暂时破坏 BBB 的完整性。然而，这些策略中的每一种都有局限性，例如与侵入性外科手术相关的固有风险，内源运输体系固有局限性强加的尺寸限制，与全身施用由在 CNS 外可具活性的载体基序构成的嵌合分子相关的潜在有害生物副作用和 BBB 受破坏的脑部区域内脑损伤的可能风险，这使其成为次优递送方法。

[0183] 可选地，可能以局部而非全身方式施用药物组合物，例如通过向患者的组织区域直接注射药物组合物。

[0184] 术语“组织”指由为进行一种功能或多种功能而设计的细胞组成的生物体的一部分。实例包括但不限于脑组织、视网膜、皮肤组织、肝组织、胰腺组织、骨、软骨、结缔组织、血液组织、肌肉组织、心脏组织脑组织、血管组织、肾组织、肺组织、性腺组织、造血组织。

[0185] 可通过本领域众所周知的工艺，例如借助于常规混合、溶解、造粒、糖衣制备、研磨、乳化、封装、包埋或冻干工艺生产本发明一些实施方案的药物组合物。

[0186] 因此可按传统方式使用包含利于将活性成分加工成药学上可用的制剂的赋形剂和助剂的生理上可接受的载体配制根据本发明一些实施方案使用的药物组合物。适当的制剂取决于所选施用途径。

[0187] 对于注射，可将药物组合物的活性成分配制于水溶液，优选于生理上相容的缓冲液中例如 Hank 溶液、林格氏溶液或生理盐缓冲液。对于透粘膜施用，在制剂中使用适于要透过的屏障的渗透剂。此类渗透剂通常在本领域中已知。

[0188] 对于口服施用，通过将活性化合物与本领域众所周知的药学上可接受的载体合并，可容易地配制药物组合物。此类载体使得能够将药物组合物配制成片剂、丸剂、糖衣丸、胶囊、液体、凝胶、糖浆、浆液、混悬液等，供患者口服。可使用固体赋形剂，在需要时添加适合助剂后任选研磨所得混合物，并加工颗粒混合物，以获得片剂或糖衣丸核，制成供口服使用的药物制剂。适合的赋形剂尤其为填料例如糖类，包括乳糖、蔗糖、甘露糖醇或山梨糖醇；纤维素制剂例如玉米淀粉、小麦淀粉、米淀粉、马铃薯淀粉、明胶、黄蓍胶、甲基纤维素、羟丙基甲基－纤维素、羧甲基纤维素钠；和／或生理上可接受的聚合物例如聚乙烯吡咯烷酮(PVP)。若需要，可添加崩解剂，例如交联聚乙烯吡咯烷酮、琼脂或海藻酸或其盐例如海藻酸钠。

[0189] 为糖衣丸核提供了适合包衣。为了这个目的，可使用可能任选含有阿拉伯树胶、滑石、聚乙烯吡咯烷酮、卡波姆凝胶、聚乙二醇、二氧化钛、漆溶液和适合有机溶剂或溶剂混合物的浓糖溶液。可向片剂或糖衣丸包衣添加着色剂或色素以鉴定或表征活性化合物剂量的不同组合。

[0190] 可口服使用的药物组合物包括由明胶制成的推入配合胶囊以及由明胶和增塑剂，例如甘油或山梨糖醇制成的软密封胶囊。推入配合胶囊可能含有与填料例如乳糖、粘合剂例如淀粉、润滑剂例如滑石或硬脂酸镁和任选稳定剂混合的活性成分。在软胶囊中，活性成分可溶解或悬浮于适合液体，例如脂肪油、液体石蜡或液体聚乙二醇中。另外，可添加稳定剂。供口服施用的所有制剂都应呈适合所选施用途径的剂量。

[0191] 对于口腔施用，所述组合物可呈以传统方式配制的片剂或锭剂形式。

[0192] 对于通过鼻吸入施用，根据本发明一些实施方案使用的活性成分便于呈气溶胶喷雾呈现形式，借助于适合的推进剂，例如二氯二氟甲烷、三氯氟甲烷、二氯－四氟乙烷或二氧化碳从加压包或喷雾器中递送。在为加压气溶胶的情况下，可通过提供递送计量好的量

的阀门测定剂量单位。可配制含化合物和适合粉末基质例如乳糖或淀粉的粉末混合物，例如用于分配器中的明胶胶囊和药筒。

[0193] 可配制本文所述的药物组合物供肠胃外施用，例如通过快速浓注或持续输注。注射用制剂可呈单位剂型，例如于任选添加了防腐剂的安瓿或多剂量容器中呈现。所述组合物可能是于油性或水性媒介物中的混悬液、溶液或乳液，并且可能含有配方剂，例如助悬剂、稳定剂和 / 或分散剂。

[0194] 供肠胃外施用的药物组合物包括呈水溶性形式的活性制剂的水溶液。另外，活性成分的混悬液可制备成适当的油或水基注射混悬液。适合的亲脂性溶剂或媒介物包括脂肪油例如芝麻油，或合成脂肪酸酯例如油酸乙酯、甘油三酯或脂质体。含水注射混悬液可含有增加混悬液粘度的物质，例如羧甲基纤维素钠、山梨糖醇或葡聚糖。任选地，混悬液还可能含有适合稳定剂或增加活性成分的溶解性的试剂以允许制备高浓溶液。

[0195] 可选地，活性成分可能呈粉末形式，以在使用之前用适合媒介物例如无菌、无热原水基溶液复水。

[0196] 也可使用（例如）传统栓剂基质例如可可油或其它甘油酯将本发明一些实施方案的药物组合物配制成直肠组合物，例如栓剂或保留灌肠剂。

[0197] 适合在本发明一些实施方案的情况下使用的药物组合物包括其中含实现预期目的有效的量的活性成分的组合物。更具体地，治疗有效量指活性成分 (SYNJ2 抑制剂）预防、减轻或改善病症（例如，癌症或转移癌）的症状或延长受治受试者的存活期有效的量。

[0198] 特别是根据本文提供的详细公开内容，治疗有效量的测定在本领域技术人员的能力范围内。

[0199] 对于用于本发明的方法的任何制剂而言，最初由体外和细胞培养测定法估计治疗有效量。例如，在动物模型中可配制一剂量以达到所需浓度或滴度。这种信息可用于更精确地测定在人类中的有用剂量。

[0200] 可在体外、细胞培养或实验动物中，通过标准制药程序测定本文所述活性成分的毒性和疗效。从这些体外和细胞培养测定法和动物研究获得的数据可用于配制用于人类的一系列剂量。剂量可根据采用的剂型和利用的施用途径改变。确切的配方、施用途径和剂量可由个别医师鉴于患者的状况选择（见例如，Fingl 等，1975 年在 "The Pharmacological Basis of Therapeutics"，第 1 章第 1 页）。

[0201] 可单独调节剂量和间隔以提供足以诱导或抑制生物效应的 SYNJ2 抑制剂水平的活性成分（最低有效浓度，MEC）。对于每种制剂 MEC 将不同，但是可由体外数据估计。达到 MEC 所必需的剂量将取决于个体特征和施用途径。检测测定法可用于测定血浆浓度。

[0202] 根据待治病状的严重程度和反应性，给药可分单次或多次施用，疗程持续几天至几周或直至达到治愈或实现疾病状态减轻。

[0203] 当然，要施用的组合物的量将取决于受治受试者、痛苦严重程度、施用方式、处方医师的判断等。

[0204] 若需要，本发明一些实施方案的组合物可于可能装有含活性成分的一种或多种单位剂型的包装或分配器装置，例如 FDA 批准的试剂盒中呈现。所述包装可能（例如）包含金属或塑料箔，例如泡罩包装。所述包装或分配器装置可能附有施用说明书。所述包装或分配器还可能提供有与容器相伴的呈管制医药品的生产、使用或销售的政府机关规定形式的

通知，所述通知反映组合物形式或人或兽医施用受该机关批准。例如，此类通知可能具有经美国食品和药物管理局批准用于处方药物的标记或具有经批准的产品说明书。如以上所进一步详述，也可制备包含配制于相容药物载体中的本发明制剂的组合物，置于适当容器中，并且标记用于治疗指定病状。

[0205] 根据 SYNJ2 对细胞迁移的贡献，本发明人观察到在侵袭性癌症亚型中 SYNJ2 mRNA 和蛋白质水平显著上调，表明 SYNJ2 可用作预后指标。

[0206] 因此，根据本发明的一方面，通过了一种在有需要的受试者中癌症预后的方法，所述方法包括测定受试者癌细胞中 SYNJ2 的水平或活性，其中在所述受试者癌细胞中所述 SYNJ2 的所述水平或活性与在未受影响的对照样品的细胞中相比上调，表明预后不良。

[0207] 如本文所使用，术语“预后”指确定疾病（癌症）的结果。

[0208] 如本文所使用，“预后不良”指疾病复发风险增加和 / 或因疾病死亡的风险增加。

[0209] 如本文所使用，术语“水平”指 DNA（基因扩增）、RNA 或蛋白质的表达水平。

[0210] 如本文所使用，“SYNJ2 活性”主要指其磷酸酶活性，即将 PI(3,4,5)P_3转化为 PI(3,4)P_2。

[0211] 根据一个特定实施方案，使用体外活性测定法测定活性。

[0212] 体外活性测定法：在这些方法中，测量从细胞提取的蛋白质混合物中特定酶（在这种情况下为磷酸酶）的活性。可在分光光度计孔内使用比色法测量活性或可在非变性丙烯酰胺凝胶（即，活性凝胶）中测量。电泳后，将凝胶浸入含底物和比色试剂的溶液中。所产生的染色带与目标蛋白质的酶活性相对应。如果精确校准并且在线性反应范围内，则样品中存在的酶量与产生颜色的量成正比。通常采用酶标准品来提高定量精确性。

[0213] 以上描述了 SYNJ2 的特定测定法，其中试验了 PI(3,4,5)P_3向 PI(3,4)P_2的转化活性。

[0214] 检测蛋白质表达和 / 或活性的方法

[0215] 可使用本领域中已知的方法测定 SYNJ2 的蛋白质表达。

[0216] 酶联免疫吸附测定法 (ELISA)：这种方法涉及将含有蛋白质底物的样品（例如，固定细胞或蛋白质溶液）固定到表面例如微量滴定板的孔上。涂覆与酶偶联的底物特异性抗体并使其与底物结合。然后检测抗体的存在并采用与抗体偶联的酶，通过比色反应进行量化。这种方法中常用的酶包括辣根过氧物酶和碱性磷酸酶。如果精确校准并且在线性反应范围内，则样品中存在的底物的量与产生颜色的量成正比。通常采用底物标准品来提高定量精确性。

[0217] 蛋白质印迹：这种方法涉及借助于丙烯酰胺凝胶将底物与其它蛋白质分开，接着将底物转移到膜（例如，尼龙或 PVDF）上。然后用对底物有特异性的抗体检测底物的存在，依次用抗体结合试剂检测抗体的存在。例如，抗体结合试剂可为蛋白 A 或其它抗体。如上文所述，抗体结合试剂可经放射性标记或经酶联。可通过放射自显影术、比色反应或化学发光检测。这种方法允许通过膜上表示电泳期间在丙烯酰胺凝胶中的迁移距离的相对位置量化底物的量并测定其同一性。

[0218] 放射免疫测定法 (RIA)：在一种型式中，这种方法涉及用特定抗体和固定在可沉淀载体例如琼脂糖珠粒上的放射标记抗体结合蛋白（例如经 I^{125}标记的蛋白 A）沉淀所需蛋白（即，底物）。沉淀团粒中计数数量与底物的量成正比。

[0219] 在 RIA 的替代型式中，采用标记底物和未标记的抗体结合蛋白。按不同量添加含未知量的底物的样品。从标记底物沉淀的计数减少量与所加样品中底物的量成正比。

[0220] 荧光激活细胞分选术 (FACS)：这种方法涉及用底物特异性抗体检测细胞内的原位底物。底物特异性抗体与荧光团相联。通过在每个细胞穿过光束时读取从每个细胞发射的光的波长的细胞分选仪检测。

[0221] 免疫组织化学分析：这种方法涉及用底物特异性抗体检测固定细胞内的原位底物。底物特异性抗体经酶联或与荧光团相联。通过显微镜术和主观或自动评价检测。如果采用酶联抗体，则可能需要比色反应。将认识到免疫组织化学分析后常常是使用例如苏木精或姬姆萨染液 (Giemsa stain) 对细胞核复染。

[0222] 原位活性测定法：根据这种方法，在含有活性酶的细胞上涂生色底物并且所述酶催化分解底物以生成用光或荧光显微镜可见的生色产物的反应。

[0223] 可选地或另外，使用本领域众所周知并且一些在下文中有描述的方法，在 RNA 水平检测 SYNJ2 的水平。

[0224] 检测 RNA 表达水平的方法

[0225] 可使用本领域中已知的方法测定本发明一些实施方案的细胞中 RNA 的表达水平。

[0226] RNA 印迹分析：这种方法涉及检测 RNA 混合物中的特定 RNA。通过用防止碱基对之间氢键结合的试剂（例如，甲醛）处理使 RNA 样品变性，确保所有 RNA 分子具有未折叠、线性构象。然后根据尺寸通过凝胶电泳分离单个的 RNA 分子并转移到变性 RNA 所附着的硝化纤维素或尼龙膜上。然后将膜暴露于标记 DNA 探针。可使用放射性同位素或酶联核苷酸标记探针。可使用放射自显影术、比色反应或化学发光检测。这种方法允许通过膜上表示电泳期间在凝胶中的迁移距离的相对位置量化特定 RNA 分子的量并测定其同一性。

[0227] RT-PCR 分析：这种方法使用相对罕见 RNA 分子的 PCR 扩增。首先，从细胞中纯化 RNA 分子并使用逆转录酶（例如 MMLV-RT）和引物例如寡聚 dT、随机六聚体或基因特异性引物转化成互补 DNA(cDNA)。然后通过应用基因特异性引物和 Taq DNA 聚合物酶，在 PCR 仪中进行 PCR 扩增反应。本领域的技术人员能够选择基因特异性引物的长度和序列及适合检测特定 RNA 分子的 PCR 条件（即，退火温度、循环次数等）。应认识到，可通过调节 PCR 循环次数并将扩增产物与已知对照比较，采用半定量 RT-PCR 反应。

[0228] RNA 原位杂交染色：在这种方法中，DNA 或 RNA 探针附着于细胞内存在的 RNA 分子上。通常，首先将细胞固定到显微镜载玻片上以保护细胞结构并防止 RNA 分子降解，然后受到含标记探针的杂交缓冲液。杂交缓冲液包括使得 DNA 或 RNA 探针与其原位靶 mRNA 分子的特异性杂交成为可能，同时避免探针非特异性结合的试剂，例如甲酰胺和盐（例如，氯化钠和柠檬酸钠）。本领域的技术人员能够为特定探针和细胞类型调节杂交条件（即，温度、盐和甲酰胺的浓度等）。杂交后，洗掉所有未结合的探针并使用已知方法检测结合的探针。例如，如果使用放射性标记探针，则使载玻片受到显示出使用放射性标记探针生成的信号的照相乳胶；如果用酶标记探针，则添加酶特异性底物以形成比色反应；如果使用荧光标记物标记探针，则使用荧光显微镜显示结合的探针；如果使用标签（例如，地高辛、生物素等）标记探针，则可按照可使用已知方法检测的与标签特异性抗体的相互作用检测结合的探针。

[0229] 原 位 RT-PCR 染 色： 在 Nuovo GJ 等 [Intracellular localization of

polymerase chain reaction(PCR)-amplified hepatitis C cDNA.Am J Surg Pathol.1993,17:683-90] 和 Komminoth P 等 [Evaluation of methods for hepatitis C virus detection in archival liver biopsies.Comparison of histology,immuno histochemistry,in situ hybridization,reverse transcriptase polymerase chain reaction(RT-PCR)and in situ RT-PCR.Pathol Res Pract.1994,190:1017-25] 中描述了这种方法。简言之,通过将标记核苷酸并入 PCR 反应中,对固定细胞进行 RT-PCR 反应。使用特定原位 RT-PCR 仪例如可从 Arcturus Engineering(Mountainview,CA) 获得的激光捕获显微切割 PixCell I LCM 系统进行反应。

[0230] DNA 微阵列 /DNA 芯片 :

[0231] 可使用 DNA 微阵列同时分析成千上万个基因的表达,允许分析特定发育过程或生理反应期间生物体的完整转录程序。DNA 微阵列由成千上万个附着于支撑物例如显微镜载玻片表面上的密集区的单独基因序列组成。已经研发出各种方法用于制备 DNA 微阵列。在一种方法中,单独 PCR 扩增用于分析的每个基因的编码区的约 1kb 片段。采用机器人装置将每种扩增 DNA 样品涂到显微镜载玻片表面上的密集区域,随后通过热和化学处理加工以使 DNA 序列与支撑物表面结合并使其变性。通常,此类阵列约 2×2cm 并且含有约 6000 个单独核酸斑点。在所述技术的变型中,由与支撑物表面共价结合的初始核苷酸合成通常长度为 20 个核苷酸的多个 DNA 寡核苷酸,以致在支撑物表面的小正方形区域内合成数万个相同寡核苷酸。在载玻片的相邻区域合成来自单个基因的多个寡核苷酸序列,以分析该基因的表达。因此,在一张载玻片上可出现成千上万个基因。此类合成寡核苷酸阵列在本领域中可称为“DNA 芯片”,与上述“DNA 微阵列”相对 [Lodish 等 (编辑) 第 7.8 章 :DNA Microarrays:Analyzing Genome-Wide Expression.In:Molecular Cell Biology,第4版,W.H.Freeman,New York.(2000)]。

[0232] 可使用金标法例如成像法、活检取样、标志表达、免疫组织化学等证实预后。

[0233] 以下是乳腺癌的具体实例,但决非意为限制性。通常在评估肿瘤大小 (T)、向相邻淋巴结转移的状态 (N) 和存在或不存在向其它器官远距离转移 (M) 的手术后,按疾病分期 (TNM 分期) 确定乳腺癌预后。即使在同一分期,根据 TNM 分期分类的患者的预后也不同。换言之,在乳腺癌的同一分期,可通过雌激素或孕激素受体 (ER 或 PR) 表达和 HER2 蛋白过表达或基因扩增确定预后。

[0234] 在诊断试剂盒 / 制品中,可能优选连同适当使用说明书和表明 FDA 批准用于诊断和 / 或评估癌症分期和 / 或预后的标签一起,包括上文描述的本发明一些实施方案的用于检测 SYNJ2 的试剂。

[0235] 例如,此类试剂盒可包括至少一个包括上述至少一种诊断剂 (例如,抗 SYNJ2,例如连同抗 HER2 和 / 或抗 ER 或这些靶标的寡核苷酸探针 / 引物一起) 的容器和包装在另一容器中的显像剂 (例如,酶、二次抗体、缓冲液、生色底物、荧光材料)。所述试剂盒还可能包括用于提高试剂盒保质期的适当缓冲液和防腐剂。

[0236] 术语“包含”、“包括”、“具有”及其同根词意为“包括但不限于”。

[0237] 术语“由……组成”意为“包括并且限于”。

[0238] 术语“基本上由……组成”意为所述组合物、方法或结构可能包括另外的成分、步骤和 / 或部分,但只有在另外的成分、步骤和 / 或部分不会实质上改变要求保护的组合物、

方法或结构的基本和新型特征时。

[0239] 如本文所使用，除非上下文中另有明确规定，单数形式“一种”、“一个”和“所述”包括复数个指示物。例如，术语“一种化合物”或“至少一种化合物”可包括多种化合物。在本申请各处，本发明的各实施方案可呈范围形式呈现。应理解，呈范围形式的描述仅仅是为了方便和简洁而不得解释为对本发明范围的硬性限制。相应地，对范围的描述应视为具有特别公开的所有可能子范围以及该范围内的单个数值。例如，对范围例如 1-6 的描述应视为具有特别公开的子范围，例如 1-3、1-4、1-5、2-4、2-6、3-6 等，以及该范围内的单个数，例如 1、2、3、4、5 和 6。不管范围宽度如何，这都适用。

[0240] 当本文指出数值范围时，意为包括指定范围内引用的所有数字（分数或整数）。短语“范围 / 范围介于”第一指定数量和第二指定数量之间和“范围 / 范围从”第一指定数量“到”第二指定数量可交换使用并且意为包括第一和第二指定数量及之间的所有分数和整数数字。

[0241] 如本文所使用，术语“方法”指完成给定认为的方式、手段、技术和程序，包括但不限于化学、药理学、生物学、生物化学和医学领域的从业者已知或易于由已知方式、手段、技术和程序研发的方式、手段、技术和程序。

[0242] 如本文所使用，术语“治疗”包括消除、大体上抑制、减缓或逆转病状的进展，大体上改善病状的临床或审美症状，或大体上预防病状的临床或审美症状出现。

[0243] 应认识到，为清楚起见在单独实施方案的上下文中描述的本发明的某些特征也可在单个实施方案组合提供。相反，为简洁起见在单个实施方案的上下文中描述的本发明的各种特征也可单独地或在任何适合的子组合或如其所应在所述本发明任何其它实施方案中提供。除非没有那些要素，实施方案无效，否则不得将各实施方案的上下文中描述的某些特征视为那些实施方案的基本特征。

[0244] 上文描绘了本发明的各实施方案和方方面面并且如以下权利要求部分中所要求那样，在下列实施例中找到实验支持。

[0245] 实施例

[0246] 现参考下列实施例，其连同以上描述一起以非限制性方式说明了本发明的一些实施方案。

[0247] 通常，本文使用的命名法和本发明利用的实验室程序包括分子、生物化学、微生物学和重组 DNA 技术。文献中透彻讲解了此类技术。见，例如，"Molecular Cloning:A laboratory Manual"Sambrook 等，(1989)；"Current Protocols in Molecular Biology"I-III 卷 Ausubel,R.M. 编 辑 (1994)；Ausubel 等，"Current Protocols in Molecular Biology"，John Wiley 和 Sons,Baltimore,Maryland(1989)；Perbal，"A Practical Guide to Molecular Cloning"，John Wiley&Sons,New York(1988)；Watson等，"Recombinant DNA"，Scientific American Books,New York；Birren 等（编辑）"Genome Analysis:A Laboratory Manual Series"，1-4 卷，Cold Spring Harbor Laboratory Press,New York(1998)；美国专利第 4,666,828、4,683,202、4,801,531、5,192,659 和 5,272,057 号中提出的方法；"Cell Biology:A Laboratory Handbook"，I-III 卷 Cellis,J.E. 编 辑 (1994)；"Current Protocols in Immunology"I-III 卷 Coligan J.E. 编 辑 (1994)；Stites 等（编辑），"Basic and Clinical Immunology"（第 8 版），Appleton&L

ange,Norwalk,CT(1994);Mishell 和 Shiigi(编辑),"Selected Methods in Cellular Immunology",W.H.Freeman and Co.,New York(1980);在专利和科学文献中广泛描述了可用免疫测定法,见,例如,美国专利第 3,791,932、3,839,153、3,850,752、3,850,578、3,853,987、3,867,517、3,879,262、3,901,654、3,935,074、3,984,533、3,996,345、4,034,074、4,098,876、4,879,219、5,011,771 和 5,281,521 号;"Oligonucleotide Synthesis"Gait,M.J. 编 辑 (1984);"Nucleic Acid Hybridization"Hames,B. D. 和 Higgins S.J. 编 辑 (1985);"Transcription and Translation"Hames,B.D. 和 Higgins S.J. 编 辑 (1984);"Animal Cell Culture"Freshney,R.I. 编 辑 (1986);"Immobilized Cells and Enzymes"IRL Press,(1986);"A Practical Guide to Molecular Cloning"Perbal,B.,(1984) 和 "Methods in Enzymology"1-317 卷,Academic Press;"PCR Protocols:A Guide To Methods And Applications",Academic Press,San Diego,CA(1990);Marshak 等,"Strategies for Protein Purification and Characterization-A Laboratory Course Manual"CSHL Press(1996);其全部以引用的方式如同本文完全阐述一样并入。在本文件各处提供了一般参考文献。据信其中的程序在本领域众所周知并且是为了方便读者而提供。其中所含的全部信息以引用的方式并入本文。

[0248] 实施例 1

[0249] 材料和方法

[0250] 细胞迁移、侵袭和趋化性测定

[0251] 将细胞接种在 Transwell 盘 (BD Bioscience) 的上部隔室内,一式三份,并使其迁移 18h 通过插入膜。之后,将细胞固定于多聚甲醛 (3%) 中,于 Triton X-100(0.05%) 中透性化并用甲基紫 (0.02%) 染色。去除在滤器上侧生长的非迁移细胞并且为迁移细胞拍照。使用 BioCoat Matrigel 小室进行侵袭测定。使用来自 ibidi(München,Germany) 的趋化小室和延时成像。使用 ImageJ 追踪细胞核的位置。

[0252] 磷酸肌醇分析

[0253] 在无肌醇的培养基中培养细胞 30min,将无肌醇的培养基更换为补充了 [^{3}H]- 肌醇和透析血清 (10%) 的培养基。细胞培养 3 天,冲洗并且于 1M HCl 中提取,接着用 1M 甲醇提取。然后刮取细胞并于氯仿,然后于甲醇 0.1M EDTA pH8.0 中提取,并蒸发有机相。之后,提取物去乙酰化,使用两个串联的 partisphere SAX 柱 (Whatman) 和磷酸铵四步梯度 pH 6.0,通过阴离子交换 HPLC(Agilent 1200) 分离。用在线流量闪烁分析仪检测放射性标记洗脱物并且使用 ProFSA 软件 (Perkin-Elmer) 量化。

[0254] 明胶酶谱法

[0255] 为检测 MMP-2 活性,在 10% 聚丙烯酰胺 /0.1% 明胶包埋凝胶上电泳分离生物样品。然后于 2.5% Triton X-100 中洗涤凝胶,并且在 37℃下于含有 0.2M NaCl、5mM $CaCl_2$、1μM $ZnCl_2$、0.02% Brij 35 和 1mM 对氨基苯汞乙酸盐的 50mM Tris-HCl(pH 7.5) 中温育 36h。

[0256] 动物转移试验

[0257] 雌性 CB-17SCID 小鼠 (Harlan Laboratories,Haslett,MI;15 只 / 组) 在脂肪垫内植入 MDA-MB-231 细胞 1.4×10^{6} 个细胞 / 只小鼠)。植入 2 周和 6 周后,麻醉小鼠,测量肿瘤大小并使用荧光双目镜目测淋巴结内的转移。对于肺部转移,处死小鼠,取出肺部、洗

涤，并使用荧光双目镜获取图像。用双侧费歇尔精确检验 (Two-sided Fischer's exact test) 分析淋巴结转移。肿瘤生长测量使用精确显著性 [2x1- 尾])Mann-Whitney 检验。

[0258] 试剂

[0259] 除非有指示，人重组生长因子和其它材料购自 Sigma(St.Louis,MO,USA)。用于免疫印迹的放射性物质和化学发光试剂盒从 Amersham(Buckinghamshire,UK) 获得。EGFR- 激酶抑制剂 AG1478、MEK 抑制剂 U0126 和 PI3K 抑制剂渥曼青霉素来自于 Calbiochem(San Diego,CA)。用于伤口愈合测定的板来自于 ibidi(Munich,Germany)。用于延时成像的 35-mm 玻璃底盘购自 MaTek(Ashland,MA)。在我们的实验室内生成 EGF 受体的鼠单克隆抗体 (mAb)111.6。用于蛋白质印迹分析的抗 EGFR 来自于 Alexis(Lausen,Switzerland)。抗 Ras-GAP 和抗 AKT 抗体来自于 Santa Cruz Biotechnology(Santa Cruz,CA)。抗 EEA1、抗 Rab5、抗 Rab4 和抗 Rac1 来自于 BD Transduction Laboratories(Franklin Lakes,NJ)。抗 SYNJ2mAb 来自于 Abnova(Taipei,Taiwan)。使用下列二次抗体 :与辣根过氧物酶偶联的山羊抗小鼠 IgG 和山羊抗兔 IgG 抗体购自 Jackson ImmunoResearch Laboratories(Bar Harbor,Maine)。德克萨斯红转铁蛋白 (Texas-red transferrin)、山羊抗小鼠 Alexa-488、Alexa-555 和 Alexa-647 二次抗体来自于 Invitrogen(Carlsbad,CA)。

[0260] siRNA 对照来自于“Thermo scientific Dharmacon”产品目录号 D-001810-10-05 ；针对 SYNJ2 的 siRNA 序列如 SEQ ID NO:6-GGACAGCACUGCAGGUGUU 所示 ；所有 shRNA 均来自于 SIGMA Israel :shRNA 对照 - 产品目录号 SHC002 ；使用的针对 SYNJ2 的 shRNA 序列为 CCGGCCGGAAGAACAGTTTGAGCAACTCGAGTTGCTCAAACTGTTCTTCCGGTTTTTG(SEQ ID NO:9)。

[0261] 细胞系和转染

[0262] 在补充了抗生素、胰岛素 (10μg/mL)、霍乱毒素 (0.1μg/mL)、氢化可的松 (0.5μg/mL)、热灭活马血清 (5％ vol/vol) 和 EGF(10ng/mL) 的 DMEM:F12(1:1) 培养基中培养 MCF10A 细胞。在补充了 10％热灭活胎牛血清 (Gibco)、1mM 丙酮酸钠和青霉素 - 链霉素混合物 (100 单位 /ml ；0.1mg/ml ；Beit Haemek,Israel) 的 RPMI-1640(Gibco BRL ；Grand Island,NY) 中培养人乳腺 MDA-MB-231 细胞。MDA-MB-231-RFP 稳定细胞系是来自于 Prof. Hadasa Degani(The Weizmann Institute of Science,Israel) 的一种礼物。根据生产商的指导 (Roche,Mannheim,Germany)，使用 Fugen-HD 进行质粒转染。可选地，对于使用 siRNA 寡核苷酸的瞬时 mRNA 敲减实验，用 Oligofectamine(Invitrogen) 转染细胞。

[0263] 慢病毒载体和病毒生产

[0264] 按照生产商的指导 (Sigma)，在 HEK-293T 细胞中生成非靶向 shRNA 发夹 (对照) 和针对人 SYNJ2 的发夹。用补充了聚凝胺 (8μg/mL) 的 shRNA 编码慢病毒感染靶细胞，并且在嘌呤霉素 (2μg/mL) 的存在下培养 4 天。按照生产商的指导，使用 ViraPower 慢病毒表达系统 (Invitrogen) 进行人 SYNJ2 的稳定基因特异性递送。

[0265] 免疫荧光和图象处理

[0266] 在涂有纤连蛋白的盖玻片上培养细胞 48h。处理后，洗涤细胞，使用 0.02％ Triton X-100 和 3％多聚甲醛透性化，并固定 20min。使用 Zeiss LSM-710 显微镜或转盘式显微镜 (Zeiss 100×，NA 1.45 ；Yokogawa CSU-22 ；Zeiss 全自动化、倒置 inverted 200M ；Photometrics HQ-CCD 相机) 和固态激光器 (473、561 和 660nm，曝光时间 :0.25-1s)，在 Slidebook™的命令下进行共聚焦显微镜检查。每 70-300ms 沿 Z 轴，通过改变压电控制工作

台的位置（步长：0.1-0.4μm）获取 3D 图像栈。可选地，使用 DeltaVision 系统（Applied Precision, Issaqua, WA）进行活细胞荧光显微镜检查并且使用 priism 软件处理图像。

[0267] EGF 的放射性标记

[0268] 如下用 IODOGEN（非水溶性碘化试剂）标记人重组 EGF：使 EGF(5μg) 在涂有 Iodogen 的管（1mg 试剂）内与 $Na^{125}I$(1mCi) 混合。在 23℃下温育 15min 后，添加白蛋白至 0.1mg/ml 的最终浓度，并且在 Excellulose GF-5 柱上分离混合物。

[0269] 受体下调测定

[0270] 每个时间点将 MDA-MB-231 细胞接种于 24 孔板上，一式三份，另外的孔接种用于对照。48h 后，使细胞饥饿 4h 并在 37℃下用 EGF(2ng/ml) 刺激指定时间间隔。随后，将其置于冰上，用结合缓冲液（DME 培养基、白蛋白 1％、Hepes 20mM，pH 7.5）冲洗一次，并且经受弱酸 / 盐洗涤（0.2M 醋酸钠缓冲液 pH 4.5，0.5M NaCl）以去除表面结合的 EGF。之后，在 4℃下用放射性标记 EGF 培养细胞 1.5h 并用结合缓冲液冲洗。用放射性标记 EGF 和过量的未标记 EGF 培养对照。最后，用 1M NaOH 裂解细胞，并使用 γ - 计数仪测定放射性。数据表示相对于时间 0，细胞表面上受体的百分比。

[0271] 表面 EGF- 受体的测定

[0272] 在 24 孔板上接种细胞（2×10^4个 / 孔），一式三份，另外的孔接种用于对照。之后，在 4℃下用放射性标记 EGF 培养细胞 1.5h 并用结合缓冲液冲洗。用放射性标记 EGF 和过量的未标记 EGF 培养对照孔。最后，用 1M NaOH 裂解细胞并测定放射性。数据表示相对于对照细胞，所述细胞表面上受体的百分比。

[0273] 免疫印迹分析

[0274] 用冰冷盐水简单地洗涤细胞，并且于缓冲洗涤剂溶液（25mM HEPES(pH 7.5)、150mM NaCl、0.5％ Na- 脱氧胆酸盐、1％ NP-40、0.1％ SDS、1mM EDTA、1mM EGTA、0.2mM Na_3VO_4 和按 1:1000 稀释的蛋白酶抑制剂混合物）刮取细胞。为了凝胶上样相等，使用 BCA(Pierce) 试剂测定蛋白质浓度。凝胶电泳后，将蛋白质转移到硝化纤维素膜上。膜于含 10％低脂奶的 TBST 缓冲液（0.02M Tris-Hcl(pH 7.5)、0.15M NaCl 和 0.05％吐温 20(Tween 20)）中封闭，沾一次抗体 1h，用 TBST 洗涤并且用与 HRP 偶联的二次抗体温育 30min。

[0275] 伤口愈合（刮伤）测定

[0276] 根据生产商的方法（iBidi, Germany）进行伤口愈合测定。简言之，MCF10A 细胞胰蛋白酶化，重新悬浮于缺乏 EGF 的培养基（7.0×10^5个细胞 /mL）中并向每个孔接种 70μl，24h 内产生汇合层。之后，通过使用无菌镊子取出培养插件并且使细胞迁移 2h。

[0277] 扫描和透射电子显微镜术

[0278] 于补充了 4％多聚甲醛和 2％蔗糖的盐水中固定细胞。洗涤样品并经受第二种固定液（于补充了 1％蔗糖和 5mM $CaCl_2$ 的 0.1M 二甲胂酸盐缓冲液中的 3％多聚甲醛和 2.5％戊二醛，pH 7.4）。于 0.1M 二甲胂酸盐缓冲液中洗涤细胞并且用 1％于二甲胂酸盐缓冲液中的四氧化锇后固定 1h。对于扫描电子显微镜术（SEM），洗涤后固定样品两次并用 1％鞣酸处理 5min，接着再次洗涤并用 1％乙酸铀酰处理 30min。样品于分级乙醇中脱水，并且通过用金 - 钯合金膜溅射使样品具导电性。使用扫描电子显微镜（Leo Supra 55/Vp Zeiss, Thornwood, NY）为样品拍照。

[0279] 受体再循环测定

[0280] 在37℃下用Alexa Fluor 488-转铁蛋白(25μg/ml于无血清培养基中)预培养MDA-MB-231细胞30min或用Alexa Fluor 488-EGF(40ng/mL)预培养10min。通过在4℃下于酸性缓冲液(150mM NaCl、1mM $MgCl_2$、0.125mM $CaCl_2$、0.1M甘氨酸)中培养30min分离表面结合的配体,之后转移到37℃下指定时间间隔,以允许内化配体再循环。通过成像或通过FACS分析细胞。

[0281] 实时细胞阻抗分析

[0282] 使用RTCA-Xcelligence系统(Roche Diagnostics,Mannheim,Germany)记录对细胞扩散和粘着的测量。于盐水中洗涤金微电极E-plates-16一次。先接种细胞(2,500个/孔),再在指定间隔记录阻抗数据(细胞指数;按照测得电阻抗的相对变化推导)。使用生产商提供的软件包1.2分析数据。

[0283] TAPP1-PH结构域表达和纯化

[0284] 将编码TAPP1-PH结构域及N端Flag标签和C端6xHis标签的构建体(SEQ ID NO.:13,图18)克隆到pET28质粒中并且在用200μM IPTG培养后,在大肠杆菌BL21(DE3)中表达。在15℃下培养细菌,然后用细胞破碎仪裂解。通过离心去除细胞碎片并且在经50mM Tris pH 8、0.5M NaCl和20mM咪唑平衡的Ni柱(HisPrep FF 16/10,GE Healthcare)上捕获蛋白质。在含有0.5M咪唑的相同缓冲液中洗脱蛋白质。将含有TAPP1-PH结构域的部分注入经含有50mM Tris pH 8和100mM NaCl的缓冲液平衡的尺寸排阻柱(Hiload_26/60_Superdex 75,GE Healthcare)中。用20mM磷酸钠缓冲液pH 7.2稀释含TAPP1-PH结构域的汇集峰值部分3倍并载入经相同磷酸盐缓冲液平衡的阳离子交换柱(HiTrap_SP_FF_5ml,GE Healthcare)上。用线性梯度的含1M NaCl的磷酸盐缓冲液从柱上洗脱纯蛋白(TAPP1-PH结构域在200mM NaCl时洗脱)。将通过SDS-PAGE评定含纯TAPP1-PH结构域的部分汇集在一起并且通过布拉德福试剂(Bradford reagent)和OD_{280}(消光系数为20,520)量子化测定蛋白质浓度。将蛋白质分成等分试样,用液氮速冻并储存在-80℃下。

[0285] SYNJ2的5'磷酸酶活性

[0286] 根据荧光偏振,通过竞争测定法记录对SYNJ2水解来自PI(3,4,5)P3的5-磷酸生成PI(3,4)P2的能力的测量,作为读出。在玻璃管中,添加100μl的SOPS(Avanti Inc.,50mg/ml于氯仿中)和50μl胆固醇(Sigma Aldrich,10mg/ml于氯仿中)制备稳定化SOP脂质混合物(x50)。使用温和氮蒸汽蒸发氯仿,风干混合物。然后在室温下通过1min涡旋,使经蒸发的脂质混合物重新悬浮于10ml的0.25mg/ml $C_{12}E_8$(Avanti Inc.)中。反应混合物包含PBS、DTT、MgCl2(全部来自于Sigma Aldrich)、SOP脂质混合物(x50)、全长纯化SYNJ2(OriGene,产品目录号TP315160)和PI(3,4,5)P3(Echelon Bioscience,产品目录号P-3908),有或无试验化合物。一旦添加PI(3,4,5)P3,就在33℃下温育反应混合物8min以允许通过SYNJ2 5'-磷酸酶活性生成PI(3,4)P2。温育后,通过添加包含PBS、DTT、检测蛋白(TAPP1的PH结构域)、SOP脂质混合物(x50)、经荧光标记的PI(3,4)P2(Echelon Bioscience,产品目录号C34M6)和EDTA(Sigma Aldrich)的检测混合物终止反应。使用适当的酶标仪和设置与BODIPY® TMR染料相容的滤波器(550nm激发/580nm偏振发射滤波器)测量荧光偏振。未标记的PI(3,4)P2对照购自Echelon Bioscience(产品目录号P-3408)。

[0287] 实施例2

[0288] EGF 诱导的 SYNJ2 表达升高促进乳腺细胞侵袭

[0289] 用 EGF 家族配体培养时，人乳腺上皮细胞 (MCF10A) 表现出强烈的迁移和侵袭表现型 (图 1A 和 1B)，但是用血清处理不足以推进细胞运动。EGF 连同 EGFR(AG1478)、MEK(U0126) 或 PI3K(渥曼青霉素) 的抑制剂一起共同培养降低了运动性 (图 1C)，表明 MEK/ERK 和 PI3K 活性对于 EGF 诱导的迁移必不可少。重要的是，EGF 诱导的运动表现型与 425 个基因的转录上调相关联 (Amit 等，2007)。为鉴定推进转移的基因，使该基因集与体内选择乳腺癌细胞的转移性亚克隆期间经历上调的较大基因集交叉 (Minn 等，2005)。23 个重叠基因的组 (图 1D) 包括编码突触囊泡磷酸酶 -2(SYNJ2)，神经胶质瘤细胞侵袭所涉及的一种脂质磷酸酶的基因 (Chuang 等，2004)。通过 PCR 和免疫印迹验证 EGF 诱导的 SYNJ2 上调 (图 2A 和 2B)。

[0290] 接下来，转化并亚克隆 MCF10A 细胞以稳定地过表达 SYNJ2(作为 GFP 融合物；SYNJ2-OX，图 1E)。当接种于缺乏 EGF 的培养基时，SYNJ2-OX 细胞表现出特征在于膜边缘波动 (图 2C)，连同基本和 EGF 诱导的迁移和侵袭能力增强的迁移前表现型 (图 2D 和 2C)。相反，使用小干扰 RNA(siRNA ；图 1G) 敲减 SYNJ2 显著减少了细胞侵袭，以及单独和集体迁移 (图 2E、1H 和 1J)。总之，EGF 诱导的 SYNJ2 上调驱动了乳腺细胞的稳健侵袭表现型。

[0291] 实施例 3

[0292] SYNJ2 的磷酸酶活性对于乳腺细胞的侵袭性必不可少

[0293] 为使体内实验成为可能，用高转移性 MDA-MB-231 乳腺癌红色荧光蛋白 (RFP) 表达细胞生成过表达 SYNJ2 或 LacZ(对照) 的亚克隆，以及表达 shControl 或 SYNJ2 特异性发夹 (shSYNJ2 ；图 3A) 的亚克隆。SYNJ2 的表达增强在 2D 培养 (图 3B) 中赋予了细长形态，并且在 3D 培养 (图 4A) 中培养细胞时赋予了广泛的侵袭臂。相反，SYNJ2 敲减消除了侵袭模式 (图 4B)。类似地，过表达使侵袭能力增强～ 3.2 倍 (图 3B)，而敲减 (图 3C) 抑制迁移和侵袭 (图 3D)。为检查磷酸酶催化活性的作用，使用具有编码 WT SYNJ2 或在磷酸酶 / 核酸酶结构域 (Pfam:PF03372) 内每个保守 WXGDXN(F/Y)R 基序 (Jefferson 和 Majerus，1996) 中隐匿有点突变的催化死亡形式 (D388A 和 D726A ；图 4C) 的慢病毒颗粒的 shSYNJ2 细胞。与 WT SYNJ2 不同，突变体重新表达不能恢复侵袭能力 (图 4D)，表明 SYNJ2 的磷酸酶活性对侵袭表现型必不可少。

[0294] shSYNJ2 细胞不能迁移进一步受扫描电子显微镜术 (图 4E) 和 F- 肌动蛋白染色支持，其揭示了肌动蛋白组织严重缺陷和细胞高度增加 (图 4F)。重要的是，还注意到在圆形部分周围簇集的肌动蛋白补丁 (图 4F ；箭头)。相应地，shSYNJ2 细胞的延时显微镜分析确认了细胞内异常囊泡的存在，表明 SYNJ2 敲减使囊泡运输脱轨。接下来，检查了 SYNJ2 的亚细胞定位。表达 GFP-SYNJ2 的 MDA-MB-231 细胞的延时图像 (图 3E) 以及使用抗 SYNJ2 抗体的免疫荧光 (图 3F)，反映了 SYNJ2 分布的两种主要模式 ：定位于前缘的小外周组件 (图 3E 中的黑色箭头) 和第二群更靠近细胞中心定位的较大组件 (蓝色箭头)。显著地，用 EGFR 配体 (TGF- α) 刺激 MDA-MB-231 细胞后不久，SYNJ2 迅速在新出现的板状伪足的基部，在形成的前缘下方装配 (图 3E、3F)。有趣的是，用 MCF10A 细胞进行的类似分析表明 SYNJ2 最初与 F- 肌动蛋白共同定位在细胞间连接处，但是在用 EGF 刺激后易位到前缘，通常易位到板状伪足基部 (图 3G)。总之，这些观察资料表明生长因子不但调节 SYNJ2 表达水平，而且调节其向前缘的动态募集。

[0295] 实施例 4

[0296] SYNJ2 向腹侧膜的募集取决于发动蛋白和 Rac1

[0297] 为研究 SYNJ2 定位位点的动力学特性，生成稳定表达 GFP-SYNJ2MDA-MB-231 亚克隆 (GFP-SYNJ2 细胞）并分析 GFP-SYNJ2 斑点的形成和消耗。将这些斑点分为动力学上不同的亚群 :定位到边缘波动膜的动态斑点和定位到靠近细胞中心的离散区域的斑点（图 5A)。显著地，GFP-SYNJ2 斑点显示出与 RFP- 网格蛋白轻链 A（图 5A) 或 RFP- 小窝蛋白 1（图 6A) 标记的组件的最小重叠，表明较少定位到网格蛋白有被小窝或细胞膜穴样内陷。重要的是，新形成的外周斑点预示初生板状伪足，因为其在局部形成板状伪足之前出现。相反，与肌动蛋白共同定位的更多中心和稳定的斑点簇持续～ 30min（图 5B)。相应地，追踪单独组件（图 6B ;左）揭示非常广泛的寿命分布 :短寿命（～ 20-40s，60％的组件）、中间寿命和长寿命组件（～ 10％的组件）。中间组起始后荧光强度不断增大，而组件在运动方面保持静止（图 6B ;右）。这种动态模式类似于网格蛋白有被小窝 (Ehrlich 等，2004) 并且表明了运输中间体的形成和消耗。

[0298] 在采用落射荧光（红色 ;对维数 Z 的变化相对不敏感）和全内反射显微术 (TIRF，绿色 ;限于～ 200nm 深度）的实验中，通过荧光信号的同步出现和消失加强腹侧膜处 GFP-SYNJ2 的主要双峰区域化。因为斑点在其整个寿命期呈黄色（图 5C)，所以本发明人推断 SYNJ2 装配在腹侧质膜的平面上。采用一组抑制剂，发现组件明显受胆固醇损耗抑制（图 6C ;左)，表明 SYNJ2 募集到腹侧膜需要富胆固醇的膜微结构域。渥曼青霉素诱导类似抑制效应（图 6C ;右)，表明了对 PI3K 的作用。采用 Dyngo-4a，一种发动蛋白抑制剂揭示了另一种需要，介导网格蛋白依赖性和网格蛋白非依赖性载体的分离步骤并且其抑制导致 U 形内陷中间体积聚的大 GTP 酶 (Macia 等，2006)。因为 Dyngo-4a 强有力地将 SYNJ2 的动态组件阻止在质膜处（图 5D)，所以本发明人推断 SYNJ2 募集到受发动蛋白调节的初生运输中间体上。因为已经暗示发动蛋白为细胞迁移和侵袭的促进因子 (Kruchten 和 McNiven，2006)，所以测试了其与 SYNJ2 的物理相互作用。该实验确认活性发动蛋白和 SYNJ2 之间的复合物形成（图 5E)，对于发动蛋白而言与在胞吞作用和基于肌动蛋白的迁移中的扩展作用一致。

[0299] SYNJ2 可与载 GTP 的 Rac1 物理性相互作用 (Malecz 等，2000)，并且诱导性激活 Rac1 需要内化和后续再循环 (Palamidessi 等，2008)。因此，测试了 SYNJ2 外周斑点与 Rac1 的重合。实际上，对内源 Rac1 的免疫染色揭示了与 GFP-SYNJ2 外周斑点的共同定位（图 5F)。而且，抑制向 Rac1 的 GTP 负载（使用 NSC-23766) 明显减少了 GFP-SYNJ2 斑点的数量（图 5G)。互补地，SYNJ2 敲减降低了 MDA-MB-231 细胞中载 GTP 的 Rac1 水平（图 5H)。根据在将 SYNJ2 募集到膜上时，对 Rac1 和肌动蛋白细胞骨架的调节作用，用拉春库林抑制肌动蛋白动力学特性消除了 GFP-SYNJ2 动力学特性（图 6D)。总之，这些结果将外周 SYNJ2 组件与取决于胆固醇、3’- 磷酸肌醇、肌动蛋白和活性 Rac1 的发动蛋白介导的胞吞途径联系起来。显著地，该途径与使得在迁移性成纤维细胞前缘处，快速膜和粘着物周转成为可能的网格蛋白非依赖性载体共有几种属性 (Howes 等，2010)。

[0300] 实施例 5

[0301] SYNJ2 控制细胞表面受体的囊泡运输

[0302] 虽然经 EGF 处理的 shSYNJ2-MCF10A 细胞相对于对照细胞显示出较高的总 EGFR 和磷酸化 EGFR 水平，这转化成 ERK 低而非高度激活（图 7A)。沿着这样的思路，注意到 SYNJ2

敲减将 EGFR 捕集于扩大的细胞内囊泡中（图 7B)。与捕集一致，MDA-MB-231 细胞的免疫印迹类似地揭示，在 siSYNJ2 细胞中 EGFR 水平稳定（图 8A)，但是使用两种方法对表面 EGFR 的量化显示出明显更低的表面水平（图 8B)。EGFR 的细胞内捕集带来功能结果：与其良好表征的趋化功能一致 (Mouneimne 等，2006；van Rheenen 等，2007)，EGFR 定位到乳腺细胞前缘，但是 shSYNJ2 细胞的 EGFR 失去其极化分布并积聚在大的肌动蛋白装饰囊泡内（图 8C)。显著地，可通过 WT SYNJ2，但不是催化死亡形式挽救在 shSYNJ2 细胞中观察到的 EGFR 运输缺陷（图 7C)，表明 SYNJ2 的磷酸酶活性对 EGFR 向和从前缘的囊泡运输至关重要，在前缘其介导对 EGF 梯度的趋化反应。与这种模型一致，shSYNJ2 细胞严重丧失沿 EGF 梯度迁移的能力（图 8D)。

[0303] EGFR 在 SYNJ2 缺乏型细胞内的异常积聚可以反映 EGFR 递送、受阻再循环或降解分选受损、受泛素化调节的过程上的缺陷 (Goh 等，2010)。与受损分选一致，SYNJ2 缺乏型细胞表现出明显更高的基本 EGFR 泛素化，这响应于 EGF 仅微弱改变（图 8E 和 7D)。此外，尽管通过磷酸化酪氨酸 1045（泛素连接酶 c-Cbl 的停泊位点；图 8F) 标记进行降解，但是 EGF 刺激实验确认在 shSYNJ2 细胞中正常激活（酪氨酸 1068 磷酸化)，但降解有缺陷（图 8G)。为解决再循环缺陷，采用荧光配体跟踪转铁蛋白受体 (TfR) 的广泛再循环，以及 EGFR 较弱的再循环。虽然 TfR 内化不受影响，但是在 shSYNJ2 细胞中再循环明显降低，并且相反，在 SYNJ2-OX 细胞中明显加速（图 8H 和 7E)。同样，流式细胞术分析表明荧光 EGF 的再循环有缺陷（图 8I)，并且干细胞成像确认了在 SYNJ2 缺乏型细胞的大囊泡内配体积聚。总之，这些结果表明 SYNJ2 对于 EGFR 和 TfR 的适当再循环必不可少。

[0304] 实施例 6

[0305] SYNJ2 敲减扰乱磷酸肌醇脂质的体内平衡并且改变胞吞作用和粘着

[0306] 胞吞系统保持了几个由特定磷酸肌醇 (PI) 限定的不同隔室 (Gruenberg 和 Stenmark，2004)，并且本分析揭示了对 SYNJ2 的强依赖性。例如，通过探测 EEA1 的初级内体，一种 PI(3)P- 结合剂，发现其空间结构在 SYNJ2 缺乏型细胞中明显改变（图 9A)。类似地，使用 GFP 标记的 Rab4 探测再循环隔室，揭示了与 shSYNJ2 细胞的环状肌动蛋白补丁的强烈关联（图 10A)。初级内体另一种标志物 Rab5 的分布也反映了对 SYNJ2 的依赖性（图 10B)。然而在 shSYNJ2 缺乏型细胞中 Rab5 阳性囊泡的数量明显更低，其平均尺寸增大并且其部分定位到环状肌动蛋白补丁处（图 9A)。为揭示磷酸肌醇的根本改变，比较了经生物合成标记的 shCtrl 和 shSYNJ2 MDA-MB-231 细胞，之后提取其磷酸肌醇（图 10C)。结果显示，主要是 PI(3)P，但还有 PI(4,5)P_2和 PI(3,5)P_2在 shSYNJ2 细胞中以较高水平存在，而 PI(4)P 水平保持不变并且通过这种方法很难检测 PI(3,4)P_2和 PI(3,4,5)P_3的水平。虽然这些结果确认了 SYNJ2 主要靶向 PI 的 D5 位置的想法，但是本发明人假定观察到的相当有限的效应表示较大的局部差异。总之，这些观察资料再次确认 SYNJ2 控制在初级内体以及后续再循环步骤的货物分选。

[0307] 随着 RTK 如 EGFR 再循环，整联蛋白的囊泡运输及其与下游伴侣例如桩蛋白的相互作用在细胞迁移和粘着斑 (FA) 突变中起重要作用 (Guo 和 Giancotti，2004)。相应地，β-1 整联蛋白和磷酸化 -EGFR(pEGFR) 定位到 MDA-MB-231 细胞的 FA 处。相反，由于在大囊泡内异常积聚，两种蛋白都难以定位到 SYNJ2 缺乏型细胞的外周（图 10D、S5B 和 S5C)。而且，使用桩蛋白作为成熟 FA 的标志，发现 FA 在 shSYNJ2 细胞中呈现圆形且相对较短的外观（图

9D)。总的来说,这些观察资料暗示底物粘着需要SYNJ2,这是通过使用两种方法测量细胞扩散审查的情况(图10E和10F)。结果证明shSYNJ2细胞的粘着力减弱,这归因于整联蛋白和RTK向FA的递送有缺陷。

[0308] 实施例7

[0309] SYNJ2调节侵袭伪足的装配

[0310] MDA-MB-231细胞基于基质的3D培养一般显示出楔形突出,但是shSYNJ2细胞显示出圆形伸出部分(图11A),表明基质降解有缺陷。为测试这一点,获得MMP-9的共聚焦免疫荧光图像,并且注意到shSYNJ2球体显示MMP-9丰度在其边界处相对急剧降低(图11A),这很可能是由于分泌削弱。实际上,对条件培养基进行的明胶酶谱测定确认经siSYNJ2寡核苷酸处理的细胞的MMP-9分泌有缺陷,但是MMP-2分泌保持不变(图12A)。相反,以过表达SYNJ2的细胞为条件的培养基显示MMP-9活性大幅提高(图11B),与MMP分泌中涉及SYNJ2一致。

[0311] 为可视化焦点蛋白水解,将细胞接种在交联荧光明胶上并探测称为侵袭伪足的以肌动蛋白为中心的基质降解细胞器(Murphy和Courtneidge,2011)。根据先前报道,活性基质蛋白水解与定位于细胞体下面的肌动蛋白点相对应。重要的是,SYNJ2-GFP斑点与这些结构共同定位(图11C,箭头),这类似于图5B中呈现的肌动蛋白关联的长寿命斑点。SYNJ2的表达水平与侵袭伪足出现明显相关;而SYNJ2过表达使含侵袭伪足的细胞几乎翻倍,siSYNJ2显著降低了侵袭伪足的发生率(图11D),暗示了因果关系。接下来,检查了SYNJ2和侵袭伪足良好表征的标志皮动蛋白之间的潜在物理性关联并且发现SYNJ2和皮动蛋白共免疫沉淀(图12B),以及共同定位到侵袭伪足和前缘(图12C)。为牢固确定对SYNJ2的驱动作用,观察TKS5,PI(3,4)P_2和用作侵袭伪足标志物的皮动蛋白结合剂(Courtneidge等,2005)。不出所料,内源TKS5定位到对照MDA-MB-231细胞内基质降解的多个腹侧位点,但是在siSYNJ2细胞内几乎未发现活性位点,并且TKS5失去其腹侧位置(图6E;X-Y和Z组)。此外,因为侵袭伪足TKS5锚定在PI(3,4)P_2上(Oikawa等,2008),所以将PI(3,4)P_2结合结构域,即Tapp1的PH结构域用作探针。与先前报道一致,PH结构域的异位表达减少了侵袭伪足的数量,然而其余信号与TKS5和肌动蛋白核共同定位(图12D)。总之,SYNJ2似乎在先于TKS5接合的步骤不可缺少,与分别生成PI(3,4,5)P_3,再生成PI(3,4)P_2,以将TKS5锚定在PI3K的EGFR诱导活化位点的PI3K(Yamaguchi等2011)和SYNJ2顺次作用一致。

[0312] 与EGFR-PI3K-SYNJ2情况一致,在具蛋白水解活性的侵袭伪足中检测到EGFR(pEGFR)活性形式,但是SYNJ2缺乏型细胞的EGFR定位到肿胀囊泡(图11F)。造成局部受体活化的机制仍未知。根据一种模型,通过包含MMP-7和CD44的复合物裂解前配体例如肝素结合EGF(HB-EGF),可能局部刺激EGFR(Yu等,2002)。与这种模型一致,SYNJ2丰度与EGFR配体的分泌相关(图11G),并且检测到CD44与侵袭伪足的肌动蛋白核共同定位(图12E)。同样,使用流式细胞术,发现相对于对照细胞,在shSYNJ2细胞中CD44的表面表达受强烈抑制(图12F)。侵袭伪足突变的另一关键步骤是激活可溶性MMP的膜1型基质金属蛋白酶(MT1-MMP)的募集(Wang和McNiven,2012)。相应地,发现在对照细胞中MT1-MMP与侵袭伪足突出位点相对应,但是siSYNJ2细胞的MT1-MMP分子形成与基质降解无关的大聚集物(图9E)。总的来说,这些观察资料暗示SYNJ2对侵袭伪足引发以及使两种蛋白酶和

先前未识别的两种驻留物 CD44 和活性 EGFR 靶向这种细胞器必不可少。

[0313] 实施例 8

[0314] 在哺乳动物模型中 SYNJ2 促进肿瘤生长和转移扩散

[0315] 为评估 SYNJ2 对体内转移性传播的影响，将 MDA-MB-231-RFP 细胞（和衍生细胞）植入雌性小鼠的乳腺脂肪垫内，并且在 2 或 6 周后测量肿瘤大小（图 13A）和转移（图 13B）。相对于 shSYNJ2 和‘非活性救援’(shSYNJ2+SYNJ2^{CD}) 组，在 shCtrl 和 shSYNJ2+SYNJ2^{WT}（‘活性救援’）组中原发性肿瘤生长明显更快。转移性行为类似地与 SYNJ2 相关 ：shSYNJ2 和‘非活性救援’组显示向局部和远侧淋巴结的转移扩散明显减少（图 13B 和 14）。为了检查远侧转移，处死小鼠并评价其肺部。与接种了 shCtrl 或‘活性救援’细胞的动物相比，植入了 shSYNJ2 细胞或‘非活性救援’细胞的动物的肺部显示在转移数量和大小上显著减小（图 13C）。总之，这些结果使 SYNJ2 涉及转移促进。

[0316] 类似地，监测过表达 SYNJ2 的异种移植物。不出所料，SYNJ2-OX 细胞产生增长更快的肿瘤（图 13D），并且还显示淋巴结转移更早开始（图 13E）。与稳健的淋巴侵袭一致，植入了 SYNJ2-OX 细胞的动物的肺部显示转移数量增加（图 13F）。接下来，检验 SYNJ2 对内渗或外渗的影响。因此，，向雌性小鼠的循环（尾静脉）直接注射 MDA-MB-231-RFP 细胞的亚克隆并对肺部定植（外渗）评分，或将其植入脂肪垫并且在血液中作为循环肿瘤细胞 (CTC ；内渗）评分。注意这些实验考虑到了各原发性肿瘤之间的大小差异。归一化结果表明 SYNJ2 对于内渗 ($p = 0.0031$) 和外渗 ($p = 0.0082$ ；图 13G) 而言不可缺少。使用 GFP-SYNJ2 过表达细胞进一步检验了这个结论（图 13H）。显著地，在该实验中获得的内渗结果显示出统计显著性，但是 SYNJ2-OX 细胞更好地外渗和定植远侧器官的能力未达到显著性，表明观察到的 SYNJ2 对局部和远侧转移的强烈影响主要是由于向淋巴和血管的内渗增强。

[0317] 实施例 9

[0318] SYNJ2 与侵蚀性人乳腺肿瘤相关

[0319] 为解决 SYNJ2 与人类癌症的相关性，在 60 个人类癌细胞系的 NCI-60 组中分析了 SYNJ2 的转录水平。与对运动表现型的贡献一致，发现 SYNJ2 的高转录产物水平与间充质表现型相关。接下来，为一组 331 个乳腺癌 NJ2 石蜡包埋样品免疫染色（图 16A）。重要的是，SYNJ2 的表达强度与由 HER2 过表达 ($p<0.001$) 和 / 或缺乏雌激素受体 ($p<0.001$) 定义的预后不利分型呈正相关。然而，在 SYNJ2 丰度与年龄、组织学分型、腋淋巴结状态和分化程度之间未发现显著关联。有趣的是，SYNJ2 的染色模式也改变 ；而 HER2+ 肿瘤显示主要为膜染色，管腔和三阴性肿瘤显示胞浆染色（图 16B）。为支持所述发现，在两个乳腺癌样本组群中分析 SYNJ2 mRNA 水平并且以更短的患者存活率发现关联（图 16C）。总而言之，这些观察资料支持在乳腺癌进展中涉及 SYNJ2，但使得在转录产物上调之后的机制悬而未决。

[0320] 总之，在动物中所做的观察，连同临床数据和体外实验一起，清楚地表明肌醇脂质受 SYNJ2 的脱磷酸作用对于转移过程至关重要，主要是由于磷酸肌醇在细胞表面分子向和从侵袭伪足和前缘的运输中所起到的主要作用。下面介绍了一种工作模型（图 15）并且讨论了 SYNJ2 在广泛的肿瘤进展情况下的多种功能。

[0321] 实施例 10

[0322] SYNJ2 的 5’磷酸酶活性的选择性抑制剂

[0323] 为了鉴定 SYNJ2 磷酸酶活性的选择性抑制剂，本发明人利用荧光偏振竞争测定

法，其依靠分子在空间上不断旋转和移动，但是一旦与另一较大元件（例如，蛋白质）结合，其运动就受到极大限制的原理。可使用呈未结合状态，产生极低偏振读数的荧光分子（即，探针）检测和测量运动上的这些变化，但是向溶液中添加结合这些分子的检测剂（例如，结合蛋白）时，荧光分子在增大溶液中的偏振读数的受限组合物中稳定（见图 17A）。

[0324] 在进行的筛选中，本发明人测量了在不同化合物的存在下，SYNJ2 为 PI(3,4,5,)P3 的 5’位置脱磷酸以生成 PI(3,4)P2 的酶活性。一旦酶促反应完成 / 停止，就将含有 PI(3,4)P2 产物的溶液与 PI(3,4)P2 结合蛋白（检测剂）和荧光 PI(3,4)P2（探针）的混合物混合。所用的检测剂蛋白为选择性结合 PI(3,4)P2(SEQ ID NO.:15) 的 Tapp1 纯化 PH 结构域。正如图 17B 所展示，该测定法中测得的偏振值在由通过 SYNJ2 酶活性生成的未标记 PI(3,4)P2 置换结合的荧光 PI(3,4)P2 荧光探针时减小并且溶液中未结合的荧光探针的量增加。

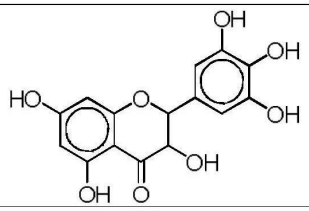
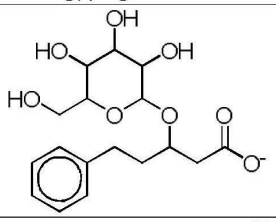
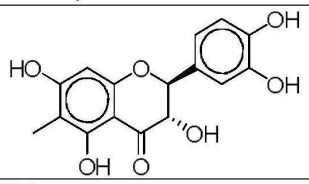
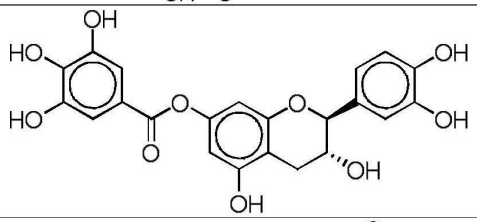
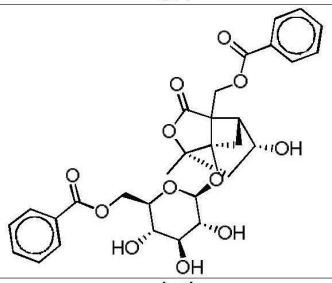
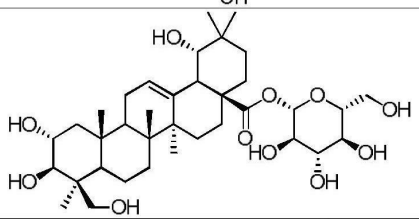
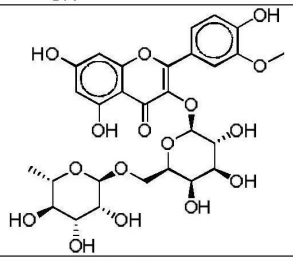
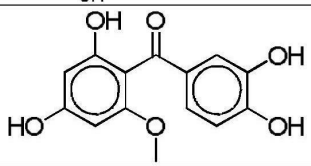
[0325] 下面表 2 描绘了使用这种方法鉴定的能够抑制 SYNJ2 生成 PI(3,4)P2 的各种化合物。

[0326] 表 2 :鉴定的 SYNJ2 选择性抑制剂

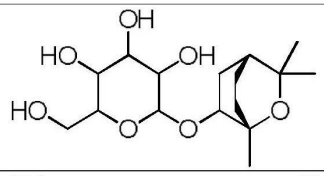
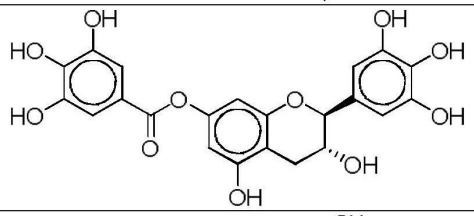
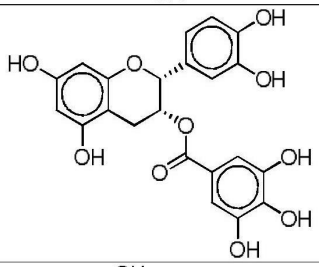
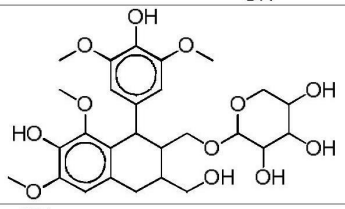
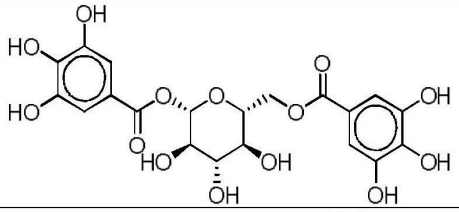
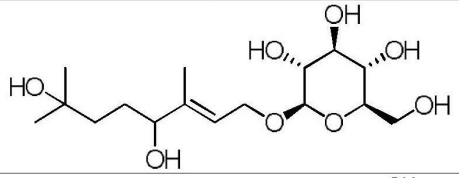
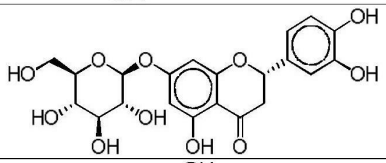
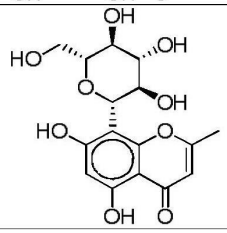
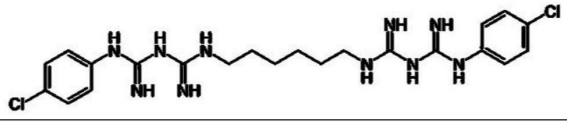
[0327]

编号	AnalytiCon 产品目录号 [化学文摘登	化合物名称	IC50 (μM)	结构

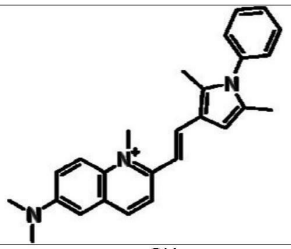
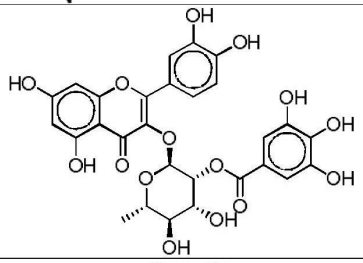
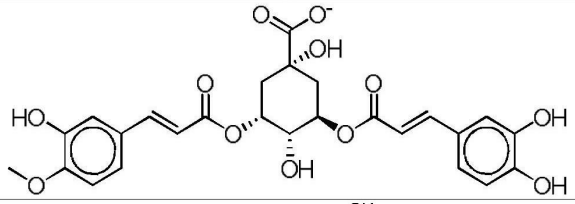
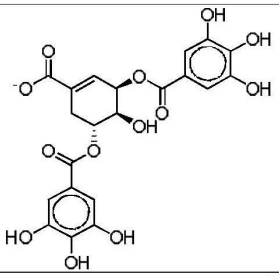
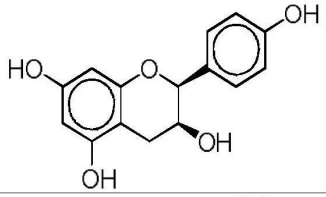
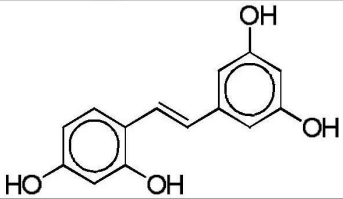
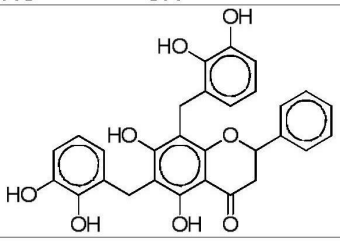
[0328]

	记号]			
1	NP-001872	白蔹素 (Ampelopsin)	3.17	
2	NP-003195	3-羟基-5-苯基戊酸 3-D-吡喃葡萄糖苷	1.87	
3	NP-012228	6-甲基二氢槲皮素 (Cedeodarin)	2.19	
4	NP-014109	(-)-八茶素-7-没食子酸酯	3.44	
5	NP-015304	Paeonivayin	6.02	
6	NP-003491	绢毛榄仁苷 (Sericoside)	3.52	
7	NP-000303	铃兰黄酮苷 (Keioside)	3.4	
8	NP-005201	2,4,3',4'-四羟基-6-甲氧基-二苯甲酮	2.84	

[0329]

9	NP-001585	2-羟基-1,8-桉树脑-吡喃葡萄糖苷	1.98	
10	NP-014110	(-)-没食子儿茶素-7-没食子酸酯	1.075	
11	NP-002326	(-)-表儿茶素-3-没食子酸酯	2.88	
12	NP-000360		1.67	
13	NP-002973	1,6-二没食子酰葡萄糖	7.53	
14	NP-015222	3,7-二甲基-2-辛烯-1,4,7-三元醇 1-β-D-吡喃葡萄糖苷	1.24	
15	NP-000181	Pyracanthoside	0.874	
16	NP-008708	异双花母草素 (Isobiflorin)	2.71	
17	[CAS: 55-56-1]	洗必泰 (Chlorhexidine)	10	

[0330]

18	[CAS: 3546-41-6]	恩波维铵 (Pyrvinium pamoate)	1.25	
19	NP-003299	没食子酰基槲皮素苷	2.94	
20	NP-013254	4'-O-甲基-3,5-二-O-咖啡酰奎宁酸	3.17	
21	NP-012429	3,5-二-O-没食子酸莽草酸	3.75	
22	NP-002325	表阿夫儿茶精 (Epiafzelechin)	15.9	
23	NP-003143	氧化白藜芦醇 (Oxyresveratrol)	6.65	
24	NP-012649	6,8-二-(2,3-二羟基苄基)- 生松素	10.3	

[0331] 讨论

[0332] SYNJ2 作为细胞迁移和肿瘤转移的主要综合调节因子的功能得以证明,很可能是由于其能够控制起第二信使和决定特定膜子结构域的同一性的标志物作用的 PI 磷脂的水平。对 SYNJ2 作用多样性的另一反映是向侵袭伪足和板状伪足主要为双峰腹侧定位。相应地,SYNJ2 与肌动蛋白动力学特性的主要调节因子(例如,发动蛋白、皮动蛋白和 Rac1)形成

物理复合物。了解 SYNJ2 作用的一个关键是控制胞吞运输的能力。质膜不断供给基于肌动蛋白的突出的部分在囊泡内的频繁包装推进细胞迁移 (Ridley,2011)。板状伪足的 SYNJ2 表现出显著活力 (图 3B),并且活细胞成像暗示 SYNJ2 募集标记了新板状伪足形成的位点。因为位于前缘的 SYNJ2 分子依赖发动蛋白、Rac1、肌动蛋白聚合和胆固醇,但是其分布与小窝蛋白 -1 和网格蛋白不同,所以本发明人假定其表示维持在前缘的膜翻转的网格蛋白非依赖性载体 (CLIC) 的发动蛋白依赖性变体 (Howes 等,2010)。

[0333] 一系列一流研究使 SYNJ1 牵连于神经元中的突触囊泡再循环 (Cremona 等,1999)。在小鼠中,SYNJ1 缺失引起稳态 PI(4,5)P_2上升、网格蛋白包被囊泡积聚和胞吞后囊泡可再用性延迟 (Mani 等,2007)。这些观察资料表明,将使囊泡外壳脱落的 PI(4,5)P_2脱磷酸化是所述表现型的原因。与此类似,本 SYNJ2 缺乏型乳腺细胞显示活性 EGFR 细胞内积聚。受体泛素化状态和胞吞途径标志表明运输在分选内体时受阻,其中内化受体通常分流进行再循环或降解。令人信服地,所述缺陷是由于不能分解与囊泡外壳或与其肌动蛋白彗尾缔合的 PI(4,5)P_2- 结合蛋白 (Kaksonen 等,2003)。因此,与在 SYNJ1 切除后观察到的突触传导缺陷类似,由于对运动性必不可少的表面分子的运输受阻,SYNJ2 丢失严重损害了细胞迁移和侵袭。

[0334] 在 SYNJ2 缺乏型细胞内分别观察到的初级和次级内体的调节因子,PI(3)P 和 PI(3,5)P_2升高,提出了另外的运输机制。已经通过鉴定多种结合剂,例如整联蛋白和几种 Rab 蛋白,加强了对 PI(3,5)P_2的调节作用 (Catimel 等,2008)。PI(3)P 受在内体和反式高尔基体网络之间循环,将 MT1-MMP 递送到侵袭伪足的途径所涉及的一种 5- 激酶 PIKfyve 磷酸化 (Poincloux 等,2009)。因此,除 PI(4,5)P_2的脱磷酸化外,SYNJ2 很可能加工 PI(3,5)P_2以微调初级内体的 PI(3)P 池并且协调 MT1-MMP 的胞吐作用和整联蛋白以及 EGFR 的再循环。

[0335] 引入动物体内时,shSYNJ2 MDA-MB-231 细胞由于到达淋巴结和血管的能力降低而严重丧失转移潜能 (图 13A-H)。为了将这些结果和体外表现型相结合,图 15 中列出了为 SYNJ2 在细胞运动性中的作用基础的机制。相应地,关键事件需要 EGF 诱导的 SYNJ2 上调及由此引起的 3 种磷酸肌醇损耗 :PI(4,5)P_2、PI(3,4,5)P_3和 PI(3,5)P_2。SYNJ2 介导的 PI(4,5)P_2脱磷酸与 PI(4,5)P_2受磷脂酶 C-γ 降解和受 PI3K 磷酸化并行,这样生成 PI(3,4,5)P_3。总的来说,3 种酶受 EGF 的刺激使一组 PI(4,5)P_2结合剂从质膜离解,并且还生成无 PI(4,5)P_2的内吞囊泡。同时,SYNJ2 将 PI(3,4,5)P_3转化成侵袭伪足形成所必需的 PI(3,4)P_2。与这种模型一致,已经报道侵袭伪足形成需要 PI3K。一旦到位,PI(3,4)P_2就结合 TKS5 并且使以发动蛋白和皮动蛋白为中心的复合物成核,该复合物使得丝切蛋白能够在侵袭伪足内产生肌动蛋白钩端。根据本结果,SYNJ2 也涉及下一侵袭伪足突变步骤,即 MMP 分泌及 MT1-MMP 和其它表面分子例如 CD44 的递送。以类似方式,SYNJ2 控制 EGFR 和整联蛋白向前缘的递送,并且很可能激活丝切蛋白,这是指示板状伪足突出形成的关键事件。

[0336] 与动物中 SYNJ2 对体外细胞迁移和转移的贡献一致,目前对乳腺癌样本的调查在疾病的侵蚀性亚型中观察到 SYNJ2 mRNA 和蛋白质水平明显上调。另外,使用来自两个组群的数据,观察到高 SYNJ2 mRNA 表达和乳腺癌患者存活期更短之间的关联。

[0337] 总之,本研究将转移引发的基本事件归因于 EGF 诱导的 PI3K 局部激活和 SYNJ2 总体上调,SYNJ2 对 PI(4,5)P_2的顺次作用调节前缘处的肌动蛋白动力学特性,以及生成侵袭

伪足的标志物PI(3,4)P_2。此外，本研究鉴定了选择性抑制SYNJ2生成PI(3,4)P2的各种化合物。

[0338] 虽然已经连同其特定实施方案描述了本发明，但是显然许多替代方案、修改和变化将对本领域的技术人员显而易见。相应地，旨在包括属于所附权利要求的精神和广泛范围内的所有此类替代方案、修改和变化。

[0339] 本说明书中提到的所有出版物、专利和专利申请均以引用的方式整体并入本说明书中，如同特别单独地指出将每个单独的出版物、专利或专利申请以引用方式并入本文一样。另外，本说明书中对任何参考文献的补充、引用或鉴定不得解释为承认此参考文献可用作本发明的先前技术。就使用章节标题来说，不得将其解释为必要的限制。

[0340] 参考文献

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尼尔·本切特里特

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Gln Glu  Ala Glu Ala Ala Ile  Arg Cys Leu Leu Glu  Ala Arg Gly
    1175                1180                1185

Gly Ala  Ser Glu Glu Ala Leu  Ser Ala Val Ala Pro  Arg Asp Leu
    1190                1195                1200

Glu Ala  Ser Ser Glu Pro Glu  Pro Thr Pro Gly Ala  Ala Lys Pro
    1205                1210                1215

Glu Thr  Pro Gln Ala Pro Pro  Leu Leu Pro Arg Arg  Pro Pro Pro
    1220                1225                1230

Arg Val  Pro Ala Ile Lys Lys  Pro Thr Leu Arg Arg  Thr Gly Lys
    1235                1240                1245

Pro Leu  Ser Pro Glu Glu Gln  Phe Glu Gln Gln Thr  Val His Phe
    1250                1255                1260

Thr Ile  Gly Pro Pro Glu Thr  Ser Val Glu Ala Pro  Pro Val Val
    1265                1270                1275

Thr Ala  Pro Arg Val Pro Pro  Val Pro Lys Pro Arg  Thr Phe Gln
    1280                1285                1290

Pro Gly  Lys Ala Ala Glu Arg  Pro Ser His Arg Lys  Pro Ala Ser
    1295                1300                1305

Asp Glu  Ala Pro Pro Gly Ala  Gly Ala Ser Val Pro  Pro Pro Leu
    1310                1315                1320

Glu Ala  Pro Pro Leu Val Pro  Lys Val Pro Pro Arg  Arg Lys Lys
    1325                1330                1335

Ser Ala  Pro Ala Ala Phe His  Leu Gln Val Leu Gln  Ser Asn Ser
    1340                1345                1350

Gln Leu  Leu Gln Gly Leu Thr  Tyr Asn Ser Ser Asp  Ser Pro Ser
    1355                1360                1365

Gly His  Pro Pro Ala Ala Gly  Thr Val Phe Pro Gln  Gly Asp Phe
```

[0012]

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    1370                1375                1380

Leu Ser  Thr Ser Ser Ala Thr  Ser Pro Asp Ser Asp  Gly Thr Lys
    1385                1390                1395

Ala Met  Lys Pro Glu Ala Ala  Pro Leu Leu Gly Asp  Tyr Gln Asp
    1400                1405                1410

Pro Phe  Trp Asn Leu Leu His  His Pro Lys Leu Leu  Asn Asn Thr
    1415                1420                1425

Trp Leu  Ser Lys Ser Ser Asp  Pro Leu Asp Ser Gly  Thr Arg Ser
    1430                1435                1440

Pro Lys  Arg Asp Pro Ile Asp  Pro Val Ser Ala Gly  Ala Ser Ala
    1445                1450                1455

Ala Lys  Ala Glu Leu Pro Pro  Asp His Glu His Lys  Thr Leu Gly
    1460                1465                1470

His Trp  Val Thr Ile Ser Asp  Gln Glu Lys Arg Thr  Ala Leu Gln
    1475                1480                1485

Val Phe  Asp Pro Leu Ala Lys  Thr
    1490                1495
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<210> 3
<211> 21
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<213> 人工序列

<220>
<223> 小干扰 RNA 分子序列的例子，靶向编码区域 1612-1633 (SJ2-1)

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<210> 4
<211> 21
<212> DNA
<213> 人工序列

<220>
<223> 小干扰 RNA 分子序列的例子，靶向
3? 非编码区(SJ2-2)

<400> 4
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<210> 5

[0013]

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<212> RNA
<213> 人工序列

<220>
<223> 成功下调 SYNJ2 mRNA 水平的 siRNA 序列的例子

<400> 5
gaagaaacau cccuuugau 19

<210> 6
<211> 19
<212> RNA
<213> 人工序列

<220>
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ggacagcacu gcagguguu 19

<210> 7
<211> 59
<212> DNA
<213> 人工序列

<220>
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<210> 8
<211> 58
<212> DNA
<213> 人工序列

<220>
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<213> 人工序列

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[0014]

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<220>
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1494-1475

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<223> 硫代骨干

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<212> DNA
<213> 人工序列

<220>
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<222> (1)..(19)
<223> 硫代骨干

<400> 11
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<210> 12
<211> 20
<212> DNA
<213> 人工序列

<220>
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<220>
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<210> 13
<211> 738
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[0015]

<220>
<223> 来自智人的重组 TAPP1 PH 区域

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caagatagta cggttatcaa agctggatat tgtgtaaaac aaggagcagt gatgaaaaac 120

tggaagagaa gatattttca attggatgaa aacacaatag gctacttcaa atctgaactg 180

gaaaaggaac ctcttcgcgt aataccactt aaagaggttc ataaagtcca ggaatgtaag 240

caaagcgaca taatgatgag ggacaacctc tttgaaattg taacaacgtc tcgaactttc 300

tatgtgcagg ctgatagccc tgaagagatg cacagttgga ttaaagcagt ctctggcgcc 360

attgtagcac agcggggtcc cggcagatct gcgtcttctg agcatccccc cggtccttca 420

gaatccaaac acgctttccg tcctaccaac gcagccaccg ccacctcaca ttccacagcc 480

tctcgcagca actctttggt ctcaaccttt accatggaga agcgaggatt ttacgagtct 540

cttgccaagg tcaagccagg gaacttcaag gtccagactg tctctccaag agaaccagct 600

tccaaagtga ctgaacaagc tctgttaaga cctcaaagta aaaatggccc tcaggaaaaa 660

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caccaccacc accactga 738

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<211> 245
<212> PRT
<213> 人工序列

<220>
<223> 来自智人的重组 TAPP1 PH 区域

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1 5 10 15

Pro Lys Pro Pro Gln Asp Ser Thr Val Ile Lys Ala Gly Tyr Cys Val
20 25 30

Lys Gln Gly Ala Val Met Lys Asn Trp Lys Arg Arg Tyr Phe Gln Leu
35 40 45

Asp Glu Asn Thr Ile Gly Tyr Phe Lys Ser Glu Leu Glu Lys Glu Pro
50 55 60

Leu Arg Val Ile Pro Leu Lys Glu Val His Lys Val Gln Glu Cys Lys
65 70 75 80

Gln Ser Asp Ile Met Met Arg Asp Asn Leu Phe Glu Ile Val Thr Thr

[0016]

85 90 95

Ser Arg Thr Phe Tyr Val Gln Ala Asp Ser Pro Glu Glu Met His Ser
100 105 110

Trp Ile Lys Ala Val Ser Gly Ala Ile Val Ala Gln Arg Gly Pro Gly
115 120 125

Arg Ser Ala Ser Ser Glu His Pro Pro Gly Pro Ser Glu Ser Lys His
130 135 140

Ala Phe Arg Pro Thr Asn Ala Ala Thr Ala Thr Ser His Ser Thr Ala
145 150 155 160

Ser Arg Ser Asn Ser Leu Val Ser Thr Phe Thr Met Glu Lys Arg Gly
165 170 175

Phe Tyr Glu Ser Leu Ala Lys Val Lys Pro Gly Asn Phe Lys Val Gln
180 185 190

Thr Val Ser Pro Arg Glu Pro Ala Ser Lys Val Thr Glu Gln Ala Leu
195 200 205

Leu Arg Pro Gln Ser Lys Asn Gly Pro Gln Glu Lys Asp Cys Asp Leu
210 215 220

Val Asp Leu Asp Asp Ala Ser Leu Pro Val Ser Asp Val Leu Glu His
225 230 235 240

His His His His His
245

<210> 15
<211> 225
<212> PRT
<213> 智人

<400> 15

Pro Tyr Phe Thr Pro Lys Pro Pro Gln Asp Ser Thr Val Ile Lys Ala
1 5 10 15

Gly Tyr Cys Val Lys Gln Gly Ala Val Met Lys Asn Trp Lys Arg Arg
20 25 30

Tyr Phe Gln Leu Asp Glu Asn Thr Ile Gly Tyr Phe Lys Ser Glu Leu
35 40 45

[0017]

```
Glu Lys Glu Pro Leu Arg Val Ile Pro Leu Lys Glu Val His Lys Val
    50                  55                  60

Gln Glu Cys Lys Gln Ser Asp Ile Met Met Arg Asp Asn Leu Phe Glu
65                  70                  75                  80

Ile Val Thr Thr Ser Arg Thr Phe Tyr Val Gln Ala Asp Ser Pro Glu
                85                  90                  95

Glu Met His Ser Trp Ile Lys Ala Val Ser Gly Ala Ile Val Ala Gln
            100                 105                 110

Arg Gly Pro Gly Arg Ser Ala Ser Ser Glu His Pro Pro Gly Pro Ser
        115                 120                 125

Glu Ser Lys His Ala Phe Arg Pro Thr Asn Ala Ala Thr Ala Thr Ser
    130                 135                 140

His Ser Thr Ala Ser Arg Ser Asn Ser Leu Val Ser Thr Phe Thr Met
145                 150                 155                 160

Glu Lys Arg Gly Phe Tyr Glu Ser Leu Ala Lys Val Lys Pro Gly Asn
                165                 170                 175

Phe Lys Val Gln Thr Val Ser Pro Arg Glu Pro Ala Ser Lys Val Thr
            180                 185                 190

Glu Gln Ala Leu Leu Arg Pro Gln Ser Lys Asn Gly Pro Gln Glu Lys
        195                 200                 205

Asp Cys Asp Leu Val Asp Leu Asp Asp Ala Ser Leu Pro Val Ser Asp
    210                 215                 220

Val
225
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<210> 16
<211> 675
<212> DNA
<213> 智人

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aaacaaggag cagtgatgaa aaactggaag agaagatatt ttcaattgga tgaaaacaca 120

ataggctact tcaaatctga actggaaaag gaacctcttc gcgtaatacc acttaaagag 180

gttcataaag tccaggaatg taagcaaagc gacataatga tgagggacaa cctctttgaa 240

[0018]

attgtaacaa cgtctcgaac tttctatgtg caggctgata gccctgaaga gatgcacagt 300

tggattaaag cagtctctgg cgccattgta gcacagcggg gtcccggcag atctgcgtct 360

tctgagcatc cccccggtcc ttcagaatcc aaacacgctt tccgtcctac caacgcagcc 420

accgccacct cacattccac agcctctcgc agcaactctt tggtctcaac ctttaccatg 480

gagaagcgag gattttacga gtctcttgcc aaggtcaagc cagggaactt caaggtccag 540

actgtctctc caagagaacc agcttccaaa gtgactgaac aagctctgtt aagacctcaa 600

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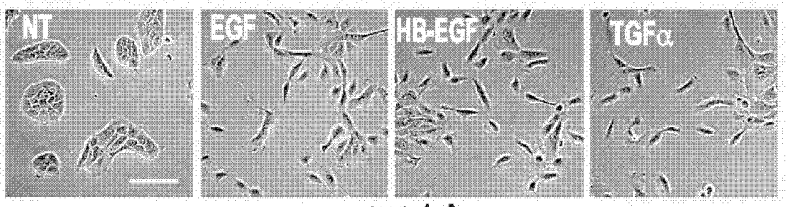
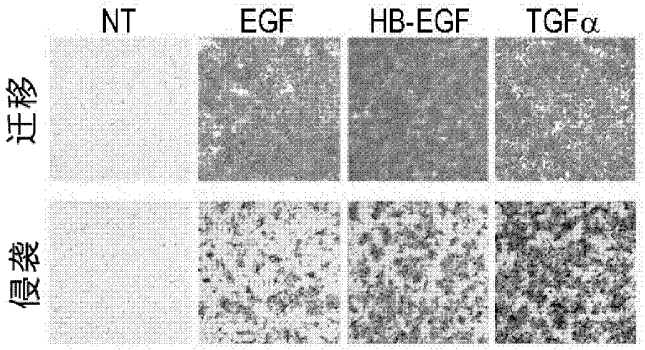
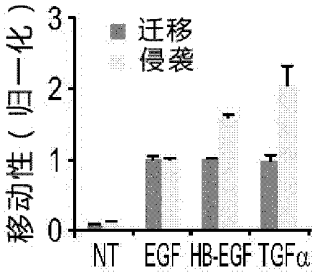


图1A

图1B





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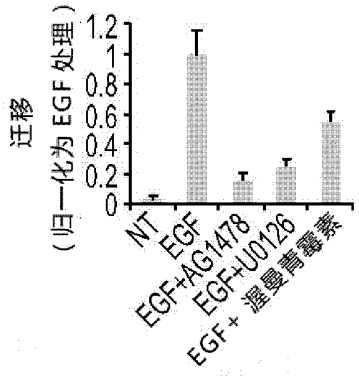


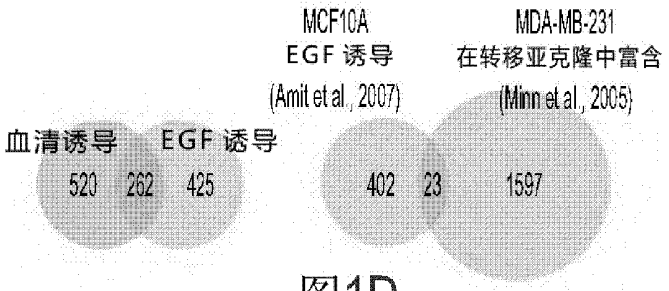


图1D

图1I

图1E

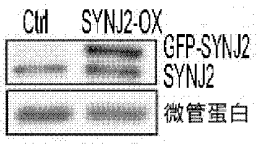


图1F

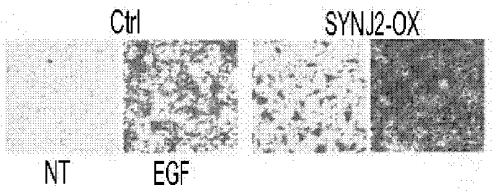


图1G

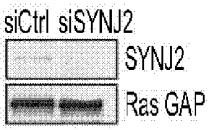
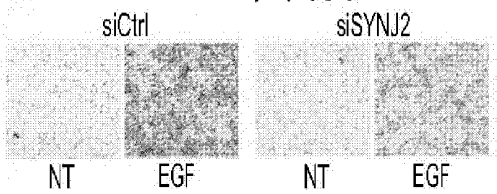
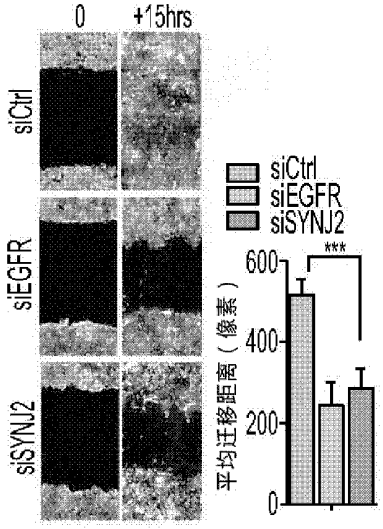


图1H





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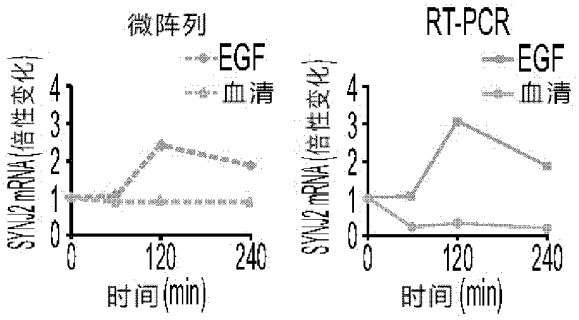


图2B

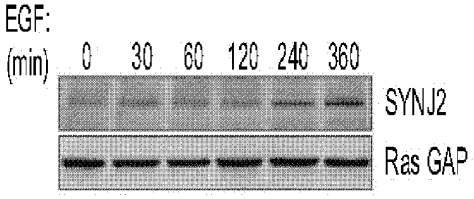
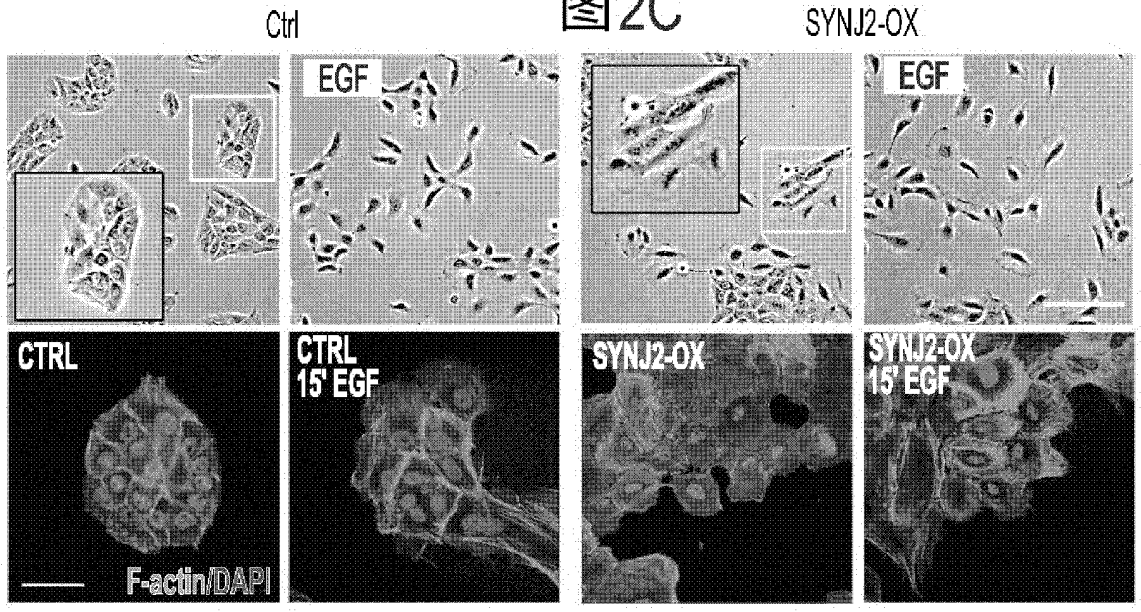


图2C



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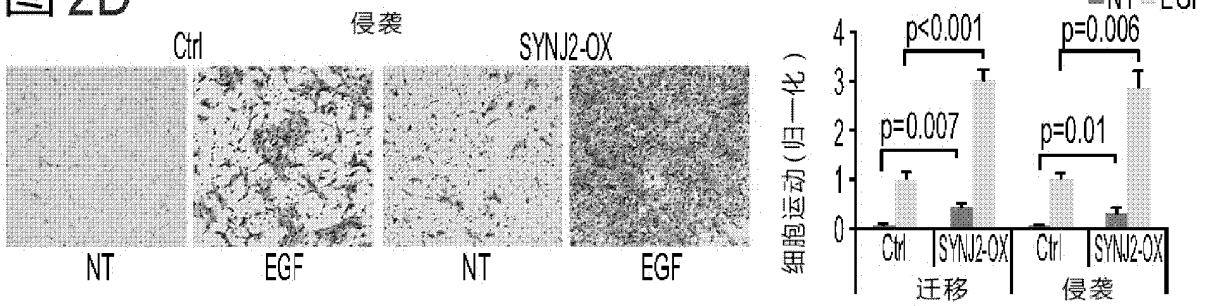

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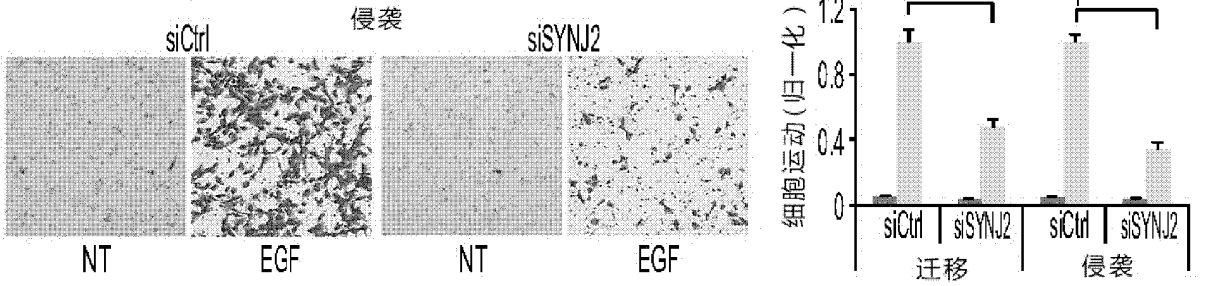

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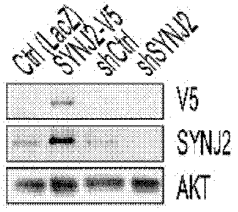

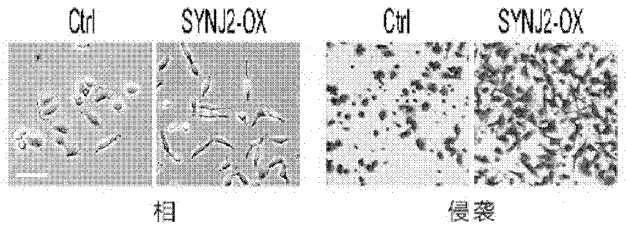

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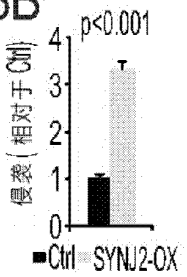

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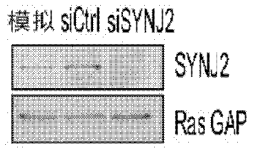

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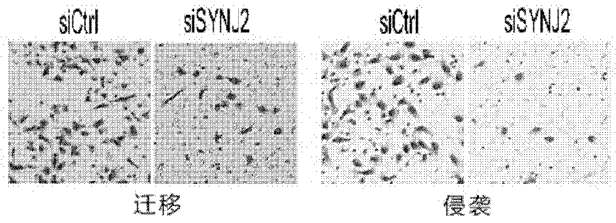

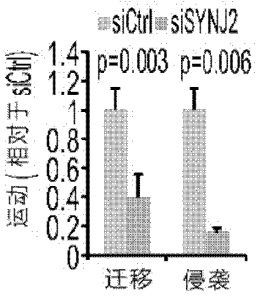

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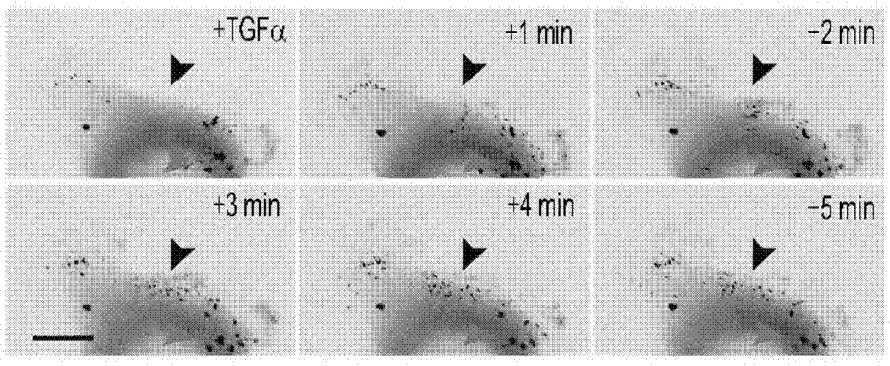


图3F

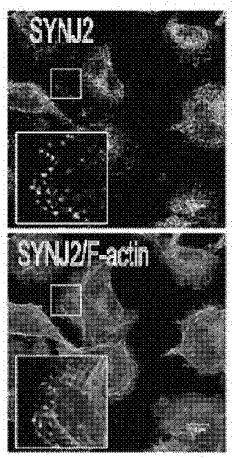


图3G

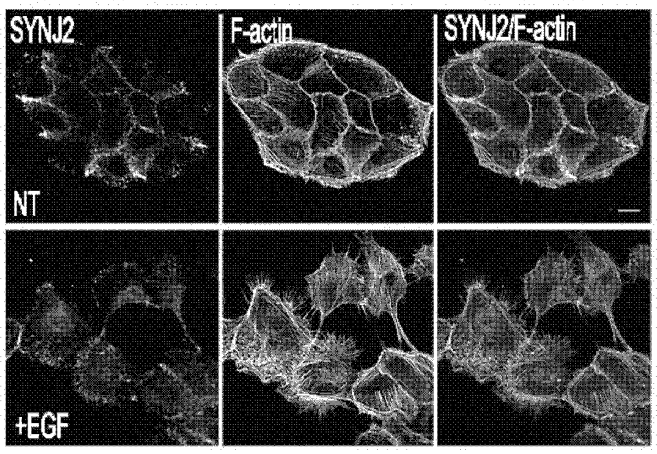
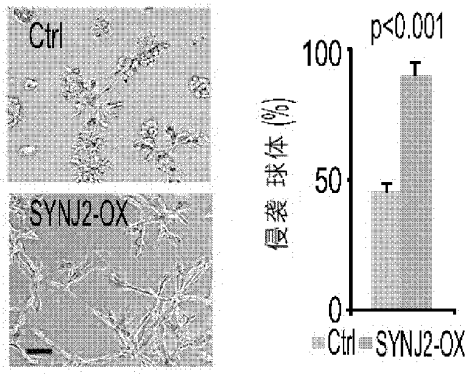


图 4A



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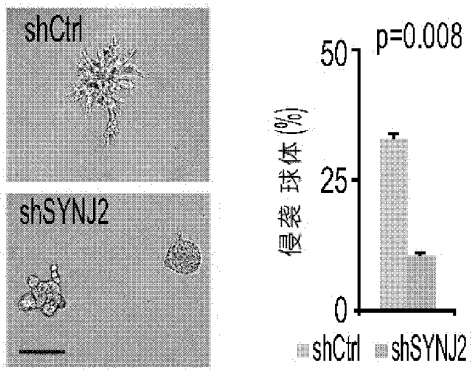


图 4C

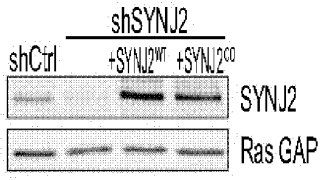
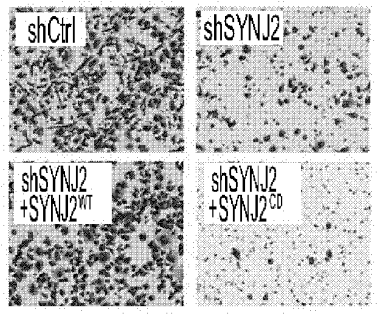


图 4D



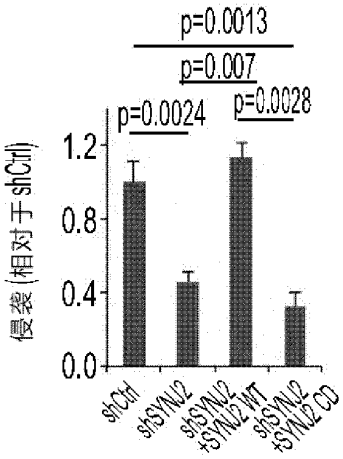
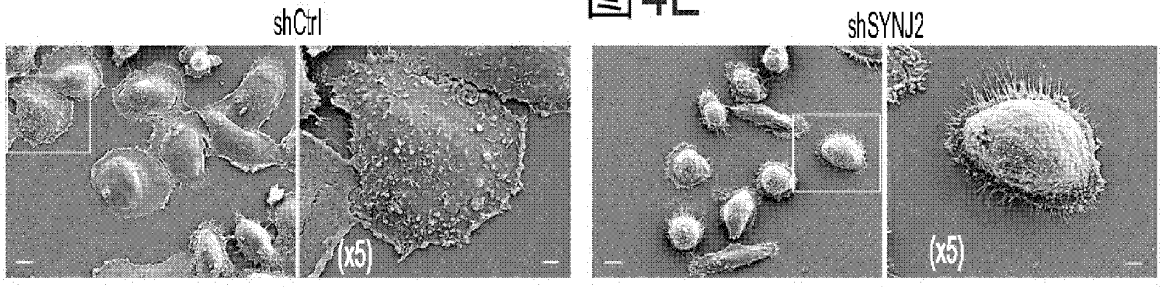


图 4E



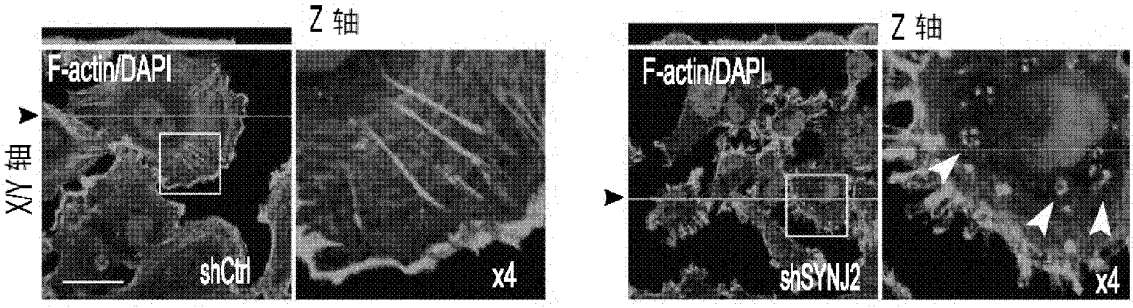


图 4F

图5A

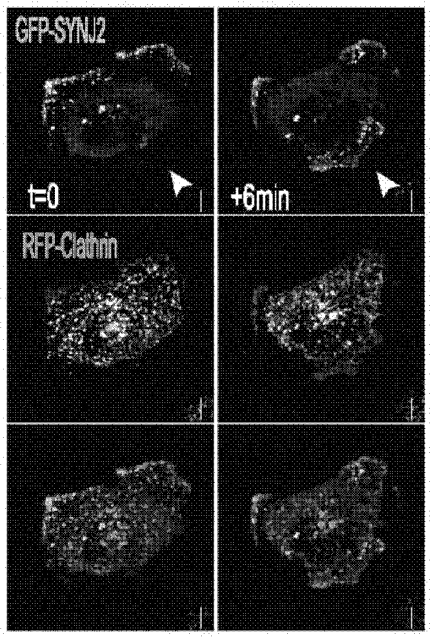
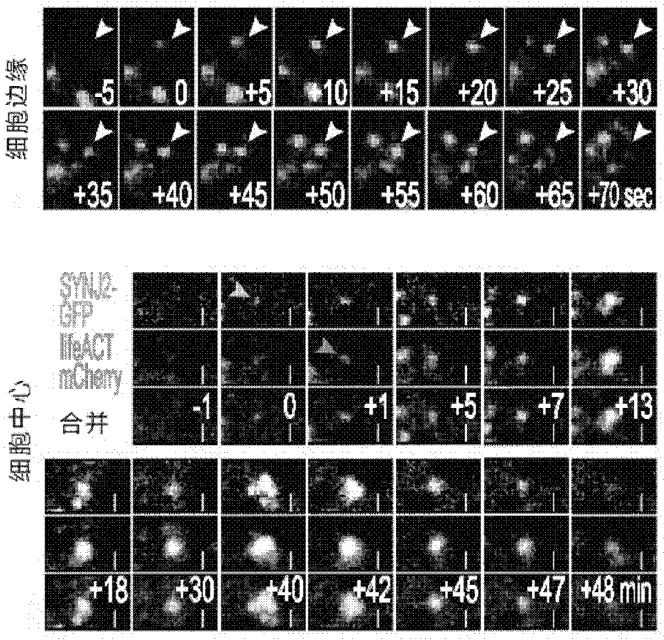


图5B



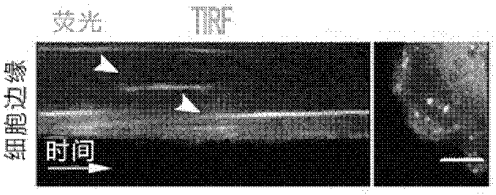


图5C

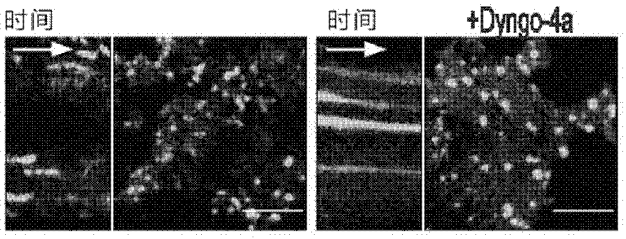


图5D

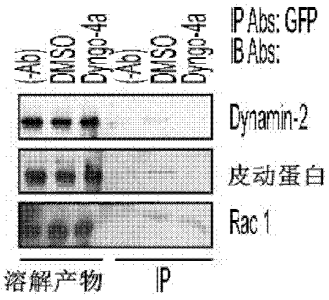


图5E

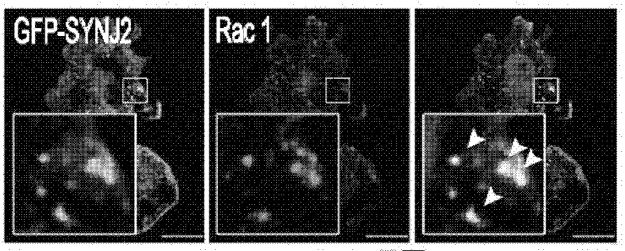


图5F

图5G

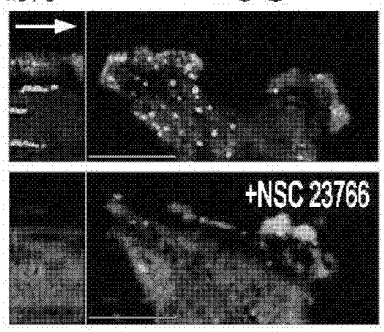


图5H

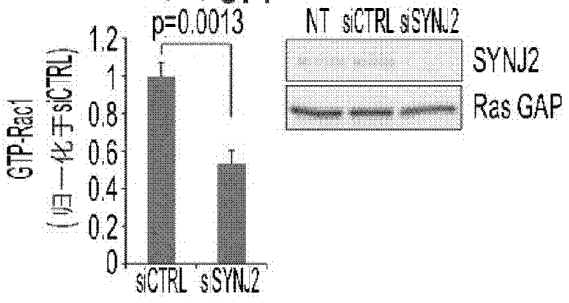
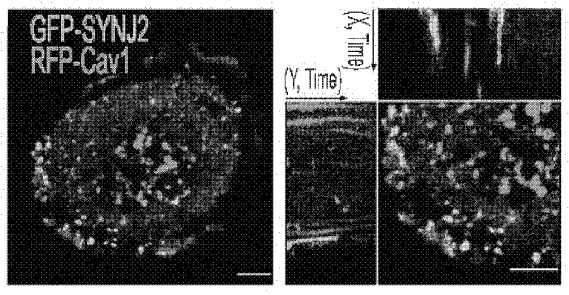
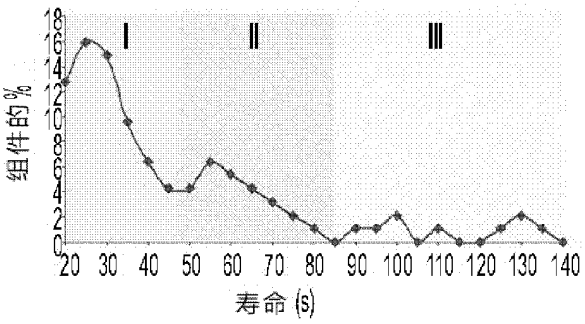


图 6A





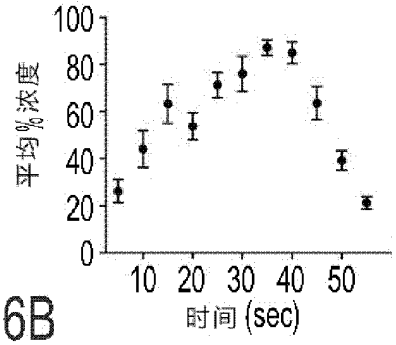
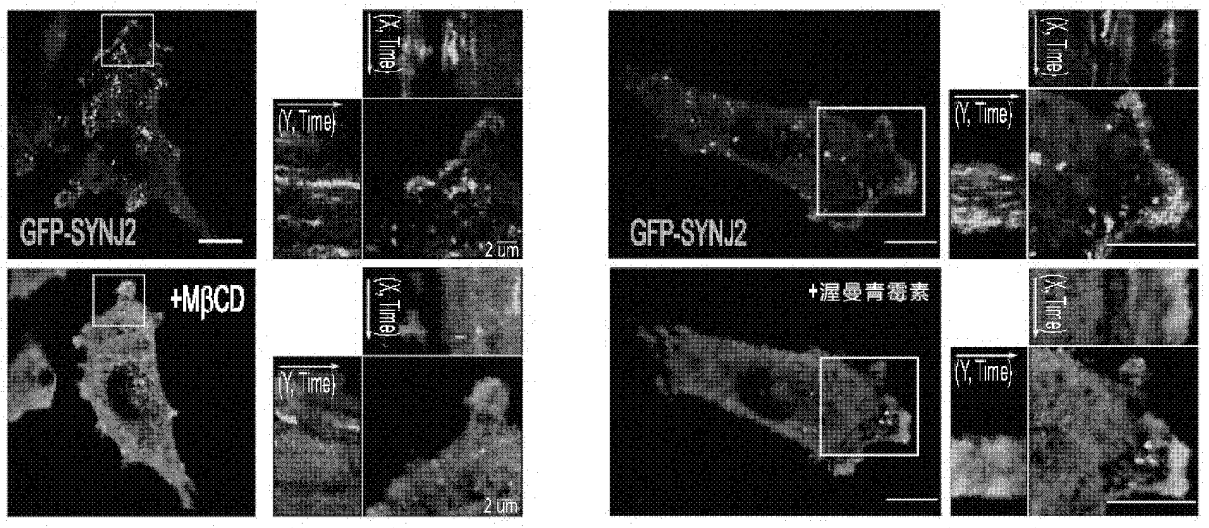


图 6B

图 6C



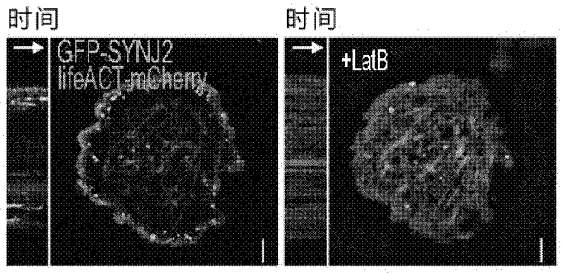


图 6D

图 7A

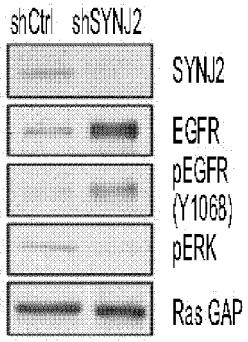


图 7B

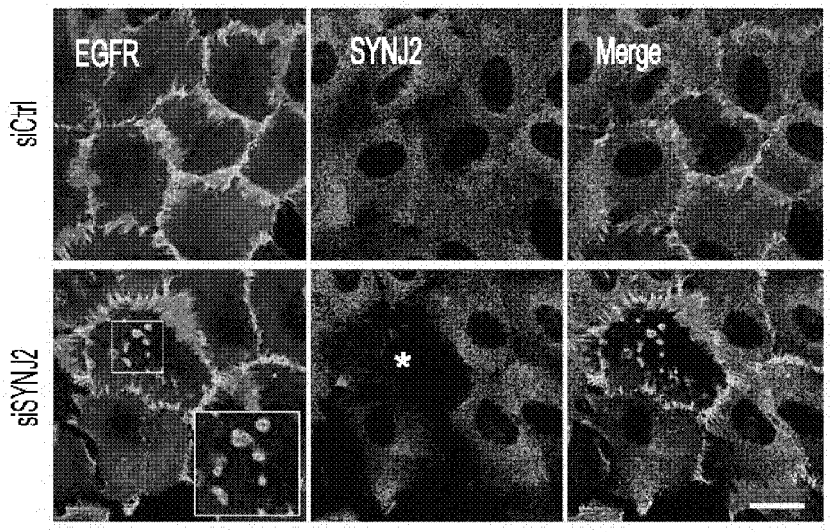


图 7C

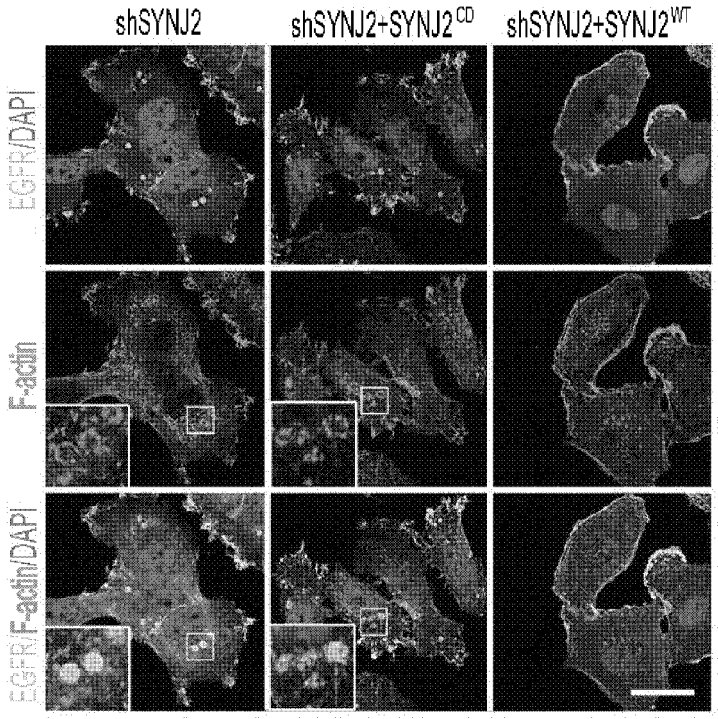
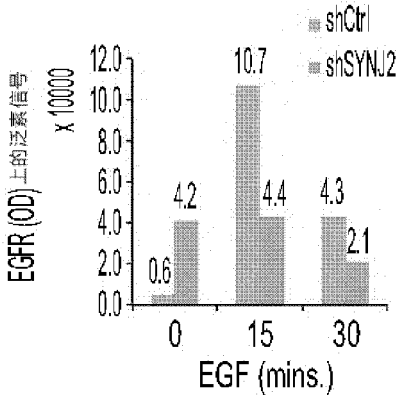


图 7D



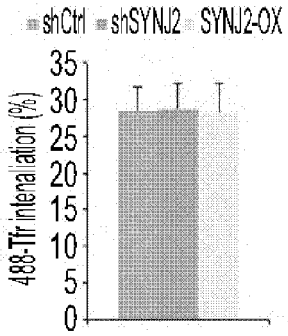
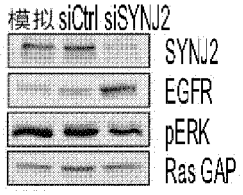


图 7E

图 8A



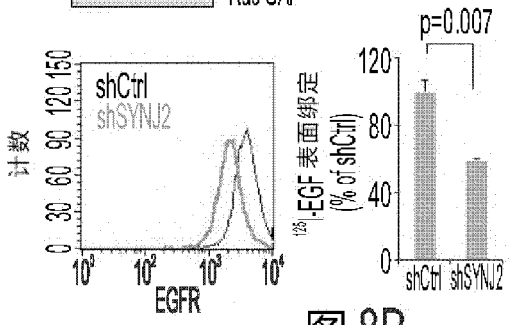
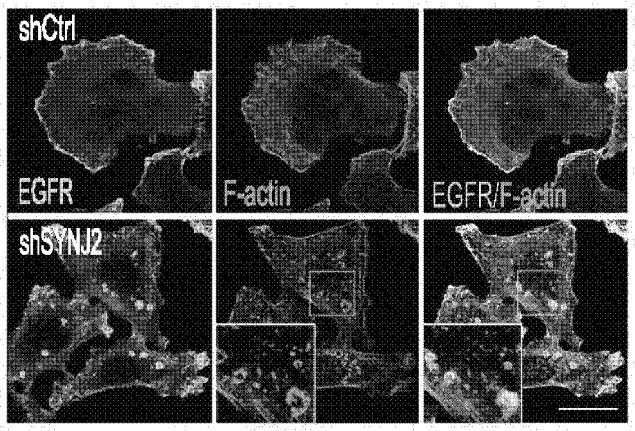


图 8B

图 8C



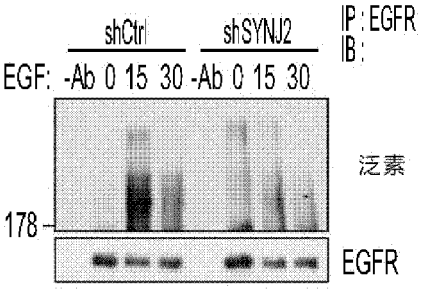


图 8E

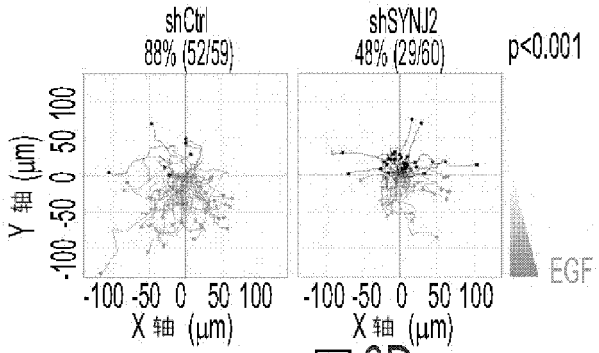


图 8D

图 8F

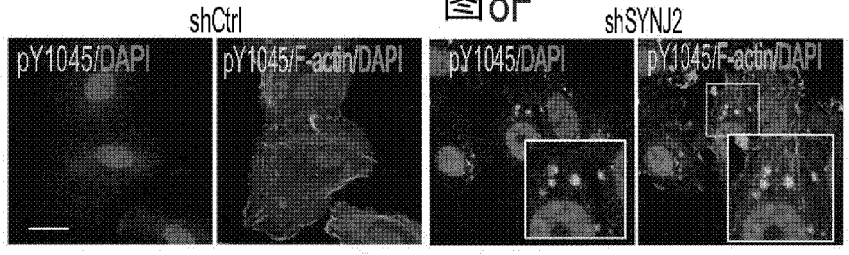
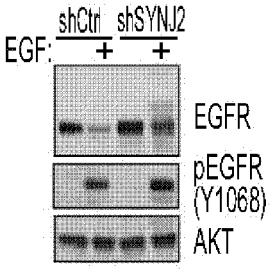


图 8G



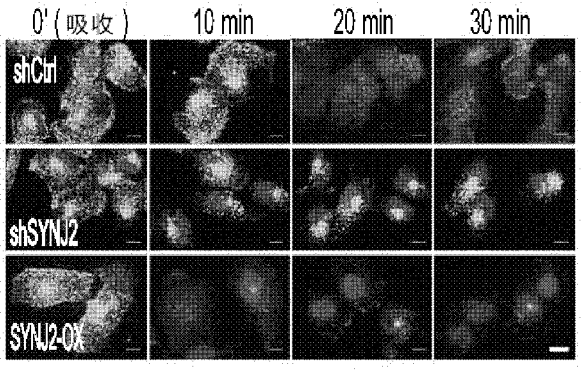
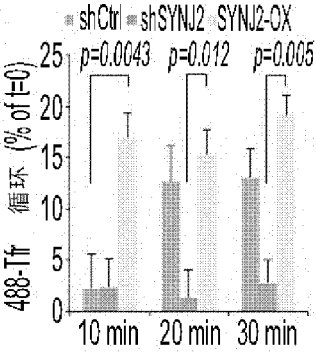


图 8H



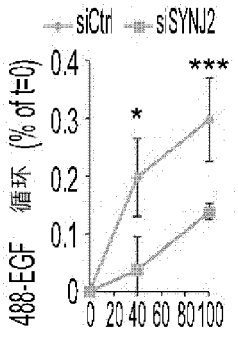


图 8I

图9A

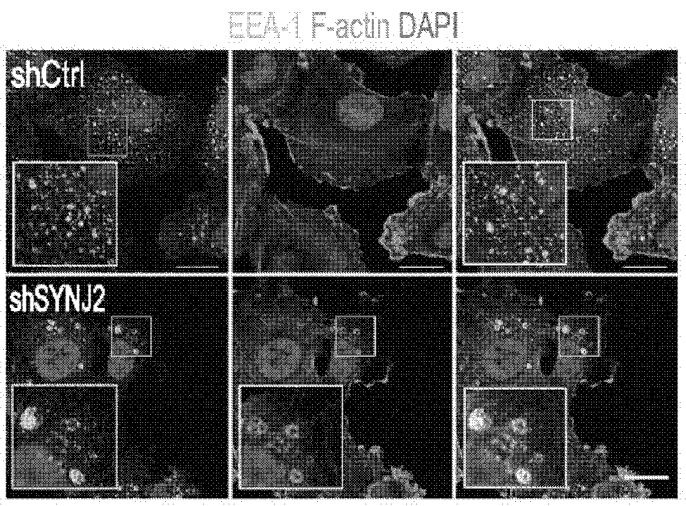


图9B

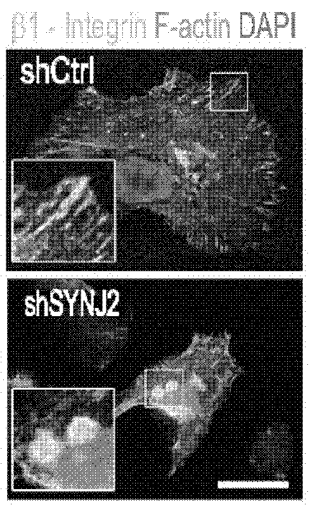


图9C

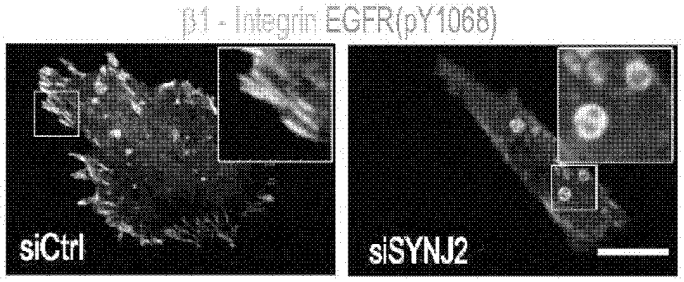
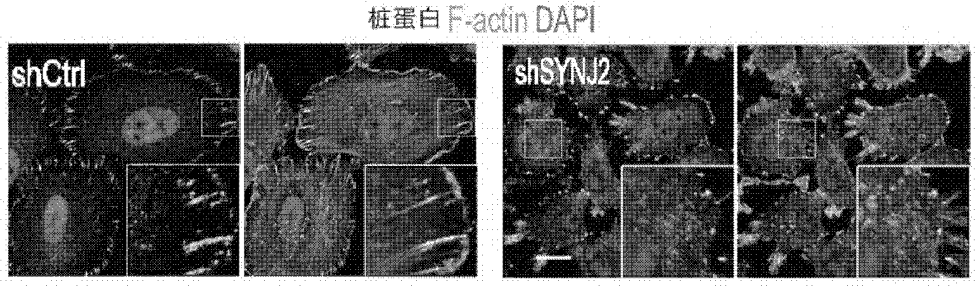


图9D



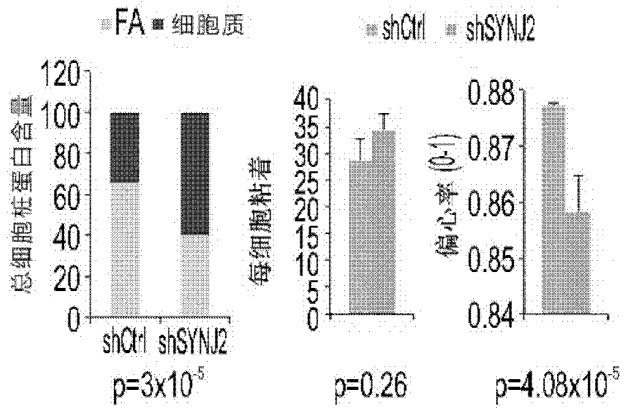


图10A

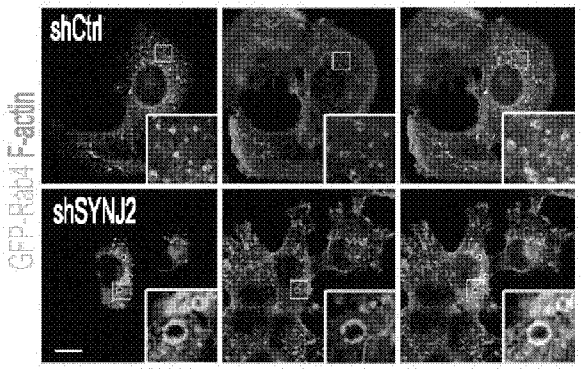
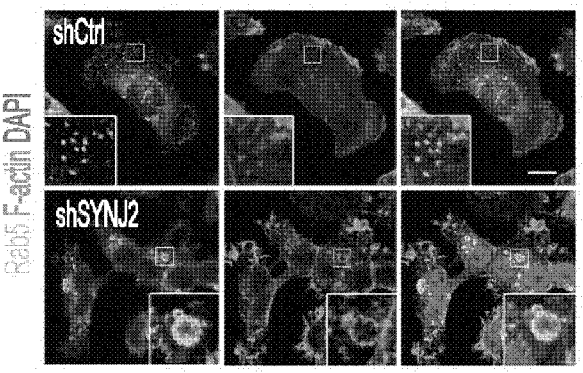


图10B



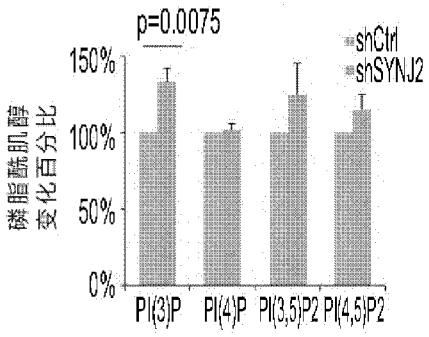
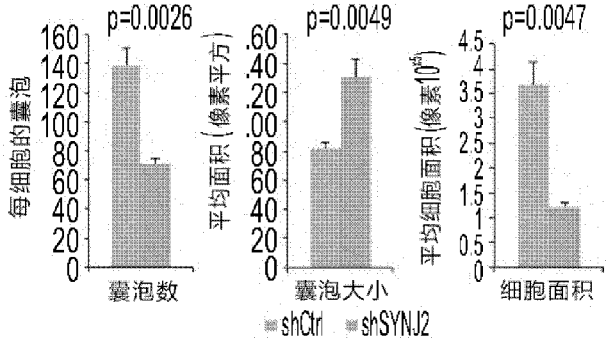


图10C



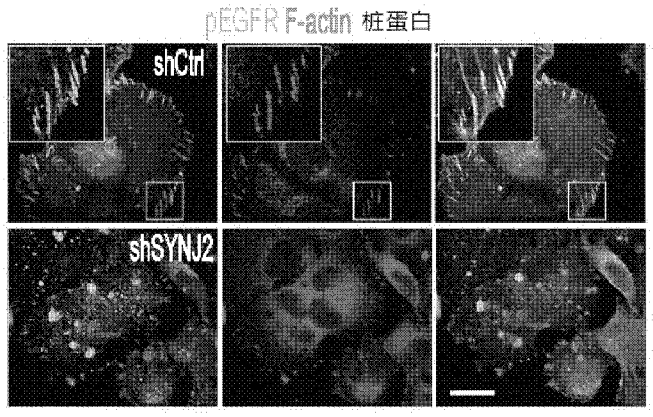


图10D

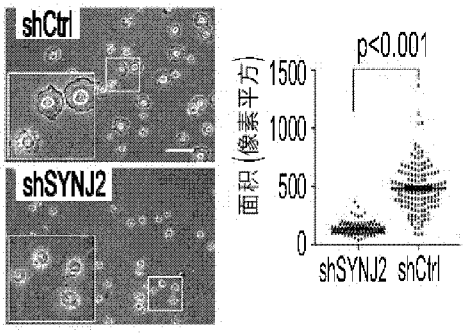


图10E

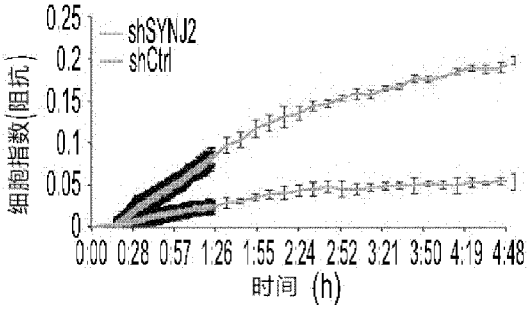


图10F

图11A

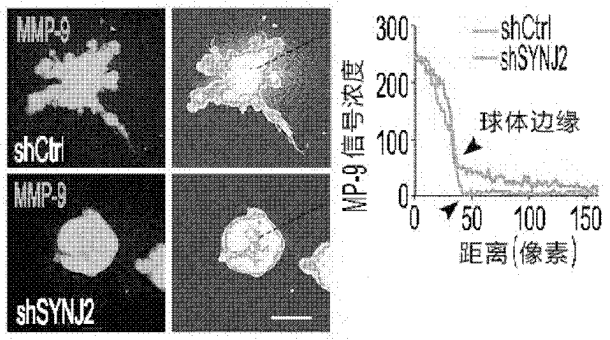


图11B

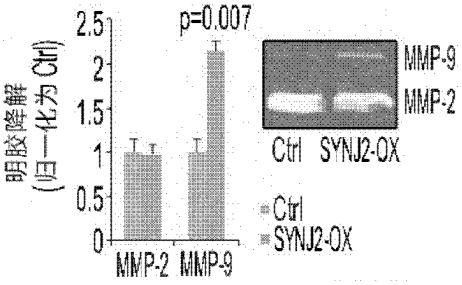


图11C

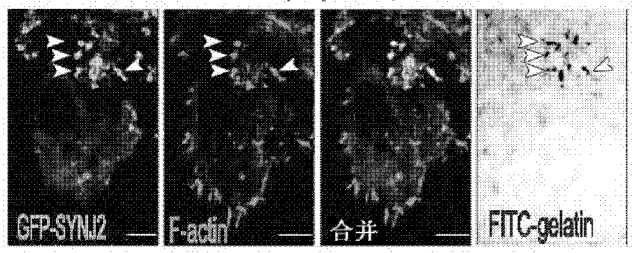
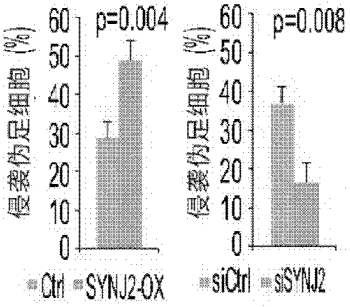


图11D



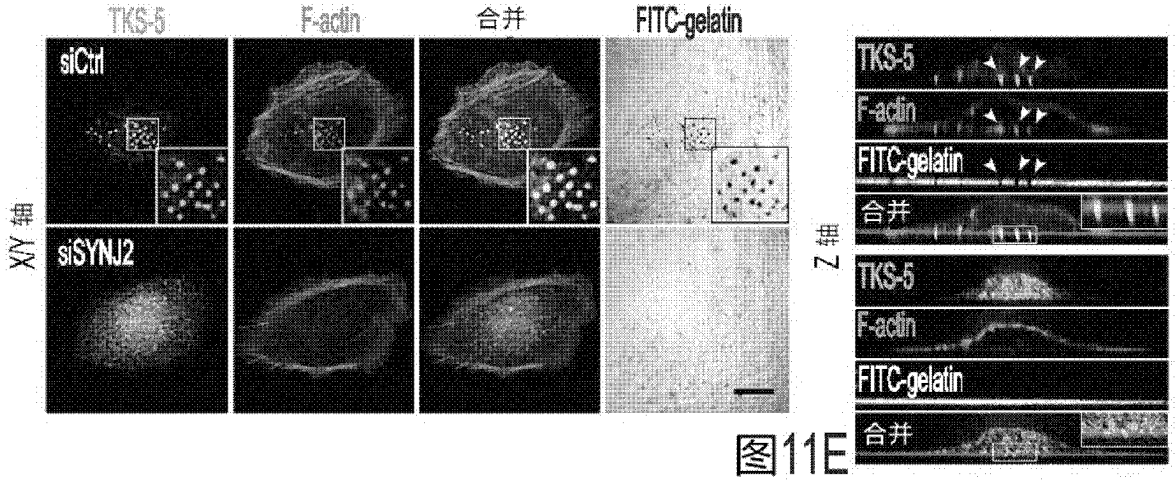


图11E

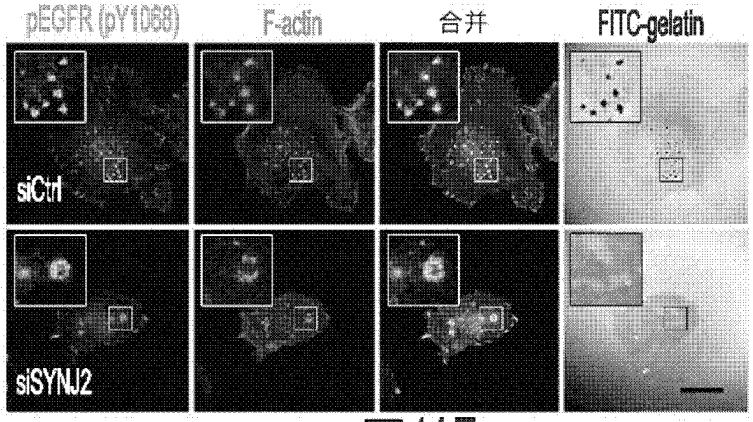


图11F

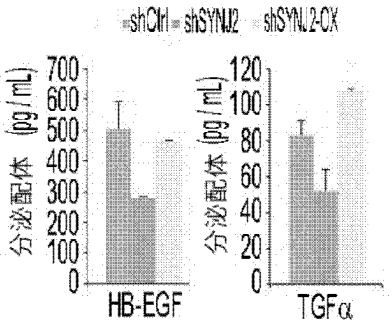


图11G

图12A

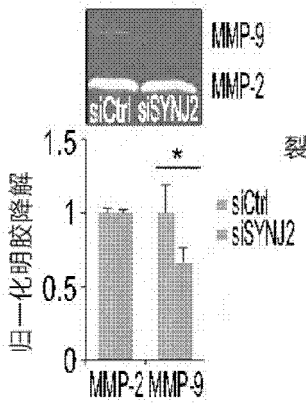


图12B

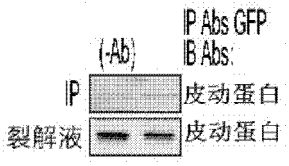


图12C

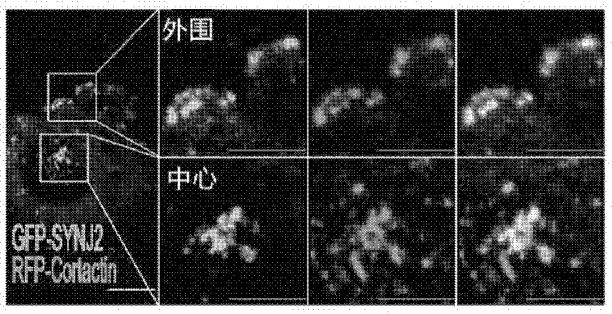


图12D

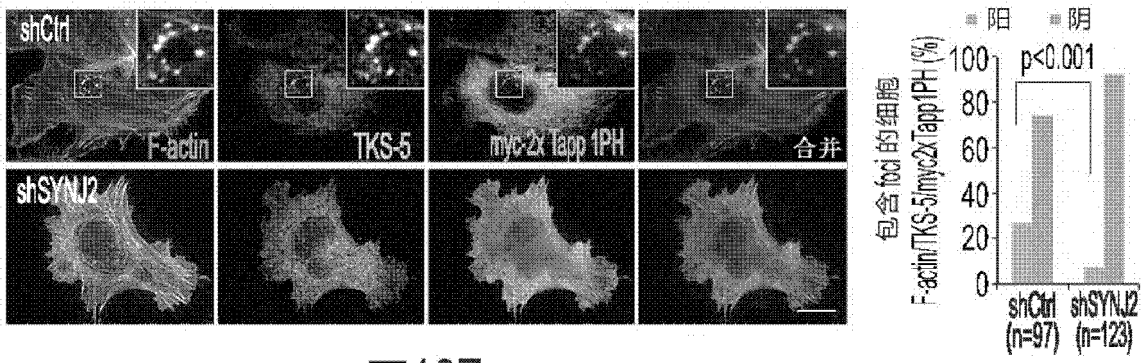


图12E

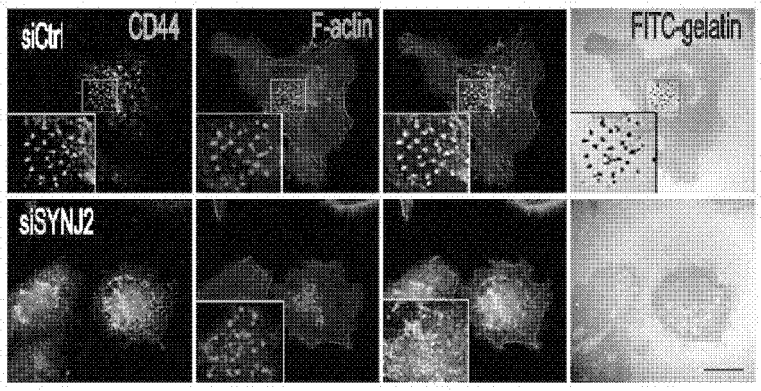


图12F

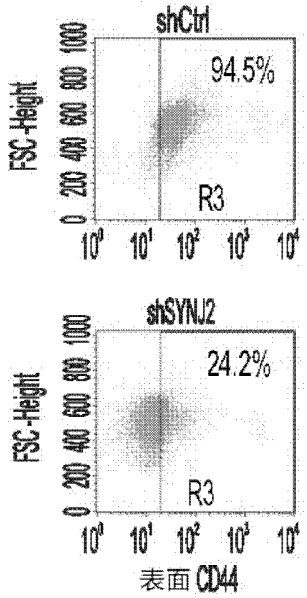
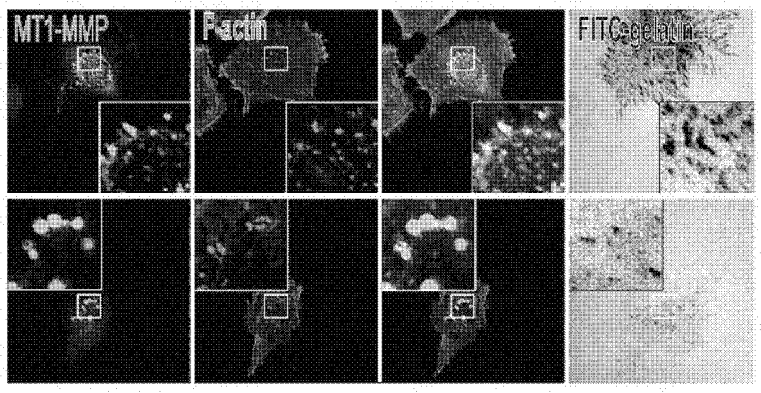


图12G



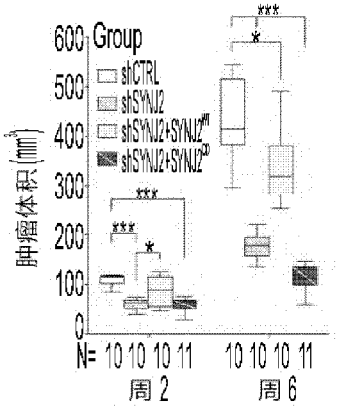


图13A

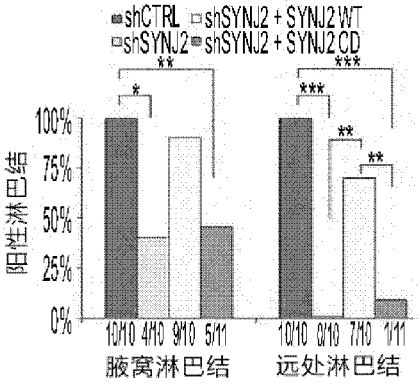


图13B

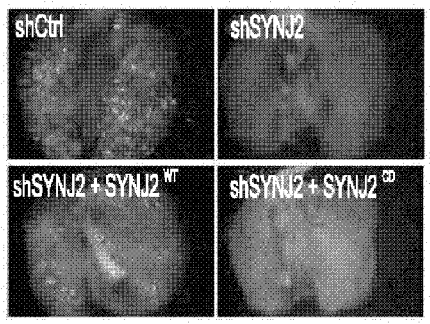


图13C

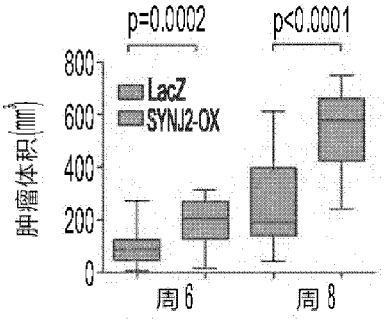


图13D

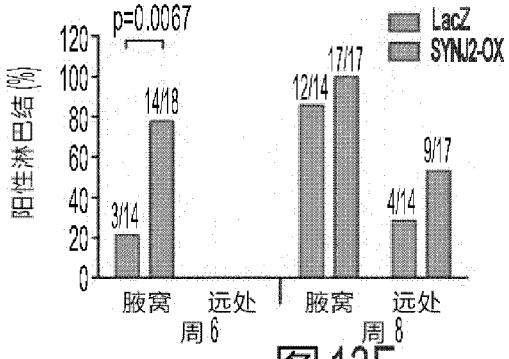
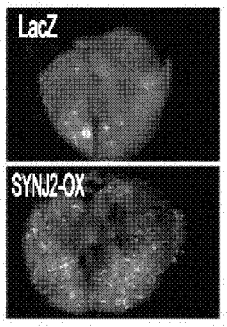


图13E



周 8

图13F

图13G

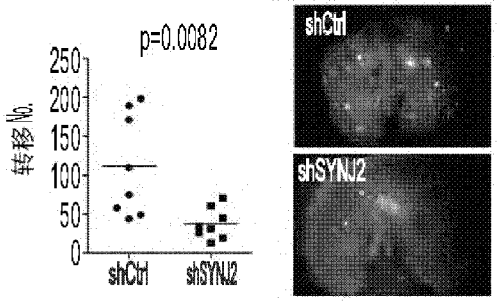
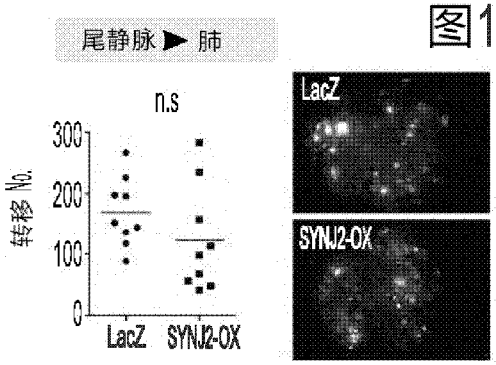


图13H



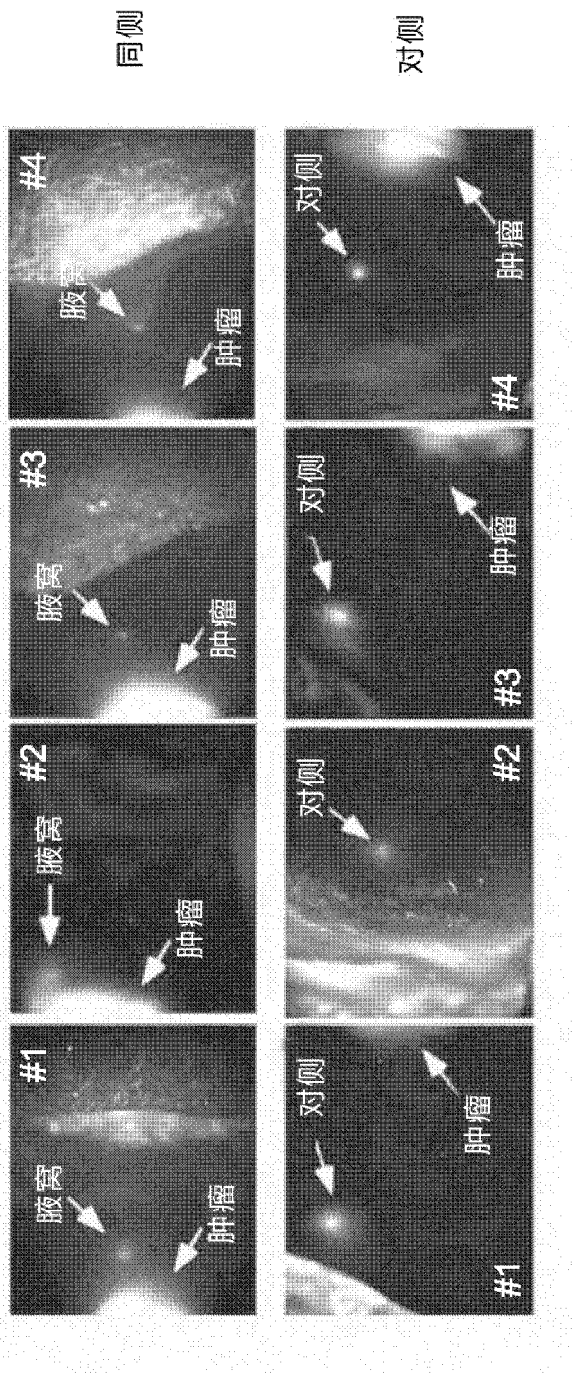


图 14

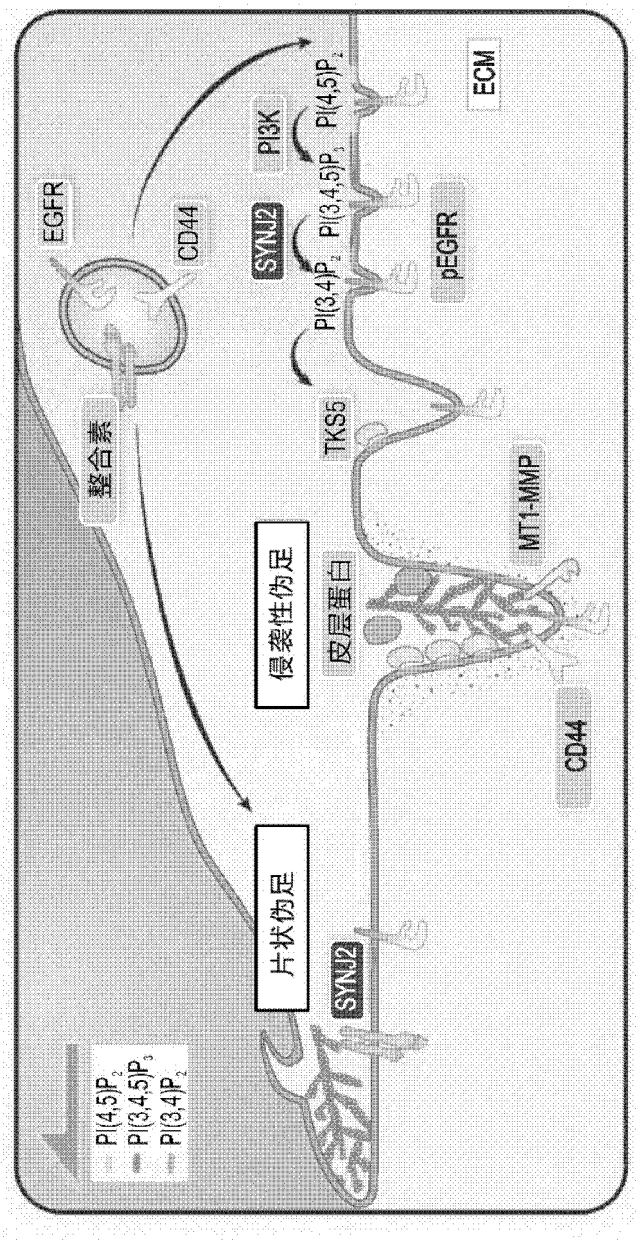

EGFR
CD44
整合素
SYNJ2
PI3K
PI(4,5)P2
PI(3,4,5)P3
PI(3,4)P2
TKS5
侵袭性伪足
皮层蛋白
片状伪足
SYNJ2
pEGFR
ECM
MT1-MMP
CD44
PI(4,5)P2
PI(3,4,5)P3
PI(3,4)P2

图 15

图16A

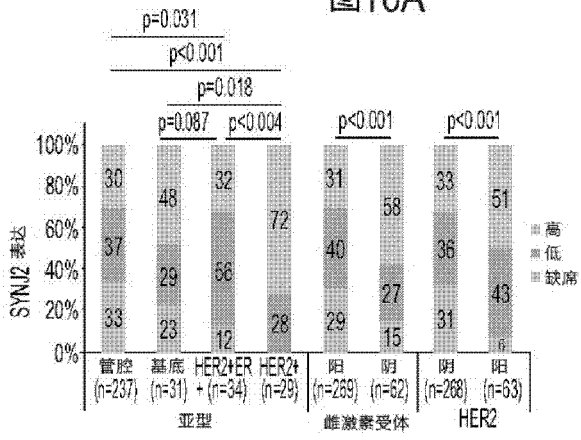


图16B

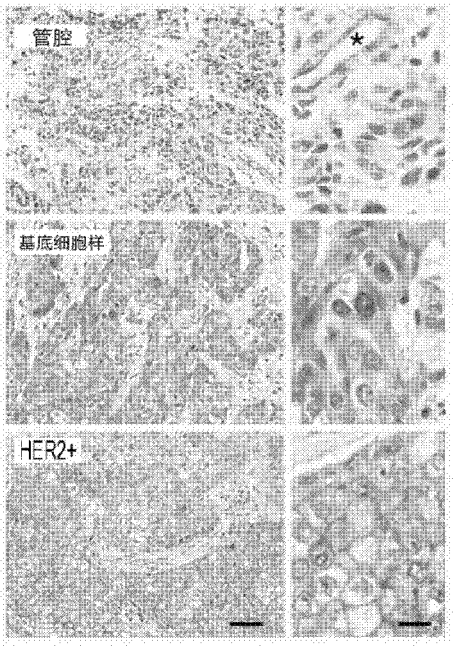


图16C

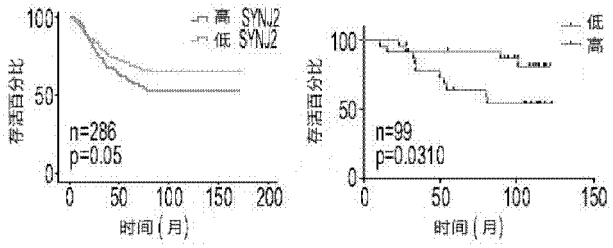


图17A

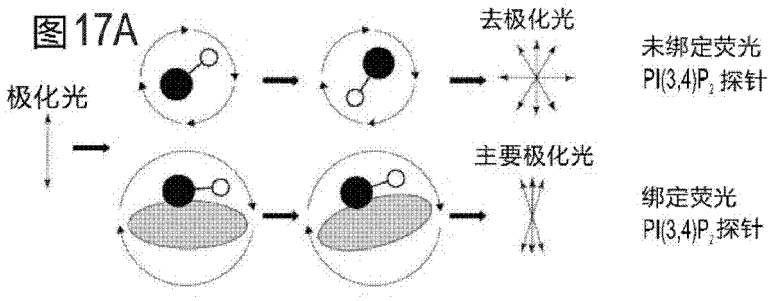
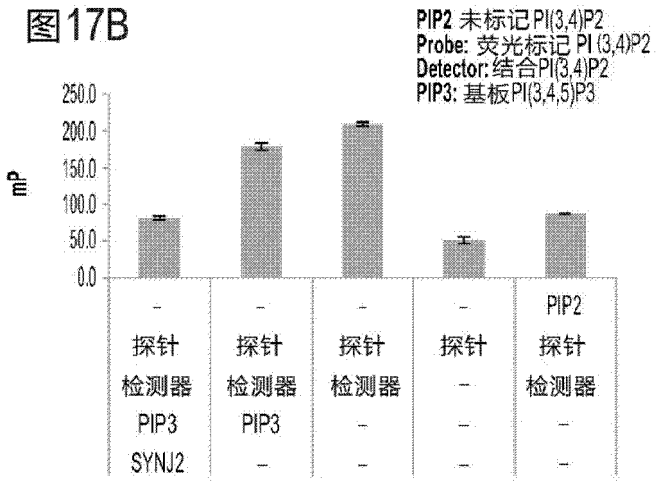

极化光
去极化光
未绑定荧光
PI(3,4)P2 探针
主要极化光
绑定荧光
PI(3,4)P2 探针

图17B


PIP2 未标记PI(3,4)P2
Probe: 荧光标记 PI(3,4)P2
Detector: 结合PI(3,4)P2
PIP3: 基板PI(3,4,5)P3
mP
250.0
200.0
150.0
100.0
50.0
0.0
- - - - PIP2
探针 探针 探针 探针 探针
检测器 检测器 检测器 - 检测器
PIP3 PIP3 - - -
SYNJ2 - - - -

1/1
31/11
61/21
91/31
121/41
151/51
181/61
211/71
241/81
271/91
301/101
331/111
361/121
391/131
421/141
451/151
481/161
511/171
541/181
571/191
601/201
631/211
661/221
691/231
721/241

图 18

Abstract

A method of preventing tumor metastasis with the proviso that the tumor is not glioma is provided. The method comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2), thereby preventing tumor metastasis. Also, provided is a method of treating cancer. The method comprising, administering to a subject in need thereof a therapeutically effective amount of an inhibitor of synaptojanin 2 (SYNJ2) and an inhibitor of a cell surface receptor associated with an onset or progression of cancer, thereby treating cancer.