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(54) Title: NEW METHOD FOR TREATING CANCER

(57) Abstract: The present invention relates to methods and pharmaceutical compositions for use in the prevention and treatment of cancer and cancer metastasis.

NEW METHOD FOR TREATING CANCER

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for use in the prevention and treatment of cancer and cancer metastasis.

BACKGROUND OF THE INVENTION:

Breast cancer is one of the most frequent malignancies which affects about 10% of women over the course of their lives. Breast cancers can be classified in different subgroups depending on the expression of markers such as ERBB2 or estrogen (ER) and progesterone (PR) receptors. In ERBB2+ or ER+/PR+ (referred as luminal A and B) subtypes, the use of targeted therapeutics has allowed a significant improvement of the patient outcome. This is not the case in the basal-like breast cancer subtype which is characterized by the lack of ERBB2 and ER/PR expression, a high degree of cell proliferation and metastatic development, and frequent relapse. Major efforts are thus required to uncover novel markers and targets for this poor prognosis cancer in order to improve the care of patients.

Recent data have revealed the importance of the planar cell polarity (PCP) pathway in breast cancer dissemination (Anastas, J. N. et al. 2012; Belotti, E. et al. 2013 and Luga, V. et al. 2012). This pathway is best known for its physiological role in the shaping of epithelial tissues during embryonic development of invertebrates and vertebrates. The organization of PCP signaling relies on a set of evolutionarily conserved molecules whose most prominent members are Wnts, Frizzled, Vang Gogh, Flamingo, Dishevelled, Prickle and Diego in Drosophila. In vertebrates, the homologous genes regulate convergent-extension, a phenomenon taking place at the early stage of gastrulation, by allowing the convergence of cells toward the midline, their intercalation and the elongation of the structure to form the body axis. In adults, the PCP pathway contributes to epidermal homeostasis and repair. Breast cancer cells mainly use this pathway to promote tumoral dissemination, in particular by hijacking VANGL1, homologue of Vang Gogh, and its associated molecules DISHEVELLED and PRICKLEl, and FRIZZLED2 which constitutes a potential target for antibody-based drugs. Overexpression of the VANGL1 and FRIZZLED2 genes has been consistently demonstrated in breast cancer and correlated with tumor aggressiveness. However, no such finding has yet been reported for most other PCP genes, including PRICKLEl.

Prickle 1 is known to regulate PCP in Drosophila as well as convergent-extension in Zebrafish and Xenopus. The Prickle 1 encoded cytoplasmic protein is conserved in these species as well as in humans and is composed of an amino-terminal Prickle Espinas Testis (PET) domain followed by three zinc finger-like domains called LIM domains and a carboxy-terminal farnesylation site. PRICKLE 1 has been shown to interact with Vang Gogh and is localized asymmetrically at the anterior pole during gastrulation and neurulation of polarized cells. The inventors have previously demonstrated that the asymmetric localization of PRICKLE 1 occurs upon phosphorylation triggered by its bona fide binding partner MINK1, a Ste20-like serine/threonine protein kinase. However, it remains unknown whether PRICKLE 1 also contributes to cancer and cell motility.

SUMMARY OF THE INVENTION:

Here, the inventors study the expression levels of PRICKLE 1 and purify novel PRICKLE 1 binding partners in breast cancer. They find that up-regulation of PRICKLE 1 is restricted to the basal-like subtype and associated with poor prognosis. Among the PRICKLEl interactors, they identify a protein complex made of RICTOR, SIN1 and LST8, three members of the mTORC2 complex. This group of molecules is responsible for the phosphorylation of the serine-threonine kinase AKT at serine 473, leading to its activation, through its interaction with mTOR, another serine-threonine kinase. The AKT-mTOR pathway plays a central role in cell signaling and is involved in many cellular processes including cell survival, apoptosis, cell growth, metabolism, and cell migration. In addition, extensive studies have demonstrated its pivotal role in tumor progression and metastatic dissemination. They show that the PRICKLE l-MINKl-mTORC2 complex controls the phosphorylation of AKT at serine 473 and contributes to cancer cell proliferation and migration in vitro and in vivo. These data suggest that targeting of this novel protein complex could be an interesting strategy to fight breast cancer progression.

Thus, the invention relates to an inhibitor of the PRICKLE l-MINKl-mTORC2 complex for use in the prevention and treatment of cancer and cancer metastasis.

A further aspect of the invention relates to a method for predicting the survival time of a patient suffering from breast cancer, comprising a step of determining in a sample obtained from the patient the expression level of PRICKLE.

The present invention also relates to a method for predicting overall survival (OS), event-free survival (EFS) and metastasis-free survival (MFS) of a patient suffering from

breast cancer, comprising a step of determining in a sample obtained from the patient the expression level of PRICKLE.

DETAILED DESCRIPTION OF THE INVENTION:

Therapeutic methods:

The invention relates to a compound which is an inhibitor of the PRICKLE 1-MINKl-mTORC2 complex for use in the prevention and treatment of cancer and cancer metastasis in a patient in need thereof.

As used herein, the term "patient" denotes a mammal. Typically, a patient according to the invention refers to any patient (preferably human) afflicted with or susceptible to be afflicted with a cancer. Typically a patient according to the invention is a patient afflicted or susceptible to be afflicted with cancer metastasis. The patient may suffer from any solid cancer. Typically, the cancer may be selected from the group consisting of bile duct cancer (e.g. periphilar cancer, distal bile duct cancer, intrahepatic bile duct cancer), bladder cancer, bone cancer (e.g. osteoblastoma, osteochrondroma, hemangioma, chondromyxoid fibroma, osteosarcoma, chondrosarcoma, fibrosarcoma, malignant fibrous histiocytoma, giant cell tumor of the bone, chordoma, lymphoma, multiple myeloma), brain and central nervous system cancer (e.g. meningioma, astocytoma, oligodendrogliomas, ependymoma, gliomas, medulloblastoma, ganglioglioma, Schwannoma, germinoma, craniopharyngioma), breast cancer (e.g. ductal carcinoma in situ, infiltrating ductal carcinoma, infiltrating, lobular carcinoma, lobular carcinoma in, situ, gynecomastia), Castleman disease (e.g. giant lymph node hyperplasia, angio follicular lymph node hyperplasia), cervical cancer, colorectal cancer, endometrial cancer (e.g. endometrial adenocarcinoma, adenocanthoma, papillary serous adnocarcinroma, clear cell), esophagus cancer, gallbladder cancer (mucinous adenocarcinoma, small cell carcinoma), gastrointestinal carcinoid tumors (e.g. choriocarcinoma, chorioadenoma destruens), Hodgkin's disease, non-Hodgkin's lymphoma, Kaposi's sarcoma, kidney cancer (e.g. renal cell cancer), laryngeal and hypopharyngeal cancer, liver cancer (e.g. hemangioma, hepatic adenoma, focal nodular hyperplasia, hepatocellular carcinoma), lung cancer (e.g. small cell lung cancer, non-small cell lung cancer), mesothelioma, plasmacytoma, nasal cavity and paranasal sinus cancer (e.g. esthesioneuroblastoma, midline granuloma), nasopharyngeal cancer, neuroblastoma, oral cavity and oropharyngeal cancer, ovarian cancer, pancreatic cancer, penile cancer, pituitary cancer, prostate cancer, retinoblastoma, rhabdomyosarcoma (e.g. embryonal rhabdomyosarcoma, alveolar rhabdomyosarcoma, pleomorphic rhabdomyosarcoma), salivary

gland cancer, skin cancer (e.g. melanoma, nonmelanoma skin cancer), stomach cancer, testicular cancer (e.g. seminoma, nonseminoma germ cell cancer), thymus cancer, thyroid cancer (e.g. follicular carcinoma, anaplastic carcinoma, poorly differentiated carcinoma, medullary thyroid carcinoma, thyroid lymphoma), vaginal cancer, vulvar cancer, and uterine cancer (e.g. uterine leiomyosarcoma). In a particular embodiment, the cancer is a colorectal cancer.

In one embodiment, the cancer is breast cancer.

In another embodiment, the cancer is a breast metastatic cancer.

The terms "prevention", "preventing", "preventative" or "prophylactic" refer to keeping from occurring, or to hinder, defend from, or protect from the occurrence of a condition, disease, disorder, or phenotype, including an abnormality or symptom. A patient in need of prevention may be prone to develop the condition.

The terms "treatment", "treat" or "treating" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the aim is to prevent or ameliorate cancer and cancer metastasis or slow down cancer and cancer metastasis. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

As used herein the term "PRICKLE1-MINK1-mTORC2 complex" denotes a complex of proteins which controls the phosphorylation of AKT, a serine-threonine kinase, leading to its activation. The inventors have demonstrated that this complex contributes to the control of focal adhesion turnover and cancer cell dissemination.

As used herein, the term "PRICKLE1" also known as REST/NRSF-interacting LIM domain protein, has its general meaning in the art and refers to a scaffold cytoplasmic protein located at the plasma membrane. Prickle is part of the non-canonical Wnt signaling pathway that establishes planar cell polarity. A gain or loss of function of Prickle1 (Entrez Gene ID number: 144165) causes defects in the convergent extension movements of gastrulation. Prickle1 plays an important role in the development of the nervous system by regulating the movement of nerve cells.

As used herein, the term "MINK1" for "Misshapen-like kinase 1" (Entrez Gene ID number: 50488 and UniProt sequence: Q8N4C8) has its general meaning in the art and refers to a serine/threonine kinase belonging to the germinal center kinase (GCK) family. The protein is structurally similar to the kinases that are related to NIK and may belong to a distinct subfamily of NIK-related kinases within the GCK family. Studies of the mouse homolog indicate an up-regulation of expression in the course of postnatal mouse cerebral

development and activation of the cJun N-terminal kinase (JNK) and the p38 pathways. Alternative splicing occurs at this locus and four transcript variants encoding distinct isoforms have been identified.

As used herein the term "mTORC2" for "mTOR Complex 2" has its general meaning in the art and refers to a protein complex that regulates cellular metabolism as well as the actin cytoskeleton. It is defined by the interaction of mTOR and the rapamycin-insensitive companion of mTOR (RICTOR), and also includes LST8 and mammalian stress-activated protein kinase interacting protein 1 (SIN1).

The invention relates to a compound which is an inhibitor of the PRICKLE 1 for use in the prevention and treatment of cancer and cancer metastasis.

The invention relates to a compound which is an inhibitor of the MINK1 for use in the prevention and treatment of cancer and cancer metastasis.

The invention relates to a compound which is an inhibitor of the mTORC2 complex for use in the prevention and treatment of cancer and cancer metastasis.

The term "PRICKLE l-MINKl-mTORC2 complex inhibitor" has its general meaning in the art and refers to a compound that selectively blocks or inactivates PRICKLE 1, MINK1 and/or mTORC2.

The term "mTORC2 inhibitor" also refers to a compound that selectively blocks the binding of mTOR, the central catalytic subunit of mTORC2, to additional protein components of mTORC2 complex such as RICTOR, SIN1 and LST8. The term "mTORC2 inhibitor" also refers to a compound able to prevent the action of mTORC2, for example by inhibiting the mTORC2 complexes controls of downstream effectors such as PKC, AKT and SGK.

As used herein, the term "selectively blocks or inactivates" refers to a compound that preferentially binds to and blocks or inactivates PRICKLE 1, MINK1 and/or mTORC2 with a greater affinity and potency, respectively, than its interaction with the other sub-types of the mTORC2 complex family. Compounds that block or inactivate PRICKLE 1, MINK1 and/or mTORC2, but that may also block or inactivate other mTORC2 complex sub-types, as partial or full inhibitors, are contemplated. The term "mTORC2 inhibitor" also relates to a compound that selectively blocks mTORC2 downstream effectors such as PKC, AKT and SGK.

The terms "PRICKLEl inhibitor", "MINK1 inhibitor" and "mTORC2 inhibitor" also refers to compounds which inhibit the expression of the gene encoding for PRICKLEl, MINK1 and the molecules of the mTORC2 complex like RICTOR, LST8 and SIN1.

The inventors showed that PRICKLE 1, MINKl and mTORC2 interact together and are involved in tumor growth and cancer cell migration.

Thus, the invention also relates to an inhibitor of the interaction of PRICKLE 1, MINKl and mTORC2 for use in the prevention and treatment of cancer and cancer metastasis.

As used herein, "an inhibitor of the interaction of PRICKLEl, MINKl and mTORC2" denotes a molecule able to inhibit the interaction or the binding between PRICKLEl, MINKl and mTORC2 and particularly between PRICKLEl and MINKl, between PRICKLEl and mTORC2 and between PRICKLEl and RICTOR.

Typically, a PRICKLEl inhibitor, a MINKl inhibitor or an mTORC2 inhibitor compound is a small organic molecule, a peptide, a polypeptide, an aptamer or an intra-antibody.

Such inhibitors of PRICKLEl -MINKl -mTORC2 complex may be selected among small molecule, siRNA, shRNA, anti-sense DNA and the like.

In one embodiment, such inhibitor of PRICKLEl -MINKl -mTORC2 complex is selected from the group consisting of siRNA, shRNA, anti-sense oligonucleotides and ribozymes.

Small inhibitory RNAs (siRNAs) can function as inhibitors of gene expression of a component of PRICKLEl -MINKl -mTORC2 complex. For example, gene expression of PRICKLEl, MINKl or a member of mTORC2 complex can be reduced by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that said gene expression of PRICKLEl, MINKl or a member of mTORC2 complex is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see for example Tuschl, T. et al. Genes Dev. 1999 Dec 15; 13(24):3 191-7; Elbashir, S. M. et al Nature. 2001 May 24;41 1(6836):494-8; Hannon, GJ. Nature. 2002 Jul 11;418(6894):244-51); McManus, MT. et al. J Immunol 169, 5754-5760 (2002).; Brummelkamp, TR. et al. Science. 2002 Apr 19; 296(5567):550-3; U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836). All means and methods which result in a decrease in PRICKLEl gene expression, MINKl gene expression or in a member of mTORC2 complex gene expression, in particular by taking advantage of specific siRNAs (i.e siRNAs that target specifically mRNA) may be used in the present invention.

Methods for generating and preparing siRNA(s) as well as method for inhibiting the expression of a target gene are also described for example in WO02/055693.

siRNAs or related nucleic acids useful as inhibitors of PRICKLE 1, MINK1 or a member of mTORC2 complex gene expression, such as anti-sense oligonucleotides can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramadite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-0-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone. Those modification includes the use of nucleosides with modified sugar moieties, including without limitation, 5'-vinyl, 5'-methyl (R or S), 4'-S, 2'-F, 2'-OCH3 and 2'-0(CH2)20CH3 substituent groups. The substituent at the 2' position can also be selected from allyl, amino, azido, thio, O-allyl, O-CI-CIO alkyl, OCF3, 0(CH2)2SCH3, 0(CH2)2-0-N(Rm)(Rn), and O-CH2-C(=0)-N(Rm)(Rn), where each Rm and Rn is, independently, H or substituted or unsubstituted C1-C10 alkyl.

Antisense oligonucleotides and siRNAs or related nucleic acids useful as inhibitors of PRICKLE l-MINKl-mTORC2 complex may be delivered *in vivo* alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide or siRNA or related nucleic acids to the target cells, preferably those with deficient expression of SMN gene, such as muscular cells. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, transposon-based vectors or other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide or siRNA or related nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to, nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses;

Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Particular viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Varmus, Harold; Coffin, John M.; Hughes, Stephen H., ed (1997). "Principles of Retroviral Vector Design". Retroviruses. Plainview, N.Y: Cold Spring Harbor Laboratory Press. ISBN 0-87969-571-4.

Particular viruses for certain applications are the adeno-viruses and adeno-associated viruses or retroviral vectors such as lentiviruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. Examples of such viral vectors includes vectors originated from retroviruses such as HIV (Human Immunodeficiency Virus), MLV (Murine Leukemia Virus), ASLV (Avian Sarcoma/Leukosis Virus), SNV (Spleen Necrosis Virus), RSV (Rous Sarcoma Virus), MMTV (Mouse Mammary Tumor Virus), etc, lentivirus, Adeno-associated viruses, and Herpes Simplex Virus, but are not limited to. These viral vectors can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hematopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions.

Other vectors include plasmid vector, cosmid vector, bacterial artificial chromosome (BAC) vector, transposon-based vector. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, eye, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis

or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

In a preferred embodiment, the antisense oligonucleotide, siRNA, shRNA or related nucleic acid sequence is under the control of a heterologous regulatory region, e.g., a heterologous promoter. The promoter can also be, e.g., a viral promoter, such as CMV promoter or any synthetic promoters.

siRNA can also be directly conjugated with a molecular entity designed to help targeted delivery. Examples of conjugates are lipophilic conjugates such as cholesterol, or aptamer-based conjugates. Cationic peptides and proteins are also used to form complexes with a negatively charged phosphate backbone of the siRNA.

In one embodiment, the siRNA used to target MINK1 are GGAACAAACUGCGGGUGUA (SEQ ID NO: 1) and GAAGUGGUCUAAGAAGUUC (SEQ ID NO: 2) and the shRNA used to target MINK1 are AGCGGCTCAAGGTCATCTATG (SEQ ID NO: 3) and GCTACTGAAGTTTCCCTTCAT (SEQ ID NO: 4).

In one embodiment, the siRNA used to target PRICKLE1 are GAGAGAAGCAUCGGAUUAA (SEQ ID NO: 5) and GAAGAUAAAUGGAGGUGAA (SEQ ID NO: 6) and the shRNA used to target PRICKLE1 are GCTCAGCATGTGACGAGATAA (SEQ ID NO: 7) and CCACCACATGATAATGAGGTA (SEQ ID NO: 8).

In another embodiment, such inhibitor of PRICKLE l-MINKl-mTORC2 complex is a small molecule. Such inhibitors and notably mTORC2 are well known in the art (see for instance Mohindra et al, 2014; Nelson et al., 2013; Pal and Quinn, 2013 (the content of which is incorporated herein by reference). Non-limiting examples of inhibitors of PRICKLE1-MINKl-mTORC2 complex includes PRICKLE1 inhibitors, MINK1 inhibitors and mTORC2 inhibitors.

mTORC2 inhibitors:

In one particular embodiment, the PRICKLE l-MINKl-mTORC2 complex inhibitor is a mTORC2 inhibitor.

As used herein, the term "mTORC2 inhibitor" refers to a compound (natural or synthetic) that inhibits at least one activity of a molecule of the mTORC2 complex such as RICTOR, LST8 and sinl. A person skilled in the art can readily determine whether a

compound, such as rapamycin or an analogue or derivative thereof, is an mTORC2 inhibitor. A specific method of identifying such compounds is disclosed in U.S. Patent Application Publication No. 2003/0008923.

In a preferred embodiment, the mTORC2 inhibitor is selected from the group consisting of rapamycin (also called sirolimus and described in U.S. Pat. No. 3,929,992), temsirolimus, deforolimus, everolimus, tacrolimus and rapamycin analogue or derivative thereof.

As used herein, the term "rapamycin analogue or derivative thereof includes compounds having the rapamycin core structure as defined in U.S. Patent Application Publication No. 2003/0008923 (which is herein incorporated by reference), which may be chemically or biologically modified while still retaining mTOR inhibiting properties. Such derivatives include esters, ethers, oximes, hydrazones, and hydroxylamines of rapamycin, as well as compounds in which functional groups on the rapamycin core structure have been modified, for example, by reduction or oxidation. Pharmaceutically acceptable salts of such compounds are also considered to be rapamycin derivatives. Specific examples of esters and ethers of rapamycin are esters and ethers of the hydroxyl groups at the 42- and/or 31-positions of the rapamycin nucleus, and esters and ethers of a hydroxyl group at the 27-position (following chemical reduction of the 27-ketone). Specific examples of oximes, hydrazones, and hydroxylamines are of a ketone at the 42-position (following oxidation of the 42-hydroxyl group) and of 27-ketone of the rapamycin nucleus.

Examples of 42- and/or 31-esters and ethers of rapamycin are disclosed in the following patents, which are hereby incorporated by reference in their entireties: alkyl esters (U.S. Pat. No. 4,316,885); aminoalkyl esters (U.S. Pat. No. 4,650,803); fluorinated esters (U.S. Pat. No. 5,100,883); amide esters (U.S. Pat. No. 5,118,677); carbamate esters (U.S. Pat. No. 5,118,678); silyl ethers (U.S. Pat. No. 5,120,842); aminoesters (U.S. Pat. No. 5,130,307); acetals (U.S. Pat. No. 551,413); aminodiesters (U.S. Pat. No. 5,162,333); sulfonate and sulfate esters (U.S. Pat. No. 5,177,203); esters (U.S. Pat. No. 5,221,670); alkoxyesters (U.S. Pat. No. 5,233,036); O-aryl, -alkyl, -alkenyl, and -alkynyl ethers (U.S. Pat. No. 5,258,389); carbonate esters (U.S. Pat. No. 5,260,300); arylcarbonyl and alkoxycarbonyl carbamates (U.S. Pat. No. 5,262,423); carbamates (U.S. Pat. No. 5,302,584); hydroxyesters (U.S. Pat. No. 5,362,718); hindered esters (U.S. Pat. No. 5,385,908); heterocyclic esters (U.S. Pat. No. 5,385,909); gem-disubstituted esters (U.S. Pat. No. 5,385,910); amino alkanoic esters (U.S. Pat. No. 5,389,639); phosphorylcarbamate esters (U.S. Pat. No. 5,391,730); carbamate esters (U.S. Pat. No. 5,411,967); carbamate esters (U.S. Pat. No. 5,434,260); amidino carbamate esters (U.S.

Pat. No. 5,463,048); carbamate esters (U.S. Pat. No. 5,480,988); carbamate esters (U.S. Pat. No. 5,480,989); carbamate esters (U.S. Pat. No. 5,489,680); hindered N-oxide esters (U.S. Pat. No. 5,491,231); biotin esters (U.S. Pat. No. 5,504,091); O-alkyl ethers (U.S. Pat. No. 5,665,772); and PEG esters of rapamycin (U.S. Pat. No. 5,780,462).

Examples of 27-esters and ethers of rapamycin are disclosed in U.S. Pat. No. 5,256,790, which is hereby incorporated by reference in its entirety.

Examples of oximes, hydrazones, and hydroxylamines of rapamycin are disclosed in U.S. Pat. Nos. 5,373,014, 5,378,836, 5,023,264, and 5,563,145, which are hereby incorporated by reference. The preparation of these oximes, hydrazones, and hydroxylamines is disclosed in the above listed patents. The preparation of 42-oxorapamycin is disclosed in U.S. Pat. No. 5,023,263, which is hereby incorporated by reference.

Other compounds within the scope of "rapamycin analog or derivative thereof include those compounds and classes of compounds referred to as "rapalogs" in, for example, WO 98/02441 and references cited therein, and "epirapalogs" in, for example, WO 01/14387 and references cited therein.

Another compound within the scope of "rapamycin derivatives" is everolimus, a 4-0-(2-hydroxyethyl)-rapamycin derived from a macrolide antibiotic produced by Streptomyces hygroscopicus (Novartis). Everolimus is also known as Certican, RAD-001 and SDZ-RAD. Another preferred mTOR inhibitor is zotarolimus, an antiproliferative agent (Abbott Laboratories). Zotarolimus is believed to inhibit smooth muscle cell proliferation with a cytostatic effect resulting from the inhibition of mTOR. Another preferred mTOR inhibitor is tacrolimus, a macrolide lactone immunosuppressant isolated from the soil fungus Streptomyces tsukubaensis. Tacrolimus is also known as FK 506, FR 900506, Fujimycin, L 679934, Tsukubaenolide, PROTOPIC and PROGRAF. Other preferred mTOR inhibitors include AP-23675, AP-23573, and AP-23841 (Ariad Pharmaceuticals).

Preferred rapamycin derivatives include everolimus, CCI-779 (rapamycin 42-ester with 3-hydroxy-2-(hydroxymethyl)-2-methylpropionic acid; U.S. Pat. No. 5,362,718); 7-epi-rapamycin; 7-thiomethyl-rapamycin; 7-epi-trimethoxyphenyl-rapamycin; 7-epi-thiomethyl-rapamycin; 7-demethoxy-rapamycin; 32-demethoxy-rapamycin; 2-desmethyl-rapamycin; and 42-0-(2-hydroxy)ethyl rapamycin (U.S. Pat. No. 5,665,772).

Additional mTORC2 inhibitors may be OSI-027 (OSI Pharmaceuticals), a small molecule mTORC2 inhibitor. OSI-027 inhibits mTORC2 signaling complexes, allowing for the potential for complete truncation of aberrant cell signaling through this pathway.

In addition, torkinibs, ATP-competitive mTOR kinase domain inhibitors and inhibitors of mTORC2 may also be used according to the invention. Exemplary torkinibs include PP242 and PP30 (see, Feldman et al. (2009) PLoS Biology 7:371) and Torinl (Thoreen et al. (2009) J Biol Chem 284:8023).

Identification of the interaction domain between PRICKLE1 and mTORC2:

The inventors identified a region in PRICKLE 1 (called CI domain) which interact with the complex mTORC2 and more precisely RICTOR. Thus, a molecule which bind to the CI domain and inhibits the interaction between PRICKLE 1 and RICTOR may be used for the prevention and treatment of cancer and cancer metastasis.

Accordingly, the present invention also relates to isolated, synthetic or recombinant PRICKLE 1 CI polypeptide.

As used herein the term "PRICKLE 1 CI" refers to the region in PRICKLE 1 polypeptide (called CI domain) which interacts with the complex mTORC2 and more precisely RICTOR. The term "PRICKLE 1 CI" also refers to the region in PRICKLE 1 polypeptide having the sequence set forth as SEQ ID NO: 9.

SEQ ID NO: 9 for PRICKLE 1 CI polypeptide

D S S D S A F Q S A R S R D S R R S V R M G K S S R S A D Q C R Q S L L L S P A L N Y K F P G L S G N A D D T L S R K L D D L S L S R Q G T S F A S E E F W K G R V E Q E T P E D P E E W A D H E D Y M T Q L L L K F G D K S L F Q P Q P N E M D I R A S E H W I S D N M V K S K T E L K Q N N Q S L A S K K Y Q S D M Y W A Q S Q D G L G D S A Y G S H P G P A S S R R L Q E L E L D H G A S G Y N H D E T Q W Y E D S L E C L S D L K P E Q S V R D S M D S L A L S N I T G A S V D G E N K P R

SEQ ID NO: 10 for PRICKLE 1 CI nucleic acid sequence

gattcttccgactctgcatttcagtcagctcgatcaagagactcccgaagaagtgtccgaatgggcaagagcagccggtcag
cagatcagtgtagacagtctctcctcttatcgcctgctctgaactacaagtttcctggcctctcaggcaatgctgatgacacccctttctcgaa
aattggatgatctgagtctctccagacaaggaacaagttttgccagtgaagaattttggaaaggcagagtagagcaggaaactccagaa
gaccctgaagaatgggctgatcatgaagattatatgacgcagctcctcctcaagtttggtgataaaagcctctttcagccacagcccaat
gagatggatattcgagccagtgagcactggatatctgataacatggttaaaagtaagaccgagttaaagcaaaataaccagagccttgc
aagtaaaaaataccagtctgatatgtactgggcacagtcacaagatggactgggcgattctgcttatggcagccacccaggccctgcaa
gcagtagaaggcttcaggaattggaactggaccatggggcttcagggtataatcatgatgaaacacagtggtatgaagattccctggag

tgtctgtcagacctgaaaccagagcaaagtgttcgggattcgatggattctttggcattgtccaatatcacaggggcttcggtggatgga gaaaacaagccaagg

In one embodiment, the present invention relates to a polypeptide comprising or consisting of:

i) an amino acid sequence SEQ ID NO: 9, or,

ii) an amino acid sequence having at least 70% of identity with the amino acid sequence SEQ ID NO: 9,

wherein the polypeptide does not consist of the PRICKLE 1 polypeptide.

According to the invention a first amino acid sequence having at least 70% of identity with a second amino acid sequence means that the first sequence has 70; 71; 72; 73; 74; 75; 76; 77; 78; 79; 80; 81; 82; 83; 84; 85; 86; 87; 88; 89; 90; 91; 92; 93; 94; 95; 96; 97; 98; or ***99%*** of identity with the second amino acid sequence and conserving biological properties of said second amino acid sequence. Amino acid sequence identity is preferably determined using a suitable sequence alignment algorithm and default parameters, such as BLAST P (Karlin and Altschul, 1990). In particular the polypeptide of the invention is a functional conservative variant of the polypeptides according to the invention. As used herein the term "function-conservative variant" are those in which a given amino acid residue in a polypeptide has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, polarity, hydrogen bonding potential, acidic, basic, hydrophobic, aromatic, and the like). Accordingly, a "function-conservative variant" also includes a polypeptide which has at least 70 % amino acid identity and which has the same or substantially similar properties or functions as the native or parent polypeptide to which it is compared (i.e. interaction properties with the complex mTORC2 and more precisely RICTOR). Functional properties of the polypeptide of the invention could typically be assessed in any functional assay as described in the EXAMPLE.

Fusion proteins of the invention

A further aspect of the invention relates to a fusion protein comprising the polypeptide according to the invention that is fused to at least one heterologous polypeptide.

The term "fusion protein" refers to the polypeptide according to the invention that is fused directly or via a spacer to at least one heterologous polypeptide.

According to the invention, the fusion protein comprises the polypeptide according to the invention that is fused either directly or via a spacer at its C-terminal end to the N-terminal end of the heterologous polypeptide, or at its N-terminal end to the C-terminal end of the heterologous polypeptide.

As used herein, the term "directly" means that the (first or last) amino acid at the terminal end (N or C-terminal end) of the polypeptide is fused to the (first or last) amino acid at the terminal end (N or C-terminal end) of the heterologous polypeptide.

In other words, in this embodiment, the last amino acid of the C-terminal end of said polypeptide is directly linked by a covalent bond to the first amino acid of the N-terminal end of said heterologous polypeptide, or the first amino acid of the N-terminal end of said polypeptide is directly linked by a covalent bond to the last amino acid of the C-terminal end of said heterologous polypeptide.

As used herein, the term "spacer" refers to a sequence of at least one amino acid that links the polypeptide of the invention to the heterologous polypeptide. Such a spacer may be useful to prevent steric hindrances.

In some embodiments, the heterologous polypeptide is a VENUS polypeptide having the sequence set forth as SEQ ID NO: 11.

SEQ ID NO: 11 for VENUS polypeptide

M V S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G K L T L K L I C T T G K L P V P W P T L V T T L G Y G L Q C F A R Y P D H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I K A N F K I R H N I E D G G V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S Y Q S A L S K D P N E K R D H M V L L E F V T A A G I T L G M D E L Y R A R P

SEQ ID NO: 12 for VENUS nucleic acid sequence

atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccaca
agttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagctgatctgcaccaccggcaagctgccc
gtgccctggcccaccctcgtgaccaccctgggctacggcctgcagtgcttcgcccgctaccccgaccacatgaagcagcacgacttct
tcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtg
aagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagct
ggagtacaactacaacagccacaacgtctatatcaccgccgacaagcagaagaacggcatcaaggccaacttcaagatccgccaca
acatcgaggacggcggcgtgcagctcgccgaccactaccagcagaacaccccccatcggcgacggccccgtgctgctgcccgacaa

ccactacctgagctaccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgc
cgggatcactctcggcatggacgagctgtacagggcgcgccct

In one embodiment, the fusion protein of the present invention comprises or consists of a fusion protein having the sequence set forth as SEQ ID NO: 13.

SEQ ID NO: 13 for VENUS-C1 amino acid sequence

M V S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A
T Y G K L T L K L I C T T G K L P V P W P T L V T T L G Y G L Q C F A R Y P D H M
K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F E G D T L
V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I T A D K Q K N G I
K A N F K I R H N I E D G G V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S
Y Q S A L S K D P N E K R D H M V L L E F V T A A G I T L G M D E L Y R A R P D S
S D S A F Q S A R S R D S R R S V R M G K S S R S A D Q C R Q S L L L S P A L N Y K
F P G L S G N A D D T L S R K L D D L S L S R Q G T S F A S E E F W K G R V E Q E T
P E D P E E W A D H E D Y M T Q L L L K F G D K S L F Q P Q P N E M D I R A S E H
W I S D N M V K S K T E L K Q N N Q S L A S K K Y Q S D M Y W A Q S Q D G L G D
S A Y G S H P G P A S S R R L Q E L E L D H G A S G Y N H D E T Q W Y E D S L E C
L S D L K P E Q S V R D S M D S L A L S N I T G A S V D G E N K P R

SEQ ID NO: 14 for VENUS-C1 nucleic acid sequence

atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccaca
agttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagctgatctgcaccaccggcaagctgccc
gtgccctggcccaccctcgtgaccaccctgggctacggcctgcagtgcttcgcccgctaccccgaccacatgaagcagcacgacttct
tcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtg
aagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagct
ggagtacaactacaacagccacaacgtctatatcaccgccgacaagcagaagaacggcatcaaggccaacttcaagatccgccaca
acatcgaggacggcggcgtgcagctcgccgaccactaccagcagaacaccccatcggcgacggccccgtgctgctgcccgacaa
ccactacctgagctaccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgc
cgggatcactctcggcatggacgagctgtacagggcgcgccctgattcttccgactctgcatttcagtcagctcgatcaagagactccc
gaagaagtgtccgaatgggcaagagcagccggtcagcagatcagtgtagacagtctctcctcttatcgcctgctctgaactacaagtttc
ctggcctctcaggcaatgctgatgacaccctttctcgaaaattggatgatctgagtctctccagacaaggaacaagttttgccagtgaaga
attttggaaaggcagagtagagcaggaaactccagaagaccctgaagaatgggctgatcatgaagattatatgacgcagctcctcctc
aagtttggtgataaaagcctctttcagccacagcccaatgagatggatattcgagccagtgagcactggatatctgataacatggttaaaa

gtaagaccgagttaaagcaaaataaccagagccttgcaagtaaaaaataccagtctgatatgtactgggcacagtcacaagatggact
gggcgattctgcttatggcagccacccaggccctgcaagcagtagaaggcttcaggaattggaactggaccatggggcttcagggtat
aatcatgatgaaacacagtggtatgaagattccctggagtgtctgtcagacctgaaaccagagcaaagtgttcgggattcgatggattctt
tggcattgtccaatatcacaggggcttcggtggatggagaaaacaagccaagg

In some embodiments, the heterologous polypeptide is a cell-penetrating peptide, a Transactivator of Transcription (TAT) cell penetrating sequence, a cell permeable peptide or a membranous penetrating sequence.

The term "cell-penetrating peptides" is well known in the art and refers to cell permeable sequences or membranous penetrating sequences such as penetratin, Transactivator of Transcription (TAT) cell penetrating sequence and compounds described in Bechara and Sagan, 2013; Jones and Sayers, 2012; Khafagy el and Morishita, 2012; and Malhi and Murthy, 2012.

In another embodiment, the heterologous polypeptide is a cancer therapeutic polypeptide.

The term "cancer therapeutic polypeptide" refers to any polypeptide that has anti-cancer activities (e.g., proliferation inhibiting, growth inhibiting, apoptosis inducing, metastasis inhibiting, adhesion inhibiting, neovascularization inhibiting). Several such polypeptides are known in the art. (See. e.g., (Boohaker et al, 2012; Choi et al, 2011; Janin, 2003; Li et al, 2013; Sliwkowski and Mellman, 2013)).

In some embodiment, the heterologous polypeptide is a tumor targeting agent.

Tumor targeting agent include but are not limited to antibodies directed against the EDB domain of fibronectin, antibodies or agents binding Vascular endothelial growth factor receptor 2, antibodies or molecules binding fibroblast growth factor receptor- 1, antibodies or agents that interact with CD31, antibodies or agents interacting with tumor lymphatic endothelium (Podoplanin, Lyve-1), or antibodies or agents binding to $\alpha v \beta 3$ integrin such as RGD peptides, or antibodies or agents interacting with tumor membrane-bound and intracellular targets.

Polypeptides and fusion proteins production

The polypeptides or fusion proteins of the invention may be produced by any technique known per se in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination.

Knowing the amino acid sequence of the desired sequence, one skilled in the art can readily produce said polypeptides or fusion proteins, by standard techniques for production of amino acid sequences. For instance, they can be synthesized using well-known solid phase method, preferably using a commercially available peptide synthesis apparatus (such as that made by Applied Biosystems, Foster City, California) and following the manufacturer's instructions.

Alternatively, the polypeptides or fusion proteins of the invention can be synthesized by recombinant DNA techniques as is now well-known in the art. For example, these fragments can be obtained as DNA expression products after incorporation of DNA sequences encoding the desired (poly)peptide into expression vectors and introduction of such vectors into suitable eukaryotic or prokaryotic hosts that will express the desired polypeptide, from which they can be later isolated using well-known techniques.

Polypeptides or fusion proteins of the invention can be used in an isolated (e.g., purified) form or contained in a vector, such as a membrane or lipid vesicle (e.g. a liposome or an erythrocyte).

In specific embodiments, it is contemplated that polypeptides or fusion proteins according to the invention may be modified in order to improve their therapeutic efficacy. Such modification of therapeutic compounds may be used to decrease toxicity, increase circulatory time, or modify biodistribution. For example, the toxicity of potentially important therapeutic compounds can be decreased significantly by combination with a variety of drug carrier vehicles that modify biodistribution.

Accordingly, the polypeptide according to the invention may be a retro-inverso amino acid sequence. The term "retro-inverso amino acid sequence" relates to an isomeric form of an amino acid sequence in which the direction of the amino acid sequence is reversed and the chirality of each amino acid residue is inverted. Retro-inverso amino acid sequence of the present invention may be composed by D-amino acids assembled in the reverse order from that of the parental amino acid sequence.

A strategy for improving drug viability is the utilization of water-soluble polymers. Various water-soluble polymers have been shown to modify biodistribution, improve the mode of cellular uptake, change the permeability through physiological barriers; and modify the rate of clearance from the body. To achieve either a targeting or sustained-release effect, water-soluble polymers have been synthesized that contain drug moieties as terminal groups, as part of the backbone, or as pendent groups on the polymer chain.

For example, Pegylation is a well-established and validated approach for the modification of a range of polypeptides (Chapman, 2002). The benefits include among others: (a) markedly improved circulating half-lives in vivo due to either evasion of renal clearance as a result of the polymer increasing the apparent size of the molecule to above the glomerular filtration limit, and/or through evasion of cellular clearance mechanisms; (b) reduced antigenicity and immunogenicity of the molecule to which PEG is attached; (c) improved pharmacokinetics; (d) enhanced proteolytic resistance of the conjugated protein (Cunningham-Rundles et.al, 1992); and (e) improved thermal and mechanical stability of the PEGylated polypeptide.

Therefore, advantageously, the polypeptides of the invention may be covalently linked with one or more polyethylene glycol (PEG) group(s). One skilled in the art can select a suitable molecular mass for PEG, based on how the pegylated polypeptide will be used therapeutically by considering different factors including desired dosage, circulation time, resistance to proteolysis, immunogenicity, etc.

In one embodiment, the PEG of the invention terminates on one end with hydroxy or methoxy, i.e., X is H or CH3 ("methoxy PEG"). In addition, such a PEG can consist of one or more PEG side-chains which are linked together. PEGs with more than one PEG chain are called branched PEGs. Branched PEGs can be prepared, for example, by the addition of polyethylene oxide to various polyols, including glycerol, pentaerythriol, and sorbitol. For example, a four-armed branched PEG can be prepared from pentaerythriol and ethylene oxide. One form of PEGs includes two PEG side-chains (PEG2) linked via the primary amino groups of a lysine (Monfardini et al, 1995).

To effect covalent attachment of PEG groups to the polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i. e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl proprionate (SPA), succinimidyl carboxymethylate (SCM),benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitable activated polymer molecules are commercially available, e. g. from Shearwater Polymers, Inc., Huntsville, AL, USA, or from PolyMASC Pharmaceuticals pic, UK. Alternatively, the polymer molecules can be activated by conventional methods known in the art, e. g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and

pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference). Specific examples of activated PEG polymers include the following linear PEGs : NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS.

The conjugation of the polypeptides or fusion proteins and the activated polymer molecules is conducted by use of any conventional method. Conventional methods are known to the skilled artisan. The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the polypeptides as well as the functional groups of the PEG molecule (e.g., being amine, hydroxyl, carboxyl, aldehyde, ketone, sulfhydryl, succinimidyl, maleimide, vinylsulfone or haloacetate).

In one embodiment, polypeptides are conjugated with PEGs at amino acid D and E (for COOH), T, Y and S (for OH), K (for NH_2), C (for SH if at least one cysteine is conserved) and/or Q and N (for the amide function).

In one embodiment, additional sites for PEGylation can be introduced by site-directed mutagenesis by introducing one or more lysine residues. For instance, one or more arginine residues may be mutated to a lysine residue. In another embodiment, additional PEGylation sites are chemically introduced by modifying amino acids on polypeptides of the invention.

In one embodiment, PEGs are conjugated to the polypeptides or fusion proteins through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al, 1977; US 4,179, 337).

Conventional separation and purification techniques known in the art can be used to purify pegylated polypeptides of the invention, such as size exclusion (e.g. gel filtration) and ion exchange chromatography. Products may also be separated using SDS-PAGE.

In one embodiment, the pegylated polypeptides provided by the invention have a serum half-life *in vivo* at least 50%, 75%, 100%, 150% or 200% greater than that of an unmodified polypeptide.

Nucleic acids, vectors and recombinant host cells

A further object of the present invention relates to a nucleic acid sequence encoding for a polypeptide or a fusion protein according to the invention.

As used herein, a sequence "encoding" an expression product, such as a RNA or polypeptide, is a nucleic acid sequence that, when expressed, results in the production of that

RNA or polypeptide. A coding sequence for a polypeptide may include a start codon (usually ATG) and a stop codon.

These nucleic acid sequences can be obtained by conventional methods well known to those skilled in the art.

Typically, said nucleic acid is a DNA or RNA molecule, which may be included in a suitable vector, such as a plasmid, cosmid, episome, artificial chromosome, phage or viral vector.

So, a further object of the present invention relates to a vector and an expression cassette in which a nucleic acid molecule encoding for a polypeptide or a fusion protein of the invention is associated with suitable elements for controlling transcription (in particular promoter, enhancer and, optionally, terminator) and, optionally translation, and also the recombinant vectors into which a nucleic acid molecule in accordance with the invention is inserted. These recombinant vectors may, for example, be cloning vectors, or expression vectors.

As used herein, the terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and translation) of the introduced sequence.

Any expression vector for animal cell can be used. Examples of suitable vectors include pAGE107 (Miyaji et al, 1990), pAGE103 (Mizukami and Itoh, 1987), pHSG274 (Brady et al, 1984), pKCR (O'Hare et al, 1981), pSGl beta d2-4 (Miyaji et al, 1990) and the like.

Other examples of plasmids include replicating plasmids comprising an origin of replication, or integrative plasmids, such as for instance pUC, pcDNA, pBR, and the like.

Other examples of viral vectors include adenoviral, lentiviral, retroviral, herpes virus and AAV vectors. Such recombinant viruses may be produced by techniques known in the art, such as by transfecting packaging cells or by transient transfection with helper plasmids or viruses. Typical examples of virus packaging cells include PA317 cells, PsiCRIP cells, GPenv+ cells, 293 cells, etc. Detailed protocols for producing such replication-defective recombinant viruses may be found for instance in WO 95/14785, WO 96/22378, US 5,882,877, US 6,013,516, US 4,861,719, US 5,278,056 and WO 94/19478.

Examples of promoters and enhancers used in the expression vector for animal cell include early promoter and enhancer of SV40 (Mizukami and Itoh, 1987), LTR promoter and

enhancer of Moloney mouse leukemia virus (Kuwana et al, 1987), promoter (Mason et al, 1985) and enhancer (Gillies et al., 1983) of immunoglobulin H chain and the like.

A further aspect of the invention relates to a host cell comprising a nucleic acid molecule encoding for a polypeptide or a fusion protein according to the invention or a vector according to the invention. In particular, a subject of the present invention is a prokaryotic or eukaryotic host cell genetically transformed with at least one nucleic acid molecule or vector according to the invention.

The term "transformation" means the introduction of a "foreign" (i.e. extrinsic or extracellular) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. A host cell that receives and expresses introduced DNA or RNA has been "transformed".

In a particular embodiment, for expressing and producing polypeptides or fusion proteins of the invention, prokaryotic cells, in particular *E. coli* cells, will be chosen. Actually, according to the invention, it is not mandatory to produce the polypeptide or the fusion protein of the invention in a eukaryotic context that will favour post-translational modifications (e.g. glycosylation). Furthermore, prokaryotic cells have the advantages to produce protein in large amounts. If a eukaryotic context is needed, yeasts (e.g. *saccharomyces* strains) may be particularly suitable since they allow production of large amounts of proteins. Otherwise, typical eukaryotic cell lines such as CHO, BHK-21, COS-7, C127, PER.C6, YB2/0 or HEK293 could be used, for their ability to process to the right post-translational modifications of the fusion protein of the invention.

The construction of expression vectors in accordance with the invention, and the transformation of the host cells can be carried out using conventional molecular biology techniques. The polypeptide or the fusion protein of the invention, can, for example, be obtained by culturing genetically transformed cells in accordance with the invention and recovering the polypeptide or the fusion protein expressed by said cell, from the culture. They may then, if necessary, be purified by conventional procedures, known in themselves to those skilled in the art, for example by fractional precipitation, in particular ammonium sulfate precipitation, electrophoresis, gel filtration, affinity chromatography, etc. In particular, conventional methods for preparing and purifying recombinant polypeptides may be used for producing the polypeptides in accordance with the invention.

A further aspect of the invention relates to a method for producing a polypeptide or a fusion protein of the invention comprising the step consisting of: (i) culturing a transformed

host cell according to the invention under conditions suitable to allow expression of said polypeptide or fusion protein; and (ii) recovering the expressed polypeptide or fusion protein.

A further aspect of the invention relates to the polypeptide or the fusion protein of the invention for use as a medicament.

Polypeptides and fusion proteins of the invention find use as an inhibitor of the interaction between PRICKLE 1 and RICTOR and accordingly as an inhibitor of the PRICKLE l-MINKl-mTORC2 complex.

Accordingly, the present invention also relates to the polypeptide or the fusion protein of the invention for use in the prevention and treatment of cancer and cancer metastasis in a subject in need thereof.

In a further aspect, the present invention also relates to a method for preventing and treating cancer and cancer metastasis in a patient comprising administering to said patient a therapeutically effective amount of an inhibitor of the PRICKLE l-MINKl-mTORC2 complex.

By a "therapeutically effective amount" of an inhibitor of the PRICKLE 1-MINKl-mTORC2 complex as above described is meant a sufficient amount of the inhibitor to prevent or treat cancer and cancer metastasis. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidential with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to

about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

Pharmaceutical compositions of the invention:

The PRICKLE l-MINKl-mTORC2 complex inhibitor as described above may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

Accordingly, the present invention also relates to a pharmaceutical composition for use in the prevention or treatment of cancer and cancer metastasis comprising a PRICKLE 1-MINKl-mTORC2 complex inhibitor according to the invention and a pharmaceutically acceptable carrier.

In a particular embodiment, the PRICKLE l-MINKl-mTORC2 complex inhibitor is rapamycin (sirolimus).

In a particular embodiment, the PRICKLE l-MINKl-mTORC2 complex inhibitor such as rapamycin may be used in high doses. Typically, the term "high doses" refers to PRICKLE l-MINKl-mTORC2 complex inhibitor concentration (such as rapamycin) higher than 20, 30, 40, 50, 60, 70, 80, 90 and 100 nM. Particularly, the term "high doses" refers to PRICKLE l-MINKl-mTORC2 complex inhibitor concentration such as rapamycin of 100 nM per day. Particularly, an effective amount of the inhibitor is supplied at a dosage level of 10 mg/kg of body weight per day.

In one embodiment, the pharmaceutical composition for use according to the invention further comprises an additional therapeutic agent.

In one embodiment said therapeutic active agent is an anti-cancer agent. For example, said anticancer agents include but are not limited to vemurafenib, fotemustine, fludarabine, gemcitabine, capecitabine, methotrexate, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, platinum complexes such as cisplatin, carboplatin and oxaliplatin, mitomycin, dacarbazine, procarbazine, teniposide, campathecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, L-asparaginase, epimbicm, 5-fluorouracil, taxanes such as docetaxel and paclitaxel, leucovorin, levamisole, irinotecan, estramustine, etoposide, nitrogen mustards, BCNU, nitrosoureas such as carmustme and lomustine, vinca alkaloids such as vinblastine, vincristine and vinorelbine, imatimb mesylate, hexamethyhnelamine, topotecan, kinase inhibitors, phosphatase inhibitors, ATPase inhibitors, tyrphostins, protease inhibitors, inhibitors herbimycm A, genistein, erbstatin, and lavendustin A. In one embodiment,

additional anticancer agents may be selected from, but are not limited to, one or a combination of the following class of agents: alkylating agents, plant alkaloids, DNA topoisomerase inhibitors, anti-folates, pyrimidine analogs, purine analogs, DNA antimetabolites, taxanes, podophyllotoxin, hormonal therapies, retinoids, photosensitizers or photodynamic therapies, angiogenesis inhibitors, antimitotic agents, isoprenylation inhibitors, cell cycle inhibitors, actinomycins, bleomycins, anthracyclines, MDR inhibitors and Ca2+ ATPase inhibitors.

Additional anticancer agents may be selected from, but are not limited to, cytokines, chemokines, growth factors, growth inhibitory factors, hormones, soluble receptors, decoy receptors, monoclonal or polyclonal antibodies, mono-specific, bi-specific or multi-specific antibodies, monobodies, polybodies.

Additional anticancer agent may be selected from, but are not limited to, growth or hematopoietic factors such as erythropoietin and thrombopoietin, and growth factor mimetics thereof.

In the present methods for treating cancer the further therapeutic active agent can be an antiemetic agent. Suitable antiemetic agents include, but are not limited to, metoclopromide, domperidone, prochlorperazine, promethazine, chlorpromazine, trimethobenzamide, ondansetron, granisetron, hydroxyzine, acethylleucine monoemanolamine, alizapride, azasetron, benzquinamide, bietanautine, bromopride, buclizine, clebopride, cyclizine, dunenhydrinate, diphenidol, dolasetron, meclizme, methallatal, metopimazine, nabilone, oxypemdyl, pipamazine, scopolamine, sulpiride, tetrahydrocannabinols, thiefhylperazine, thioproperazine and tropisetron. In a preferred embodiment, the antiemetic agent is granisetron or ondansetron.

In another embodiment, the further therapeutic active agent can be an hematopoietic colony stimulating factor. Suitable hematopoietic colony stimulating factors include, but are not limited to, filgrastim, sargramostim, molgramostim and epoietin alpha.

In still another embodiment, the other therapeutic active agent can be an opioid or non-opioid analgesic agent. Suitable opioid analgesic agents include, but are not limited to, morphine, heroin, hydromorphone, hydrocodone, oxymorphone, oxycodone, metopon, apomorphine, nomioiphine, etoipbine, buprenorphine, mepeddine, lopermide, anileddine, ethoheptazine, piminidine, betaprodine, diphenoxylate, fentanil, sufentanil, alfentanil, remifentanil, levorphanol, dextromethorphan, phenazodne, pemazocine, cyclazocine, methadone, isomethadone and propoxyphene. Suitable non-opioid analgesic agents include, but are not limited to, aspirin, celecoxib, rofecoxib, diclofmac, diflusinal, etodolac,

fenoprofen, flurbiprofen, ibuprofen, ketoprofen, indomethacin, ketorolac, meclofenamate, mefanamic acid, nabumetone, naproxen, piroxicam and sulindac.

In yet another embodiment, the further therapeutic active agent can be an anxiolytic agent. Suitable anxiolytic agents include, but are not limited to, buspirone, and benzodiazepines such as diazepam, lorazepam, oxazapam, chlorazepate, clonazepam, chlordiazepoxide and alprazolam.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described, in an amount sufficient to cure or at least partially stop the symptoms of the disease and its complications. An appropriate dosage of the pharmaceutical composition is readily determined according to any one of several well-established protocols. For example, animal studies (for example on mice or rats) are commonly used to determine the maximal tolerable dose of the bioactive agent per kilogram of weight. In general, at least one of the animal species tested is mammalian. The results from the animal studies can be extrapolated to determine doses for use in other species, such as humans for example. What constitutes an effective dose also depends on the nature and severity of the disease or condition, and on the general state of the patient's health.

In prophylactic applications, compositions containing, for example the PRICKLE 1-MINKl-mTORC2 complex inhibitors, are administered to a patient susceptible to or otherwise at risk of APS. Such an amount is defined to be a "prophylactically effective" amount or dose. In this use, the precise amount depends on the patient's state of health and weight.

In both therapeutic and prophylactic treatments, the inhibitor contained in the pharmaceutical composition can be administered in several dosages or as a single dose until a desired response has been achieved. The treatment is typically monitored and repeated dosages can be administered as necessary. Compounds of the invention may be administered according to dosage regimens established whenever inactivation of the the PRICKLE 1-MINKl-mTORC2 complex is required.

The daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 10 mg/kg of body weight per day. It will be understood, however, that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability, and length of action of that compound, the age, the body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

Predictive methods:

A second aspect of the invention relates to a method for predicting the survival time of a patient suffering from breast cancer comprising the steps of: i) determining in a sample obtained from the patient the expression level of PRICKLE 1 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

In one embodiment, the gene expression level of PRICKLE 1 is determined.

In one embodiment, the breast cancer is a breast metastatic cancer.

The invention also relates to a method for predicting the overall survival (OS) of a patient suffering from breast cancer comprising the steps of: i) determining in a sample obtained from the patient the expression level of PRICKLE 1 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

The invention also relates to a method for predicting the event-free survival (EFS) of a patient suffering from breast cancer comprising the steps of: i) determining in a sample obtained from the patient the expression level of PRICKLE 1 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

The invention also relates to a method for predicting the metastasis-free survival (MFS) of a patient suffering from breast cancer comprising the steps of: i) determining in a sample obtained from the patient the expression level of PRICKLE 1 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

As used herein, the term "Overall survival (OS)" denotes the percentage of people in a study or treatment group who are still alive for a certain period of time after they were diagnosed with or started treatment for a disease, such as breast cancer (according to the invention). The overall survival rate is often stated as a five-year survival rate, which is the percentage of people in a study or treatment group who are alive five years after their diagnosis or the start of treatment.

As used herein, the term "Event-Free Survival (EFS)" denotes the length of time after primary treatment for a cancer ends that the patient remains free of certain complications or events that the treatment was intended to prevent or delay. These events may include the return of the cancer or the onset of certain symptoms, such as bone pain from cancer that has spread to the bone.

As used herein, the term "Metastasis Free Survival (MFS)" denotes the length of time after primary treatment for a cancer ends that the patient develop metastasis.

As used herein, the term "Good Prognosis" denotes a patient with more than 50% chance of survival for the next 5 years after the treatment.

As used herein and according to all aspects of the invention, the term "sample" denotes biopsy and particularly breast cancer biopsy, blood, serum or plasma.

Measuring the expression level of a gene can be performed by a variety of techniques well known in the art.

Typically, the expression level of a gene may be determined by determining the quantity of mRNA. Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the patient) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis, in situ hybridization) and/or amplification (e.g., RT-PCR).

Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In certain embodiments, it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization.

Typically, the nucleic acid probes include one or more labels, for example to permit detection of a target nucleic acid molecule using the disclosed probes. In various applications, such as in situ hybridization procedures, a nucleic acid probe includes a label (e.g., a detectable label). A "detectable label" is a molecule or material that can be used to produce a detectable signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or concentration of a target nucleic acid sequence (e.g., genomic target nucleic acid sequence) (to which the labeled uniquely specific nucleic acid molecule is bound or hybridized) in a sample. A label associated with one or more nucleic acid molecules (such as a probe generated by the disclosed methods) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including

absorption, emission and/ or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons). Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

Particular examples of detectable labels include fluorescent molecules (or fiuorochromes). Numerous fiuorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies (formerly Invitrogen), e.g., see, The Handbook—A Guide to Fluorescent Probes and Labeling Technologies). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Pat. No. 5,866, 366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2' disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl) aminonaphthalene-1 -sulfonic acid (EDANS), 4-amino -N- [3 vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-l-naphthyl)maleimide, antllranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifiuoromethylcouluarin (Coumarin 151); cyanosine; 4',6-diarninidino-2-phenylindole (DAPI); 5',5''dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7 -diethylamino -3 - (4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulforlic acid; 5-[dimethylamino] naphthalene- 1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfiuorescein (FAM), 5-(4,6diclllorotriazin-2-yDarnino fluorescein (DTAF), 2'7'dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC Q(RITC); 2',7'-difiuorofiuorescein (OREGON GREEN®); fiuorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives such as pyrene, pyrene butyrate

and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives. Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 mn (Heyduk and Heyduk, Analyt. Biochem. 248:216-27, 1997; J. Biol. Chem. 274:3315-22, 1999), as well as GFP, LissamineTM, diethylaminocoumarin, fluorescein chlorotriazinyl, naphtho fluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No. 5,800,996 to Lee et al.) and derivatives thereof. Other fluorophores known to those skilled in the art can also be used, for example those available from Life Technologies (Invitrogen; Molecular Probes (Eugene, Oreg.)) and including the ALEXA FLUOR® series of dyes (for example, as described in U.S. Pat. Nos. 5,696,157, 6, 130, 101 and 6,716,979), the BODIPY series of dyes (dipyrrometheneboron difluoride dyes, for example as described in U.S. Pat. Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,1 13, 5,338,854, 5,451,663 and 5,433,896), Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Pat. No. 5,132,432) and Marina Blue (U.S. Pat. No. 5,830,912).

In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, e.g., a QUANTUM DOTTM (obtained, for example, from Life Technologies (QuantumDot Corp, Invitrogen Nanocrystal Technologies, Eugene, Oreg.); see also, U.S. Pat. Nos. 6,815,064; 6,682,596; and 6,649, 138). Semiconductor nanocrystals are microscopic particles having size-dependent optical and/or electrical properties. When semiconductor nanocrystals are illuminated with a primary energy source, a secondary emission of energy occurs of a frequency that corresponds to the handgap of the semiconductor material used in the semiconductor nanocrystal. This emission can he detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can he coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in, for example, Bruchez et al, Science 281 :20132016, 1998; Chan et al, Science 281:2016-2018, 1998; and U.S. Pat. No. 6,274,323. Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927, 069; 6,914,256; 6,855,202;

6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,1 14,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Publication No. 2003/0165951 as well as PCT Publication No. 99/26299 (puhlished May 27, 1999). Separate populations of semiconductor nanocrystals can he produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can he produced that emit light of different colors hased on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 mn, 655 mn, 705 mn, or 800 mn emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlshad, Calif).

Additional labels include, for example, radioisotopes (such as 3 H), metal chelates such as DOTA and DPTA chelates of radioactive or paramagnetic metal ions like Gd3+, and liposomes.

Detectable labels that can he used with nucleic acid molecules also include enzymes, for example horseradish peroxidase, alkaline phosphatase, acid phosphatase, glucose oxidase, beta-galactosidase, beta-glucuronidase, or beta-lactamase.

Alternatively, an enzyme can he used in a metallographic detection scheme. For example, silver in situ hyhridization (SISH) procedures involve metallographic detection schemes for identification and localization of a hybridized genomic target nucleic acid sequence. Metallographic detection methods include using an enzyme, such as alkaline phosphatase, in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. The substrate is converted to a redox-active agent by the enzyme, and the redoxactive agent reduces the metal ion, causing it to form a detectable precipitate. (See, for example, U.S. Patent Application Publication No. 2005/0100976, PCT Publication No. 2005/ 003777 and U.S. Patent Application Publication No. 2004/ 0265922). Metallographic detection methods also include using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to form a detectable precipitate. (See, for example, U.S. Pat. No. 6,670,1 13).

Probes made using the disclosed methods can be used for nucleic acid detection, such as ISH procedures (for example, fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) and silver in situ hybridization (SISH)) or comparative genomic hybridization (CGH).

In situ hybridization (ISH) involves contacting a sample containing target nucleic acid sequence (e.g., genomic target nucleic acid sequence) in the context of a metaphase or

interphase chromosome preparation (such as a cell or tissue sample mounted on a slide) with a labeled probe specifically hybridizable or specific for the target nucleic acid sequence (e.g., genomic target nucleic acid sequence). The slides are optionally pretreated, e.g., to remove paraffin or other materials that can interfere with uniform hybridization. The sample and the probe are both treated, for example by heating to denature the double stranded nucleic acids. The probe (formulated in a suitable hybridization buffer) and the sample are combined, under conditions and for sufficient time to permit hybridization to occur (typically to reach equilibrium). The chromosome preparation is washed to remove excess probe, and detection of specific labeling of the chromosome target is performed using standard techniques.

For example, a biotinylated probe can be detected using fluorescein-labeled avidin or avidin-alkaline phosphatase. For fluorochrome detection, the fluorochrome can be detected directly, or the samples can be incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin. Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat antiavidin antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples can be incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline phosphatase, washed again and pre-equilibrated (e.g., in alkaline phosphatase (AP) buffer). For a general description of in situ hybridization procedures, see, e.g., U.S. Pat. No. 4,888,278.

Numerous procedures for FISH, CISH, and SISH are known in the art. For example, procedures for performing FISH are described in U.S. Pat. Nos. 5,447,841; 5,472,842; and 5,427,932; and for example, in Pirlkel et al, Proc. Natl. Acad. Sci. 83:2934-2938, 1986; Pinkel et al, Proc. Natl. Acad. Sci. 85:9138-9142, 1988; and Lichter et al, Proc. Natl. Acad. Sci. 85:9664-9668, 1988. CISH is described in, e.g., Tanner et al, Am. .1. Pathol. 157:1467-1472, 2000 and U.S. Pat. No. 6,942,970. Additional detection methods are provided in U.S. Pat. No. 6,280,929.

Numerous reagents and detection schemes can be employed in conjunction with FISH, CISH, and SISH procedures to improve sensitivity, resolution, or other desirable properties. As discussed above probes labeled with fluorophores (including fluorescent dyes and QUANTUM DOTS®) can be directly optically detected when performing FISH. Alternatively, the probe can be labeled with a nonfluorescent molecule, such as a hapten (such as the following non-limiting examples: biotin, digoxigenin, DNP, and various oxazoles, pyrrazoles, thiazoles, nitroaryls, benzofurazans, triterpenes, ureas, thioureas, rotenones, coumarin, courmarin-based compounds, Podophyllotoxin, Podophyllotoxin-based

compounds, and combinations thereof), ligand or other indirectly detectable moiety. Probes labeled with such non-fluorescent molecules (and the target nucleic acid sequences to which they bind) can then be detected by contacting the sample (e.g., the cell or tissue sample to which the probe is bound) with a labeled detection reagent, such as an antibody (or receptor, or other specific binding partner) specific for the chosen hapten or ligand. The detection reagent can be labeled with a fluorophore (e.g., QUANTUM DOT®) or with another indirectly detectable moiety, or can be contacted with one or more additional specific binding agents (e.g., secondary or specific antibodies), which can be labeled with a fluorophore.

In other examples, the probe, or specific binding agent (such as an antibody, e.g., a primary antibody, receptor or other binding agent) is labeled with an enzyme that is capable of converting a fluorogenic or chromogenic composition into a detectable fluorescent, colored or otherwise detectable signal (e.g., as in deposition of detectable metal particles in SISH). As indicated above, the enzyme can be attached directly or indirectly via a linker to the relevant probe or detection reagent. Examples of suitable reagents (e.g., binding reagents) and chemistries (e.g., linker and attachment chemistries) are described in U.S. Patent Application Publication Nos. 2006/0246524; 2006/0246523, and 2007/ 0 1 17153.

It will he appreciated by those of skill in the art that by appropriately selecting labelled probe-specific binding agent pairs, multiplex detection schemes can he produced to facilitate detection of multiple target nucleic acid sequences (e.g., genomic target nucleic acid sequences) in a single assay (e.g., on a single cell or tissue sample or on more than one cell or tissue sample). For example, a first probe that corresponds to a first target sequence can he labelled with a first hapten, such as biotin, while a second probe that corresponds to a second target sequence can be labelled with a second hapten, such as DNP. Following exposure of the sample to the probes, the bound probes can he detected by contacting the sample with a first specific binding agent (in this case avidin labelled with a first fluorophore, for example, a first spectrally distinct QUANTUM DOT®, e.g., that emits at 585 mn) and a second specific binding agent (in this case an anti-DNP antibody, or antibody fragment, labelled with a second fluorophore (for example, a second spectrally distinct QUANTUM DOT®, e.g., that emits at 705 mn). Additional probes/binding agent pairs can he added to the multiplex detection scheme using other spectrally distinct fluorophores. Numerous variations of direct, and indirect (one step, two step or more) can he envisioned, all of which are suitable in the context of the disclosed probes and assays.

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and

700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. The probes and primers are "specific" to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature Tm, e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

In a particular embodiment, the methods of the invention comprise the steps of providing total RNAs extracted from cumulus cells and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.

In another preferred embodiment, the expression level is determined by DNA chip analysis. Such DNA chip or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be about 10 to about 60 base pairs. To determine the expression level, a sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids that are complementary to probe sequences attached to the microarray surface. The labelled hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, e.g. by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man skilled in the art (see e.g. the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210).

Expression level of a gene may be expressed as absolute expression level or normalized expression level. Typically, expression levels are normalized by correcting the absolute expression level of a gene by comparing its expression to the expression of a gene

that is not a relevant for determining the cancer stage of the patient, e.g., a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene ACTB, ribosomal 18S gene, GUSB, PGK1 and TFRC. According to the invention the housekeeping genes used were GAPDH, GUSB, TBP and ABL1. This normalization allows the comparison of the expression level in one sample, e.g., a patient sample, to another sample, or between samples from different sources.

Predetermined reference values used for comparison may comprise "cut-off or "threshold" values that may be determined as described herein. Each reference ("cut-off) value for each gene of interest may be predetermined by carrying out a method comprising the steps of

a) providing a collection of samples from patients suffering of breast cancer;

b) determining the expression level of PRICKLE 1 for each sample contained in the collection provided at step a);

c) ranking the tumor tissue samples according to said expression level

d) classifying said samples in pairs of subsets of increasing, respectively decreasing, number of members ranked according to their expression level,

e) providing, for each sample provided at step a), information relating to the responsiveness of the patient or the actual clinical outcome for the corresponding cancer patient (i.e. the duration of the event-free survival (EFS), metastasis- free survival (MFS) or the overall survival (OS) or both);

f) for each pair of subsets of samples, obtaining a Kaplan Meier percentage of survival curve;

g) for each pair of subsets of samples calculating the statistical significance (p value) between both subsets

h) selecting as reference value for the expression level, the value of expression level for which the p value is the smallest.

For example the expression level of a gene X has been assessed for 100 AML samples of 100 patients. The 100 samples are ranked according to their expression level. Sample 1 has the best expression level and sample 100 has the worst expression level. A first grouping provides two subsets: on one side sample Nr 1 and on the other side the 99 other samples. The next grouping provides on one side samples 1 and 2 and on the other side the 98 remaining samples etc., until the last grouping: on one side samples 1 to 99 and on the other side sample Nr 100. According to the information relating to the actual clinical outcome for the

corresponding AML patient, Kaplan Meier curves are prepared for each of the 99 groups of two subsets. Also for each of the 99 groups, the p value between both subsets was calculated.

The reference value is selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other terms, the expression level corresponding to the boundary between both subsets for which the p value is minimum is considered as the reference value. It should be noted that the reference value is not necessarily the median value of expression levels.

In routine work, the reference value (cut-off value) may be used in the present method to discriminate AML samples and therefore the corresponding patients.

Kaplan-Meier curves of percentage of survival as a function of time are commonly to measure the fraction of patients living for a certain amount of time after treatment and are well known by the man skilled in the art.

The man skilled in the art also understands that the same technique of assessment of the expression level of a gene should of course be used for obtaining the reference value and thereafter for assessment of the expression level of a gene of a patient subjected to the method of the invention.

Such predetermined reference values of expression level may be determined for any gene defined above.

According to the invention, the level of the protein PRICKLE 1 may also be measured to determine the survival time of a patient affected with breast cancer.

Thus, the invention also relates to a method for predicting the survival time of a patient suffering from breast cancer i) determining in a sample obtained from the patient the concentration of the PRICKLE 1 protein ii) comparing the concentration determined at step i) with its predetermined reference value and iii) providing a good prognosis when the concentration determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the concentration determined at step i) is higher than its predetermined reference value.

Measuring the level of the protein can be performed by a variety of techniques well known in the art.

Typically protein concentration may be measured for example by capillary electrophoresis-mass spectroscopy technique (CE-MS) or ELISA performed on the sample.

Detection of protein concentration in the sample may also be performed by measuring the level of protein (PRICKLEl). In the present application, the "level of protein" means the

quantity or concentration of said proteins. In another embodiment, the "level of protein" means the level of protein fragments (PRICKLEl fragments).

Such methods comprise contacting a sample with a binding partner capable of selectively interacting with proteins present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal.

The presence of the protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; Immunoelectrophoresis; immunoprecipitation, capillary electrophoresis-mass spectroscopy technique (CE-MS).etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies against the proteins to be tested. A sample containing or suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule is added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate is washed and the presence of the secondary binding molecule is detected using methods well known in the art.

Methods of the invention may comprise a step consisting of comparing the proteins and fragments concentration in circulating cells with a control value. As used herein, "concentration of proteins" refers to an amount or a concentration of a transcription product, for instance the proteins Chkl or Pol theta. Typically, a level of a protein can be expressed as nanograms per microgram of tissue or nanograms per milliliter of a culture medium, for

example. Alternatively, relative units can be employed to describe a concentration. In a particular embodiment, "concentration of proteins" may refer to fragments of the proteins (Chkl or Pol theta). Thus, in a particular embodiment, fragments of Chkl or Pol theta may also be measured.

A further object of the invention relates to kits for performing the methods of the invention, wherein said kits comprise means for measuring the expression level of CHK1 and/or POLQ genes of the invention in the sample obtained from the patient.

The kits may include probes, primers macroarrays or microarrays as above described. For example, the kit may comprise a set of probes as above defined, usually made of DNA, and that may be pre-labelled. Alternatively, probes may be unlabelled and the ingredients for labelling may be included in the kit in separate containers. The kit may further comprise hybridization reagents or other suitably packaged reagents and materials needed for the particular hybridization protocol, including solid-phase matrices, if applicable, and standards. Alternatively the kit of the invention may comprise amplification primers that may be pre-labelled or may contain an affinity purification or attachment moiety. The kit may further comprise amplification reagents and also other suitably packaged reagents and materials needed for the particular amplification protocol.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: Overexpression of Prickle 1 mRNA in breast cancer is correlated with poor prognosis.

A. Box plot of PRICKLE 1 expression across 36 breast cancer cell lines. The data show an increase of PRICKLE 1 mRNA expression in basal/mesenchymal cell lines compared with luminal cell lines. B. Kaplan-Meier curves of the metastasis free survival among basal breast cancers having overexpression (Up) of PRICKLE 1 mRNA with at least higher than 2 fold SD vs no overexpression (noUp) of PRICKLEl mRNA. *P <0.05; ***P<0.001.

Figure 2: PRICKLE and MINK1 are involved in cell migration and cell proliferation ofMDA-MB-231 cells.

A. Inhibition of PRICKLEl expression decreases MDA-MB-231 cell motility. Two independent siRNAs targeting PRICKLEl or a control siRNA (Non Targeting, NT) were

transfected into MDA-MB-231 cells. Loss of PRICKLE1 mRNA expression was confirmed by quantitative PCR (left panel). Transfected cells were subjected to a cell migration assay using Boyden chambers (right panel). Inhibition of PRICKLE1 expression decreased MDA-MB-231 cell motility. B. Same as (A) using two independent siRNAs targeting MINK1 expression. C-D. PRICKLE1 (C) and MINK1 (D) play a role in cell proliferation. Two independent siRNAs directed against PRICKLE1 and MINK1 were transfected in MDA-MB-231 cells. Cell proliferation was measured at the indicated times.

Figure 3: Interaction of PRICKLE1 with MINK1 is required for cell migration.

A. MDA-MB-231cells were stably transfected by two independent shRNAs directed against PRICKLE1 and cells were subjected to Boyden chamber assays (top panel). Downregulation of PRICKLE mRNA was confirmed by semi-quantitative PCR shown in the bottom panel. B. MDA-MB-231 stably expressing low level of venus-PRICKLE1 or venus-PRICKLE1 resistant to shRNA PRICKLE1 (#405) (VENUS-PRICKLE1 res) were transfected with a shRNA (#405) targeting PRICKLE1. MDA-MB-231 cells were then subjected to migration assay using boyden chamber assay. Migration of MDA-MB-231 cells expressing VENUS-PRICKLE1 was inhibited upon shRNA treatment in contrast to MDA-MB-231 cells expressing VENUS-PRICKLE1 resistant to the shRNA. C. Interaction between PRICKLE1 and MINK1 is required for MDA-MB-231 cell motility. MDA-MB-231 cells stably expressing VENUS-PRICKLE1 or VENUS-PRICKLE1 resistant to the shRNA lacking the LIM2 domain (VENUS-PRICKLE1 ΔLIM2 res) or mutated for the MINK1 phosphorylation site (VENUS-PRICKLE1 P mutant res) were subjected to shRNA treatment targeting PRICKLE1 (#405). Expression of the constructs was evaluated by western blot with anti-GFP antibody. Cells were subjected to Boyden chamber assays: PRICKLE1 mutants lacking the MINK1 interaction domain (LIM2) or mutated on the MINK1 phosphorylation site were unable to rescue PRICKLE1 downregulation. A-C. Statistics were performed using the results of three independent experiments using ANOVA and Tukey post-test. ***P<0.001.

Figure 4: MINK1 plays a role in tumor growth and cancer cell dissemination.

A. MDA-MB-231 cells subjected to stable downregulation of MINK1 were assayed in Boyden chamber assays: downregulation of MINK1 was associated with a decrease of cell proliferation. B. MDA-MB-231 cells subjected to stable downregulation of MINK1 were evaluated in cell proliferation assays: downregulation of MINK1 was associated with a decrease of cell proliferation. C. Luciferase-positive MDA-MB-231 cells stably expressing shRNAs were xenografted into the fat pads of SCID mice. After 35 days, the volume of

primary tumors was measured: downregulation of MINK1 was associated with a decrease of tumor volume.

Figure 5: Dominant negative effect of the CI domain of PRICKLE 1 on tumor growth and formation of metastasis. A. MDA-MB-23 1 cells expressing VENUS or VENUS-Cl of PRICKLE 1 were assayed for cell proliferation. B. Mice were xenografted with lucifierease-positive MDA-MB-23 1 cells expressing VENUS or VENUS-Cl of PRICKLE 1. The volume of primary tumors was measured after 35 days. Mice xenografted with MDA-MB-23 1 expressing VENUS-Cl have lung metastasis in 5 over 8 cases. The luminescent signals were quantified and results are represented in (C). A similar study was done for liver metastasis and quantification was reported in (D). ANOVA with Dunnett's correction for multiple testing was used to assess the significance of differences among the different groups of animals. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

EXAMPLE:

Material & Methods

Cell culture, transfection and antibodies

HEK293T, MDA-MB-23 1 cells were obtained from the ATCC. Cells were grown in DMEM containing 10%FCS. Transfections were performed using Polyethyleimine (PEI) or Lipfectamine 2000 (Invitrogen). Antibodies targeting MINK1 were obtained from Bethyl. The following antibodies were obtained from Cell Signaling: a-AKT, a-pS473-AKT, a-pT308-AKT, a-RICTOR, a-mTOR.

DNA constructs, siRNAs and shRNAs

The following sequence of siRNA were used to target MINK1 (#9) GGAACAAACUGCGGGUGUA (SEQ ID NO: 1), MINK1 (#10) GAAGUGGUCUAAGAAGUUC (SEQ ID NO: 2), PRICKLE 1 (#02) GAGAGAAGCAUCGGAUUAA (SEQ ID NO: 5), PRICKLE 1 (#04) GAAGAUAAAUGGAGGUGAA (SEQ ID NO: 6). shRNA were cloned into PLKO backbone and lentiviral particles were produced using PSPAX and VSV-G system in HEK293T cells. The following sequence of shRNA were used to target MINK1 (#03) AGCGGCTCAAGGTCATCTATG (SEQ ID NO: 3), MINK1 (#06) GCTACTGAAGTTTCCCTTCAT (SEQ ID NO: 4), PRICKLE 1 (#403) GCTCAGCATGTGACGAGATAA (SEQ ID NO: 7) and PRICKLE 1 (#405) CCACCACATGATAATGAGGTA (SEQ ID NO: 8). Lentiviral vector coding for shRNA targeting RICTOR were obtained from Addgene (plasmid #1854 and #1853). PSL9-Venus-

Paxillin construct is described elsewhere. AKT1 was cloned under Ascl/Ecorl site of PGEX4 backbone. Myc-RICTOR and myc-RAPTOR were obtained from Addgene (plasmid #11367 and #1859).

Boyden chamber assays

50,000 cells were counted and loaded in serum starve condition in the upper chamber of a Boyden chamber. Lower chamber is filled with media supplemented with 5%FCS. After 12 hours, migrating cells were gently recover from the bottom side of the chamber by trypsin treatment and counted using Promega cell titer assay.

In vitro kinase assay

PRICKLE 1 complex or RADIL complex were purified from HEK293T cells stably expressing FLAG-PRICKLE 1. FLAG-PRICKLE 1 or FLAG-RADIL protein complexes were incubated with soluble recombinant GST-AKT1 protein purified from Escherichia coli. Phosphorylation reactions were performed in kinase buffer (25 mMHepes, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCL2, 0.1 mM Na3V04, 0.5 mM DTT) supplemented with 20μM ATP at 37°C for 1 hour. Reactions were stopped by addition of 4xLaemmli sample buffer. Proteins were resolved by SDS-PAGE and analyzed by a-Serine 473-AKT antibody.

Immunopurification

5X107 HEK293T cells expressing either FLAG-PRICKLE 1 were used for affinity purification procedure. Briefly, cells were lysed and solubilised in TAP lysis buffer (0.1% Igepal CA 630, 10% glycerol, 50mM Hepes-NaOH; pH 8.0, 150mM NaCl, 2mM EDTA, 2mM DTT, lOmM NaF, 0.25mM NaOV03, 50mM β-glycerophosphate, and protease inhibitor cocktail (Calbiochem). After 30 min centrifugation at 40,000xg (18,000rpm in a Beckman JA20 rotor), the soluble fraction is incubated overnight at 4°C with anti-FLAG M2 beads (Sigma). Beads were washed with TAP lysis buffer followed by 50mM ammonium bicarbonate. Finally, proteins were eluted twice from the beads using 200μL of 500mM ammonium hydroxide at pH 11.0. The eluted fraction is evaporated by speed vac and the remaining pellet is then washed with IOOμL of milliQ water. The pellet is then resuspended in 50mM ammonium bicarbonate, reduced with 25mM DTT and alkylated with lOOmM iodoacetamide (Sigma) and supplemented with ImM CaC12 (Bioshop) prior to digestion with 1μg of sequencing-grade trypsin (Promega).

Mass spectrometry analysis

Mass spectrometry analysis was carried out by LC-MSMS using a LTQ-Velos-Orbitrap (Thermo Electron, Bremen, Germany) online with a nanoLC Ultimate 3000 chromatography system (Dionex, Sunnyvale, CA). 5 microliters corresponding to l/5th of

whole sample were injected on the system. After pre-concentration and washing of the sample on a Dionex Acclaim PepMap 100 CI8 column (2 cm x 100 μιη i.d. 100 A, 5 μιη particle size), peptides were separated on a Dionex Acclaim PepMap RSLC C18 column (15 cm x 75 μιη i.d., 100 A, 2 μιη particle size) at a flow rate of 300 nL/min a two steps linear gradient (4-20% acetonitrile/H20; 0.1 % formic acid for 90 min and 20-45-45% acetonitrile/H20; 0.1 % formic acid for 30 min. The separation of the peptides was monitored by a UV detector (absorption at 214 nm). For peptides ionisation in the nanospray source, spray voltage was set at 1.4 kV and the capillary temperature at 275 °C. All samples were measured in a data dependent acquisition mode. Each run was preceded by a blank MS run in order to monitor system background. The peptide masses are measured in a survey full scan (scan range 300-1700 m/z, with 30 K FWHM resolution at m/z=400, target AGC value of 1.00x106 and maximum injection time of 500 ms). In parallel to the high-resolution full scan in the Orbitrap, the data-dependent CID scans of the 10 most intense precursor ions were fragmented and measured in the linear ion trap (normalized collision energy of 35 %, activation time of 10 ms, target AGC value of 1.00×104, maximum injection time 100 ms, isolation window 2 Da). Parent masses obtained in orbitrap analyser were automatically calibrated on 445.1200 locked mass. The fragment ion masses are measured in the linear ion trap to have a maximum sensitivity and the maximum amount of MS/MS data. Dynamic exclusion was implemented with a repeat count of 1 and exclusion duration of 30 s. Raw files generated from mass spectrometry analysis were processed with Proteome Discoverer 1.4 (ThermoFisher Scientific). This software was used to search data via in-house Mascot server (version 2.4.1; Matrix Science Inc., London, UK) against the Human subset (20,194 sequences) of the SwissProt database (version 2014_10). Database search were done using the following settings: a maximum of two trypsin miscleavage allowed, methionine oxidation, N-terminal protein acetylation as variable modifications, cysteine carbamido-methylation as fixed modification. A peptide mass tolerance of 6 ppm and a fragment mass tolerance of 0.8 Da were used for search analysis. Only peptides with high stringency Mascot score threshold (identity, FDR<1%) were used for protein identification.

Affinity purification, immunoprecipitation and western blot

48 hours post-transfection, cells were lysed with the TAP lysis buffer and incubated at 4°C for 1 hour to solubilize proteins. Affinity purification and immunoprecipitations were performed using either streptavidin resin (GE Healthcare) or anti-FLAG-M2 beads (Sigma) for 3 hours at 4°C. After extensive washes with lysis buffer, proteins were eluted with 2X Laemmli sample buffer and heated at 95°C for 5 min in the presence of β-mercaptoethanol

(Sigma). Whole cell lysates or purified protein samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Biotrace NT Nitrocellulose Transfer Membranes (Pall Corporation). Western blotting were performed with antibodies as indicated in the figures legends, followed by chemiluminescent detection using appropriate HRP-conjugated antibodies and the SuperSignal West Pico (Thermo Scientific) reagent.

TIRF experiments

For live-cell TIRF microscopy, engineered MDA-MB-23 1 stably expressing venus-PAXILLIN cells were siRNA transfected with the indicated siRNA and seeded 48 hours later on collagen coated glass bottom Petri dishes. Cells were imaged the next day with a Roper Scientic ILas2 laser illuminator for TIRF Microscopy with 491 nm laser excitation (Cobolt Calyso 100 mW) on a Axio Observer Z1 microscpe (ZEISS) and TIRF objective alpha plan neoFluar x100/1.48 with EM-CCD Evolve 512 Camera (photmetrics), and drivern by MetaMorph 7 software (Molecular devices).

Quantitative RT-PCR

Primers for PRICKLEl and MINK1 were used to amplify these genes.

Mouse experiments

NOD/SCID (non obese diabetic/severe combined immunodeficient)/ gc null mice (NSG) were obtained from Charles River Laboratory Charles River France or bred in-house and maintained under specific pathogen- free conditions. 5x105 Luciferase-expressing MDAMB-23 1 cells were transplanted (IOOμL PBS lx/matrigel, vol/vol) into mammary fat pads of mice (6-8 week-old female). After completion of the analysis, autopsy of mice was done, the tumors were weighed and organ luminescence was assessed. Bioluminescence analysis was performed using PhotonlMAGER (BiospaceLab), following intraperitoneal injection of luciferin (30 mg/kg).

Results

PRICKLEl as a biomarker

To investigate if PRICKLEl is altered in human breast cancer, we analyzed PRICKLEl mRNA expression in a panel of 45 breast cancer cell lines and 5410 primary invasive breast carcinomas. Basal/mesenchymal cell lines (N=19) showed higher PRICKLEl expression level than luminal cell lines (N=17; p=1.78E-02, Student t-test; Figure 1A), expression levels being similar between basal and mesenchymal cell lines (p=0.95, Student t-test; data not shown). In clinical samples, PRICKLEl was also overexpressed in basal breast

cancers when compared with luminal A, luminal B and ERBB2-positive breast cancers. We assessed the prognostic value of PRICKLEl expression (binary variable) in terms of metastasis-free survival (MFS). Within the 5,410 breast cancer samples tested, 860 tumors (16%) showed PRICKLEl upregulation when compared to normal breast (ratio T/NB >2; "PRICKLEl-up" group), and 4,550 (84%) did not show any upregulation (ratio <2; "PRICKLEl-no up" group). MFS data were available for 1,037 patients, including 613 who remained metastasis-free during a median follow-up of 83 months (median MFS not reached) and 424 who displayed metastatic relapse. The 5-year MFS rate was 61% [95CI, 58-65]. In the whole population, PRICKLEl expression was not associated with poor MFS (p=0.49, log-rank test). The same analysis was performed in each molecular subtype separately. As shown in Figure IB, PRICKLEl overexpression was observed to be associated with high MFS in the basal subtype with 61% 5-year MFS [95CI, 54-68] in the "PRICKLEl-no up" group and 41% [95CI, 31-55] in the "PRICKLEl-up" group (p=0.008; log-rank test). By contrast, no significant correlation with PRICKLEl overexpression was seen in the luminal A, luminal B, ERBB2-positive, and normal-like subtypes (data not shown). Together these data show overexpression of PRICKLEl in basal breast cancer and its correlation with poor prognosis in this molecular subtype.

PRICKLEl and MINK1 are involved in breast cancer cell migration

To investigate the role of PRICKLEl in breast cancer, we knocked-down PRICKLEl expression by siRNA in MDA-MB-23 1, a highly invasive basal breast cancer cell model. We determined by qPCR that two independent siRNAs yielded at least 80%> of downregulation of PRICKLEl expression (Figure 2A, left panel). We next determined the role of PRICKLEl in cancer cell migration by performing Boyden chamber assays. Downregulation of PRICKLEl expression was correlated with a strong decrease of MDA-MB-23 1 migration (Figure 2A, right panel). In a previous study, we identified MINK1 as protein kinase that specifically binds to- and phosphorylates PRICKLEl. We designed two siRNAs that led to at least 70%> of MINK1 mRNA dowregulation in MDA-MB-23 1 cells (Figure 2B, left panel) and to a strong decrease of cell motility (Figure 2B, right panel). We also investigated the role of PRICKLEl and MINK1 in MDA-MB-23 1 cell proliferation and found that downregulation of both genes had an effect on this process (Figure 2C, 2D). MDA-MB-23 1 cells treated with PRICKLEl or MINK1 siRNAs were next plated on coverslips treated with collagen, fixed and stained with phalloidin to examine cell morphology. We observed that downregulation of PRICKLEl and MINK1 led to a robust increase of cell spreading correlated with the formation of thick actin bundles. PRICKLEl- or MINK 1-depleted cells were flattened with an increase of the size of

the nucleus and an absence of lamellipodium, suggesting a lack of polarization. We provide quantitative measurements of these data by measuring the area of MDA-MB-23 1 cells treated with two independent siRNAs per gene. Using a similar approach, we confirmed the importance of PRICKLEl and MINKl in cell motility and cytoskeleton reorganization in two other basal breast cancer cell lines (SUM149 and SUM159, data not shown). To further investigate this cytoskeleton reorganization, we stained the cells with anti-VINCULIN antibody, a marker of focal adhesions, and phalloidin. Downregulation of PRICKLEl or MINKl expression was associated to the presence of larger focal adhesion patches mainly localized at the tips of actin bundles compared to the control siRNA condition. We provide quantitative data by measuring the area of more than 390 focal adhesions observed in one experiment which is representative of three independent experiments using two independent siRNAs per gene. Our findings demonstrate that PRICKLEl and MINKl regulate migration, proliferation and actin cytoskeleton organization of MDA-MB-23 1 cells.

The interaction of PRICKLEl with MINKl and phosphorylation of PRICKLEl are required for cell migration

In a previous work, we showed that the second LIM domain of PRICKLEl (LIM2) is able to directly bind to MINKl, allowing its phosphorylation by the protein kinase. We next aimed to determine whether the interaction of PRICKLEl with MINKl and PRICKLEl phosphorylation is important for cell migration. We engineered MDA-MB-23 1 cell populations stably downregulated for PRICKLEl mRNA expression following transfection with two independent shRNAs (Figure 3A). As with siRNAs (Figure 2A), cell migration was decreased in Boyden chamber assays using shRNAs in a proportion correlated with PRICKLEl downregulation (Figure 3A). We demonstrated the specificity of the shRNA-PRICKLEl in a rescue experiment whereby we expressed a VENUS-PRICKLE 1 construct resistant to shRNA-PRICKLEl (VENUS-PRICKLE 1 res) (Figure 3B). The decrease of cell migration induced by shPRICKLEl was rescued by expression of VENUS-PRICKLE 1 res in MDA-MB-23 1 cells (Figure 3B). To determine the contribution of the interaction between PRICKLEl and MINKl in cell migration, we used a similar rescue strategy and expressed a version of PRICKLEl (VENUS-PRICKLE 1 ALIM2) unable to bind MINKl or a version of PRICKLEl (VENUS-PRICKLE 1 P mutant) resistant to MINKl phosphorylation in MDA-MB-23 1 cells expressing shRNA-PRICKLEl 19. These two constructs were unable to rescue cell motility of PRICKLEl -deficient MDA-MB-23 1 cells (Figure 3C) suggesting that both the interaction between PRICKLEl and MINKl and PRICKLEl phosphorylation are required for cell motility.

MINK 1 plays a role in tumor growth and cancer cell dissemination

PRICKLE 1 has previously been implicated in the metastatic development using MDA-MB-23 1 cells in xenograft experiments. No such data are available for MINK1. We thus generated MDA-MB-23 1 (previously engineered to stably express the firefly luciferase) cell populations stably downregulated for MINK1 expression by two independent shRNAs, as controlled by western blot analysis. We first confirmed that downregulation of MINK1 with shRNAs led to a similar decreased cell motility (Figure 4A) and cell proliferation (Figure 4B) as with siRNAs (Figure 2B, D). Next, MDA-MB-23 1 cell populations were orthotopically transplanted into the mammary fat pad of NOD/SCID/yc mice, and tumor growth and metastatic dissemination were followed by caliper and luciferase activity measurement, respectively. After 33 days, we observed a significant decrease of the primary tumor volume measured in mice xenografted with MINK1-deficient MDA-MB-23 1 cells compared to control cells (Figure 4C). As expected MDA-MB-23 1 cells stably expressing the non-targeting shRNA (shRNA NT) dramatically invaded the lung and the liver. However, MDA-MB-231 downregulated for MINK1 expression by shRNA MINK1 #06 and shRNA MINK1 #03 showed a lower invasion rate, as fewer metastases or no metastases were detected in the lung and in the liver, respectively. The intensity of bioluminescence was quantified in each dissected lung and liver. Taken together, these in vivo data demonstrate the importance of MINK1 in breast cancer growth and metastatic dissemination.

PRICKLE 1 forms a complex with mTORC2 and MINK1

In order to understand the signaling pathway involved in the function of the MINK1-PRICKLEl complex, we used proteomics to identify novel components of this complex. As described previously (Daulat A. M. et al, 2012), we generated a stable cell line stably expressing FLAG-PRICKLE 1 and performed anti-FLAG immunoprecipitation followed by mass spectrometry analysis. Among the proteins identified which included MINK1, our attention was dran to RICTOR, SIN1 and LST8, three members of the mTORC2 complex. It is known that this protein complex also contains mTOR, a serine-threonine kinase responsible for the phosphorylation of AKT. To assessed the presence of mTOR in the complex, we transiently transfected HEK293T cells with FLAG-PRICKLE 1 or a negative control (FLAG-RADIL). After anti-FLAG immunoprecipitation, we confirmed the presence of RICTOR and mTOR in the complex associated with PRICKLE 1 but not with RADIL. mTORCl and mTORC2 are protein complexes presenting distinct substrate specificities due to the respective presence of RAPTOR (in mTORCl) and RICTOR (in mTORC2) subunits. We transiently expressed Myc-RICTOR or Myc-RAPTOR in HEK293T cells and assessed their

interaction with FLAG-PRICKLE 1. We detected the presence of FLAG-PRICKLE 1 in the complex immunopurified with RICTOR, but not with RAPTOR, confirming the specific interaction of PRICKLE 1 with the mTORC2 complex. We then asked whether MINK1 belongs to the complex containing PRICKLE 1 and RICTOR. In order to test that, we immunopurified FLAG-MINK1 from HEK293T cells and assessed the presence of endogenous RICTOR in the complex by western blot analysis. We observed a weak but reproducible MINK1-RICTOR copurification, which was increased by overexpression of VENUS-PRICKLE 1, suggesting that the MINK1-RICTOR interaction is dependent on the presence of PRICKLE 1. In order to prove this, we downregulated expression of PRICKLE 1 with two independent siRNAs, performed the immunoprecipitation of FLAG-MINK 1, and looked for the presence of RICTOR in the protein complexes. We observed that the interaction between MINK1 and RICTOR was decreased under PRICKLE 1 siRNA treatment. We thus provide evidence that PRICKLEl function as a scaffold to recruit MINK1 to the mTORC2 complex.

The PRICKLEl complex regulates mTORC2 activity

As mentioned above, AKT is a substrate for mTORC2. In order to measure the contribution of PRICKLEl to AKT phosphorylation, we first determined the kinetics of AKT phosphorylation in MDA-MB-231 cells. Serum starved cells were stimulated with 5% FCS and AKT phosphorylation was monitored using specific anti-phosphoSerine 473 (pS473-AKT) and -Threonine 308 (pT308-AKT) antibodies. Maximal phosphorylation of both sites was detected after 30 min of serum treatment. Downregulation of PRICKLEl expression using two independent siRNAs led to a robust decrease of AKT phosphorylation at Serine 473 and Threonine 308 compared to the control after 30 minutes of serum stimulation. We next examined to what extend MINK1 contributed to AKT phosphorylation using MDA-MB-23 1 downregulated for MINK1 expression. We observed a strong inhibition of AKT phosphorylation at Serine 473 and Threonine 308 after 30 minutes of serum stimulation demonstrating a role of MINK1 in the phosphorylation of AKT.

We next aimed to evaluate more directly the contribution of the PRICKLEl complex to AKT phosphorylation in an in vitro assay. We purified FLAG-PRICKLE 1 from HEK293T cells and performed in vitro kinase assays using recombinant GST-AKT added to the FLAG immunoprecipitate as a substrate. AKT phosphorylation was monitored by western blot using anti-pS473-AKT antibody. We observed that the PRICKLEl complex promoted AKT phosphorylation in comparison to a control RADIL complex. In this assay, we did not observe AKT phosphorylation on Threonine 308. To test whether AKT phosphorylation observed in

this assay was due to the presence of mTORC2 complex associated wih PRICKLEl, we generated stable HEK293T cell populations downregulated for RICTOR expression using two independent shRNAs. Purification of the PRICKLEl complex from these cell populations and incubation with recombinant GST-AKT led to a strong decrease of AKT phosphorylation. We thus conclude that the PRICKLEl complex contributes to AKT phosphorylation through its interaction with mTORC2.

Stability of focal adhesions is increased by the targeting of the PRICKLE l-mTORC2 complex activity

We next examined the cellular basis of the cell migration defect observed in the absence of PRICKLEl and MINK1 (Figure 2). Interestingly, previous work has shown the involvement of MINK1 and mTORC2 in modulation of cytoskeleton organization. Accordingly, downregulation of RICTOR in MDA-MB-23 1 cells with two independent siRNAs led to decreased cell migration and increased cell and focal adhesion area, as was shown for PRICKLEl and MINK1 (Figure 2). We next examined the dynamics of focal adhesions, since it can correlate with the capacity for a cell to migrate. Migrating cells form two major populations of focal adhesions when adhering on cellular matrices such as collagen. Nascent focal adhesions are present in the lamellipodia of migratory cells and are highly dynamics with a short half-life. Some of these structures can mature by binding to actin filaments under the regulation of the RHO small GTPase and its effector ROCK to form larger and more stable patches in the cytoplasm of the cells 26. As we showed that depletion of PRICKLEl or MINK1 increases the presence of large focal adhesions in MDA-MB-23 1 cells, we decided to monitor the stability of focal adhesion by Total Internal Resonance Fluorescence (TIRF) microscopy which is well suited for the analysis of the localization and dynamics of events at the plasma membrane. MDA-MB-23 1 cells were thus engineered to stably express VENUS-PAXILLIN, a marker of focal adhesion, and subsequently transfected with specific siRNAs directed against PRICKLEl, MINK1 or RICTOR. Cells were seeded on collagen and the focal adhesion patches were followed over a period of 11 minutes. Results shows a typical image of migratory MDA-MB-23 1 cells in which focal adhesions were localized every minute from 1 minute (red) to 11 minutes (pink). Each focal adhesion was tracked and processed by a Matlab-based tracking software. Focal adhesions were classified into stable and unstable categories. Stable focal adhesions are circled in white and are present throughout the 11 min acquisition, conversely transient focal adhesions which are considered unstable since they disappear within 11 min are circled in black. The analysis of stable versus unstable focal adhesions showed that in the control condition 20± 3% of focal adhesions were

stable. In conditions where MINK1, PRICKLE 1 or RICTOR were downregulated by siRNAs, we observed an increase of focal adhesion stability by 34± 2% (MINK1 siRNA), 38± 4% (PRICKLEl siRNA) and 32± 4% (RICTOR). We monitored the disassembly of focal adhesions by determining the half-life in each condition. We observed that downregulation of PRICKLEl, MINK1 and RICTOR led to stabilization of focal adhesions: the half-life: is increased from 6.9 min for control to 8.2 min for PRICKLEl, 8.2 min for MINK1 and 7.6 min for RICTOR. These data demonstrate the importance of the MINK1-PRICKLE 1-RICTOR signaling pathway in controlling focal adhesion dynamics. To confirm our observation, we analyzed the phosphorylation state of PAXILLIN at Tyrosine 118 which represents a faithful marker of mature focal adhesions 28. Decreased expression of PRICKLEl, MINK1 and RICTOR yielded an increase of PAXILLIN phosphorylation as asses by western blot, confirming the conclusion of the TIRF experiments. Our results suggest that the PRICKLEl complex controls cell migration of cancer cells by decreasing focal adhesion dynamics.

The interaction between PRICKLEl and mTORC2 is required for AKT phosphorylation and cell migration

In order to investigate whether the interaction between PRICKLEl and mTORC2 is functionally relevant, we next decided to determine more precisely how PRICKLEl is associated with RICTOR. To do so, we generated deleted versions of PRICKLEl lacking the PET and/or the LIM domains and a construct encompassing the PRICKLEl C-terminus region (PRICKLE C2). We expressed the different constructs in HEK293T cells and performed anti-FLAG immunoprecipitation to assess the presence of RICTOR in the complex. We found that the PET and LIM domains were dispensable for the interaction, and that the PRICKLE C2 construct was not able to bind RICTOR. We thus concluded that the region between LIM3 domain and the PRICKLE C2 region was most likely involved in the interaction with RICTOR. To prove that, we fused this region (hereafter named CI domain) to VENUS (VENUS-C1) and transiently expressed the construct in HEK293T cells. VENUS-Cl, but not VENUS, was able to co-immunoprecipitate with endogenous RICTOR. To assess the possibility of using VENUS-C1 as a dominant negative inhibitor of the PRICKLEl-RICTOR interaction, we transiently expressed FLAG-PRICKLE 1 with VENUS or VENUS-Cl in HEK293T cells. We observed that, in anti-FLAG immunoprecipitates, expression of VENUS-C1 led to a strong decrease of PRICKLEl -RICTOR association, highlighting a dominant-negative effect. We next assessed whether this effect is functionally relevant by overexpressing VENUS-C1 domain in MDA-MB-23 1 cells and by monitoring AKT

phosphorylation and cell migration. Upon serum stimulation, we observed that overexpression of VENUS-C1 led to a robust inhibition of AKT phosphorylation at Serine 473 and Threonine 308 and cell migration in Boyden chamber assays. In addition, expression of VENUS-C1 phenocopied the increased cell area observed following downregulation of PRICKLEl. In conclusion, we have identified a region in PRICKLEl required for the PRICKLE 1-RICTOR association and demonstrated that disruption of this interaction decreases AKT phosphorylation and cell migration.

Disruption of the PRICKLE 1-RICTOR interaction decreases tumor growth and metastatic dissemination

To address the role of the PRICKLE 1-RICTOR association in tumorigenesis, we transplanted VENUS and VENUS-C1 in MDA-MB-231 cells stably expressing firefly luciferase for in vivo experiments. VENUS-C1 expression in MDA-MB-231 cells led to decreased cell proliferation (Figure 5A), correlated with impaired AKT phosphorylation (data not shown), and with compromised tumor growth following orthotopical transplantation of cancer cells in fat pads of NOD/SCID/yc mice (Figure 5B). In addition, metastasis formation was observed in the lungs of all mice of the control group (7 out of 7 mice) whereas only 3 out of 8 mice developed pulmonary tumors when VENUS-C1 was expressed (Figure 5C). Comparable results were obtained when considering metastasis in the liver (7 out of 7 mice for VENUS as compared to 2 out of 8 mice for VENUS-C1) (Figure 5D). In conclusion, we have demonstrated the importance of the PRICKLE 1-RICTOR association in tumor growth and metastatic dissemination.

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

Daulat, A. M. et al. Minkl regulates beta-catenin-independent Wnt signaling via Prickle phosphorylation. Mol Cell Biol 32, 173-185, doi:10.1 128/MCB.06320-1lMCB.06320-1 1 [pii] (2012).

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Pal SKI, Quinn DI. Differentiating mTOR inhibitors in renal cell carcinoma. Cancer Treat Rev. 2013 Nov;39(7):709-19.

CLAIMS:

1. A polypeptide comprising or consisting of:

i) an amino acid sequence SEQ ID NO: 9, or,

ii) an amino acid sequence having at least 70% of identity with the amino acid sequence SEQ ID NO: 9,

wherein the polypeptide does not consist of the PRICKLE1 polypeptide.

2. A fusion protein comprising the polypeptide according to claim 1 that is fused to at least one heterologous polypeptide.

3. The fusion protein of claim 2 wherein the heterologous polypeptide is a VENUS polypeptide having the sequence set forth as SEQ ID NO: 11.

4. An inhibitor of the PRICKLE1-MINK1-mTORC2 complex for use in the prevention and treatment of cancer and cancer metastasis in a patient in need thereof.

5. The inhibitor for use according to claim 4 wherein said inhibitor of the PRICKLE1-MINK1-mTORC2 complex is the polypeptide according to claim 1 or the fusion protein according to any of claims 2 or 3.

6. The inhibitor for use according to claim 4 wherein said inhibitor of the PRICKLE1-MINK1-mTORC2 complex is selected from the group consisting of siRNA, shRNA, anti-sense oligonucleotides and ribozymes.

7. The inhibitor for use according to claim any of claims 4 or 6 wherein said inhibitor of the PRICKLE1-MINK1-mTORC2 complex is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8.

8. The inhibitor for use according to claim 4 wherein said inhibitor of the PRICKLE1-MINK1-mTORC2 complex is a mTORC2 inhibitor.

9. The inhibitor for use according to claim 8 wherein said mTORC2 inhibitor is selected from the group consisting of rapamycin, temsirolimus, deforolimus, everolimus, tacrolimus and rapamycin analogue or derivative thereof.

10. A method for predicting the overall survival (OS) of a patient suffering from breast cancer comprising the steps of: i) determining in a sample obtained from the patient the expression level of PRICKLE 1 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

11. A method for predicting the event-free survival (EFS) of a patient suffering from breast cancer comprising the steps of: i) determining in a sample obtained from the patient the expression level of PRICKLE 1 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

12. A method for predicting the metastasis-free survival (MFS) of a patient suffering from breast cancer comprising the steps of: i) determining in a sample obtained from the patient the expression level of PRICKLE 1 ii) comparing the expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

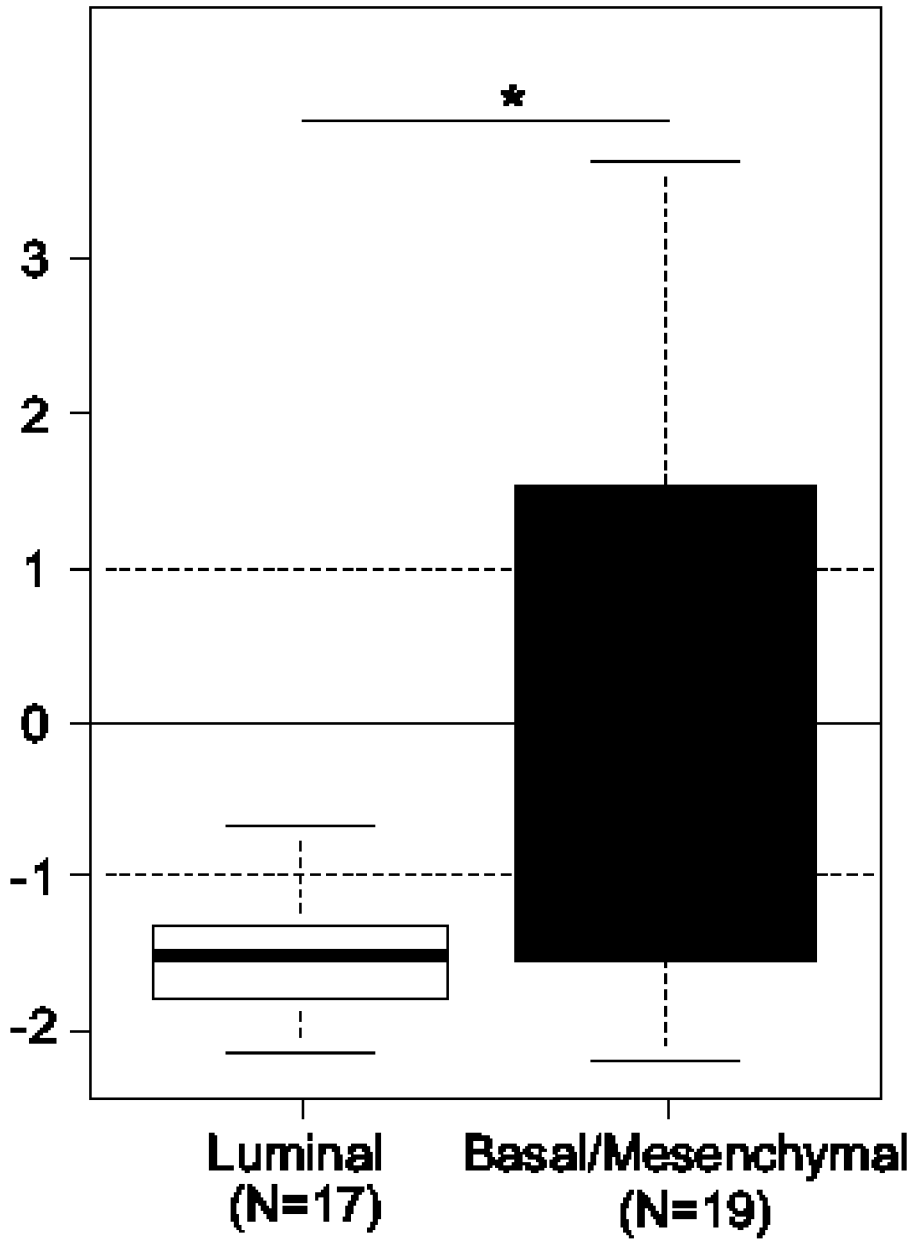


Figure 1 A

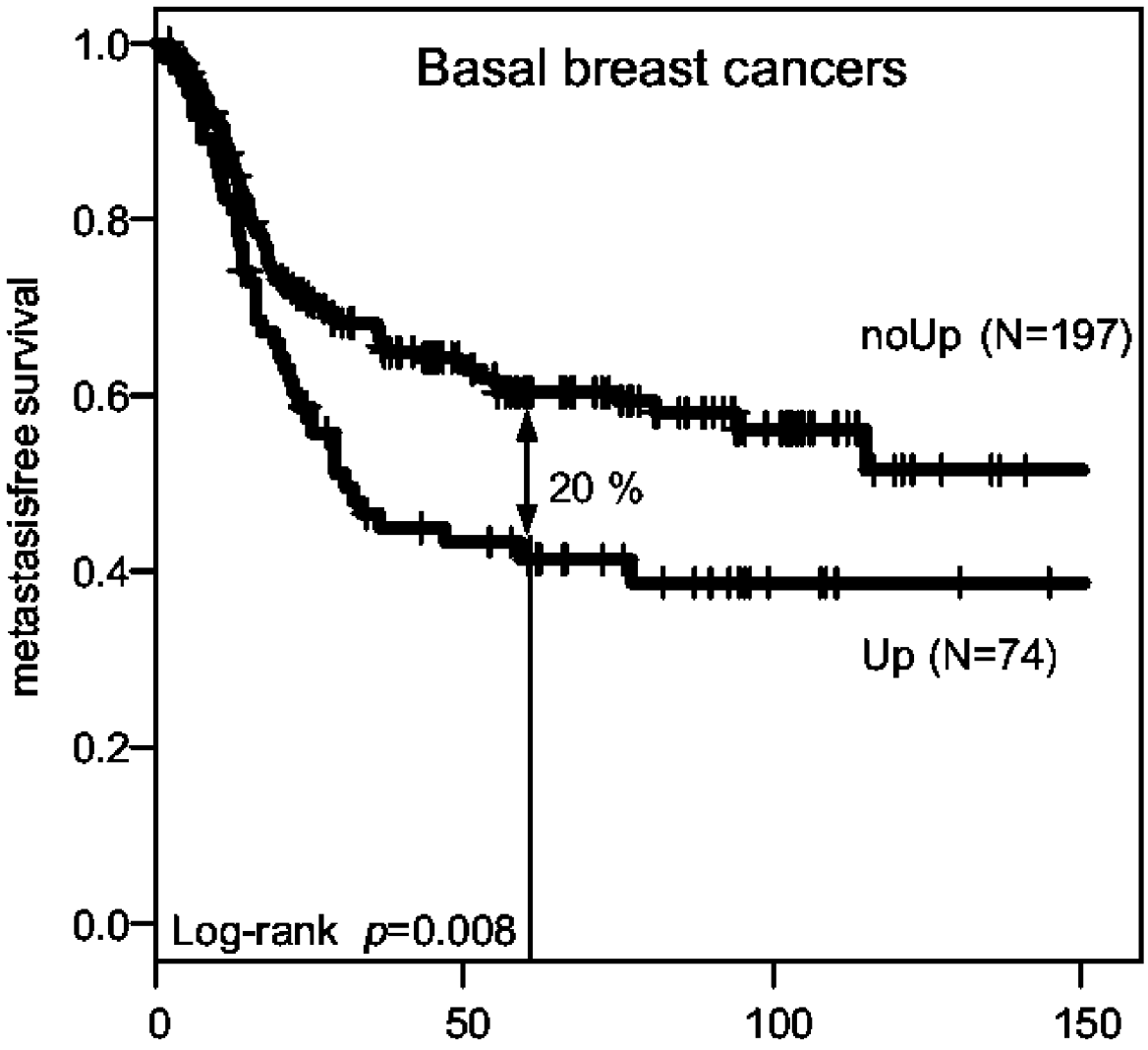
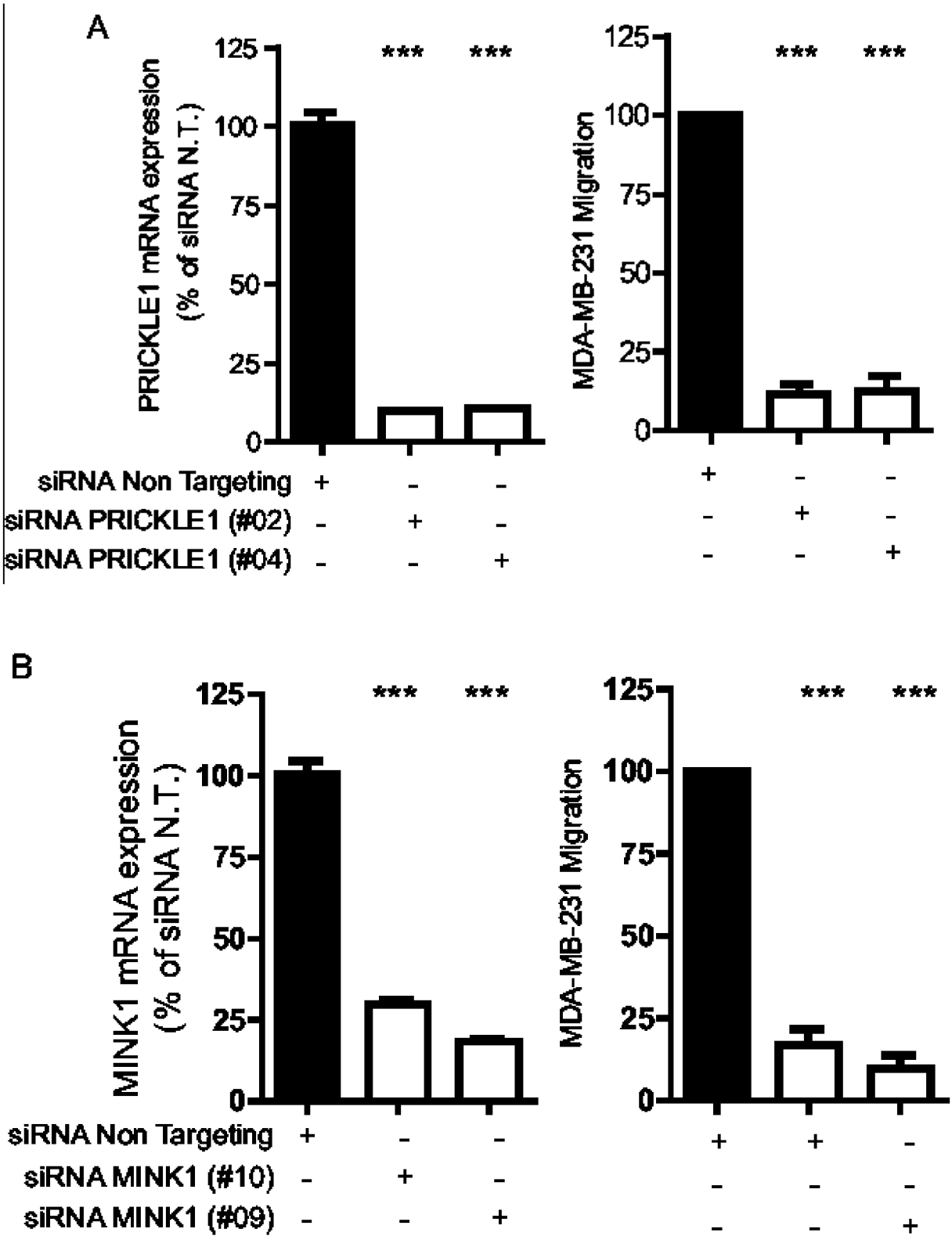
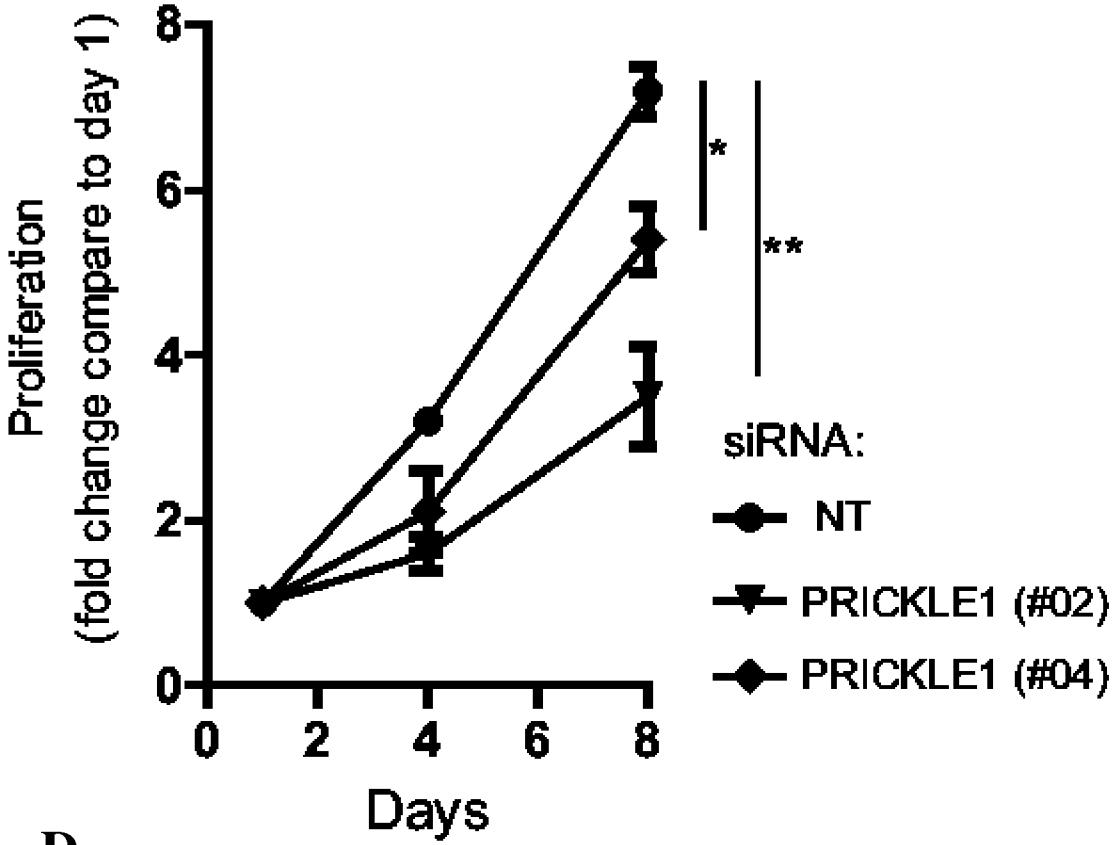

Basal breast cancers
metastasisfree survival
1.0
0.8
0.6
0.4
0.2
0.0
noUp (N=197)
20 %
Up (N=74)
Log-rank p=0.008
0
50
100
150

Figure 1 B

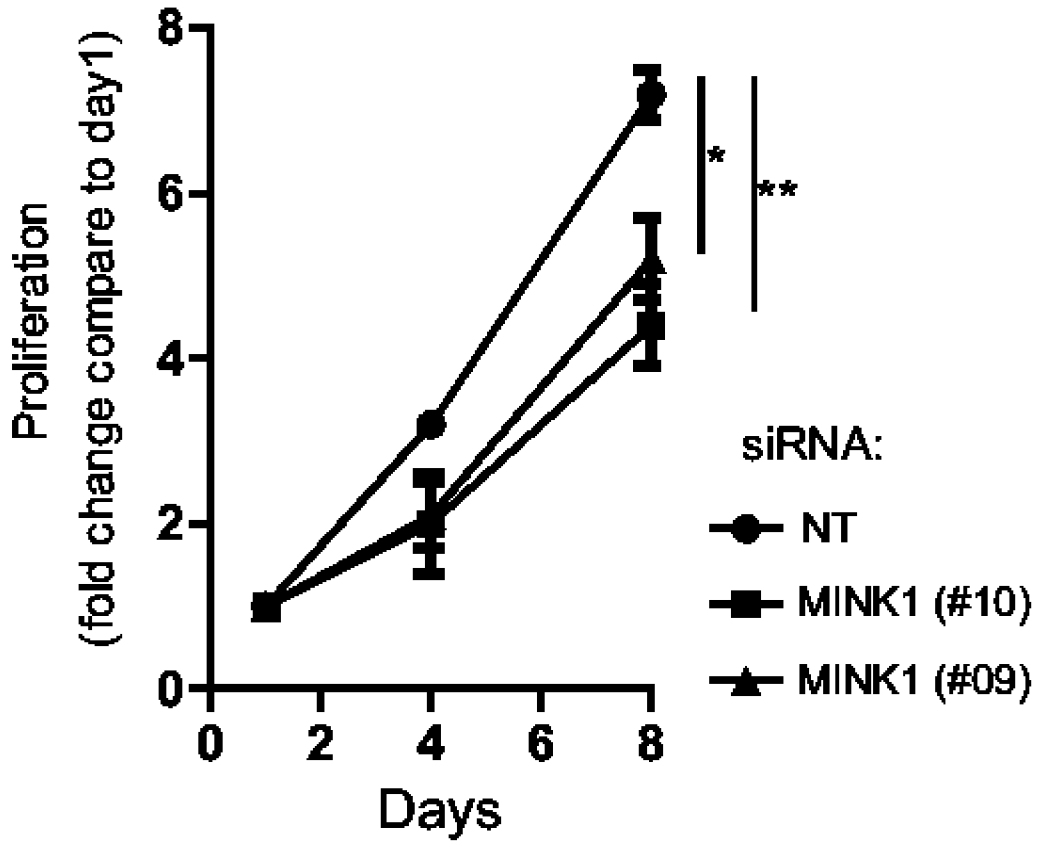


Figures 2 A and B

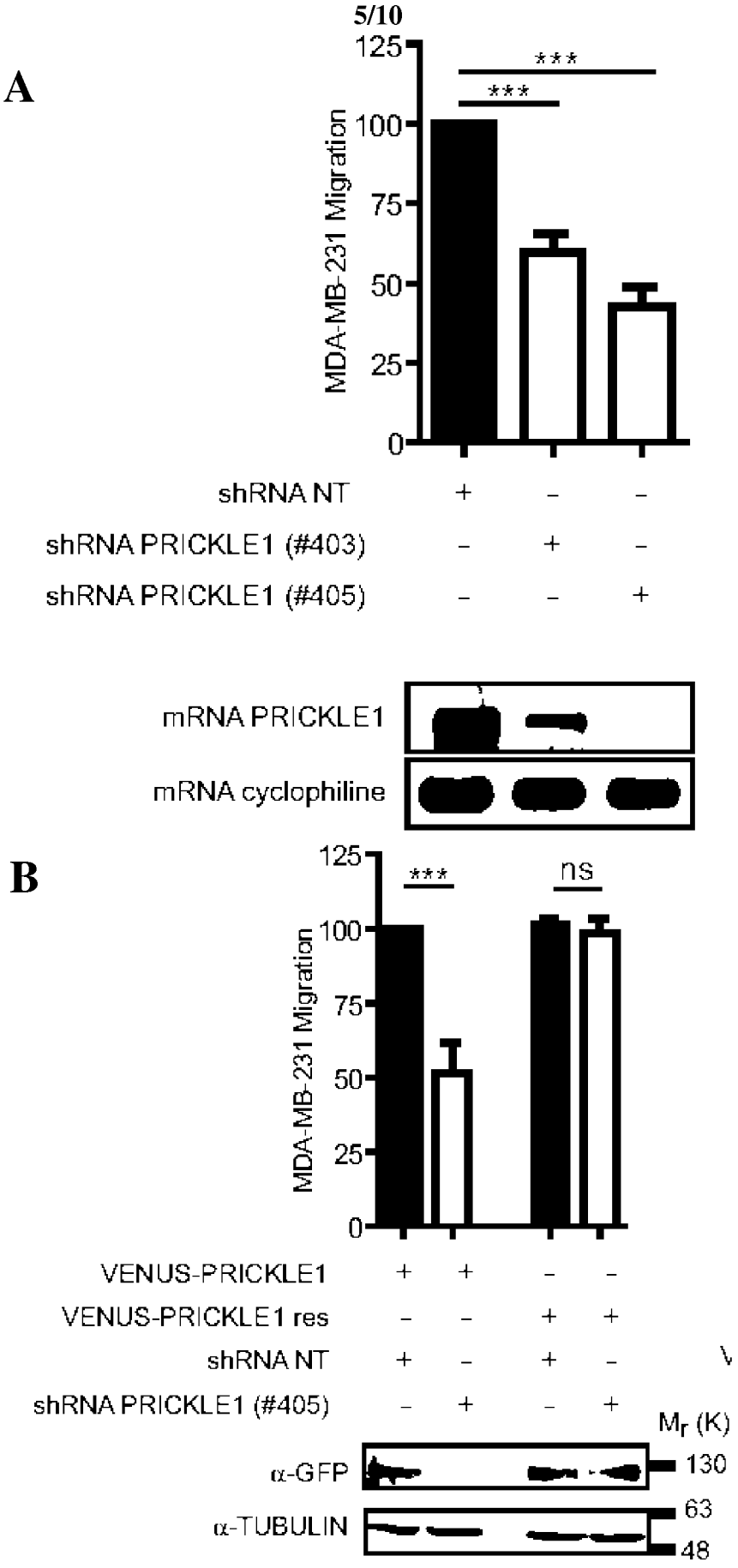
C



D



Figures 2 C and D



Figures 3 A and B

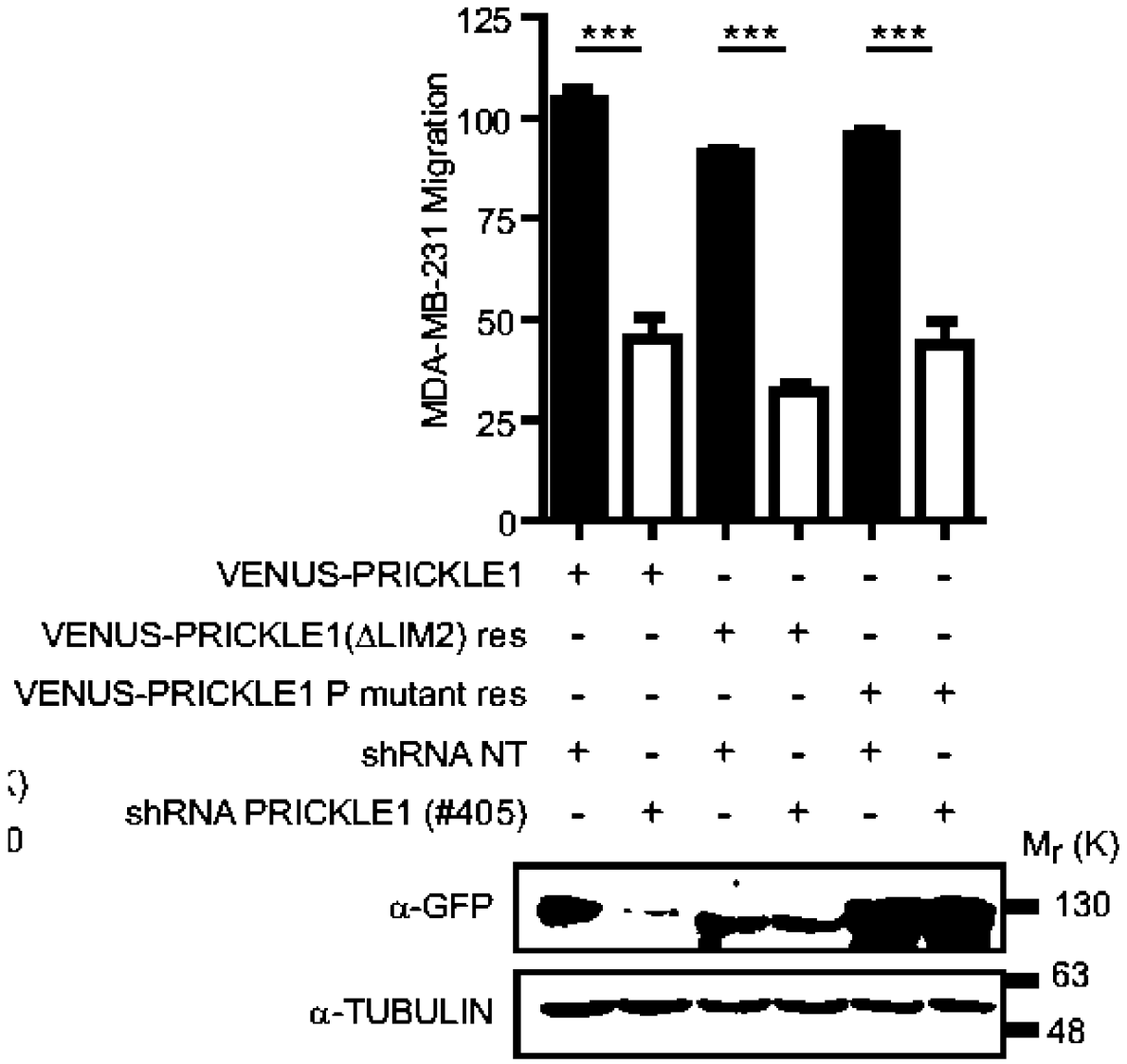
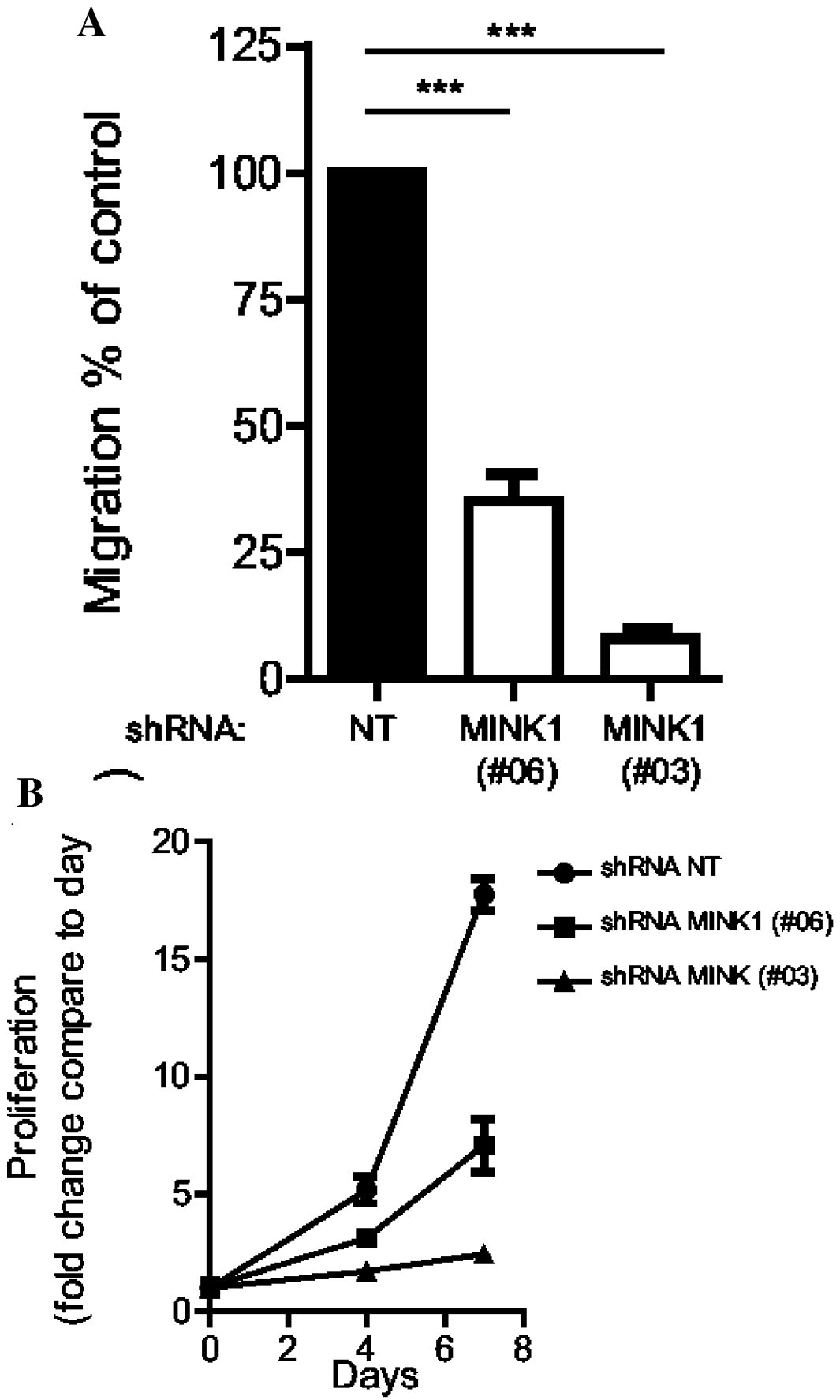

125
100
75
50
25
0
MDA-MB-231 Migration
***
***
***
VENUS-PRICKLE1 + + - - - -
VENUS-PRICKLE1(ΔLIM2) res - - + + - -
VENUS-PRICKLE1 P mutant res - - - - + +
shRNA NT + - + - + -
shRNA PRICKLE1 (#405) - + - + - +
Mr (K)
α-GFP
130
α-TUBULIN
63
48

Figure 3 C



Figures 4 A and B

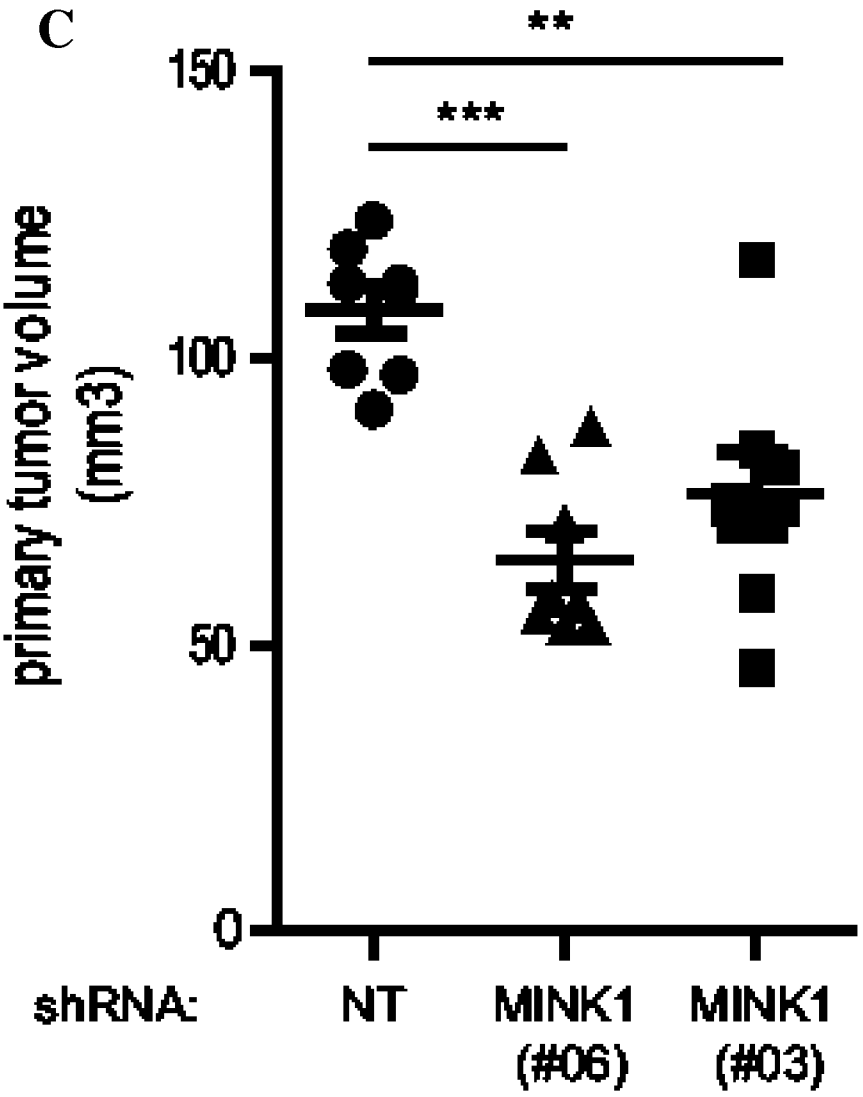
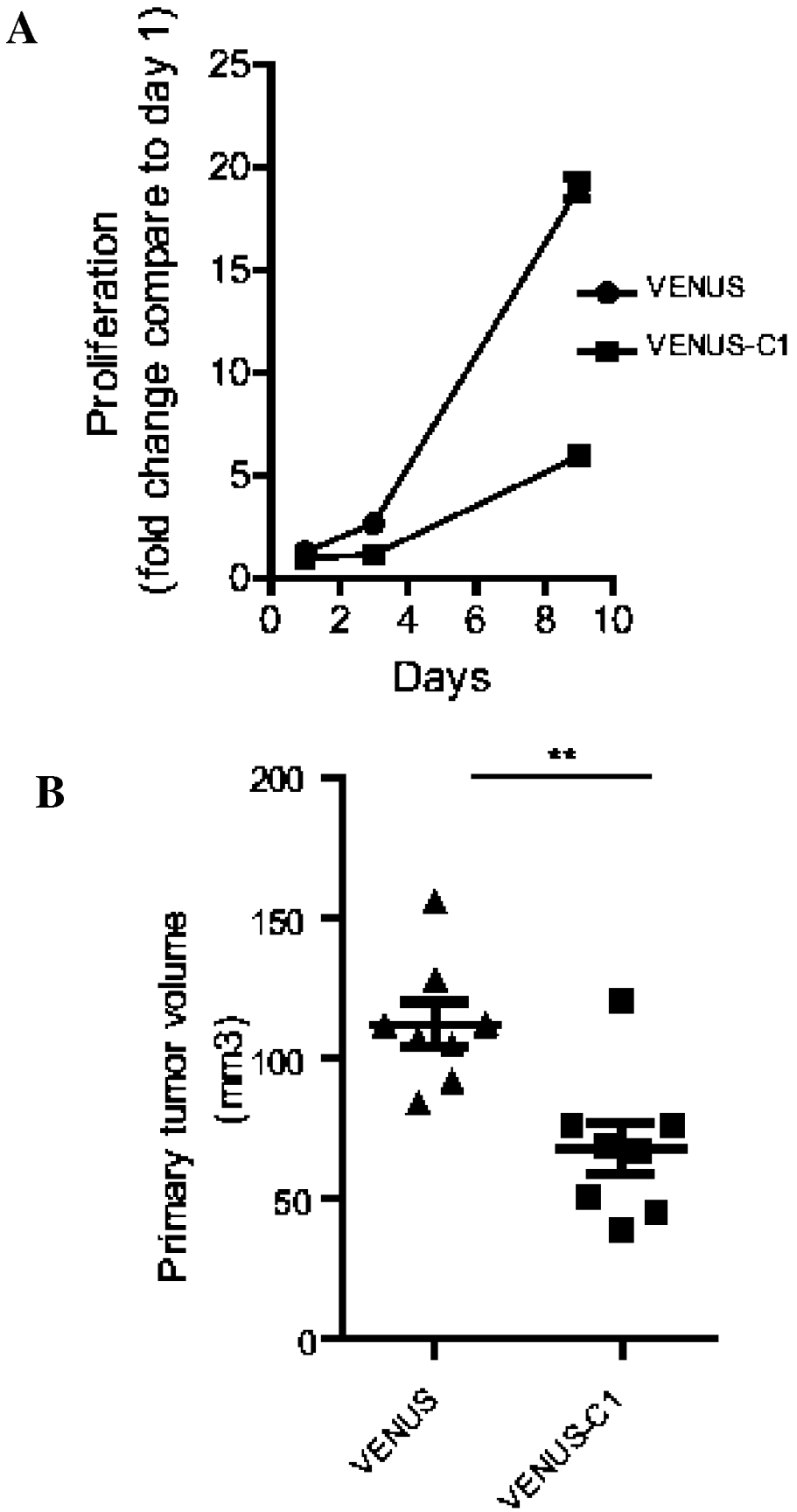
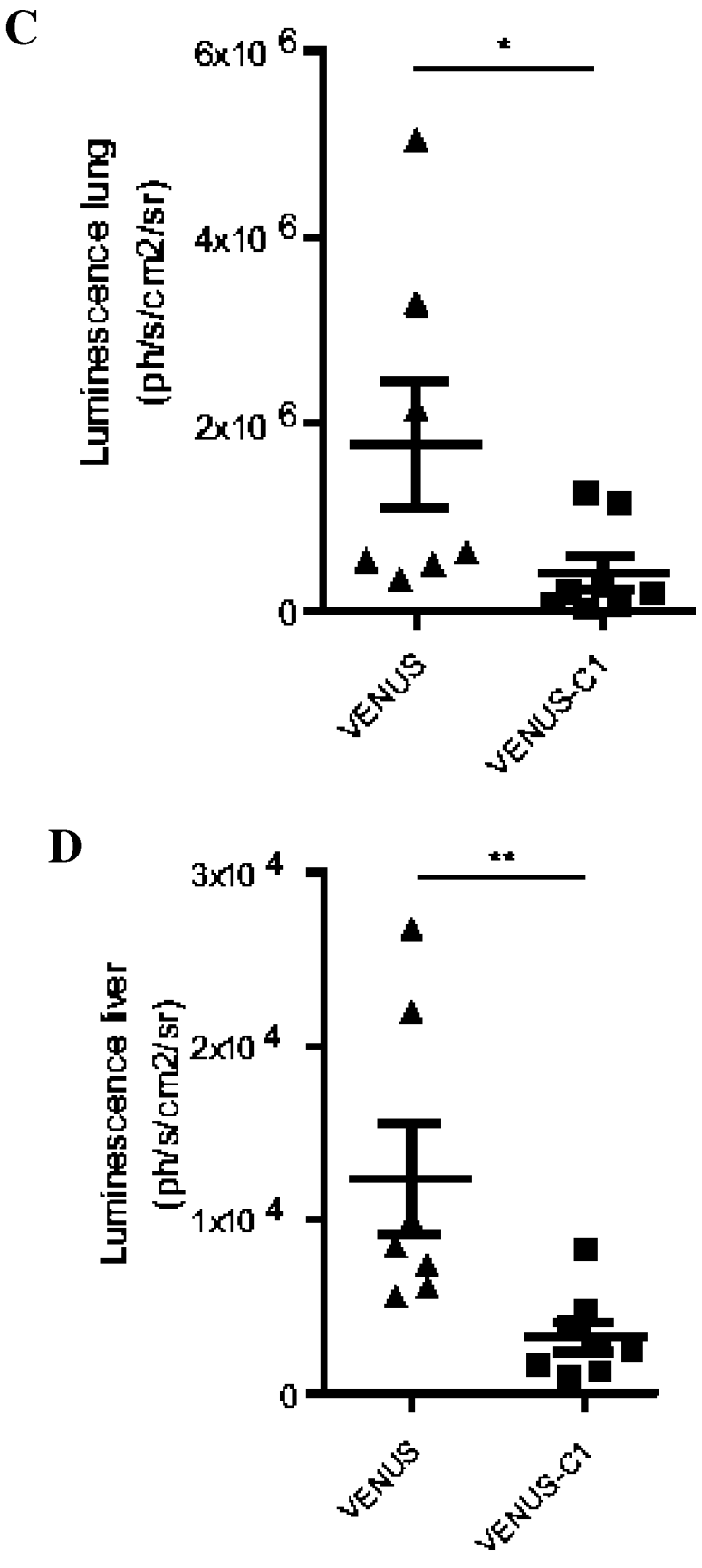

C
**
***
150
100
50
0
primary tumor volume (mm3)
shRNA:
NT
MINK1 (#06)
MINK1 (#03)

Figure 4 C



Figures 5 A and B



Figures 5 C and D

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/069690

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/11 C12Q1/68 C12N15/62 A61K38/17 C07K14/47 G01N33/50
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)
C07K C12Q C12N A61K G01N

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[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" documentwhich may throw doubts on priority claim(s) orwhich 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
26 October 2016	07/11/2016

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	Smalt, Rolf

2

Form PCT/ISA/210 (second sheet) (April 2005)

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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