METHODS OF CONTROLLING TUMORIGENESIS AND DIAGNOSING THE RISK THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application makes reference to and claims the benefit of priority of an application for "Preventing, inhibiting, arresting or reversing tumorigenesis by modulation of a rab binding protein and related uses of a rab binding protein" filed on April 13, 2007 with the US Patent and Trademark Office and there duly assigned serial number 60/911,573. The content of said application filed on April 13, 2007 is incorporated herein by reference for all purposes, including an incorporation of any element or part of the description, claims or drawings not contained herein and referred to in Rule 20.5(a) of the PCT, pursuant to Rule 4.18 of the PCT.

FIELD OF THE INVENTION

[0002] The present invention relates to methods of controlling tumorigenesis and diagnosing the risk thereof. The methods rely on an altered amount and/or activity of a Rab binding protein. The invention also relates to methods of identifying a compound capable of forming a complex with a Rab binding protein, and of modulating the amount, the expression and/or the activity of a Rab binding protein. The invention also provides a method of determining whether a neoplasm is sensitive to an alteration of intracellular calcium levels.

BACKGROUND OF THE INVENTION

[0003] Cancer is a major cause of death worldwide, being the second-leading cause of death in developed countries, and even the number one cause of death in e.g. Australia, Japan, Korea, Singapore and the male population of the UK and Spain. The number of people who develop cancer each year is increasing. Nevertheless cancer therapy has not managed to decrease cancer mortality in the last three decades.

[0004] Autonomous cell growth resulting in tissue invasion and metastasis is the defining feature of all malignant neoplasms. Cancers do not necessarily arise solely as a result of an accelerated rate of cell proliferation. Rather they are the consequence of an imbalance between the rate of cell-cycle progression (cell division) and cell growth (cell mass) on one hand and programmed cell death (apoptosis) on the other.

[0005] Resolving the identity of the oncogenic drivers of tumorigenesis and metastatic growth is crucial not only for understanding the pathobiology of cancer, but also for treating patients, as such genes are increasingly being exploited for therapeutic benefit (Herceptin,

Avastin, Gleevec). In human cancers, oncogenes are frequently activated by aberrant overexpression resulting from genomic amplification, sequence mutations or promoter hypomethylation. Chromosomal loci that are frequently amplified in cancer, known as amplicons, are thought to be driven by one or more oncogenes, and though many recurrent amplicons have been observed, their oncogenic determinants remain largely unknown. These loci have traditionally been interrogated by cytogenetics techniques such as comparative genomic hybridization (CGH) to assess the extent and frequency of copy number gains, and although this strategy has provided many gene candidates for consideration, the extensive size of the gene lists, together with a paucity of functional information, has limited progress towards oncogene discovery. More frequently, the discovery of oncogenes has been a slow and empirical process – depending mostly on the generation of hypotheses derived from cumulative observations of gene function in different experimental contexts.

[0006] It is an object of the present invention to provide means of controlling, including preventing tumorigenesis and of identifying a cell with a predisposition to turn tumorigenic.

SUMMARY OF THE INVENTION

[0007] In a first aspect the present invention provides methods of controlling tumorigenesis, including preventing, inhibiting, arresting or reversing tumorigenesis. The present invention also provides methods of treatment, prevention and diagnosis of neoblastoma, including cancer, such as metastatic cancer.

[0008] One such method includes modulating the amount or activity of a Rab binding protein, in particular of a Rab11FIP protein (Rab11 family-interacting protein).

[0009] A further method of the invention relates to identifying one or more cells having a predisposition to turn tumorigenic. This method includes detecting in the one or more cells an altered amount, an altered subcellular localisation or an altered activity of a Rab binding protein, in particular a Rab11FIP protein.

[0010] The invention also provides a method of diagnosing the risk of developing a neoplasm. This method includes determining at least one of the expression level, the activity level and the subcellular localisation of a Rab binding protein, in particular a Rab11FIP protein.

[0011] The application is also concerned with the identification of suitable compounds capable of modulating the activity of a Rab binding protein, in particular a Rab11FIP protein, and thereby for example of acting as anti-cancer agents.

[0012] In this regard the invention provides an *in-vitro* method of identifying a compound capable of forming a complex with a Rab binding protein, in particular a Rab11FIP protein. The method includes contacting the components that form the respective complex with each other.

[0013] In a further aspect the invention provides a method for identifying a compound capable of modulating the activity of a Rab binding protein, in particular a Rab11FIP protein, in vivo. The method includes providing a microorganism expressing the Rab binding protein or a functional fragment thereof. The method further includes adding a compound to the microorganism. The method also includes monitoring a change in cell phenotype or activity of a Rab binding protein.

[0014] In another aspect the invention provides methods of identifying a compound capable of modulating the amount or the activity of a Rab binding protein, in particular a Rab11FIP protein.

[0015] One such method includes providing a host organism. The host organism is capable of accommodating and growing a cancer cell. The method also includes introducing a cancer cell into the host organism. Furthermore the method includes administering a compound suspected to be capable of modulating the expression and/or the activity of a Rab binding protein. The method further includes monitoring the growth of any tumor (including a plurality thereof) in the host organism.

[0016] A further such method also includes providing a host organism. The host organism is capable of accommodating and growing a cancer cell. The method also includes introducing a cancer cell into the host organism. The cancer cell comprises a compound suspected to be capable of modulating the amount or the activity of a Rab binding protein. The method further includes monitoring the growth of any tumor (including a plurality thereof) in the host organism.

[0017] In yet a further aspect the invention provides a method of determining whether a neoplasm is sensitive to an alteration of intracellular calcium levels. The method includes determining the expression and/or the activity of a Rab binding protein. An increased expression or an increased activity of the Rab binding protein is an indication that the neoplasm is sensitive to an alteration of intracellular calcium levels.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the detailed description when

considered in conjunction with the non-limiting examples and the accompanying drawings.

[0018] Figure 1 depicts a multi-step schematic of the TRIAGE methodology.

[0019] Figure 2 is a table showing the clinical characteristics of the breast cancer cohorts used to construct the integrated microarray dataset.

[0020] Figure 3 depicts the LSVD analysis of the breast cancer oncogenome. Eigen values (y-axis) are plotted along the length of each chromosome (x-axis). The genomic locations of the highest-scoring principle eigen peaks are highlighted.

[0021] Figure 4 shows the proliferation signature and distant metastasis-free survival (DMFS). **A,B**: All probe sets (n=2,377) correlated with poor DMFS ($P<0.05$; likelihood ratio test) in the Uppsala cohort (n=251) are shown hierarchically clustered. The predominant gene cluster reflects the proliferation signature. The central core genes of the proliferation signature are, in clustered order are named in the examples below. Below the clustergram, tumors that metastasized (white circles) and did not (dark circles) are shown as a function of time. **C**: Probe sets associated with poor DMFS (n=2,377) were binned according to P-value and the fraction of probesets correlated with the proliferation signature at >0.4 are indicated in white and shown as a percentage of each bin. Approximately 35 % of all DMFS-associated probesets are correlated with the proliferation signature at >0.4.

[0022] Figure 5 depicts TRIAGE analysis of 17q12 and 8p11 amplicons. **A-B**: Expression heatmaps of the probable expression amplicons (PEAs) at (A) 17q12 and (B) 8p11 are shown. Tumors are arranged in columns and genes identified by LSVD are organized in rows (in order along the chromosome). Tumors are ranked by the absolute value of the eigen weight (shown below heatmap). Above-mean expression is shown in white. Genes with significant survival associations at TRIAGE step 3 (see Figure 1) are shown on the right. Genes in white letters indicate top oncogene candidates by TRIAGE. **Fig. 5C** and **Fig. 5D** depict the same data as Figures 5A and 5B, with below-mean expression shown in white. **Fig. 5E** summarises the obtained data. NS indicates "not significant". Asterisks indicate >0.4 correlation with the proliferation signature. Black vertical bars above the heatmap in panel A denote ERBB2 (HER2/neu) positivity by immunohistochemistry.

[0023] Figure 6 illustrates that RCP overexpression in MCF10A cells induces oncogenic phenotypes. **A**: Endogenous RCP levels assessed by Western blot in MCF10A and breast cancer cell lines. **B**: Impact of RCP overexpression on cell morphology. (Scale bars, 100 μm.). **C**: Effects of RCP overexpression on localization of E-cadherin at cell-cell junctions (top), organization of F-actin (middle) and expression of fibronectin under serum starvation (bottom) by immunofluorescence. (Scale bars, 50 μm.) **D-F**: Effects of RCP overexpression on (**D**)

growth factor-independent proliferation of MCF10A cells by WST assay (mean +/- s.d.), (**E**) anchorage-independent colony formation in soft agar (magnification, 5x), and (**F**) cell migration by wound healing (scratch) assay. (Scale bars, 100 μm.)

[0024] Figure 7 illustrates the mechanisms of RCP action. **A**: Cell cycle analysis of serum-starved MCF10A-RCP and control cells by flow cytometry. **B**: Western blots showing Erk and Akt phosphorylation status in response to RCP overexpression (MCF10A cells) and RNAi-mediated knock down of RCP (MCF7 cells). **C**: Inhibition of RCP-mediated Erk phosphorylation by the MAPK inhibitor, U0126 (upper panel), and U0126-mediated inhibition of the RCP proliferation phenotype (lower panel; mean +/- s.d.).

[0025] Figure 8 shows oncogenic phenotypes of RCP in breast cancer cell lines. **A**: Effects of RCP overexpression and RNAi-mediated knock down of RCP on cell proliferation by WST assay. Left panel, results at 6 days post-transfection. Middle panel, proliferation time course of RCP overexpression in MCF10A cells. Right panel, proliferation time course of RCP inhibition in MCF7 cells using two different RNAi RCP constructs and (scramble sequence) controls. **B**: Effects of RCP overexpression and inhibition on anchorage-independent colony formation in soft agar. **C-D:** Effects of RCP overexpression and inhibition on (**C**) cell invasion through matrigel, and (**D**) cell migration. All error bars computed from mean +/- s.d. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$, Student's t-test (two-tailed).

[0026] Figure 9 illustrates that RCP regulates tumor formation in nude mice. **A-B:** Effect of RCP inhibition on tumor growth *in vivo* using MCF7 cells (**Fig. 9A, Fig. 9B, Fig. 9C**) and MB231 cells (**Fig. 9D, Fig. 9E, Fig. 9F**). Figures 9A and 9D depict average tumor volume plotted as a function of time (mean +/- s.e.m.). Figures 9B and 9E depict tumors plotted by weight at 5 weeks (MCF7) and 7 weeks (MB231); mean weight indicated by solid line. In Figures 9C and 9F resected whole tumors are shown. **Fig. 9G** depicts an immunohistochemical staining for RCP in tumor samples. (Scale bars, 100 μm.). **Fig. 9H** shows the apoptotic effects of RCP inhibition in serum-starved MB231 cells by TUNEL staining. (Scale bars, 100 μm.)

[0027] Figure 10 depicts the derivation of the proliferation signature. 1,134 IMAGE clones identified by Whitfield et al., ((2002) *Mol Biol Cel*l, 13, 1977-2000) as cell cycle regulated genes (in HeLa cells) were mapped to 884 UniGene IDs (build #187), corresponding to 2,113 Affymetrix probe sets (U133A and U133B). Using these probe sets, expression profiles of the Uppsala tumor cohort (n=251) were hierarchically clustered (Pearson correlation, average linkage) and visualized in an Eigen clustergram heatmap (left side). White signals in Fig. 10A indicate above-mean expression, white signals in Fig. 10B reflect below-

mean expression. The respective degree of brightness reflects the magnitude of expression. (Due to space limitations, the gene dendrograms have been omitted.) The dark bar designates a cluster of genes mostly expressed in G2/M that was previously identified by Whitfield and Perou as being associated with proliferation in human breast tumors. 78 probe sets corresponding to 67 genes of the proliferation cluster had an average correlation of >0.8 (designated by the white bar) and are shown to the right hierarchically clustered. These probe sets (right side) were designated as the core proliferation signature genes, and averaged to derive Pearson correlations between all probe sets and the proliferation signature (i.e., step 6 of TRIAGE).

DETAILED DESCRIPTION OF THE INVENTION

[0028] The present invention is based on the surprising finding that Rab binding proteins and in particular Rab11FIP proteins (Rab11 family-interacting proteins) are able to promote the formation of neoplasms, including tumors, and in particular cancer.

[0029] The present inventors hypothesized that the pathophysiologic involvement of genes at genomic sites of recurrent amplification might be inferred with greater resolving capacity through the integration and triangulation of genomic position, gene expression and clinical data, where the latter allows for statistical associations to be drawn between gene expression and clinical measures of tumor aggressiveness. To this end, a multi-step data mining strategy was developed that led to the finding of Rab11FIP proteins as oncogene. The methods of the present invention are based on this finding.

[0030] This finding of the inventors also provides an explanation of the data reported on the involvement of the gene localizations, on which the proteins of the RAB11FIP are encoded, to an increased probability of cancers. For example, the human RAB11FIP1 gene (accession No ENSG00000156675 of the Ensembl database) is found on Chromosome 8 in the p12 region, at location 37,835,628-37,876,161. Likewise, the macaque RAB11FIP1 gene (accession No ENSMMUG00000003213 of the Ensembl database) is found on Chromosome 8, at location 38,309,752-38,349,824. The dog RAB11FIP1 gene (accession No ENSCAFG 00000006176 of the Ensembl database) is found on Chromosome 16, at location 30,456,385-30,484,441. The chicken RAB11FIP1 gene (accession No ENSGALG00000003129 of the Ensembl database) is found on Chromosome 22, at location 2,258,738-2,267,830. The human chromosomal region 8p12 has previously been shown to be amplified in a certain number of analyzed breast cancers (Letessier, A., et al., (2006) *BMC Cancer* (2006) 6, 1-13, available at http://www.biomedcentral.com/content/pdf/1471-2407-6-245.pdf). Losses, gains, ampli-

fications and rearrangements of the short arm of chromosome 8 have also been shown to be found in a set of breast, colon and pancreatic cancer cell lines (Pole, J.C.M., et al. (2006) *Oncogene* 25, 5693–5706). A correlation of an alteration of the human chromosomal region 8p12 with lung cancers in patients who underwent surgical resection has been observed (Kim, T.-M., et al., (2005) *Clinical Cancer Research*, 11, 23, 8235-8242). Using comparative genomic hybridization, amplifications and deletions in the gene locus were also shown to occur in pancreatic and lung cancer subtypes (Tonon, G., et al., (2005) *Proc Natl Acad Sci U S A*, 102, 27, 9625-9630). Copy number losses at the same locus were also observed in colon cancer samples obtained from patients (Yaginuma, Y, et al. (2006) *Journal of International Medical Research*, 34, 4, 390-396).

[0031] Rab11 family interacting proteins (FIPs) proteins share a homologous Rab11-binding domain, which is located at or near the C terminus (see e.g. Hales, C.M, et al., *J. Biol. Chem.* (2001) 276, 42, 39067-39075). Based on their domain architecture Rab11FIP proteins have been divided into two subfamilies, which are class I (FIP5/Rip11, FIP1and FIP 2) and class II (FIP3/eferin, and FIP4). The term "Rab11FIP proteins" as used herein thus refers to any member of the family of Rab11 family interacting proteins, in particular to Rab11FIP1, Rab11FIP2, Rab11FIP3 (eferin), Rab11FIP4, Rab11FIP5 (Rip11), and isoforms thereof. Examples of a Rab11FIP1 protein include, but are not limited to, the proteins of the SwissProt accession numbers Q05A58 (Q05A58_MOUSE), Q8K028 (Q8K028_MOUSE), Q6WKZ4 (RFIP1_HUMAN), Q9D620 (RFIP1_MOUSE), Q3B7T9 (RFIP1_RAT), Q6KAN5 (Q6KAN5_MOUSE), Q307T1 (Q307T1_HUMAN), Q28BL8 (Q28BL8_XENTR), (from Xenopus tropicalis), A4IHL6 (A4IHL6_XENTR) (from Xenopus tropicalis) e.g. with the genes of the SwissProt accession numbers ENSG00000156675 (human), ENSMUSG00000031488 (mouse), ENSPTRG00000020164 (chimpanzee), ENSPPYG00000018515 (orangutan), ENSMMUG00000003213 (rhesus monkey), ENSCAFG00000006176 (dog), ENSGALG 00000003129 (chicken), ENSMODG00000010813 (gray short-tailed opossum), ENSMLUG 00000007472 (little brown bat), ENSMICG00000010174 (gray mouse lemur), ENSOCUG 00000004502 (rabbit), ENSCPOG00000008298 (guinea pig), ENSETEG00000007044 (small Madagascar hedgehog), ENSECAG00000016134 (horse), ENSOANG00000003030 (platypus), ENSOPRG00000010860 (American pika), ENSSTOG00000005565 (thirteen-lined ground squirrel), ENSDNOG00000008299 (nine-banded armadillo), ENSLAFG00000004250 (African savanna elephant), ENSOGAG00000005737 (small-eared galago), e.g. the nucleic acid sequences of the SwissProt accession numbers DQ236342 (human), BC125400 (mouse), BC132094 (mouse), BC034201 (mouse), BC077720 (human), CX078607 (rhesus monkey), DR005339 (human), BC135580 (Xenopus tropicalis), including the coding sequences of the

SwissProt accession numbers AAH34201 (mouse), AAI25401 (mouse), AAI32095 (mouse), ABB43161 (human), AAH77720 (human), EAW63349 (human), EAW63347 (human), EAW63348 (human), AAI35581 (Xenopus tropicalis) and CAJ81459 (Xenopus tropicalis). Examples of a Rab11FIP2 protein include, but are not limited to, the proteins of the SwissProt accession numbers Q5HZI0 (Q5HZI0_MOUSE), Q7L804 (RFIP2_HUMAN), Q3U366 (Q3U366_MOUSE), e.g. with the genes of the SwissProt accession numbers ENSMUSG00000040022 (mouse), ENSG00000107560 (human), ENSGALG00000009304 (chicken), ENSPTRG00000002982 (chimpanzee), ENSMODG00000009385 (gray short-tailed opossum), ENSXETG00000009541 (Xenopus tropicalis), ENSMMUG00000021805 (rhesus monkey), ENSCAFG00000011991 (dog), ENSPPYG00000002692 (orangutan), ENSEEUG00000006651 (western European hedgehog), ENSMLUG00000015200 (little brown bat), ENSMICG00000001976 (gray mouse lemur), ENSOCUG00000009241 (rabbit), ENSCPOG00000000453 (guinea pig), ENSETEG00000019294 (small Madagascar hedgehog), ENSECAG00000019791 (horse), ENSSARG00000001794 (European shrew), IGI00014376 (Integr8 database, human), IGI00031145 (Integr8 database, mouse) and IGI02699308 (Integr8 database, bovine), and e.g. the nucleic acid sequences of the SwissProt accession numbers BC089010 (mouse), BC075073 (human), BC075074 (human) and DN994254 (human), including the coding sequences of the SwissProt accession numbers AAH89010 (mouse), EAW49423 (human), AAH75073 (human) and AAH75074 (human). Examples of a Rab11FIP3 protein include, but are not limited to, the proteins of the SwissProt accession numbers Q75154 (RFIP3_HUMAN), Q8JZT3 (Q8JZT3_MOUSE), A2IDE0 (A2IDE0_HUMAN), A2A2G1 (A2A2G1_HUMAN), A2IDD9 (A2IDD9_HUMAN), A2A2G0 (A2A2G0_HUMAN), and Q8CHD8 (Q8CHD8_MOUSE), e.g. with the genes of the SwissProt accession numbers ENSG00000090565 (human), ENSMUSG00000037098 (mouse), ENSPTRG00000007543 (chimpanzee), ENSMMUG00000000613, IGI00010332 (Integr8 database, human) and IGI00087092 (Integr8 database, chicken), IGI00026472 (Integr8 database, mouse) e.g. the nucleic acid sequences of the SwissProt accession numbers AL049542 (human), Z98882 (human), BC023364 (mouse), BC037132 (mouse), BC051360 (human), and AL023881 (human), including the coding sequences of the SwissProt accession numbers AAH23364 (mouse), AAH37132 (mouse), CAI95788 (mouse), CAI95591 (human), CAI95593 (human), CAM26382 (human), CAM26383 (human), CAQ09643 (human), CAQ11003 (human) CAM28374 (human), AAH51360 (human), EAW85805 (human), EAW85806 (human), EAW85807 (human), EAW85808 (human), EAW85809 (human), and EAW85810 (human). Examples of a Rab11FIP4 protein include, but are not limited to, the proteins of the SwissProt accession numbers Q6DGI2 (Q6DGI2_BRARE), Q86YS3 (RFIP4_

HUMAN), Q8BQP8 (RFIP4_MOUSE), Q5SYH4 (Q5SYH4_MOUSE), Q3LGD4 (Q3LGD4_BRARE), Q3U4R6 (Q3U4R6_MOUSE) and Q3LGD3 (Q3LGD3_BRARE), e.g. with the genes of the SwissProt accession numbers ENSDARG00000053855 (zebrafish), ENSG 00000131242 (human), ENSMUSG00000017639 (mouse), ENSMODG00000019209 (gray short-tailed opossum), ENSPTRG00000008974 (chimpanzee), ENSMMUG00000008127 (rhesus monkey), ENSOCUG00000007443 (rabbit), ENSMLUG00000016407 (little brown bat), ENSCPOG00000011634 (guinea pig), IGI00013025 (Integr8 database, human), IGI00029750 (Integr8 database, mouse), IGI02693035 (Integr8 database, zebrafish) and IGI00051410 (Integr8 database, zebrafish), e.g. the nucleic acid sequences of the SwissProt accession numbers BC076363 (zebrafish), BC093914 (human), BC101517 (human), AL591174 (mouse), BC135989 (Xenopus tropicalis) and DR000701 (human), including the coding sequences of the SwissProt accession numbers AAH76363 (zebrafish), AAI35990 (Xenopus tropicalis), CAI24833 (mouse), CAI24836 (mouse), EAW80265 (human), AAI01518 (human) and AAH93914 (human). Examples of a Rab11FIP5 protein include, but are not limited to, the proteins of the SwissProt accession numbers Q6ZQ33 (Q6ZQ33_MOUSE), Q9BXF6 (RFIP5_HUMAN), Q8R361 (RFIP5_MOUSE) and Q3UM85_MOUSE, e.g. with the genes of the SwissProt accession numbers ENSG00000135631 (human), ENSMUSG00000051343 (mouse), ENSGALG00000016092 (chicken), ENSBTAG00000006162 (cattle), ENSXETG00000011781 (Xenopus tropicalis), ENSOCUG00000000840 (rabbit) ENSPTRG00000012057 (chimpanzee), ENSMMUG00000002301 (rhesus monkey), ENSOGAG00000009150 (small-eared galago), ENSECAG00000007084 (horse), IGI00035642 (Integr8 database, mouse) and IGI00006075 (Integr8 database, human), e.g. the nucleic acid sequences of the SwissProt accession numbers DQ890991 (synthetic construct of human clone), DQ894168 (synthetic construct of human clone), BC035013 (human), BC026473 (mouse), BC044833 (mouse), BC051063 (mouse), DR770942 (rhesus macaque monkey), including the coding sequences of the SwissProt accession numbers AAH26473 (mouse), AAH44833 (mouse), AAH51063 (mouse), ABM81917 (synthetic construct), ABM85094 (synthetic construct), AAH35013 (human), EAW99750 (human), EAW99749 (human) and EAW99751 (human).

[0032] The small GTPase proteins of the Rab family are known to have crucial roles in regulating cellular activity including intracellular membrane trafficking, signal transduction, receptor recycling and the recruitment of effector proteins to cellular membranes. Similar roles have accordingly been ascribed to Rab family interacting proteins such as Rab11 family interacting proteins. Rab11-FIP3 has for example been shown to play a critical role in the

function of the endosomal recycling compartment (see e.g. Horgan, C.P., et al., (2007), *Traffic*, 8, 414-430).

[0033] The Rab proteins belong to the larger family of Ras-related small G-proteins (including the Rho-related proteins) that regulate a wide range of basic cell functions including cytoskeletal organization and cell morphology, cytokinesis, vesicle transport and secretion, cell-cycle progression and cell differentiation. Several of these proteins and their regulators and effectors have been implicated in carcinogenesis and cancer progression. Among these, RAB25, which is frequently amplified in breast and ovarian cancers, has been shown to enhance proliferation and apoptotic resistance in vitro, and tumor formation in vivo. The RAB family of proteins play a central role in membrane trafficking and organelle compartmentalization, and RAB11, RCP's primary binding partner, is specifically involved in membrane protein recycling. RAB11 has been implicated in cell migration, and disruption of RAB11 pathways compromise migration in different cell types (Jones, M.C., et al., (2006) *Curr Opin Cell Biol* 18, 549-557; Powelka, A. M., et al., (2004) *Traffic* 5, 20-36; Yoon, S.O., et al., (2005) *Cancer Res* 65, 2761-2769). Furthermore, the competitive inhibition of RAB11 function decreases hypoxia-induced invasiveness of breast cancer cells (Yoon et al., 2005, supra) and alters trafficking of membrane receptors involved in invasion such as Integrin alpha 6 beta 4, EGFR, CXCR2 and PKC alpha (Jones et al., 2006, supra). Dysregulation of RAB gene expression is implied as a generalized component of many human tumors. However, the role of Rab interacting proteins in tumor progression has not been studied.

[0034] The present inventors have identified a broad range of cancer-promoting effects linked to Rab11FIP1expression. In noncancerous breast epithelial cells, Rab11FIP1 overexpression led to malignant transformation, and increased cell motility and migration. In breast cancer cell lines, attenuation of endogenous Rab11FIP1 by RNA inhibition reduced proliferation, anchorage-independent growth, invasion and apoptotic resistance, and diminished tumor formation in nude mice. Physiologically, Rab11FIP1 is thought to interact primarily with Rab11 to regulate protein sorting in tubular endosomes (Peden, A.A., et al., (2004) *Mol Biol Cell* 15, 3530-3541). Rab11-Rab11FIP1 complexes have been shown to channel transferrin receptor away from lysosomes (the degradation pathway) to recycling endosomes (the recycling pathway), thereby promoting a relative increase in the level of membrane-bound receptor (Peden et al., 2004, supra). Though speculative, it is plausible that the transforming effects of Rab11FIP1 may be mediated by this shift towards the recycling pathway, prolonging the membrane localization (and thus function) of certain membrane proteins such as the integrins or growth factor receptors with oncogenic roles. The strong correlation the present inventors observed between Rab11FIP1 expression and ERK

phosphorylation may reflect this possibility, as it may indicate a specific link between Rab11FIP1 (in the following also named RCP) and growth factor receptor-mediated MAPK activation.

[0035] Without being bound by theory, immunoprecipitation data obtained by the present inventors (not shown) show that there is a direct interaction between Rab11FIP1 and the small GTPase Ras, indicating the formation of a complex between the two proteins. The Ras proteins are a family of small monomeric proteins that are in the art known for their central position in apoptosis, cellular proliferation, and cancer. At least 11 distinct functional classes of proteins have been shown to be effectors of Ras proteins. Accordingly, Ras proteins are thought to be centrally located in complex signal transaction networks, in which they activate at least three overlapping and cooperative protein kinase cascades, namely Raf/MEK/ERK, Ral GTPase, and PI3K/Akt. As one randomly selected further illustrative example they act via the Rb/E2F and Dnmt-1, thereby causing epigenetic changes by methylation, such as inactivation of p16, which activates tumor cell growth. In turn, ras proteins are subject to complex regulatory mechanisms. Point mutated Ras genes are frequently found in one-third of various tumors and Ras genes are known to be oncogenes.

[0036] Some methods according to the present invention are methods of controlling tumourigenesis. These methods include in particular methods of preventing, inhibiting, arresting or reversing tumourigenesis. Tumourigenesis may for example be carcinogenesis, including the formation of malignant forms of carcinomas. Accordingly, the method may for example be included in a treatment or prevention of a proliferative disease or disorder, such as cancer.

[0037] Using the present method of the invention, tumourigenesis may be prevented, inhibited, arrested or reversed in any organism, including for instance a mammal, a fish, an amphibian, a bird or a microorganism. A respective microorganism is in some embodiments a cell. Such a cell may be obtained from, including directly taken or isolated from, an organism, such as for instance one of the examples above, such as a mammal. Examples of a suitable mammal include, but are not limited to, a rat, a mouse, a rabbit, a Guinea pig, an opossum, a dog, a cat, a chimpanzee, a rhesus monkey, a macaque, an orang-utan, a cattle (cow), a marmoset, an American pika, a galago ("bushbaby"), a squirrel, a nine-banded armadillo, an elephant and a human. The cell may also be a cell of a conventional cell line, for instance a cancer cell line. The cell is in some embodiments cultured. A respective cell may in other embodiments be included, for example part of, an organism, such as for instance one of the examples above.

[0038] Some embodiments of the present method according to the invention include modulating the amount of a Rab binding protein, such as a Rab11FIP protein. Modulating the

amount of the Rab binding may include allowing a portion of the total number of the Rab binding protein molecules, present in a cell, in an *in vitro* set-up such as a test tube or in an organism, to be degraded. Degradation of the Rab binding protein may for instance be allowed to be carried out by a protease, such as a calpain protease (also termed calcium-activated protease or calpain). As an illustrative example, Rab11FIP1 has been disclosed to contain three PEST (Pro, Gly and Ser/Thr-rich) motifs, which serve as targets for calpains, and to be cleaved by calpains *in vivo* (Nicolas, M., et al. (2005) *Biochem. J.*, 389, 223-231). Modulating the amount of the Rab binding protein may also include modulating the expression of the Rab11FIP protein. This may for example be carried out by administering a compound that modulates the expression of the Rab11FIP protein. The expression of the Rab11FIP protein may be increased or reduced. As an illustrative example, the expression of the Rab11FIP protein may be reduced by means of a nucleic acid molecule, such as a non-coding nucleic acid molecule.

[0039] The term "nucleic acid molecule" as used herein refers to any nucleic acid in any possible configuration, such as single stranded, double stranded or a combination thereof. Nucleic acids include for instance DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA), analogues of the DNA or RNA generated using nucleotide analogues or using nucleic acid chemistry, and PNA (protein nucleic acids). DNA or RNA may be of genomic or synthetic origin and may be single or double stranded. In the present method of the invention typically, but not necessarily, an RNA or a DNA molecule will be used. Such nucleic acid can be e.g. mRNA, cRNA, synthetic RNA, genomic DNA, cDNA, synthetic DNA, a copolymer of DNA and RNA, oligonucleotides, etc. A respective nucleic acid may furthermore contain non-natural nucleotide analogues and/or be linked to an affinity tag or a label. In some embodiments the nucleic acid molecule may be isolated, enriched, or purified. The nucleic acid molecule may for instance be isolated from a natural source by cDNA cloning or by subtractive hybridization. The natural source may be mammalian, such as human, blood, semen, or tissue. The nucleic acid may also be synthesized, e.g. by the triester method or by using an automated DNA synthesizer.

[0040] Many nucleotide analogues are known and can be used in nucleic acids and oligonucleotides used in the present method of the invention. A nucleotide analogue is a nucleotide containing a modification at for instance the base, sugar, or phosphate moieties. Modifications at the base moiety include natural and synthetic modifications of A, C, G, and T/U, different purine or pyrimidine bases, such as uracil-5-yl, hypoxanthin-9-yl, and 2-aminoadenin-9-yl, as well as non-purine or non-pyrimidine nucleotide bases. Other nucleotide analogues serve as universal bases. Universal bases include 3-nitropyrrole and 5-nitroindole.

Universal bases are able to form a base pair with any other base. Base modifications often can be combined with for example a sugar modification, such as for instance 2'-O-methoxyethyl, e.g. to achieve unique properties such as increased duplex stability.

[0041] In some embodiments of the present method of the invention the nucleic acid molecule is a non-coding nucleic acid molecule, such as for example an aptamer or a Spiegelmer® (described in WO 01/92655). A non-coding nucleic acid molecule may also be an nc-RNA molecule (see e.g. Costa, FF, *Gene* (2005), 357, 83-94 for an introduction on natural nc-RNA molecules). Examples of nc-RNA molecules include, but are not limited to, an anti-sense-RNA molecule, an L-RNA Spiegelmer®, a silencer-RNA molecule (such as the double-stranded Neuron Restrictive Silencer Element), a micro RNA (miRNA) molecule, a short hairpin RNA (shRNA) molecule, a small interfering RNA (siRNA) molecule, a repeat-associated small interfering RNA (rasiRNA) molecule or an RNA that interacts with Piwi proteins (piRNA) (for a brief review see e.g. Lin, H., *Science* (2007) 316, 397). An illustrative example of suitable shRNA is given as SEQ ID NO: 3 and SEQ ID NO: 4 in the examples below.

[0042] The use of small interfering RNAs has become a tool to "knock down" specific genes. It makes use of gene silencing or gene suppression through RNA interference (RNAi), which occurs at the posttranscriptional level and involves mRNA degradation. RNA interference represents a cellular mechanism that protects the genome. SiRNA molecules mediate the degradation of their complementary RNA by association of the siRNA with a multiple enzyme complex to form what is called the RNA-induced silencing Complex (RISC). The siRNA becomes part of RISC and is targeted to the complementary RNA species which is then cleaved. This leads to the loss of expression of the respective gene (for a brief overview see Zamore, PD, & Haley, B (2005) *Science* 309, 1519-1524). This technique has for example been applied to silencing parasitic DNA sequences, such as the cleavage of HIV RNA, as disclosed in US patent application 2005/0191618.

[0043] A typical embodiment of such a siRNA for the current invention includes an in vitro or in vivo synthesized molecule of 10 to 35 nucleotides, in some embodiments 15 to 25 nucleotides. A respective si-RNA molecule may be directly synthesized within a cell of interest (including a cell that is part of a microorganism and an animal). It may also be introduced into a respective cell and/or delivered thereto. An illustrative example of delivering a siRNA molecule into selected cells in vivo is its non-covalent binding to a fusion protein of a heavy-chain antibody fragment (Fab) and the nucleic acid binding protein protamin (Song, E. et al. (2005), *Nature Biotech.* 23, 6, 709-717). In an embodiment of the present invention

siRNA molecules are used to induce a degradation of mRNA molecules encoding one or more transcription factors involved in the (e.g. *in vivo*) synthesis of the Rab binding protein.

[0044] Some embodiments of the present method according to the invention include modulating the activity of a Rab binding protein, e.g. of a Rab11FIP protein. Modulating the activity of the Rab binding protein may include forming a complex between the Rab binding protein and a compound. This may for example be carried out by administering a compound that modulates the activity of the Rab binding protein. The activity of the Rab binding protein (e.g. the Rab11FIP protein) may be increased or reduced.

[0045] A respective compound can be administered by any suitable means. If the host organism is a mammal, the compound may for example be administered parenterally or non-parenterally (enterally). In a typical embodiment of administering to a mammal, the application ensures a delivery to blood and liver, for instance by administering a preparation of the compound orally, intravenously or by inhalation. Examples for preparations for an oral application are tablets, pills or drinking solutions, examples for preparations for intravenous administrations are injection or infusion solutions, examples of preparations for administration by inhalation are aerosol mixtures or sprays. If the host organism is a recombinant microorganism, examples of administration are the injection or addition of the compound to the environment of the microorganism. In case of the microorganism being a single cell, the latter form of administration may possibly be performed in combination with a technique that modifies the microorganism. Such a technique may comprise electroporation or a permeabilisation of the cell membrane.

[0046] Modulating the activity of a Rab binding protein, e.g. a Rab11FIP protein, may also include altering a posttranslational modification of the Rab binding protein, including altering the pattern of posttranslational modifications of the Rab binding protein. In some embodiments altering a posttranslational modification of the Rab binding protein, such as the Rab11FIP protein, includes the use of a compound that alters the pattern of posttranslational modifications of the Rab binding protein. Illustrative example of a respective posttranslational modification are phosphorylation, dephosphorylation, acetylation, and ubiquitylation. As a result of a respective posttranslational modification, e.g. phosphorylation, the activity of the Rab binding protein changes. As an illustrative example, Rab11-FIP2 function has been shown to be regulated by phosphorylation on serine 227 (Ducharme, N.A., et al., *Molecular Biology of the Cell* (2006) 7, 8, 3625-3637). Phosphorylated by the kinase MARK2/EMK1/Par-1B (MARK2), Rab11-FIP2 serves in establishing epithelial cell polarity (ibid.).

[0047] A compound that modulates the activity, expression, amount or subcellular

localisation of a Rab binding protein, such as a Rab11FIP protein, may be any compound. Examples include, but are not limited to, a nucleic acid (see above), a peptide, a peptoid, an inorganic molecule and a small organic molecule. Peptoids can have a much higher cell permeability than peptides (see e.g. Kwon, Y.-U., and Kodadek, (2007) T., *J. Am. Chem. Soc.* 129, 1508-1509). A peptide may be of synthetic origin or isolated from a natural source by methods well-known in the art. The natural source may be mammalian, such as human, blood, semen, or tissue. A peptide, including a polypeptide may for instance be synthesized using an automated polypeptide synthesizer. Illustrative examples of polypeptides are an antibody, a fragment thereof and a proteinaceous binding molecule with antibody-like functions. Examples of (recombinant) antibody fragments are Fab fragments, Fv fragments, single-chain Fv fragments (scFv), diabodies or domain antibodies (Holt, L.J., et al., (2003) *Trends Biotechnol.*, 21, 11, 484-490). An example of a proteinaceous binding molecule with antibody-like functions is a mutein based on a polypeptide of the lipocalin family (WO 03/029462, Beste et al., (1999) *Proc. Natl. Acad. Sci. U.S.A.*, 96, 1898-1903). Lipocalins, such as the bilin binding protein, the human neutrophil gelatinase-associated lipocalin, human Apolipoprotein D or glycodelin, posses natural ligand-binding sites that can be modified so that they bind to selected small protein regions known as haptens. Examples of other proteinaceous binding molecules are the so-called glubodies (see WO 96/23879), proteins based on the ankyrin scaffold (Mosavi, L.K., et al., (2004) *Protein Science* 13, 6, 1435-1448) or crystalline scaffold (WO 01/04144) the proteins described in Skerra, (2000) *J. Mol. Recognit.* 13, 167-187, AdNectins, tetranectins, and avimers. Avimers contain so called A-domains that occur as strings of multiple domains in several cell surface receptors (Silverman, J., et al., (2005) *Nature Biotechnology* 23, 1556-1561). Adnectins, derived from a domain of human fibronectin, contain three loops that can be engineered for immunoglobulin-like binding to targets (Gill, D.S. & Damle, N.K., (2006) *Current Opinion in Biotechnology* 17, 653-658). Tetranectins, derived from the respective human homotrimeric protein, likewise contain loop regions in a C-type lectin domain that can be engineered for desired binding (ibid.). Peptoids, which can act as protein ligands, are oligo(N-alkyl) glycines that differ from peptides in that the side chain is connected to the amide nitrogen rather than the α carbon atom. Peptoids are typically resistant to proteases and other modifying enzymes and can have a much higher cell permeability than peptides (see e.g. Kwon, Y.-U., and Kodadek, T., (2007) *J. Am. Chem. Soc.* 129, 1508-1509).Where desired, a modifying agent may be used that further increases the affinity of the respective moiety for any or a certain form, class etc. of target matter.

[0048] A respective compound may also alter a posttranslational modification of a Rab binding protein (such as a Rab11-FIP protein).The compound may for instance be able to

change the phosphorylation status of cellular components, including the Rab binding protein. A respective compound may also be a modulator of the degree of the phosphorylation of cellular proteins, including proteins that differ from the Rab binding protein. Such a protein may in turn alter a posttranslational modification of the Rab binding protein. An illustrative example of a respective compound is tyrosine kinase inhibitor. A large number of tyrosine kinase inhibitors are commercially available such as tyrphostins, quinazolines, quinoxalines, quinolines, 2-phenylaminopyrimidines, flavonoids, benzoquinoids, aminosalicylates or stilbenes (which are described in e.g. WO 9618738, WO 03035621 and references cited therein, for an example of their experimental identification see e.g. US 6,740,665). Examples of tyrphostins are AG213, AG490, AG 879, AG 1295, AG 1478, AG 1517, AGL 2043, tyrphostin 46 and methyl 2,5-dihydroxycinnamate. Quinazolines are for instance PD153035, PD 156273, gefitinib or lapatinib; quinoxalines are for example PD153035 or ZD1839. An example for a quinoline is 5-methyl-5H-indolo[2,3-β]quinoline, an example for a 2-phenylaminopyrimidine is imatinib, examples for flavonoids are genistein or quercetin, an example for a benzoquinoid is herbimycin A, an example for an aminosalicylate is lavendustin A, and an example for a stilbene is piceatannol. Other suitable compounds may comprise a receptor tyrosine kinase inhibitor such as the tyrphostin erbstatin, an EGFR specific receptor tyrosine kinase inhibitor such as WHI-P97 or the tyrphostin AG 592, a tyrosine phosphorylation stimulator such as aurin tricarboxylic acid or a tyrosine phosphatase inhibitor such as sodium pervanadate or isoxazole carboxylic acids.

[0049] Further examples of compounds that have been reported to inhibit the function of protein kinases include, but are not limited to, bis monocyclic, bicyclic or heterocyclic aryl compounds (international patent application WO 92/20642) vinylene-azaindole derivatives (PCT WO 94/14808), 1-cyclopropyl-4-pyridyl-quinolones (U.S. Patent No. 5,330,992), styryl compounds (U.S. Patent No. 5,217,999), styryl-substituted pyridyl compounds (U.S. Patent No. 5,302,606), certain quinazoline derivatives (European patent application No. 0 566 266 A1), seleoindoles and selenides (international patent application WO 94/03427), tricyclic polyhydroxylic compounds (international patent application WO 92/21660), and benzyl-phosphonic acid compounds (international patent application WO 91/15495). Yet other compounds that could be used as modulators include oxindolinones such as those described in U.S. patent application Serial No. 08/702,232 filed August 23, 1996.

[0050] A further example of a compound modulating a posttranslational modification of a Rab binding protein is an agonist or antagonist for a cell surface molecule that is able to induce the regulation of a protein kinase or protein phosphatase.

[0051] Another example of a compound that modulates the activity of a Rab binding protein such as a Rab11FIP proteins is a member of the D vitamins, such as vitamin D3 or vitamin D4, for example. Vitamin D3 (also called cholecalciferol, calciol or arachitol, IUPAC name (1S, 3Z)-3-[(2E)-2-[(1R,3aS,7aR)-1-[(1R)-1,5-dimethylhexyl]octahydro-7a-methyl-4H-inden-4-ylidene]ethylidene]-4-methylene-cyclohexanol, also termed (3β,5Z,7E)-9,10-Secocholesta-5,7,10(19)-trien-3-ol) in its active form has the ability to induce growth arrest, differentiation, and cell death in malignant cells of various origins, including breast cancer. This observation has led to the development of vitamin D analogs as possible anticancer agents (see e.g. Baudet, C., et al. (1996) *Cancer Lett.* 100, 3-10; Hsieh, T., & Wu, J. M., (1997) *Biochem. Biophys. Res. Commun.* 235, 539-544; James, S. Y., et al., (1999) *Gen. Pharmacol.* 32, 143-154; Mathiasen, I. S., et al., (1999) *Cancer Res.* 59, 4848-4856). Compound EB 1089 is one such analog and is currently in phase III clinical trials for the treatment of cancer (see e.g. Mathiasen, I.S., et al., (1993) *J. Steroid Biochem. Mol. Biol.* 46, 365-371; Colston, K.W., et al., (1992) *Biochem. Pharmacol.* 44, 2273-2280; Hansen, C.M., et al., (2000) *Curr. Pharm. Des.* 6, 803-828). The anti-cancer activity of vitamin D compounds is directly related to its effects on intracellular free calcium ($[Ca^{2+}]$). The elevation of free Ca^{2+} in cells treated with vitamin D compounds correlates with the induction of apoptosis in breast cancer cells (Sergeev, I. N., et al., (2000) in *Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects* (Norman, A. W., Bouillon, R., and Thomasset, M., eds) pp. 399-402, University of California, Riverside, CA; Vandewalle, B., et al. (1995) *Int. J. Cancer* 61, 806-811; Sergeev, I.N., & Rhoten, W.B., (1998) *Endocrine* 9, 321-327), and has been shown to be a primary mechanism in the activation of the calcium-dependent cysteine proteases, also known as calpains (Nakagawa, T., & Yuan, J., (2000) *J. Cell Biol.* 150, 887-894; Ray, S.K., et al., (2000) *Brain Res.* 852, 326-334; Ishihara, I., et al., (2000) *Neurosci. Lett.* 279, 97-100).

[0052] Without wishing to be bound by theory, recent evidence from studies in breast cancer cells suggests that the calpains may be the primary execution proteases in apoptotic death induced by vitamin D3 and EB 1089 (Mathiasen, I.S., et al., (2002) *J. Bio. Chem.* 277, 30738-30745). The protease (protein degradation) function of calpains depend on PEST (Pro, Gly, and Ser/Thr-rich) amino acid motifs present in the peptide sequence of protein targets of calpains (Dice, J. F. (1987) *FASEB J.* 1, 349-357; Tompa, P., et al., (2004) *J. Biol. Chem.* 279, 20775-20785; Rechsteiner, M., & Rogers, S.W., (1996) *Trends Biochem. Sci.* 21, 267-271; Shumway, S.D., et al., (1999) *J. Biol. Chem.* 274, 30874-30881; Fukuda, M., & Itoh, T., (2004) *J. Biol. Chem.* 279, 22314-22321). RAB11FIP1, contains for example three such PEST motifs located between the C2 domain and the RBD (Rab binding domain). In a recent study, Marie, et. al. sought to determine if the PEST motifs in RAB11FIP1 target it for regulated

degradation by calpains (Marie, N., et al., (2005) *Biochem. J.* 389, 223-231). They observed that treating cells with increasing concentrations of calcium triggers the degradation of RAB11FIP1, and this degradation can be abolished by addition of specific calpain inhibitors. Furthermore, the involvement of PEST sequences in this calcium-regulated degradation was validated using an RAB11FIP1 mutant in which the PEST sequences were removed. Thus, the authors concluded that the PEST sequences of RAB11FIP1 target the protein for calpain-mediated degradation, presumably as a mechanism of physiological turnover in the cell. Given that for example RAB11FIP1 contains active PEST domains sensitive to calpain action, and that calpains are activated for proteolytic activity via vitamin D3 and its chemotherapeutic analogs such as EB 1089, the methods of the invention allow for RAB11FIP1 mRNA or protein levels to be used as a biomarker for selecting patients for chemotherapy regimens based on vitamin D3 and its related analogs including EB 1089, and any other treatment regimens that biochemically act to elevate intracellular free calcium levels or activate the calpains.

[0053] As noted above, in some embodiments of a method of controlling, e.g. a method of preventing, arresting or reversing tumourigenesis in a cell, the levels, the expression and/or the activity of a Rab binding protein are analysed. The cell may be an (e.g. isolated) individual cell, a cell of an organism, which may harbor cancerous tissue, a cell of a tissue, including a cancerous tissue, or a cell of a cell culture. A cancer cell may for instance be a neuronal, glial, lung, liver, brain, breast, bladder, blood, leukemic, colon, endometrial, stomach, skin, ovarian, fat, bone, cervical, esophageal, pancreatic, prostate, kidney, or thyroid cell In some aspects a cancer includes, but is not limited to astrocytoma, acute myelogenous leukemia, breast carcinoma, bladder carcinoma, cervical carcinoma, colorectal carcinoma, endometrial carcinoma, esophageal squamous cell carcinoma, glioma, glioblastoma, gastric carcinoma, hepatocellular carcinoma, Hodgkin lymphoma, leukemia, lipoma, melanoma, mantle cell lymphoma, myxofibrosarcoma, multiple myeloma, neuroblastoma, non-Hodgkin lymphoma, lung carcinoma, non-small cell lung carcinoma, ovarian carcinoma, esophageal carcinoma, osteosarcoma, pancreatic carcinoma, prostate carcinoma, squamous cell carcinoma of the head and neck, thyroid carcinoma and urothelial carcinoma.

[0054] An altered amount, an altered expression or an altered activity of a Rab binding protein in a cell may be adjusted to a level corresponding to about an average level or to a commonly found level. Altering the amount of the Rab binding protein in the cell may be achieved via the modulation of the activity or of the expression of a protease. In some embodiments such a protease is a calcium dependent protease (calpain), e.g. calpain I or II. In some embodiments the activity and/or the expression of a calpain protease is modulated via an

alteration of intracellular calcium levels, e.g. free intracellular calcium.

[0055] In some embodiments the activity and/or the expression of a calpain protease is modulated, e.g. activated, by the use of a member of the vitamin D family, a prodrug of such a member of the vitamin D family or an analog of such a member of the vitamin D family, including by application thereof. A respective vitamin D, analog or prodrug thereof may for example be contacted with a respective cell or be administered to a respective organism, including a respective subject. The activity and/or the expression of a protease may also be modulated by other factors such as a protease modulator that is in turn modulated via signal transduction cascades. Numerous proteases are known to be subject to regulation both in terms of their activity and their expression (see e.g. Turk, B., (2006) *Nature Reviews Drug Discovery*, 5, 9, 785-799). Apoptotic protease activating factor-1 or tissue inhibitors of metalloproteinase may serve as illustrative examples.

[0056] A "prodrug" of a member of the vitamin D family or an analog of a member of the vitamin D family is understood to be a compound that is transformed (e.g gradually or rapidly) *in vivo* to yield the parent vitamin D/ vitamin D analog compound, for example by metabolization in the host, including e.g. by hydrolysis in blood or by oxidation, by either enzymatic action or by general acid or base solvolysis, to form an active ingredient. Typically a prodrug is a derivative of the parent vitamin D/ vitamin D analog, which on use *in vivo* releases the original compound through a chemical or physiological process. A prodrug may for instance be converted into the parent vitamin D/ vitamin D analog when a physiological pH is reached, by spontaneous chemical reaction(s), enzyme catalyzed chemical reaction(s), photolysis, and/ or metabolic chemical reaction(s). Common examples are compounds with biologically labile protecting groups on a functional moiety of the parent compound, including, but not limited to, ester and amide forms of a compound having an active form bearing a carboxylic acid moiety. Further examples include moieties of compounds that can be oxidized, reduced, aminated, deaminated, esterified, deesterified, alkylated, dealkylated, acylated, deacylated, phosphorylated, dephosphorylated, photolyzed, hydrolyzed, or other functional group change or conversion involving forming or breaking chemical bonds on the prodrug. A respective prodrug may as such not be fully active or physiologically available until converted *in vivo* to its therapeutically active or available form. A "prodrug" may also include a product formed, *in vitro* or physiologically, by a covalent or non-covalent bond between the respective drug molecule (in this case the parent vitamin D/ vitamin D analog) and a carrier moiety. It is also to be understood that certain compounds can exist in solvated as well as unsolvated forms such as, for example, hydrated forms. A solvated form is also simply referred to as a "solvate".

[0057] As further noted above, in some embodiments of a method of identifying a cell having a predisposition to turn tumorigenic an altered amount, an altered expression or an altered activity of a Rab binding protein in the cell, which may be an (e.g. isolated) individual cell, a cell of an organism or a cell of a cell culture, may be determined. Likewise, in some embodiments of a method of diagnosing the risk of developing a neoplasm in a subject, e.g. a patient, an altered amount, an altered expression or an altered activity of a Rab binding protein may be determined. In such cases determining a respective alteration may be an indication that a respective neoplasm or tumor will be sensitive to an alteration of the activity and/or amount of a protease in the cell or in cells of the subject. In some embodiments such a protease is a calcium dependent protease (calpain) such as the calcium-dependent proteases CDPI or CDP II, also known as calpain proteases calpain I or II (also termed calcium activated neutral proteases I and II). In some embodiments determining an altered activity and/or expression of a Rab binding protein, may be an indication that a respective neoplasm or tumor will be sensitive to an alteration of intracellular calcium levels.

[0058] In this regard, a respective method can further include obtaining a sample from the subject as well as isolating nucleic acids from the sample. The nucleic acids may be amplified as well as labelled. Methods of determining the amount of a selected protein are well known in the art. A respective method may for instance rely on proteomics-based techniques. It may involve the use of an antibody, a fragment thereof or a proteinaceous binding molecule with antibody-like functions, which binds to the respective Rab binding protein. Methods of analyzing the expression protein of a protein are well established in the art. mRNA expression may for instance be quantified using northern blotting and in situ hybridization, RNAse protection assays and PCR-based methods. As a further example, an antibody, a fragment thereof or a proteinaceous binding molecule may be employed that can recognise specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. Illustrative examples of methods of sequencing-based gene expression analysis include Serial Analysis of Gene Expression (SAGE), and gene expression analysis by massively parallel signature sequencing (MPSS).

[0059] Examples of (recombinant) antibody fragments are Fab fragments, Fv fragments, single-chain Fv fragments (scFv), diabodies, triabodies (Iliades, P., et al., *FEBS Lett* (1997) 409, 437-441), decabodies (Stone, E., et al., *Journal of Immunological Methods* (2007) 318, 88–94) and other domain antibodies (Holt, L.J., et al., *Trends Biotechnol.* (2003), 21, 11, 484-490). Single-chain Fv fragments are for instance fusions of variable regions from one heavy chain and one light chain of an immunoglobulin molecule. An example of a proteinaceous binding molecule with antibody-like functions is a mutein based on a polypeptide of the

lipocalin family (WO 2003/029462; WO 2005/019254; WO 2005/019255; WO 2005/019256; *Beste et al., Proc. Natl. Acad. Sci. USA* (1999) 96, 1898-1903). Lipocalins, such as the bilin binding protein, the human neutrophil gelatinase-associated lipocalin, human Apolipoprotein D, human tear lipocalin, or glycodelin, posses natural ligand-binding sites that can be modified so that they bind to selected small protein regions known as haptens. Other non-limiting examples of further proteinaceous binding molecules so-called glubodies (see WO 96/23879), proteins based on the ankyrin scaffold (Mosavi, L.K., et al., *Protein Science* (2004) 13, 6, 1435-1448) or the crystalline scaffold (WO 2001/04144), the proteins described by Skerra (*J. Mol. Recognit.* (2000) 13, 167-187), AdNectins, tetranectins, avimers and peptoids. Avimers contain so called A-domains that occur as strings of multiple domains in several cell surface receptors (Silverman, J, et al., *Nature Biotechnology* (2005) 23, 1556-1561). Adnectins, derived from a domain of human fibronectin, contain three loops that can be engineered for immunoglobulin-like binding to targets (Gill, D.S. & Damle, N.K., *Current Opinion in Biotechnology* (2006) 17, 653-658). Tetranectins, derived from the respective human homo-trimeric protein, likewise contain loop regions in a C-type lectin domain that can be engineered for desired binding (ibid.). Peptoids, which can act as protein ligands, are oligo(N-alkyl) glycines that differ from peptides in that the side chain is connected to the amide nitrogen rather than the α carbon atom. Peptoids are typically resistant to proteases and other modifying enzymes and can have a much higher cell permeability than peptides (see e.g. Kwon, Y.-U., and Kodadek, T., *J. Am. Chem. Soc.* (2007) 129, 1508-1509). Where desired, a modifying agent may be used that further increases the affinity of the respective moiety for any or a certain form, class etc. of target matter.

[0060] PCR (polymerase chain reaction) is a standard technique useful to amplify and detect transcripts from a cell or from a tissue sample such as a melanoma sample. Reverse Transcriptase PCR (RT-PCR), is for instance a sensitive quantitative method that can be used to compare mRNA levels in different samples (e.g., non- metastatic and metastatic melanoma samples, or benign cutaneous and melanoma samples) to examine gene expression signatures. To perform RT-PCR, mRNA is isolated from a sample (e.g., total RNA isolated from a human melanoma sample). mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin- fixed) tissue samples. Purification kits for RNA isolation are commercially available. The RT-PCR technique may also be used in the embodiment of real time quantitative PCR, which measures PCR product accumulation through a dual-labeled fluorigenic probe. Real time PCR is compatible both with quantitative competitive PCR, where internal competitor for each target sequence is used for normalization, and with quantitative comparative PCR using a normalization gene contained within the sample, or a

housekeeping gene for RT-PCR. As a further example, the competitive PCR design may be used for gene expression analysis, possilby including automated, high-throughput matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry detection and quantification of nucleic acid molecules. Further examples of techniques that may be included in PCR-based methods suitable for gene expression analysis may include, but are not limited to, differential display, amplified fragment length polymorphism (iAFLP), the BeadAmiy™ technology, BeadsArvay for Detection of Gene Expression (BADGE) and high coverage expression profiling (HiCEP) analysis. As a further example, determining gene expression of a tissue sample can also be performed with microarrays.

[0061] In some embodiments determining an altered activity and/or expression of a Rab binding protein, in particular an increased activity and/or expression of a Rab binding protein may be an indication that a respective neoplasm or tumor will be sensitive to treatment with a member of the D vitamins, an analog or a prodrug thereof. In this regard the present invention also provides a method of determining whether a neoplasm (including a tumor, e.g. cancer) is sensitive to an alteration of the activity and/or amount of a protease. The protease may be present, including expressed, therein, in particular in cells thereof. In some embodiments the method is a method of determining whether a neoplasm is sensitive to member of the vitamin D family, an analog or a prodrug thereof.

[0062] A large number of vitamin D analogs, including vitamin D3 analogs are known in the art that may be used in a method according to the present invention (for an overview see e.g. Kubodera, N., (2006) *Current Bioactive Compounds*, 2(3), 301-315). Suitable examples including e.g. calcitriol, maxacalcitol, tacalcitol, secalciferol, alfacalcidol or seocalcitol (EB 1089) have for instance been listed in international patent application WO 2007/082542. Seocalcitol has previously been shown to potentiate the response to ionizing radiation in breast cancer tumor cells, which has been suggested to be a consequence of alterations in cell signalling pathways downstream of the initial induction of DNA damage (DeMasters, G., et al., (2006) *Mol Cancer Ther*, 5, 11, 2786-2797). Further recent examples are 16-en-22-oxa-vitamin D3 analogs such as SMD-429, the formation of which has been described by Shimizu et al. ((2006) *Bioorganic & Medicinal Chemistry Letters*, 16, 3323-3329 & references cited therein), 1α,25-dihydroxy-20*S*,21(3-hydroxy-3-methylbutyl)-23-yne-26,27-hexafluorochole-calciferol as described by e.g. Lee et al. ((2007), *Cancer Research*, 67(24), 11840-11847) or the 1α,25-dihydroxyvitamin D3 analogs presented by Yoon et al. ((2008) *International Journal of Oncology*, 23, 387-396), 19-functionalized derivatives of 1α-hydroxy-5,6-trans-vitamin D3 (Wojtkielewicz, A., (2007) *Steroids*, 72(6-7), 552-558.), the analogs with elongated side chains described by Ono et al. ((2007), Journal of Combinatorial Chemistry

9(4), 711-716) or the difluoro analogs described by Posner et al. ((2007) *Journal of Steroid Biochemistry & Molecular Biology*, 103, 213-221).

[0063] For some embodiments of the invention, compounds may be used in form of a library. Examples of such libraries are collections of various small organic molecules, chemically synthesized as model compounds, or nucleic acid molecules containing a large number of sequence variants.

[0064] In some embodiments the present method of the invention includes detecting the expression, the subcellular localisation and/or the activity of the Rab11-FIP protein, for example in an organism or in a tissue or a cell thereof. As an illustrative example, the cellular amount, the expression, the subcellular localisation and/or the activity of the Rab11-FIP protein may be monitored over a period of time.

[0065] The present method of the invention may furthermore include comparing the results of measuring the cellular amount, the expression, the subcellular localisation and/or the activity of the Rab11-FIP protein with results of a control measurement (or "reference" measurement). A control measurement may include the use of conditions that do not modulate the cellular amount, the expression, the subcellular localisation and/or the activity of a Rab11FIP protein. In comparing cellular amount, expression, and/or activity, detected levels may for example be compared to a control level. The term "control level" as used herein refers to the number of molecules of the respective protein, e.g. in a cell, a mRNA or protein expression level of a Rab11FIP protein, as well as to an activity level of a Rab11FIP protein in a control sample. The term thus includes both a normal control level and a cancer control level (see also below). The term can refer to a single reference measurement or to a plurality of reference measurements. In some embodiments the control level may be a database of expression or activity values from previously conducted measurements. The term "customary level" refers to a level of expression of a Rab11FIP protein or an activity level of a Rab11FIP protein detected in a normal, healthy individual or in a population of individuals known not to be suffering from a neoplasm, including cancer. A normal individual is one with no clinical symptoms of a respective neoplasm.

[0066] According to the present invention, a gene expression level or an activity level or an amount of a protein, e.g. in a cell, is deemed to be "altered" or to "differ" when gene expression / activity / amount is increased or decreased by about 10 %, about 25 %, about 50 %, about 75 %, about 100 %, or higher, as compared to the control level. Alternatively, an expression level or an activity level is deemed "increased" or "decreased" when gene expression / or an activity is increased or decreased by at least about 0.1, at least about 0.2, at

least about 1, at least about 2, at least about 5, or at least about 10 or more fold as compared to a control level.

[0067] The present method of the invention may include altering the activity of extracellular signal-regulated kinase (ERK). In some embodiments the method includes preventing, inhibiting, arresting or reversing activation of ERK. In typical embodiments, modulating the amount or the activity of a Rab11-FIP protein may result in modulating the activity of ERK.

[0068] A further method of the invention is a method of identifying a cell that has a predisposition to turn tumorigenic, including cancerogenic. The method includes detecting in the cell an altered amount, an altered expression, an altered subcellular localisation or an altered activity of a Rab11FIP protein. As already explained above for a related method, the method may include measuring the cellular amount, the expression, the subcellular localisation and/or the activity of the Rab11-FIP protein. In some embodiments the method may also include comparing the results of measuring the cellular amount, the expression, the subcellular localisation and/or the activity of the Rab11-FIP protein with results of a control measurement (see above).

[0069] The present invention also relates to a method of diagnosing the risk of developing a neoplasm, such as a tumor, including cancer, in a subject. A respective tumor may for example be a breast tumor, a lung tumor, a colorectal tumor, a tumor of the urinary bladder or a tumor of the fallopian tube (also termed oviduct). Likewise, a respective cancer may for instance be breast cancer, lung cancer, colorectal cancer, cancer of the urinary bladder or cancer of the fallopian tube (also termed oviduct), including one of the corresponding carcinomas. An illustrative example of a carcinoma of lung cancer is a non-small cell lung carcinoma. The method includes determining at least one of the amount, the expression level, the activity level and the subcellular localisation of a Rab11FIP protein. The measurement may in some embodiments be carried out in a sample, such as a tissue sample or a cell sample, from the subject. In some embodiments the method may also include comparing the results of measuring the cellular amount, the expression, the subcellular localisation and/or the activity of the Rab11-FIP protein with results of a control measurement (see above). For a respective control measurement a sample may be used, in which the expression and/or the activity of the Rab binding protein is on a customary ("normal") level. In typical embodiments an altered amount, expression level, activity level or subcellular localisation of a Rab binding protein as compared to the control measurement indicates that the subject suffers from or is at risk of developing a neoplasm.

[0070] Further methods of the invention are methods, both *in-vivo* and *in-vitro* methods, of identifying a respective compound. The compound may be capable of preventing, inhibiting, arresting or reversing tumourigenesis, including carcinogenesis. The compound may be capable of forming a complex with a Rab binding protein, such as a Rab11FIP protein. Typically these methods include exposing the components of this complex to each other, whether *in-vitro* or *in-vivo*. One such method is an in-vitro method, which includes contacting the components that form, or are suspected to form, a complex with each other. For example a Rab11FIP protein and a drug candidate molecule, which is suspected to form a complex with the Rab11FIP protein, may be contacted with each other. In some embodiments the method further includes detecting the formation of the complex.

[0071] Any suitable method of detecting a complex formation may be used. A detection method may for instance include electrophoresis, HPLC, flow cytometry, fluorescence correlation spectroscopy or a modified form of these techniques. Other techniques involve a measurement of the biomolecular binding itself. Such measurements may for instance rely on spectroscopic, photochemical, photometric, fluorometric, radiological, enzymatic or thermodynamic means, or on cellular effects. An example for a spectroscopic detection method is fluorescence correlation spectroscopy (Thompson, N.L., et al, *Curr. Opin. Struct. Biol.* (2002) 12 (5), 634-641). A photochemical method is for instance photochemical cross-linking (Steen, H., Jensen, O.N., *Mass Spectrom Rev.* (2002) 21, 3, 163-182). The use of photoactive, fluorescent, radioactive or enzymatic labels respectively (for an overview see: Rippe, R.A., et al., *Methods Mol. Biol.* (2001) 160, 459-479) are examples for photometric, fluorometric, radiological and enzymatic detection methods. An example for a thermodynamic detection method is isothermal titration calorimetry (ITC, for an overview see: Velazquez-Campoy, A., et al., *Methods Mol. Biol.* (2004) 261, 35-54). An example of a method using cellular effects is the measurement of cell proliferation or cell viability including its enzymatic detection or cellular replication.

[0072] Further related methods are *in-vivo* methods that include providing a microorganism. The microorganism expresses the Rab binding protein, with which the compound is suspected to be capable of forming a complex, or the expression of which the compound is suspected to be capable of altering (e.g. suppressing). The microorganism may in some embodiments endogenously express the Rab binding protein. In some embodiments the microorganism is a recombinant cell or a transgenic microorganism. In some embodiments the microorganism is a single cell, such as a cancer cell, including a breast cancer cell or a lung cancer cell. In some embodiments the microorganism is a cell obtained from an organism. A respective organism may for instance be a mammal, a fish, an amphibian or a bird. A cell is

typically derived from tissue of a respective organism, such as skin tissue or breast tissue. In some embodiments the microorganism is a cell of a cell line, such as a cancer cell line, including a breast cancer cell line or a lung cancer cell line. Examples of a suitable breast cancer cell line include, but are not limited to, a HMEC cell line, 3D, Bcap-37, BT-20, BT-474, BT474M1, Hs578T, Hs578Bst, JIMT-1, MCF-7, M231, MDA-231, MDA-MB-134, MDA-MB-175, MDA-MB-231, MDA-MB-361, MDA-MB-361DYT2, MDA-MB-435, MDA-MB-435HM, MDA-MB-468, MB231, MW1, PMC42, SK-BR-3, SK-BR-7, SUM149, SUM159, SUM1315, SUM-52PE, T-47D, ZR-75, ZR-75-1 and ZR-75-30. A lung cancer cell line may for example be an adenocarcinoma cell line, a squamous-cell carcinoma cell line or a small-cell carcinoma cell line. Examples of a suitable lung cancer cell line include, but are not limited to, ABC-1, A549, Calu-6, EBC-1, H322, H446, H460, HCC2279, HTB-56R, HUT29, IMEC-2, KU-T1, L9981, LCD, LCOK, LK-2, LC-1/sq, Lu135, MS-1, NCI-H69, NCI-H82, NCI-H209, NCI-H446, NCI-H1395, NCI-H1437, NCI-H1770, NCI-H2009, NCI-H2087, NCI-H2122, NCI-H2126, NCI-H2171, NCI-N226, NCI-N231, PC1, PC3, PC7, PC9, PC10, PC14, PLA-801D, QG56, RERF-LCAI, RERF-LCMS, RERF-LCKJ, SBC3, SPC-A1, SQ5 and YTLMC-90. Numerous other cancer cell lines are known in the art, e.g. the additional cell lines named in Pole et al. (2006, supra).

[0073] In some embodiments the microorganism is a cell of a colorectal cancer cell line, an urinary bladder cancer cell line and a fallopian tubal carcinoma cell line. Examples of a suitable colorectal cancer cell line include, but are not limited to, Colo-320, HCT116, HT-29, L0, L0-200, L0-500, L0-1000, LoVo, LS1034, LST-R1, RKO, SW480, and WiDr, as well as further colorectal cancer cell lines referred to by Douglas et al. ((2004) *Cancer Research* 64, 14, 4817-4825). Examples of a suitable bladder cancer cell line include, but are not limited to, BIU-87, EJ, KoTCC-1, MBT-2, RT4, ScaBER, T24 and UM-UC-2. Two illustrative examples of a fallopian tubal carcinoma cell line are FT-MZ-1 (Runnebaum, I.B., et al., (1994) *FEBS Lett.*, 353, 1, 29-32) and HEPFT (see Ishiwata, I., et al., (2007) *Human Cell*, 20,119-130).

[0074] In some embodiments the microorganism is a cell of a non-tumorigenic, including non-cancerous tissue cell line, such as the breast cancer epithelial cell line MCF10A, overexpressing a Rab binding protein, such as a Rab11FIP, e.g. RAB11FIP1. Likewise, any cell – whether of a cell line, an individual cell, a cell within a multicellular organism, a cell obtained (including directly obtained and isolated) from a multicellular organism – may be selected and transformed into a cancer cell by overexpressing a Rab binding protein such as RAB11FIP1. The examples below and e.g. Fig. 6 illustrate how a cell can be transformed into a cancer cell accordingly. In this regard the method also provides a method of obtaining a cancer cell, which includes a method of modulating, in particular increasing, the expression of

a Rab binding protein in a cell. Thereby the cell is transformed into a cancer cell. The respective Rab binding protein may for example be a Rab11 binding protein such as RAB11FIP1. It may be an endogenous or an heterologous protein, i.e. a protein with an encoding nucleic acid sequence naturally occurring in the cell or a protein of which the encoding nucleic acid sequence is being/has been introduced into the respective cell. The invention also relates to the use of such a cell for identifying a compound capable of modulating the expression of the corresponding Rab binding protein *in vivo* (see below).

[0075] The present methods of the invention include adding the compound, which is suspected to be capable of forming a complex with the Rab binding protein, or to be capable of altering its expression, to the microorganism. In some embodiments of a respective method, the expression of the Rab binding protein, e.g. a Rab11FIP protein, is monitored. In some embodiments of a respective method the activity of the the Rab binding protein is monitored. In some embodiments a change in cell phenotype of the Rab binding protein is monitored. In some embodiments such a method includes a control measurement (see also above). The results of the control measurement are compared to the results obtained using the compound. A control measurement may for example include the use of a compound that is known not to affect the expression or the activity of the Rab binding protein.

[0076] Yet other related methods are an *in-vivo* methods that include providing a host organism. Any desired host organism may be provided as long as it is capable of accommodating and growing a cancer cell. Examples of a host organism include, but are not limited to, a mammal, a fish, an amphibian and a bird. Examples of a suitable mammal include, but are not limited to, a rat, a rabbit, a Guinea pig, an opossum, a dog and a cat.

[0077] Any desired cancer cell may be used for this purpose (see above for examples).The method further includes introducing a cancer cell into the host organism. Furthermore the method includes the use of a compound as described above, i.e. a compound that is suspected to be capable of forming a complex with a Rab binding protein, to be capable of modulating the amount of a Rab binding protein, or to be capable of modulating the activity of a Rab binding protein. In some embodiments the cancer cell includes the compound. Accordingly the compound may be introduced into the cancer cell before introducing the same into the host organism. In some embodiments the compound is administered to the host organism, before, after or concurrently with introducing the cancer cell therein. Typically the compound is introduced into the cancer cell at a certain stage of the method. The method further includes monitoring the growth of tumors in the host organism.

[0078] In order that the invention may be readily understood and put into practical effect,

particular embodiments will now be described by way of the following non-limiting examples.

EXAMPLES

[0079] The particularly relevant aspects and features of the TRIAGE method developed by the inventors can be summarized as follows (see also Fig. 1):

TRIAGE steps:	*TRIAGE steps:*
Correlating amplicon genes with distant metastasis-free survival	**RATIONALE**
1. Putative expression amplicons are mapped by genome-wide LSVD; those that overlap with chromosomal regions of known recurrent amplification are further investigated.	1. Expression footprints of recurrent genomic amplicons can be detected by LSVD.
2. Patients are stratified by each amplicon gene into below-mean and above mean expression groups.	2. & 3. Some metastasis-promoting oncogenes frequently activated by amplification should have expression patterns that correlate with poor DMFS in breast cancer patients, reflecting their functional involvement in metastatic progression.
3. Cox proportional hazards regression (2-group survival analysis) is performed and the significance of the hazards ratio (p-value) is reported for each gene.	
Testing for robustness; discriminating oncogenes from passenger genes	4. Passenger genes located on survival-associated amplicons may correlate with poor DMFS without functional involvement. Oncogenes activated by multiple cancer mechanisms not limited to amplification should be associated with poor outcome in the absence of the amplicon, whereas passenger genes should not.
4. Tumors are ranked by eigen weight to estimate the amplicon-containing tumors. Steps 2 & 3 are repeated excluding those patients with amplicon containing tumors.	
5. Genes within the amplicon are ranked by their average expression in the amplicon-containing tumors.	5. The oncogenic driver(s) of an amplicon should be relatively highly and consistently expressed in amplicon-containing tumors.
6. The Pearson correlation between	6. DMFS-associated genes also correlated

expression pattern and the proliferation signature is calculated for each gene.	with proliferation are considered with caution, as proliferation itself is associated with metastasis of breast cancer.

Cells and drug treatment

[0080] Microarray data and tumor samples. Four previously described breast cancer microarray datasets were used in this study, and all expression profiles were generated using the Affymetrix U133A and U133B genechips as described in the original publications: Uppsala cohort (Miller, L. D., et al. (2005) *Proc Natl Acad Sci U S A* 102, 13550-13555.), Stockholm cohort (Pawitan, Y., et al. (2005) *Breast Cancer Res* 7, R953-964), Belgium cohort (Loi, S., et al. (2007) *J Clin Oncol* 25, 1239-1246) and Singapore cohort (Ivshina, A. V., et al. (2006) *Cancer Res* 66, 10292-10301). All Affymetrix CEL files were retrieved from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), normalized using the global mean method (Miller et al., 2005, supra), and probe set signal intensities were natural log transformed and scaled by adjusting the mean intensity to a target signal value of log 500. To remove cohort-dependent array biases, arrays within each cohort were mean-centered prior to combining them into one dataset. Clinical characteristics of all patients and tumor samples are summarized in Fig. 2.

[0081] Local Singular Value Decomposition (LSVD). Microarray probe sets were mapped to genomic coordinates according to the UCSD Genome Browser. Candidate PEAs were identified as the localized coordinated overexpression of genes in a corresponding subset of tumors. First, a gene was defined as overexpressed in a sample if its expression in that sample was >2.5 times the adjusted median absolute deviation (aMAD) from its median expression across all samples (Fig. 1, step 1). Second, localized coordinated overexpression was quantified by the principal eigen value obtained by SVD applied on a sliding window of 50 probes. Probes corresponding to the same gene (according to UniGene ID, build #177) were averaged to represent a single gene measurement. Candidate PEAs were identified as contiguous chromosomal regions having local principal eigen values above a baseline threshold. Candidate PEAs were scored and ranked by the peak principal eigen value. Highest scoring candidate PEAs were subjected to a second round of LSVD using a window size of 10 probes to better resolve peak structure (ie, to distinguish one from multiple sub-peaks). Finally, highest scoring sub-peaks were then subjected to SVD to derive eigen weights for the individual genes and tumors. Eigen weights reflect the relative contribution of genes and tumors to the magnitude of a principal eigen peak. For a given peak, genes with nonzero eigen weights were considered components of the PEA. Tumors with an eigen weight greater than

the median plus 2 standard deviations were labeled as amplicon-containing tumors (TRIAGE, step 4).

[0082] Local Singular Value Decomposition (LSVD) to identify recurrent genomic amplifications. LSVD analysis of gene expression profiles from a cohort of tumor samples is a key first step in identifying probable expression amplicons (PEAs), from which oncogenes contributing to metastasis may be discovered by the TRIAGE methodology (Triangulating Oncogenes through Clinico-Genomic Intersects). LSVD identifies PEAs based on the hypothesis that a recurrent amplicon will manifest as the coordinated overexpression of genomically localized genes in a subset of tumor samples. LSVD is built around singular value decomposition (SVD) (Strang, G., (1998) "Introduction to Linear Algebra", Section 6.7. 3rd ed., Wellesley-Cambridge Press. ISBN 0-9614088-5-5) and predicts PEAs as peaks in the coordinated local overexpression plot which is a local principal eigen value (LPEV) plot obtained by the local application of SVD on a chromosome-by-chromosome basis. LSVD is achieved by deriving local expression matrices for each genomic position on the genome, transforming it to a binary connectivity matrix and applying SVD on the binary matrix. The principal eigen value obtained upon such application is a measure of the coordinated overexpression of genes at that locus (Kluger, Y., et al., (2003) *Genome Research*, 13, 703-716; Kleinberg, J.M., (1999) *Jl of the ACM*, 46, 5, 604-632). Eigen value "peaks" that exceed a certain basal threshold in the plot of LPEV vs. genomic location (LPEV plot) demarcate the PEAs. The detailed description of the LSVD procedure is provided below.

[0083] Let E_c be the gene expression matrix of chromosome C_c; E_{cij} is the $\log_2$ of the expression of gene at location l_i in tumor T_j, where $i = 1, 2, ..., N_c$ and $j = 1, 2, ..., M$. *Probable Expression Amplicons (PEAs)* on C_c are predicted by analyzing E_c using LSVD which has the following sequence of steps: (1) Transforming E_c to binary connectivity matrix A_c; (2) Deriving Chromosome Localized Matrices, Ap_c; (3) SVD on Ap_c; and, (4) Identifying PEAs.

[0084] 1. Transforming E_c to binary connectivity matrix A_c: E_c is transformed to a binary connectivity matrix A_c through a boolean descretization rule as follows:

$$A_{cij} = 1 \text{ if } E_{cij} > \mu_i + x*v_i$$
$$A_{cij} = 0 \text{ if } E_{cij} \leq \mu_i + x*v_i$$

where μ_i and v_i are the median and adjusted Median Absolute Deviation (aMAD) of $E_{ci} = \{E_{ci1}, E_{ci2}, ..., E_{ciM}\}$. aMAD is 1.4826 times the MAD. x is 2.5 for the first pass of coarse map and the 2 for the finer map obtained in the second pass.

[0085] 2. Deriving Localized Matrices, Ap_c: A local matrix Ap_c at position Lp_c is derived from A_c using genes at positions from p-w to p+w on chromosome C_c where w is the window size, a predefined parameter. w is 50 for the first pass coarse map and 10 for the second pass finer map.

[0086] 3. Singular Value Decomposition (SVD): The application of SVD on Ap_c is a crucial step to predict PEAs. The SVD of Ap_c decomposes Ap_c into a product of three matrices Up_c, Σp_c and Vp_c i.e. $Ap_c = Up_c \times \Sigma p_c \times Vp_c^T$. Up_c and Vp_c are of $(2w+1)\times(2w+1)$ and $M \times M$ matrices respectively while Σp_c is $(2w+1)\times M$ diagnol matrix. The column vectors of Up_c and Vp_c are the eigen vectors of $Ap_cAp_c^T$ and $Ap_c^TAp_c$ respectively while the diagonal elements of Σp_c are the respective eigen values. The highest eigen value is called the principal eigen value (denoted by λp_c) and the respective eigen vectors are called the principal eigen vectors. *Eigen weight of tumor* T_j at position Lp_c is denoted by Tp_{cj} and it defined as the absolute of the j^{th} component of the principal eigen vector of the matrix $Ap_c^TAp_c$. Similarly, *eigen weight of a gene* G_i at position Lp_c, denoted by Gp_{cj} is the absolute of the j^{th} component of the principal eigen vector of the matrix $Ap_cAp_c^T$.

[0087] To improve the contrast between true PEA signal and background, in our LSVD implementation, we use SVD on $(Ap_cAp_c^T)^3$ instead of on Ap_c to find λp_c. The eigen weights of tumors and genes are obtained, following the above definitions, from the principal eigen vectors of $(Ap_c^TAp_c)^4$ and $(Ap_cAp_c^T)^4$ respectively. Now, λp_c is the ratio of the fourth root of the principal eigen value of $(Ap_cAp_c^T)^4$ to M^4; the eigen weights of genes and tumors are the fourth root of the respective components of the principal eigen vectors of $(Ap_cAp_c^T)^4/M^4$ and $(Ap_c^TAp_c)^4/M^4$ respectively.

[0088] 4. Predicting PEAs: Plotting λp_c vs. Lp_c gives the plot of LPEVs, this is called *LPEV plot*. The peaks in this plot show the PEAs. Higher the value of λp_c at the peak, higher the confidence of PEA at around Lp_c. Finally, a PEA is reported at a peak with range that covers a contiguous region with $\lambda p_c > T_\lambda = 20$ and centered around the peak.

[0089] TRIAGE gene-survival analysis. For each probe set, the mean expression level was first computed for a given cohort. Patients were then stratified into below-mean and above-mean groups, and the Cox proportional hazards regression model was fit to this data to compute a hazard ratio based on distant metastasis-free survival (DMFS). The null hypothesis was that the two groups would have the same risk of recurrence, and the likelihood ratio test p-value was computed as evidence against this hypothesis. Cox model fitting and statistical calculations were performed using the survival package in R. In the case of multiple probe sets mapping to the same gene, the results of the probe set with the most significant association

was reported. DMFS was defined as the time interval from surgery until the first distant recurrence event or last date of follow-up. All patients with bilateral and contralateral cancers, and those with recurrence or disease-specific death >10 years post-diagnosis were systematically censored for events. The Uppsala cohort was used to generate the survival data in TRIAGE as it was our largest population-based cohort with longest and most complete patient follow-up.

[0090] The proliferation signature and gene correlations. Using cDNA Microarrays, Whitfield et al previously identified >850 cell cycle regulated genes in HeLa cervical carcinoma cells (Whitfield, M. L., et al., (2002) *Mol Biol Cell*, 13, 1977-2000). The authors discovered a fraction of these genes that displayed highly correlated expression in breast tumors and were associated with tumor cell proliferation in the Perou et al breast cancer dataset (Perou, C.M., et al. (2000) *Nature* 406, 747-752). To approximate this proliferation signature, we mapped the cell cycle regulated genes (corresponding to 1,134 IMAGE clones in the Whitfield study) to Affymetrix probe sets via UniGene build #187, and hierarchically clustered the Uppsala cohort tumors. A distinctive cluster containing the proliferation associated genes emerged (see **Fig. 10**) and was found to be highly correlated with markers of proliferation in the Uppsala cohort including histologic grade, mitotic index, and KI67 scores (data not shown). Probe sets central to this cluster having an average correlation of >0.8 (n=78 probe sets corresponding to 67 genes [see **Fig. 10**]) were designated the "core proliferation signature" genes, and their average expression was used to calculate Pearson correlations between the proliferation signature and all other probe sets for the TRIAGE analysis, step #7. Notably, this step may not be essential for TRIAGE analysis of other cancer types, depending on the clinical association between proliferation and cancer recurrence.

[0091] Selection and cloning of RAB11FIP1 (RCP). Three splice variants of RCP were identified in GenBank: variant 1 (NM_025151), variant 2 (NM_001002233) and variant 3 (NM_001002814). Of these, only variants 1 and 3 could be detected consistently in tumor samples and cell lines by RT-PCR (data not shown), and only variant 1 was observed by Western blot with the polyclonal RCP antibody (GenWay). Thus, the full-length variant 1 of RCP was amplified from normal human blood by RT-PCR (forward primer: 5'-ACCATG TCCCTAATGGTCTCGGCT-3' (SEQ ID NO: 1); reverse primer: 5'-TGCTGATTTACA TCTTTCCTCT-3') (SEQ ID NO: 2), sequence verified, and used to investigate RCP function in this study.

[0092] Cell lines and transient transfection. Human mammary cell lines, MCF10A, MCF7 and MDA-MB231 were obtained from American Type Culture Collection (ATCC) and

maintained at 37 °C with 5 % CO_2 with growth medium recommended by ATCC. Full-length RCP was ligated into the BamH1 and EcoR1 site of pcDNA3.1. Two RCP-specific RNAi constructs were designed as previously described (Reynolds, A., et al., (2004) *Nat Biotechnol*, 22, 326-330) and manufactured by Ambion. Transient plasmid and RNAi transfection on MCF7 was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

[0093] Generation of stable cell lines by lentiviral infection. For the generation of cell lines stably overexpressing shRNA, oligonucleotides encoding the target sequence (forward: 5'-TCGATAAGCAAGAAGGAGTTTTCAAgAgAAACTCCTTCTTGCTTATCGTTTTTTC-3' (SEQ ID NO: 3), and reverse: 5'-TCGAGAAAAAACGATAAGCAAGAAGGAGTTTCT CTTGAAAACTCCTTCTTGCTTATCGA-3') (SEQ ID NO: 4) were annealed and cloned into the LentiLox pLL3.7 vector. For control shRNA, a non-targeting "scramble" sequence was cloned into pLL3.7. (forward: 5'-TGAACGGCATCAAGGTGAACttcaagagaGTTCA CCTTGATGCCGTTCTTTTTTC-3' (SEQ ID NO: 5), and reverse: 5'-TCGAGAAAAAAG AACGGCATCAAGGTGAACTCTCTTGAAGTTCACCTTGATGCCGTTCA-3') (SEQ ID NO: 6). For overexpression of RCP by lentivirus, RCP was first cloned into the Invitrogen gateway entry vector pENTR3C and further recombined into the lentivirus vector, pLenti6/V5-DEST (Invitrogen) by LR recombination reaction according to the manufacturer's protocol. For lentivirus production, pLL3.7 or pLenti6/V5-DEST was co-transfected with packaging vectors into 293FT cells and the supernatant was harvested after 48 h. Virus was concentrated by ultracentrifugation for 2 h at 25,000 rpm in a Beckman SW28 rotor and resuspended in phosphate-buffered saline. Titers were determined by infecting NIH/3T3 cells with a serial dilution of the concentrated virus. For a typical preparation, the titer was approximately $1\text{-}5\times10^8$ particles for pLL3.7 and $4\text{-}10\times10^7$ particles for pLenti6/V5-DEST vector. For infection of MCF10A, MCF7 and MB231 cells, 2×10^5 cells were incubated in suspension with $1\text{x}10^7$ particles and 8 μg/ml polybrene for 3 h at 37 °C. The cells were then re-plated and cultured as previously described. To obtain a pure population of RCP knock-down stable cells, GFP-positive cells were sorted after 4 days of culture. For generating RCP overexpressing stable lines, cells were selected with blastacidin for 2 weeks after infection.

[0094] Western blot analysis. Whole cell lysates were prepared using RIPA buffer. The proteins were separated by SDS-PAGE and transferred to Hybond-P PVDF membrane (GE Healthcare). Antibodies to RCP (Genway, Catalog Number (CN): 15-288-21574A), β-actin (Sigma, CN: A5441), phospho-Akt (Cell Signaling Technology, CN: 9271), Akt (Cell Signaling Technology, CN: 9272), phospho-Erk (Cell Signaling Technology, CN: 9101), and Erk (Cell Signalling, CN: 9102) were used to probe the membrane, and antibody-protein

complex was detected by HRP-conjugated antibodies and ECL (Amersham Biosciences).

[0095] Cell proliferation and colony formation. Cells were plated at a density of 5000cells/well in 96-well plates, and cell proliferation was measured in quadruplicate (ie, 4 wells per condition) using WST-1 (Roche) according to the manufacturer's protocol. For the MAPK inhibition assays, cells were treated with MEK inhibitor U0126 (Promega) overnight in medium with 0.5 % serum before being re-plated into 96-well plates. To test the effect of RCP on anchorage-independent colony formation, cells were suspended in 250 µl of 0.3 % agar (Sigma) dissolved in complete medium containing 25 % FBS, and plated in quadruplicate (ie, 4 wells per condition) in 24-well plates pre-coated with 500ul of 0.6 % agar base. Colony forming efficiency was examined 21 days or more after plating by staining with Iodonitro-tetrazolium chloride (Sigma). Colonies of size >50um were counted using Leica QWin software.

[0096] ***In vitro*** **invasion and migration**. Transwell migration and invasion assays were performed using Falcon FluoroBlok 24-Multiwell inserts (BD Biosciences) with 8 µm pores. For invasion assays, the inserts were coated with 20 µg Matrigel (BD Biosciences) in 80 µL serum-free growth medium. For both assays, 5000 cells in 200 µL serum-free growth medium were loaded into each transwell insert with 750 µL complete growth medium with 10 % fetal bovine serum in the lower chamber. After 24 hours, cells that had migrated or invaded through the pores of the inserts were fixed with 3.7 % formaldehyde, stained with 2.5 µg/mL Hoechst 33342 (Invitrogen) for 15 mins, washed with PBS and counted using the Target Activation Bioapplication on an ArrayScan VTI (Cellomics). Field size was 1 mm^2. For invasion assays, experiments were performed with 4- to 5-fold replication, with 10-16 fields scanned per experiment. For migration assays, experiments were performed with 3-fold replication, with 10-16 fields scanned per experiment.

[0097] Immunofluorescence microscopy of cells. Cell cultures were fixed in 4 % para-formaldehyde and permeabilized with 0.25 % Triton X-100, followed by blocking with 1 % goat serum in PBS. Cells were stained with anti E-Cadherin (BD Pharmingen, CN: 610404) and fibronectin antibody (BD Pharmingen, CN: 610077) at 1:500 dilution, followed by the appropriate secondary antibodies detecting mouse and rabbit IgG conjugated with Alexa Fluor 594 or 488 (Molecular Probes), respectively. F-actin was labeled with Texas Red-X phalloidin (Molecular Probes). DAPI was used to stain the nucleus. Images were captured with a confocal microscope (LSM 510 META, Zeiss).

[0098] Cell cycle analysis. MCF10A cells were starved in 1 % serum for 48 h. Cells were then fixed in 75 % ethanol, treated with RNase A (0.25 mg/ml), stained with propidium iodide

(10 μg/ml) and analyzed on a LDR2 flow cytometer (BD Biosciences). Sorted cells was analyzed using FACS DiVa software (BD Biosciences).

[0099] Apoptosis assay. MB231 cells were maintained in complete medium or starved with 1 % serum for 48 h. Apoptotic cells were detected with the In Situ Cell Death Detection Kit (Roche). Cells with DNA strand breaks were labeled with TMR (red) by TUNEL (TdT-mediated dUTP nick end labeling) reactions and analyzed by fluorescence microscopy and flow cytometry.

[0100] Tumorigenicity in nude mice. Female BALB/c-nu/nu (nude) mice 6 - 8 weeks old were housed under pathogen-free conditions in a temperature controlled room on 12/12 h light/dark schedule with food and water *ad libitum*. All procedures involving animals and their care were in accordance with national and international regulations. For MCF-7 tumor formation experiments, a 1.7 mg 17β-estradiol pellet (90-day release, Innovative Research of America) was implanted in each mouse 1 week prior to tumor cell injection to supplement the estrogen requirements of MCF-7 cells. The experimental groups were: MCF7-RNAi RCP (stable RCP knock down), MCF7-RNAi Ctrl (control), MB231-RNAi RCP, MB231-RNAi Ctrl. For each experimental group, 8 mice were injected subcutaneously (above the right and left hind legs) with 2.0×10^6 cells. Subcutaneous tumors were measured with a digital caliper once a week. Tumor volume was calculated as $ab^2/2$ in mm^3, where a and b are the longest and the shortest perpendicular diameters of the tumor, respectively. Tumor weights were taken at termination and mice were killed in compliance with Singapore animal holding rules.

[0101] Immunohistochemistry. Four μm thick paraffin-embedded tumor tissue sections were stained using ABC immunohistochemistry reagents and avidin-biotin blocking reagents (Vector Laboratories) according to the manufacturer's protocols. The slides were incubated with anti-RCP antibody at a 1:500 dilution and counterstained with hematoxylin.

Results

[0102] The above data demonstrates that the coordinate analysis of genomic, transcriptomic and clinical information can successfully direct the discovery of novel genes in cancer that likely contribute to metastatic recurrence and patient death, thereby facilitating the rational prioritization of genes for downstream functional investigations. The exemplary validation of RAB11FIP1 as a multifunctional and frequently occurring oncogene in human breast cancer underscores its importance as a therapeutic target and provides new insight into the emerging role of endocytic recycling pathways in carcinogenesis.

[0103] For illustration purposes, a functionally validation of RAB11FIP1, located on

chromosome 8p11, as a novel oncogene with multiple pathological roles in breast tumorigenesis is herein provided. RAB11FIP1 was previously identified as the Rab4 and Rab11 interacting protein, also called Rab coupling protein (RCP), which functions in endosomal recycling and receptor sorting (Lindsay, A.J., et al. (2002) *J Biol Chem* 277, 12190-12199; Peden, A.A., et al. (2004) *Mol Biol Cell* 15, 3530-3541). Though dysregulation of RAB11 and RAB25 expression has been implied as a generalized component of many human tumors(see e.g. Cheng, K.W., et al. (2004) *Nat Med* 10, 1251-1256; Cheng, K.W., et al. (2005) *Methods Enzymol* 403, 202-215; Gebhardt, C., et al. (2005) *Am J Pathol* 167, 243-253; Goldenring, J.R., et al. (1999) *Yale J Biol Med* 72, 113-120; Yoon, S.O., et al. (2005) *Cancer Res* 65, 2761-2769), a role for Rab interacting proteins in tumor progression has not been described. The present inventors devised an integrated analytical strategy for triangulating critical oncogenes in cancer forms through clinical and genomic intersects, which takes into account expression frequency, coexpression in an amplicon, association with patient disease-free survival, and which discounts fortuitous association with tumor proliferation. Using this multi-step data mining approach, abbreviated TRIAGE (Triangulating Oncogenes through Clinico-Genomic Intersects, see below), the present inventors have identified RAB11FIP1 as an oncogene.

Oncogene deduction by TRIAGE

[0104] To identify oncogenes contributing to aggressive breast cancer, it was hypothesized that a particular subset of such genes might be discovered through triangulation of transcriptomic, genomic and clinical information. Specifically, the inventors sought to uncover oncogenes that are: 1) located in regions of recurrent amplification, 2) activated by gene dysregulation, and 3) associated with metastatic dissemination of cancer. To this end, we developed a multi-step data mining approach termed TRIAGE (Triangulating Oncogenes through Clinico-Genomic Intersects), that identifies expression footprints of recurrent genomic amplicons in expression microarray data, relates gene expression to risk of metastatic recurrence, and evaluates the candidacy of genes based on survival correlations and comparative mRNA dynamics (**Fig. 1**).

[0105] In the first step, microarray expression profiles of 737 primary invasive breast tumors (**Fig. 2**) were analyzed by Local Singular Value Decomposition (LSVD) to infer the presence and location of recurrent genomic amplicons. This approach is based on the premise that recurrent amplicons can be detected by the coordinate overexpression of involved genes (Chin, K., et al. (2006) *Cancer Cell* 10, 529-541; Reyal, F., et al. (2005) *Cancer Res* 65, 1376-1383), and is supported by evidence that the majority of highly amplified genes in breast cancer are concurrently overexpressed (Pollack, J. R., et al., (2002) *Proc Natl Acad Sci U S A*, 99,

12963-12968). LSVD allows for the simultaneous identification of genomic loci enriched for gene overexpression, and the fraction of tumors where "locus-specific" overexpression occurs. The output is viewed as *principal eigen peaks* that reflect both the degree of locus-specific overexpression and the number of tumors involved. The highest-scoring *principal eigen peaks* discovered by whole-genome LSVD mapped predominantly to loci corresponding with known regions of recurrent copy number gain (see **Fig. 3**). Though not all peaks identified by LSVD would be expected to represent recurrent amplification events, those with high magnitude and precise genomic overlap with known recurrent amplicons were termed *probable expression amplicons* (PEAs), and were further investigated by the TRIAGE process.

[0106] Based on the precept that the expression level of an oncogene of interest would correlate with poor patient outcome, genes within PEAs were assessed for significant associations with distant relapse. For each gene, tumors (and corresponding patients) were dichotomized into low expression (below mean) or high expression (above mean) groups and analyzed by Cox proportional hazards regression for distant metastasis-free survival (DMFS) (**Fig. 1**, steps 2&3). Likelihood ratio test *P*-values reflecting the significance of the resulting hazard ratios were used as an initial measure of the oncogenic potential of the PEA genes (**Figure 1**, step 3, *unselected survival*). However, since recurrent genomic amplicons may correlate with poor patient survival, benign "passenger genes" physically present on the amplicon may also correlate with poor DMFS without functionally contributing to tumor aggression, whereas the driving oncogenes (which may be activated in cancer by mechanisms other than copy number gain) would be expected to remain correlated with poor DMFS in tumors lacking the amplicon, while passenger genes would not. Therefore, the correlations between gene expression and DMFS with the amplicon-containing tumors censored from the analysis were re-examined (**Fig. 1**, step 4, *selected survival*). In this manner, only those candidate oncogenes robustly associated with poor DMFS independent of an amplification event will emerge.

[0107] Candidate oncogenes significant at both steps of survival analysis were further scrutinized through comparisons of mRNA expression patterns (**Fig. 1,** steps 5&6). Following the assumption that the driving oncogenes should be relatively highly and consistently expressed in amplicon-containing tumors, the expression levels of genes within PEAs were averaged, and the genes were subsequently ranked by mean expression (where a rank of 1 denotes the highest average expression level). In this way, the expression ranking serves as an additional relative gauge of oncogenic potential at a given locus.

[0108] Highly proliferative breast tumors are more likely to relapse, and genes associated

with proliferation are often correlated with poor DMFS. This is exemplified in the proliferation signature comprised of many hundreds of highly correlated genes enriched for cell cycle functions, and which has been found to be positively correlated with recurrence of many cancer types (Dai, H., et al., (2005) *Cancer Res* 65, 4059-4066; Ivshina, A. V., et al., (2006) *Cancer Res* 66, 10292-10301; Perou, C. M., et al., (2000) *Nature* 406, 747-752; Rosenwald, A., et al. (2003) *Cancer Cell* 3, 185-197; Whitfield, M. L., et al., (2002) *Mol Biol Cell* 13, 1977-2000; Whitfield, M. L., et al., (2006) *Nat Rev Cancer* 6, 99-106). As in the search was for genes with unique contributions to oncogenesis independent of association with proliferation, in the final step of TRIAGE, the expression patterns of candidate genes were examined for correlation with the proliferation signature to consider the extent to which survival associations might be explained by tumor proliferation rate. Probe sets in the Uppsala breast tumor cohort (n=251 tumors) correlating with poor DMFS are depicted in Fig. 4. Of the 2,377 probe sets found to be significantly correlated with poor DMFS ($p<0.05$), ~35% were also found to be members of this proliferation signature at a Pearson correlation of >0.4 (see **Figure 4**). The central core genes of the proliferation signature are, in clustered order: B.222958_s_at DEPDC1, A.204170_s_at CKS2, B.222680_s_at DTL, A.209680_s_at KIFC1, A.202503_s_at CSNK1G1, A.201291_s_at TOP2A, A.201292_at TOP2A, A.204033_at TRIP13, B.225687_at FAM83D, A.200853_at H2AFZ, A.213911_s_at H2AFZ, A.203362_s_at MAD2L1, B.228729_at CCNB1, A.214710_s_at CCNB1, A.203755_at BUB1B, B.228323_at CASC5, A.204825_at MELK, A.204822_at TTK, A.207828_s_at Unknown, A.202870_s_at CDC20, A.222039_at LOC146909, A.204962_s_at CENPA, B.228273_at Unknown, A.209408_at KIF2C, A.202705_at CCNB2, A.222077_s_at RACGAP1, A.218542_atCEP55, A.203213_at CDC2, A.203214_x_at CDC2, A.210559_s_at CDC2, A.218039_at NUSAP1, A.218009_s_at PRC1, A.204444_at KIF11, A.210052_s_at TPX2, A.219918_s_at Unknown, A.203764_at DLG7, B.223307_at CDCA3, A.202095_s_at BIRC5, A.202954_at UBE2C, A.202580_x_at FOXM1, A.218726_at DKFZp762E1312, A.203554_x_at PTTG1, A.209642_at BUB1, A.209773_s_at RRM2, A.201890_at RRM2, A.204641_at NEK2, A.218355_at KIF4A, A.208079_s_at Unknown, A.204092_s_at AURKA, A.38158_at SP1 and A.218755_at KIF20A. As approximately one third of the probe sets significantly correlating with poor DMFS were identified to be members of the proliferation signature, in breast cancer, approximately one in three poor survival-associated genes may be best explained by association with proliferation, rather than an independent oncogenic mechanism. We therefore de-prioritized or discounted candidate genes with significant survival associations yet correlated with the proliferation signature (at >0.4) in the selection process.

[0109] To test the validity of this analytical approach, TRIAGE was applied to the

principal eigen peak of greatest magnitude identified by whole-genome LSVD. This corresponded to a 1 Mb region on chromosome 17q12 encompassing about 30 genes. The gene components of this PEA mapped precisely to the well-characterized 17q12 amplicon housing the *EGFR*-related oncogene *ERBB2* (*HER2/neu*) and known to occur in ~20-30% of breast cancer cases. Notably, the expression footprint of this amplicon has previously been observed in microarray studies and shown to correlate well with the boundaries of the physical amplicon as discerned by array-CGH (Chin et al., 2006, supra; Pollack et al., 2002, supra). In tumors exhibiting this PEA, we found a strong enrichment for HER2 positivity by immuno-histochemistry ($P<1.0x10^{-26}$; Chi-square test), consistent with the evidence that this PEA corresponds with the physical 17q12 amplicon (**Fig. 5A**, **Fig. 5C**, black vertical bars). Initial (*unselected*) gene-survival analysis identified 6 genes within this PEA significantly associated with DMFS (**Fig. 5A**). Upon re-analysis of survival in the absence of patients with tumors harboring the PEA (ie, *selected* survival), only 2 genes remained significantly associated with DMFS: *TCAP* and *ERBB2*. While neither was strongly correlated with the proliferation signature, *ERBB2* had the highest expression ranking (**Fig. 5E**). Thus, TRIAGE identified *ERBB2* (encoding the precursor of receptor tyrosine-protein kinase erbB-2) as a "top candidate" oncogenic driver at the 17q12 locus, independent of its known roles as a metastasis-promoting oncogene and therapeutic target in breast cancer.

[0110] A second large principal eigen peak identified by LSVD mapped to the p-arm of chromosome 8 to a region spanning 1.5 Mb and encompassing 22 genes. This PEA was found to precisely overlap at the gene level with the previously described 8p11-12 amplicon known to occur in 10-25% of breast cancers (Garcia, M. J., et al., (2005) *Oncogene* 24, 5235-5245; Gelsi-Boyer, et al., (2005) *Mol Cancer Res* 3, 655-667; Letessier, A., et al. (2006) *BMC Cancer* 6, 245; Ray, M. E., et al., (2004) *Cancer Res* 64, 40-47). The oncogenic drivers of this amplicon have not been well characterized. By TRIAGE, we identified 3 genes on this amplicon significantly associated with increased risk of distant metastasis: *RAB11FIP1*, *EIF4EBP1*, and *WHSC1L1* (**Fig. 5B**). Upon analysis for *selected* gene-survival correlations, only two genes remained significantly associated with distant metastasis, *RAB11FIP1* and *WHSC1L1* (encoding Histone-lysine N-methyltransferase NSD3), with *RAB11FIP1* having the most significant association with distant recurrence and highest expression rank (**Fig. 5E**). Based on this finding, we investigated the functional impact of RAB11FIP1 on oncogenic properties of breast cancer.

RCP overexpression transforms MCF10A cells

[0111] To verify if RAB11FIP1 (herein also referred to as RCP) is amplified in mammary

tumors at the protein level, western blot analysis was performed on a panel of cell lines with different grades of tumor progression using a commercially available antibody. RCP was the lowest in the non-tumorigenic (normal) mammary epithelial cell line MCF10A, but was expressed at 4- to 10-fold higher levels in the breast cancer cell lines CAMA-1, MDA-MB231, MCF7 and T47D (**Fig. 6A**).

[0112] Next the role of RCP amplification in breast cancer was investigated by establishing MCF10A cell lines stably over-expressing full length RCP (MCF10A-RCP) (Genbank Accession: NM_025151) or the control vector. While the control cells exhibited a flattened morphology and monolayer growth characteristic of normal epithelium (**Fig. 6B**; left panels), RCP overexpression resulted in dramatic morphological changes, including spindle-like projections and a scattered growth pattern occasionally interspersed with foci-like formations reminiscent of transformed fibroblasts (**Fig. 6B**; right panels). The control MCF10A cells, on the other hand, exhibited a flattened morphology and monolayer growth in epithelial-type islands - more characteristic of normal epithelium. Furthermore, at confluence, the MCF10A RCP overexpressing cells displayed loss of contact inhibition and exhibited continuous multilayer growth. Immunofluorescent staining of cells revealed that RCP overexpression was associated with loss of E-cadherin at cell-cell junctions and disorganization of beta-actin. Additionally, fibronectin was observed to be more abundant in MCF10A-RCP cells than control cells under serum starvation conditions (**Fig. 6C**). Loss of E-cadherin at sites of cell-cell contact (an epithelial marker) and increased expression of fibronectin (a mesenchymal marker) are both consistent with an RCP-dependent epithelial to mesenchymal (EMT) transition.

[0113] To further evaluate the effects of RCP, the inventors took advantage of the stringent requirement of MCF10A cells for serum and supplemental growth factors to support their proliferation. The stable lines were maintained both in the presence and absence of growth factors. MCF10A-RCP cells showed significant increased rate of proliferation in both culture conditions than control cells (**Fig. 6D** and **Fig. 8A**). Most interestingly, in the most stringent medium of 1 % FBS without supplemented growth factors (EGF, insulin), the control lines died within 6 days, while MCF10A-RCP cells could be maintained for over a month indicating a RCP-mediated loss of growth factor dependence (**Fig. 6D**). The cell cycle analysis by propidium iodide staining revealed that after 48 hr of serum and growth factor starvation, 93 % of the control cells had arrested at G1 phase compared to only 3 % of the cells at G2M stage, whereas in MCF10A-RCP cells, about 80 % of cells were at G1 phase and 12 % of cells were in G2M stage, confirming the role of RCP in regulating cell cycle progression (**Fig. 7A**).

[0114] Next, the capacity of RCP to drive anchorage-independent growth in MCF10A

cells in soft agar colony formation assays was examined. As expected, the control cells failed to produce colonies in soft agar. In marked contrast, however, MCF10A-RCP cells formed numerous colonies after 4 weeks, demonstrating that RCP is able to induce a transformed phenotype (**Figure 6E** and **Figure 8B**). Additionally, we observed that RCP overexpression enhanced the migratory potential of MCF10A cells as demonstrated by wound-healing assays (**Figure 6F**) and transwell assays (**Figure 8D**).

[0115] To gain insight into the mechanism(s) by which RCP exerts its oncogenic phenotypes, it was examined whether RCP expression could activate growth signaling through either ERK or AKT, two of the major pathways regulating oncogenic growth. MCF10A-RCP cells displayed strong activation of ERK but not AKT (**Figure 7B**). To test whether RCP-induced Erk activation was associated with the proliferative advantage of MCF10A-RCP cells, MCF10A cells were treated with U0126, a potent inhibitor of both active and inactive MEK1/2. While control cells displayed low phosphorylation of ERK across a range of U0126 concentrations, ERK phosphorylation status in MCF10A-RCP cells was concentration dependent (**Fig. 7C**). At 30 μM of U0126, RCP-induced phosphorylation of ERK was reduced to baseline (same level as control), while RCP levels remained unaltered. Importantly, the MCF10A-RCP cells attenuated for ERK phosphorylation by 30 uM U0126, showed a reduction of proliferation down to control levels (**Fig. 7D**) despite high RCP expression, suggesting that the RCP proliferation phenotype is dependent on activation of the MAPK pathway via phosphorylation of ERK.

RCP enhances tumorigenicity in multiple breast cancer cell lines

[0116] Overexpression of RCP in MCF7 (estrogen receptor-positive, p53-wild type) breast cancer cells moderately increased cell proliferation rate, colony forming activity in soft agar and invasion ability (**Figure 8A-C**). In MDA-MB-231 (MB231; estrogen receptor-negative, p53 negative) cells, RCP overexpression increased colony forming activity but not the cell proliferation rate, invasion or migration ability (**Figure 8A-D**). To further verify the role of RCP in regulating tumorigenicity, we attenuated the expression of endogenous RCP with RNAi technology. RCP was "knocked down" either by transient transfection with an siRNA construct or by stable transduction of a lentivirus vector expressing an shRNA targeting RCP. RNAi constructs with scrambled sequence were used as controls. Two independent and non-overlapping siRNAs directed against RCP led to a 40-80% reduction in protein levels as well as a significant decrease in cell proliferation in MCF7 cells (**Figure 8A**, right panel). Knock down of RCP in MCF7 cells also significantly reduced colony formation in soft agar (**Figure 8B**). In MB231 cells, RCP reduction inhibited colony formation but not cell proliferation rate

(**Figure 8A,B**). In both cell lines, RCP knock down markedly attenuated invasion and migration (**Figure 8C,D**). Furthermore, consistent with observations in MCF10A cells, reduction of RCP in MCF7 cells also led to dephosphorylation of ERK (**Figure 7B**). Together, these results suggest that expression of endogenous RCP in tumor cells is crucial for maintaining capacity for proliferation, migration and invasion, and the targeted attenuation of RCP function might represent a viable therapeutic strategy.

RCP knock down inhibits tumor formation *in vivo*

[0117] To investigate the effects of RCP attenuation in cancer cells *in vivo*, we compared the tumor forming abilities of MCF7 and MB231 cells stably expressing shRNA against RCP (RNAi RCP) to that of controls (RNAi Ctrl) in nude mice. Each cell type was implanted subcutaneously above the left and right hind legs of 8 mice. MCF7 is an estrogen-dependent cell line which forms moderately malignant tumors in nude mice in the presence of supplemental estrogen. While all estrogen-supplemented mice implanted with MCF7-RNAi Ctrl (n=8) or MCF7-RNAi RCP (n=8) developed overt tumors, the RCP-inhibited cells developed significantly smaller sized tumors (P=0.00007; Student's t-test) (**Fig. 9A, Fig. 9B, Fig. 9C**). MB231, on the other hand, is an estrogen-independent breast cancer cell line that forms highly malignant and metastatic tumors in nude mice. All of the mice injected with MB231-RNAi Ctrl cells developed large tumors, however, only 2 of 8 mice injected with MB231-RNAi RCP cells developed tumors (i.e, 2 tumors in one mouse and 1 tumor in another) (**Fig. 9D, Fig. 9E, Fig. 9F**). Notably, these tumors were much smaller than that of the control mice, the average weight being only 1% of the controls. The knock down of RCP in these tumors was verified by immunohistochemistry (**Fig. 9G**).

[0118] The fact that RCP knock down in MB231 cells had no effect on proliferation *in vitro* suggests that the strong anti-tumor effects observed for RCP inhibition *in vivo* may be related to induction of cell death under serum deprivation, given that tumor vascularization of the subcutaneous implant takes several days to manifest. Accordingly, we investigated the sensitivity of the MB231 stable cell lines to cell death and apoptosis under different culture conditions. In complete medium, the MB231-RNAi RCP and control lines showed no difference in cell death as evidenced by very few apoptotic cells in either line. Under serum-starved conditions, however, the controls remained resistant to cell death while the RNAi RCP cells did not. Significantly more RCP-attenuated cells died as a consequence of serum starvation, among which, apoptosis could be detected in ~13 % (**Figure 9H**). This suggests that RCP reduction enhances the vulnerability of MB231 cells to a poor nutrient environment, which may explain the dramatic anti-tumor effect of RCP knockdown *in vivo*. Intriguingly, we did

not observe an increase in tumorigenicity associated with RCP overexpression *in vivo* (data not shown), perhaps reflecting a maximal pathological effect on tumor formation achieved by endogenous levels of RCP. Overall, the results indicate that the targeted knock down of RCP suppresses the *in vivo* growth of both estrogen-dependent and independent breast tumors, and implicates RCP as a potentially valuable new target for therapeutic intervention.

Discussion

[0119] Genome-wide microarray analysis of primary tumors has enabled the discovery of novel, clinically-relevant tumor subtypes defined by unique patterns of gene expression. More recently, however, the inverse of this concept has been explored through bottom-up analytical strategies that seek to identify *gene subtypes* with functional roles in tumorigenesis. These strategies probe the conditional relationships between gene expression and clinical and genomic features of cancer. Adler and colleagues used a genetic linkage approach (stepwise linkage analysis of microarray signatures) to uncover transcriptional mechanisms driving the expression of a prognostic "wound signature" in breast cancer (Adler, A.S., & Chang, H.Y., (2006) *Cell Cycle* 5, 1148-1151). By identifying changes in both copy number and gene expression that were significantly associated with tumors positive or negative for the wound signature, the authors deduced and biochemically verified that MYC (at 8q24) and CSN5 (at 8q13) functionally interact to regulate transcription of the wound response genes. In the present work, we have built on this concept of data integration and functional discovery, and developed a novel correlation-based strategy to deduce genes with mechanistic roles in cancer progression.

[0120] The above described approach, termed TRIAGE, combines gene expression characteristics with positional information (genomic location), clinical data (distant metastasis-free survival of patients) and predictive tumor biology (proliferative capacity) to pinpoint over-expressed and survival-correlated genes at sites of recurrent amplification. While TRIAGE does not detect all types of functional oncogenes, by design, it has the potential to resolve those that drive malignant processes contributing to distant metastasis in human patients. TRIAGE is also optimized to discover oncogenes that are activated with high frequency in cancer, as well as those that are associated with resistance to routine therapies. For example, our search for oncogenes is prioritized by the magnitude of principal eigen peaks identified by the LSVD procedure. Since this magnitude is positively related to the frequency of an amplification event, high frequency amplicons (corresponding to high magnitude peaks) are given greater priority. While LSVD allows the robust application of TRIAGE to any comprehensive collection of tumor expression profiles, it has inherent limitations that should be noted. LSVD provides only an approximation of tumors that possess a given amplification

event, with poor resolution of the actual amplicon boundaries. Corresponding copy number data, such as that afforded by high-resolution array-CGH, could substantially enhance the performance of TRIAGE by improving the accuracy and precision of amplicon determination.

[0121] Additional factors that influence TRIAGE performance include cohort size, duration of patient follow-up, and treatment characteristics – all of which impact candidate gene selection. Our gene-survival associations were calculated using our largest population-based cohort, the Uppsala cohort (n=251 patients), which also has the longest and most complete patient follow-up data. Importantly, the tumors in this collection span a range of aggressive types (Fig. 2), from early-stage (small diameter, lymphnode negative, untreated) to late-stage (large diameter, lymphnode positive, aggressively treated) breast cancer. Thus, this cohort reflects a variety of cancer pathophysiologies presumably driven by a diverse range of oncogenes. The mixed therapeutic background of the patients, however, favors the identification of genes with survival associations independent of therapeutic type. Thus, oncogenes with treatment-dependent or bifunctional survival associations (Andre, F., et al., (2007) *Clin Cancer Res* 13, 2061-2067) may be unrecognized in this cohort, but potentially identified in others.

[0122] LSVD analysis of our integrated breast tumor dataset resulted in the discovery of a large principal eigen peak centered on a group of genes located in a frequently amplified region of 8p11-12. Amplification of this locus has been observed in ~10-25 % of breast tumor cases (Adnane, J., et al. (1991) *Oncogene* 6, 659-663; Garcia, M. J., et al., (2005) *Oncogene* 24, 5235-5245; Letessier et al., 2006, supra; Theillet, C., et al., (1993) *Genes Chromosomes Cancer* 7, 219-226), and has been associated with poor patient survival and short interval to distant metastasis in multiple independent studies (Chin et al., 2006, supra; Cuny, M., et al., (2000) *Cancer Res* 60, 1077-1083; Gelsi-Boyer, V., et al., (2005) *Mol Cancer Res* 3, 655-667; Letessier et al., 2006, supra). Recently, this amplicon has been the focus of several intensive functional genomics investigations involving primary breast tumors and cell lines (Garcia et al., 2005, supra; Gelsi-Boyer et al., 2005, supra; Ray et al., 2004, supra). Using a high-resolution BAC microarray specific for chromosome 8p, Gelsi-Boyer and colleagues (2005, supra) determine that the 8p11-12 amplicon actually comprises of four well demarcated sub-amplicons, termed A1, A2, A3 and A4, with minimal overlap. The authors of this and other studies have proposed a number of candidate oncogenes in this region based on strong correlations between gene amplification and mRNA overexpression (Garcia et al., 2005, supra; Gelsi-Boyer et al., 2005, supra; Ray et al., 2004, supra). Cummulatively, these studies have identified *ZNF703 (FLJ14299), ERLIN2 (SPFH2), PROSC, BRF2, RAB11FIP1 (RCP),* and *LSM1* as having the strongest correlations between amplification and overexpression, with

all but *LSM1* localizing to a 1-Mb "minimal amplicon" (Garcia et al., 2005, supra) and overlapping precisely with the A1 sub-amplicon (Gelsi-Boyer et al., 2005, supra). Notably, the genes identified by LSVD in our study specifically comprise the A1 and A2 sub-amplicons and thus include all the "best candidate" oncogenes described by these earlier studies.

[0123] Application of TRIAGE to 8p11-12 resulted in the identification of *RAB11FIP1 (RCP)* as the strongest candidate oncogene at this amplicon, potentially driving metastaticdissemination in patients. Of the other candidate oncogenes in this region previously shown to possess some transforming properties in breast epithelial cells (Streicher, K. L., et al., (2007) *Oncogene* 26, 2104-2114; Yang, Z. Q., et al., (2006) *Cancer Res* 66, 11632-11643), *LSM1*, *BAG4* and *C8orf4 (TC1)* all reside primarily on the A2 and A3 sub-amplicons (Gelsi-Boyer et al., 2005, supra). *RAB11FIP1 (RCP)*, in contrast, is located closer to the core of the A1 amplicon on the 1-Mb minimal amplicon (Garcia et al., 2005, supra), and is the first in this region to be functionally characterized as an oncogene.

[0124] Using their novel approach, the inventors have discovered a broad range of cancer-promoting effects linked to RCP expression. In noncancerous breast epithelial cells, RCP overexpression led to malignant transformation, and increased cell motility and migration. In breast cancer cell lines, attenuation of endogenous RCP by RNA inhibition reduced proliferation, anchorage-independent growth, invasion and apoptotic resistance, and diminished tumor formation in nude mice. Physiologically, RCP is thought to interact primarily with Rab11 to regulate protein sorting in tubular endosomes (Peden et al., 2004, supra). Rab11-RCP complexes have been shown to channel transferrin receptor away from lysosomes (the degradation pathway) to recycling endosomes (the recycling pathway), thereby promoting a relative increase in the level of membrane-bound receptor (Peden et al., 2004, supra). Though speculative, it is plausible that the transforming effects of RCP may be mediated by this shift towards the recycling pathway, prolonging the membrane localization (and thus function) of certain membrane proteins such as the integrins or growth factor receptors with oncogenic roles. The strong correlation we observed between RCP expression and ERK phosphorylation may reflect this possibility, as it may indicate a specific link between RCP and growth factor receptor-mediated MAPK activation. The above data indicate that RAB11FIP1 may represent a key driving force behind the recurrent selection of the 8p11-12 amplicon, and functionally contribute to the metastatic spread of breast cancer which is associated with this amplified region and the expression of RAB11FIP1 itself. That this amplicon has also been observed with frequency in other solid tumors including non-small cell lung carcinomas (Balsara, B. R., et al., (1997) *Cancer Res* 57, 2116-2120), colorectal cancer

(Nakao, K., et al., (2004) *Carcinogenesis* 25, 1345-1357), and tumors of the urinary bladder (Simon, R., et al. (2001) *Cancer Res* 61, 4514-4519) and fallopian tube (Snijders, A.M., et al., (2003) *Oncogene* 22, 4281-4286) suggests that RAB11FIP1 acting alone, or in synergy with neighboring oncogenes, may represent a ubiquitous path to cancer progression.

[0125] The listing or discussion of a previously published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

[0126] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0127] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. Further, it will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

[0128] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" etc. shall be read expansively and without limitation, and are not limited to only the listed components they directly reference, but include also other non-specified components or elements. As such they may be exchanged with each other. Additionally, the terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed.

[0129] Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

Claims

What is claimed is:

1. A method of preventing, inhibiting, arresting or reversing tumorigenesis in a cell, the method comprising modulating the amount or the activity of a Rab binding protein.

2. The method of claim 1, wherein the Rab binding protein is a Rab11 binding protein.

3. The method of claim 2, wherein the Rab11 binding protein is a Rab11FIP protein (Rab11 family-interacting protein).

4. The method of any one of claims 1 - 3, wherein modulating the amount of the Rab binding protein in a cell comprises modulating the expression of the Rab binding protein.

5. The method of any one of claims 1 - 3, wherein modulating the amount of the Rab binding protein in a cell comprises degradation of the Rab binding protein.

6. The method of method of any one of claims 1 - 3, wherein modulating the activity of the Rab binding protein in a cell comprises forming a complex between the Rab binding protein and a compound.

7. The method of claim 6, wherein the compound is selected from the group consisting of a peptide, a peptoid, an inorganic molecule and a small organic molecule.

8. The method of claim 5, wherein a calpain protease is allowed to carry out the degradation of the Rab binding protein.

9. The method of any one of claims 1 - 8, wherein said tumorigenesis is carcinogenesis.

10. The method of any one of claims 1 - 8, wherein the method is comprised in a treatment or prevention of a proliferative disease or disorder.

11. The method of claim 10, wherein the proliferative disease or disorder to be treated is cancer.

12. The method of any one of claims 1 - 11, comprising administering a compound that modulates the expression and/or the activity of a Rab binding protein.

13. The method of claim 12, wherein the Rab binding protein is the Rab binding protein the amount or the activity of which is to be modulated.

14. The method of any one of claims 1 to 13, wherein the expression of the Rab binding protein is reduced by means of a nucleic acid molecule.

15. The method of claim 14, wherein the nucleic acid molecule is a non-coding nucleic acid molecule.

16. The method of claims 14 or 15, wherein the nucleic acid molecule is RNA or DNA.

17. The method of claims 15 or 16, wherein the nucleic acid molecules is selected from the group consisting of an aptamer, an anti-sense-RNA molecule, a silencer-RNA molecule, a short hairpin RNA (shRNA) molecule, a micro RNA (miRNA) molecule, a small interfering RNA (si-RNA) molecule and a repeat-associated small interfering RNA (rasiRNA) molecule.

18. The method of any one of claims 1 - 17, the method being a method of preventing, inhibiting, arresting or reversing tumorigenesis in a cell, wherein the cell is comprised in an organism selected from the group consisting of a mammal, a fish, an amphibian, and a bird.

19. The method of any one of claims 1 - 17, the method being a method of preventing, inhibiting, arresting or reversing tumorigenesis in a cell, wherein the cell is obtained from an organism selected from the group consisting of a mammal, a fish, an amphibian, and a bird.

20. The method of claim 19, wherein the cell is cultured.

21. The method of any one of claims 18 to 20, wherein the mammal is selected from the group consisting of a rat, a mouse, a rabbit, a Guinea pig, an opossum, a squirrel, a nine-banded armadillo, a dog, a cat, an elephant, a chimpanzee, a rhesus monkey, a macaque, an orang-utan, a cattle (cow), a marmoset, an American pika, a galago, and a human.

22. The method of any one of claims 1 - 21, comprising preventing, inhibiting, arresting or reversing activation of extracellular signal-regulated kinase (ERK).

23. The method of any one of claims 1 - 22, further comprising detecting the expression and/or activity of the Rab binding protein.

24. The method of claim 23, wherein detecting the expression and/or activity comprises measuring the expression and/or activity over a period of time.

25. The method of claims 23 or 24, further comprising:
comparing the obtained results with those of a control measurement.

26. The method of claim 25, wherein the control measurement comprises the use of conditions that do not modulate the expression and/or the activity of a Rab binding protein.

27. A method of identifying a cell having a predisposition to turn tumorigenic, the method comprising detecting in the cell an altered amount, an altered expression, an altered subcellular localisation or an altered activity of a Rab binding protein.

28. The method of claim 27, wherein the Rab binding protein is a Rab11 binding protein.

29. The method of claim 28, wherein the Rab11 binding protein is a Rab11FIP protein.

30. The method of any one of claims 27 - 29, wherein at least one of an altered expression and an altered activity of a Rab binding protein is determined and wherein an increased expression or an increased acitivity of the Rab binding protein is an indication that a respective tumor will be sensitive to an alteration of intracellular calcium levels.

31. The method of claim 30, wherein the increase of intracellular calcium levels is achievable by the use of a member of the D vitamins, a prodrug of a member of the D vitamins and an analog of a member of the D vitamins.

32. The method of claim 32, wherein the member of the D vitamins is vitamin D3.

33. A method of diagnosing the risk of developing a neoplasm in a subject, the method comprising determining at least one of the expression level, the activity level and the subcellular localisation of a Rab binding protein.

34. The method of claim 33, wherein the Rab binding protein is a Rab11 binding protein.

35. The method of claim 34, wherein the Rab11 binding protein is a Rab11FIP protein.

36. The method of claim 33, wherein said neoplasm is a tumor.

37. The method of claim 36, wherein said tumor is one of a breast tumor, a lung tumor, a colorectal tumor, a tumor of the urinary bladder and a tumor of the fallopian tube.

38. The method of claim 36 or 37, wherein said tumor is cancer.

39. The method of claim 33, wherein determining at least one of the expression level, the activity level and the subcellular localisation of a Rab binding protein is carried out in a sample from the subject.

40. The method of claim 33, further comprising:
comparing the obtained results with those of a control measurement.

41. The method of claim 40, wherein the control measurement comprises the use of a sample in which the expression and/or the activity of the Rab binding protein is on a customary level.

42. The method of claim 41, wherein an altered expression level, activity level or subcellular localisation of a Rab binding protein as compared to the control measurement indicates that the subject suffers from or is at risk of developing a neoplasm.

43. The method of any one of claims 33 - 42, wherein at least one of an altered expression and an altered activity of a Rab binding protein is determined and wherein an increased expression or an increased acitivity of the Rab binding protein is an indication that a respective neoplasm will be sensitive to an alteration of intracellular calcium levels.

44. The method of claim 43, wherein the increase of intracellular calcium levels is achievable by the use a member of the D vitamins, a prodrug of a member of the D vitamins and an analog of a member of the D vitamins.

45. The method of claim 44, wherein the member of the D vitamins is vitamin D3

46. The method of any one of claims 1 - 45, wherein the Rab binding protein is a Rab11FIP protein selected from the group consisting of Rab11FIP1, Rab11FIP2, Rab11FIP3 (eferin), Rab11FIP4, Rab11FIP5 (Rip11), and isoforms thereof.

47. An *in-vitro* method of identifying a compound capable of forming a complex with a Rab binding protein, the method comprising contacting the components that form said complex with each other.

48. The method of claim 47, wherein the Rab binding protein is a Rab11 binding protein.

49. The method of claim 48, wherein the Rab11 binding protein is a Rab11FIP protein.

50. The method of any one of claims 47 - 49, comprising:
(a) providing the Rab binding protein in a test tube,
(b) adding a compound to the test tube suspected to be capable of forming a complex with the Rab binding protein, and
(c) detecting the said complex formation.

51. The method of claim 50, wherein the detection is performed by a suitable spectroscopic, photochemical, photometric, fluorometric, radiological, enzymatic or thermodynamic method, or is based on cellular effects.

52. The method of any one of claims 49 - 51, wherein said method is a method of identifying a compound capable of preventing, inhibiting, arresting or reversing tumourigenesis.

53. A method for identifying a compound capable of modulating the activity of a Rab binding protein *in vivo* comprising:
(a) providing a microorganism expressing the Rab binding protein or a functional fragment thereof,
(b) adding a compound to said microorganism; and
(c) monitoring a change in cell phenotype or activity of a Rab binding protein.

54. The method of claim 53, wherein the Rab binding protein is a Rab11 binding protein.

55. The method of claim 54, wherein the Rab11 binding protein is a Rab11FIP protein.

56. The method of any one of claims 53 - 55, further comprising:
comparing the obtained results with those of a control measurement.

57. The method of claim 56, wherein the control measurement comprises the use of a compound that does not modulate the expression and/or the activity of the Rab11FIP protein.

58. A method for identifying a compound capable of modulating the expression of a Rab binding protein *in vivo* comprising:

(a) providing a microorganism expressing the Rab11FIP protein or a functional fragment thereof,

(b) adding a compound to said microorganism; and

(c) monitoring the expression of the Rab binding protein.

59. The method of claim 58, wherein the Rab binding protein is a Rab11 binding protein.

60. The method of claim 59, wherein the Rab11 binding protein is a Rab11FIP protein.

61. The method of any one of claims 58 - 60, further comprising:
comparing the obtained results with those of a control measurement.

62. The method of claim 61, wherein the control measurement comprises the use of a compound that does not modulate the expression of the Rab binding protein.

63. The method of any one of claims 53 - 62, wherein the microorganism is a cancer cell.

64. The method of claim 63, wherein the cancer cell is one of a breast cancer cell, a lung cancer cell, a colorectal cancer cell, a cancer cell of the urinary bladder and a cancer cell of the fallopian tube.

65. The method of any one of claims 53 - 64, wherein the microorganism is a cell derived from one of breast tissue, lung tissue, colorectal tissue, urinary bladder tissue and tissue of the fallopian tube.

66. The method of claim 63, wherein the cell is of a cell line selected from the group consisting of a HMEC cell line, 3D, ABC-1, A549, Bcap-37, BIU-87, BT-20, BT-474, BT474M1, Calu-6, EBC-1, Colo-320, EJ, FT-MZ-1, H322, H446, H460, HCC2279, HCT116, HEPFT, Hs578T, Hs578Bst, HT-29, HTB-56R, HUT29, IMEC-2, JIMT-1, KoTCC-1, KU-T1, L0, L0-200, L0-500, L0-1000, L9981, LC-1/sq, LCD, LCOK, LK-2, LoVo, LS1034, LST-R1, Lu135, MBT-2, MCF-7, M231, MDA-231, MDA-MB-134, MDA-MB-175, MDA-MB-231, MDA-MB-361, MDA-MB-361DYT2, MDA-MB-435, MDA-MB-435HM, MDA-MB-468, MB231, MS-1, MW1, NCI-H69, NCI-H82, NCI-H209, NCI-H446, NCI-H1395, NCI-H1437, NCI-H1770, NCI-H2009, NCI-H2087, NCI-H2122, NCI-H2126, NCI-H2171, NCI-N226, NCI-N231, PC1, PC3, PC7, PC9,

PC10, PC14, PMC42, PLA-801D, QG56, RERF-LCAI, RERF-LCMS, RERF-LCKJ, RKO, RT4, SBC3, ScaBER, SK-BR-3, SK-BR-7, SPC-A1, SQ5, SUM149, SUM159, SUM1315, SUM-52PE, SW480, T24, T-47D, UM-UC-2, WiDr, YTLMC-90, ZR-75, ZR-75-1, and ZR-75-30.

67. The method of claim 63, wherein the cell is obtained from a non-cancerous cell by overexpressing a Rab binding protein.

68. The method of claim 63, wherein the cell is overexpressing RAB11FIP1.

69. The method of any one of claims 55 - 64, wherein the microorganism is a recombinant cell or a transgenic microorganism.

70. A method of identifying a compound capable of modulating the amount or the activity of a Rab binding protein, the method comprising:

(a) providing a host organism, wherein the host organism is capable of accommodating and growing a cancer cell,

(b) introducing a cancer cell into the host organism,

(c) administering a compound suspected to be capable of modulating the expression and/or the activity of a Rab binding protein,

(c) monitoring the growth of tumors in the host organism.

71. The method of claim 70, wherein the Rab binding protein is a Rab11 binding protein.

72. The method of claim 71, wherein the Rab11 binding protein is a Rab11FIP protein.

73. The method of any one of claims 70 - 72, further comprising:
comparing the obtained results with those of a control measurement.

74. The method of claim 73, wherein the control measurement comprises the use of a compound that does not modulate the expression and/or the activity of the Rab11FIP protein.

75. A method of identifying a compound capable of modulating the amount or the activity of a Rab binding protein, the method comprising:

(a) providing a host organism, wherein the host organism is capable of accommodating and growing a cancer cell,

(b) introducing a cancer cell into the host organism, wherein said cancer cell comprises a compound suspected to be capable of modulating the amount or the activity of a Rab binding protein,

(c) monitoring a the growth of tumors in the host organism.

76. The method of claim 75, wherein the Rab binding protein is a Rab11 binding protein.

77. The method of claim 76, wherein the Rab11 binding protein is a Rab11FIP protein.

78. The method of any one of claims 75 - 77, further comprising:
comparing the obtained results with those of a control measurement.

79. The method of claim 78, wherein the control measurement comprises the use of a compound that does not modulate the expression and/or the activity of the Rab11FIP protein.

80. The method of any one of claims 70 - 79, wherein the host organism is selected from the group consisting of a mouse, a rat, an elephant, a rabbit, a Guinea pig, an opossum, a squirrel, a nine-banded armadillo, a dog, a cat, an American pika, a galago, a chimpanzee, a rhesus monkey, a macaque, an orang-utan, a cattle (cow) and a marmoset.

81. A method of determining whether a neoplasm is sensitive to an alteration of intracellular calcium levels, the method comprising determining at least one of the expression and the activity of a Rab binding protein, wherein an altered expression or an altered activity of the Rab binding protein is an indication that the neoplasm is sensitive to an alteration of intracellular calcium levels.

82. The method of claim 81, wherein the neoplasm is comprised in a mammal.

83. The method of claim 81 or 82, wherein the neoplasm is a tumor.

84. The method of claim 83, wherein said tumor is one of a breast tumor, a lung tumor, a colorectal tumor, a tumor of the urinary bladder and a tumor of the fallopian tube.

85. The method of claims 81 or 82, wherein said tumor is cancer.

86. The method of any one of claims 81-85, wherein the alteration of intracellular calcium levels is an increase of free intracellular calcium.

87. The method of claim 86, wherein the increase of free intracellular calcium is achievable by the use of one of a member of the vitamin D family, a prodrug of a member of the D vitamins and an analog of a member of the D vitamins, and wherein an increased expression or an increased acitivity of the Rab binding protein is an indication that the neoplasm is sensitive to treatment with a member of the D vitamins, a prodrug or an analog thereof.

88. The method of claim 87, wherein the member of the D vitamins is vitamin D3.