(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 November 2001 (15.11.2001)

PCT

(10) International Publication Number
WO 01/85980 A2

(51) International Patent Classification[7]: C12Q 1/00

(21) International Application Number: PCT/EP01/05404

(22) International Filing Date: 11 May 2001 (11.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0011439.7 12 May 2000 (12.05.2000) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/85980 A2

(54) **Title:** ENZYMATIC ASSAYS FOR SCREENING ANTI-CANCER AGENTS

(57) **Abstract:** Methods of identifying anti-cancer agents are provided based on ligand binding or enzymatic activity of TIP49 family members. The assays may include the measurement of ATPase and/or helicase activity. Also provided are anti-cancer agents identified by the screening methods of the invention, as well as the use of the agent for the prophylaxis or treatment of cancer. A complex of a TIP49 family member with various proteins that regulate transcription is also provided.

ENZYMATIC ASSAYS FOR SCREENING ANTI-CANCER AGENTS

The invention relates to the field of cancer diagnosis and therapy. The invention also relates to the screening of compounds for potential anti-cancer activity, whether prophylactic or therapeutic. The screening assays concerned are those which seek to mimic a part of the biochemical machinery of intact cells *in vivo* involved in processes of cell division, gene expression and transformation which gives rise to cancers.

In the more affluent countries of the world cancer is the cause of death of roughly one person in five. The American Cancer Society in 1993 reported that the five most common cancers are those of the lung, stomach, breast, colon/rectum and the uterine cervix. Cancer is not fatal in every case and only about half the number of people who develop cancer die of it. The problem facing cancer patients and their physicians is that seeking to cure cancer is like trying to get rid of weeds. Although cancer cells can be removed surgically or destroyed with toxic compounds or with radiation, it is very hard to eliminate all of the cancerous cells. A general goal is to find better ways of selectively killing cancer cells whilst leaving normal cells of the body unaffected. Part of that effort involves identifying new anti-cancer agents.

Cancer cells have lost the normal control of the cell cycle and so divide out of control compared to normal cells. The sub-cellular machinery which controls the cell cycle is a complex biochemical device made up of a set of interacting proteins that induce and co-ordinate the essential processes of duplication and division of the contents of a cell. In the normal cell cycle, the control system is regulated such that it can stop at specific points in the cycle. The

stopping points allow for systems of feedback control from the processes of duplication or division. They also provide points for regulation by environmental signals.

Gene expression plays an integral part in cell division and its control. Loss of control of cell division may in certain instances have its origin in an alteration in gene expression. Analysis of genetic alterations in cancer cells has revealed many genes which encode proteins involved in the control of cell division in some way.

Oncogenes are one family of such genes. Oncogenes are either expressed in cancer cells in a mutated form or they are over-expressed. The products of such oncogenes promote cell proliferation. The non-mutated or normally expressed version of an oncogene is known as a proto-oncogene and this is expressed in normal cells and encodes a constituent protein of the normal cellular machinery.

Another kind of gene product connected with cancer is that expressed by tumour-suppressor genes and the gene products serve to restrain cell proliferation. Mutation of a tumour-suppressor gene or loss of function of the gene product results in a loss of the normal control on proliferation and the cell divides out of control.

The study of cancer cells and their oncogenes or tumour-suppressor genes has helped to show how growth factors regulate cell proliferation in normal cells through a complex network of intracellular signalling cascades. These cascades ultimately regulate gene transcription and the assembly and activation of the cell cycle control system. As knowledge increases about the component parts of the cell cycle control machinery and how it operates, the possibilities for correcting the loss of control in cancer cells are increased. Essential points of control and essential proteins can be identified in the

control hierarchy and potentially targetted with drugs to act as promoters or inhibitors, as required.

The cell cycle control system is based on two main families of proteins. The first is the family of cyclin-dependent protein kinases (CDK) of which there are a number of varieties, e.g. CDK 1 and CDK 2. CDK phosphorylates selected proteins at serine and threonine residues. The second sort of protein is a family of specialised activating proteins called cyclins that bind to CDK molecules and control their ability to phosphorylate targets. Cyclins themselves undergo a cycle of synthesis and degradation within each division of the cell cycle. There are a variety of species of cyclin, e.g. cyclin A and cyclin B.

Chao Y *et al* (1998) Cancer Research 58: 985-990 report a correlation between over-expression of cyclin A in patients and proliferative activity of tumour cells compared to those patients expressing a normal cyclin A level. Patients over-expressing cyclin A had a shorter median disease-free survival time than those who did not over-express. Chao *et al* (1998) also report that a cyclin A-interacting protein (Skp 2) did not exhibit the same correlation with tumour cell activity as cyclin A when over-expressed. Chao *et al* (1998) remark on how expression of Skp 2 appears to be involved in the control of cell cycle progression but caution that the actual biochemical function of Skp 2 is still not known.

In a more recent paper, Chao Y *et al* (1999) Cancer Letters 139: 1-6 conclude that cyclin A may provide a useful target for the exploration of new anti-hepatocelluar carcinoma (HCC) therapeutics. In particular, Chao *et al* (1999) showed that an over-expression of cyclin A in HCC cells could be inhibited with antisense mRNA for the cyclin A gene. Although an over-expression of Skp 2 is apparently also associated with HCC cell proliferation, Chao *et al* (1999) indicate that the biochemical function of Skp 2 remains unknown. For example, the results of an experiment seeking to block over-

expression of Skp 2 using antisense mRNA suggests that abnormal Skp 2 expression has no direct correlation with HCC proliferation.

The activity of CDK is subject to regulation in the cell and a CDK inhibitor protein (p27) has been identified. In normal cells p27 has been shown to regulate the action of CDK's that are necessary for DNA replication. Levels of p27 are found to be high in quiescent cells and low in cells stimulated to divide. p27 appears to act as a brake on cell division by inhibiting activated CDK which itself drives cells to divide. A reduction in the level of p27 frees activated CDK from inhibition and drives cells to divide. Consistent with this activity of p27 is the way in which its destabilisation correlates generally with tumour aggressiveness and poor prognosis for cancer patients.

The cell cycle control system is a dynamic system and p27 itself does not remain at a constant level in the cell. The level is different depending on the point in the cell cycle. Lower levels of p27 arise due to breakdown via ubiquitination and subsequent proteasome-mediated degradation. A requirement for ubiquitin-mediated degradation of p27 is phosphorylation of the threonine residue 187 (T187) by activated CDK. The enzymes needed for ubiquitination of phosphorylated p27 are not known, although from knowledge of ubiquitination in systems such as yeast it is expected that there may be a human ubiquitin-protein ligase (E3) specific for p27.

Sutterlüty H *et al* (1999) Nature Cell Biology 1: 207-214 report that Skp 2 promotes the degradation of p27 in cells via the ubiquitination pathway. Skp 2 is a protein member of the F-Box-Protein (FBP) family. Skp 2 appears to be a p27 specific receptor of a Skp 1, CulA (Cdc53), F-Box Protein (SCF) complex. Such complexes are known in yeast and act as ubiquitin-protein ligases (E3) in which the FBP subunit has specificity for the substrate for ubiquitination. E3 facilitates the transfer of an activated ubiquitin molecule from a ubiquitin-conjugating enzyme (E2) to the substrate to be degraded. Similarly, in humans there are SCF complexes and Skp 2 is an FBP which

has an ability to interact specifically with p27 and which appears to be essential in the ubiquitin-mediated degradation of p27. Both *in vivo* and *in vitro*, Skp 2 is found to be a rate-limiting component of the cellular machinery which ubiquitinates and degrades phosphorylated p27.

Skp 2 appears to be the product of a single gene and as such has an unusual ability in that it is able to drive cells to divide. This ability is shared with only a few other known gene products, e.g. E2F-1, c-Myc and cyclin E-CDK2 complexes. Timely accumulation of Skp 2 at the G1/S transition of the cell cycle may be one of the few rate-limiting steps controlling the initiation of DNA replication in mammalian cells. Sutterlüty *et al* (1999) found that a mutant of Skp 2 which does not assemble into an SCF complex was defective in promoting the elimination of ectopically produced wild-type p27. Also, mutant Skp 2 produced an activation of cyclin-E/A associated kinases and an induction of the S phase. Skp 2 also appears to have an independent binding site for CDK and activated CDK is involved in the phosphorylation of the T187 residue of p27. Sutterlüty *et al* (1999) also note how normal Skp 2 induces an accumulation of cyclin A protein, even when activation of cyclin-E/A-dependent kinases and entry into S phase are blocked by the expression of a non-degradable p27 mutant. What is concluded is that Skp 2 up-regulates cyclin A and independently of this down-regulates p27. The mechanism by which Skp 2 up-regulates cyclin A is not known. There is a suggestion that observed increased levels of Skp 2 in transformed cells might contribute to the process of tumourigenesis, at least partly, by causing an increase in the rate of degradation of the tumour suppressing agent p27. A lack of p27 expression correlates with a reduced disease-free survival of patients with colorectal and breast cancer. Also, p27 has been found to be haplo-insufficient for tumour suppression.

Carrano A.C. *et al* (1999) Nature Cell Biology 1: 193-199 report how Skp 2 interacts physically with phosphorylated p27 both *in vitro* and *in vivo*. Whilst every component of the ligase machinery required for p27 ubiquitination

remains to be discovered, Carrano *et al* (1999) demonstrate that Skp 2 is a critical part of this machinery and provides substrate recognition and specificity for p27. Antisense oligonucleotides against Skp 2 were found to decrease Skp 2 expression in cells and thereby result in increased levels of endogenous p27. Carrano *et al* (1999) also confirm an additional need for cyclin E-CDK 2 or cyclin A-CDK 2 for ubiquitination of p27 to take place. p27 degradation in cells appears to be subject to dual control by accumulation of both Skp 2 and cyclins following mitogenic stimulation.

Of interest to scientists elucidating the molecular bases of cancer is a field of study relating to the molecular basis of the control of gene expression. Previously unconnected with the apparently essential roles of Skp 2 and p27 in cancer is the protein Pontin 52 reported in Bauer A. *et al* (1998) Proc. Natl. Acad. Sci. USA 95: 14787-14792. Pontin 52 is a nuclear protein which has a binding site for the TATA box binding protein (TBP). Pontin 52 also has a binding site for β-catenin. Pontin 52 is a ubiquitous and highly conserved ATP-dependent helicase protein. β-catenin is normally a cytoplasmic protein which has one role of providing a cytoplasmic anchor for other molecules involved in intercellular connections. β-catenin is also known to be a participant in the Wnt signalling pathway. In the Wnt signalling pathway, β-catenin becomes stabilised in the cytoplasm and can therefore interact with transcription factors of the lymphocyte enhancer factor-1/T-cell factor (LEF-1/TCF) family. Interaction with these transcription factors causes β-catenin to become localised in the nucleus. Binding of β-catenin with Pontin 52 provides the necessary molecular bridge between β-catenin and the TATA box binding protein. The TATA box binding protein binds to DNA, particularly in the TATA box region of gene promoters.

A protein equivalent to Pontin 52 is found in rats and is called TIP49. Wood M.A. *et al* (2000) Molecular Cell 5: 321-330 observe that c-Myc oncogenic transformation of cultured rat embryo fibroblasts required TIP49 as an

essential co-factor. TIP49 and a similar protein, TIP 48, were found to complex with c-Myc *in vivo* and binding was dependent on the MbII domain of c-Myc. TIP49 is a highly conserved protein and has ATPase and DNA helicase activity. In the present specification reference to either TIP48 or TIP49 are to be construed as references to the relevant proteins in humans or in any animal species.

Genebank sequence AF083242 comprises 726 base pairs and is shown in Figure 1 as SEQ ID NO:2. The protein sequence is set forth as SEQ ID NO:1 in figure 2.

The inventors have screened a variety of different cancer cell types for levels of expressed Skp 2 and p27. The inventors have also carried out co-transformation of primary rodent fibroblasts with both Skp 2 and H-RAS^{G12V}. Out of these experiments the inventors have discovered that Skp 2 is an oncogene responsible for many human cancers.

In exploring the oncogenic function of Skp 2 the inventors have unexpectedly discovered a novel protein called Skp 2-associated protein one (STAP1). The inventors generated antibodies against STAP1 and used these antibodies to immunoprecipitate STAP1 from HeLa cells. The immunoprecipitates were surprisingly found to contain several STAP1-co-immunoprecipitating proteins. The proteins including STAP1 were found to form a complex. The molecular weights of proteins were determined by mass spectrometry and then databases of proteins and gene sequences were searched to try and identify the proteins. Quite unexpectedly the STAP1-containing complex of proteins is found to include TIP48, TIP49, RPB 5 (RNA pol II subunit 5), RMP1 (RNA pol II mediator protein or RPB5-mediating protein) as well as other hitherto unknown proteins.

Without wishing to be bound by any particular theory, the inventors have realised that Skp 2 represents an oncogene which can interact through

STAP1 and its complex with known elements of a transcriptional control apparatus, in particular TIP49 (and TIP48) and that this link provides a new point of attack for inhibitors of protein-protein binding and enzymic activities. Such inhibitors are expected to have anti-proliferative and therefore anti-cancer properties. In the light of these discoveries, suitable screening assays can now be developed to identify new anti-cancer agents.

In one aspect, the invention therefore provides a TIP 49 family member complexed to at least one other protein selected from the group of STAP1, prefoldin, RPB 5 and RMP1. Preferably, the complex comprises STAP1, TIP48 or TIP49, RPB 5, and RMP 1 in a ratio of about 1:1:1:1:1. In a further aspect of the invention, a transcription regulatory protein complex is provided comprising a TIP49 family member and three or more other proteins or polypeptides. Thus, a TIP 49 family member, preferably TIP48 or TIP49, can be used for assembly of a complex *in vitro*, although complexes formed *in vivo* can also be useful in the present invention.

A complex is preferably provided substantially free of other cellular contaminants. In particular, an isolated complex of at least 80% purity, preferably 90% purity, more preferably 95% purity, even more preferably 99% purity.

Also provided by the invention is a method for identifying an agent active against cancer cells whereby a member of the TIP49 family, a fragment or variant thereof, is contacted with a test compound. Enzymatic or ligand binding activity of the TIP 49 family member is measured and the test compound is identified as a potential candidate agent active against cancer cells that do not express c-Myc, if the test compound results in a change in enzymatic or ligand binding activity of the TIP 49 family member relative to when the test compound is absent. TIP48 or TIP49 are preferably employed in the screening methods of the invention. Preferably, the cancer cells express Skp 2.

The enzymatic activity will typically involve measuring ATPase activity and/or helicase activity. The ligand binding activity will typically involve detecting binding to a protein, a test compound, a nucleic acid or enzyme substrate, such as nucleotide triphosphates or their analogues, such as non-hydrolysable nucleotide triphosphate analogues. The TIP 49 family member, fragment or variant thereof, or ligand may be labelled, such as with a fluorescent label, an enzyme label, biotin, a metal sol particle or a radiolabel. The assays can be carried out with at least one member (that is, the TIP 49 family member, its ligand or other interacting agent, such as in a complex) linked to a solid surface, where the solid surface is preferably nickel or nickel coated. Alternatively, the assay can be a liquid phase assay, preferably employing labelling of at least one of a TIP 49 family member, a ligand or other interacting agent, preferably involving fluorescent labelling of one of the listed members.

An anti-cancer agent is most likely to be identified as a test compound that inhibits enzymatic or ligand binding activity. Therefore, also provided by the invention is an anti-cancer agent identified by the screening methods of the invention, preferably an anti-proliferative agent. Such anti-cancer agents can be a nucleic acid complementary to all or a part of a nucleic acid encoding a TIP 49 family member, for example an antisense or RNAi molecule. An antibody or antibody fragment specific for a TIP 49 family member can also be used as an anti-cancer agent.

Also provided by the present invention is the use of an agent identified by the screening methods for the manufacture of a medicament for the prophylaxis or treatment of cancer, as well as a method of preventing or treating cancer comprising administering to an individual an effective amount of a compound identified by a screening method of the invention.

DETAILED DESCRIPTION

The term "TIP 49 family member" refers to TIP49 (EP 092615A1), TIP48 (EP 092615A1), Pontin 52 (Bauer et al. (1998)) or a protein having a sequence substantially homologous therewith, particularly a degree of identity of at least 60%, at least 70%, preferably at least 80%, more preferably at least 90%, even more preferably 95%, most preferably at least 99%, or a fragment thereof. The percentage identity of two sequences can be easily determined using standard computer alignment software.

"TIP 49 family member" therefore encompasses variants of the native proteins, which may have one or more amino acids deleted or substituted. Preferred variants have enzymatic activity, such as ATPase or helicase activity, or ligand binding activity, such as binding affinity for, and/or association affinity with, one or more of STAP1, prefoldin, RPB5 and RMP1, a transcriptional regulatory factor, and optionally other proteins or polypeptides. Thus, variants preferably do not exhibit any change in sequence in the regions responsible for ligand binding or enzymatic activity compared to the native sequence. ATPase and helicase motifs are easily ascertainable in the art using programmes designed to analyse nucleic acid and/or protein sequences (see also Wood, M.A. et al.). Any changes involving substitution of amino acids are preferably neutral or conservative substitutions.

Other variants include proteins or polypeptides comprising at least one additional amino acid in the sequence, or an additional amino acid sequence or domain. Synthesis of fusion proteins, for example, fusion proteins with green fluorescent protein or antigenic/affinity tags are well known in the art.

Further variants are proteins or polypeptides with at least one natural or unnatural analogue of an amino acid of the native sequence. Also, one or more amino acids in the sequence may be chemically modified, e.g. to

increase physical stability or to lower susceptibility to enzymatic, particularly protease or kinase, activity.

In one aspect of the invention, the TIP49 family member is complexed to at least one other protein selected from the group of Skp2 binding protein (STAP1 or SKAP 1), prefoldin, RPB 5 (Cheong et al., EMBO J. 14 (1), 143-150 (1995)) and RMP1 (WO9960115 or a variant thereof, for example comprising additional amino acids at its N-terminal: MEAPTVETPPDPSPPSAPAPALVPLRAPDVARLREEQEKVVTNCQERIQH WKKVDNDYNALRERLSTLPDKLSYNI), and optionally one or more further proteins or polypeptides. The sequence of the Skp 2 binding protein (STAP1) is provided in Figure 2 (SEQ ID NO: 1), and it is encoded by a nucleic acid sequence substantially as set forth in Figure 1 (SEQ ID NO:2).

Preferably, the complex comprises the subunits, TIP48 and/or TIP49, STAP1, RPB 5, prefoldin and RMP 1 in a ratio of about (1:) 1:1:1:1:1, although other ratios are possible. Optionally, the additional proteins or polypeptides may also be in a stoichiometric ratio of 1:1, but again other ratios are possible. In a further aspect of the invention, a transcription regulatory protein complex is provided comprising a TIP49 family member and three or more other proteins or polypeptides. Thus, a TIP 49 family member, preferably TIP48 or TIP49, can be used for assembly of a complex *in vitro*, although complexes formed *in vivo* can also be useful in the present invention.

The invention also provides a transcription regulatory protein complex comprising TIP48 and/or TIP49 and three or more other proteins or polypeptides. These other proteins or polypeptides may be as hereinbefore described.

In any of the complexes of the invention hereinbefore described the constituent protein or polypeptide subunits may each have a molecular

weight in the range 5 to 500kD, preferably 5 to 300kD, more preferably, 10 to 200kD, even more preferably 10 to 100kD. SDS-PAGE or mass spectrometry provide ways of establishing molecular weights.

Complexes of the invention as hereinbefore described may be obtainable by immunoprecipitation using an antibody reactive against a TIP 49 family member. Ideally, complexes of the invention are substantially free of other cellular contaminants. Thus, isolated complexes may be of at least 80% purity, preferably 90% purity, more preferably 95% purity, even more preferably 99% purity. Purity can be determined by various methods, e.g. SDS-PAGE or size exclusion chromatography.

Alternative ways of producing complexes of the invention may be to assemble them from constituent protein or polypeptide subunits. One way is to have a cell transformed to overexpress each of the constituent subunits so that assembly of the complex takes place in the cell. A preferred expression system employs transformed insect cells.

Another way is to mix the constituent subunits together *in vitro* under conditions sufficient for self-assembly of the complex. Preferably, the mixing of subunits occurs substantially simultaneously. There are many other possibilities of mixing including assembly of partial complexes in transformed cells followed by isolating and mixing them with the remaining subunits *in vitro* under conditions promoting self assembly of the whole complex. Also, partial complexes can be made *in vitro* by mixing and then mixed with the remaining subunits. The order of mixing subunits or partial complexes *in vitro* is not believed to be critical in order to yield complexes.

Also provided by the invention is a method for identifying an agent active against cancer cells whereby a member of the TIP49 family, a fragment or variant thereof, preferably TIP 48 or TIP 49, is contacted with a test compound. Enzymatic or ligand binding activity of the TIP 49 family member

is measured and the test compound is identified as a potential candidate agent active against cancer cells, in particular for cancer cells that do not express c-Myc, if the test compound results in a change in enzymatic or ligand binding activity of the TIP 49 family member relative to when the test compound is absent. TIP48 or TIP49 are preferably employed in the screening methods of the invention. Preferably, the cancer cells express Skp 2.

The enzymatic activity will typically involve measuring ATPase activity and/or helicase activity, preferably enzymatic activity resulting from the use of TIP 48 or TIP 49. The ligand binding activity will typically involve detecting binding to the test compound, a protein, nucleic acid or enzyme substrate, such as nucleotide triphosphates or their analogues, such as non-hydrolysable nucleotide triphosphate analogues, or even a test compound. The assays may optionally comprise using a control, such as measuring binding or enzymatic acitivity in the presence of a control compound or comparing values to a control assay carried out in the absence of a test compound.

The screening methods of the present invention, whether based on enzymatic assays or ligand binding assays, may employ a TIP 49 family member complexed to at least one other protein, in particular the complexes described above.

The TIP 49 family member, fragment or variant thereof, or ligand may be labelled, such as with a fluorescent label, an enzyme label, biotin, avidin, a metal sol particle, a radiolabel, or a tag, such as HIS6. In preferred embodiments, the label is europium.

The assays can be carried out with at least one member (that is, the TIP 49 family member, its ligand or other interacting agent, such as in a complex) linked to a solid surface, where the solid surface is preferably nickel or nickel

coated, e.g., nickel coated microtiter plates allowing attachment of His6-tagged proteins to a solid surface. Alternatively, the assay can be a liquid phase assay, preferably employing labelling of at least one of a TIP 49 family member, a ligand or other interacting agent, preferably involving fluorescent labelling of one of STAP1, RPB 5, prefoldin and RMP 1.

An anti-cancer agent is typically identified as a test compound that inhibits enzymatic or ligand binding activity, although activators are also encompassed by the present invention.

The invention therefore includes the use of a TIP 49 family member in a method of identifying anti-cancer agents (or any other condition dependent on TIP49 or TIP48 activity) as hereinbefore described.

In another aspect the invention provides a method of identifying an anti-cancer agent comprising contacting an amount of a complex as hereinbefore described with a test compound and then determining one or more of: (a) the amount of intact complex remaining, (b) the amount of intact complex lost, or (c) the amount(s) of free protein or polypeptide subunit(s) released from the complex.

The amount of complex may be determined by measuring one or more activities of the complex, preferably an enzymic and/or ligand binding activity, as described above.

In methods which determine the amount(s) of free protein or polypeptide subunits lost from the complex then the free protein or polypeptide subunit(s) may be one or more of RBP 5, RMP 1, TIP48, TIP49, SKP2, prefoldin or a STAP1. Free protein or polypeptide subunit amounts may be determined by measuring an enzymic and/or ligand binding activity, as described above, or by using an antibody specific for the free protein, for example. In some

embodiments, the free protein is separated from the complex prior to measuring activity.

In the methods of anti-cancer agent screening there may be the further step of forming the complex from its protein subunit components prior to contact with the test compound.

Another aspect of the invention is the use of a TIP49 family member in a method of screening for anti-cancer agents, preferably any of the methods hereinbefore described. Allied to this aspect of the invention is the use of a TIP 49 family member for *in vitro* assembly of a complex as hereinbefore described.

The invention permits the identification of anti-cancer agents by performance of any of the methods of screening described herein. Preferred anti-cancer agents are those which inhibit proliferation of the cancer cells and which may be general anti-proliferative agents. The invention includes all agents identified by performing the methods and the use of these agents as pharmaceuticals, particularly as medicaments for the prophylaxis or treatment of cancer.

The invention includes a method of preventing or treating cancer comprising administering to an individual an effective amount of a compound identified by a screening method of the invention described above.

Therefore, also provided by the invention is an anti-cancer agent identified by the screening methods of the invention, preferably an anti-proliferative agent. Such anti-cancer agents can be a nucleic acid complementary to all or a part of a nucleic acid encoding a TIP 49 family member, for example an antisense or double-stranded RNA (RNAi) molecule. An antibody or antibody fragment specific for a TIP 49 family member can also be used as an anti-cancer agent. (See EP 092615A1 for description of TIP 48 and TIP 49 sequences,

antisense and antibody molecules useful in the methods of the present invention).

Nucleic acids comprising all or a part of a nucleic acid sequence encoding a TIP 49 family member (or non-coding regulatory sequences), sequences having at least 70% homology thereto, or their complementary sequences are particularly useful in the present invention. A sequence having at least 70% homology (or identity) to a reference sequence means a nucleic acid that is able to hybridise with the reference sequence under low stringency conditions, conditions for which are well known in the art depending on nucleotide composition, probe length, temperature and the like. The nucleic acid preferably has at least 80% homology, preferably at least 90%, more preferably at least 95%, even more preferably at least 95%, most preferably at least 99% to its reference sequence (for example, TIP 48 or TIP 49 sequence).

Nucleic acids are preferably to be at least 10 bases long, more preferably at least 15 even more preferably at least 50 bases long. The nucleic acid can be single stranded or double stranded, antisense or sense, RNA or DNA. In certain embodiments at least some of the nucleotide residues of the nucleic acid may be made resistant to nuclease degradation and these can be selected from residues such as phophorothioates and/or methylphosphonates for routine chemical synthesis of the nucleic acid.

The nucleic acids described above can also be used as probes for determining expression of a TIP 49 family member in a cell. This may be of practical utility in circumstances where host cells have been transfected with the TIP 49 family member gene and it is desired to check for transcription of the gene. Also the antisense nucleic acid can be used as a research tool to identify transcription levels of the TIP 49 family member gene in cancer cell samples.

Nucleic acid primers may be of use in performing PCR amplification of samples comprising nucleic acids encoding a TIP 49 family member. PCR can be used as an analytical tool, optionally in conjunction with nucleic acid probes specific for a TIP 49 family member 1, for detection of the TIP 49 family member gene and/or its expression.

The nucleic acids as hereinbefore described can advantageously be used as pharmaceuticals, preferred pharmaceutical applications being for the manufacture of a medicament for the prophylaxis or treatment of cancer. Without wishing to be bound to any particular theory, the inventors believe that an antisense inhibition of TIP49/48 expression in cancer cells, or indeed other expression products such as those proteins present *in vivo* complexed to TIP 48 or TIP 49, may reduce the level of the transcription regulatory complex containing TIIP48 or TIP 49. This in turn may switch off genes involved in proliferation. Similarly, sense nucleic acids or double stranded nucleic acids (in particular double-stranded RNA) may also be use as agents active against cancer activity, for example, through a mechanism of sense suppression.

Also useful for carrying out the present invention are nucleic acid constructs or vectors comprising the nucleic acids as hereinbefore described and at least one nucleic acid sequence not encoding a TIP 49 family member. Constructs are not naturally occurring sequences in that they comprise a hybrid of at least two sequences. For example, they may include nucleic acid sequences that function as linkers or restriction sites. Constructs also lack essential sequences of DNA which might permit them to function as vectors. Preferred constructs are synthesised using methods of oligonucleotides synthesis well known to those of skill in the art, although other techniques well known to the molecular biologist, such as the polymerase chain reaction, can also be used. Preferred vectors are expression vectors, preferably plasmids or viruses although cloning vectors are also provided for, optionally in the form of plasmids, which can be made using routine procedures.

Host cells containing the vectors, preferably where the host cell expresses a TIP 49 family member are also useful. Preferred host cells are eukaryotic cells, more preferably insect cells or mammalian cells.

Also encompassed by the present invention is therefore the use of nucleic acids, constructs, vectors and transformed host cells as hereinbefore described as pharmaceuticals particularly as a medicament for the prophylaxis or treatment of cancer.

Antibodies reactive against a TIP 49 family member are also useful as pharmaceuticals, preferably the antibodies are specifically reactive against the STAP1 protein or polypeptide. The antibodies may be monoclonal or polyclonal and other forms e.g. humanised are possible within the scope of the invention.

The invention therefore provides a method of preventing or treating cancer comprising administering to an individual an effective amount of a nucleic acid, a construct, vector, host cell or antibody as described above.

Preferred embodiments of the invention will now be described by way of example and where convenient with reference to drawings in which:

Figure 1 shows a nucleotide sequence of STAP1 (SEQ ID NO:2).

Figure 2 shows a derived amino acid sequence of STAP1 (SEQ ID NO:1).

Figure 3 shows a nucleotide sequence (SEQ ID NO:3) and derived protein sequence (SEQ ID NO:4) of TIP48.

Figure 4 shows a nucleotide sequence (SEQ ID NO:5) and derived protein sequence (SEQ ID NO:6) of TIP49.

Example 1 – Skp 2 and H-Ras$^{(G12V)}$ transfection of cells transforms them.

Skp 2 co-operates with H-Ras^{G12V} to cause cellular transformation of primary rodent fibroblasts as scored by colony formation in soft agar and tumour formation in nude mice. Such transformants express significantly lower levels of p27 than normal fibroblasts or E1A/H-Ras^{G12V}-transformed derivatives.

A sensitive assay of functional properties of candidate oncogenes derives from the use of embryo cell cultures that can be transfected with these genes singly or in combination. When introduced into rat embryo fibroblasts, oncogenes such as E1A or E2F1 are able to transform them only in the presence of a co-introduced, collaborating oncogene like the oncogenic version of H-Ras in which Gly^{12} was changed to Val (G12V). Mammalian expression plasmids encoding Skp 2 and H-Ras^{G12V} were transfected either alone or in combination into primary rat embryo fibroblasts (REFs). After selection in G418 for 3 weeks, plates were scored for the presence of morphologically transformed colonies. In the absence of H-Ras^{G12V}, Skp 2 alone failed to give rise to morphologically transformed foci. In contrast, addition to H-Ras^{G12V} together with the Skp 2 gene gave rise to substantially increased number of morphologically transformed colonies, ranging on average from 70-110 colonies pre plate. Colonies produced by transfection of Skp 2 and H-Ras^{G12V} were easily established and gave rise to cell lines that grew rapidly in culture. These Skp 2/H-Ras^{G12V}-expressing cells were plated into semisolid medium (fresh medium containing 0.3% agar). After 2 weeks plates were analysed for the presence of colonies. Skp 2/H-Ras^{G12V}-expressing cells readily formed colonies in soft agar, which is a strong criterion for cultured cell transformation. In addition, 1 X 10^6 Skp 2/H-Ras^{G12V}-expressing cells were injected in the flank of 2-3 week old nude mice. Mice were scored for the presence of tumours at the injection site. At two weeks thereafter, tumour formation was detected in all experimental animals injected with Skp 2/H-Ras^{G12V}-expressing cells but not with control

REFs. The results of the cotransfection experiments shows that Skp 2 can act as an oncogene.

Example 2 – Immunohistochemical analysis of cells shows a significant inverse relationship between the levels of Skp 2 and p27 in tumour cells.

Skp 2 expression was analysed in a series of human primary oral squamous cell carcinomas, breast carcinomas, lymphomas and prostate cancers. In general, 5 micrometer thick formalin fixed and paraffin embedded tissue sections were stained for p27 and Skp 2 protein by immunohistochemistry using a monoclonal antibody against p27 and polyclonal antibody against Skp 2.

Monoclonal antibodies against p27 are available from Transduction Laboratories. Polyclonal antibodies against Skp 2 are readily raised by persons of average skill in the art by immunisation of an animal with a suitably purified Skp 2 preparation. The polyclonal antibodies can additionally be affinity purified as described by Lisztwag J *et al* (1998) EMBOJ 17: 368 – 363.

The results showed that the expression of p27 and Skp 2 is inversely related in all cancers tested. This confirms that Skp 2 is most likely to function as an oncogene.

These results implicate a substrate-recognition subunit of an SCF ubiquitin protein ligase complex in the development of human cancer.

Example 3 – Isolation and cloning of a cDNA encoding an Skp 2 associated protein (STAP1).

A yeast-two hybrid screen was performed using Skp 2 as a bait. From this a cDNA was cloned that encodes for a protein of about 18 kDa that we now

refer to as STAP1 (for Skp 2-associated protein one). The STAP1 protein is hitherto unknown.

About 1 x 10^6 clones were screened from a HeLa cell library constructed in pGAD-GH (Clontech) which baits encoding residues 101-423 of human Skp 2 cloned in the GAL4 DNA-binding domain vector pAS2-1. Interacting clones were identified after selection on triple-dropout media (minus Leu/Trp/His with 25 mM 3-amino-triazole), and assaying for strong-galactosidase activity. 35 positive clones were sequenced. Sequence comparison revealed that all cloned cDNAs encode for the novel protein STAP1, having a molecular weight of about 18 kD.

Example 4 – Production of recombinant STAP1.

Human STAP1 full-length version was expressed in *Escherichia coli* BL21 as glutathione-S-transferase (GST) fusion proteins and purified on glutathione-sepharose, eluted with glutathione. Methodology is described in Kaelin *et al* (1991) Cell. 64: 521-532 and also Krek *et al* (1994) Cell. 78: 161-172.

Example 5 – Preparation of antibodies reactive against STAP1.

Eluted STAP1 material from example 4 above was injected into mice to generate monoclonal antibodies. A routine monoclonal antibody production protocol was undertaken as will be well known to those of skill in the art. Polyclonal antiserum and antibodies against STAP1 were also generated by injection of the STAP1 eluted material of example 4 above into rabbits following a standard form of protocol which will be familiar to those of skill in the art.

Example 6 – Immunoprecipitation and electrophoretic separation of a complex containing STAP1 from HeLa cells.

Large scale immunoprecipitation was carried out with HeLa whole cell extracts. 100 µg of monoclonal anti-STAP1 antibody coupled to protein A was added to 50 ml of HeLa nuclear extracts (from about 2 to 10^9) and rotated for 2hr at 4ºC. The immunoprecipitates were then washed in 25ml of TNN [20 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40] four times. The precipitated proteins were eluted with 300 µl 0.2M Glycine (pH 2.5) into Laemmli buffer and separated on a 10% SDS-polyacrylamide gel. The gel was then stained with silver.

Example 7 – Analysis of STAP1-associated protein by mass spectrometry.

The SDS-PAGE separated proteins were excised from the gel of example 6, reduced with DTT, alkylated with iodoacetamide and cleaved with trypsin (Promega, sequencing grade) as described by Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Anal. Chem., 68: 850-858. The extracted tryptic peptides were desalted with 5% formic acid, 5% Methanol in H_2O on a 1 µl Poros P20 column and concentrated to 1 µl with 5% formic acid, 50% Methanol in H_2O directly into the Nanoelectrospray ionisation (NanoESI) needle. NanoESI mass spectrometry (MS) was performed according to the published method of Wilm, M. and Mann, M. (1996) Anal. Chem., 68: 1-8. The mass spectra was acquired on an API 300 mass spectrometer (PE Sciex, Toronto, Ontario, Canada) equipped with a NanoESI source (Protana, Odense, Denmark). See also W.R. Pearson & D.J. Lipman (1998) PNAS, 85: 2444-2448.

The STAP1-containing complex is found to contain a large number, about 20 or so proteins. As well as STAP1, the complex has also been found to comprise TIP48, TIP49 (two evolutionarily conserved ATPases and DNA

helicases), RPB5 (RNA pol II subunit 5), RMP1 (RNA pol II mediator protein) and at least three other hitherto unknown proteins.

Example 8 – Analysis of the STAP-containing complex by sucrose density gradient centrifugation and Western blotting.

A crude HeLa cell extract was subjected to 5 – 30% and 10 – 30% (w/v) density centrifugation. The sample was loaded in TNN buffer made up of 10mM Tris (pH 7.5), 250 mM Na Cl, 0.5% NP40, 1MM DTT, sodium vanadate, PMSF and aproteinin. The buffer was also used in the sucrose gradient but the NP40 was omitted. Figure 3 shows the protein profile of fractions taken from the gradient following centrifugation.

Each of the fractions was mixed with sample buffer and subjected to standard Laemlli denaturing SDS-PAGE at 12%. A number of gels were run and then each was blotted with an antibody. Polyclonals against RMP1 and TIP49 were used, as were monoclonals against RPB5, TIP48, STAP1 and Skp 2. The lanes of the blotted gels are aligned in figure 3 with their respective sucrose fractions and what is apparent is that the components of the STAP1-containing complex are clearly associated together and do not form part of the main peak of protein in the gradient. The components of the complex are all found in fractions where higher molecular weight proteins sediment. Skp 2 has a different pattern in the gradient compared to the STAP1-containing complex and this is consistent with Skp 2 being a binding partner for STAP1.

Also noted for the first time is how TIP49 antibodies recognise a doublet on SDS-PAGE. There is an immunologically related TIP49 variant of slightly higher molecular weight.

Example 9 – Screening for anti-cancer agents which are inhibitors of a STAP1-associated DNA helicase complex.

Small molecule compounds that disrupt specific interactions between the components of a STAP1-containing TIP49, TIP48, RPB5, RMP1, STAP1 and Skp 2, for example are putative anti-cancer agents. The component proteins of the complex are expressed in Sf9 insect cells using recombinant baculoviruses. All possible combinations of pairwise interactions between subunits of the complex are constructed and used to screen synthetic and natural compounds. In practice, coinfection of insect cells followed by immunoprecipitation with the appropriate antibody provides the complex substrate used in the screening assays. Coimmunoprecipitation between two of the above-noted components indicates a direct interaction and hence a target for disruption of interaction by putative anti-cancer agents. For example, STAP1 and Skp 2 coimmunoprecipiate when coexpressed in this system and provide a binding pair suitable as the basis of a screening assay for synthetic or natural compounds which disrupt that binding in some way. To screen for small molecular compounds, recombinant hexahistidine-tagged STAP1 is purified from insect cells and immobilized to the surface of nickel-coated 96-well plates. Immobilized STAP1 is incubated with purified biotinylated Skp 2 and washed. Subsequently, europium-labelled streptavidin is added. Then, time-resolved fluorescence of europium is monitored in the absence of presence of synthetic chemical libraries and natural products.

Example 10 – Screening for anti-cancer agents which are inhibitors of TIP48 and/or TIP49 ATPase activity.

Recombinant TIP48 and TIP49 are expressed in *E. coli* using experimental procedures as described in Makino Y *et al* (1999) J. Biol. Chem. 274: 15329 – 15335. Purification of recombinant TIP48 and TIP49, as well as assays for ATPase activity and DNA helicase activity are also as described in Makino Y

et al (1999). The purified recombinant proteins are used to screen for natural products or synthetic compounds which interfere with the normal enzymic activities of TIP48 and/or TIP49.

The screening assay is conveniently carried out in microtiter plates. TIP48 and/or TIP49 proteins are placed in the wells and one or both of the enzyme assays are carried out in the presence or absence of compounds from natural or synthetic chemical libraries. Advantageously, an ATPase microassay format can be used as described in Henkel R D *et al* (1988) Anal. Biochem. 169: 312 – 318. A suitable helicase assay is described in Example 9 of EP 0926157A1.

All references referred to herein, as well as priority application GB 0011439.7 filed 12 May 2000, are hereby incorporated by reference, to the same extent as if each was referred to individually.

Claims:

1. A method for identifying an agent active against cancer cells said method comprising:

contacting a member of the TIP49 family, a fragment or variant thereof, with a test compound;

measuring enzymatic or ligand binding activity of said TIP 49 family member; and

identifying said test compound as a potential candidate agent active against cancer cells that do not express c-Myc, if said test compound results in a change in enzymatic or ligand binding activity of said TIP 49 family member relative to when said test compound is absent.

2. A method as claimed in claim 1, comprising measuring ATPase activity.

3. A method as claimed in claim 1 or 2, wherein said test compound inhibits said enzymatic or ligand binding activity.

4. A method as claimed in any of the preceding claims, wherein the cancer cells express Skp 2.

5. A method as claimed in any of the preceding claims, wherein said TIP 49 family member is complexed to at least one other protein.

6. A method as claimed in claim 5, wherein said protein is one or more of a transcription regulatory factor, RBP 5, RMP 1, prefoldin or STAP1.

7. A method as claimed in any of the preceding claims, comprising detecting TIP 48 ATPase activity.

8. A method as claimed in any of the preceding claims, comprising detecting TIP 49 ATPase activity.

9. A method as claimed in any of the preceding claims, comprising detecting TIP 48 helicase activity.

10. A method as claimed in any of the preceding claims, comprising detecting TIP 49 helicase activity.

11. A method as claimed in any of the preceding claims, comprising detecting TIP 48 ligand binding activity.

12. A method as claimed in any of the preceding claims, comprising detecting TIP 49 ligand binding activity.

13. A method as claimed in claim 11 or claim 12, wherein the ligand is selected from the group consisting of a nucleotide triphosphate, a nucleic acid and a protein.

14. A method as claimed in any of the preceding claims, wherein said TIP 49 family member, fragment or variant thereof is immobilised to a solid surface, more preferably wherein the substrate is nickel or nickel coated.

15. A method as claimed in claim 14, wherein said TIP 49 family member, fragment or variant thereof, or a ligand is labelled.

16. A method as claimed in claim 15, wherein the label is selected from a fluorescent label, an enzyme label, biotin, a metal sol particle or a radiolabel.

17. A method as claimed in claim 16, wherein the label is europium.

18. A method as claimed in any of the preceding claims, said method being a liquid phase assay, preferably employing fluorescent labelling of TIP 49 or a ligand.

19. The use of TIP48 or TIP49 in a method of screening for an agent active against cancers that are not mediated by c-Myc.

20. The use of TIP48 or TIP49 for assembly of a complex *in vitro*.

21. An anti-cancer agent identified by a method of any of claims 1 to 18, preferably an anti-proliferative agent.

22. The anti-cancer agent of claim 21, wherein said agent comprises a nucleic acid complementary to all or a part of a nucleic acid encoding a TIP 49 family member.

23. The anti-cancer agent of claim 21, wherein said agent is an antibody or antibody fragment specific for a TIP 49 family member.

24. The use of an agent identified by a method of any of claims 1 to 18 for the manufacture of a medicament for the prophylaxis or treatment of cancer.

25. A method of preventing or treating cancer comprising administering to an individual an effective amount of a compound identified by a method of any of claims 1 to 18.

25. A complex comprising a TIP49 family member and one or more other proteins or polypeptides selected from the group of STAP1, prefoldin, RPB 5 and RMP1.

26. A complex as claimed in claim 25, wherein the complex comprises STAP1, TIP48 or TIP49, RPB 5, and RMP 1 in a ratio of about 1:1:1:1:1.

27. A transcription regulatory protein complex comprising TIP48 and/or TIP49 and three or more other proteins or polypeptides.

28. A complex as claimed in any of claims 25 to 27 substantially free of other cellular contaminants.

29. An isolated complex as claimed in any of claims 25 to 28 of at least 80% purity, preferably 90% purity, more preferably 95% purity, even more preferably 99% purity.

1: AF083242 . Homo sapiens HSPC0...[gi:5106778] Protein, Related Sequences

```
LOCUS       AF083242      726 bp    mRNA            PRI       21-JUN-1999
DEFINITION  Homo sapiens HSPC024-iso mRNA, complete cds.
ACCESSION   AF083242
VERSION     AF083242.1  GI:5106778
KEYWORDS    FLI_CDNA.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;
            Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 726)
  AUTHORS   Zhou,J., Ye,M., Fu,G., Zhang,Q., Shen,Y., Huang,Q., Xu,S., He,K.,
            Chen,S., Mao,M. and Chen,Z.
  TITLE     Human HSPC024-iso gene, complete cds
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 726)
  AUTHORS   Zhou,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-AUG-1998) Shanghai Second Medical University, Rui-Ji
            Hospital, Shanghai Institute of Hematology, 197, Rui-Jin Road II,
            Shanghai, P. R. China, 200025
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                     /db_xref="GI:5106779"
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                     VALGYGFFLELTLAEALKFIDRKSSLLTELSNSLTKDSMNIKAHIHMLLEGLRELQG
                     QNFPEKPHH"
BASE COUNT      193 a    186 c    198 g    149 t
ORIGIN
        1 ggaggtaaag gccgcgcttg ggtgtccctg ggtggtcggg tccccgagtt gggaggggcg
       61 gaaggctgaa cctccagctt gagccggaca agccgattcc cagcgttgag agggtagaga
      121 tgaactgtgt gtgaggccaa actggatcgg tcaacatggt cttccccctc cccactcccc
      181 aggagcccat catggcgacg ccccctaagc ggcgggcggt ggaggccacg ggggagaaag
      241 tgctgcgcta cgagaccttc atcagtgacg tgctgcagcg ggacttgcga aaggtgctgg
      301 accatcgaga caaggtatat gagcagctgg ccaaatacct tcaactgaga aatgtcattg
      361 agcgactcca ggaagctaag cactcggagt tatatatgca ggtggatttg ggctgtaact
      421 tcttcgttga cacagtggtc ccagatactt cacgcatcta tgtggccctg ggatatggtt
      481 ttttcctgga gttgacactg gcagaagctc tcaagttcat tgatcgtaag agctctctcc
      541 tcacagagct cagcaacagc ctcaccaagg actccatgaa tatcaaagcc catatccaca
      601 tgttgctaga ggggcttaga gaactacaag gcctgcagaa tttcccagag aagcctcacc
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      721 aaaaaa
//
```

FIGURE 1

Program: blastp
Database: nrcrc
Number:
5083
e-mail:
Format: plain_text
Sequence:
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DLGCNFFVD

TVVPDTSRIYVALGYGFFLELTLAEALKFIDRKSSLLTELSNSLTKDSMNIKAHIHMLLEGLRELQGLQNF
PEKPHH

FIGURE 2

TIP48 protein sequence

1 matvtattkv peirdvtrie rigahshirg lglddalepr qasqgmvgql aarraagvvl
61 emiregkiag ravliagqpg tgktaiamgm aqalgpdtpf taiagseifs lemsktealt
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241 tvslheidvi nsrtqgflal fsgdtgeiks evreqinakv aewreegkae iipgvlfide
301 vhmldiesfs flnralesdm apvlimatnr gitrirgtsy qsphgipidl ldrllivstt
361 pysekdtkqi lrirceeedv emsedaytvl trigletslr yaiqlitaas lvcrkrkgte
421 vqvddikrvy slfldesrst qymkeyqdaf lfnelkgetm dts

TIP 48 coding sequence (NCBI Accession No. XM_009417)

1 gttggtgagc atcatggcaa ccgttacagc cacaaccaaa gtcccggaga tccgtgatgt
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121 cttggagcct cggcaggctt cgcaaggcat ggtgggtcag ctggcggcac ggcgggcggc
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241 tggccagccg ggcacgggga agacggccat cgccatgggc atggcgcagg ccctgggccc
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1021 gggcaccagc taccagagcc ctcacggcat ccccatagac ctgctggacc ggctgcttat
1081 cgtctccacc acccccctaca gcgagaaaga cacgaagcag atcctccgca tccggtgcga
1141 ggaagaagat gtggagatga gtgaggacgc ctacacggtg ctgacccgca tcgggctgga
1201 gacgtcactg cgctacgcca tccagctcat cacagctgcc agcttggtgt gccggaaacg
1261 caagggtaca gaagtgcagg tggatgacat caagcgggtc tactcactct tcctggacga
1321 gtcccgctcc acgcagtaca tgaaggagta ccaggacgcc ttcctcttca acgaactcaa
1381 aggcgagacc atggacacct cctgagttgg atgtcatccc ccgaccccac cctgttttcc
1441 accagagttc tgacactgtg actctgtata aaatggttgg gaagctgc

FIGURE 3

TIP49 protein sequence:

1 mkieevkstt ktqriashsh vkglgldesg lakqaasglv gqenareacg vivelikskk
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121 glriketkev yegevteltp cetenpmggy gktishviig lktakgtkql kldpsifesl
181 qkerveagdv iyieansgav krqgrcdtya tefdleaeey vplpkgdvhk kkeiiqdvtl
241 hdldvanarp qggqdilsmm gqlmkpkkte itdklrgein kvvnkyidqg iaelvpgvlf
301 vdevhmldie cftylhrale ssiapivifa snrgncvirg teditsphgi pldlldrvmi
361 irtmlytpqe mkqiikiraq teginiseea lnhlgeigtk ttlrysvqll tpanllakin
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TIP 49 coding sequence (NCBI Accession No. AF070735)

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301 caagacagct ctggctctgg ctattgctca ggagctgggt agtaaggtcc ccttctgccc
361 aatggtgggg agtgaagttt actcaactga gatcaagaag acagaggtgc tgatggagaa
421 cttccgcagg gccattgggc tgcgaataaa ggagaccaag gaagtttatg aaggtgaagt
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541 ccatgtgatc ataggactca aaacagccaa aggaaccaaa cagttgaaac tggaccccag
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781 ccaagatgtg accttgcatg acttggatgt ggctaatgcg cggccccagg ggggacaaga
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FIGURE 4