The antagonist antagonises the factor earlier application (Rule 4.17(Hi))

Published:
— with international search report (Art. 21(3))
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

Declarations under Rule 4.17:
— as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(Hi))

Title: EIF6 PHOSPHORYLATION ANTAGONISTS FOR USE IN MEDICINE

Abstract: An antagonist of phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in medicine, wherein the antagonist antagonises phosphorylation of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.
FIELD OF THE INVENTION

The present invention relates to an antagonist of phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in medicine, pharmaceutical compositions comprising the antagonist and methods comprising the use of the antagonist. The invention also relates to diagnostic and prognostic methods involving detection of phosphorylation of eIF6 polypeptide.

BACKGROUND TO THE INVENTION

Protein synthesis plays a pivotal role in cell growth and transformation. Initiation of translation is a rate-limiting step of protein synthesis (1). Initiation factors (IF) regulate initiation of translation (2).

This process requires three sequential steps: formation of 43S pre-initiation complex by binding of ternary complex eIF2-GTP-tRNAiMet to 40S; formation of 48S pre-initiation complex by binding of 43S subunits to mRNA, assisted by eIF4F, the cap binding complex; formation of active 80S by recruitment of free 60S subunits.

eIF4F complex controls 48S formation by favoring binding of mRNAs to the 40S and unwinding of their 5’UTRs, downstream of growth factor activation (3). Genetic and biochemical studies have shown that eIF4F complex formation is critically dependent from mTORc1 activity and constitutes an important rate-limiting step in tumor growth (4).

Formation of active, translating 80S requires recruitment of free 60S subunits at the start codon. eIF6 affects 80S formation by regulating 60S ribosomal availability (5).

Eukaryotic Initiation Factor 6 (eIF6) is an evolutionarily conserved protein with two activities: nucleolar eIF6 is necessary for ribosome biogenesis; cytoplasmic eIF6 is involved in insulin-stimulated translation.
eIF6 binds 60S ribosomal subunits and prevents their premature association with 40S ribosomal subunits (16). Regulated binding of eIF6 to 60S is therefore a crucial step in translational control, and is partially controlled by RACK1, that acts as a stimulator of eIF6 release (5).

Blockade of RACK1 interaction to eIF6 may lead to inactive 60S subunits, unable to properly bind 40S subunits. Similarly, interfering with eIF6 binding to 60S leads to an increase of inactive 80S couples, because 60S subunits may prematurely bind 40S subunits (16).

Tumour cells overexpress eIF6, but is unknown whether this gives them a competitive advantage.

_in vitro_, eIF6 haploinsufficient (eIF6<sup>+/−</sup>) cells are impaired in insulin-stimulated translation, and refractory to transformation (6). eIF6<sup>+/−</sup> mice have a significant reduction in postnatal growth due to impaired growth of specific organs such as fat and liver. Further, mouse embryonic fibroblasts (MEFs) from heterozygous mice have impaired G1/S phase progression and growth. eIF6<sup>+/−</sup> MEFs have reduced insulin-stimulated translation and are resistant to transformation. The phenotype can be restored by re-expressing eIF6. Thus, eIF6 acts in conditions of stimulated growth as a rate-limiting initiation factor transducing extracellular signals to translational rate (6, 16).

eIF6 may also be involved in the modulation of other diseases, such as Shwachman-Diamond syndrome (S-D syndrome). S-D syndrome has been shown to be due to mutations of the SBDS gene (Woloszynek JR et al, Blood. 2004 Dec 1;104(12):3588-90.). It has been demonstrated that point-mutations of eIF6, that change the affinity of eIF6 binding to 60S ribosomal subunits, are able to restore the molecular alteration of the SBDS gene, in the simple model _S. cerevisiae_ (Menne TF et al, The Shwachman-Bodian-Diamond syndrome protein mediates translational activation of ribosomes in yeast., Nat Genet. 2007 Apr;39(4):486-95).
However, whether and how eIF6 activity can be modulated *in vivo* has previously remained unknown. It was previously not possible to modulate eIF6 activity for medical use.

There is a need for novel therapeutics that target this area. The present invention addresses this need.

The present invention provides novel therapeutics for use in medicine, novel methods of treatment and novel diagnostic and prognostic methods.

**Summary**

In the present application, we disclose results obtained from using the Eμ-Myc lymphoma model to understand how eIF6 affects tumorigenesis and tumor growth *in vivo*. In this model, we can assess whether eIF6 is downstream of the Myc oncogene, if it acts as a rate-limiting factor in tumorigenesis in a mTORc1 dependent fashion or as an independent modulator of 80S formation.

We disclose the analysis of Myc-induced lymphomagenesis in eIF6 haploinsufficient mice (eIF6<sup>+/−</sup>). We disclose results which indicate eIF6<sup>+/−</sup> mice are resistant to Myc-induced lymphomagenesis, resulting in prolonged survival times. eIF6<sup>+/−</sup> mice are protected also by p53 loss of function. eIF6 haploinsufficiency reduces Myc-induced cell cycle progression, but does not affect Myc-induced apoptosis.

We demonstrate eIF6 is phosphorylated by tumor-derived PKCβII, but not by the general translational activator mTORc1. We show for the first time that mutation of serine 235 of eIF6, the PKCβII consensus site, results both in reduced tumorigenesis *in vitro* and in impaired tumor growth *in vivo*. We conclude that eIF6 is the second initiation factor controlling tumorigenesis and tumor growth. Its mechanism of action is unique since is independent from Myc and mTOR, and acts at the level of 80S formation.
Thus the present invention opens new avenues for antagonising phosphorylation of eIF6 at the PKCβII consensus site \textit{in vivo}. These avenues may include antagonising phosphorylation of eIF6 at sites such as serine 230 and 243 which are thought to be co-regulated with phosphorylation at the PKCβII consensus site.

In particular, the present invention relates to such antagonists being for use in medicine for the first time. These antagonists open new avenues for treatment of cancer and other diseases associated with eIF6.

**Statements of the Invention**

According to a first aspect of the present invention, there is provided an antagonist of phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in medicine, wherein the antagonist antagonises phosphorylation of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

In one embodiment the antagonist antagonises phosphorylation of an amino acid serine at the position corresponding to position 235 in the polypeptide shown in Figure 5. This antagonist may also antagonise phosphorylation of an amino acid serine at the position corresponding to position 230 and/or 243 in the polypeptide shown in Figure 5.

The antagonist may be a polypeptide or a polypeptide fragment.

According to a second aspect of the present invention, there is provided an isolated Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in medicine, wherein the amino acid serine at the position corresponding to position 235, 230 and/or 243 of the sequence in Figure 5 is replaced with another amino acid.

In one embodiment the amino acid serine at the position corresponding to position 235 of the sequence in Figure 5 is replaced with another amino acid. This isolated Eukaryotic Initiation Factor 6 (eIF6) polypeptide may also have the amino acid...
corresponding to position 230 and/or 243 of the sequence in Figure 5 replaced with another amino acid.

In one embodiment, the isolated Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in medicine is a homologue which naturally has the amino acid serine at the position corresponding to position 235, 230 and/or 243 of the sequence in Figure 5 is replaced with another amino acid. The homologue may be a homologue from a non-mammalian organism such as Drosophila or yeast for example.

According to another aspect of the present invention, there is provided an isolated Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in medicine, wherein the polypeptide does not comprise the amino acid serine at the position corresponding to position 235, 230 and/or 243 of the sequence in Figure 5.

According to a further aspect of the present invention, there is provided an isolated Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in medicine, wherein the amino acid serine at the position corresponding to position 235, 230 and/or 243 of the sequence in Figure 5 has been removed.

According to another aspect of the present invention, there is provided an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment for use in medicine, wherein the fragment is a fragment of the polypeptide defined in the paragraph above and wherein the fragment has the amino acid serine at the position corresponding to position 235, 230 and/or 243 of the sequence in Figure 5 removed. According to a third aspect of the present invention, there is provided an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment for use in medicine, wherein the fragment is a fragment of the polypeptide defined in the second aspect of the present invention and wherein the fragment comprises the amino acid of the polypeptide which is replaced with another amino acid.

The fragment may comprise the amino acid corresponding to position 235 of the sequence in Figure 5 that has been replaced with another amino acid. This fragment may also comprise the amino acid corresponding to position 230 and/or 243 of the sequence in Figure 5 that has been replaced with another amino acid.
The fragment may comprise the amino acid corresponding to position 235 of the sequence in Figure 5 replaced with another amino acid, wherein the fragment comprises the replaced amino acid embedded in a low-stringency consensus sequence for PKC phosphorylation. The fragment may therefore comprise the sequence IAT*MRD, wherein * represents the replaced amino acid.

According to a further aspect of the present invention, there is provided an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment for use in medicine, wherein the fragment is a fragment of Eukaryotic Initiation Factor 6 (eIF6) polypeptide which does not comprise the amino acid serine at the position corresponding to position 235, 230 and/or 243 of the sequence in Figure 5.

Embodiments of such fragments for use in the invention include truncated portions of Eukaryotic Initiation Factor 6 (eIF6) polypeptide. An example of a preferred fragment is one lacking a portion of eIF6 polypeptide corresponding to the amino acids 223-245 of the polypeptide shown in Figure 5.

Fragments for use in the present invention are able to antagonise phosphorylation of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5. In one embodiment the fragment antagonises phosphorylation of an amino acid serine at the position corresponding to position 235 in the polypeptide shown in Figure 5. This fragment may also antagonise phosphorylation of an amino acid serine at the position corresponding to position 230 and/or 243 in the polypeptide shown in Figure 5.

It is also possible to employ molecules which can elicit the release of the phosphate group from eIF6 and these can be used in place of the antagonists in the methods and uses described herein. Thus references to antagonists apply equally well to agents which promote the release of phosphate groups from eIF6.
We have also found evidence of the effect of eIF6 deletion on the modulation of a cluster of putative targets of miR467e. Thus administration of miR467e may mimic the effects of eIF6 inhibition or phosphomutation.

In one embodiment the antagonist, polypeptide or fragment, or miR467e is administered in an effective amount to a subject in need of having the phosphorylation of serine 235, 230 and/or 243 in the polypeptide shown in Figure 5 antagonised. The subject may be in need of having phosphorylation of eIF6 by PKCπI antagonised. The subject may be in need of having phosphorylation of an amino acid serine at the position corresponding to position 235 in the polypeptide shown in Figure 5 antagonised. The subject may additionally need phosphorylation of an amino acid serine at the position corresponding to position 230 and/or 243 in the polypeptide shown in Figure 5 antagonised.

In one embodiment there is provided the antagonist, polypeptide or fragment, or miR467e for use in medicine, wherein the antagonist, polypeptide or fragment is cell-permeable. The antagonist, polypeptide or fragment may be operably or functionally linked to a cell-penetrating peptide sequence.

In one embodiment the antagonist, polypeptide or fragment, or miR467e is for use in modulating cellular translation in a subject or for use in modulating the immune system or for use in the treatment of proliferative disorders.

In one embodiment the antagonist, polypeptide or fragment, or miR467e is for use in modulating gene silencing.

In one embodiment the antagonist, polypeptide or fragment, or miR467e is for use in modulating apoptosis.

In one embodiment the antagonist, polypeptide or fragment, or miR467e is for use in the treatment of neoplastic disease, adipogenesis disorders or liver disorders.
In one embodiment the antagonist, polypeptide or fragment, or miR467e is for use in the treatment of cancer.

In one embodiment there is provided a product comprising i) the antagonist, polypeptide or fragment, or miR467e and ii) a cancer drug as a combined preparation for simultaneous, contemporaneous, separate or sequential use for use in the treatment of cancer.

In one embodiment the antagonist, polypeptide or fragment, or miR467e is for use in modulating an immune response to an allergen or transplant, or for use in treatment of autoimmune disease or inflammation.

In one embodiment the antagonist, polypeptide or fragment, or miR467e is for use in dampening or preventing an immune response. The antagonist, polypeptide or fragment may for use in dampening or preventing activation of B cells.

In one embodiment the antagonist, polypeptide or fragment, or miR467e is for use in treating cancer, type II diabetes, liver failure, obesity or Shwachman-Diamond syndrome.

The antagonist, polypeptide or fragment, or miR467e for use in the invention may antagonise phosphorylation of eIF6 by PKCβiI.

In one embodiment there is provided a polypeptide for use in medicine, wherein the polypeptide comprises the polypeptide as defined in the second aspect of the invention or the fragment defined in the third aspect of the invention. The polypeptide may be for the same medical uses described in the above statements of the invention. A pharmaceutical composition comprising such a polypeptide and a pharmaceutically acceptable carrier, diluent or excipient is also provided by the present invention. A polynucleotide encoding such a polypeptide is also provided for use according to the invention.
In another embodiment there is provided a fusion protein for use in medicine, wherein the fusion protein comprises the polypeptide as defined in the second aspect of the invention or the fragment defined in the third aspect of the invention. The fusion protein may be for the same medical uses described in the above statements of the invention. A pharmaceutical composition comprising such a fusion protein and a pharmaceutically acceptable carrier, diluent or excipient is also provided by the present invention. A polynucleotide encoding such a fusion protein is also provided for use according to the invention.

A pharmaceutical composition comprising such a polynucleotide, or miR467e and a pharmaceutically acceptable carrier, diluent or excipient is also provided.

According to a further aspect of the present invention, there is provided an antagonist of Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in treating cancer.

According to a fourth aspect of the present invention, there is provided an isolated polynucleotide for use in medicine, wherein the polynucleotide comprises a nucleotide sequence encoding the polypeptide defined in the second aspect of the present invention.

According to a fifth aspect of the present invention, there is provided an isolated polynucleotide for use in medicine, wherein the polynucleotide comprises a nucleotide sequence encoding the Eukaryotic Initiation Factor 6 (eIF6) fragment defined in the third aspect of the present invention.

In one embodiment the polynucleotide defined in the fourth or fifth aspect of the present invention is administered in an effective amount to a subject in need of having the phosphorylation of serine 235, 230 and/or 243 antagonised. The subject may be in need of having phosphorylation of eIF6 by PKCβII antagonised. The subject may be in need of having phosphorylation of an amino acid serine at the position corresponding to position 235 in the polypeptide shown in Figure 5 antagonised. The subject may additionally need phosphorylation of an amino acid serine at the position...
corresponding to position 230 and/or 243 in the polypeptide shown in Figure 5 antagonised.

In one embodiment, the polynucleotide defined in the fourth or fifth aspect of the present invention is for use in modulating cellular translation in a subject or for modulating the immune system or for use in the treatment of proliferative disorders.

In one embodiment the polynucleotide defined in the fourth or fifth aspect of the present invention is for use in modulating gene silencing.

In one embodiment the polynucleotide defined in the fourth or fifth aspect of the present invention is for use in modulating apoptosis.

In one embodiment, the polynucleotide defined in the fourth or fifth aspect of the present invention is for use in the treatment of neoplastic disease, adipogenesis disorders or liver disorders.

In one embodiment, the polynucleotide defined in the fourth or fifth aspect of the present invention is for use in the treatment of cancer.

A polynucleotide for use in the invention may encode a polypeptide, fragment or fusion protein that antagonises phosphorylation of eIF6 by PKCβII.

In one embodiment there is provided a product comprising i) the polynucleotide defined in the fourth or fifth aspect of the present invention and ii) a cancer drug as a combined preparation for simultaneous, contemporaneous, separate or sequential use for use in the treatment of cancer.

In one embodiment, the polynucleotide defined in the fourth or fifth aspect of the present invention is for use in modulating an immune response to an allergen or transplant, or for use in treatment of autoimmune disease or inflammation.
In one embodiment the polynucleotide is for use in dampening or preventing an immune response. The polynucleotide may be for use in dampening or preventing activation of B cells.

In one embodiment, the polynucleotide defined in the fourth or fifth aspect of the present invention is for use in treating cancer, type II diabetes, liver failure, obesity or Shwachman-Diamond syndrome.

The polynucleotides defined above may be contained in a gene transfer vector. The gene transfer vector may be used to deliver the polynucleotide according to the fourth or fifth aspect of the present invention to a site or cell of interest. The vector of the may be delivered to a cell or target site by a viral or non-viral vector.

A vector is a tool that allows or facilitates the transfer of an entity from one environment to another. By way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

In another embodiment the gene transfer vector is in the form of a non-viral gene transfer vector. In this embodiment, the gene transfer vector may comprise, or be in the form of, an expression vector or plasmid which comprises the polynucleotide defined in the fourth or fifth aspect of the present invention.

The polynucleotides defined in the fourth and fifth aspects of the present invention may be contained in a viral vector.

Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, retroviral vector, lentiviral vector, baculoviral vector.
The viral vector may be a lentiviral vector. The viral vector may be in the form of an integrated provirus.

Preferably, the viral vector preferentially transduces a certain cell type or cell types.

The viral vector may be a targeted vector, that is it has a tissue tropism which is altered compared to the native virus, so that the vector is targeted to particular cells.

The present invention also encompasses the use of cells into which a polypeptide, fragment or polynucleotide as defined above is introduced.

Polynucleotides for use in the invention include polynucleotides encoding the antagonists, polypeptides, fragments or fusion proteins as described above. These polynucleotides may be used as described above. A pharmaceutical composition comprising such a polynucleotide and a pharmaceutically acceptable carrier, diluent or excipient is also provided.

According to a sixth aspect of the present invention, there is provided a pharmaceutical composition comprising:

(i) an antagonist as defined in the first aspect of the present invention, an isolated polypeptide as defined in the second aspect of the present invention or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in the third aspect of the present invention,

(ii) a liposome comprising an antagonist as defined in the first aspect of the present invention, an isolated polypeptide as defined in the second aspect of the present invention or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in the third aspect of the present invention, or

(iii) a cell comprising an antagonist as defined in the first aspect of the present invention, an isolated polypeptide as defined in the second aspect of the present invention or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in the third aspect of the present invention.
Factor 6 (eIF6) fragment as defined in the third aspect of the present invention,
and a pharmaceutically acceptable carrier, diluent or excipient.

According to a seventh aspect of the present invention, there is provided a pharmaceutical composition comprising:

(i) an isolated polynucleotide as defined in the fourth or fifth aspect of the present invention,
(ii) a liposome comprising an isolated polynucleotide as defined in the fourth or fifth aspect of the present invention,
(iii) a vector as defined above,
(iv) a viral particle obtainable from a viral vector as defined above, or
(v) a cell comprising an isolated polynucleotide as defined in the fourth or fifth aspect of the present invention,

and a pharmaceutically acceptable carrier, diluent or excipient.

According to an eighth aspect of the present invention, there is provided a method of diagnosing cancer, said method comprising detecting the level of Eukaryotic Initiation Factor 6 (eIF6) polypeptide expression in a sample from subject, the method further comprising detecting the occurrence of phosphorylation of an amino acid in said polypeptide in the sample, wherein the phosphorylation detected is that of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

The level of expression in the sample may be compared to a control sample e.g. one from a subject patient known not to have cancer.

According to an ninth aspect of the present invention, there is provided a method of detecting remission of a tumour after therapy, said method comprising detecting the level of Eukaryotic Initiation Factor 6 (eIF6) polypeptide expression in a sample from subject, the method further comprising detecting the occurrence of phosphorylation of an amino acid in said polypeptide in the sample, wherein the phosphorylation detected
is that of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

According to a tenth aspect of the present invention, there is provided a prognostic method for determining whether a subject will be susceptible to treatment with a polypeptide, fragment or polynucleotide for use according to the present invention or a pharmaceutical composition according to the sixth or seventh aspect of the present invention, said method comprising detecting the occurrence of phosphorylation of an amino acid in Eukaryotic Initiation Factor 6 (eIF6) in a sample from said subject, wherein the phosphorylation detected is that of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

For example, if phosphorylation is detected by a method according to the eighth or tenth aspect of the invention, this can be used as a guide to treatment options and performance, i.e. a prognostic in personalised medicine applications, to select subjects that are likely to be susceptible to treatment with antagonist for use in the present invention. The treatment may involve treatment of cancer or other diseases disclosed herein.

In the methods according to the eighth, ninth and tenth aspects of the present invention the phosphorylation detected may be that of an amino acid serine at the position corresponding to position 235 in the polypeptide shown in Figure 5. The phosphorylation detected may additionally include phosphorylation of an amino acid serine at the position corresponding to position 230 and/or 243 in the polypeptide shown in Figure 5.

The occurrence of phosphorylation of the amino acid serine at the position corresponding to position 235, 230 and/or 243 may be detected using an antibody. Such an antibody forms part of the invention.

The above methods may be used for in applications relating to cancer treatment and diagnosis, for example to select subjects that are likely to need more aggressive anti-cancer surgical, chemotherapeutic or radiotherapeutic treatment.
The above methods may involve comparing the level of phosphorylation detected with that detected in to a control sample e.g. one from a subject patient known not to have a disease e.g. cancer disclosed herein.

According to an eleventh aspect of the present invention, there is provided an *ex vivo* method of antagonising phosphorylation of Eukaryotic Initiation Factor 6 (eIF6), comprising contacting a cell or part of an organism or eIF6 with an antagonist as defined in the first aspect of the present invention, an isolated polypeptide as defined in the second aspect of the present invention or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in the third aspect of the present invention.

According to a twelfth aspect of the present invention, there is provided an *ex vivo* method of antagonising phosphorylation of Eukaryotic Initiation Factor 6 (eIF6), comprising contacting a cell or part of an organism or eIF6 with an isolated polynucleotide as defined above.

The cell or part of an organism produced by methods according to the eleventh and twelfth aspects of the present invention may be for use in medicine. The cell or part of an organism may be introduced into a patient. The cell or part of an organism may be used for the same uses described above for the antagonist, polypeptide or fragments defined in the first, second and third aspects of the present invention.

According to a thirteenth aspect of the present invention, there is provided a method of identifying an agent that prevents phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide, wherein the phosphorylation is of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5, the method comprising contacting a cell expressing Eukaryotic Initiation Factor 6 (eIF6) with said agent and detecting the occurrence of phosphorylation of Eukaryotic Initiation Factor 6 (eIF6), wherein the phosphorylation is of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.
Said agent may be a known inhibitor of translation. The occurrence of phosphorylation may be detected using the same methods used for the eighth, ninth and tenth aspects of the present invention.

In the methods according to the thirteenth aspect of the present invention, the agents identified may prevent phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide, wherein phosphorylation is of an amino acid serine at the position corresponding to position 235 in the polypeptide shown in Figure 5. The identified agent may additionally prevent phosphorylation of an amino acid serine at the position corresponding to position 230 and/or 243 in the polypeptide shown in Figure 5.

According to the fourteenth aspect of the present invention there is provided an agent identified using a method according to the thirteenth aspect of the present invention. The agent may be an antagonist that antagonises phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide, wherein the phosphorylation is of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5. The agent may be a polynucleotide encoding such an antagonist.

The agent may be used for the same uses as an antagonist for use according to the invention or a polynucleotide for use according to the invention. A pharmaceutical composition comprising said agent and a pharmaceutically acceptable carrier, diluent or excipient is also provided.

Further particular and preferred aspects of the present invention are set out in the accompanying independent and dependent claims. Features of the dependent claims may be combined with features of the independent claims as appropriate, and in combinations other than those explicitly set out in the claims.

The present invention also envisages the use of biomarkers in the test methods e.g. diagnostic and prognostic methods of the present invention. Suitable biomarkers are set out in Figures 16, 17, 18 and 19.
The foregoing and other objects and features of the disclosures will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

**Fig. 1. eIF6 is a limiting factor in Myc-induced lymphomagenesis and protects from p53 loss of function.** (A) Representative results of Western Blot analysis on control mice and tumors from Eμ-Myc tg mice: c-Myc and eIF6 are overexpressed in tumors, but levels of eIF6 protein are still reduced in heterozygous tumors. (B) Western Blot analysis on eIF6+/+ and eIF6+/− MEFs expressing MycER, in the absence or presence of OHT. c-Myc overexpression increases eIF4E but not eIF6 protein levels. Corresponding densitometric analysis, normalised to actin levels, is indicated. (C) Kaplan-Meier curves show that the onset of lymphomas and death in the Eμ-Myc / eIF6+/− mice (n = 82) is markedly delayed compared to the Eμ-MycZ eIF6+/+ animals (n = 106) (***p < 0.0001). (D) Weight of spleens and lymph nodes of Eμ-Myc tg mice: eIF6+/− mice exhibit a significant reduction in splenomegaly and tumor mass at early stages of the disease (*P < 0.05 and **P < 0.01). (E) Kaplan-Meier curves show a survival time increase in p53+/− Eμ-Myc / eIF6+/− mice (n = 24) compared to p53+/− Eμ-Myc tg / eIF6+/− ones (n = 21), (**P < 0.0001).

**Fig. 2. eIF6 reduction affects Myc oncogenesis by reducing pre-B cell proliferation and methionine incorporation.** (A) FACS analysis on primary pre-B lymphocytes isolated from spleens and bone marrow of 4-weeks-old control mice (c) and Eμ-Myc tg ones (tg), previously injected i.p. with 1 mg BrdU (n = 3 per genotype). eIF6 heterozygosity reduced the percentage of pre-B lymphocytes in S-phase during the cell cycle. *P < 0.05 and **P < 0.01. (B) TUNEL assay on purified pre-B lymphocytes from spleens and bone marrow shows an increase of apoptotic rate in Eμ-Myc tg mice, but no significant differences between eIF6+/+ and eIF6+/− (n = 3 per genotype). (C) Representative results of 35S-Methionine labelling experiment in purified pre-B lymphocytes either unstimulated or upon stimulation with LPS/IL4. Increase of global translation is detected in Eμ-Myc tg B cells but is not evident in conditions of eIF6 haploinsufficiency.
Fig. 3 eIF6 is phosphorylated in Myc-expressing lymphomas containing PKCβII. (A) Lymphomas express PKCpII. Representative Western Blot of proteins recovered from tumors of Eμ-Myc tg mice presenting comparable weight. (B-C) Kinase assay on tumor samples at indicated conditions. eIF6 is directly phosphorylated by PKCβII (B), but not by mTOR (C). Corresponding densitometric analyses were normalised to background and appropriate controls indicated. (D) Representative 2D gel electrophoresis of a lymphoma sample: black arrows indicate eIF6 phosphorylation points.

Fig. 4 eIF6 haploinsufficiency and mutation of eIF6^{S235A} reduce transformation and tumor growth. (A) Transformation rate of wt and eIF6^{+/−} fibroblasts with indicated retroviruses (n = 6). (B) eIF6 levels were determined by Western Blotting in wt and heterozygous eIF6 tumor cells. Anti H-Ras antibody was used as a control for retroviral infection. (C) Transformation assay on eIF6^{+/−} and eIF6^{−/−} primary fibroblasts transformed with DNp53 plus H-ras
^{V12}. Both genotypes were then infected with lentivirus carrying GFP as an internal control and mutant eIF6^{S235A}, while eIF6^{+/−} MEFs received also a lentivirus bearing full-length eIF6 (eIF6^{wt}) to rescue normal eIF6 protein levels. Single retroviral infection is indicated as a control (100%). Mutant eIF6^{S235A} dramatically reduced transformation efficiency both in eIF6^{+/−} and eIF6^{−/−} MEFs, while eIF6 rescue in heterozygous MEFs led to increase in the transformation rate (n = 6 per genotype). (D-F) Subcutaneous injection of transformed eIF6^{+/−} and eIF6^{−/−} MEFs, (DN) reconstituted with either wt eIF6 or eIF6^{S235A}, showed that eIF6^{−/−} tumors growth is inhibited in vivo (D), whilst the expression of eIF6^{S235A} affects growth of eIF6^{+/−} tumors (E), as well of eIF6^{−/−} tumors (F). Reconstitution with wt eIF6 accelerates tumor growth (F). n=3 for D, n = 6 for E-F. (G-H) Proliferation rate was measured by BrdU incorporation: eIF6^{−/−} tumors have significant reduction in the percentage of BrdU positive cells (**P < 0.01) (G). Corresponding representative immunohistochemical staining (H). Scale bar is indicated.

Fig. 5 shows the amino acid sequence of a Eukaryotic Initiation Factor 6 (eIF6) polypeptide. The amino acid serine at positions 235, 230 and 243 are indicated by bold type.
Fig. 6 shows the nucleotide sequence of the polypeptide shown in Fig. 5. The start codon is indicated in bold. The codon encoding Serine 235 is underlined. The stop codon is indicated in italics.

Fig. 7 shows the amino acid sequence of the Ser235Ala eIF6 mutant polypeptide described in the materials and methods below. The mutated amino acid residue at position 235 is indicated by **bold** type.

Fig. 8 shows the nucleotide sequence of the polypeptide shown in Fig. 7. The start codon is indicated in bold. The codon encoding alanine (the mutated amino acid residue) is underlined. The stop codon is indicated in italics.

Fig. 9. E\(\mu\)-Myc tg / eIF6\(^{+/-}\) mice show a reduction of splenomegaly and GATA1 staining at early stages of disease. (A) Representative staining of spleens of 4-week-old E\(\mu\)-Myc tg mice. Spleen morphology was analyzed with H&E staining, while proliferating cells were detected by anti-PCNA antibody. Changes in proliferation rate involve primarily the spleen red pulp (as shown in insets), suggesting an altered hemopoietic development. Hemopoiesis in spleens was analyzed with an anti GATA1 antibody. Scale bar is indicated. (B) Representative result of western blot analysis of spleen extracts. Overexpression of eIF6 and GATA1 is evident in E\(\mu\)-Myc tg / eIF6\(^{+/-}\) spleen. Corresponding densitometric analysis, normalised to actin levels, is also shown.

Fig. 10. Heterozygosity does not affect B cell development. B cells of 4-week-old mice were isolated from spleen and bone marrow of control (c) mice and E\(\mu\)Myc tg ones (tg). Total bone marrow lymphocytes were stained with FITC-anti-mouse B220 and PE-anti-mouse IgM, while total spleen cells were stained with FITC-anti-mouse IgD and and PE-anti-mouse IgM; nuclei were counterstained with 7-AAD marker and all samples were analyzed by FACS. Data, expressed in percentage, show no significant differences in B cell maturation (n = 3).
Fig. 11. Transformation efficiency is impaired in eIF6<sup>+/−</sup> MEFs. Transformation assay of asynchronous MEFs infected with retrovirus carrying DNp53-H-Ras<sup>v12</sup>. The number of transformed colonies indicates that cellular potential to undergo transformation is impaired in eIF6<sup>+/−</sup> MEFs.

Fig. 12. Western blot analysis of eIF6 levels in eIF6 and eIF6<sup>S235A</sup> transduced cells. Anti H-Ras and anti-GFP antibody were used as a control for retroviral and lentiviral infection, respectively.

Fig. 13. eIF6<sup>S235</sup> activity is critical for tumorigenesis. eIF6<sup>+/+</sup> primary fibroblasts were infected with retrovirus bearing DNp53-H-Ras<sup>v12</sup> and with three different lentiviral vectors expressing mutant eIF6<sup>S235A</sup>, eIF6<sup>wt</sup> and GFP as a control. While mutant eIF6<sup>S235A</sup> dramatically inhibits transformation efficiency, rescue with eIF6<sup>wt</sup> increases the number of transformed colonies.

Fig. 14. eIF6 haploinsufficiency affects cellular density and angiogenesis, but not apoptosis, in tumors. Representative staining of tumors recovered from nude mice s.c. injected with eIF6<sup>+/+</sup> and eIF6<sup>+/−</sup> transformed MEFs. Tissue morphology was analysed with Hematoxilin and Eosin staining; apoptosis was detected both by TUNEL assay and by immunohistochemistry for Cleaved-Caspase 3; staining for CD31 revealed reduced positive vessels in heterozygous tumors. Scale bar: 50 µm.

Fig. 15. Blood cell counts of eIF6 het. mice, versus wt. mice (n=39). Blood was extracted and analyzed for single cell types. WBC, white blood cells; RBC, red blood cells; PLT, platelets; HGB, haemoglobin.

Fig. 16. GO analysis of metabolic changes due to eIF6 impairment of activity. The main classes of genes affected by eIF6 depletion are represented by cell cycle progression.

Fig. 17. shows the genes down-regulated by the miRNA467e.
Fig. 18. shows genes down-regulated by eIF6\(^{+/−}\) and miRNA467e.

Fig. 19. Gene expression changes validated by real time Qt-PCR. Total RNA was extracted with TRIzol reagent (Invitrogen) from tissues of wild-type and eIF6 het. mice. After treatment of total RNA with RQ1 RNase-free DNase (Promega), reverse transcription was performed with M-MLV reverse transcriptase enzyme (Promega) according to the manufacturer’s instructions. One hundred ng of reverse transcribed complementary DNA was amplified with the specific probes for the indicated genes in an ABI PRISM 7900HT Sequence Detection System. Gapdh was used as an internal control of gene expression (TaqMan gene expression assay 4352932E from Applied Biosystems). Each sample was analyzed at least in biological triplicate. Note that changes are observed both in the polysomal column (left) and in the total RNA column (right) indicating that they occur also at the protein level.

**Detailed description**

As used herein, "Eukaryotic Initiation Factor 6 (eIF6) polypeptide" is understood within the scope of the invention to include the Eukaryotic Initiation Factor 6 (eIF6) polypeptide shown in Figure 5 and homologues thereof as a skilled person will readily appreciate that eIF6 sequences vary among different organisms. Thus, Eukaryotic Initiation Factor 6 (eIF6) polypeptides may include an isolated polypeptide which comprises an amino acid sequence which has at least 85% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, most preferably at least 97-99% or exact identity, to that shown in Figure 5. This % identity may be over the entire length of the sequence shown in Figure 5.

It should be noted that in this application amino acid positions are identified by those 'corresponding' to a particular position in the consensus sequence of Figure 5. This is not to be interpreted as meaning the sequences of the present invention must include sequences present in Figure 5. Reference to this Figure is used merely to enable identification of a particular amino acid location within any particular eIF6 polypeptide. Such amino acid locations can be routinely identified using sequence alignment programs, the use of which are well known in the art.

As used herein, "antagonist" is understood within the scope of the invention to include any agent, which may include any compound, substance or molecule, e.g. a protein, polypeptide or polypeptide fragment capable of antagonising phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide. An antagonist for use in the invention may antagonise phosphorylation of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5. An antagonist for use in the invention may antagonise phosphorylation of eIF6 by PKCβII. An antagonist for use in the invention may antagonise phosphorylation of an amino acid serine at the position corresponding to position 235 in the polypeptide shown in Figure 5. An antagonist for use in the invention may also antagonise phosphorylation of an amino acid serine at the position corresponding to position 230 and/or 243 in the polypeptide shown in Figure 5.
As used herein, the terms "polypeptide" and "peptide" refer to a polymer in which the monomers are amino acids and are joined together through peptide or disulfide bonds. The polypeptide or peptide may be a protein or part of a protein. A "protein" is understood within the scope of the invention to include single-chain polypeptide molecules, as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by covalent or non-covalent means. The present invention envisages that peptides may be used in the same way as polypeptides that are for use in the invention.

The term "fragment" typically refers to a selected region of the polypeptide that is of interest. A "fragment" is a polypeptide having an amino acid sequence that is entirely the same as part but not all of any amino acid sequence of a polypeptide as defined in the second aspect of the invention or antagonist as defined in the first aspect of the invention. Preferred fragments for use in the invention include an isolated polypeptide comprising an amino acid sequence having at least 15, 20, 30, 40, 50 or 100 contiguous amino acids from the amino acid sequence of a polypeptide for use in the invention or an isolated polypeptide comprising an amino acid sequence having at least 15, 20, 30, 40, 50 or 100 contiguous amino acids truncated or deleted from the amino acid sequence of a polypeptide for use in the invention. The fragments may have portions the C terminal end and/or the N-terminal leader sequence of eIF6 polypeptide truncated or deleted.

Fragments that have the same function as an antagonist of the first aspect of the invention are disclosed herein. These may antagonise phosphorylation of an amino acid serine at the position corresponding to position 235 in the polypeptide shown in Figure 5 are disclosed. They may also antagonise phosphorylation of an amino acid serine at the position corresponding to position 230 and/or 243 in the polypeptide shown in Figure 5. They may antagonise phosphorylation of eIF6 by PKCπI.

Methods of generating fragments of a known polypeptide are known in the art (see for example Curr Protoc Immunol. 2002 May; Chapter 9:Unit 9.1. Introduction to peptide synthesis.). Further, methods for testing whether such fragments have the required antagonist properties are known in the art and can be performed using routine
experimentation. Methods of testing whether generated fragments have such properties may for example include the use of SPOT arrays consisting of synthesized peptides 12- to 18-amino acids long, with overlapping sequences that cover the entire sequence of a full length antagonist polypeptide, covalently linked to a solid support. *Curr. Protoc. Protein Sci. 51:18.10.1-18.10.9. © 2008 by John Wiley & Sons, Inc.* describes how to construct peptide SPOT arrays, biotinylate recombinant proteins, and conduct overlay assays to identify binding interactions. In addition, directions describing how to analyze results to determine single amino acid binding contributions are included. The two techniques in this reference describe how to scan protein sequences to find binding motifs and how to conduct site-directed mutagenesis studies.

In addition to the specific polypeptides, fragments and polynucleotides mentioned herein, the present invention also encompasses the use of variants.

In the context of the present invention, a variant of any given sequence is a sequence in which the specific sequence of residues (whether amino acid or nucleic acid residues) has been modified in such a manner that the polypeptide or polynucleotide in question retains at least one of its endogenous functions. A variant sequence can be obtained by addition, deletion, substitution modification replacement and/or variation of at least one residue present in the naturally-occurring protein. Typically, amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the required activity or ability. Amino acid substitutions may include the use of non-naturally occurring analogues.

Such variants may be prepared using standard recombinant DNA techniques such as site-directed mutagenesis. Where insertions are to be made, synthetic DNA encoding the insertion together with 5' and 3' flanking regions corresponding to the naturally-occurring sequence either side of the insertion site. The flanking regions will contain convenient restriction sites corresponding to sites in the naturally-occurring sequence so that the sequence may be cut with the appropriate enzyme(s) and the synthetic DNA ligated into the cut. The DNA is then expressed in accordance with the invention to make the encoded protein. These methods are only illustrative of the numerous
standard techniques known in the art for manipulation of DNA sequences and other known techniques may also be used.

Polypeptides and fragments for use according to the present invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent protein. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the transport or modulation function is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

<table>
<thead>
<tr>
<th>ALIPHATIC</th>
<th>Non-polar</th>
<th>GAP</th>
<th>ILV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polar – uncharged</td>
<td></td>
<td>CSTM</td>
<td></td>
</tr>
<tr>
<td>Polar – charged</td>
<td></td>
<td>DE</td>
<td>K</td>
</tr>
<tr>
<td>Aromatic</td>
<td></td>
<td>HFWY</td>
<td></td>
</tr>
</tbody>
</table>

The polypeptides and fragments for use in the invention may be "free-standing," or comprised within a larger polypeptide of which they form a part or region, most preferably as a single continuous region in a single larger polypeptide.
The polypeptides and fragments for use in the invention may be in the form of the "mature" protein or may be a part of a larger protein such as a precursor or a fusion protein.

The terms "polypeptide" and "fragment" include polypeptides and fragments that have been modified e.g. by the addition of sugar residues or polypeptides and fragments that have been modified e.g. to make them cell-permeable. The polypeptide or fragment may include a cell- penetrating peptide sequence that facilitates delivery of the polypeptide or fragment to the intracellular space. e.g., HIV-derived TAT peptide, penetratins, transportans, or hCT derived cell-penetrating peptides, see, e.g., Caron et al., (2001) Mol Ther 3(3):310-8; Langel, Cell-Penetrating Peptides: Processes and Applications (CRC Press, Boca Raton FL 2002); El-Andaloussi et al, (2005) Curr Pharm Des. 11 (28): 3597-61; and Deshayes et al, (2005) Cell Mol Life Sci. 62(16): 1839-49.

The polypeptide or fragment may be "operably" or "functionally" linked to a cell-penetrating peptide sequence. "Operably" or "functionally" linked means they are connected so that the cell- penetrating peptide sequence can direct import of the polypeptide or fragment comprising a cell- penetrating peptide into a cell.

A polypeptide or fragment comprising a cell- penetrating peptide sequence can be prepared by standard peptide synthesis methods known to those skilled in the art. They may also be produced using an expression vector having a nucleotide sequence encoding the modified polypeptide or fragment and a cell- penetrating peptide sequence operably linked to appropriate promoter, terminator, and other functional sequences, such as a sequence encoding a purification tag, to facilitate expression and purification of the peptides. The polypeptide or fragment can be operably or functionally linked to a cell- penetrating peptide, for example, by one or more peptide bonds. The cell- penetrating peptide can be immediately C-terminal or N-tenninal to the polypeptide or fragment, and more than one cell- penetrating peptide can be used, more than one polypeptide or fragment can be used, and/or the cell- penetrating peptide and polypeptide or fragment amino acid sequences can be separated by one or
more amino acids in the region between the cell-penetrating peptide and polypeptide or fragment. The polypeptide or fragment that is operably or functionally linked to a cell-penetrating peptide sequence can comprise additional amino acids either C-terminal or N-terminal, or both.

The term "polynucleotide" comprises DNA or RNA and other types of polynucleotides known in the art. Polynucleotides for use in the invention may be single-stranded or double-stranded. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides used in the invention to reflect the codon usage of any particular host organism in which the polypeptides are to be expressed.

The term "polynucleotide encoding a polypeptide or fragment" as used herein encompasses polynucleotides that include a sequence encoding a polypeptide or fragment for use in the invention. The term also encompasses polynucleotides that include a single continuous region or discontinuous regions encoding the polypeptide (for example, polynucleotides interrupted by integrated phage, an integrated insertion sequence, an integrated vector sequence, an integrated transposon sequence, or due to RNA editing or genomic DNA reorganization) together with additional regions, that also may contain coding and/or non-coding sequences.

**Pharmaceutical Compositions**

According to the sixth and seventh aspects of the present invention, there are provided pharmaceutical compositions comprising the specified ingredients admixed with one or more pharmaceutically acceptable diluents, excipients or carriers. Other active materials may also be present, as may be considered appropriate or advisable for the disease or condition being treated or prevented.
Even though the specified antagonists, polypeptides, fragments, liposomes, cells, polynucleotides, vectors and viral particles can be administered alone, they will generally be administered in admixture with a pharmaceutical carrier, excipient or diluent, particularly for human therapy. The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine.

Examples of such suitable excipients for the various different forms of pharmaceutical compositions described herein may be found in the "Handbook of Pharmaceutical Excipients, 2nd Edition, (1994), Edited by A Wade and PJ Weller. The carrier, or, if more than one be present, each of the carriers, must be acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient.

Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985).

Examples of suitable carriers include lactose, starch, glucose, methyl cellulose, magnesium stearate, mannitol, sorbitol and the like. Examples of suitable diluents include ethanol, glycerol and water.

The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as, or in addition to, the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

Examples of suitable binders include starch, gelatin, natural sugars such as glucose, anhydrous lactose, free-flow lactose, beta-lactose, corn sweeteners, natural and synthetic gums, such as acacia, tragacanth or sodium alginate, carboxymethyl cellulose and polyethylene glycol.
Examples of suitable lubricants include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like.

Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

According to a further aspect of the invention, there is provided a process for the preparation of a pharmaceutical or veterinary composition as described above, the process comprising bringing the active compound(s) into association with the carrier, for example by admixture.

In general, the formulations are prepared by uniformly and intimately bringing into association the active agent with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product. The invention extends to methods for preparing a pharmaceutical composition comprising bringing a compound of general formula (I) in conjunction or association with a pharmaceutically or veterinarily acceptable carrier or vehicle.

Administration

Even though the antagonists, polypeptides, fragments, liposomes, cells, polynucleotides, vectors and viral particles used in the present invention can be administered alone, they will generally be administered in admixture with a pharmaceutical carrier, excipient or diluent, particularly for human therapy.

These compositions and the pharmaceutical compositions of the present invention may be adapted for rectal, nasal, intrabronchial, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intraarterial and intradermal), intraperitoneal or intrathecal administration. The formulations may conveniently be presented in unit dosage form, i.e., in the form of discrete portions
containing a unit dose, or a multiple or sub-unit of a unit dose. By way of example, the formulations may be in the form of tablets and sustained release capsules, and may be prepared by any method well known in the art of pharmacy.

Formulations for oral administration may be presented as: discrete units such as capsules, gellules, drops, cachets, pills or tablets each containing a predetermined amount of the active agent; as a powder or granules; as a solution, emulsion or a suspension of the active agent in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion; or as a bolus etc. Preferably, these compositions contain from 1 to 250 mg and more preferably from 10-100 mg, of active ingredient per dose.

For compositions for oral administration (e.g. tablets and capsules), the term "acceptable carrier" includes vehicles such as common excipients e.g. binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth, polyvinylpyrrolidone (Povidone), methylcellulose, ethylcellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, sucrose and starch; fillers and carriers, for example corn starch, gelatin, lactose, sucrose, macrocrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride and alginic acid; and lubricants such as magnesium stearate, sodium stearate and other metallic stearates, glycerol stearate stearic acid, silicone fluid, talc waxes, oils and colloidal silica. Flavouring agents such as peppermint, oil of wintergreen, cherry flavouring and the like can also be used. It may be desirable to add a colouring agent to make the dosage form readily identifiable. Tablets may also be coated by methods well known in the art.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active agent in a free flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets
may be optionally be coated or scored and may be formulated so as to provide slow or
controlled release of the active agent.

Other formulations suitable for oral administration include lozenges comprising the active agent in a flavoured base, usually sucrose and acacia or tragacanth; pastilles comprising the active agent in an inert base such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active agent in a suitable liquid carrier.

Other forms of administration comprise solutions or emulsions which may be injected intravenously, intraarterially, intrathecally, subcutaneously, intradermally, intraperitoneally or intramuscularly, and which are prepared from sterile or sterilisable solutions. Injectable forms typically contain between 10 - 1000 mg, preferably between 10 - 250 mg, of active ingredient per dose.

The pharmaceutical compositions of the present invention may also be in form of suppositories, pessaries, suspensions, emulsions, lotions, ointments, creams, gels, sprays, solutions or dusting powders.

An alternative means of transdermal administration is by use of a skin patch. For example, the active ingredient can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. The active ingredient can also be incorporated, at a concentration of between 1 and 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilisers and preservatives as may be required.

**Dosage**

A person of ordinary skill in the art can easily determine an appropriate dose of one of the instant compositions to administer to a subject without undue experimentation. Typically, a physician will determine the actual dosage which will be most suitable for an individual patient and it will depend on a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that
compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the individual undergoing therapy. The dosages disclosed herein are exemplary of the average case. There can of course be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

No unacceptable toxicological effects are expected when antagonists, polypeptides, fragments, liposomes, cells, polynucleotides, vectors and viral particles and pharmaceutical compositions are administered in accordance with the present invention. The antagonists, polypeptides, fragments, liposomes, cells, polynucleotides, vectors and viral particles and pharmaceutical compositions, which may have good bioavailability, may be tested in one of several biological assays to determine the concentration of a compound which is required to have a given pharmacological effect.

**Combinations**

In a particularly preferred embodiment, the one or more polypeptides, fragments, liposomes, cells, polynucleotides, vectors and viral particles for use according to the invention and pharmaceutical compositions of the invention or combinations thereof are administered in combination with one or more other active agents, for example, existing drugs available on the market. In such cases, the polypeptides, fragments, liposomes, cells, polynucleotides, vectors and viral particles or pharmaceutical compositions or combinations thereof may be administered consecutively, simultaneously or sequentially with the one or more other active agents.

Drugs in general are more effective when used in combination. In particular, combination therapy is desirable in order to avoid an overlap of major toxicities, mechanism of action and resistance mechanism(s). Furthermore, it is also desirable to administer most drugs at their maximum tolerated doses with minimum time intervals between such doses. The major advantages of combining chemotherapeutic drugs are
that it may promote additive or possible synergistic effects through biochemical interactions and also may decrease the emergence of resistance.

Beneficial combinations may be suggested by studying the antagonistic activity of the test polypeptides, fragments, liposomes, cells, polynucleotides, vectors and viral particles or pharmaceutical compositions with agents known or suspected of being valuable in the treatment of a particular disorder. This procedure can also be used to determine the order of administration of the agents, i.e. before, simultaneously, or after delivery. Such scheduling may be a feature of all the active agents identified herein.

Thus, one aspect of the present invention further comprises administering another active pharmaceutical ingredient, such as a chemotherapeutic agent, either in combined dosage form with a polypeptide, fragment, liposome, cell, polynucleotide, vector or viral particle for use in the invention or a pharmaceutical composition of the present invention or in a separate dosage form. Such separate chemotherapeutic agent dosage forms may include solid oral, oral solution, syrup, elixir, injectable, transdermal, transmucosal, or other dosage form. The compound and the other active pharmaceutical ingredient can be combined in one dosage form or supplied in separate dosage forms that are usable together or sequentially.

Examples of chemotherapeutic agents which may be used in the present invention include, but are not limited to, cytotoxic antibiotics such as aclarubicin, bleomycin, dactinomycin, daunorubicin, doxorubicin, epirubicin, idarubicin, mitomycin, and mitoxantrone (mitozantrone); alkylating agents such as busulfan, carmustine, chlorambucil, chloromethine hydrochloride, mustine hydrochloride, cyclophosphamide, estramustine phosphate, ifosfamide, lomustine, melphalan, thiotepa, and treosulfan; antimetabolites such as capecitabine, cladribine, cytarabine, fludarabine, fluorouracil, gemcitabine, mercaptopurine, methotrexate, raltitrexed, tegafur, and thioguanine; vinca alkaloids, such as etoposide, vinblastine, vincristine, vindesine, and vinorelbine; other antineoplastic drugs such as amsacrine, altretamine, crisantaspase, dacarbazine, temozolomide, hydroxycarbamide, hydroxyurea, and pentostatin; platinum compounds such as carboplatin, cisplatin, and oxaliplatin; porfimer sodium; procarbazine;
razoxane; taxanes such as docetaxel and paclitaxel; topoisomerase I inhibitors such as irinotecan and topotecan; trastuzumab; tretinoin.

**Introduction of polypeptides, polypeptide fragments and nucleic acid sequences into cells**

Where the invention makes use of a polypeptide or fragment, they may be administered directly e.g. as the polypeptide itself or by introducing nucleic acid constructs/viral vectors encoding the polypeptide or fragment into cells under conditions that allow for expression of the polypeptide or fragment in a cell of interest.

Transfer of the polynucleotide, polypeptide or fragment may be performed by any of the methods mentioned below which physically or chemically permeabilize the cell membrane or by the use of liposomes. Cell-penetrating peptides may also be used to transfer a polypeptide or fragment into a cell.

The polynucleotides for use in the invention may be contained in a gene transfer vector. The vector of the may be delivered to a target site by a viral or non-viral vector. The vector may be transferred into a cell as described below to provide for expression of a polypeptide or polypeptide fragment.

The vector may be an expression vector. Expression vectors as described herein comprise regions of nucleic acid containing sequences capable of being transcribed. Thus, sequences encoding mRNA, tRNA and rRNA are included within this definition.

Expression vectors preferably comprise a polynucleotide for use in the invention is preferably operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell. The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under
condition compatible with the control sequences. The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

Non-viral delivery systems include but are not limited to DNA transfection methods and calcium phosphate precipitation. Here, transfection includes a process using a non-viral vector to deliver a gene to a target mammalian cell. Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), and combinations thereof.

Convenient non-limiting methods for introducing polynucleotides, polypeptides and polypeptide fragments into cells are discussed below. However, any suitable method of transferring the polynucleotides, polypeptides and polypeptide fragments into cells may be used.

1. Electroporation

In certain preferred embodiments of the present invention, the antigen is introduced into the cells via electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge.

It is contemplated that electroporation conditions for cells may be optimized. One may particularly with to optimize such parameters as the voltage, the capacitance, the time and the electroporation media composition. The execution of other routine adjustments will be known to those of skill in the art.

2. Particle Bombardment
One method for transferring a naked DNA construct into cells involves particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them. The microprojectiles used have consisted of biologically inert substances such as tungsten, platinum or gold beads.

It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using particle bombardment. It is contemplated that particles may contain DNA rather than be coated with DNA. Hence it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force. Another method involves the use of a Biolistic Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as stainless steel or Nytex screen, onto a filter surface covered with cells in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectile aggregates and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters, or alternatively on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded.

It is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance and
helium pressure. One may also optimize the trauma reduction factors by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art.

3. Adenovirus Assisted Transfection

In certain embodiments, the nucleic acid construct is introduced into the cell using adenovirus assisted transfection. Increased transfection efficiencies have been reported in cell systems using adenovirus coupled systems, and the inventors contemplate using the same technique to increase transfection efficiencies.

4. Viral Transformation

a. Adenoviral Infection

One method for delivery of the nucleic acid constructs involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a construct that has been cloned therein.

The vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization or adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb. In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal
manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are cis elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off. The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

b. AAV Infection

Adeno-associated virus (AAV) is an attractive vector system for use in the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue culture. AAV has a broad host range for infectivity. Details concerning the generation and use of rAAV vectors are described in U.S. Pat. No. 5,139,941 and U.S. Pat. No. 4,797,368, each incorporated herein by reference.

Recombinant AAV vectors have been used successfully for in vitro and in vivo transduction of marker genes and genes involved in human diseases.

c. Retroviral and lentiviral vectors
In one embodiment the present invention involves the use of lentiviral vectors.

A large number of different retroviruses have been identified. Examples include: murine leukemia virus (MLV), human T-cell leukemia virus (HTLV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV). A detailed list of retroviruses may be found in Coffin et al. (1997) "Retroviruses", Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763.

Retroviruses are RNA viruses that replicate through an integrated DNA intermediate. Retroviral particles encapsidate two copies of the full-length viral RNA, each copy containing the complete genetic information needed for virus replication. Retroviruses possess a lipid envelope and use interactions between the virally encoded envelope protein that is embedded in the membrane and a cellular receptor to enter the host cells. Using the virally encoded enzyme reverse transcriptase, which is present in the virion, viral RNA is reverse transcribed into a DNA copy. This DNA copy is integrated into the host genome by integrase, another virally encoded enzyme. The integrated viral DNA is referred to as a provirus and becomes a permanent part of the host genome. The cellular transcriptional and translational machinery carries out expression of the viral genes. The host RNA polymerase II transcribes the provirus to generate RNA, and other cellular processes modify and transport the RNA out of the nucleus. A fraction of viral RNAs are spliced to allow expression of some genes whereas other viral RNAs remain full-length. The host translational machinery synthesizes and modifies the viral proteins. The newly synthesized viral proteins and the newly synthesized full-length viral RNAs are assembled together to form new viruses that bud out of the host cells.
Retroviruses may be broadly divided into two categories: namely, "simple" and "complex". Retroviruses may even be further divided into seven groups. Five of these groups represent retroviruses with oncogenic potential. The remaining two groups are the Antiviruses and the spumaviruses. A review of these retroviruses is presented in Coffin et al (1997) ibid.

The basic structure of retrovirus and lentivirus genomes share many common features such as a 5' LTR and a 3' LTR, between or within which are located a packaging signal to enable the genome to be packaged, a primer binding site, integration sites to enable integration into a host cell genome and gag, pol and env genes encoding the packaging components - these are polypeptides required for the assembly of viral particles. Lentiviruses have additional features, such as rev and RRE sequences in HIV, which enable the efficient export of RNA transcripts of the integrated provirus from the nucleus to the cytoplasm of an infected target cell.

In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. LTRs also serve as enhancer-promoter sequences and can control the expression of the viral genes.

The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

In a defective retroviral vector genome gag, pol and env may be absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.
In a typical retroviral vector of the present invention, at least part of one or more protein coding regions essential for replication may be removed from the virus. This makes the viral vector replication-defective.

Viral vectors for use in the invention may include but are not limited to integration defective retroviral vectors. Such a vector can be produced, for example, by packaging the vector with catalytically inactive integrase (such as an HIV integrase bearing the D64V mutation in the catalytic site; Naldini et al, Science 1996, and PNAS USA 1996, Leavitt et al. J Virol. 1996) or by deleting essential att sequences from the vector LTR (Nigthingale et al. Mol Ther 2006), or by a combination of the above. These modifications reduce integration to baseline level leaving unaffected the other steps of the transduction process (Naldini et al. Science 1996, Nigthingale et al. Mol Ther 2006, Vargas et al. Hum Gene Ther 2004, Yanez-Munoz et al. Nat Med 2006; Philippe et al. PNAS 2006).

Lentivirus vectors are part of a larger group of retroviral vectors. A detailed list of lentiviruses may be found in Coffin et al (1997) "Retroviruses" Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763). In brief, lentiviruses can be divided into primate and non-primate groups. Examples of primate lentiviruses include but are not limited to: the human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

The lentivirus family differs from retroviruses in that lentiviruses have the capability to infect both dividing and non-dividing cells.

A lentiviral vector, as used herein, is a vector which comprises at least one component part derivable from a lentivirus. Preferably, that component part is involved in the
biological mechanisms by which the vector infects cells, expresses genes or is replicated.

The lentiviral vector may be a "non-primate" vector, i.e., derived from a virus which does not primarily infect primates, especially humans.

The examples of non-primate lentivirus may be any member of the family of lentiviridae which does not naturally infect a primate and may include a feline immunodeficiency virus (FIV), a bovine immunodeficiency virus (BIV), a caprine arthritis encephalitis virus (CAEV), a Maedi visna virus (MW) or an equine infectious anaemia virus (EIAV).

d. Other Viral Vectors

Other viral vectors may be employed as constructs in the methods and compositions described here. Vectors derived from viruses such as vaccinia and herpesviruses may be employed.

5. Calcium Phosphate Co-Precipitation or DEAE-Dextran Treatment

In other preferred embodiments, polynucleotide is introduced to the cells using calcium phosphate co-precipitation. In another embodiment, the expression construct is delivered into the cell using DEAE-dextran followed by polyethylene glycol.

6. Direct Microinjection or Sonication Loading

Further embodiments include the introduction of the polynucleotide, polypeptide or polypeptide fragment by direct microinjection. Sonication loading may also be used.

7. Liposome Mediated Transfer
In a further embodiment, the polynucleotide, polypeptide or polypeptide fragment may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers. Also contemplated is a nucleic acid construct comprising a polynucleotide for use in the invention, wherein the construct is complexed with Lipofectamine (Gibco BRL).

A nucleic acid construct comprising a polynucleotide for use in the invention may be fully encapsulated in a lipid formulation e.g. to form a stable nucleic acid lipid particle (SNALP). A stable nucleic acid lipid particle can be a vesicle of lipids coating a reduced aqueous interior comprising a nucleic acid (e.g., ssDNA, dsDNA, ssRNA, dsRNA, siRNA, or a plasmid, including plasmids from which an interfering RNA is transcribed). Such particles include a stabilized plasmid-lipid particle (SPLP) that consists of a single plasmid encapsulated within a bilayer lipid vesicle.

In certain embodiments for use in the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA. In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1.

In certain embodiments, it is desirable to target the liposomes of the invention using targeting moieties that are specific to a cell type or tissue. Targeting of liposomes using a variety of targeting moieties, such as ligands, cell-surface receptors, glycoproteins, and monoclonal antibodies, has been previously described.

8. Cell-penetrating peptides
As described above a polypeptide or fragment may be "operably" or "functionally" linked to a cell-penetrating peptide sequence so that the cell-penetrating peptide sequence can direct import of the polypeptide or fragment comprising a cell-penetrating peptide into a cell.

**Cancers**

The present invention is useful in treating cancer.

Examples of types of cancer, include, but are not limited to, non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukemia (e.g., acute leukemia such as acute lymphocytic leukemia, acute myelocytic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, multiple myeloma), colon carcinoma, rectal carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, cervical cancer, testicular cancer, lung carcinoma, bladder carcinoma, melanoma, head and neck cancer, brain cancer, cancers of unknown primary site, neoplasms, cancers of the peripheral nervous system, cancers of the central nervous system, tumors (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, seminoma, embryonal carcinoma, Wilms' tumor, small cell lung carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, and retinoblastoma.

**Immune disorders**
In one embodiment the antagonist, polypeptide or fragment is for use in modulating an immune response to an allergen or transplant, or for use in treatment of autoimmune disease or inflammation.

In one embodiment the antagonist, polypeptide or fragment is for use in dampening or preventing an immune response to an allergen or transplant.

The term "allergen" is used to describe an antigen that elicits an unwanted immune hypersensitivity or allergic reaction.

An "immune response to an allergen" can include, but is not limited to immune hypersensitivity means a state of altered reactivity in which the body reacts with an exaggerated immune response to a foreign substance (allergen). There are four types of hypersensitivity reaction (Types I, II, III and IV). The first three are antibody-mediated; the fourth is mediated mainly by T cells and macrophages.

Type I, anaphylactic or immediate-type hypersensitivity is an allergic reaction provoked by re-exposure to an allergen. Exposure may be by ingestion, inhalation, injection or direct contact. The reaction is mediated by IgE antibodies and produced by the immediate release of histamine, arachidonate and derivatives by basophils and mast cells. This causes an inflammatory response leading to an immediate (seconds to minutes) reaction which may be followed by a late phase response or reaction, causing, for example, asthma, hay fever, systematic anaphylaxis or contact dermatitis.

An "immune response to a transplant" can include, but is not limited to, transplant rejection.

An "autoimmune disease" is understood to include, but not limited to myasthenia gravis, neuromyelitis optica, multiple sclerosis, Lambert-Eaton Myasthenic Syndrome (LEMS), peripheral neuropathy, Acquired Neuromyotonia, thyroiditis, Hashimoto's thyroiditis, insulitis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, insulin dependent diabetes mellitus, several forms of anemia (aplastic, hemolytic),
autoimmune hepatitis, thyroiditis, skleritis, uveitis, orchitis, idiopathic thrombocytopenic purpura, inflammatory bowel diseases (Crohn's disease, ulcerative colitis), Hashimoto's thyroiditis, Guillain-Barre syndrome, colitis, Grave's disease, pemphigus vulgaris, idiopathic myxedema, experimental autoimmune encephalomyelitis (EAE), autoimmune thrombocytopenia, sarcoidosis, experimental leishmaniasis, pernicious anemia, temporal arteritis, dermatitis herpetiformis, vitiligo, primary biliary cirrhosis, autoimmune oophoritis and orchitis, autoimmune disease of the adrenal gland, dermatomyositis, dermatomyositis, spondyloarthropathies (such as ankylosing spondylitis), Goodpasture's syndrome, glomerulonephritis, psoriasis, pemphigoid, idiopathic leukopenia, rheumatoid arthritis, juvenile arthritis, scleroderma and systemic sclerosis, Sjogren's syndrome, undifferentiated connective tissue syndrome, antiphospholipid syndrome, different forms of vasculitis (polyarteritis nodosa, allergic granulomatosis and angitis, Wegner's granulomatosis, Kawasaki disease, hypersensitivity vasculitis, Henoch-Schoenlein purpura, Behcet's Syndrome, Takayasu arteritis, Giant cell arteritis, Thrombangiitis obliterans), lupus erythematosus, polymyalgia rheumatica, essential (mixed) cryoglobulinemia, Psoriasis vulgaris and psoriatic arthritis, diffus fasciitis with or without eosinophilia, polymyositis and other idiopathic inflammatory myopathies, relapsing panniculitis, relapsing polychondritis, lymphomatomoid granulomatosis, erythema nodosum, Reiter's syndrome and different forms of inflammatory dermatitis and the like.

**Diagnostic and Prognostic Methods**

According to the eighth aspect of the present invention, there is provided a method of diagnosing cancer, said method comprising detecting the level of Eukaryotic Initiation Factor 6 (eIF6) polypeptide expression in a sample from subject. The level of eIF6 polypeptide expression can be determined using a number of different techniques which can detect expression at the RNA level or preferably at the polypeptide level. Preferably the expression level is detected at the polypeptide level.

For detection at the RNA level, RNA may be extracted from cells using RNA extraction techniques including, for example, using acid phenol/guanidine
isothiocyanate extraction (RNAzol B; Biogenesis), RNeasy RNA preparation kits (Qiagen) or PAXgene (PreAnalytix, Switzerland). Typical assay formats utilising ribonucleic acid hybridisation include nuclear run-on assays, RT-PCR, RNase protection assays (Melton et al., Nuc. Acids Res. 12:7035), Northern blotting and In Situ hybridization. Gene expression can also be detected by microarray analysis. Such techniques are well known in the art.

For detection at the polypeptide level, altered protein expression may also be detected by measuring the eIF6 polypeptides. This may be achieved by using molecules which bind to the eIF6 polypeptides. Suitable molecules/agents which bind either directly or indirectly to the polypeptides in order to detect the presence of the protein include naturally occurring molecules such as peptides and proteins, for example antibodies, or they may be synthetic molecules.

Antibodies for the eIF6 polypeptides may be derived from commercial sources or through techniques which are familiar to those skilled in the art. Methods for production of antibodies are known by those skilled in the art. If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing an epitope(s) from a polypeptide. Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an epitope from a polypeptide contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order to generate a larger immunogenic response, polypeptides or fragments thereof may be haptenised to another polypeptide for use as immunogens in animals or humans.

Monoclonal antibodies directed against epitopes in polypeptides can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels
of monoclonal antibodies produced against epitopes in the polypeptides of the invention can be screened for various properties; i.e., for isotype and epitope affinity.

An alternative technique involves screening phage display libraries where, for example the phage express scFv fragments on the surface of their coat with a large variety of complementarity determining regions (CDRs). This technique is well known in the art.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes whole antibodies, or fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and F(ab')2 fragments, as well as single chain antibodies (scFv).

Standard laboratory immunoassays such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunofluorescence assay (IFA), enzyme linked assay (EIA), luminescence immunoassay (LIA), immuno-precipitation, and other comparable techniques can be used to detect altered levels of eIF6 polypeptide expression compared to control sample(s).

The expressed polypeptides may also be analysed using mass-spectroscopy analysis techniques known in the art. (See for example: Ruedi Aebersold & Matthias Mann, review article Mass spectrometry-based proteomics, Nature 422, 198-207 (13 March 2003).) In particular, it is envisaged that eIF6 polypeptides may be immunopurified and then analysed by liquid chromatography electrospray ionisation tandem mass spectrometry (LS-ESI/MS) techniques known in the art. Such techniques may also be used to analyse phosphorylation of the polypeptides. (See for example: Blethrow JD, Tang C, Deng C, Krutchinsky AN (2007) Modular Mass Spectrometric Tool for Analysis of Composition and Phosphorylation of Protein Complexes. PLoS ONE 2(4): e358. doi:10.1371/journal.pone.0000358.)

The methods according to the eighth to tenth aspects of the present invention involve detecting the occurrence of phosphorylation of an amino acid in Eukaryotic Initiation Factor 6 (eIF6) in a sample from said subject, wherein the phosphorylation detected is
that of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5. The phosphorylation can be detected using antibodies specific for eIF6 having phosphorylated serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5. These antibodies can be generated as described above and then used in immunoassays as described above in order to detect phosphorylation. These techniques are well known in the art (see for example Yao Z, Seger R., Immunological detection of phosphorylation., Curr Protoc Cell Biol. 2001 May; Chapter 14:Unit 14.2.). Phosphorylation may also be detected using 2D-gel analysis e.g. as described in the Experimental Results section and shown in Figure 3D.

The diagnostic and prognostic methods may also employ monitoring the up- or down-regulation of the biomarkers of the present invention.

Methods of identifying further antagonists

The present invention provides methods of identifying an agent that prevents phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide, wherein the phosphorylation is of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

One such method comprises contacting a cell expressing Eukaryotic Initiation Factor 6 (eIF6) with an agent and detecting the occurrence of phosphorylation of amino acid in Eukaryotic Initiation Factor 6 (eIF6) polypeptide, wherein the phosphorylation is that of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

A person skilled in the art will appreciate that further such methods can be envisaged. For example cell-free translation systems can also be employed instead of cells. These systems may involve the use of cellular compartments, such as a membrane, cell envelope or cell wall.
The agent may be a naturally occurring macromolecule or a synthetic one such as a drug. The agent may be an inhibitor of translation.

The above methods may involve high-throughput screening techniques (which are known in the art) of available large-compound libraries or through known structure-based (de novo) ligand design methodologies (see e.g. Lenz GR, Nash HM, Jindal S 2000 Chemical ligands, genomics and drug discovery. Drag Discov Today 5:145-156).

It is also possible to design assays which develop molecules able to inhibit the activation of eIF6, and hence mimic the effect of phosphomutants. Specifically, one can design an antibody able to recognize phospho-eIF6, and generate recombinant phospho eIF6. It is thus possible screen for molecules that can elicit the release of the phosphate group from eIF6 or inhibit its phosphorylation, by combining technology in which we will for example preadsorb either anti-phospho-eIF6 antibodies or phospho-eIF6 to microwells, deploy combinatorial libraries, administer a second compound, and evaluate modulators of the phosphorylation and/or phosphomutants analogs; alternatively, by deploying non-phosphorylated eIF6, eIF6 phosphorylating kinase, a compound library and detecting anti-phospho eIF6.

The following Examples further illustrate, but do not limit, the invention.

**Materials and Methods**

*Experimental animals*

All experiments were approved by the Ethical Committee of San Raffaele and comply with E.C. regulations (IACUC authorization SK397).

eIF6<sup>−/−</sup> mice were generated as previously described (6), and backcrossed to C57BL6/N strain for a minimum of 8 generations.
eIF6<sup>+/−</sup>, p53<sup>−/−</sup> and Eµ-Myc transgenic mice were intercrossed to obtain the genotypic combinations. Mice were monitored daily for the tumor development until they died spontaneously or were sacrificed if showing evident signs of distress. Kaplan-Meier curve was used to examine the survival rate of all considered animals. Mice with tumors larger than 600 mm<sup>3</sup> were sacrificed; cervical, axillary and inguinal lymph nodes and spleens of these mice were recovered, weighted and used for further analysis.

8-week old CD1 athymic nude mice were used for detecting tumor growth after a subcutaneous (s.c.) injection of transformed MEFs cells. Mice with tumors larger than 600 mm<sup>3</sup> were sacrificed.

**Genotype analysis**

Genotyping of the offspring mice was detected by PCR using AmpliTaq Gold (Roche) according to the manufacturer's protocol. The PCR primers for genotyping of eIF6 and Eµ-Myc transgenic mice were previously reported (6), (C. Gorrini et al., Nature 448, 1063 (Aug 30, 2007)); p53 genotyping was performed using the specific primers: 5'-ACAGCGTGGTGGTACCTTAT-3' (wt allele), 5'-TATACTCAGAGCCGGCCT-3' (common primer) and 5'-CTATCAGGACATAGCGTTGG-3' (mutant allele). PCR products were resolved on 2% agarose gels.

**Primary Cell Culture**

Primary MEFs were isolated from 13.5 d.p.c. embryos as previously described (6). Primary B-lymphocytes were isolated from spleen and bone marrow of 4-week-old mice by labeling with CD45R(B220) microbeads and using an autoMACS separator (Milteny Biotec), according to manufacturer's instruction and (C. Gorrini et al., Nature 448, 1063 (Aug 30, 2007)). All the analyses were performed at least three times on different genetic backgrounds.

**Antibodies and reagents**

The following antibodies were used: rabbit polyclonal antibodies against eIF6 (27), c-Myc, Cleaved Caspase 3, RACK1, ERK1/2 (Cell Signaling), PKCβII, H-Ras (Santa
Cruz), PCNA, GATA1 (Abeam); mouse monoclonal antibodies against BrdU (Sigma), p53 (Cell Signaling), β-Actin (Sigma) and eIF6. LPS, IL4, 4-hydroxy-tamoxifen (OHT) and all powders and reagents were from Sigma.

**Histological staining, immunohistochemistry and In Situ Tunel Assay**

Immunohistochemical and histological analysis were performed on paraﬁne-embedded sections obtained from spleens of eIF6^{+/+}/Eμ-Myc tg and eIF6^{−/−}/Eμ-Myc tg mice and from tumors derived from nude mice. Staining with Hematoxylin-Eosin (H&E) was performed on all tissues for morphological analysis, while immunohistochemistry for the considered targets was done using the Vectastain Elite ABC kit (Vector), according to the manufacturer's instructions.

Apoptotic cells were identiﬁed on tumor sections using a commercially available In Situ Cell Death Detection Kit, AP (Roche), according to manufacturer's protocol.

**Flow Cytometry; apoptotic rate of primary B-lymphocytes**

Cell cycle rate was analyzed on freshly isolated B-lymphocytes from 4 weeks-old mice (eIF6^{+/+}/Eμ-Myc tg and eIF6^{−/−}/Eμ-Myc tg mice). These mice received 1 mg of BrdU (Sigma) by intraperitoneal (i.p.) injection 2 h before being sacriﬁced. Primary B-lymphocytes were recovered from spleen and bone marrow, ﬁxed in 95% ethanol, and labeled with FITC-conjugated anti-BrdU, using the BD Biosciences BrdU Flow Kit, following manufacturer's instructions.

B-lymphocytes development was examined by labeling speciﬁc surface markers with FITC-anti-mouse IgD, FITC-anti-mouse B220 and PE-anti-mouse IgM (BD Pharmigen). Nuclei were counterstained with 7-AAD marker. For both analyses, 10,000 events per tube were acquired by BD FACS CANTO II ﬂow cytometer and then analyzed using the FCS Express software (BD).

The apoptotic rate was also measured on the same cells. B-lymphocytes were ﬁxed in paraformaldehyde, penneabilised and stained using In Situ Cell Death Detection Kit, AP (Roche), following the manufacturer's instructions.
All the results were expressed as percentage and each experiment was done in triplicate.

Analysis of global and IRES-dependent translation rate

Global protein synthesis was measured on primary B-lymphocytes isolated from spleen and bone marrow of Eμ-Myc tg mice. Cells were seeded at sub-confluency and stimulated with LPS/IL4 overnight at 10 μg/ml and 500 U/mL, respectively. 35S-Methionine labeling was performed both on controls and stimulated cells as previously described (6), (S. Campaner et al., Nat Cell Biol. 2010 Jan;12(l):54-9; sup pp 1-14). Results, derived from the mean of four independent experiments, were normalized to total protein content and expressed as percentage of relative, untreated controls.

IRES-dependent translation was analysed on primary MEFs, wt and heterozygous for eIF6. MEFs were infected with a recombinant retrovirus expressing a puromycin resistance marker along with the 4-hydroxy-tamoxifen (OHT)-inducible Myc-oestrogen receptor chimaera MycER (S. Campaner et al., Nat Cell Biol. 2010 Jan;12(l):54-9; sup pp 1-14). Asynchronous and 48h starved MEFs were transfected with the HCV-IRES bicistronic vector, (12). After 24 h, cells were collected and Firefly and Renilla activities were quantified using the Glomax luminometer. Results were normalized on Renilla activity and derived from the mean of three experiments.

Transformation Analysis

Primary fibroblasts were infected at early passage with a retrovirus carrying DNp53 + oncogenic H-rasV12 as previously described (6). After 2 days these cells were also infected with other lentiviral and retroviral vectors. The lentiviral vectors used in this study express full-length wt eIF6, mutant eIF6S235A and GFP as internal control. Packaging plasmid VSV-G, PMDLg/pRRE, pREV and transfer vector pCCL-PPT-hPGK-pre used in this paper was previously described and full-length wt eIF6, mutant eIF6S235A and GFP cloning was performed as previously described (6). Foci were counted 2-3 weeks after infections and transformed cells were recovered for subcutaneous injection in nude mice (500,000 cells / mouse ).
**eIF6\(^{S235A}\) mutant**

The eIF6\(^{S235A}\) mutant used in the experiments is described in reference (5) at page 582. The amino acid sequence of this mutant is provided in Figure 7 and the nucleotide sequence of this mutant is provided in Figure 8.

**Kinase assay**

Proteins from Eu-myc tumoral lymphonodes were extracted in lysis buffer (50mM TrisHCl, 150 mM NaCl, 0.1 Tween20, 1X Protease Inhibitors (Sigma), 1 mM NaF, 10 mM \(\beta\)-glycerophosphate), clarified by centrifugation and protein concentration was quantified by the BiChinonic Acid (BCA) protein assay (EuroClone). For PKC assays, lysates were subjected to pre clearing with Protein G (GE Healthcare) for 2 hours at 4°C, and pre cleared protein extracts were incubated with antibody against PKC\(\beta\)II (Santa Cruz Biotechnology) overnight at 4°C using constant rotation. Immunoprecipitation was performed with protein G for 2 hours. Beads were washed three times and resuspended in lysis buffer. The kinase assay was performed by adding 3 \(\mu\)g of eIF6 recombinant protein, 3 \(\mu\)g of recombinant RACK1 (5) or 5 \(\mu\)g of GST-MARCKS (Myristoylated Alanine-Rich C-Kinase Substrate (J. W. Soh, I. B. Weinstein, J Biol Chem 278, 34709 (Sep 5, 2003)) in the PKC\(\beta\)I specific buffer (100 mM MgCl\(_2\), 10 mM CaCl\(_2\), lipid mixture) either in the presence or absence of immunoprecipitated PKC\(\beta\)I. mTOR kinase assay was instead performed by adding either 3 \(\mu\)g of eIF6 or 11 \(\mu\)g of GST-4E-BP1 recombinant protein to immunoprecipitated mTOR in a specific buffer (10 mM HEPES, 50 mM \(\beta\)-glycerophosphate, 150 mM NaCl). The negative control was an immunoprecipitate obtained with irrelevant antibodies.

4 \(\mu\)Ci of \(\gamma\)32-ATP (Perkin Elmer) were added to each sample. The reaction was run at 30 °C for 1 h and terminated by adding one volume of sample buffer. The samples were boiled 5 min, separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. Autoradiography was performed on dried gels.
Recombinant eIF6 was prepared in *E. coli*. N-terminal histidine tagged human eIF6 was co-expressed with a mixture of molecular chaperones (A. de Marco et al., BMC Biotechnol 7, 32 (Jun 12, 2007)). Protein production was induced at 16 °C overnight and the recombinant protein was first recovered by metal affinity chromatography. Monomeric active eIF6 was separated by inactive, dimeric and aggregated eIF6 by gel filtration. GST-4E-BP1 was a kind gift of Dr. C. Proud (University of Southampton, UK).

Two dimensional (2D) gel electrophoresis

Tumor samples were examined in 2D gel electrophoresis. Samples were lysed in RIPA buffer and proteins were TCA precipitated. Pellets were resuspended in 2D buffer (7 M Urea, 2 M Thiourea, 50 mM DTT and 4% CHAPS) and 100 µg of proteins were isoelectrofocused. The first dimension was performed on ReadyStrip IPG Strips (pH 3.9-5.1; Biorad) For the reduction/alkylation step, the strips were incubated with re-equilibration buffer (50 mM TrisHCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromphenol blue) plus DTT and re-equilibration buffer plus iodoacetamide, respectively.

Then, the strips were subjected to SDS/PAGE for the second dimension. Proteins were transferred on PVDF membrane and subsequently incubated with eIF6 monoclonal antibodies. The signal was detected with an anti-mouse secondary antibody and ECL substrate kit (GE Healthcare).

Statistical analysis

To assess the statistical significance of the results, each experiment was repeated at least three times; means and standard deviations between different experiments were calculated. Statistical *P*-values obtained by Student *t*-test were also indicated: three asterisks *** for *P*-values less than 0.001, two asterisks ** for *P*-values less than 0.01 and one asterisk * for *P*-values less than 0.05.

Kaplan-Meier curves were validated by the Log-rank test.
EXPERIMENTAL RESULTS

To understand how eIF6 affects tumorigenesis and tumor growth in vivo, we selected the Eµ-Myc lymphoma model. In this model, expression of the Myc oncogene in the B cell lineage drives a lethal lymphoma with a median survival of three months (7), associated with increased translation rate (8) and upregulation of eIF4E (9). Myc-induced lymphomagenesis is reduced by eIF4F complex inhibition (10, 11) and ribosomal protein rpL24 haploinsufficiency (12). Here, we can assess whether eIF6 is downstream of the Myc oncogene, if it acts as a rate-limiting factor in tumorigenesis in a mTORcl dependent fashion or as an independent modulator of 80S formation. We crossed eIF6+/- mice to Eµ-Myc/eIF6+/- to generate Eµ-Myc/eIF6+/- and Eµ-Myc/eIF6+/-, and analyzed Myc-induced lymphomagenesis. Myc-induced lymphomagenesis results in a 100% increase of eIF6 levels both in wild type and eIF6+/- mice (Fig. 1A). The upregulation of eIF6 in Myc-driven lymphomas is higher in wt mice than in eIF6+/- mice, indicating that gene dosage represents a strong determinant of eIF6 expression. We overexpressed Myc in primary fibroblasts to establish whether eIF6 upregulation was directly regulated by Myc. Transient Myc overexpression did not change eIF6 levels, indicating that eIF6 is not a direct Myc target. This is different from eIF4E (Fig. 1A) which is transcriptionally regulated by Myc (9) and is rate-limiting in lymphomagenesis (10, 11). We then compared survival of Eµ-Myc/eIF6+/- and Eµ-Myc/eIF6+/- transgenic mice. Eµ-Myc/eIF6+/- survived longer than Eµ-Myc/eIF6+/- wt mice (Fig. 1C). At 200 days, when virtually all Eµ-Myc/eIF6+/- mice had succumbed to the disease, 40% of Eµ-Myc/eIF6+/- mice were still alive. One early sign of lymphomagenesis is the enlargement of the spleen, due to extramedullary hematopoiesis, and of the lymph nodes due to expansion of tumor cells (13). At two months, Eµ-Myc/eIF6+/- mice had little splenomegaly and lymph node enlargement, suggesting a retard in tumorigenesis (Fig. 1C). The reduction in splenomegaly observed in Eµ-Myc/eIF6+/- mice was due to reduced extramedullary hemopoiesis, as shown by lower expression of hemopoietic GATA-1 transcription factor (Supplementary Figure 1). However, just one month later (90 days of age), the size of lymph nodes and spleen was identical in Eµ-Myc/eIF6+/- wt and Eµ-Myc/eIF6+/- (Fig. ID). Summarizing, although splenomegaly of Eµ-Myc/eIF6+/- was
delayed only 30 days compared to wt mice, the increased mean survival was highly appreciable, suggesting that eIF6 haploinsufficiency could limit both tumor onset and growth (see below).

p53 deletion accelerates E\(_\mu\)-Myc-driven lymphomagenesis by suppressing Myc-induced apoptosis (14). It is important to define whether p53 is required for protection from tumorigenesis driven by eIF6 haploinsufficiency because haploinsufficiency of ribosomal proteins causes "ribosomal stress" and p53 induction (15), and a 20-30\% pool of eIF6 is necessary for biogenesis of the ribosomal 60S subunits (16). In general, phenotypes due to ribosomal stress are reverted by p53 deletion (15), as in the case of rpl24 haploinsufficiency (17). The protective effect of eIF6 heterozygosity was observed also in the p53 null\(^{-}\)-Myc transgenic background (Fig. ID). Thus, the protective effect of eIF6 haploinsufficiency is independent from induction of ribosomal stress and p53. These data suggest that eIF6 role in lymphomagenesis is linked to a cytoplasmic function of eIF6 unrelated to ribosome biogenesis.

The protective effect of eIF6 haploinsufficiency, both in the presence or absence of p53 may suggest that, in lymphomagenesis, eIF6 acts both as a proliferation factor and as a pro-survival factor downstream of p53. To sort out this question, we purified pre-B cells from pre-tumoral 4-week old mice and analyzed cell cycle distribution, maturation, and apoptosis. As previously reported (8), pre-B cells from E\(_\mu\)-Myc transgenics had a higher percentage of S-phase cells as compared to non-transgenics (Fig. 2A). In this context, a slight modulatory effect of eIF6 heterozygosity was observed, namely a reduction in the percentage of S-phase cells and an increase in Go/G1 cells (Fig. 2A). Early activation of Myc leads also to a strong increase in apoptosis which was, however, not affected by eIF6 haploinsufficiency (Fig. 2B). Finally, in E\(_\mu\)-Myc transgenics, pre-B cell development is affected by a reduction of mature B cells (18). We did not see a significant change in the percentages of mature and immature B cells in E\(_\mu\)-Myc transgenic wt mice, compared to E\(_\mu\)-Myc/eIF6\(^{+/+}\) mice (Supplementary Figure 2). Taken together, data suggest that eIF6 acts as a node translation factor in cell cycle progression, but plays little or no role in cell differentiation. The fact that the protective effect of eIF6 haploinsufficiency is visible
also in p53 mutants is in line with the proposed role of p53 in the expansion (proliferation) of pre-B tumor (stem) cells (19).

In mouse fibroblasts, eIF6 heterozygosity impairs growth factor and PMA stimulated translation, but does not change the rate of translation of serum-free cells (6). Myc oncogenesis increases the translational rate (8, 12). We evaluated how eIF6 haploinsufficiency impacts translation of purified pre-B cells, by methionine incorporation (Fig. 2C). We evaluated basal translation and stimulation of translation by overnight treatment with LPS/interleukin 4. Data are presented in Fig. 2C. Briefly, Myc oncogenesis caused an increase in methionine incorporation. eIF6 haploinsufficiency reduced both the increase of Myc-induced translation, and LPS/interleukin 4 induced translation (12). In conclusion, eIF6 is rate-limiting for translation upon conditions of increased growth, and by doing so it delays cell cycle progression.

To gain an insight on how the translation activity of eIF6 could be involved in the tumorigenesis process, we exploited its activation properties. RACK1, a ribosomal scaffold protein and intracellular PKC receptor RACK1 (20, 21), interacts with eIF6 (5); PKCβII is the most effective PKC isoform binding RACK1 (20). Ser235 of eIF6 is phosphorylated in vivo upon pharmacological stimulation of PKC (5), and is found in phosphorylated form in cycling cells (22). Consequently, we examined whether lymphomas express PKCβII and whether this expression was linked to the phosphorylation of eIF6. Individual tumors were analysed for the presence of PKCpII, Ras, and ERK activation. Of these, PKCβiI was expressed in all lymphomas (Fig. 3A and not shown), whereas Ras and active ERK were present only in a subset of samples, irrespective of their genotype (not shown). We then immunoprecipitated PKCpII from lymphomas and assayed the capability to phosphorylate eIF6. PKCβII immunoprecipitated from lymphomas phosphorylated eIF6, as well as its control substrate MARCKS1 (Fig. 3B). In this same condition, eIF6 was not phosphorylated by mTOR kinase (Fig. 3C). Next, we assayed by 2D-gel analysis whether endogenous eIF6 was phosphorylated in lymphomas; eIF6 from lymphomas showed 3 spots
compatible with phosphorylation patterns (Fig. 3D). These data led us to analyse the impact of Ser235 of eIF6 in tumorigenesis and tumor growth.

First, we re-evaluated in primary fibroblasts the transformation efficiency of wt eIF6, compared to eIF6<sup>S<sup>235A</sup></sup> (Fig. 4A; Figure 11). As previously reported, eIF6<sup>S<sup>235A</sup></sup> fibroblasts transformed at lower efficiency than wt cells (6). Similarly to what we saw in Myc-driven lymphomagenesis, eIF6<sup>S<sup>235A</sup></sup> transformed cells had lower levels of eIF6 than wt cells (Fig. 4B). We then proceeded to reconstitution experiments. Transformed primary fibroblasts, with DNp53 + H-ras<sup>V12</sup> were reconstituted as it follows: a) het cells with either eIF6 wt or eIF6<sup>S<sup>235A</sup></sup>, and b) wt fibroblasts with either eIF6<sup>S<sup>235A</sup></sup> or control GFP. Analysis of the levels of eIF6 in each population indicated that each construct was mildly overexpressed (Figure 12). In summary, reconstitution of het fibroblasts with wt eIF6 increased their transformation rate almost 300%, whereas the reconstitution with eIF6<sup>S<sup>235A</sup></sup> led to an inhibition of the transformation rate of 50% (Fig. 4C; Figure 13). Similarly, wt eIF6 fibroblasts infected with a lentiviral vector expressing eIF6<sup>S<sup>235A</sup></sup> showed a reduction of the transformation rate of 70% (Fig. 4C; Figure 13).

To establish if transformed eIF6<sup>S<sup>235A</sup></sup> cells with reduced eIF6 levels, or with mutant Ser235, grew as well as normal cells, we collected transformed cells from the previous experiments and injected equal amounts in nude mice. We found that eIF6 wt transformed fibroblasts grew faster than the eIF6<sup>S<sup>235A</sup></sup> heterozygous (het) counterparts (Fig. 4D). Furthermore, the expression of eIF6<sup>S<sup>235A</sup></sup> reduced the growth of tumors in the context of both wt eIF6 (Fig. 4E) and het eIF6 transformed cells (Fig. 4F). In eIF6<sup>S<sup>235A</sup></sup> transformed cells, the re-expression of wt eIF6 accelerated tumor growth (Fig. 4F). Analysis of histology, S-phase entry, apoptosis, and angiogenesis in tumor xenografts confirmed that tumors expressing wild type levels of eIF6 had more BrdU labeling, a marker of S-phase entry, than eIF6<sup>S<sup>235A</sup></sup> tumors (Fig. 4G, H). With respect to wt tumors, eIF6<sup>S<sup>235A</sup></sup> tumors also presented reduced cellularity (Fig. 4H, Figure 14). We did not detect differences in the apoptotic rate of wt and eIF6<sup>S<sup>235A</sup></sup> tumors, as determined by TUNEL and caspase 3 stainings, with both tumors presenting sporadic clusters of
apoptotic cells in necrotic or infiltrated areas (Figure 14). Thus, eIF6 levels restricted proliferation also in transformed cells.

We have presented evidence that eIF6 activity is rate-limiting in tumorigenesis and tumor growth. Several initiation factors are required for translational control (1). Of these, only eIF4E, under the control of mTORc1, affects tumorigenesis and tumor growth, in vivo (10, 11, 23). eIF6 is therefore the second rate-limiting controller of initiation of translation, able to affect tumorigenesis and tumor growth. In addition, differently from eIF4E, regulation of eIF6 activity is independent from Myc and mTOR, and acts downstream of 48S formation, controlling 60S availability and 80S formation. The model of serine 235 activation of eIF6 is based on RACK1/PKC mediated phosphorylation, by RACK1-PKCβn. Some evidence suggests that this model may be relevant in B-cells: PKCβII is upregulated in human lymphomas (24, 25), and PKCp is necessary for B-cell activation (26). The mechanism may not be totally restricted to B-cells, because in HEK293s, inhibition of PKCβII activity reduces translation without affecting mTORc1 targets (21). We suggest that the independent mode of eIF6 activity regulation from eIF4E, associated with its importance in regulating tumor growth, broadens the potential of inhibiting the translational machinery in therapeutical targeting, especially where eIF4E inhibition is not effective.

a-Evaluation of toxicity of eIF6 inhibition

A systematic analysis was performed in order to define metabolic changes that are related to the inhibition of eIF6 activity, in conditions in which eIF6 inhibition leads to a reduction of, for instance, tumor growth. First, analysis of blood cell levels of mice heterozygous for eIF6 did not show alterations in cell numbers, with the exception of a slight non-pathogenic reduction of platelets (Fig. 15). No pathological alterations are therefore envisaged by eIF6 inhibition showing that the inhibition of eIF6 phosphorylation is indeed suitable as a therapy.

Biomarkers
We performed gene expression studies aimed at the definition of biomarkers, which may be altered through the blocking of eIF6. Haploinsufficiency of eIF6 mimics administration of eIF6Ser235Ala and/or cannot be rescued by the administration of eIF6Ser235Ala (6). The gene changes that we observed in conditions of eIF6 haploinsufficiency can be summarized as it follows: 1 - reduction of the expression of the genes that act in the pathways of cell cycle progression, hence validating the crucial role that eIF6 plays in this process; 2 - increase in the expression of enzymes involved in gluconeogenesis, accompanied by the reduction of enzymes involved in glycolysis. These changes, taken together further indicate that eIF6 activity acts at the crossroad between cell cycle progression and nutrient sensing. Fig. 16 shows the main pathways altered by eIF6 inhibition. To look at gene changes that occur upon inhibition of eIF6 activity, mRNA was isolated from wt and eIF6 het mice, and analyzed by Affymetrix-based gene expression microarray. Pooled RNAs (wt, n=3; het= 3) were used for microarray analysis using the one-cycle cDNA synthesis GeneChip kit and hybridized to GeneChip® Mouse Expression Set 430 chips, according to the manufacturer's instructions. For normalization, total RNA was extracted from cells using Trizol and further purified through RNeasy mini-elute columns (QIAGEN). RNA quantity and quality were determined using a bioanalyzer (Agilent Technologies).

Additionally we have evidence for the modulation of a cluster of putative miR467e targets, and thus miR467e administration may mimic the effects of eIF6 inhibition/phosphomutant. miR467e derives from a "GO" bioinformatic analysis (Gene Ontology) of the data (Fig. 17 and Fig. 18). These genes may be relevant biomarkers which can be affected by eIF6, and represent a specific signature of eIF6 inhibition.

We then performed validation of some of these changes on multiple samples (n=3), confirming that all the changes were fully penetrant (Fig. 19).

References and Notes
All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.
CLAIMS

1. An antagonist of phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in medicine, wherein the antagonist antagonises phosphorylation of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

2. An isolated Eukaryotic Initiation Factor 6 (eIF6) polypeptide for use in medicine, wherein the amino acid serine at the position corresponding to position 235, 230 and/or 243 of the sequence shown in Figure 5 is replaced with another amino acid.

3. An isolated Eukaryotic Initiation Factor 6 (eIF6) fragment for use in medicine, wherein the fragment is a fragment of the polypeptide defined in claim 2 and wherein the fragment comprises the amino acid of the polypeptide which is replaced with another amino acid.

4. An antagonist as defined in claim 1, an isolated polypeptide as defined in claim 2 or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in claim 3, wherein the antagonist, polypeptide or fragment is for use in modulating cellular translation in a subject or for use in modulating the immune system or for use in the treatment of proliferative disorders.

5. An antagonist as defined in claim 1, an isolated polypeptide as defined in claim 2 or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in claim 3, wherein the antagonist, polypeptide or fragment is for use in the treatment of neoplastic disease, adipogenesis disorders or liver disorders.

6. An antagonist as defined in claim 1, an isolated polypeptide as defined in claim 2 or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in claim 3, wherein the antagonist, polypeptide or fragment is for use in modulating an immune response to an allergen or transplant, or for use in treatment of autoimmune disease or inflammation.
7. An antagonist as defined in claim 1, an isolated polypeptide as defined in claim 2 or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in claim 3, wherein the antagonist, polypeptide or fragment is for use in treating cancer, type II diabetes, liver failure, obesity or Shwachman-Diamond syndrome.

8. An isolated polynucleotide for use in medicine, wherein the polynucleotide comprises a nucleotide sequence encoding the polypeptide defined in claim 2.

9. An isolated polynucleotide for use in medicine, wherein the polynucleotide comprises a nucleotide sequence encoding the Eukaryotic Initiation Factor 6 (eIF6) fragment defined in claim 3.

10. An isolated polynucleotide as defined in claim 8 or 9, wherein the polynucleotide is for use in modulating cellular translation in a subject or for modulating the immune system or for use in the treatment of proliferative disorders.

11. An isolated polynucleotide as defined in claim 8 or 9, wherein the polynucleotide is for use in the treatment of neoplastic disease, adipogenesis disorders or liver disorders.

12. An isolated polynucleotide as defined in claim 8 or 9, wherein the polynucleotide is for use in modulating an immune response to an allergen or transplant, or for use in treatment of autoimmune disease or inflammation.

13. An isolated polynucleotide as defined in claim 8 or 9, wherein the polynucleotide is for use in treating cancer, type II diabetes, liver failure, obesity or Shwachman-Diamond syndrome.

14. An isolated polynucleotide for use according to any one of claims 8-13, wherein the polynucleotide is contained in a gene transfer vector.

15. An isolated polynucleotide for use according to claim 14, wherein the polynucleotide is contained in a viral vector.
16. An isolated polynucleotide for use according to claim 15, wherein the polynucleotide is contained in a lentiviral vector.

17. An isolated polynucleotide for use according to claim 15 or 16, wherein the vector is in the form of an integrated provirus.

18. A pharmaceutical composition comprising:
   (i) an antagonist as defined in claim 1, an isolated polypeptide as defined in claim 2 or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in claim 3,
   (ii) a liposome comprising an antagonist as defined in claim 1, an isolated polypeptide as defined in claim 2 or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in claim 3, or
   (iii) a cell comprising an antagonist as defined in claim 1, an isolated polypeptide as defined in claim 2 or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in claim 3, and a pharmaceutically acceptable carrier, diluent or excipient.

19. A pharmaceutical composition comprising:
   (i) an isolated polynucleotide as defined in claim 8 or 9,
   (ii) a liposome comprising an isolated polynucleotide as defined in claim 8 or 9,
   (iii) a vector as defined in any of any of claims 14 to 16,
   (iv) a viral particle obtainable from the vector of any of claims 15 to 17, or
   (v) a cell comprising an isolated polynucleotide as defined in claim 8 or 9, and a pharmaceutically acceptable carrier, diluent or excipient.

20. A method of diagnosing cancer, said method comprising detecting the level of Eukaryotic Initiation Factor 6 (eIF6) polypeptide expression in a sample from subject, the method further comprising detecting the occurrence of phosphorylation of an amino acid in said polypeptide in the sample, wherein
the phosphorylation detected is that of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

21. A method of detecting remission of a tumour after therapy, said method comprising detecting the level of Eukaryotic Initiation Factor 6 (eIF6) polypeptide expression in a sample from subject, the method further comprising detecting the occurrence of phosphorylation of an amino acid in said polypeptide in the sample, wherein the phosphorylation detected is that of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

22. A prognostic method for determining whether a subject will be susceptible to treatment with an antagonist, polypeptide, fragment or polynucleotide for use according to any one of claims 1-17 or a pharmaceutical composition according to claim 18 or 19, said method comprising detecting the occurrence of phosphorylation of an amino acid in Eukaryotic Initiation Factor 6 (eIF6) in a sample from said subject, wherein the phosphorylation detected is that of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.

23. An *ex vivo* method of antagonising phosphorylation of Eukaryotic Initiation Factor 6 (eIF6), comprising contacting a cell or part of an organism with an antagonist as defined in claim 1, an isolated polypeptide as defined in claim 2 or an isolated Eukaryotic Initiation Factor 6 (eIF6) fragment as defined in claim 3.

24. An *ex vivo* method of antagonising phosphorylation of Eukaryotic Initiation Factor 6 (eIF6), comprising contacting a cell or part of an organism with an isolated polynucleotide as defined in any one of claims 8 to 17.
25. A method of identifying an agent that prevents phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide, wherein the phosphorylation is of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5, the method comprising contacting Eukaryotic Initiation Factor 6 (eIF6) with said agent and detecting the occurrence of phosphorylation of Eukaryotic Initiation Factor 6 (eIF6), wherein the phosphorylation is of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.


27. A method of identifying an agent that promotes de-phosphorylation of Eukaryotic Initiation Factor 6 (eIF6) polypeptide, wherein the de-phosphorylation is of an amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5, the method comprising contacting phosphorylated Eukaryotic Initiation Factor 6 (eIF6) with said agent and detecting the occurrence of de-phosphorylation of Eukaryotic Initiation Factor 6 (eIF6), wherein the de-phosphorylation is of the amino acid serine at the position corresponding to position 235, 230 and/or 243 in the polypeptide shown in Figure 5.
FIGURE 5

Amino acid sequence of an elF6 polypeptide - SEQ ID NO. 1

MAVRASFENNCEIGCFAKLTNTYCLVAIGGSENFYSVFEGELSDTIPVVHASIA
GCRIRRMCVGNRHGLLYPNNTTDQELQHIRNSLPDTVQIRQVEERLSALGNV
TTCDNYVALVHPDLDRETEILADVKLKEVFRTVQADQNVLVGSYCVFNSQG
GLVHPKTSIEDQDELSSLLQVPLVAGTVNRGEVIAAGMVVNDWCAFCGLDT
TSTELSVVESVFKLNEAQPSTIATSMRDSLIDSRT
FIGURE 6

Nucleotide sequence of an eIF6 polypeptide - SEQ ID NO. 2

GGCACCGAGTGCGAGCTTTGTTACTGTGTACTTGGCTGCATGCGGCTCCGA
GCTTGTTCCGAGACACACTGAGATCGGCTCCTTTGGCCAAGCTCAACAA
CACCTACTGTCCTGGTACCGATCGGAGCTCACAAACTTACAGTGTG
TCGAGGGCGAGCTCTCCGATACCACTCCCGGTGCTGACGCTCCTATGCC
GGCTGCCCAGCATTACGAGGCGATGCTGTGAGGCCGAGGCACTTCTCTC
GGTACCAACAATACACCCGGACCGTGTCAAACATGATCAGGCTGGCGTT
GGCGGAGAACAGAAGGATAGGTGCTGAAAGGAAAGCTCAGGCTTT
TCAGCAACAGACAGTGGCCGACAGGAGTCTAGTAGGAAAGCTAGTCTTT
CAGCAATCAAGGAGGGCTGCTGATCCCAAGACATTCAATGAGAACAG
ATGAGCTGTCCTCTCCTTTCTCAGAATCCCCCTTGTGGCGGGCACTGGAAC
GACGAGTGAAGTTGCTGGTCTGGATGCTGAAAGCTAGTGCTGCC
TTCTGAGGCTGGACACACACACACAGAAGCTGCTGAGGCTGGAGAGTTG
CTTCAAGCTGAAATGCGCCAGCCACCCATGACCCACATGCTGGGCCGG
ATTTCTCTAGCACACCTCACCTAGTGATCTGCCAAGTGTTCCTATGGG
CTTCTGGCTGTGGACTGTCAGGGCACCACCTTCTCCACACATTGCCGCTGTA
CCGATGCTGCAGGAGGCTGGACAGAAGCTCACTGGACTGGAGGGCT
GGCACCACCAACCTTTCCACCTGTGCTTAATGCCCTGATCATCTATCAT
GCAAAAAACCTGGCTCTTTGTGGCTGGCAGCGCCTTGTGGCTGCCTGGCTT
GGGGTGTGTCTTCAGTCTGTCCTGGCCACACCATTTAAAGTGCAAGTTCCTC
CGGAAAAAABBABBBBBBBBBBBBBBBBBBBBBBBBBBBBBA
AAAAAAAA
FIGURE 7

Amino acid sequence of a Ser235Ala eIF6 mutant polypeptide - SEQ ID NO. 3

MAVRASFENNCEIGCFAKLNTYCLVAIGGSNFYSVFEGELSDTIPVVHASIA
GCR1IGRMCVGVRHGCLLVPNNTTDQELQHIIRNSLPDTVQRKREEERLALGNV
TTCDYVALVHPDLDRETEEIADVLKVEVFRQTVADQVLVGSYCVFSNQG
GLVHPKTSEDQDELSSLQVPLVAGTVNRGSEVIAAGMVVNDWCAFCGLDT
TSTELSVVESVFKLTEAQPSTIATAMRDSLDSLT
FIGURE 8

Nucleotide sequence of the Ser235Ala eIF6 mutant polypeptide - SEQ ID NO. 4

GGCAGGAGGTGGAGGCTGGATCTGTTATGTTAATGCTTGCCCATATGGAGGTTCGA
GCTTGTTGAGAACAAGCTATGAGATCGGCTGCTTGGCAAAGTCACCACA
CACCTACTGTCTGTATACGATACGGAAATGCTAGACTATGCTAGG
TCGAGGACGGCAGTCTCTCAGAATCCATCCAATGACACAGGTAGATGTCAGGGG
GGAGTAGGAGGAAGAAAGAATTCTGGCAGATGTCCTCAAGTGGGAAGTC
TTCAGACAGAAGTGGCGAGACCAGTGCTAGTAGGAAAGCTAGTGTCTTT
CAGCAATCGGAGGAGGTGTGGATCGATCACCAGATTTAGAGAAGG
ATGAGCTGTCCCTCTTCTTTCAAGTGCACCCTTGGCCGGCAGTGAACC
GAGGCGAGTGGTGATGCTGTGGATGTTGATTGTAATGACTGGTGCTCC
TTCTGTGCGCTGACAAACACAGACAGTCAGTCTGCTGAGTAGGGT
CTTCAAGCTGAATGAGCCAGCTAGCAGGTACGAGTATTCAGAGTGGT
CTCCTGTGCTGGACTGTGGGCAACATCCTTCCACATCCCAGAATCTGTA
CCGAGTGCTGGGACAGGAGTGGCAGAGACAGCTACTGGGAGAGGGGCC
GGCACCACCAACCTTTTCCACTGCTGTATTACGCTGAGATCATATAC
GCACAAAAATCTGCTGGTGCTGCTGGGCAGGCCCTGGGTGCTGGTGCTGGCT
GGGCTTTTGTGCTTCTGCTCTGTGGTGCACCCCAATTAAGTGCAGTTTTCTC
CGGAAAAA/AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAA
Figure 11

A

Figure 12
Figure 13

A

Figure 14

H&E

TUNEL

CD8

CD4
# Means, standard deviation and p-values calculated by a linear model (parameter ~ genotype * sex)

<table>
<thead>
<tr>
<th></th>
<th>eIF6</th>
<th>female</th>
<th></th>
<th>male</th>
<th></th>
<th>ANOVA</th>
<th>ANOVA</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>control</td>
<td>mutant</td>
<td>control</td>
<td>mutant</td>
<td>genotype</td>
<td>sex</td>
<td>genotype sex</td>
</tr>
<tr>
<td>n=10</td>
<td>n=10</td>
<td></td>
<td></td>
<td>n=10</td>
<td>n=9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mean ± sd</td>
<td>mean ± sd</td>
<td>mean ± sd</td>
<td>mean ± sd</td>
<td>p-value</td>
<td>p-value</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>WBC [10^9/mm³]</td>
<td>7.43 ± 0.93</td>
<td>6.88 ± 1.54</td>
<td>9.86 ± 1.43</td>
<td>8.72 ± 1.89</td>
<td>0.083</td>
<td>&lt; 0.001</td>
<td>0.538</td>
<td></td>
</tr>
<tr>
<td>RBC [Mio/mm³]</td>
<td>10.66 ± 0.15</td>
<td>10.61 ± 0.16</td>
<td>10.87 ± 0.35</td>
<td>10.95 ± 0.21</td>
<td>0.859</td>
<td>0.001</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>PLT [10^9/mm³]</td>
<td>1234.9 ± 83.33</td>
<td>1155.2 ± 64.84</td>
<td>1468.8 ± 92.23</td>
<td>1327.22 ± 113.15</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.287</td>
<td></td>
</tr>
<tr>
<td>HGB [g/dl]</td>
<td>15.39 ± 0.37</td>
<td>15.44 ± 0.3</td>
<td>15.71 ± 0.41</td>
<td>15.79 ± 0.2</td>
<td>0.551</td>
<td>0.004</td>
<td>0.894</td>
<td></td>
</tr>
<tr>
<td>HCT [%]</td>
<td>55.57 ± 1.03</td>
<td>55.51 ± 1.19</td>
<td>55.88 ± 1.88</td>
<td>55.73 ± 0.81</td>
<td>0.806</td>
<td>0.527</td>
<td>0.918</td>
<td></td>
</tr>
<tr>
<td>MCV [fl]</td>
<td>52 ± 0.47</td>
<td>52.5 ± 0.85</td>
<td>51.5 ± 0.53</td>
<td>50.89 ± 0.6</td>
<td>0.785</td>
<td>&lt; 0.001</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>MCH [pg]</td>
<td>14.45 ± 0.24</td>
<td>14.54 ± 0.2</td>
<td>14.46 ± 0.3</td>
<td>14.43 ± 0.26</td>
<td>0.698</td>
<td>0.554</td>
<td>0.476</td>
<td></td>
</tr>
<tr>
<td>MCHC [g/dl]</td>
<td>27.7 ± 0.28</td>
<td>27.77 ± 0.26</td>
<td>28.15 ± 0.62</td>
<td>28.32 ± 0.3</td>
<td>0.347</td>
<td>&lt; 0.001</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>RDW [%]</td>
<td>13.43 ± 0.15</td>
<td>13.27 ± 0.25</td>
<td>13.06 ± 0.24</td>
<td>13.17 ± 0.13</td>
<td>0.681</td>
<td>0.001</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>MPV [fl]</td>
<td>5.04 ± 0.05</td>
<td>5.06 ± 0.1</td>
<td>4.98 ± 0.06</td>
<td>4.97 ± 0.05</td>
<td>0.88</td>
<td>0.001</td>
<td>0.453</td>
<td></td>
</tr>
</tbody>
</table>

Figure 15
<table>
<thead>
<tr>
<th>Term</th>
<th>Count</th>
<th>Genes</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell cycle</td>
<td>47</td>
<td>***</td>
<td>2.01E-23</td>
</tr>
<tr>
<td>DNA packaging</td>
<td>18</td>
<td>Asf1b,Cdca2,Cenpa,Hist1h1a,Hist1h1b,Hist1h2an,Hist1h2bc,Hist1h3a,Hist1h3d,Hist2h3b,Ncapd2,Ncapg2,Nusap1,Smc2,Smc4,Tgm1,Top2a,Trdn</td>
<td>3.31E-11</td>
</tr>
<tr>
<td>cholesterol biosynthetic process</td>
<td>9</td>
<td>Dhcr7,Fdft1,Fdps,Hmgcr,Hmgcs1,Lss,Mvd,Nsdhl,Tm7sf2</td>
<td>3.18E-10</td>
</tr>
<tr>
<td>lipid biosynthetic process</td>
<td>21</td>
<td>0610007P14Rik,9130409I23Rik,Acacb,AcsI3,Aldeh1a1,Dhcr7,ElovI,Fdps,Hdps,Hmgcr,Hmgcs1,Hsd17b12,Hsd17b2,Lss,Mvd,Nsdhl,Scd2,Srd5a1,Stard4,Tm7sf2</td>
<td>5.02E-10</td>
</tr>
<tr>
<td>steroid metabolic process</td>
<td>16</td>
<td>0610007P14Rik,Dhcr7,Fdft1,Fdps,Hmgcr,Hmgcs1,Hsd17b12,Hsd17b2,Insig1,Ldr,Lss,Mvd,Nsdhl,Srd5a1,Stard4,Tm7sf2</td>
<td>2.05E-09</td>
</tr>
<tr>
<td>alcohol metabolic process</td>
<td>20</td>
<td>0610007P14Rik,Adh4,Aldoc,Cmah,Dhcr7,Fdft1,Fdps,Hmgcr,Hmgcs1,Hsd17b2,Insig1,Ldr,Lrrc16a,Lss,Mvd,Nsdhl,Pklr,Rdh11,Tkt,Tm7sf2</td>
<td>8.38E-07</td>
</tr>
<tr>
<td>oxidation-reduction process</td>
<td>23</td>
<td>9130409I23Rik,Adh4,Aldh1a1,Aldh1a7,Cmah,Cyp2f2,Cyp2j5,Cyp2j6,Cyp2u1,Cyp8b1,Dhcr7,Fasn,Fdft1,Hmgcr,Hsd17b12,Hsd17b2,Me1,Nsdhl,Rdh11,Rrm2,Scd2,Srd5a1,Tm7sf2</td>
<td>1.55E-06</td>
</tr>
<tr>
<td>cytokinesis</td>
<td>6</td>
<td>Anln,Aurkb,Nusap1,Plk1,Prcl,Racgap1</td>
<td>1.67E-05</td>
</tr>
<tr>
<td>chromatin assembly or disassembly</td>
<td>12</td>
<td>Asf1b,Cdca2,Cenpa,Hist1h1a,Hist1h1b,Hist1h2an,Hist1h2bc,Hist1h3a,Hist1h3d,Hist2h3b,Tgm1,Trdn</td>
<td>1.87E-05</td>
</tr>
<tr>
<td>microtubule cytoskeleton organization</td>
<td>10</td>
<td>Cenpe,F630043A04Rik,Kif11,Mid1p1,Nek2,Nuf2,Nusap1,Prkcz,Stmn1,Tacc3</td>
<td>2.19E-05</td>
</tr>
<tr>
<td>retinoid metabolic process</td>
<td>4</td>
<td>Aldh1a1,Aldh1a7,Cyp2j6,Rdh11</td>
<td>6.93E-03</td>
</tr>
<tr>
<td>coenzyme A metabolic process</td>
<td>3</td>
<td>Hmgcr,Ncapd2,Nudt7</td>
<td>7.25E-03</td>
</tr>
<tr>
<td>fatty acid biosynthetic process</td>
<td>6</td>
<td>9130409I23Rik,Acacb,AcsI3,ElovI,Fasn,Scd2</td>
<td>1.46E-02</td>
</tr>
<tr>
<td>positive regulation of nitric oxide biosynthetic process</td>
<td>3</td>
<td>Egfr,Raet1b,Raet1d</td>
<td>3.81E-02</td>
</tr>
</tbody>
</table>
**Figure 17**

**Down-regulated**

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Hits</th>
<th>Gene(s)</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-467e</td>
<td>24</td>
<td>2910417H13Rik,Acsl3,Agpm1,Bub1b,Bub1b,Cibb,Cac5,Ctnnb2,Cnet,Cnmah,Cpp2</td>
<td>2.43E-04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>117725961,2.2rpm2,2.2rpm2,6ert111a,6ert111b,99vmy,ln1,nluq1,Mus1,Mup3,Mup9,M</td>
<td></td>
</tr>
</tbody>
</table>
I. Validation of microarray data

Validation of transcriptionally modulated genes

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>p-value</th>
<th>Fold-Change</th>
<th>HET * POL vs WT * POL</th>
<th>p-value</th>
<th>Fold-Change</th>
<th>HET * TOT vs WT * TOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccnb1</td>
<td>4.0245E-03</td>
<td>-2.290</td>
<td>HET * POL down vs WT * POL</td>
<td>1.0550E-04</td>
<td>-5.283</td>
<td>HET * TOT down vs WT * TOT</td>
</tr>
<tr>
<td>Ect2</td>
<td>5.2435E-03</td>
<td>-1.708</td>
<td>HET * POL down vs WT * POL</td>
<td>3.3025E-05</td>
<td>-4.274</td>
<td>HET * TOT down vs WT * TOT</td>
</tr>
<tr>
<td>Top2a</td>
<td>1.0097E-02</td>
<td>-1.686</td>
<td>HET * POL down vs WT * POL</td>
<td>1.0806E-04</td>
<td>-3.683</td>
<td>HET * TOT down vs WT * TOT</td>
</tr>
<tr>
<td>Plk1</td>
<td>1.6886E-05</td>
<td>-2.903</td>
<td>HET * POL down vs WT * POL</td>
<td>5.1683E-06</td>
<td>-3.444</td>
<td>HET * TOT down vs WT * TOT</td>
</tr>
<tr>
<td>Egfr</td>
<td>2.1547E-02</td>
<td>-2.813</td>
<td>HET * POL down vs WT * POL</td>
<td>1.2721E-02</td>
<td>-2.963</td>
<td>HET * TOT down vs WT * TOT</td>
</tr>
<tr>
<td>Fasn</td>
<td>3.8901E-03</td>
<td>-2.683</td>
<td>HET * POL down vs WT * POL</td>
<td>5.1772E-03</td>
<td>-2.322</td>
<td>HET * TOT down vs WT * TOT</td>
</tr>
<tr>
<td>Foxo1</td>
<td>1.9776E-04</td>
<td>3.158</td>
<td>HET * POL up vs WT * POL</td>
<td>1.1037E-03</td>
<td>2.070</td>
<td>HET * TOT up vs WT * TOT</td>
</tr>
<tr>
<td>Cdkn1a/P2</td>
<td>4.3535E-03</td>
<td>2.418</td>
<td>HET * POL up vs WT * POL</td>
<td>2.0174E-03</td>
<td>2.616</td>
<td>HET * TOT up vs WT * TOT</td>
</tr>
<tr>
<td>Ppargc1a</td>
<td>2.8059E-02</td>
<td>2.229</td>
<td>HET * POL up vs WT * POL</td>
<td>2.1833E-03</td>
<td>3.980</td>
<td>HET * TOT up vs WT * TOT</td>
</tr>
<tr>
<td>Zbtb16/PLZF</td>
<td>4.6496E-06</td>
<td>6.522</td>
<td>HET * POL up vs WT * POL</td>
<td>1.8602E-06</td>
<td>7.839</td>
<td>HET * TOT up vs WT * TOT</td>
</tr>
</tbody>
</table>
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K38/17 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>SCHECHTMAN D ET AL: &quot;ISOZYME-SPECIFIC INHIBITORS AND ACTIVATORS OF PROTEIN KINASE C&quot; METHODS IN ENZYMOL...</td>
<td>18</td>
</tr>
<tr>
<td>Y</td>
<td>the whole document table 1</td>
<td>1-27</td>
</tr>
</tbody>
</table>

**Further documents are listed in the continuation of Box C.** See patent family annex.

* Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* "A" document member of the same patent family

**Date of the actual completion of the international search**

15 February 2012

**Date of mailing of the international search report**

28/02/2012

**Name and mailing address of the ISA/Office**

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

**Authorized officer**

Orlando, Michel
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>the whole document</td>
<td>1-27</td>
</tr>
<tr>
<td>Y</td>
<td>the whole document</td>
<td>1-27</td>
</tr>
<tr>
<td>Y</td>
<td>the whole document</td>
<td>1-27</td>
</tr>
<tr>
<td>Y</td>
<td>the whole document</td>
<td>1-27</td>
</tr>
<tr>
<td>Y</td>
<td>the whole document</td>
<td>1-27</td>
</tr>
</tbody>
</table>

Form PCT/ISA/210 (continuation of second sheet) [April 2005]
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>MARCELLO CECI ET AL: &quot;Release of elf6 (p27BBP) from the 60S subunit allows 80S ribosome assembly&quot;, NATURE, vol. 426, no. 6966, 4 December 2003 (2003-12-04), pages 579-584, XP55018683, ISSN : 0028-0836, DOI : 10.1038/nature02160</td>
<td>18,23, .24</td>
</tr>
<tr>
<td>Y</td>
<td>the whole document</td>
<td>1-27</td>
</tr>
<tr>
<td>Y</td>
<td>the whole document</td>
<td>1-27</td>
</tr>
<tr>
<td>A</td>
<td>the whole document</td>
<td>-----</td>
</tr>
<tr>
<td>A</td>
<td>RICHARD J FLAVIN ET AL: &quot;Altered elf6 and DICER expression is associated with clinicalopathological features in ovarian serous carcinoma patients&quot;, MODERN PATHOLOGY, vol. 21, no. 6, 1 June 2008 (2008-06-01), pages 676-684, XP55018690, ISSN : 0893-9952, DOI : 10.1038/modpathol.2008.33</td>
<td>1-27</td>
</tr>
</tbody>
</table>

Form PCT/ISA/210 (continuation of second sheet) (April 2005)
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>