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(72) Inventor; and

(71) Applicant: LATHANGUE, Nicholas [GB/GB]; Department of Oncology, University of Oxford, Old Road Campus Research Building, Old Road Campus off Roosevelt Dr, Oxford Oxfordshire OX3 7DQ (GB).

(74) Agent: ELKINGTON AND FIFE LLP; Prospect House, 8 Pembroke Road, Sevenoaks Kent TN13 1XR (GB).

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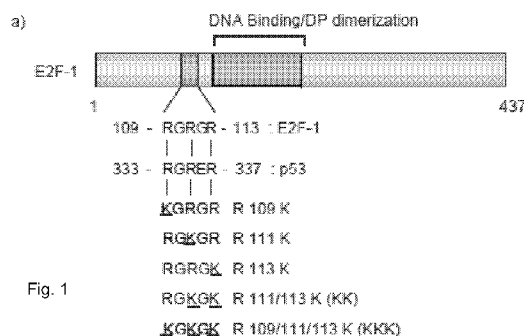
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(54) Title: METHOD OF TREATMENT AND SCREENING METHOD



(57) Abstract: A method for the treatment of a proliferative disease comprising providing a E2F-1 protein which is arginine-methylation defective or administering a substance which reduces the expression and/or activity of PRMT5. The invention also provides antibodies, screening methods and kits.

METHOD OF TREATMENT AND SCREENING METHOD

Field of the invention

5 The present invention relates to methods for preventing and treating a proliferative disease, such as cancer, by modulating the arginine methyltransferase enzyme PRMT5 or by providing a E2F-1 protein which is arginine-methylation defective. The invention also relates to a method for identifying a proliferative disease which may be susceptible to this method of treatment and a screening method for identifying
10 substances useful for the treatment of a proliferative disease.

Background of the invention

E2F is a family of transcription factors implicated in a variety of cell fates including
15 proliferation, apoptosis and differentiation (Stevens and La Thangue; 2003; Frolov and Dyson 2004, Polager and Ginsberg 2008; van den Heuvel and Dyson 2008). E2F proteins share the capacity to regulate a diverse group of target genes (Frolov and Dyson 2004; van den Heuvel and Dyson 2008). The first family member identified, E2F-1, physically interacts with the retinoblastoma tumour suppressor protein pRb,
20 which negatively regulates E2F-1 activity (Bandara and La Thangue 1991; Zamanian and La Thangue 1992; Weinberg 1995; Stevens and La Thangue 2003). Whilst it is established that E2F-1 can promote proliferation, it has also become clear that E2F-1 can prompt apoptosis (van den Heuvel and Dyson 2008, Polager and Ginsberg 2008). In Rb-/- mice, the enhanced levels of apoptosis in certain tissues reflect deregulated
25 E2F-1 activity (Tsai et al 1998; Iaquinta and Lees 2007). Further, E2F-1-/- mice suffer from an increased incidence of tumours (Field et al 1996), suggesting that E2F-1 adopts a tumour suppressor role in some tissues, perhaps reflecting its ability to induce apoptosis. However, the mechanisms that influence the diverse cellular outcomes that have been ascribed to E2F-1 activity, particularly its apoptotic activity and the cell
30 context dependency of these events, remain elusive. It is an object of the invention to identify such mechanisms.

Not only is E2F-1 regulated during cell cycle progression (Stevens and La Thangue, 2003, van den Heuvel and Dyson 2008), but also under conditions of DNA damage (Pediconi et al 2003; Stevens et al 2003; Stevens and La Thangue 2003). In DNA damaged cells, E2F-1 is induced in a fashion that follows similar kinetics to p53 (Pediconi et al 2003; Stevens and La Thangue 2003), which co-incides with activation of a diverse collection of E2F target genes (Ren et al 2002). DNA damage activates a signal transduction pathway involving protein phosphokinases, such as ATM/ATR and Chk1/Chk2, which in turn phosphorylate effector proteins that mediate the outcome of the DNA damage response (Jackson and Bartek, 2009). Both families of DNA damage responsive kinases phosphorylate E2F-1, which contributes to the regulation of E2F-1 in DNA damaged cells (Stevens et al 2003; Stevens and La Thangue 2003). Moreover, E2F-1 prompts apoptosis under DNA damage conditions and, in tumour cells which harbour compromised p53 activity, might provide an important pathway that enables apoptosis to be activated (Stevens and La Thangue 2003).

Arginine methylation is established as an important type of modification in protein control (Bedford and Richard 2005). A variety of processes are influenced by arginine methylation, including RNA splicing, chromatin and transcription control (Meister et al 2001; Pal et al 2004; Bedford and Richard 2005). It has been established that the p53 tumour suppressor protein is regulated by arginine methylation, and defined a role for the protein arginine N-methyltransferase 5 (PRMT5) (Jansson et al 2008). Significantly, arginine methylation occurred in DNA damaged cells, and influenced the outcome of the p53 response (Jansson et al 2008).

Cancer (and other hyperproliferative disease) is characterised by uncontrolled cellular proliferation. It is an object of the present invention to provide an alternative strategy for treating cancer and other proliferative diseases. Specifically it is an object of the invention to identify mechanisms that may be manipulated to lead to the death of tumour cells and other cells that proliferate abnormally. It is a further object of the invention to provide screening methods for identifying compounds that may be useful in the treatment of these conditions. It is a further object of the invention to identify a biomarker to identify responsive tumours.

Summary of the invention

According to a first aspect the invention provides a method for the treatment of a proliferative disease comprising administering a substance which reduces the expression and/or activity of the enzyme PRMT5. Preferably the substance reduces the catalytic activity of PRMT5. In particular the substance preferably reduces or abolishes the ability of PRMT5 to methylate E2F1 protein. Advantageously, this method provides a new therapeutic strategy which is not currently exploited by any available therapeutic agent. This will also allow combination therapies with existing drugs which have very different mechanisms of action.

In one embodiment, the method comprises antisense or RNA interference (RNAi). Preferably, the method comprises administering an antisense molecule, an shRNA or an siRNA, for example an oligonucleotide comprising the sequence:

5' CCGCUAUUGCACCUUGGAA (SEQ ID NO:1), or a sequence with at least 90% identity thereto, or 5' CAACAGAGAUCCUAUGAUU (SEQ ID NO:2), or a sequence with at least 90% identity thereto. Such oligonucleotides may be modified to improve their stability and/or potency and/or may be modified to enable systemic delivery.

In another embodiment the method comprises administering a small molecule inhibitor of PRMT5.

According to another aspect the invention provides a method for the treatment of a proliferative disease comprising providing an E2F-1 protein which is arginine-methylation defective. This approach relates directly to the E2F-1 protein whereas the PRMT5 approach has an indirect effect on the E2F-1 protein with equivalent end results.

According to another aspect the invention provides a substance which reduces the expression or activity of the enzyme PRMT5 for the treatment of a proliferative disease and the use of a substance which reduces the expression or activity of the enzyme PRMT5 for the manufacture of a medicament for the treatment of a proliferative disease.

According to a further aspect the invention provides an antibody which specifically binds to arginine-methylated E2F-1 protein, for example an antibody which specifically binds to a peptide comprising a sequence according to SEQ ID NO:5 or SEQ ID NO:6.

5 Such an antibody may be used to detect E2F-1 methylation and in a method of identifying a proliferative disease which may be susceptible to treatment by the inhibition of PRMT5.

According to yet another aspect the invention provides a method of determining if a
10 subject will benefit from treatment with a PRMT5 inhibitor comprising evaluating a biological sample from said subject for the presence and/or concentration of methylated E2F-1 protein, wherein the presence of methylated E2F-1 protein indicates that the subject will benefit from treatment with a PRMT5 inhibitor, or wherein an elevated concentration of methylated E2F-1 protein in the biological sample indicates that the
15 subject will benefit from treatment with a PRMT5 inhibitor. Alternatively the presence and/or concentration of PRMT5 protein may be used as a biomarker for identifying responsive tumours in such a method.

According to another aspect, the invention provides a method of determining the
20 prognosis of a subject comprising evaluating a biological sample from said subject for the presence and/or concentration of methylated E2F-1 protein and/or PRMT5 protein.

Further, the invention provides a kit for use in identifying a proliferative disease which may be susceptible to treatment by the inhibition of PRMT5, or for determining if a
25 subject will benefit from treatment with a PRMT5 inhibitor, or for determining the prognosis of a subject, comprising an antibody as defined herein, or epitope-binding fragment thereof.

Further, the invention provides a method for screening for a substance, or a salt or a
30 solvate thereof, to be used in the prevention or treatment of a proliferative disease, which comprises the following steps:

- (i) incubating PRMT5 and E2F-1 with and without a substance of interest, and
- (ii) measuring the amount of arginine methylation of E2F-1,

(iii) comparing the amount of arginine methylation of E2F-1 without incubation of the test substance with the amount of arginine methylation of E2F-1 with incubation of the test substance, wherein a decrease in the amount of arginine methylation of E2F-1 indicates that the substance may be useful in the prevention or treatment of cancer.

5

Brief description of the drawings

Figure 1 shows that E2F-1 undergoes arginine methylation by PRMT5.

Figure 2 shows that E2F-1 is methylated on arginine residues under physiological conditions.

10

Figure 3 shows the properties of arginine methylated E2F-1.

Figure 4 shows the functional properties of arginine methylated E2F-1.

Figure 5 shows a model for arginine methylation in E2F-1 control.

Figure 6 shows that PRMT5 regulates apoptosis by suppressing E2F-1.

Figure 7 shows PRMT5 and E2F-1 expression in clinical tumour samples.

15

Detailed description of the invention

The invention is based on a novel mechanism for targeting and controlling the E2F pathway, by arginine methylation. It is shown herein that PRMT5 methylates E2F-1 and that arginine methylation is a key regulator of E2F-1 activity. Specifically, an arginine methylation defective E2F-1 protein exhibits increased protein stability, and activates transcription and induces apoptosis more effectively than wild-type E2F-1. Moreover, PRMT5 methylation down-regulates E2F-1 levels, which in turn enables PRMT5 to suppress E2F-1-dependent apoptosis. These results establish a new mechanism that controls the apoptotic properties of E2F-1, and more generally suggest that arginine methylation contributes to the context dependency and cellular consequence of E2F-1 activation.

20

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Accordingly, methods are described herein in which the expression and/or activity of the PRMT5 enzyme is reduced, thereby promoting E2F-1-dependent apoptosis. Advantageously, this method may be useful in killing tumour cells, and other cells which proliferate abnormally, or hyperproliferate, which contain methylated E2F-1.

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According to a first aspect the present invention provides a method for the treatment of a proliferative disease comprising administering a substance which reduces the expression and/or activity of the enzyme PRMT5. Such a substance may be referred to herein as a 'PRMT5 inhibitor'.

5

The sequence information for PRMT5 may be found at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/pubmed/>) under accession numbers NM_006109 (nucleotide) and NP_006100 (protein).

10 By 'reduces the expression and/or activity of the enzyme' it is meant that expression of the enzyme is reduced or inhibited and/or that the activity of the enzyme is reduced partially or completely. Expression of the enzyme may be altered by gene therapy or by disrupting transcription of the gene encoding the enzyme or by destruction of the gene transcript or by disrupting translation or by degradation of the enzyme. The
15 activity of the enzyme may be altered by a competitive or non-competitive inhibitor, or by genetic modification. Preferably the substance reduces the catalytic activity of PRMT5. In particular the substance preferably reduces or abolishes the ability of PRMT5 to methylate E2F1 protein.

20 Preferably the method provides a reduction in the amount of active enzyme of from 10% to 100% based on the amount of of active enzyme in the cell prior to treatment. Most preferably the method provides a reduction in the amount of active enzyme of at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99%, based on the amount of of active
25 enzyme in the cell prior to treatment. The amount of active enzyme may be quantitated by measuring the enzyme activity in the cell or in an *in vitro* assay. For example, a recombinant PRMT5 enzyme may be expressed and the activity of methyltransferases measured *in vitro*, as described, for example, in Example 1, Fig 1b and the Materials and Methods.

30

In accordance with the invention, the substance to be administered may comprise a small molecule drug or a peptide which may be designed to inhibit the activity of PRMT5 or to block the methylation of E2F-1 at one or more of the arginine residues at

positions 109, 111 or 113 of the protein. Methylated E2F-1 refers to an E2F-1 protein which is methylated on 1 or more of residues R109, R111 and R113. Preferably, methylated E2F-1 is di-methylated on two residues of E2F-1 selected from residues R109, R111 and R113. Most preferably methylated E2F-1 is di-methylated on residues
5 R111 and R113. Preferably the methylation is symmetric.

Alternatively the method may comprise the use of antisense or RNA interference (RNAi) technology to reduce expression of the PRMT5 protein. For example, an antisense molecule, a short hairpin RNA (shRNA) or a small interfering RNA (siRNA)
10 may be administered in accordance with the method of the invention, in order to downregulate expression of PRMT5. Antisense, shRNA and siRNA technologies are known to the skilled person and are described in Goyal 2009, Hajeri 2009, Oh 2009, Singh 2009, Rao 2009, Tilesi 2009, Singer 2008 and Bernards 2006, all of which are incorporated herein by reference.

15

An antisense molecule preferably comprises a single-stranded oligonucleotide of approximately 12 to 20 nucleotides in length, most preferably about 16 nucleotides. A small interfering RNA (siRNA) preferably comprises a double stranded oligonucleotide of approximately 14 to 22 base pairs in length, most preferably 16 to 20 base pairs in
20 length. For example the siRNA may comprise a double stranded oligonucleotide of 16, 17, 18, 19 or 20 base pairs in length. The siRNA may have a single or double overhang at one or both ends, i.e. a 3' or 5' overhang consisting of one or two bases.

An antisense or siRNA molecule may be designed based on the mRNA sequence that
25 encodes the target protein, namely the PRMT5 enzyme. Preferably the antisense or siRNA molecule is complementary to a section of the mRNA sequence that encodes the target protein. The design of siRNA molecules is known in the art (e.g. Tilesi 2009) and there are websites which may be used for designing siRNA, for example:

<http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx>.

30 A series of oligonucleotides may be designed and tested against the target mRNA (i.e. PRMT5 mRNA) and a reduction in the amount of target mRNA looked for. The levels of mRNA can be measured directly or indirectly by monitoring PRMT5 protein levels, by known techniques.

A preferred siRNA for use in accordance with the invention comprises the sequence 5' CCGCUAUUGCACCUUGGAA (SEQ ID NO:1) or a sequence with at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% identity thereto.

5 Another preferred siRNA for use in accordance with the invention comprises the sequence 5' CAACAGAGAUCCUAUGAUU (SEQ ID NO:2) or a sequence with at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% identity thereto.

10 An 'oligonucleotide' or 'oligo' shall mean multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). The term 'oligonucleotide' as used herein refers to both oligoribonucleotides and
15 oligodeoxyribonucleotides. The term 'oligonucleotide' shall also include oligonucleosides (i.e. an oligonucleotide minus the phosphate) and any other organic base containing polymer. Oligonucleotides can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis).

20

In preferred embodiments the oligonucleotide may be modified to improve its stability and/or potency. A 'stabilized oligonucleotide' shall mean an oligonucleotide that is more resistant to *in vivo* degradation (e.g. via an exo- or endo-nuclease) than the same oligonucleotide which is not stabilized, for example it may be degraded at least 2, 3, 4
25 or 5 times more slowly than the non-stabilized oligonucleotide. Preferred stabilized oligonucleotides of the invention have a modified phosphate backbone. Especially preferred oligonucleotides have a phosphorothioate modified phosphate backbone (i.e. at least one of the phosphate oxygens is replaced by sulfur). Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl- phosphonates
30 (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain a diol, such as tetraethyleneglycol or

hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

Other ways to increase the stability and/or potency of the oligonucleotide include 2'-O
5 modifications and/or LNA modifications.

In other preferred embodiments the oligonucleotide may be modified to promote local, topical or systemic delivery. For example, it may be conjugated to an aptamer, formulated in a lipid nanoparticle such as a liposome, polyconjugated, conjugated to a
10 lipophilic molecule such as cholesterol, contained in a cyclodextrin nanoparticle or complexed with an antibody. To promote systemic delivery the oligonucleotide is preferably modified in one of these ways. These and other suitable modifications are described by Toudjarska and de Fougerolles 2009.

15 In an alternative embodiment of the invention, a gene therapy approach may be taken, in which an E2F-1 protein which is arginine-methylation defective is provided. The sequence information for E2F-1 may be found at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/pubmed/>) under accession numbers NM_005225 (nucleotide) and NP_005216 (protein). The protein sequence is
20 reproduced here as SEQ ID NO:3:

1 MALAGAPAGG PCAPALEALL GAGALRLLDS SQIVIISAAQ DASAPPAPTG
PAAPAAGPCD

61 PDLLEFATPQ APRPTPSAPR PALGRPPVKR RLDLETDHQY LAESSGPARG
RGRHPGKGVK

25 121 SPGEKSRYET SLNLTTRKFL ELLSHSADGV VDLNWAAEVL
KVQKRRIYDI TNVLEGIQLI

181 AKKSKNHIQW LGSHTTVGVG GRLEGLTQDL RQLQESEQQL
DHLMNICTTQ LRLLSEDTDS

241 QRLAYVTCQD LRSIADPAEQ MVMVIKAPPE TQLQAVDSSE
30 NFQISLKSKQ GPIDVFLCPE

301 ETVGGISPGK TPSQEVTSSE ENRATDSATI VSPPPSSPPS SLTTDPSQSL
LSLEQEPLLS

361 RMGSLRAPVD EDRLSPLVAA DLSLEHVRED FSGLLP EEFI SLSP PHEALD
YHFGLEEGEG
421 IRDLFDCDFG DLTP LDF

- 5 An E2F-1 protein which is arginine-methylation defective is mutated so that one or more of residues R109, R111 and R113 are substituted with residues that are resistant to methylation. Preferably the arginine (R) residue(s) are substituted with lysine (K) residue(s). Preferably the methylation-defective E2F-1 protein contains the mutations R111K and R113K (the 'KK mutant') or R109K, R111K and R113K (the 'KKK mutant').
- 10 Accordingly the invention also provides a methylation-defective E2F-1 protein for the treatment of a proliferative disease and the use of a methylation-defective E2F-1 protein for the manufacture of a medicament for the treatment of a proliferative disease. Further the invention provides an oligonucleotide which encodes a methylation-defective E2F-1 protein for the treatment of a proliferative disease and
- 15 the use of an oligonucleotide which encodes a methylation-defective E2F-1 protein for the manufacture of a medicament for the treatment of a proliferative disease.

The methods of the invention may be used in the treatment of a proliferative disease. As such, the methods may result in the killing of cells which proliferate abnormally,

20 such as cancerous cells, including tumour cells, and other (non-malignant) tumour cells. The methods are particularly effective in killing cells which contain high levels of E2F-1 methylation. Thus, the invention provides methods for reducing E2F-1-mediated cell proliferation (e.g. tumour growth) in a patient.

- 25 High E2F-1 methylation may be defined by pathologists upon examining a biological sample (e.g. a tumour biopsy) from a patient. For example, a biological sample may be examined and given a total staining score (TSS). Total staining score is a parameter which is well-known to the skilled person. It is based upon the number of cells stained by an antibody specific for the antigen (in this case, methylated E2F-1 protein), the
- 30 intensity of the staining in the cells and the overall pathology of the tumour (e.g. for bowel cancer the overall pathology may be defined as Dukes' stage A, B, C or D). A high level of E2F-1 methylation may then be defined as a TSS of at least 50%, preferably at least 60% or at least 70% or at least 80%.

The term 'proliferative disease' as used herein refers to both cancer and non-cancer disease. Preferably the proliferative disease is one characterized by increased methylation of the E2F-1 gene product in afflicted patients. Illustrative non-cancer

5 diseases include inflammatory and/or immunoproliferative disorders such as arthritis, fibrosis, asthma and allergies. The invention can be used to screen for risk of and/or treat a variety of different types of cancer, particularly malignant (and preferably solid) tumours of epithelial or mesenchymal cells, e.g. an advanced solid tumour as disclosed in WO 02/66019. Examples of cancers that can be screened for risk of and/or treated

10 by the present invention include brain and other central nervous system tumours (e.g. tumours of the meninges, brain, spinal cord, cranial nerves and other parts of central nervous system, e.g. glioblastomas or medulla blastomas); head and/or neck cancer; breast tumours; circulatory system tumours (e.g. heart, mediastinum and pleura, and other inbrathoracic organs, vascular tumours and tumour-associated vascular tissue);

15 excretory system tumours (e.g. kidney, renal pelvis, ureter, bladder, other and unspecified urinary organs); gastrointestinal tract tumours (e.g. oesophagus, stomach, small intestine, colon, colorectal, rectosigmoid junction, rectum, anus and anal canal), tumours involving the liver and intrahepatic bile ducts, gall bladder, other and unspecified parts of biliary tract, pancreas, other and digestive organs); head and neck;

20 oral cavity (lip, tongue, gum, floor of mouth, palate, and other parts of mouth, paroud gland, and other parts of the salivary glands, tonsil, oropharynx, nasopharynx, pyriform sinus, hypopharynx, and other sites in the lip, oral cavity and pharynx); reproductive system tumours (e.g. vulva, vagina, Cervix uteri, Corpus uteri, uterus, ovary, and other sites associated with female genital organs, placenta, penis, prostate, tests, and other

25 sites associated with male genital organs); respiratory tract tumours (e.g. nasal cavity and middle ear, accessory sinuses, larynx, trachea, bronchus and lung, e.g. small cell lung cancer or non-small cell lung cancer); skeletal system tumours (e.g. bone and articular cartilage of limbs, bone articular cartilage and other sites); skin tumours (e.g. malignant melanoma of the skin, non-melanoma skin cancer, basal cell carcinoma of

30 skin, squamous cell carcinoma of skin, mesothelioma, Kaposi's sarcoma); and tumours involving other tissues including peripheral nerves and autonomic nervous system, connective and soft tissue, retroperitoneum and peritoneum, eye and adnexa, thyroid, adrenal gland and other endocrine glands and related structures, secondary and

unspecified malignant neoplasm of lymph nodes, secondary malignant neoplasm of respiratory and digestive systems and secondary malignant neoplasm of other sites, leukemias, including hairy cell leukemia, multiple myeloma, chronic lymphocytic leukemia, chronic myeloid leukemia, acute myeloid leukemia and acute lymphocytic leukemia, lymphomas, including non-Hodgkin's lymphoma and Hodgkin's lymphoma. Where hereinbefore and subsequently a tumour, a tumour disease, a carcinoma or a cancer is mentioned, also metastasis in the original organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumour and/or metastasis is.

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By 'treating a proliferative disease' it is intended to include the inhibition of the symptoms of a disease, namely, inhibition or retardation of the progression of the disease; and the alleviation of the symptoms of a disease, namely, regression of the disease or the symptoms, or inversion of the progression of the symptoms. It may also include the prevention of the development of a disease or a symptom from a patient who may have a predisposition of the disease or the symptom but has yet been diagnosed to have the disease or the symptom. By 'treating cancer' it is intended to include the inhibition of tumour growth, including the prevention of the growth of a tumour in a subject or a reduction in the growth of a pre-existing tumour in a subject. The inhibition can also be the inhibition of the metastasis of a tumour from one site to another. It is preferred to treat a tumour exhibiting high levels of PRMT5 protein and low levels of E2F-1 protein with a PRMT5 inhibitor in accordance with the invention, in order to inactivate PRMT5 and thereby activate E2F-1.

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In other aspects the invention provides a substance which reduces the expression or activity of the enzyme PRMT5 for the treatment of a proliferative disease, for example cancer, and the use of a substance which reduces the expression or activity of the enzyme PRMT5 for the manufacture of a medicament for the treatment of a proliferative disease, for example cancer. Such substances are described above and may be formulated and administered as described herein.

Administration

In the treatment comprising the administration of a substance which reduces the expression and/or activity of the enzyme PRMT5, such substance may be mixed with a pharmaceutically acceptable carrier, so that it can be provided in the form of a pharmaceutical composition. Any ratio of active ingredient to carrier may be used and
5 the ratio of an active ingredient to a carrier may preferably be between 1% and 90% by weight. In addition, the pharmaceutical composition of the present invention can be administered to humans or organisms other than humans [for example, non-human mammals (e.g. a bovine, a monkey, a chicken, a cat, a mouse, a rat, a hamster, a swine, a canine, etc.), birds, reptiles, amphibians, fish, insects, etc.] in various forms via either
10 a topical, local or systemic administration route. For example, an oral administration route (including gavage, or admixture with food or drink) or a parenteral administration route (e.g. intravenous injection, intramuscular injection, subcutaneous administration, rectal administration, intraperitoneal, and dermal administration) may be used. Accordingly, as the pharmaceutical composition of the present invention, an active
15 ingredient can be used singly. However, it is also possible to formulate such an active ingredient with a pharmaceutically acceptable carrier or diluent by a method commonly used depending on an administration route, so as to manufacture a formulation having a suitable dosage form. Any dosage form known to the skilled person may be used. Any appropriate carrier or diluent may be used, for example isotonic saline solution, buffers,
20 etc. Such pharmaceutical carriers are well known in the art and the selection of a suitable carrier is deemed to be within the scope of those skilled in the art from the teachings contained herein.

The substance is administered *in vivo* in an amount effective to affect a decrease in the
25 expression and/or activity of the enzyme PRMT5, as discussed above, and thereby induce apoptosis.

The term 'an effective amount' for purposes of this application shall mean that amount of substance capable of producing the desired effect. In this case, the desired effect
30 may be the slowing of tumour growth, the death of tumour cells, reduction in the size of the tumour, regression of the condition, for example. The amount of substance which is given depends upon a variety of factors including the age, weight and condition of the patient, the administration route, the properties of the pharmaceutical

composition, the condition of the patient, the judgment of a doctor, the condition and the extent of treatment or prevention desired. The substance may be administered to the individual as a short-term therapy or long-term therapy depending on the condition and the extent of treatment or prevention desired.

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The substance which reduces the expression and/or activity of the enzyme PRMT5 may be employed alone or in combination with other techniques, drugs or compounds for preventing, controlling or treating the proliferative disease. For example, for the treatment of cancer the substance may be administered in combination (e.g. contemporaneously or sequentially) with chemotherapeutic agents, cytostatic agents, antiangiogenic agents, radiation or anti-neoplastic agents, such as doxorubicin, cisplatin, taxotere, 5-fluoruracil, methotrexat, gemcitabin and cyclophosphamide.

10

The oligonucleotides used in the methods of the invention may be formulated as a pharmaceutical composition as described above or may be administered using gene therapy. In the gene therapy of the present invention, it may be possible to select either an *in vivo* method of directly administering a recombinant vector encoding the oligonucleotide of interest to a patient, or an *ex vivo* method of collecting a target cell from a patient body, introducing the oligonucleotide of interest, or a recombinant vector encoding the oligonucleotide of interest, into the target cell outside of the body, and returning the target cell, into which the aforementioned oligonucleotide or vector has been introduced, to the patient body. In the case of the *in vivo* method, the recombinant vector encoding the oligonucleotide of interest is directly administered to a patient by using a gene transfer vector known in the present technical field, such as a retrovirus vector. As with the pharmaceutical composition of the present invention, such an oligonucleotide or gene transfer vector used in the gene therapy of the present invention can be mixed with a pharmaceutically acceptable carrier, so as to produce a formulation. Such a formulation can be parenterally administered, for example. Fluctuation of a dosage level can be adjusted by standard empirical optimizing procedures, which are well understood in the present technical field. An alternative for *in vivo* administration is to use physical approaches, such as particle bombardment or jet injection, to directly deliver DNA encoding heavy and light chains of an engineered agonist antibody (Walther *et al* Mole Biotechnology 28:121-128, 2004; Yang *et al*

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PNAS 87:9568-72, 1990). In the case of the *ex vivo* method, such an oligonucleotide of interest can be introduced into a target cell according to a method known in the present technical field, such as the calcium phosphate method, the electroporation method, or the viral transduction method. Such a target cell can be collected from the affected region, e.g. tumour. In the case of selecting the *ex vivo* method an oligonucleotide of interest or a gene transfer vector to which the oligonucleotide of interest is operably linked is introduced into a cell, and the aforementioned oligonucleotide is then allowed to express in the cell. Thereafter, the cell is transplanted to a patient, so that the disease can be treated.

A gene transfer vector available for gene therapy is well known in the present technical field, and it can be selected, as appropriate, depending on a gene introduction method or a host. Examples of such a vector include an adenovirus vector and a retrovirus vector. When an oligonucleotide is ligated to a gene transfer vector, a control sequence such as a promoter or a terminator, a signal sequence, a polypeptide-stabilizing sequence, etc. may be appropriately ligated, such that the oligonucleotide can be expressed in a host. Selection or construction of such vectors is well known to the skilled person.

The administration and gene therapy techniques described above are also applicable for the gene therapy approach, in which an E2F-1 protein which is arginine-methylation defective is provided. In this case an E2F-1 gene encoding the arginine-methylation defective protein is inserted into a gene transfer vector and administered by any suitable method known to the skilled person. This approach may be employed alone or in combination with other techniques, drugs or compounds for preventing, controlling or treating the proliferative disease. For example, for the treatment of cancer the substance may be administered in combination (e.g. contemporaneously or sequentially) with chemotherapeutic agents, cytostatic agents, antiangiogenic agents, radiation or anti-neoplastic agents, such as doxorubicin, cisplatin, taxotere, 5-fluoruracil, methotrexat, gemcitabin and cyclophosphamide.

Biomarker

In a further aspect, the invention provides an antibody which specifically binds to arginine-methylated E2F-1 protein. Preferably the antibody specifically binds to a methylated E2F-1 peptide comprising or consisting of the sequence RGRGR (SEQ ID NO:4) in which one or more of the arginine residues is methylated, for example the
5 sequence RGR(Me)GR(Me) (SEQ ID NO:5). Most preferably the antibody specifically binds to a methylated E2F-1 peptide comprising or consisting of the sequence CESSGPARGR(Me)GR(Me)HPGKG (SEQ ID NO:6).

The term 'antibody' as used herein includes all forms of antibodies such as
10 recombinant antibodies, humanized antibodies, chimeric antibodies, single chain antibodies, polyclonal antibodies, monoclonal antibodies etc. Preferably the antibody is a monoclonal antibody. The invention is also applicable to antibody fragments and derivatives that are capable of binding to the antigen.

15 The skilled person could make such antibodies by known methods. Various forms of antibodies can be made using standard recombinant DNA techniques (Winter and Milstein, Nature, 349, pp. 293-99 (1991)). For example, the production of rat proteolytic fragments of IgG antibodies is described by Rousseaux, J (Methods in Enzymology 1986; 121; 663). Antibodies: A Laboratory Manual (Ed Harlow, Edward
20 Harlow, David Lane, CSHL Press, 1988) describes obtaining fragments of human antibodies. Gilliland et al (Tissue Antigens 1996;47(1):1-20) describes a general method for isolating the variable regions of antibodies and the production of a chimeric antibody. The preparation of monoclonal antibodies is a well-known process (Kohler et al., Nature, 256:495 (1975)).

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Chimeric and humanised, e. g. CDR-grafted, antibodies may be used in accordance with the present invention. These antibodies are less immunogenic than the corresponding rodent antibodies. Thus, the antibody may have CDRs which are of different origin to the variable framework region. Similarly, the antibody may have
30 CDRs of different origin to the constant region.

Preferred antibodies according to the present invention are such that the affinity constant for the antigen is 10^5 mole^{-1} or more, for example up to $10^{12} \text{ mole}^{-1}$. Ligands

of different affinities may be suitable for different uses so that, for example, an affinity of 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} or 10^{11} mole⁻¹ or more may be appropriate in some cases. However antibodies with an affinity in the range of 10^6 to 10^8 mole⁻¹ will often be suitable. Conveniently the antibodies also do not exhibit any substantial binding
5 affinity for other antigens, in particular antigens which are not methylated but which otherwise have the same sequence as the desired antigen. Specifically, the antibody preferably specifically binds to E2F-1 which is methylated on one or more arginine residues but does not bind to unmethylated E2F-1.

10 Binding affinities of the antibody and antibody specificity may be tested by assay procedures such as radiolabelled or enzyme labelled binding assays and use of biacore with solid phase ligand. (Bindon, C.I. *et al.* 1988 Eur. J. Immunol. 18, 1507-1514; Dall'Acqua, W., *et al.* Biochemistry 35, 9667; Luo *et al.* J. Immunological Methods 275 (2203) 31-40 ; Murphy *et al.* Curr Protoc Protein Sci. 2006 Sep; Chapter 19: Unit
15 19.14).

The antibody may be used to measure E2F-1 methylation, which can be used as a biomarker. In particular, the antibody may be used in a method of identifying patients which may benefit from treatment by the method described above. Accordingly, the
20 invention provides a method of identifying a proliferative disease (for example a tumour) which may be susceptible to treatment by the inhibition of PRMT5 which comprises the use of an antibody which specifically binds to arginine-methylated E2F-1 protein.

25 The invention also provides a method of determining if a subject will benefit from treatment with a PRMT5 inhibitor comprising the steps of evaluating a biological sample from said subject for the presence and/or concentration of methylated E2F-1 protein, wherein the presence of methylated E2F-1 protein indicates that the subject will benefit from treatment with a PRMT5 inhibitor, or wherein an elevated
30 concentration of methylated E2F-1 protein in the biological sample indicates that the subject will benefit from treatment with a PRMT5 inhibitor.

The invention further provides a method of determining if a subject will benefit from treatment with a PRMT5 inhibitor comprising evaluating a biological sample from said subject for the presence and/or concentration of PRMT5 protein, wherein the presence of PRMT5 protein indicates that the subject will benefit from treatment with a PRMT5 inhibitor, or wherein an elevated concentration of PRMT5 protein in the biological sample indicates that the subject will benefit from treatment with a PRMT5 inhibitor.

The biological sample may be a solid or body fluid sample. The biological sample may include cells, blood, plasma or serum. Preferably the biological sample is a tissue biopsy or serum or circulating tumour cells. Examples of solid biological samples include samples taken from faeces, the rectum, central nervous system, bone, breast tissue, renal tissue, the uterine cervix, the endometrium, the head/neck, the gallbladder, parotid tissue, the prostate, the brain, the pituitary gland, kidney tissue, muscle, the esophagus, the stomach, the small intestine.

The method of the invention can be used to detect methylated E2F-1 protein and/or PRMT5 protein in a biological sample *in vitro* or *in vivo*. The presence and/or concentration of methylated E2F-1 protein and/or PRMT5 protein may be determined by standard immunochemical techniques which are well known to the skilled person (for example immunohistochemistry, radioimmunoassay, ELISA, Western blot, fluorescence assay, etc).

In one embodiment, the method of the invention is used to determine the level of methylated E2F-1 protein and/or PRMT5 protein in a tissue or in cells derived from the tissue. In a preferred embodiment, the tissue is a diseased tissue. In a preferred embodiment of the method, the tissue is a tumour or a biopsy thereof. In a preferred embodiment of the method, a tissue or a biopsy thereof is first excised from a patient, and the levels of methylated E2F-1 protein and/or PRMT5 protein in the tissue or biopsy can then be determined in an immunoassay with the antibodies or antibody fragments of the invention. The tissue or biopsy thereof can be frozen or fixed. The same method can be used to determine other properties of the methylated E2F-1 protein and/or PRMT5 protein, such as its cell surface levels, or its cellular localization.

This method requires assessing the presence and/or concentration of methylated E2F-1 and/or PRMT5 in order to determine whether the subject will benefit from treatment with a PRMT5 inhibitor. The skilled person will understand that clinical material may be examined to understand how levels of methylated E2F-1 protein and/or PRMT5 protein relate to cancer. In some cases, the presence of methylated E2F-1 protein and/or PRMT5 protein will be indicative that the subject will benefit from treatment with a PRMT5 inhibitor. In other cases, an elevated concentration of methylated E2F-1 protein and/or PRMT5 protein will be indicative that the subject will benefit from treatment with a PRMT5 inhibitor. An elevated concentration is intended to be relative to a predetermined base level, which may be the level of methylated E2F-1 protein and/or PRMT5 protein in normal (i.e. noncancerous) biological samples from the subject or may be the level of methylated E2F-1 protein and/or PRMT5 protein in a sample or samples obtained from non-cancer-afflicted individuals. Preferably, a total staining score (TSS) of at least 50%, preferably at least 60% or at least 70% or at least 80%, as discussed above, may be indicative of the presence of E2F-1 methylation. Preferably, a total staining score (TSS) of at least 50%, preferably at least 60% or at least 70% or at least 80%, as discussed above, may be indicative of an elevated concentration as compared to the predetermined base level, which may have a TSS of less than 50%, preferably less than 25%, preferably less than 10%, preferably close or equal to zero.

For example, the method may comprise the steps of:

- (i) contacting a biological sample of said patient with an antibody as described herein, or epitope-binding fragment thereof,
 - (ii) measuring the binding of said antibody or epitope-binding fragment thereof to said cells, and
 - (iii) comparing the binding in part (ii) with that of a normal reference subject or standard.
- The antibodies or antibody fragments of the invention can be used to detect methylated E2F-1 protein in a biological sample *in vitro* or *in vivo* as described above.

The above-described method can be used to diagnose a proliferative disease, for example cancer, in a subject known to or suspected to have a such a disease, wherein the level of methylated E2F-1 protein and/or PRMT5 protein measured in said patient is compared with that of a normal reference subject or standard. Said method can then
5 be used to determine whether a tumour contains cells in which the E2F-1 protein is methylated, or which contains increased levels of PRMT5 protein, both of which may suggest that the tumour will respond well to treatment with the substances of the present invention.

10 Methylated E2F-1 protein and/or PRMT5 protein may also be used as a prognostic indicator. In this embodiment the levels of methylated E2F-1 protein and/or PRMT5 protein may be measured prior to treatment to predict the severity and progression of the disease. Optionally, the levels of methylated E2F-1 protein and/or PRMT5 protein may also be measured over a time course during and after treatment to monitor the
15 progress of the disease and the success of any treatment. For example, high levels of methylated E2F-1 protein and/or PRMT5 protein may be indicative of a severe condition. For example, reduced levels of methylated E2F-1 protein and/or PRMT5 protein over a time period may be indicative of regression of the disease. For example, the longer the time it takes for the levels of methylated E2F-1 protein and/or PRMT5
20 protein to double (the doubling time), the better the prognosis for the patient. The levels of methylated E2F-1 protein and/or PRMT5 protein may be measured using the antibodies or antibody fragments. For example, the levels of methylated E2F-1 protein may be measured using the antibodies or antibody fragments of the invention or using any methods known to the skilled person. Accordingly, the invention further provides
25 a method of determining the prognosis of a subject comprising evaluating a biological sample from said subject for the presence and/or concentration of methylated E2F-1 protein and/or PRMT5 protein.

In accordance with the invention, it is preferred to treat a tumour which expresses high
30 levels of PRMT5 and low levels of E2F-1. It is envisaged that in these tumours PRMT5 is suppressing E2F1 activity and thereby preventing apoptosis, and keeping cells in the proliferative mode. As such, the increase in E2F1 levels in a tumour may be used as a mechanistic endpoint biomarker for the effects of a PRMT5 inhibitor. The

images in Figures 7d and 7e show that tumours exist with either high PRMT5/low E2F1 verses low PRMT5/high E2F1. It is preferred to treat the former with a PRMT5 inhibitor in accordance with the invention, in order to inactivate PRMT5 and thereby activate E2F1. Furthermore, a patient who has a tumour with high levels of PRMT5 and low levels of E2F-1 is likely to have an improved prognosis compared to a patient who has a tumour with low PRMT5/high E2F1.

The present invention further provides for monoclonal antibodies, humanized antibodies and epitope-binding fragments thereof that are further labelled for use in research, prognostic or diagnostic applications. In preferred embodiments, the label is a radiolabel, a fluorophore, a chromophore, an imaging agent or a metal ion. A method for diagnosis is also provided in which said labelled antibodies or epitope-binding fragments thereof are administered to a subject suspected of having a cancer, and the distribution of the label within the body of the subject is measured or monitored.

15

Kit

The present invention also includes kits, e.g., comprising a substance which reduces the expression and/or activity of the enzyme PRMT5 and instructions for the use of the substance for killing of particular cell types. The instructions may include directions for using the substance *in vitro*, *in vivo* or *ex vivo*.

20

Typically, the kit will contain a substance which reduces the expression and/or activity of the enzyme PRMT5 or a pharmaceutical composition comprising the substance. This may be in a lyophilized form, liquid form, or other form amendable to being included in a kit. The kit may also contain additional elements needed to practice the method described on the instructions in the kit, such a sterilized solution for reconstituting a lyophilized powder, additional agents for combining with the substance prior to administering to a patient, and tools that aid in administering the substance to a patient.

25

30

The present invention also includes a kit for use in identifying tumours which may be susceptible to treatment by the inhibition of PRMT5, or for determining if a subject will benefit from treatment with a PRMT5 inhibitor, or for determining the prognosis of a

subject. The kit comprises an antibody as described here, or epitope-binding fragment thereof. The kit may also comprise means for obtaining a biological sample, such as a spatula or a dipstick or a container for accepting the sample. The kit may also comprise an assay for detecting the presence and/or concentration of methylated E2F-1 bound to the antibody. Preferably the assay comprises an immunoassay, such as an ELISA.

Screening method

Accordingly, in another aspect the invention provides a screening method for identifying substances useful in the treatment of cancer. Specifically the invention provides a method for screening for a substance, or a salt or a solvate thereof, to be used in the prevention or treatment of cancer, which comprises the following steps:

- (i) incubating PRMT5 and E2F-1 with and without a substance of interest, and
 - (ii) measuring the amount of arginine methylation of E2F-1,
 - (iii) comparing the amount of arginine methylation of E2F-1 without incubation of the test substance with the amount of arginine methylation of E2F-1 with incubation of the test substance, wherein a decrease in the amount of arginine methylation of E2F-1 indicates that the substance may be useful in the prevention or treatment of cancer.
- This method may be carried out *in vitro*.

The type of a substance screened by the screening method of the present invention is not particularly limited. Examples of such a substance include a therapeutic agent for treating cancer or a candidate compound therefor. A compound library is preferably used to provide for a high throughput screen. Preferably, substances may be selected as potential inhibitors by rational design based on the crystal structure of PRMT5. Molecules may be selected using a variety of computational approaches (for example substructure searching, similarity searching and shape-based screening).

The method may be carried out in cells or a cell-free system. Any cell type may be used in accordance with the invention, for example 3T3 cells, 293 cells, 721 cells, B16 cells, CHO cells, COS-7 cells, NIH-3T3 cells, and so on. Preferred are cancer cell lines, for example the human osteosarcoma cell line U2OS, the cervical cancer cell lines HeLa and C33A, the breast cancer cell lines MCF-7 and MDA-MB-438, the prostate cancer lines PC3 and LNCap, Saos-2 cells, and so on. Cells are plated and

incubated to form monolayers in any manner known to the skilled person. For example 1×10^5 cells may be plated into 24-well plates and incubated for 18 hours to form monolayers.

- 5 PRMT5 and E2F-1 proteins may be obtained by expression in cells or by recombinant expression *in vitro*. For example, a plasmid containing the PRMT5 and/or E2F-1 genes may be transfected into cells. Subsequently, the cells may be lysed and the PRMT5 protein immunoprecipitated (e.g. with agarose beads) and eluted. The proteins may be expressed as fusions proteins, for example Flag-PRMT5.

10

The E2F-1 peptide and PRMT5 enzyme may then be mixed with other recombinant substrates (such as GST fusion proteins or histones) in a methylation reaction buffer and with a methyl group donor (such as ^3H labeled SAM) and incubated. For example, the methylation reaction buffer may contain 50mM Tris, 0.1mM EDTA and 50mM
15 NaCl. The $-\text{CH}_3$ group donor may be ^3H labeled SAM. The reactants may be mixed to a volume of 40 μl . The reactants may be incubated at 30°C for 90 min.

- The presence and/or concentration of methylated E2F-1 may be determined by standard immunochemical techniques which are well known to the skilled person (for example
20 immunohistochemistry, radioimmunoassay, ELISA, Western blot, fluorescence assay, DELFIA®, LANCE, FRET, etc). The method may preferably be carried out as a high throughput screen. The amount of arginine methylation of E2F-1 without incubation of the test substance is compared with the amount of arginine methylation of E2F-1 with incubation of the test substance. A decrease in the amount of arginine methylation of
25 E2F-1 indicates that the substance may be useful in the prevention or treatment of cancer.

Examples

- 30 The following examples are illustrative of the products and methods falling within the scope of the present invention. They are not to be considered in any way limitative of the invention.

Example 1: E2F-1 is methylated by PRMT5.

A comparison with p53 identified a sequence motif in E2F-1, RGRGR, with similarity to the known sites of arginine methylation in p53, namely RGRER. Figure 1A shows the location of E2F-1 RGRGR sequence motif in E2F-1, highlighting the similarity with the region in p53 targeted by PRMT5 (Jansson et al 2008). It is consistent with this observation that wild-type E2F-1 could be methylated *in vitro* by the arginine methyltransferase PRMT5.

To assess whether the RGRGR motif was a direct target for PRMT5 methylation, a series of mutant derivatives of E2F-1 were made in which each arginine (R) residue was substituted with a lysine residue (K; Figure 1a), and the ability of the mutant derivative to be methylated *in vitro* then assessed. Figure 1b shows the *in vitro* methylation of E2F-1 and the various mutant derivatives. In Figure 1b(i) each of the indicated proteins (about 1µg) was used in the methylation reaction. *In vitro* methylated samples were analysed by SDS-PAGE as described below. E2F-1 is indicated. In Figure 1b(ii) samples in Figure 1b(i) were counted (3H cpm as indicated). Histones treated with PRMT5 served as the positive control. The data shown provide one example taken from two different experiments.

It can be seen from Figure 1b that mutation of any of the three R residues resulted in reduced methylation, although R111K and R113K exhibited a greater level of reduction compared to R109K. The double R111/113K (KK) and triple R109/111/113K (KKK) mutants could not be methylated by PRMT5, suggesting that R111 and R113 are the predominant sites of methylation. Histones methylated with Set7 served as a positive control.

It was then assessed whether E2F-1 could interact with PRMT5 and thereafter the role of each R residue in mediating the interaction. In an *in vitro* binding assay where GST-E2F-1 was incubated with cell extracts, wild-type E2F-1 bound to PRMT5. Figure 1c shows the binding of E2F-1 to PRMT5. The indicated GST proteins were incubated with U2OS cell lysate, and bound proteins immunoblotted using anti-GST (for input GST), DP-1 or PRMT5 antibodies. Binding to DP-1, the major heterodimeric partner for E2F-1 (Stevens and La Thangue 2003), served as a control since it bound equally to

each of the E2F-1 proteins. The single R111K and R113K substitution mutants exhibited compromised PRMT5 binding compared to the interaction between wild-type and PRMT5, whereas R109K exhibited significant PRMT5 binding activity, although a modest reduction was apparent. Both the KK and KKK mutants were completely
5 devoid of PRMT5 binding, which co-incided with their inability to undergo methylation *in vitro*.

To establish whether E2F-1 was able to bind to PRMT5 in cells, the interaction between ectopic wild-type E2F-1 or KKK with PRMT5 was studied. Figure 1d shows
10 that E2F-1 and PRMT5 do interact in cells. Either a wild-type E2F-1 expression vector or a KKK expression vector was transfected into U2OS cells, and HA11 antibody was used for immunoprecipitation (IP) followed by immunoblotting with HA11 (E2F-1), PRMT5 or DP-1 as indicated. The level of input (In) protein is indicated. As seen in
15 Figure 1d wild-type E2F-1 co-immunoprecipitated with PRMT5, which was not apparent with the KKK mutant. In contrast, binding to DP-1 was equivalent for both wild-type E2F-1 and KKK. These results indicate that E2F-1 and PRMT5 interact in cells. Further, the RGRGR motif is necessary for the interaction, which also encompasses the site of PRMT5 methylation.

20 Next E2F-1 was immunoprecipitated with either anti-E2F-1 or anti-PRMT5 from U2OS cells and subsequently immunoblotted with anti-PRMT5 or anti-E2F-1 as indicated in Figure 1e. The input (In) and control (-) immunoprecipitations are indicated. The results show that the endogenous proteins interact.

25 **Example 2: E2F-1 is methylated in cells.**

It was investigated whether E2F-1 is methylated in cells using a site-specific methylated-arginine peptide antibody, prepared against a methylated RGRGR peptide taken from E2F-1, in which the predominant sites of methylation, R111 and R113, were symmetrically methylated. As shown in Figure 2a, anti-MeR-E2F-1 recognised
30 ectopic wild-type E2F-1 immunoprecipitated from cells, but failed to react with either the KK or KKK mutants, indicating that the antibody recognised the relevant region in E2F-1. Either wild-type E2F-1, KK or KKK expression vectors were transfected to HeLa cells and immunoprecipitated (IP) with HA11, followed by immunoblotting (IB)

with anti-MeR E2F-1, HA11 (E2F-1) or DP-1 antibodies. The level of input (In) protein is indicated.

The specificity of the antibody for arginine methylated E2F-1 was established by immunoprecipitating ectopic E2F-1, followed by immunoblotting with anti-MeR-E2F-1 in the presence of competing matched E2F-1 peptides that differed only in the methylation status of R111 and R113. In Figure 2b, either wild-type E2F-1, KK and KKK expression vectors were transfected to HeLa cells and immunoprecipitated (IP) with HA11, followed by immunoblotting with anti-MeR E2F-1 or HA11 (E2F-1), either in the presence of methylated (Me-E2F-1) or unmethylated E2F-1 peptide (1µg), as indicated. Two different exposures (short and long) of the HA11 (E2F-1) blot are shown. Anti-MeR-E2F-1 recognised the wild-type protein, and its binding activity was competed by the methylated but not the unmodified peptide (Figure 2b, compare tracks 6 and 10). As expected, neither the KK nor KKK mutants were recognised by the anti-MeR-E2F-1 antibody (Figure 2b). These results establish that the anti-MeR-E2F-1 peptide antibody detects methylated E2F-1 and, further, that E2F-1 expressed ectopically in cells undergoes arginine methylation.

The anti-MeR-E2F-1 antibody was used to study the methylation of endogenous E2F-1 by immunoprecipitation of E2F-1 followed by immunoblotting with anti-MeR-E2F-1. E2F-1 was found to be methylated in different cell types, including HeLa, C33A and U2OS cells (Figure 2c and d). In Figure 2c, HeLa or U2OS cell lysates were harvested and immunoprecipitated (IP) with anti-E2F-1 (KH95) or control antibody, followed by immunoblotting with anti-MeR E2F-1 or anti-E2F-1 antibodies. The level of input (In) E2F-1 protein is shown.

Moreover, E2F-1 methylation was regulated upon DNA damage with etoposide (Figure 2d). In Figure 2d, E2F-1 arginine methylation is regulated upon DNA damage treatment. U2OS, HeLa or C33A cells were treated with etoposide (+; 10µM for 16h) or left untreated (-), extracts prepared and subsequently immunoblotted with anti-PRMT5, anti-E2F-1, anti-MeR E2F-1 or actin (loading control) as indicated.

Further, methylated E2F-1 was present in nuclei (Figure 2e). In Figure 2E, U2OS cells transfected with E2F-1 expression vector were immunostained with anti-E2F-1 or anti-MeR E2F-1 in the presence or absence of competing methylated E2F-1 peptide as indicated. DAPI shows the location of nuclei.

5

These results indicate that endogenous E2F-1 undergoes arginine methylation, and further show that the level of arginine methylation is regulated upon DNA damage treatment.

10 **Example 3: The effect of E2F-1 methylation.**

Since arginine methylation of E2F-1 is regulated by DNA damage, and because E2F-1 is induced upon DNA damage (Stevens and La Thangue 2003; Iaquinta and Lees 2007; Polager and Ginsberg 2008), it was considered that arginine methylation may play a role in the controlling E2F-1 stability. When the level of ectopic protein was compared, both the KK and KKK mutants were expressed at increased levels compared to wild-type E2F-1. Figure 3a shows the levels of wild-type E2F-1, KK and KKK after transfection (1µg) into U2OS cells. '-' in the left lane indicates untransfected cells. In part, this difference reflected their altered protein stability, because each of the KK and KKK mutants exhibited an increased half-life compared to wild-type E2F-1. Figure 3b shows the stability of wild-type E2F-1, KK and KKK mutants. Expression vectors (1µg) encoding wild-type E2F-1 (WT), KK and KKK mutants were transfected into U2OS cells for 48h. Cells were treated with 100µg/ml of cyclohexamide, and then harvested at 0, 2h, 4h, 6h post treatment time points as indicated for subsequent immunoblotting (i), and further quantitated (ii). The HA11 antibody was used for immunoblotting and actin served as protein loading control; n=2. Both the KKK (solid line) and KK (dashed line) mutants had similarly increased half-life compared to wild-type (dotted line) E2F-1 (75 to 25 min respectively). These results suggest that arginine methylation of E2F-1 negatively regulates protein stability.

30 To explore this possibility in greater detail and establish whether PRMT5 had a direct effect on E2F-1, PRMT5 in cells was depleted using siRNA and thereafter measured E2F-1 protein levels. The depletion of PRMT5 co-incided with increased levels of E2F-1 protein (Figure 3c), which is compatible with the increased level and stability of

the KK and KKK mutants (Figure 3b). Figure 3c shows that PRMT5 siRNA increases E2F-1 protein levels. PRMT5 (P) or control (C) non-targeting siRNA was transfected into U2OS cells, and cells harvested 72h post transfection with or without etoposide treatment (10 μ M) in the last 16h. Extracts were immunoblotted with anti-PRMT5 and
5 E2F-1, and GAPDH levels served as a loading control.

Further, E2F-1 RNA was higher under conditions of PRMT5 depletion, contrasting with the control siRNA treatment. Figure 3d shows E2F-1 RNA levels in PRMT5 siRNA treated cells. RNA was extracted from cells treated as described above, and
10 RNA levels of E2F-1 examined. RNA levels of 18S and GAPDH served as controls. This result might reflect the ability of E2F-1 protein to positively auto-regulate expression of the E2F-1 gene (Stevens and La Thangue 2003). Significantly, under conditions of PRMT5 depletion and the consequent increased E2F-1 levels, a variety of E2F target genes were induced, including p73, Chk1 and Chk2 and Cdc2. Figure 3E
15 shows protein levels of E2F-1 target genes in PRMT5 siRNA treated cells. PRMT5 (P) or control (C) non-targeting siRNA was transfected into U2OS cells and cells were harvested 72h post transfection with or without etoposide (Et; 10 μ M) or doxorubicin (Dox; 2 μ M) treatment in the last 16h. Extracts were immunoblotted with anti-PRMT5, E2F-1, p73, Chk2, Chk1 and Cdc2 as indicated. Levels of GAPDH served as the
20 loading control.

These results suggest therefore that arginine methylation regulates E2F-1 protein levels, which in turn impacts on the expression of downstream E2F target genes.

25 Figures 3f and g show that PRMT5 regulates the ubiquitination of E2F1. Figure 3f shows the ubiquitination of wild-type E2F-1 and the KKK mutant. H1299 cells were transiently transfected with expression vectors encoding wild-type (WT) E2F-1 or KKK (2 μ g), together with His6-ubiquitin (4 μ g) as indicated. Cells were treated with MG132 (20nM) for 4h before harvesting. Cell lysates and Ni²⁺ pull-down eluates
30 were analysed as described. The KKK E2F1 mutant (which cannot be methylated) is ubiquitinated at a lower level than WT E2F1, which explains its increased stability upon PRMT5 knockdown (see Figure 3c).

Figure 3g shows the effect of PRMT5 on E2F-1 ubiquitination. U2OS cells were transfected with PRMT5 (P) or control (C) siRNA. After 24h, cells were transfected with expression vectors encoding wild-type (WT) E2F-1 or the KKK mutant (2 μ g) and His6-ubiquitin (4 μ g) as indicated. Cells were treated with MG132 (20nM) for 4h before being collected. Cell lysates and Ni²⁺ pull-down eluates were analysed as described. Quantitation of ubiquitin signals was performed using ImageJ 1.43u, and the input protein levels are shown underneath. It can be seen that PRMT5 knockdown affects the ubiquitination of E2F-1 (compare lanes 2 to 4).

Figure 3h shows that E2F-1 arginine methylation is regulated upon DNA damage treatment. C33A or U2OS cells were treated with etoposide (Et+; 10 μ M for 16h) or left untreated (-), extracts prepared and subsequently immunoblotted with anti-E2F-1, anti-MeR E2F-1 or actin (loading control) as indicated. Figure 3h shows that in cancer cells grown in vitro, changes in E2F-1 protein levels coincide with changes in arginine methylation of E2F-1. Thus, the top blot shows overall levels of E2F-1, and the middle blot (MeR E2F-1) the level of methylated E2F-1 (note the inverse correlation).

Example 4: Functional consequences of arginine methylation.

To further explore the role of arginine methylation in the transcription properties of E2F-1, wild-type E2F-1 was compared with the KK and KKK mutants in a reporter-based transfection assay, using E2F-responsive promoters derived from E2F target genes. The assay was performed under conditions in which each mutant protein was expressed at an equivalent level to wild-type E2F-1 (Figure 4a and b), thus ruling out any difference in activity that could be attributed to protein levels. Under these conditions, both the KK and KKK mutants exhibited increased transcriptional activity compared to wild-type E2F-1. Figures 4a and b show the transcription properties of wild-type E2F-1, KK and KKK mutants. U2OS cells were transfected with expression vectors encoding WT or mutant E2F-1 protein, together with p73-luciferase (A) or cyclinE-luciferase (B) for 48h as indicated, and pCMV- β gal to monitor transfection efficiency. Relative luciferase activity (luciferase/ β gal) is shown together with expression level of ectopic proteins; n=3. These results suggest therefore that arginine methylation impacts on the ability of E2F-1 to activate transcription, and explains in part the increased expression of E2F target genes under conditions of PRMT5

depletion. Furthermore, under conditions of PRMT5 depletion through treatment with siRNA, E2F-1 responsive promoters were more active (Figure 4c). Figure 4c shows E2F transcription upon PRMT5 depletion. PRMT5 (P) or control (C) non-targeting siRNA was transfected into U2OS cells together with p73-luciferase (i) or cyclinE-luciferase (ii) for 48h, together with pCMV- β gal to monitor transfection efficiency. Relative luciferase activity is shown together with expression level of the ectopic proteins.

Example 5: Arginine methylation regulates apoptosis.

- Whether arginine methylation influences E2F-1-dependent apoptosis was investigated by comparing the ability of wild-type E2F-1 to the KK and KKK mutants to induce cells with a sub-G1 DNA content (Stevens et al 2003). When each protein was compared, both mutants exhibited increased levels of apoptosis relative to wild-type E2F-1. Figure 4d shows apoptosis induced by wild-type E2F-1, KK and KKK mutants. U2OS cells were transfected with expression vectors encoding wild-type E2F-1, KK and KKK as indicated for 48h and analysed by FACS as described. The graph represents the relative change in sub-G1 populations compared to the empty vector control treatment; n=2.
- Moreover, an increased level of apoptosis was also apparent when PRMT5 was depleted through siRNA in different cell types, in both unperturbed and DNA damaged cells. Figure 4e shows apoptosis upon PRMT5 depletion. PRMT5 (P) or control (C) non-targeting siRNA was transfected into U2OS cells and the cells were harvested 72h post-transfection (with or without etoposide (10 μ M) for 16h) and analysed by FACS. The graph (i) represents the relative number of sub-G1 cells (compared to the control treatment) in the indicated conditions; and (ii) the level of PRMT5 and E2F-1; n=3. Figure 4f shows apoptosis upon PRMT5 depletion in C33A cells (i) and levels of PRMT5 and E2F-1 (ii).
- Most importantly, the increased level of apoptosis seen upon PRMT5 depletion was dependent on E2F-1 activity, because in conditions where E2F-1 was compromised through treatment with E2F-1 siRNA, the level of apoptosis upon PRMT5 depletion was much less. Figure 4g shows that apoptosis upon PRMT5 depletion is dependent

upon E2F-1. PRMT5 (P), E2F-1 (E) or control (C) siRNA was transfected into C33A cells as indicated, with or without etoposide treatment, and the level of PARP cleavage, reflecting apoptosis, measured. Cleaved PARP is indicated by the arrow. These results indicate that arginine methylation regulates the apoptotic properties of E2F-1, and that
5 the apoptosis induced upon PRMT5 depletion is dependent upon E2F-1.

The results presented here show that PRMT5 can influence E2F-1-dependent apoptosis, and identify the arginine residues in E2F-1 which are modified by PRMT5. Because E2F-1 mutants that are compromised in methylation exhibited heightened apoptotic
10 activity, our results argue that arginine methylation acts to negatively regulate apoptosis. It is consistent with a role for arginine methylation in regulating apoptosis that depleting PRMT5 in cells caused a co-incident increase in apoptosis. However, the enhanced apoptosis activity not only reflected increased E2F-1 protein levels, but also altered functional properties. For example, when expressed at equivalent protein
15 levels, both the KK and KKK mutants, which are devoid of arginine methylation, possessed increased activity. Arginine methylation therefore regulates a number of different properties in E2F-1, which have the combined effect to regulate E2F-1-dependent apoptosis.

20 Arginine methylation targets the p53 protein, and impacts on the physiological outcome of the p53 response (Jansson et al 2008). That arginine methylation of p53 prompts cell cycle arrest, rather than apoptosis (Jansson et al 2008), is compatible with the observations described herein. Namely, as shown in Figure 5, when E2F-1 is methylated, its apoptotic activity is held in check (through for example low protein
25 levels and reduced expression of target genes), allowing arginine methylated p53 to over-ride E2F-1 and prompt cell cycle arrest. In contrast, under conditions that limit arginine methylation (for example PRMT5 siRNA), E2F-1 levels and apoptotic activity increase, which over-rides the cell cycle arrest activity imposed by p53. Arginine methylation therefore allows a critical level of interplay to occur between the pathways
30 controlled by E2F-1 and p53. By enabling crosstalk between E2F-1 and p53, arginine methylation is able to control a key decision point, dictating whether apoptosis or cell cycle arrest is the predominant outcome. Arginine methylation therefore provides a

signal, and PRMT5 the mechanism, through which E2F-1 and p53 activity is integrated.

Example 6: Arginine methylation and apoptosis.

- 5 In this example the effect of manipulating the levels of PRMT5 in cells on apoptosis was examined, as was the effect of co-manipulating E2F-1.

Figure 6a shows apoptosis upon PRMT5 depletion. PRMT5 (P) or control (C) non-targeting siRNA (25nM) was transfected into HCT116 p53+/+ (i), HCT116 p53-/- (ii) 10 or U2OS (iii) cells and the cells were harvested 72h post-transfection and analysed by FACS. The graph represents the actual number of sub-G1 cells in the indicated conditions, and the immunoblot the level of PRMT5 and actin control; n=3. It can be seen that depleting PRMT5 in a variety of tumour cells (independent of p53 status) induces apoptosis (top graph % sub-G1 cells).

15

Figure 6b shows apoptosis upon PRMT5 depletion. PRMT5 (P) or control (C) non-targeting siRNA was transfected into SAOS2 together with an expression vector encoding wild-type E2F-1 (1µg) as indicated, harvested at 72h and processed as described in (a); n = 3. It can be seen that depleting PRMT5 causes enhanced levels of 20 apoptosis in the presence of ectopic E2F-1. This is consistent with the idea that PRMT5 mediates its effects through E2F-1.

Figure 6c shows the effect of PRMT5 siRNA in MEFs. PRMT5 (P) or control (C) non-targeting siRNA was transfected into MEFs and cells harvested at 72h post-transfection 25 as indicated. Cell extracts were immunoblotted with anti-PRMT5 and anti-E2F-1. Actin levels served as the loading control.

MEFs were treated as described in 6c and thereafter analysed by FACS. In Figure 6d the black bar represents the effect of C siRNA, and clear bar the effect of P siRNA; 30 n=3. Figures 6c and 6d show that increased apoptosis does not occur in normal non-cancer cells because no apoptosis in MEFs.

Figure 6e shows apoptosis upon PRMT5 and E2F-1 depletion. PRMT5 (P), E2F-1 (E) or control (C) non-targeting siRNA was transfected into U2OS cells and harvested 72h post-transfection and analysed by FACS as described. The graph shows the level of sub-G1 cells compared to the control treatment and the level of endogenous PRMT5 and E2F-1 is shown underneath; n = 3. It can be seen that the co-depletion of PRMT5 and E2F-1 (P/E in the figure) prevents the induction of apoptosis, in contrast to PRMT5 depletion alone, i.e. the induced apoptosis is mediated through E2F1 activity.

Figures 6f and 6g address the role of one of the key E2F target genes in the PRMT5 dependent apoptosis, i.e. p73. Figure 6f shows the effect of PRMT5 siRNA on p73. PRMT5 (P) or control (C) siRNA was transfected into U2OS cells and cells harvested at 72h post-transfection. Both RNA (i) and protein (ii) levels were measured, as indicated, for p73, 18S and GAPDH (i) and PRMT5, E2F-1, p73 and GAPDH (ii). Figure 6f shows that p73 is upregulated upon PRMT5 knockdown (both RNA and protein) and that p73 activity is also required for the apoptosis that occurs upon PRMT5 knockdown. This experiment supports activation of the E2F1 pathway leading to apoptosis upon PRMT5 knockdown, i.e. PRMT5 normally acts to suppress activation of the pathway.

Figure 6g shows apoptosis upon PRMT5 and p73 depletion. PRMT5 (P), p73 (73) or control (C) non-targeting siRNA was transfected into U2OS cells and harvested at 72h post-transfection and analysed by FACS. The graph represents the relative change in the sub-G1 population compared to the control treatment, and the level of PRMT5, E2F-1 and p73 is shown underneath; n=3.

Example 7: PRMT5 and E2F-1 expression in clinical tumour biopsies

Figure 7a shows representative images of PRMT5 and E2F-1 IHC in an MDA-MB-231 breast cancer cell xenograft showing examples of E2F-1 and PRMT5 staining cells. Note that both positive and negative E2F-1 and PRMT5 stained tumour cells are evident (indicated by arrows; 20x and 40x magnification, as indicated).

Figure 7b shows representative images of PRMT5 and E2F-1 IHC in human mantle cell lymphoma biopsy showing areas of PRMT5 positive and E2F-1 negative tumour cells.

Note that the expression level of E2F-1 was considerably less than PRMT5 in the lymphoma sections.

Figure 7c shows representative images of PRMT5 IHC in follicular lymphoma. The
5 follicles containing malignant cells which stain intensely with anti-PRMT5 are indicated (i), and at higher magnification in (ii).

Figures 7d and 7e show representative images of PRMT5 and E2F-1 IHC in CRC. Examples of tumours expressing low PRMT5/high E2F-1 (d) and high PRMT5/low
10 E2F-1 (e), and are shown, together with fields of increased magnification. The images in Figures 7d and 7e show that tumours do exist with either high PRMT5/low E2F1 versus low PRMT5/high E2F1. The present invention is particularly concerned with treating tumours with high PRMT5/low E2F1 with a PRMT5 inhibitor, in order to inactivate PRMT5 and thereby activate E2F1.

15

Materials & Methods

Cell culture

U2OS, C33A, MCF7, HCT116, SAOS2, HeLa, H1299 cells and MEFs were
20 maintained in DMEM with 5% FCS.

Antibodies

The following antibodies were used; anti-Flag peptide monoclonal antibody M2 (Sigma), anti-Flag peptide monoclonal antibody M2 coupled agarose beads (Sigma),
25 and anti-HA11 monoclonal antibody (Covance). PRMT5 antibody was from Upstate. GST, E2F1, Chk1, Cdc2 and p53 antibodies were monoclonal antibodies from Santa Cruz. DP-1, p73, Chk2 and GAPDH were polyclonal antibodies from Santa Cruz. β -actin monoclonal antibody was from Sigma. PARP monoclonal antibody was from BD Pharmingen.

30

Transfection

Gene Juice transfection reagent (Novagen, San Diego, CA) was used to transfect cells with DNA. All transfections with the indicated plasmids included a pCMV- β Gal

internal control for gauging transfection efficiency. Empty pcDNA3.1a vector was used to equalise amounts of transfected DNA where appropriate.

siRNA

5 Oligofectamine transfection reagent (Invitrogen) was used to transfect cells with siRNAs for 72h. siRNA treatment of cells was carried out using siRNA non-targeting (Dharmacon) as control or PRMT5 (5' CCGCUAUUGCACCUUGGAA (SEQ ID NO:1)). p73 smart pool siRNA was from Dharmacon. siRNA treatment was for 72h at 50nM.

10

Flow cytometry

Cells were transfected with the indicated expression vectors together with pBB14-GFP (for 48h) to monitor transfection efficiency or siRNA (for 48h or 72h) with or without treatment of the DNA damage agent for the last 16h. Then, cells were fixed and stained
15 with propidium iodide and analyzed by Flow Cytometry.

Reporter assay

Cells were transfected with expression vectors (1µg) encoding WT or the mutant E2F-1 proteins as indicated with E2F responsive luciferase reporters, namely p73-luciferase or
20 cyclinE-luciferase, together with pCMV-βgal to monitor transfection efficiency. Cells were harvested, lysed 48h post-transfection and luciferase activity measured as previously described (Jansson et al 2008).

Chromatin immunoprecipitation

25 U2OS cells were maintained in DMEM containing 5% foetal calf serum. Cells were transfected with 50nM PRMT5 siRNA or a non-targeting control (C) siRNA for 72h. Cells were cross-linked with formaldehyde (final concentration 1%). Chromatin was prepared as described previously (Zalmas et al., 2008). Antibodies used for immunoprecipitation were as follows: anti-E2F-1 (C-20, Santa Cruz Biotechnology),
30 and anti-acetyl-histone H3 (Millipore). The non-specific rabbit IgG used as a negative control in the ChIP assays was from Jackson ImmunoResearch. The recovered DNA was analyzed by semi-quantitative PCR. Primer sequences are available upon request.

Cyclohexamide half-life assay

Cells were transfected with WT or mutant HA-E2F1 plasmids for 48h as indicated. Cells were treated with 100ug/ml of cyclohexamide (Fluka) and then harvested at different time points as indicated for immunoblotting.

5

Preparation of whole cell extracts, immunoprecipitation and immunoblot analysis

Cells were either left untransfected or transfected with expression vectors as indicated. At 48h post transfection, cells were harvested in TNN buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.5% Ipegal, 50 mM NaF, 0.2 mM sodium

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orthovanadate and protease inhibitor cocktail (1mM PMSF, leupeptin [1 µg/ml], aprotinin [1µg/ml], and pepstatin A [1µg/ml])) and rotated at 4°C for 30min to 1h. The cell lysate was then homogenised, centrifuged at 11000rpm for 10min to remove cell debris and the supernatant collected. All lysates were normalized for protein concentration and, where appropriate, for transfection by β-galactosidase activity.

15

Immunoprecipitation was carried out by incubation of lysates with the indicated antibody and protein-A/G Sepharose beads or agarose-antibody conjugated at 4°C for 4h or overnight. The beads were collected and washed three times with TNN buffer (containing 0.25mg/ml Ipegal) before denaturation and SDS-PAGE. Protein was transferred to nitrocellulose and probed with the indicated antibody. Enhanced chemi-

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luminescence (Pierce Biotechnology, Rockford, IL) was used to visualize antibody binding.

Ubiquitination assays

Cells were transfected with pcDNA3.1a-ubiquitin (His-tagged) and treated as described. Briefly cells were harvested into 8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, and pH 8.0. Cell lysate was incubated with nickel (Ni²⁺-NTA)-agarose beads (Qiagen), 5 mM imidazole and 10 mM β-mercapthoethanol overnight at 4°C. The beads were collected and washed as described (Liu and Warbrick, 2006). Bound proteins were then eluted and assayed by immunoblotting.

30

Site-directed mutagenesis

The following primers were used:

5'-GAGCAGTGGGCCAGCTAAAGGCAGAGGCCGCC-3' (SEQ ID NO:8) and

5'-GGCGGCCTCTGCCTTTAGCTGGCCCCACTGCTC-3' (SEQ ID NO:9) were used for making mutant E2F-1 R109K.

5'-AGCTCGGGGCAAAGGCCGCCATC-3' (SEQ ID NO:10) was used for making mutant E2F-1 R111K.

5 5'-GGGCAGAGGCAAACATCCAGGAA-3' (SEQ ID NO:11) was used for making mutant E2F-1 R113K.

5'-AGTGGGCCAGCTCGGGGCAAAGGCAAACATCCAGGAAAAGGTGTG-3' (SEQ ID NO:12) was used for making mutant E2F-1 KK.

5'-

10 AGAGCAGTGGGCCAGCTAAGGGCAAGGGCAAGCATCCAGGAAAAGGTGTG-3' (SEQ ID NO:13) was used for making mutant E2F-1 KKK.

Quick Change Mutagenesis Kit (Stratagene) was used.

Purification of GST-E2F1 fusion proteins

15 Wild-type GST-E2F1 and the GST-E2F1 mutants were expressed in *E.coli* BL21-DE3 (Stratagene) cells by induction with isopropyl β -D-1-thiogalactopyranoside (IPTG) (0.1mM). Cell pellets resuspended in lysis buffer consisting of phosphate buffered saline containing protease inhibitor cocktail (1mM PMSF, leupeptin [1 μ g/ml], aprotinin [1 μ g/ml], and pepstatin A [1 μ g/ml]) were sonicated. 1% Triton-X100 was
20 then added and the cells lysed at 4°C for 30min. Following centrifugation at 13,000rpm for 30 min the supernatants were incubated with glutathione affinity resin (Pharmacia) for 2h at 4°C. The beads were then washed three times with lysis buffer containing 0.1% Triton-X100, once with lysis buffer without Triton-X100 and then once with elution buffer (50mM Tris-HCl pH8, 50mM NaCl). GST fusion proteins were eluted
25 from the beads by using 20mM glutathione dissolved in elution buffer.

***In vitro* methyltransferase assays**

Flag-PRMT5 plasmid was transfected into U2OS cells for 48h. Cells were lysed and Flag-PRMT5 was immunoprecipitated with agarose beads and then eluted. The elute
30 was mixed with recombinant substrates (GST fusion proteins or histones) in methylation reaction buffer (50mM Tris, 0.1mM EDTA, 50mM NaCl) with ³H labeled SAM (as -CH₃ group donor) to a volume of 40 μ l and incubated at 30°C for 90 min. Half of the reactions were then spotted onto p81 membrane circles (Whatman) and air

dried. The membranes were then washed three times, 5min each in 50ml of wash buffer (46mM NaHCO₃, 4mM Na₂CO₃, pH 9.2). After washing briefly with acetone, membranes were air dried, placed in scintillation vials, immersed in scintillation fluid (Beckman Coulter) and disintegrations per minute (DPM) measured in a scintillation counter. The other half of the reactions were run on SDS-PAGE gel and used to detect ³H auto-radioisotope/ methylation signal.

GST binding assay

GST or GST tagged proteins (10µg each) were incubated with agarose beads and damaged or undamaged U2OS cell lysates for 3h. Beads were then washed with TNN buffer 3 times. SDS sample buffer was added to beads and samples were analysed by immunoblotting.

Preparation and purification of anti-MeR E2F-1 antibody

The E2F-1 peptides, either unmethylated (CESSGPARGRGRHPGKG (SEQ ID NO:7)) or with R111 and R113 symmetrically methylated (sequence) (CESSGPARGR(Me)GR(Me)HPGKG (SEQ ID NO:6)), were injected into rabbits for antibody preparation (Alta Bioscience). At the appropriate time, rabbit serum diluted in PBS was purified by peptide affinity chromatography as described (Jansson et al 2008).

Histopathology

Paraffin-embedded formalin-fixed tissue from different tumour types were cleared of paraffin in CitrocLEAR, and rehydrated through graded alcohol baths. After a rinse in water, sections were heated at 95°C for 20min in 10mM sodium citrate, pH 6 for antigen retrieval. Slides were incubated in 0.5% hydrogen peroxide for 15min to inactivate endogenous peroxidases. Slides were washed in phosphate buffered saline/0.1% Tween 20 (PBST) for 5min before being blocked in serum for 20 min. Slides were incubated in primary antibodies; anti-PRMT5 or anti-E2F-1 overnight at 4°C. This was followed by corresponding anti-HRP secondary antibodies, ABC reagent (Vectorlabs) and then substrate (DAB; Vectorlabs). Slides were placed in hematoxylin (Sigma) for 5s for nuclear counterstaining and then mounted with coverslips using AquaTex (Merck). Sections were examined under a light microscope.

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Claims

1. A method for the treatment of a proliferative disease comprising administering a
5 substance which reduces the expression and/or activity of PRMT5.
2. A method according to claim 1 wherein the substance reduces the catalytic
activity of PRMT5, preferably reducing or abolishing the ability of PRMT5 to
methylate E2F1 protein.
- 10 3. A method according to claim 1 or 2 wherein the method comprises antisense or
RNA interference (RNAi), preferably the administration of an antisense molecule, an
shRNA or an siRNA.
- 15 4. A method according to claim 3 wherein the siRNA comprises a double stranded
oligonucleotide comprising the sequence 5' CCGCUAUUGCACCUUGGAA (SEQ ID
NO:1) or a sequence with at least 90% identity thereto, or 5'
CAACAGAGAUCCUAUGAUU (SEQ ID NO:2) or a sequence with at least 90%
identity thereto.
- 20 5. A method according to claim 3 or 4 wherein the oligonucleotide is modified to
improve its stability and/or potency and/or to enable systemic delivery.
6. A method according to claim 1 wherein the method comprises administering a
25 small molecule inhibitor of PRMT5.
7. A method for the treatment of a proliferative disease comprising providing a
E2F-1 protein which is arginine-methylation defective.
- 30 8. A method according to claim 7 which comprises administering an E2F-1 protein
which is arginine-methylation defective, or an oligonucleotide which encodes such a
protein.

9. A method according to claim 8 wherein the E2F-1 protein which is arginine-methylation defective is selected from an E2F-1 protein which is mutated so that one or more of arginine residues R109, R111 and R113 are substituted with residues that are resistant to methylation, preferably the arginine residue(s) are substituted with lysine residue(s).

10. A method according to any preceding claim wherein the proliferative disease is cancer.

10 11. An E2F-1 protein which is arginine-methylation defective, or an oligonucleotide which encodes such a protein, or a substance which reduces the expression or activity of PRMT5, for the treatment of a proliferative disease.

12. Use of (i) an E2F-1 protein which is arginine-methylation defective, or (ii) an oligonucleotide which encodes such a protein, or (iii) a substance which reduces the expression or activity of PRMT5, for the manufacture of a medicament for the treatment of a proliferative disease.

13. A pharmaceutical composition comprising (i) an E2F-1 protein which is arginine-methylation defective, or (ii) an oligonucleotide which encodes such a protein, or (iii) a substance which reduces the expression or activity of PRMT5.

14. An antibody which specifically binds to arginine-methylated E2F-1 protein, or epitope-binding fragment thereof.

15. An antibody according to claim 14 which specifically binds to a peptide comprising a sequence according to SEQ ID NO:5 or SEQ ID NO:6.

16. An antibody according to claim 14 or 15 which is a monoclonal antibody.

17. A method of identifying a proliferative disease which may be susceptible to treatment by the inhibition of PRMT5 which comprises the use of an antibody according to any of claims 14 to 16, or epitope-binding fragment thereof.

18. Use of an antibody according to any of claims 14 to 16, or epitope-binding fragment thereof, to detect E2F-1 methylation.

5 19. A method of determining if a subject will benefit from treatment with a PRMT5 inhibitor comprising evaluating a biological sample from said subject for the presence and/or concentration of methylated E2F-1 protein,

wherein the presence of methylated E2F-1 protein indicates that the subject will benefit from treatment with a PRMT5 inhibitor, or

10 wherein an elevated concentration of methylated E2F-1 protein in the biological sample indicates that the subject will benefit from treatment with a PRMT5 inhibitor.

20. A method of determining if a subject will benefit from treatment with a PRMT5 inhibitor comprising evaluating a biological sample from said subject for the presence and/or concentration of PRMT5 protein,

15 wherein the presence of PRMT5 protein indicates that the subject will benefit from treatment with a PRMT5 inhibitor, or

wherein an elevated concentration of PRMT5 protein in the biological sample indicates that the subject will benefit from treatment with a PRMT5 inhibitor.

20

21. A method of determining the prognosis of a subject comprising evaluating a biological sample from said subject for the presence and/or concentration of methylated E2F-1 protein and/or PRMT5 protein.

25 22. The method of any of claims 19 to 21 wherein the subject has a proliferative disease.

23. The method of claim 22 wherein the biological sample is from a tumour.

30 24. The method of any of claims 19 or 21 to 23 which comprises the steps of:

(i) contacting a biological sample of said patient with an antibody according to any of claims 14 to 16, or epitope-binding fragment thereof,

(ii) measuring the binding of said antibody or epitope-binding fragment thereof to its antigen, and

(iii) comparing the binding in part (ii) with that of a normal reference subject or standard.

5

25. A kit for use in identifying a proliferative disease which may be susceptible to treatment by the inhibition of PRMT5, or for determining if a subject will benefit from treatment with a PRMT5 inhibitor, or for determining the prognosis of a subject, comprising an antibody according to any of claims 14 to 16, or epitope-binding
10 fragment thereof.

26. A method for screening for a substance, or a salt or a solvate thereof, to be used in the prevention or treatment of a proliferative disease, which comprises the following steps:

- 15 (i) incubating PRMT5 and E2F-1 with and without a substance of interest, and
(ii) measuring the amount of arginine methylation of E2F-1,
(iii) comparing the amount of arginine methylation of E2F-1 without incubation of the test substance with the amount of arginine methylation of E2F-1 with incubation of the test substance, wherein a decrease in the amount of arginine methylation of E2F-1
20 1 indicates that the substance may be useful in the prevention or treatment of cancer.

27. An E2F-1 protein which is arginine-methylation defective comprising a sequence according to SEQ ID NO:3 in which one or more of residues R109, R111 and R113 are substituted with residues that are resistant to methylation.

25

28. An oligonucleotide which encodes a protein according to claim 27.

29. A double stranded oligonucleotide comprising the sequence 5' CCGCUAUUGCACCUUGGAA (SEQ ID NO:1) or a sequence with at least 90%
30 identity thereto, or 5' CAACAGAGAUCCUAUGAUU (SEQ ID NO:2) or a sequence with at least 90% identity thereto.

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Fig. 1

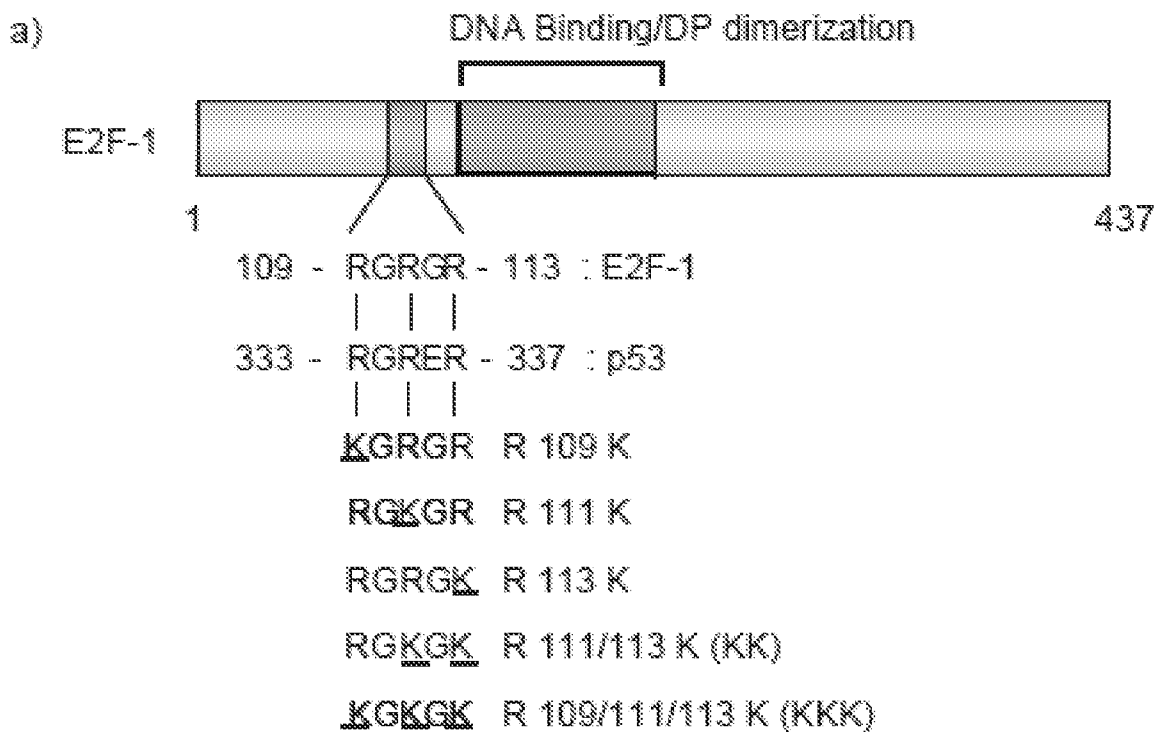


Fig. 1

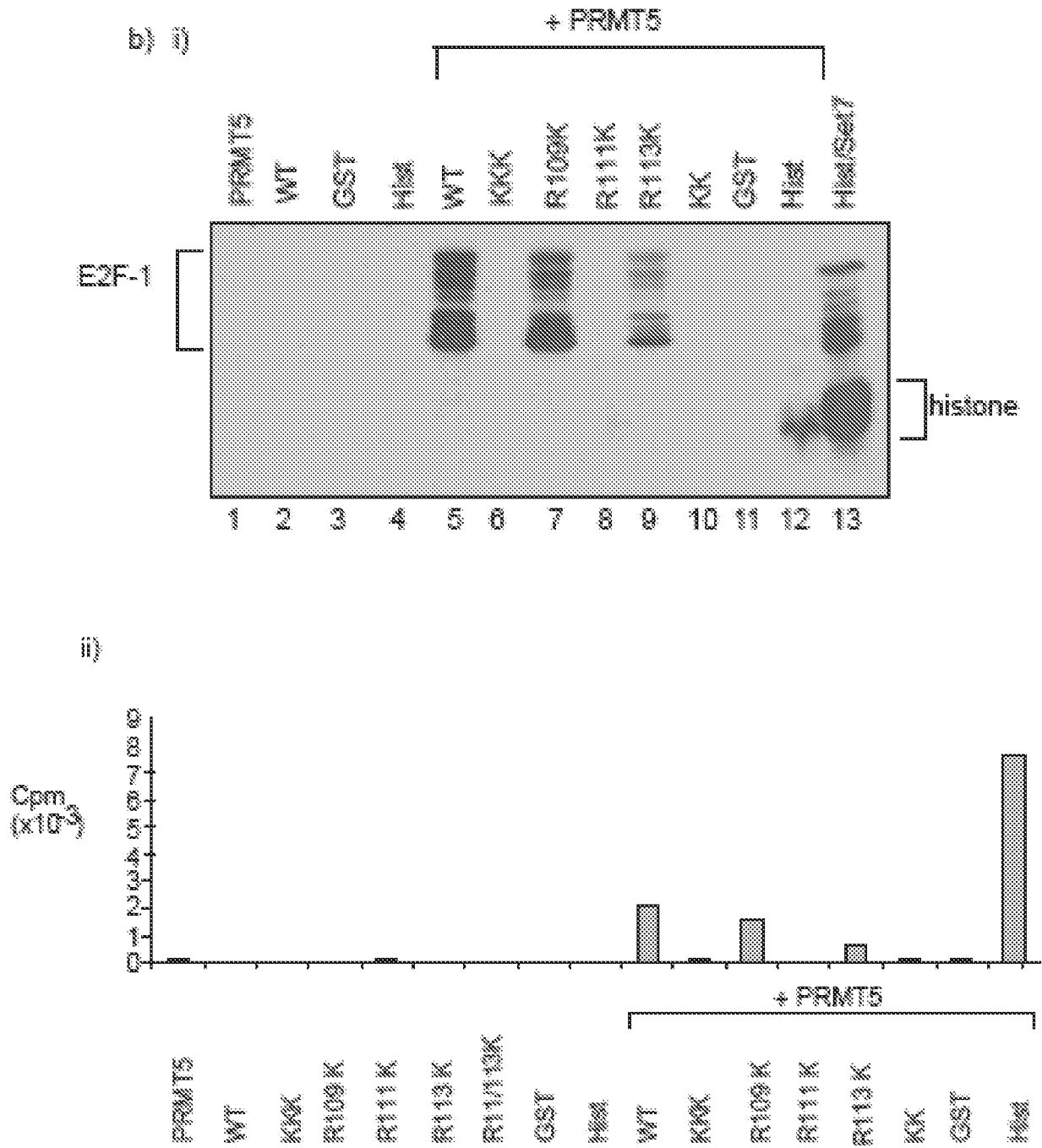
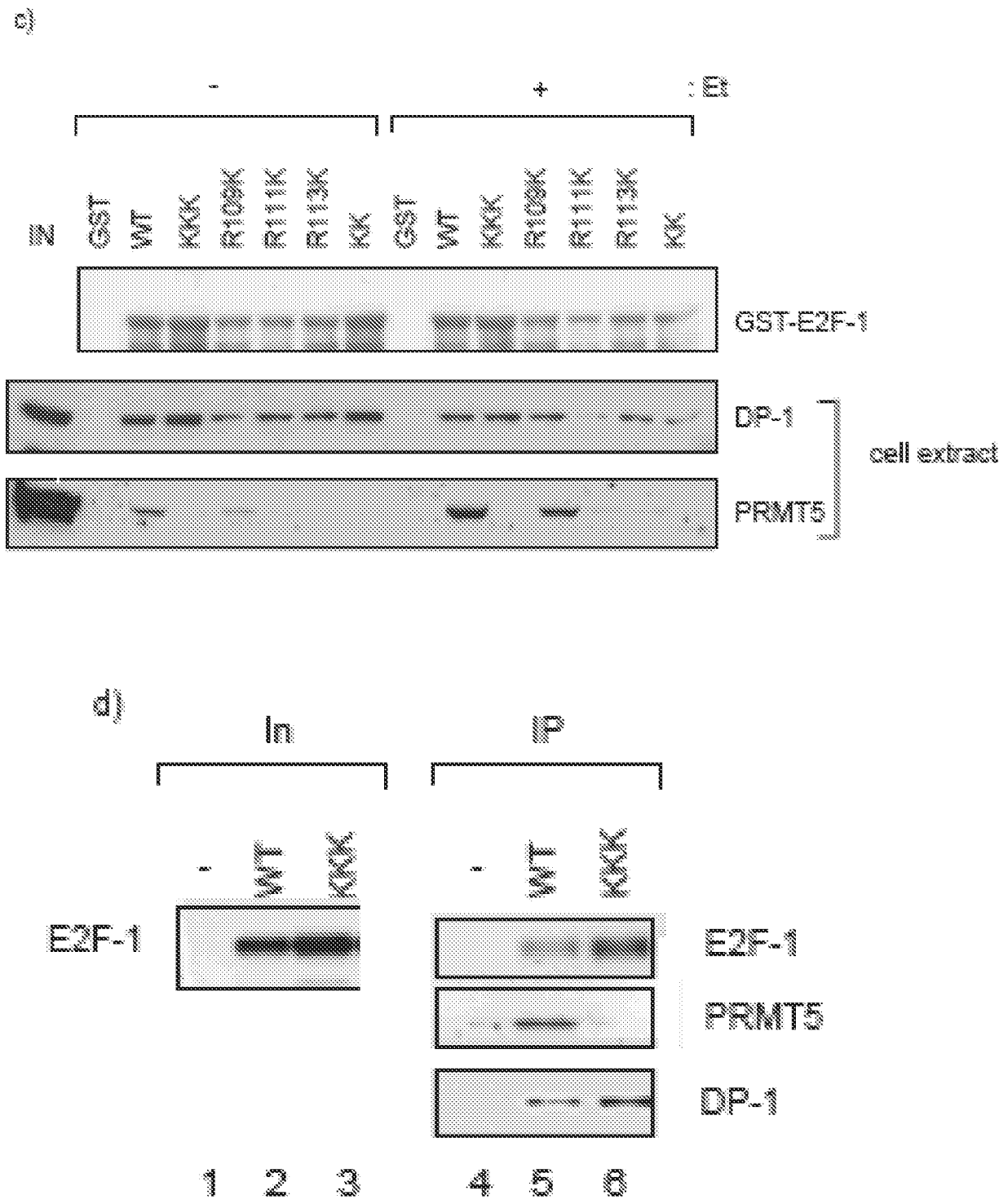
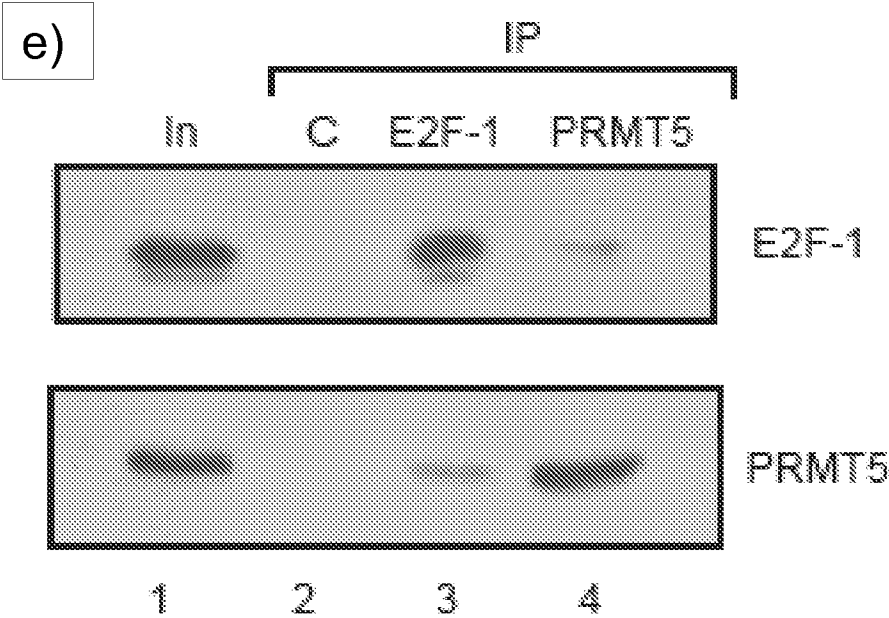


Fig. 1



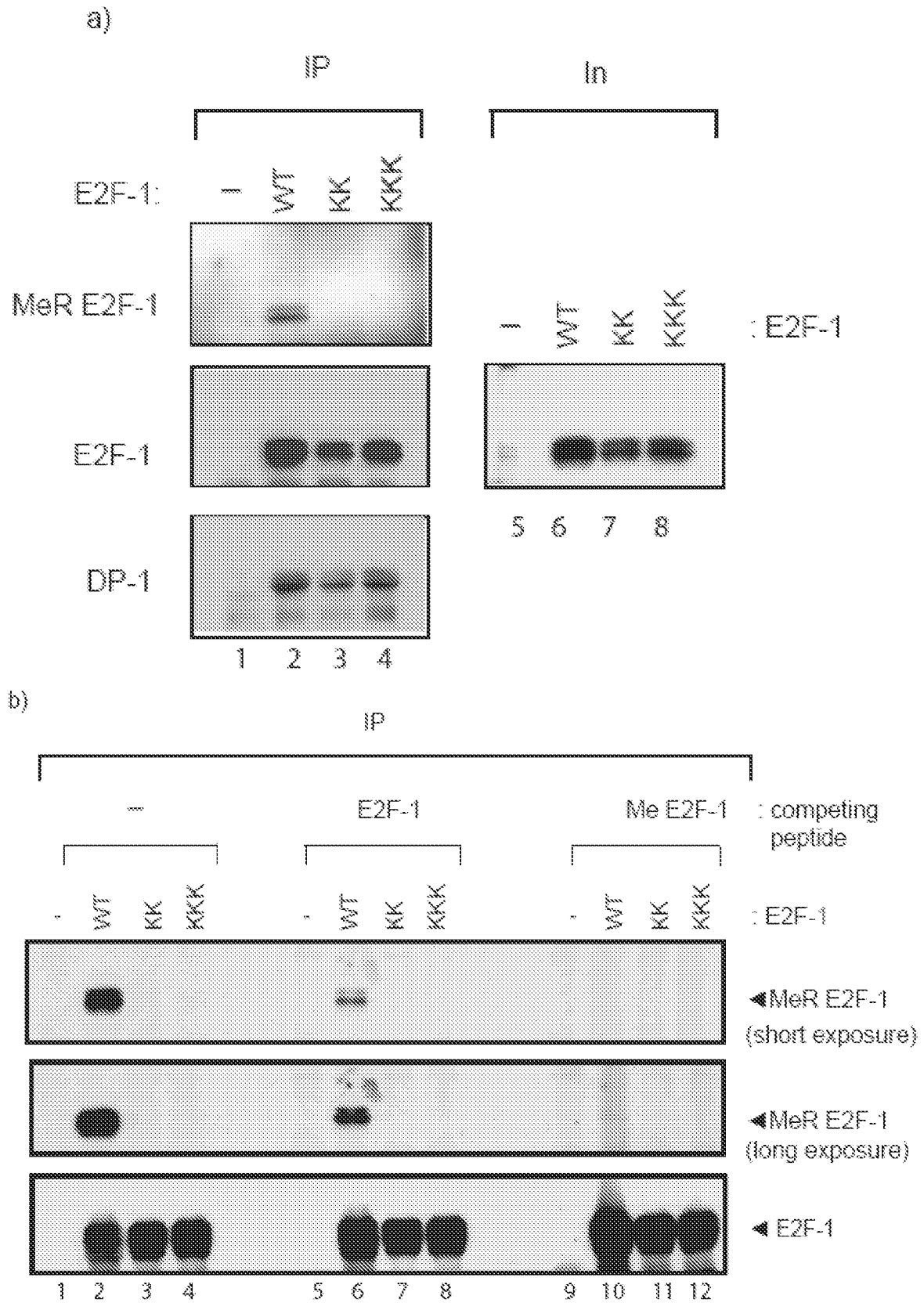
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Fig. 1



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Fig. 2



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Fig. 2

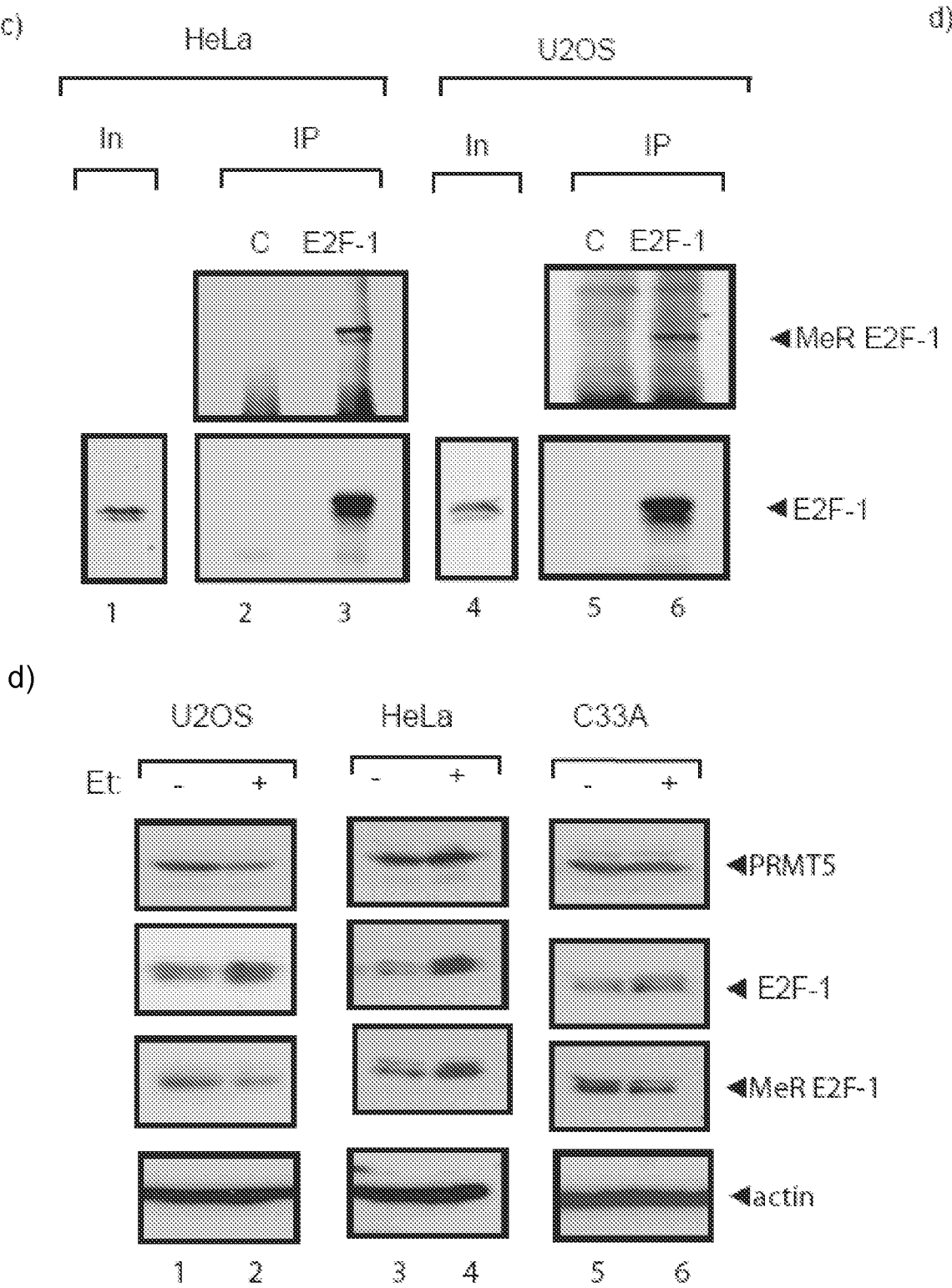
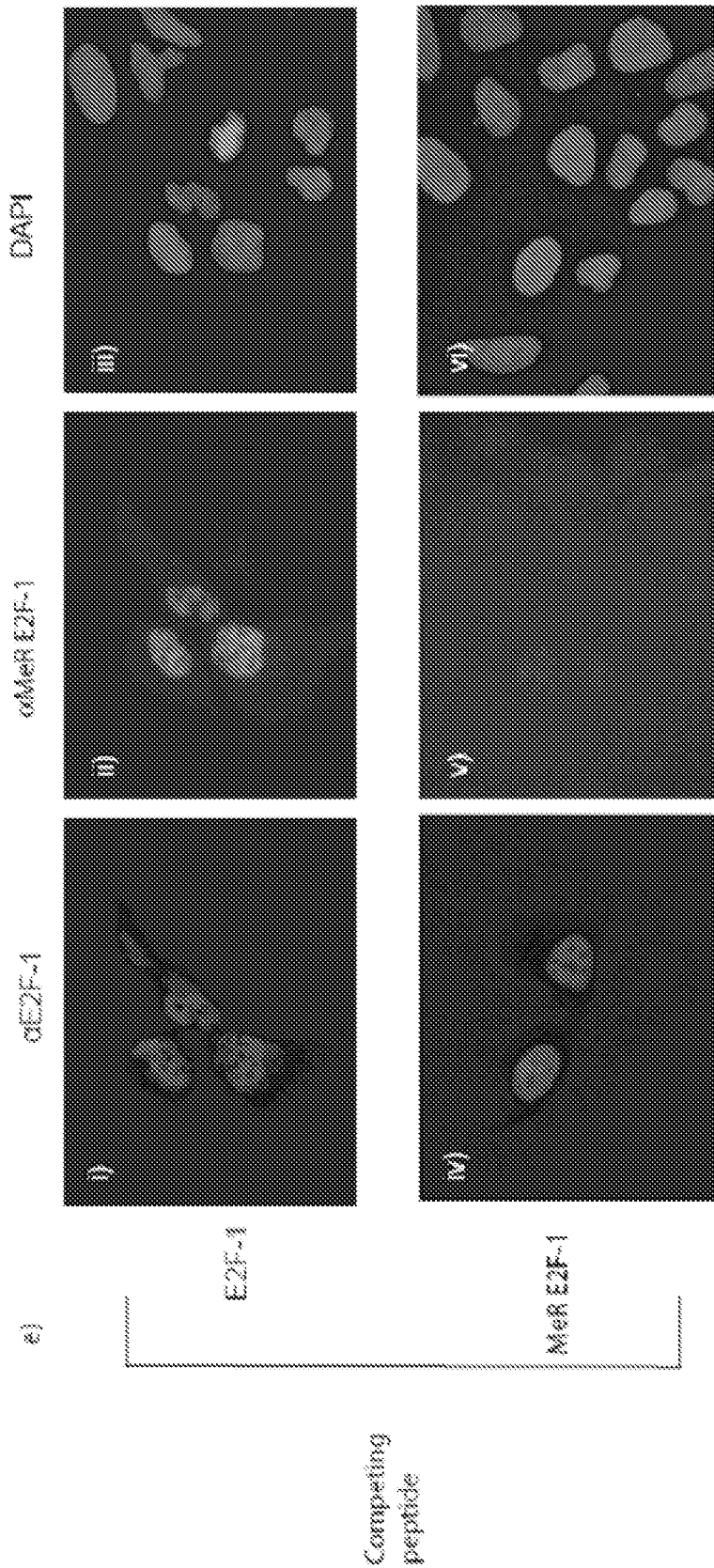


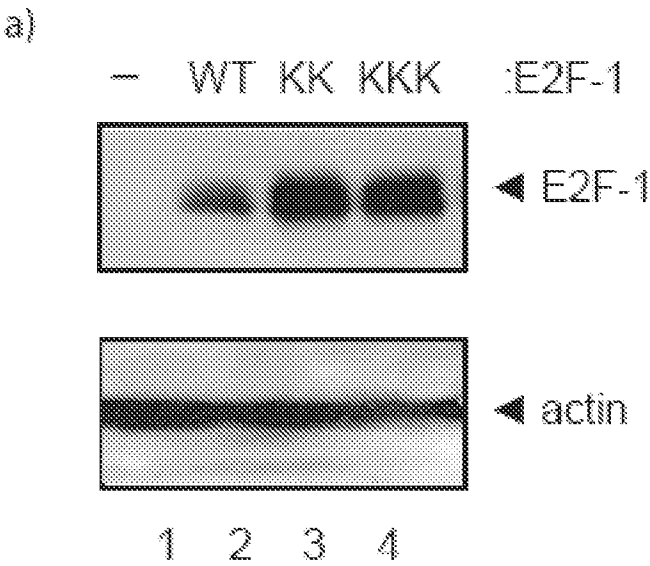
Fig. 2

e)



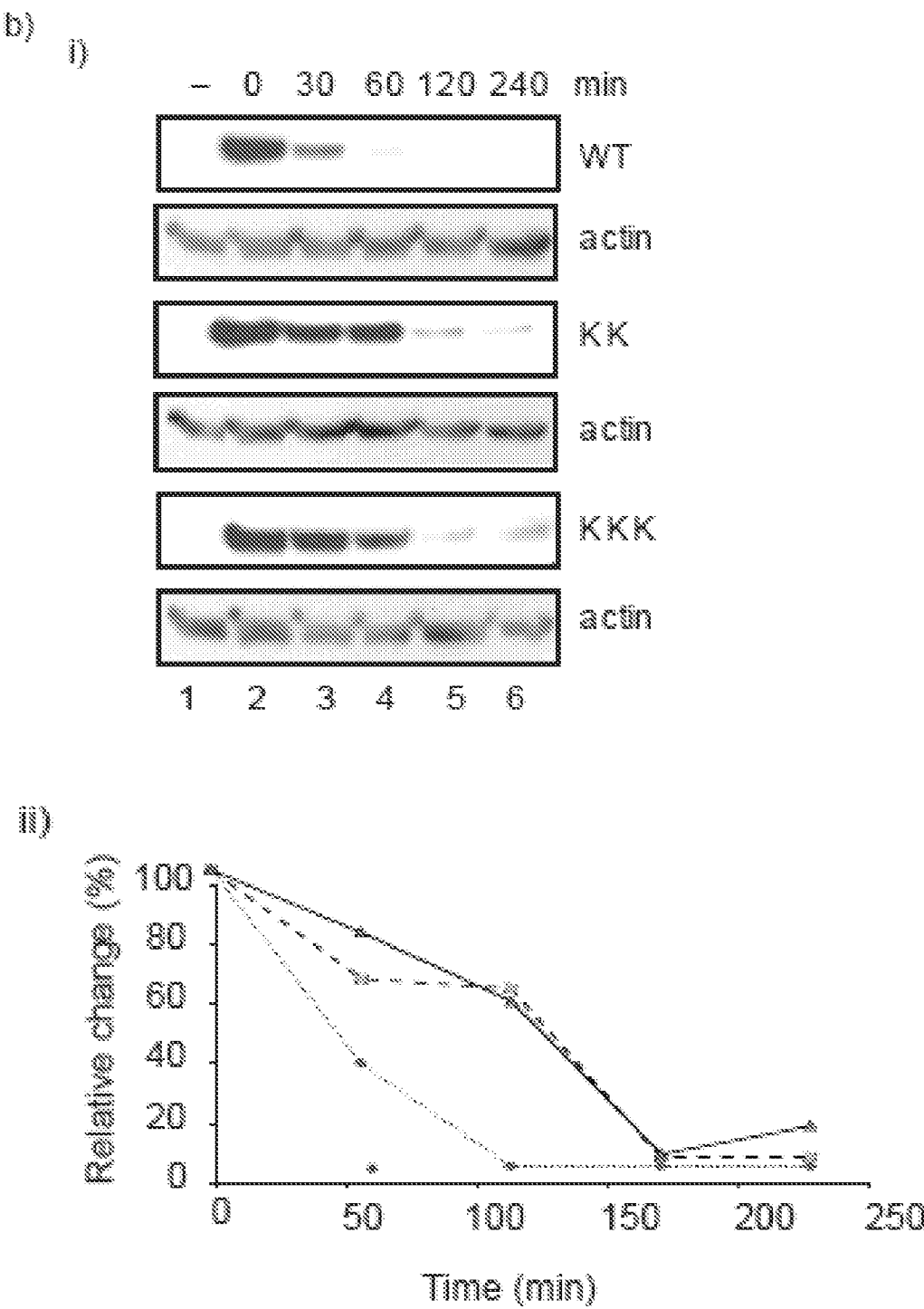
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Fig. 3



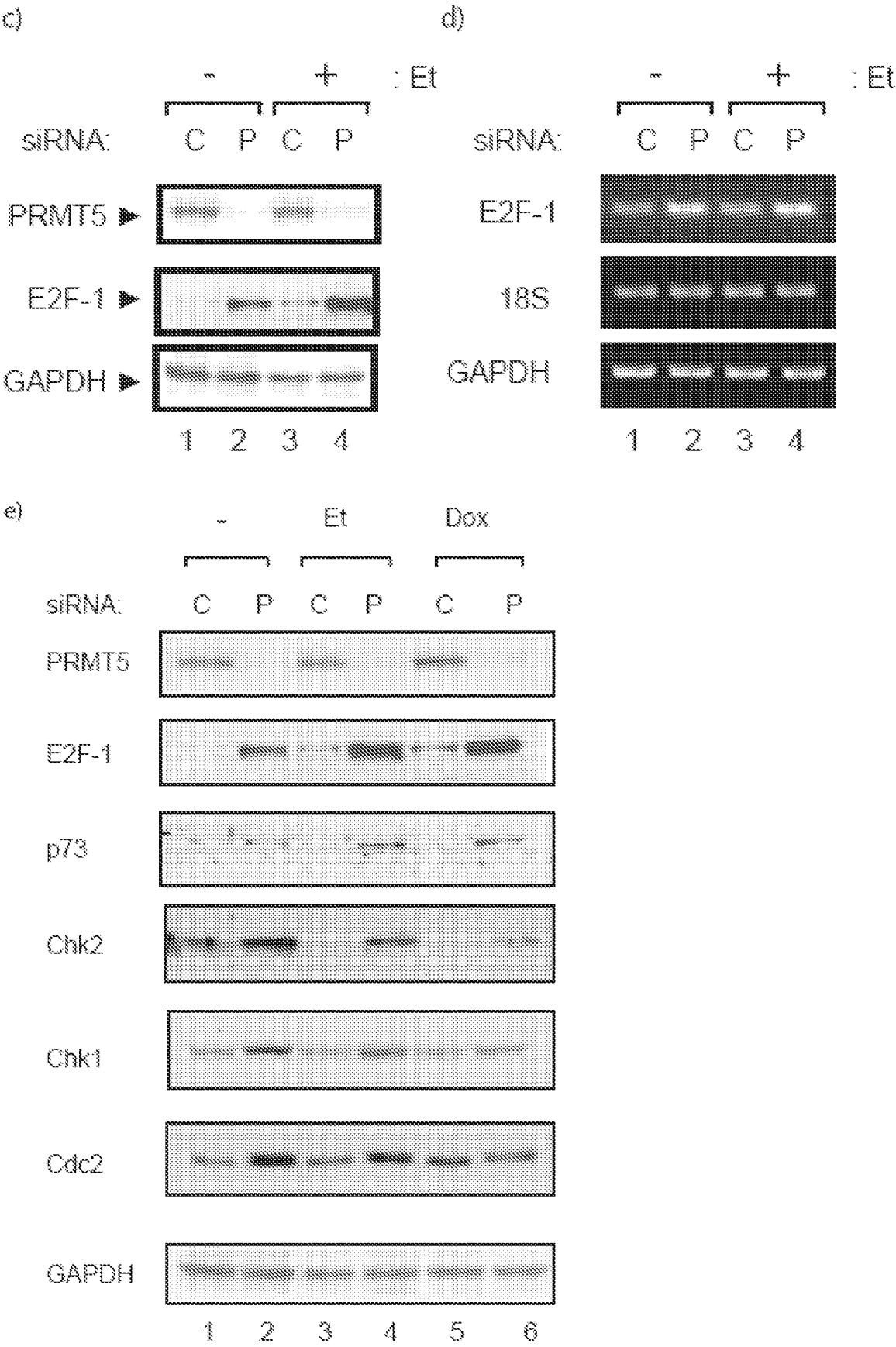
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Fig. 3



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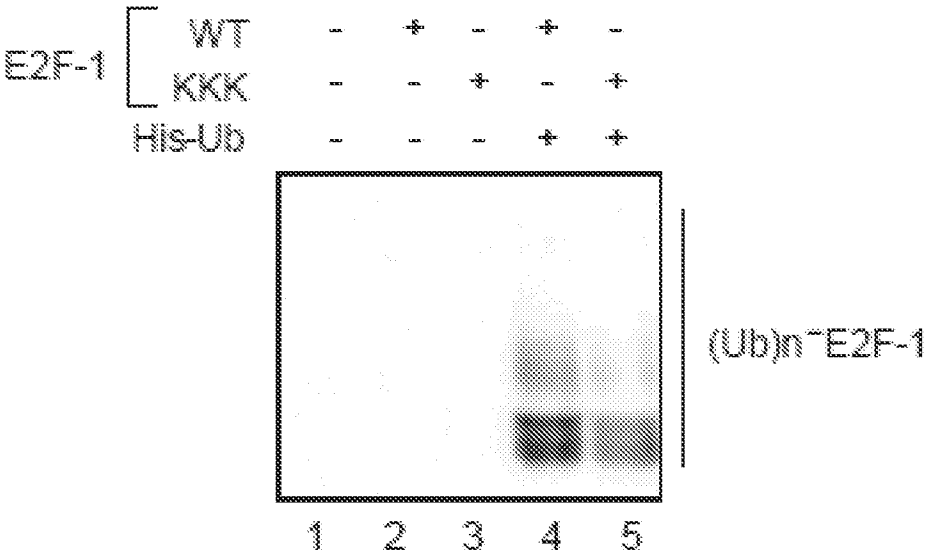
Fig. 3



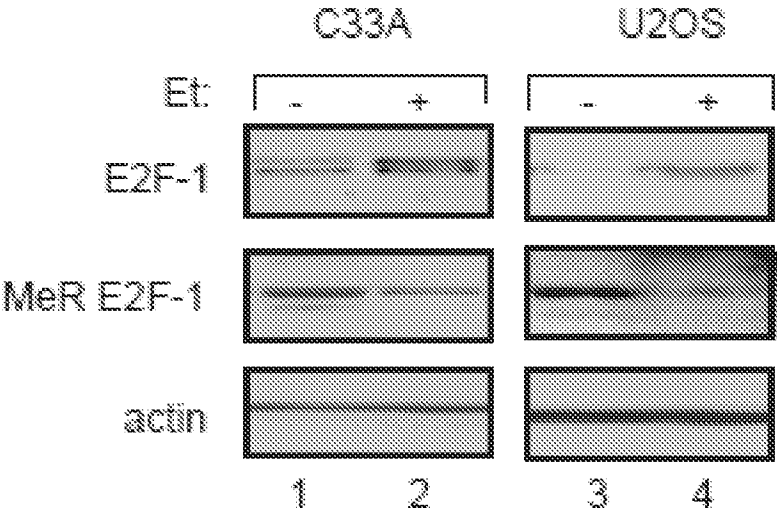
11/27

Fig. 3

f)



h)



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Fig. 3

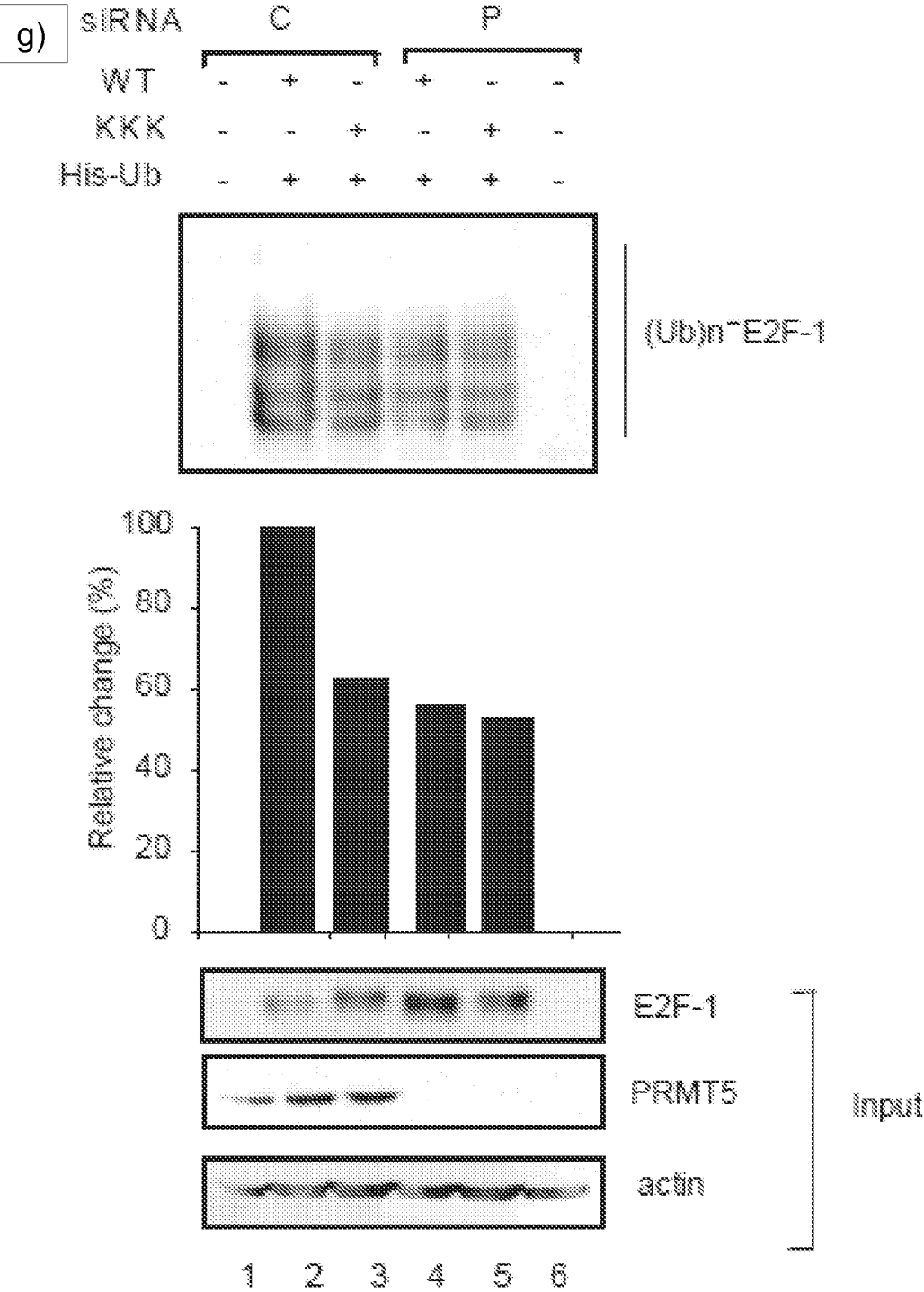
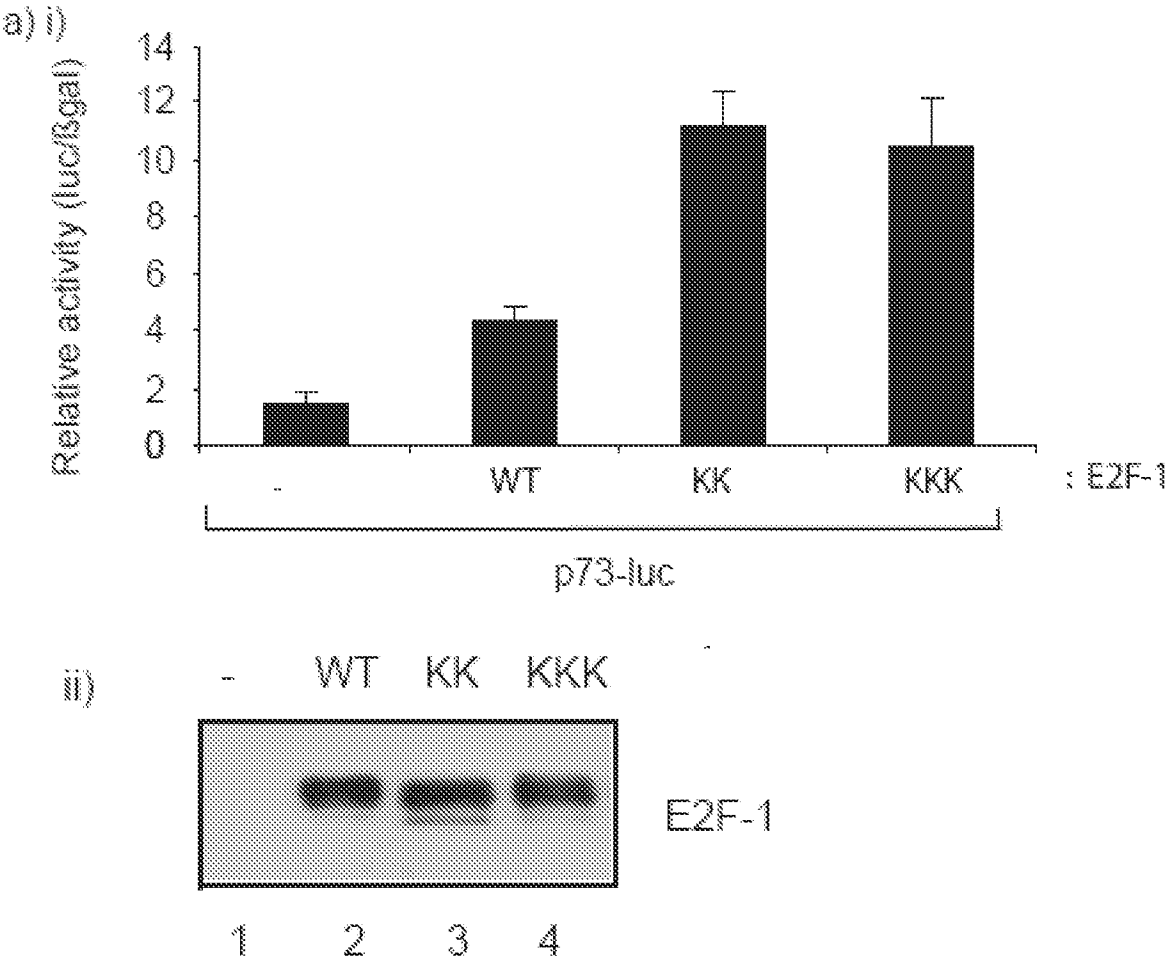


Fig. 4



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Fig. 4

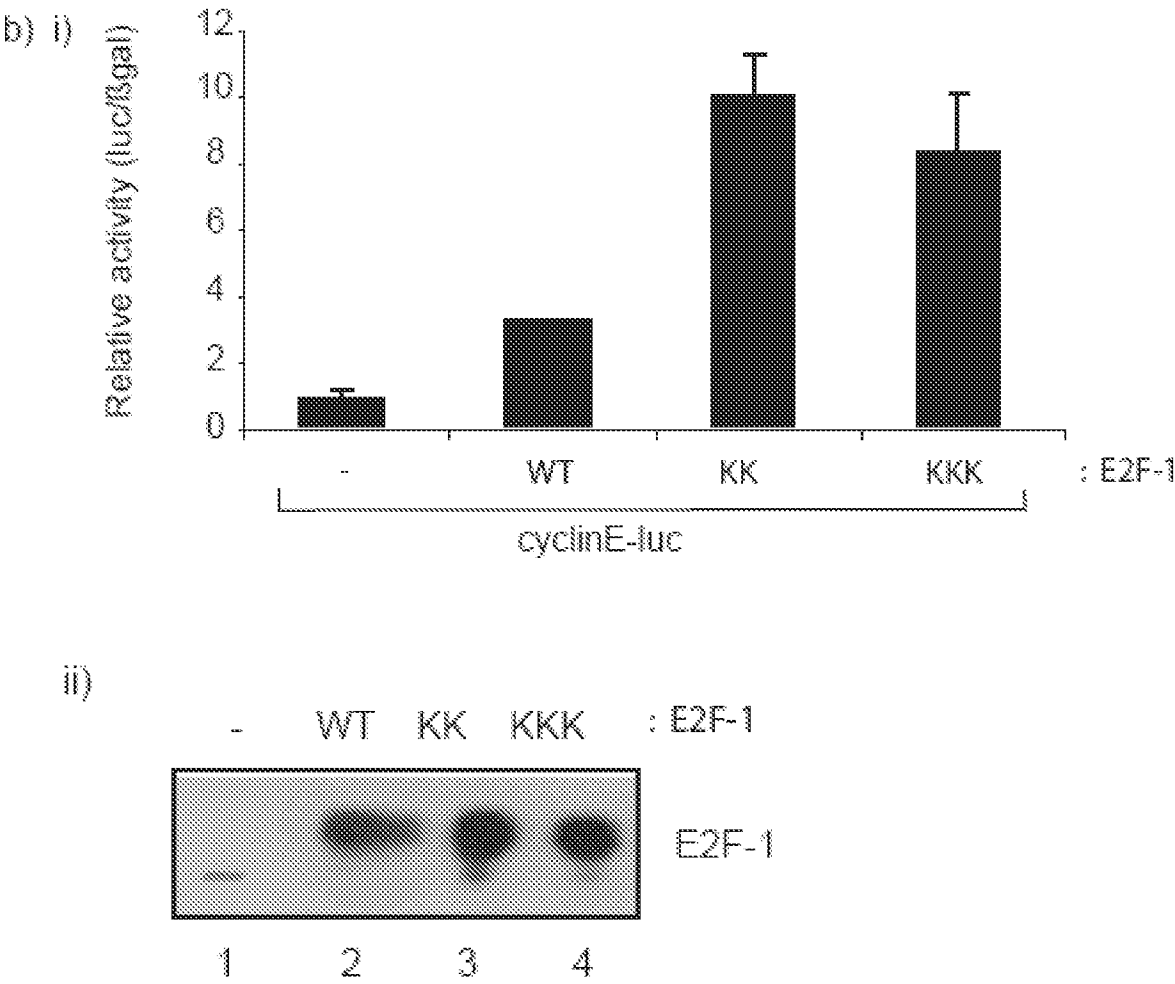


Fig. 4

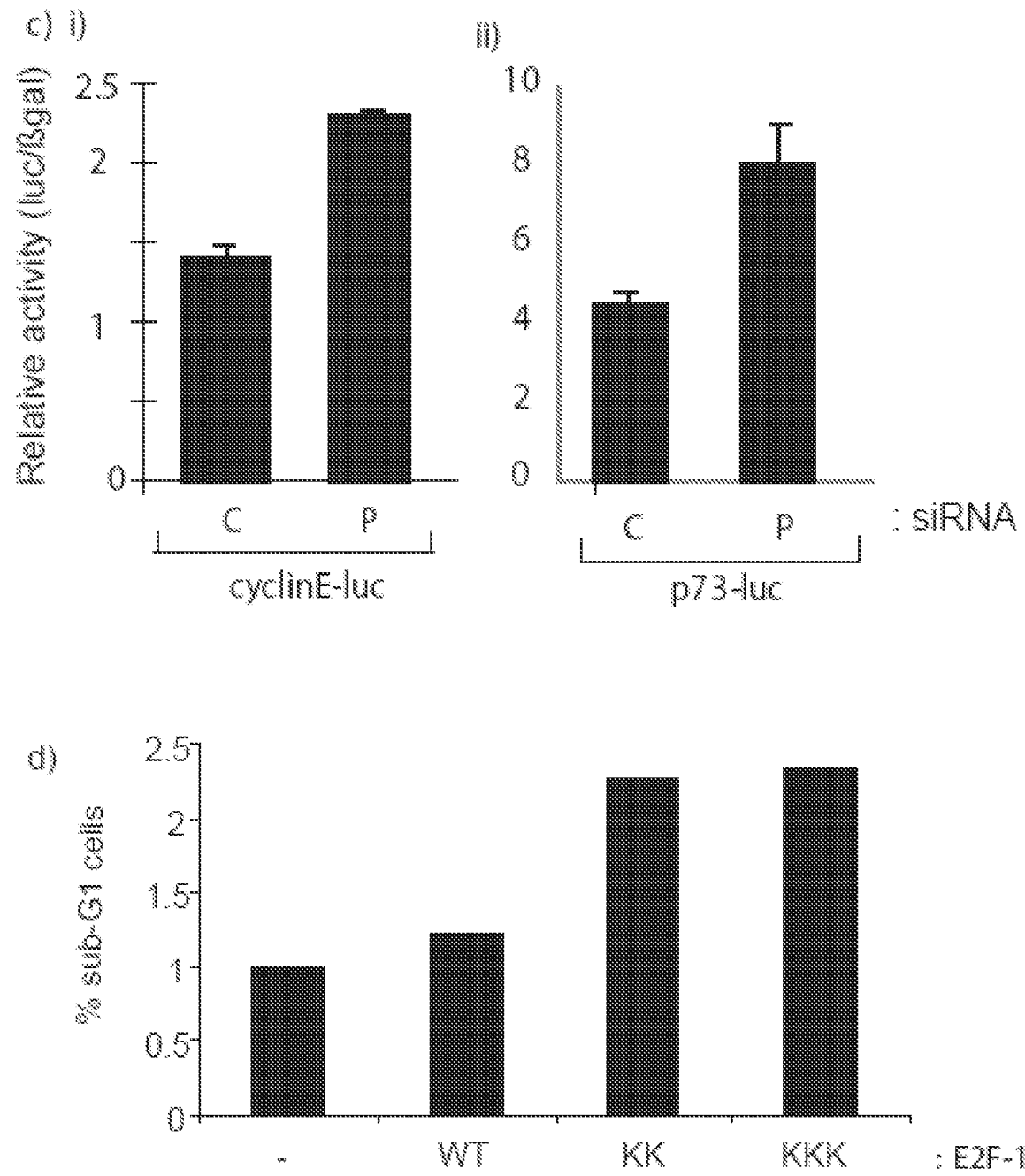


Fig. 4

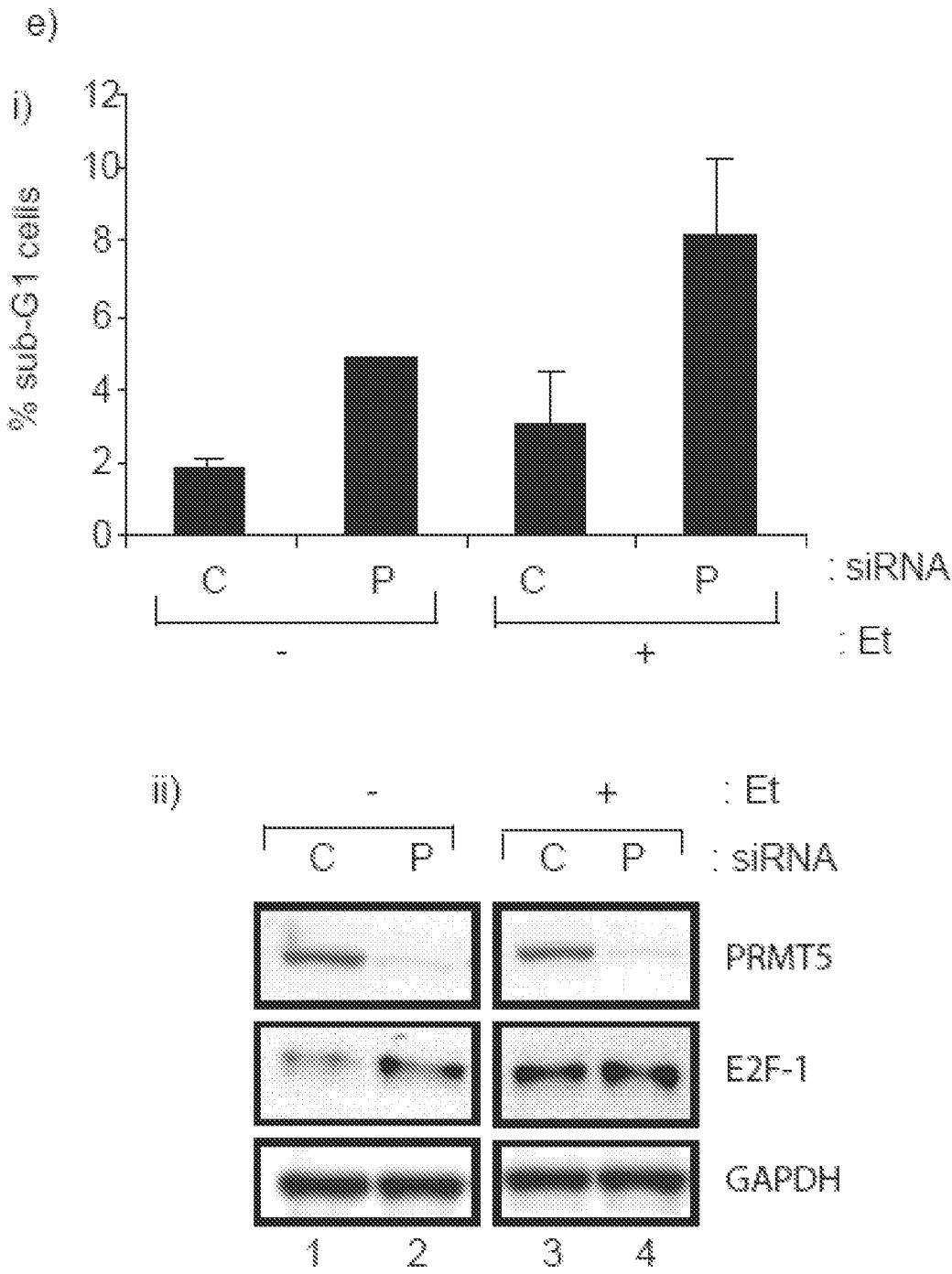


Fig. 4

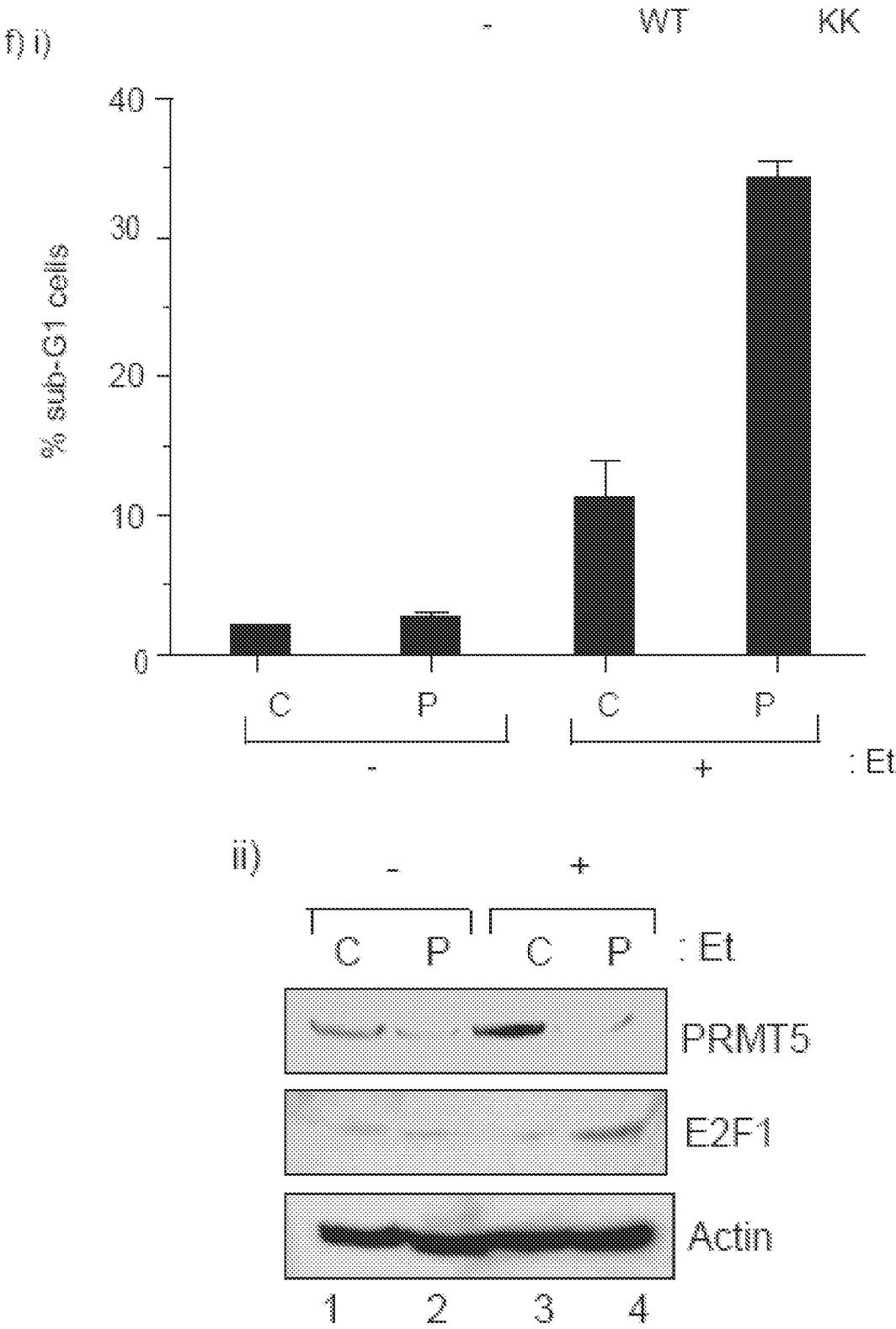
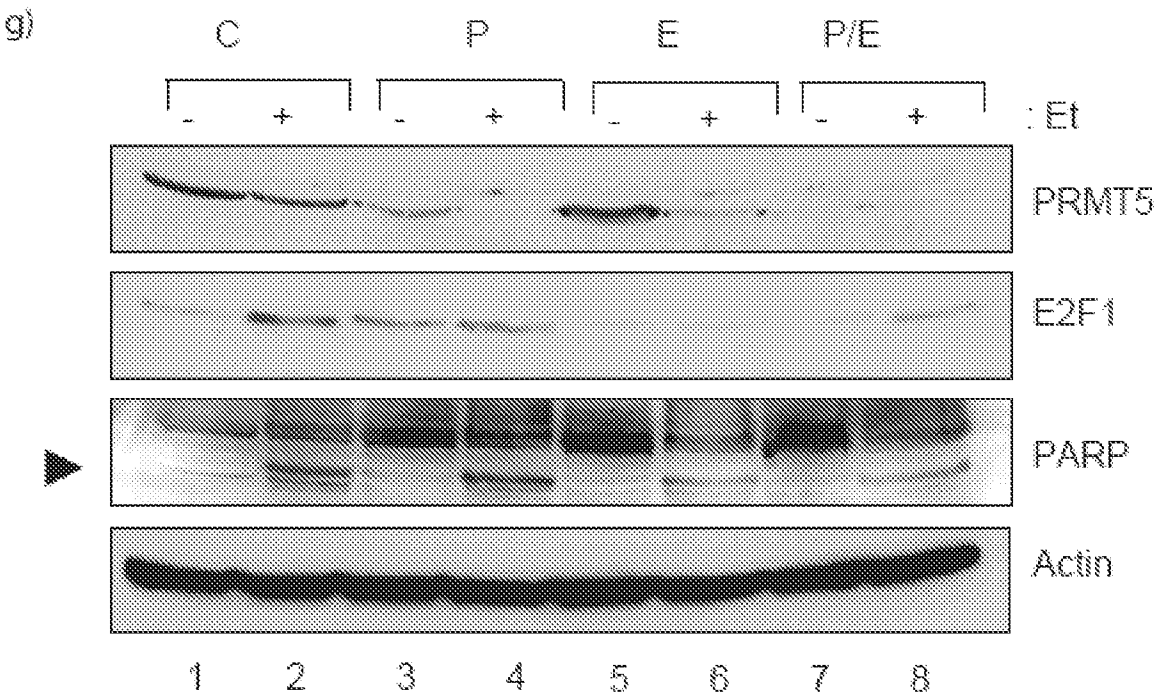


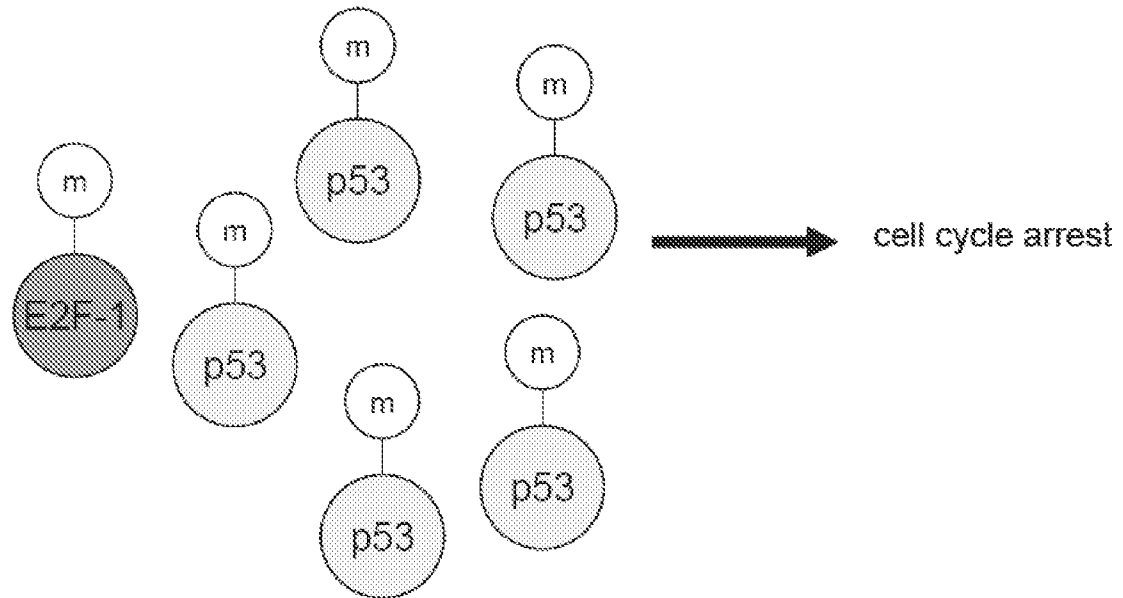
Fig. 4



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Fig. 5

Methylation



No methylation

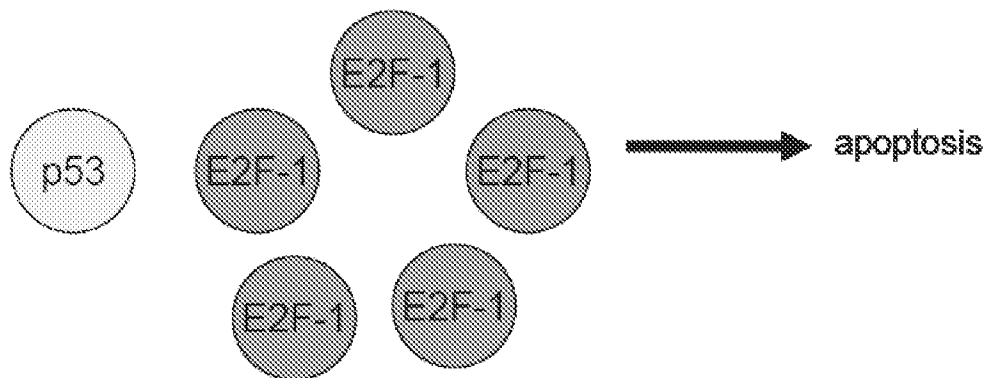


Fig. 6

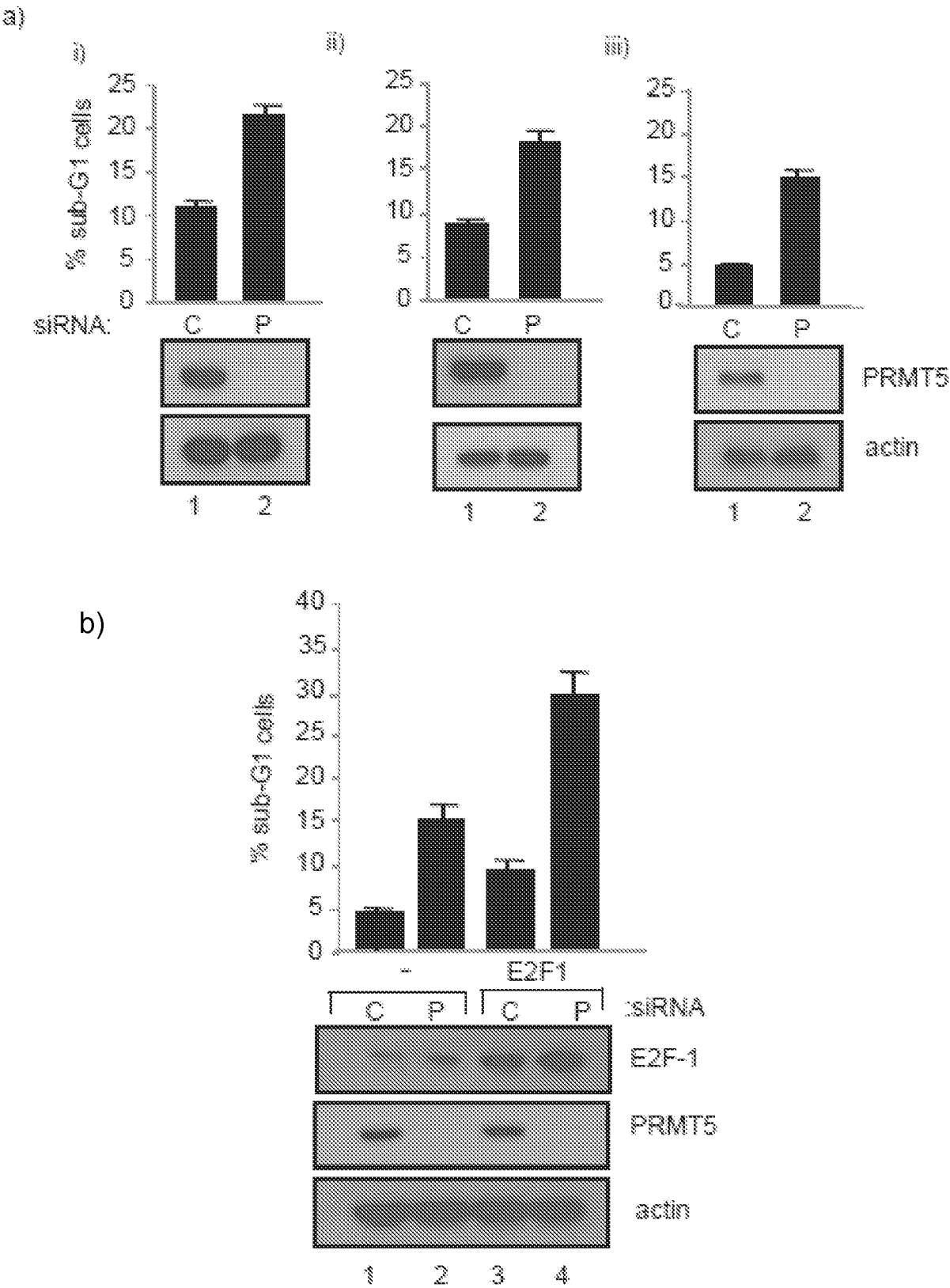


Fig. 6

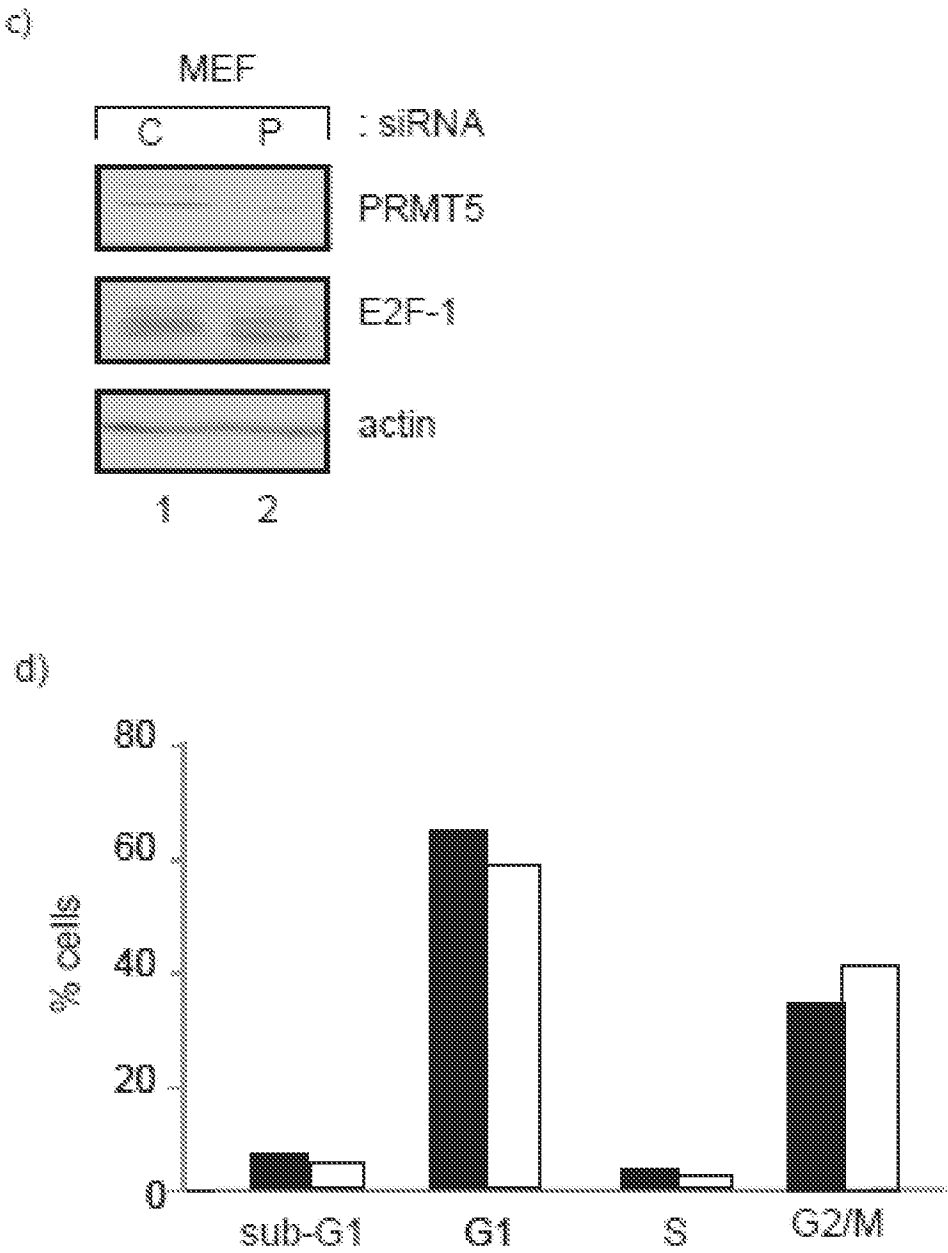


Fig. 6

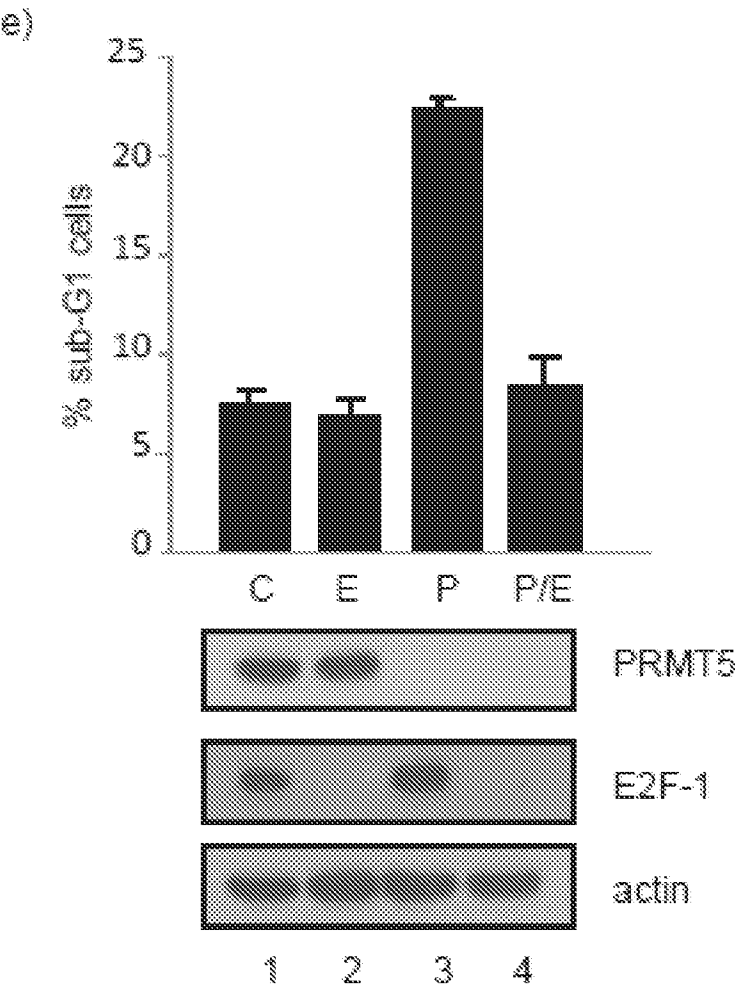
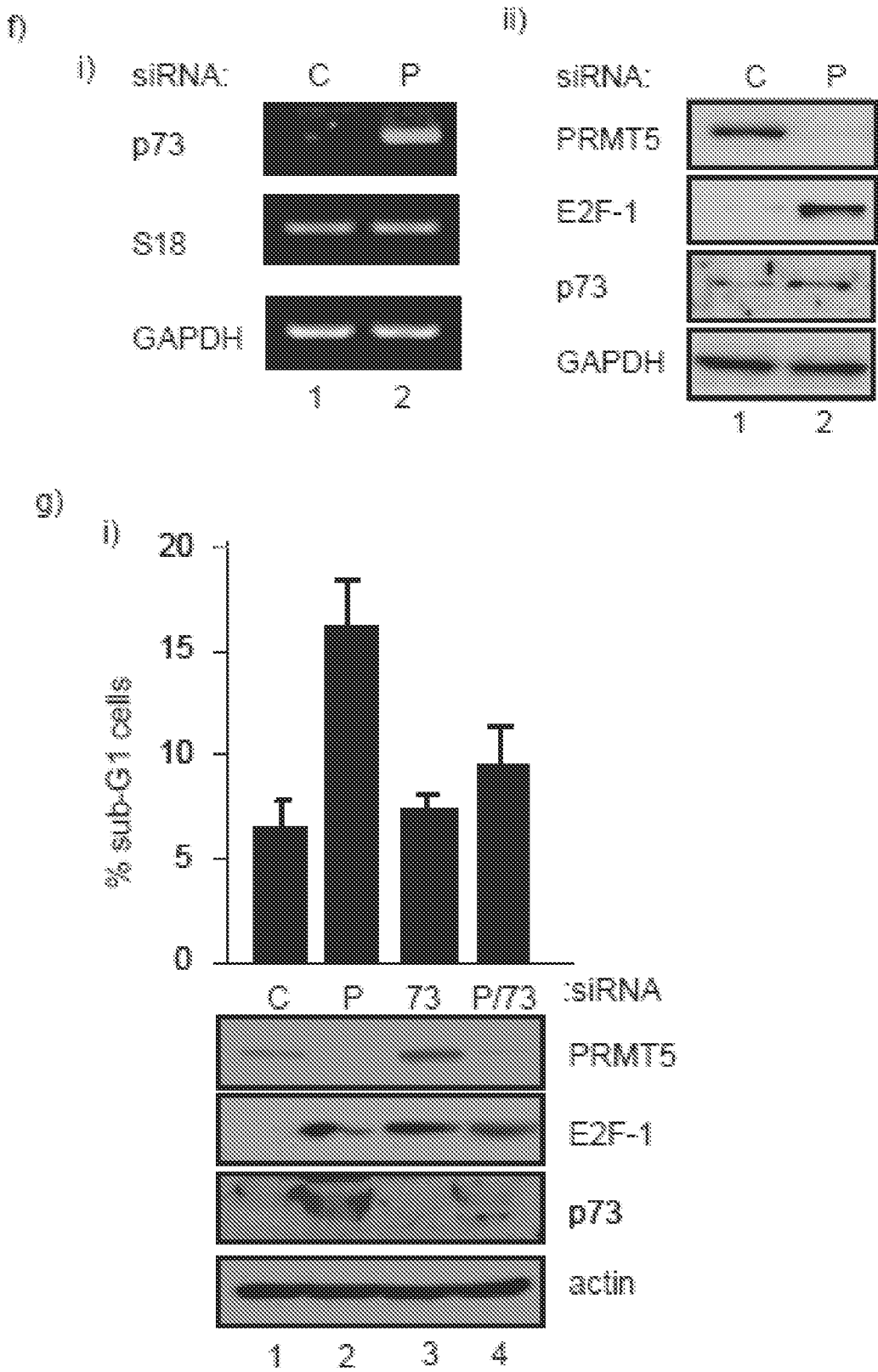
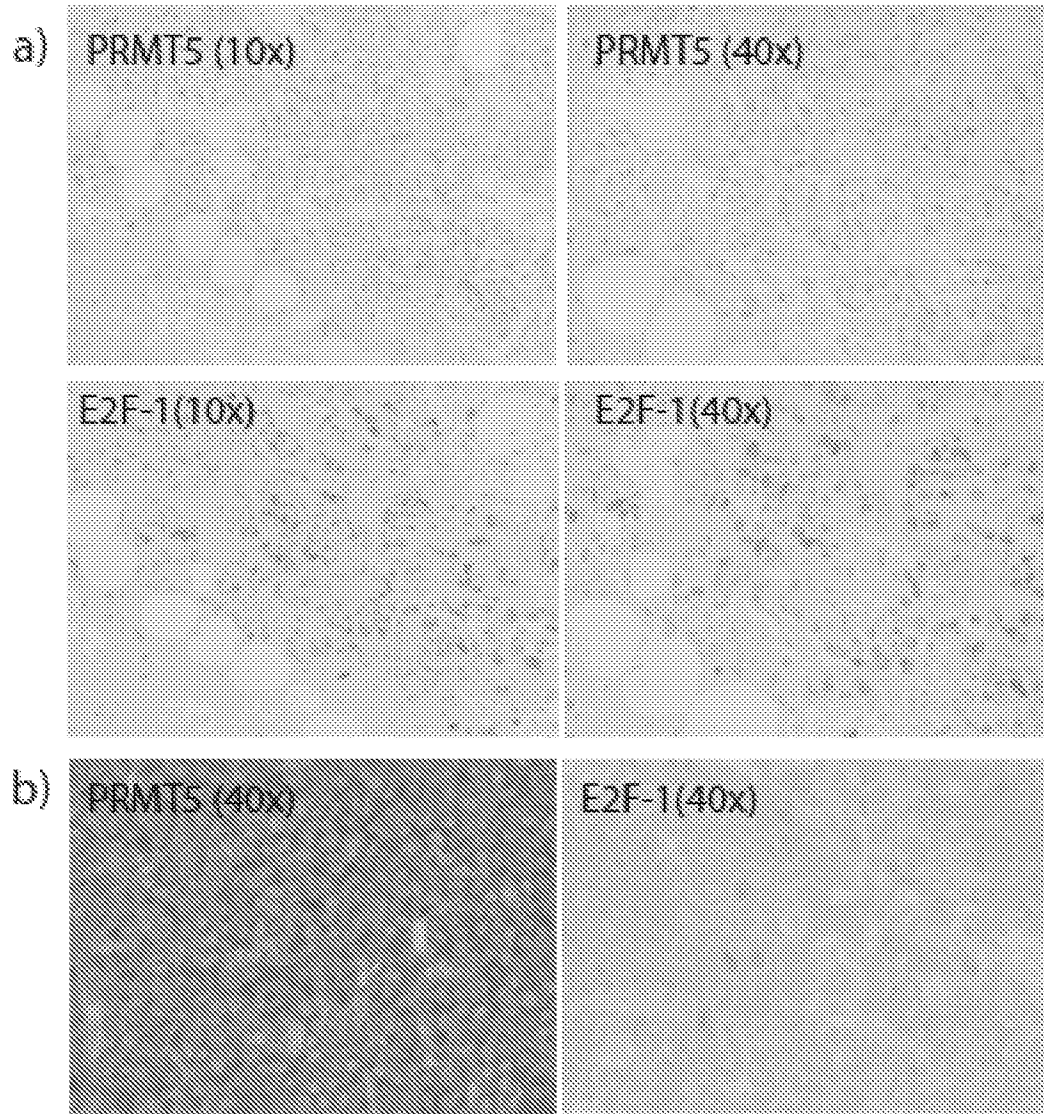


Fig. 6



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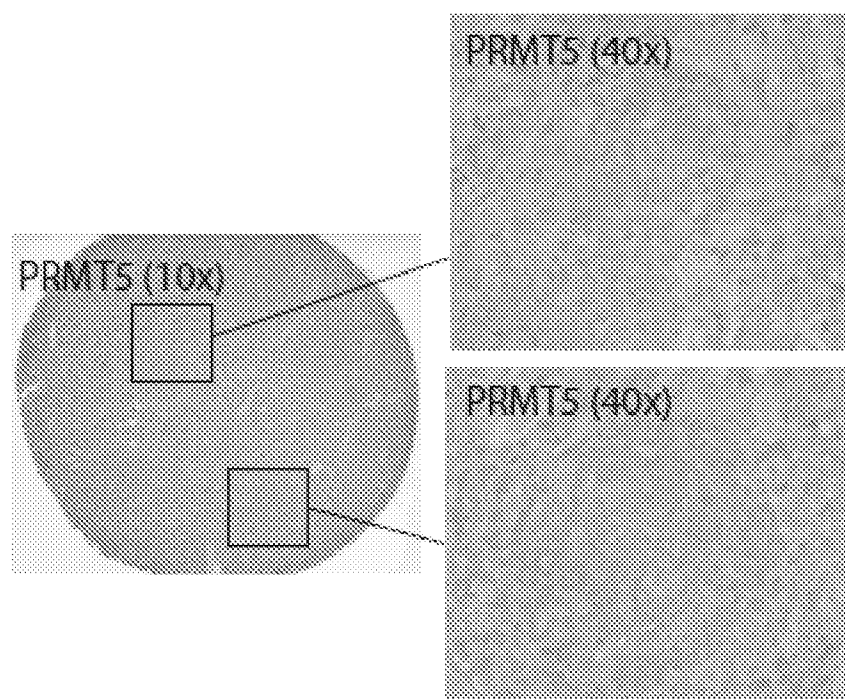
Fig. 7



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Fig. 7

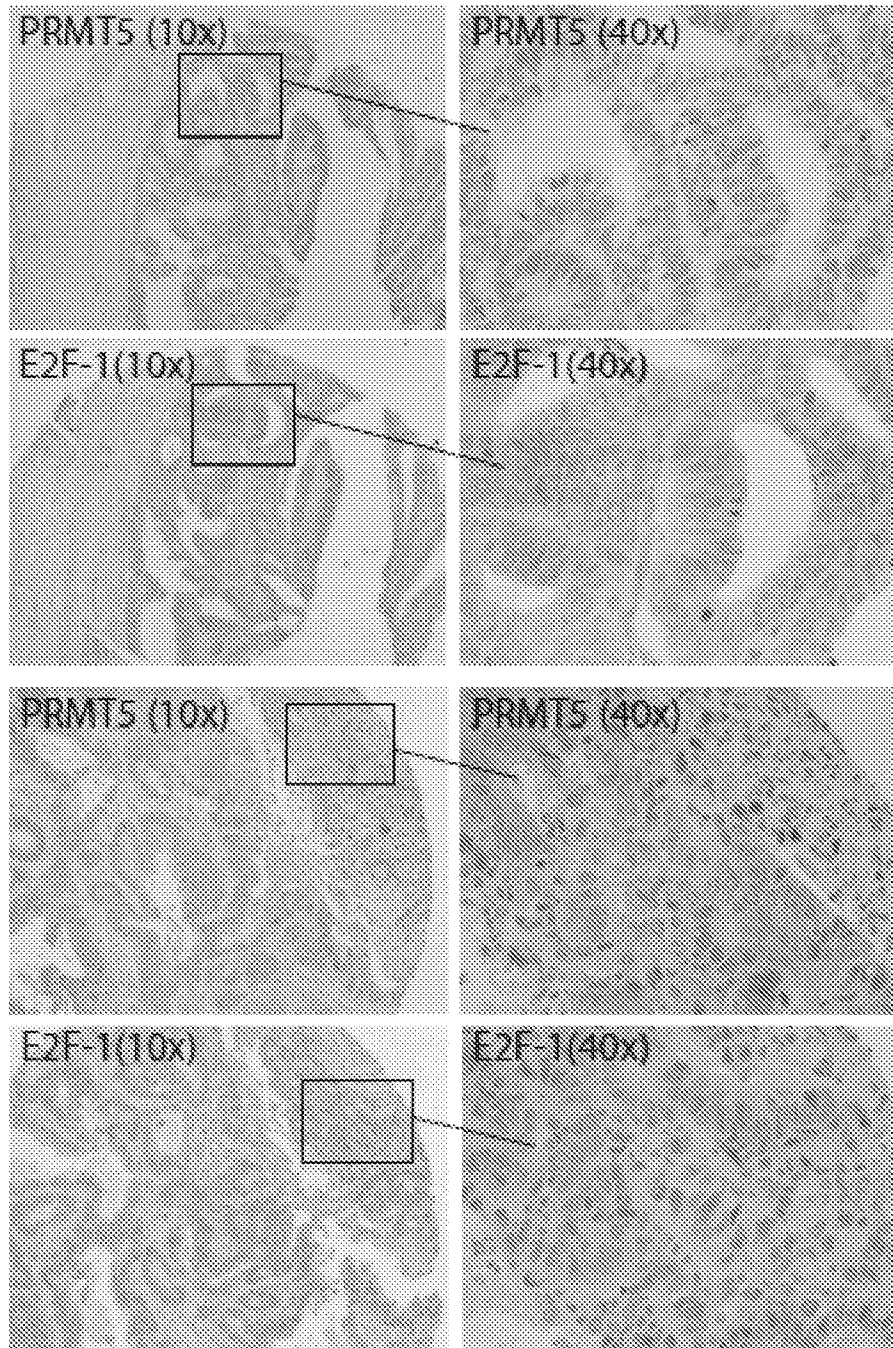
c)



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Fig. 7

d) Low PRMT5 vs High E2F-1



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Fig. 7

e) High PRMT5 vs Low E2F-1

