

Apoptosis-Inducing Factor (AIF) and Applications thereof

Field of the invention

5 The present invention relates to a method for the treatment of cancer comprising inhibiting or reducing the activity of the apoptosis-inducing factor (AIF) in the cancer cells, resulting in the suppression of the transformed phenotype and/or sensitisation of the cancer cells to apoptosis. The invention also relates to a method of screening or identification of drug candidates for
10 the inhibition or reduction of the AIF activity in cancer cells.

Background of the invention

A single X-chromosome-linked mammalian gene *aif* encodes a protein called apoptosis inducing factor (AIF), which is localised in the mitochondrial
15 membrane (Lipton and Bossy-Wetzel, Cell, 111(2):147-50, October 2002; Hansen and Nagley, Sci. STKE, 193, page 31, July 2003; Penninger and Kroemer, Nature Cell Biology, 5, 97-99, 2003; Daugas et al., FEBS Letters, Vol.476, No.3, 7 July 2000). The nucleic acid and amino acid sequence of AIF is described by Susin et al., Nature 397: 441- 446 (1999). AIF amino acid
20 sequences are well conserved (e. g. > 95%) between mice and humans. Mature AIF is a flavoprotein of 57 kDa that shares significant homology with prokaryotic oxido-reductases, in particular NADH-dependent ferredoxin reductases from both bacteria and archaeobacteria and also, with plant monodehydroascorbate reductases (Lorenzo, H. K., et al., Cell Death Differ.,
25 6: 516-24, 1999). Mouse and human AIF isoforms have also been described in WO 00/31254.

It is well established in the literature that in many types of mammalian cells induced to undergo apoptosis, the AIF is released from the mitochondria and translocates to the nucleus, where it participates in promoting a non-caspase-dependent nuclear breakdown (Susin et al., Nature, 397, 441-446, 1999; Joza et al., Nature, Vol. 410, 549-554, 2001; Cregan et al., J. Cell Biol., 158(3): 507-517, Aug 2002).

The *aif* gene was knocked out in mouse ES cells, but the loss of this gene was lethal, as the *aif* gene disrupts the first wave of morphogenetic programmed cell death during early mouse embryo development, at the pluricellular stage, shortly after the differentiation of ectoderm and endoderm. Accordingly, only a limited analysis of knockout cells derived from the dead embryo was possible (Joza et al., as above). Evidence was presented that AIF is required for the early cavitation of the mouse embryo due possibly to a failure of programmed cell death needed to sculpt the cavity. Moreover, AIF knockout cells were more resistant to serum starvation than wild type cells. Thus, the limited results of the mouse knockout are consistent with AIF functioning as an apoptosis inducing factor (Joza et al., above).

The AIF homologue, WAH-1 in the worm *C. elegans* has been shown by genetic experiments to also serve as an apoptosis inducing factor during development. WAH-1 translocates to the nucleus in combination with a DNA endonuclease CPS-6, the worm homologue of endonuclease G in mammals (Xiaochen Wang et al., Science, 298, 1587-1592, 2002). Overall, evidence suggests that in worms and mammals, AIF contributes to nuclear DNA degradation, and therefore, contributes to the dismantling of the cell in apoptosis. When AIF is present in the mitochondrion, it probably does not contribute to cell death.

The crystal structures of both the mouse and human AIF reveal two important regions (Mate et al., Nat. Struct. Biol., Vol. 9, 442-446, 2002; Alzari Pedro, WO03/089465). The first region represents a site required for DNA fragmentation - a putative DNA-binding site, and the second shows homology to a bacterial NADH-dependent ferredoxin reductase that is similar to the eukaryotic glutathione reductase family of enzymes. In functional in-vitro assays, AIF acts as an NADH oxidase or oxidoreductase, accepting electrons from NADH, and transferring them to molecular oxygen to form the superoxide free radical (Joza et al., above; Miramar et al., J. Biol. Chem. 276, 16391-16398, 2001). Superoxide in turn would be converted to hydrogen peroxide (H₂O₂) in cells. The NADH oxidase and DNA fragmenting activities of AIF are completely independent, suggesting that the NADH oxidase function of AIF is not connected to apoptosis.

Many tumour cells have significantly elevated levels of ROS (superoxide and H₂O₂ are indicated as "reactive oxygen species" or "ROS") compared to normal untransformed cells (Szatrowski and Nathan, Cancer Res. 51, 794, 1991), and there is a large body of evidence that this increase in ROS is required for the promotion and/or maintenance of the transformed state (Suh et al., Nature 401, 79-82, 1999; Arnold et al., PNAS 98, 5550-5555, 2001; Sukhdev S. Brar et al., Am. J. Physiol. Cell Physiol. 285, C353, 2003; Cerutti PA, Science 227, 375, 1985; Dreher D & Junod AF, Eur. J. Cancer 32A, 30, 1996; Oberley TD, Am. J. Pathol. 160, 403, 2002). H₂O₂ can mediate cell growth, transformation and angiogenesis caused by overexpression of the mitogenic oxidase Nox1 (Nature 401, 79, 1999; PNAS 98, 5550, 2001; PNAS 99, 715, 2002). On the other hand, superoxide may play a role in transformation or maintenance of the malignant state in certain models of carcinogenesis. Some tumour cells have higher endogenous levels of superoxide, which may be due to their lower expression of the enzyme MnSOD, a scavenger of superoxide. (Cancer Res. 61, 6082, 2001; Cancer

Res. 63, 1297, 2003; Free Rad. Res. 34, 325, 2001; PNAS 90, 3113, 1993; Mol. Cancer Ther. 2, 361, 2003).

5 Klein Jeffrey (US 2003/0228581) and Klein J. et al., Nature, 419, 367–374, 2002, indicate that decreased levels of AIF expression are correlated with oxidative stress caused by progressive increases in ROS-induced damage in neurons. In turn, the resultant stress leads to the unscheduled cell cycle reentry of these cells. However, neurons that re-enter the cell cycle cannot proliferate and thus, undergo apoptosis. The processes of oxidative stress
10 and aberrant cell cycle re-entry are critically intertwined in terminally differentiated neurons, and their correlation with decreased AIF expression levels provides a potential molecular mechanism by which free radical damage leads to neuronal death and, ultimately, to the development of late-onset neurodegenerative disorders.

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The role of AIF and its mechanism of action in inducing apoptosis is far to be clear. Because apoptosis and its regulations play a critical role in the maintenance of cellular and tissue homeostasis, there is the need for further investigations and to understand the role of AIF in apoptosis in order to
20 develop new applications useful in the cancer therapy.

Summary of the invention

25 Contrary to the well-established knowledge that AIF induces apoptosis, the present inventors have surprisingly found that in some cells AIF suppresses apoptosis.

Accordingly, the present invention is addressed to this new mechanism of action of AIF and to its applications.

According to one aspect, the invention provides a method for the treatment of cancer comprising inhibiting or reducing the activity of apoptosis-inducing factor (AIF) resulting in suppression of malignant phenotype, reversion of malignancy, increased sensitivity of cancer cells to apoptosis, induction of
5 apoptosis and/or also a partial or complete loss of tumorigenicity. Further, the method of the invention is capable of sensitizing the treated cancer cells to drug or stress-induced apoptosis.

The treated cancer is preferably a mammal cancer. For example, mouse or human cancer. As a non-limitative example of cancer wherein the inhibition or
10 reduction of AIF may suppress the malignant phenotype, reverse the malignancy, increase the sensitivity of cancer cells to apoptosis and/or induce apoptosis, is colon, breast or lung carcinoma. However, any other cancer cell line suitable and within the scope of the method of the invention can be used. In particular, the cancer cells are from a continuous (immortal) mammalian
15 cell line. For example, the continuous (immortal) mammalian cell line is DLD-1 and/or HCT-116.

It has been found that the inhibition or reduction of the activity of AIF is capable of increasing the sensitivity of the treated cancer cells to some drug, compounds and substances. Accordingly, the invention relates to a method
20 wherein the inhibition or reduction of activity of AIF is capable of increasing the sensitivity of the treated cancer cells to cytotoxic substances comprising etoposide, trichostatin A, camptothecin, t-butyl hydrogen peroxide and/or H_2O_2 .

According to another aspect, the invention relates to a method wherein the
25 inhibition or reduction of activity of AIF reduces the intracellular concentration of ROS. In particular, the inhibition or reduction of activity of AIF reduces the intracellular concentration of ROS, particularly superoxide and/or H_2O_2 .

The method of the invention can be carried out either in vivo or in vitro. Accordingly, the method of the invention comprises in vivo or in vitro administering to cancer cells a compound inhibiting or reducing the activity of AIF.

- 5 According to another aspect, the method comprises inhibiting, delaying or repressing the expression of the *aif* gene encoding the AIF polypeptide. In particular, by inhibiting, delaying or repressing the expression of the *aif* gene and/or *aif* mRNA. Accordingly, the method comprises administering to or transfecting the cancer cells with a nucleic acid construct comprising a nucleic acid molecule binding or hybridising to the *aif* gene and/or *aif* mRNA or to a portion thereof. The nucleic acid molecule is complementary or substantially complementary to the *aif* gene and/or *aif* mRNA or to a portion thereof. For example, the nucleic acid molecule is an antisense DNA and/or RNA molecule. The nucleic acid molecule may be an antisense single strand RNA (sRNA), double strand RNA (dsRNA), double strand DNA (dsDNA), double strand hybrid RNA/DNA (RNA/DNA), small interfering RNA (siRNA) and/or ribozymes. The nucleic acid construct can be any suitable vector, phage, plasmid, or nucleic acid fragment comprising the nucleic acid molecule. There is no limit in the size of the nucleic acid construct and the nucleic acid molecule. In particular, the nucleic acid molecule is 18-30, 18-25, or 19-23 nucleotides. In particular, the nucleic acid molecule is introduced into a construct or vector. The nucleic acid molecule may be complementary or hybridise to a nucleic acid molecule having the sequence of SEQ ID NO:1 or SEQ ID NO:3 or a fragment thereof (the sequences SEQ ID NO:1 and SEQ ID NO:3 representing the mouse and human *aif* gene, respectively). In particular, the nucleic acid molecule of 18-30 nucleotides may be a nucleotide sequence complementary or which hybridises to any region of SEQ ID NO:1 or SEQ ID NO:3 or a fragment thereof. More in particular, the invention provides nucleic acid molecules which are complementary to the *aif* gene and

have the sequence of SEQ ID NO:13 and/or SEQ ID NO:15, or complementary to the *aif* mRNA and have the sequence of SEQ ID NO:14 and/or SEQ ID NO:16. In particular, the nucleic acid molecules comprising the SEQ ID NOS: 13 and 15, and siRNA comprising the SEQ ID NOS: 14 and 16 are suitable for the sRNAi technology for the inhibition of *aif* mRNA. The nucleic acid molecules may be introduced into a construct and/or a vector. In particular, the vector comprises at least one double DNA targeting sequence SEQ ID NO:13 and SEQ ID NO:15, and a promoter for RNA polymerase. The vector is administered in vitro or in vivo to the cell(s). The cell RNA polymerase of the cell transcribes the siRNA molecules, which complementary to the *aif* mRNA, thereby inhibiting the AIF activity. Accordingly, one aspect of the invention is the inhibition, delay or repression of AIF carried out by siRNA technology. Furthermore, the RNAi technology can also be used to prepare knockdown cells by inhibiting the expression of the *aif* mRNA. Accordingly, the invention also provides *aif* knockdown cells, prepared in particular by siRNA technology, for example by administering the construct and/or vector as above comprising the double strand targeting sequence SEQ ID NOS:13 and 15. The cell can be any *aif* knockdown cell, for example, a mammal carcinoma cell, like a lung, breast and/or colon *aif* cell, for example, the SW480 cell line, but not limited to these cell types.

According to a further aspect, the invention relates to a method for the treatment of cancer comprising reducing the intracellular concentration of ROS of the cancer cells. ROS is superoxide and/or H₂O₂. The reduction of intracellular concentration of superoxide and/or H₂O₂ in cancer cells may result in the suppression of malignant phenotype, reversion of malignancy, increased sensitivity of the cancer cells to apoptosis and/or a reduction in the growth rate. In particular, the reduction of intracellular concentration of superoxide and/or H₂O₂ in cancer cells induces apoptosis. Further, as a consequence of the reduction of intracellular concentration of superoxide

and/or H₂O₂, the treated cancer cells may show a partial or complete loss of tumorigenicity and/or a reduction in the growth rate. The invention also provides a method comprising reducing the intracellular concentration of superoxide and/or H₂O₂ in cancer cells by administering ROS scavengers including Manganese superoxide dismutase (Mn-SOD) mimetics such as
5 EUK-8 (Calbiochem) and EUK-134; manganese porphyrins such as MnTMPyP (C₄₄H₃₆Cl₅MnN₈), MnTM-2-PyP (C₄₄H₃₆Cl₅MnN₈), MnTE-2-PyP (C₄₄H₃₆Cl₅MnN₈) (all examples of manganese porphyrins provided are from Calbiochem). The structural information about the Mn-SOD mimetics and
10 manganese porphyrins is shown in Table 1.

The cancer cells may be treated either in vitro or in vivo. The cancer cells may be mammal cancer cells. For example, mouse or human cancer cells. In particular, the cancer is colon carcinoma. In particular, the cancer cells are from a continuous (immortal) mammalian cell line. For example, the
15 continuous (immortal) mammalian cell line is DLD-1, HCT-116 or SW480.

According to a further aspect, the invention provides an isolated cell, wherein the apoptosis-inducing factor (AIF) activity has been inhibited, delayed or reduced and wherein the cell shows suppression of malignant phenotype, reversion of malignancy, increased sensitivity of cancer cells to apoptosis
20 and/or a reduction in the growth rate. The cell may be a mammal cell, like mouse or human cell. In particular it may be a colon, breast or lung carcinoma cell. However, the carcinoma cells are not limited to colon, breast and lung. Any other carcinoma cells suitable for the purposes of the invention may be used. This cell may show an increased sensitivity to some drugs, compounds
25 or substances, as mentioned above. Examples of carcinoma cells treated according to the invention are HCT-116, DLD-1 and SW480. The inhibition, delay or repression of the *aif* gene encoding the AIF polypeptide may be carried out using the construct as mentioned above. For example, by

inhibiting, delaying or repressing the expression of *aif* mRNA. More in particular, by siRNA technology.

According to another aspect, the invention provides an isolated apoptosis-inducing factor (AIF) knockout and/or knockdown carcinoma cell, wherein the
5 knockout and/or knockdown cell shows suppression of malignant phenotype, reversion of malignancy, increased sensitivity to apoptosis, and/or a reduction in the growth rate, compared to the wild type cell. The knockout cells may be prepared according to any standard technology, for example, by knocking out the *aif* gene by homologous recombination. The knockdown cell may be
10 prepared using the RNAi technology as indicated above. The knockout and/or knockdown cell may be a mammal cell, like a mouse or human cell. In particular, the cell is a colon, breast or lung carcinoma cell. However, the knockout and/or knockdown cells according to the invention are not limited to the indicated carcinoma cells. Any other carcinoma cell showing the same
15 characteristics of the carcinoma cells of the invention in showing increased sensitivity to some drugs, ability of suppressing the malignant phenotype, reversing the malignancy, increasing the sensitivity of cancer cells to apoptosis, inducing apoptosis, and/or a reduction in the growth rate, may also be used and are within the scope of the present invention.

20 The knockout and/or knockdown cell of the invention shows an increased sensitivity to some drugs, compounds and substances. Accordingly, the invention provides one or more knockout and/or knockdown cells, in particular knockout and/or knockdown cancer cells like colon, breast and lung carcinoma cells, wherein the inhibition of AIF is capable of increasing the
25 sensitivity of the treated cells to cytotoxic substances, including DNA modifying compounds, for example, etoposide, trichostatin A, camptothecin, t-butyl hydrogen peroxide and/or H₂O₂.

The knockout and/or knockdown cell may show intracellular concentrations of superoxide and/or H₂O₂ lower than the wild type cell.

The knockout and/or knockdown cell shows suppression of malignant phenotype, reversion of malignancy, increased sensitivity of cancer cells to apoptosis and/or a reduction in the growth rate.

Only as non-limiting example, an *aif* knockout and/or knockdown carcinoma cell according to the invention may be HCT-116, DLD-1 and/or SW480.

According to a further aspect, the invention provides a use for the knockout cells made according to the method of the present invention, wherein the knockout cells are implanted in an animal, for the purposes of drug testing and/or drug screening. For example, for the purpose of being used as negative control for drug testing and/or drug screening. Any knockout cells prepared according to the invention may be implanted on the animal. In particular, *aif* knockout carcinoma colon, breast, and lung cell or cell line. For example, *aif* knockout cell lines HCT-116, DLD-1, and/or SW480 prepared according to the invention can be used. However, the knockout cells to be implanted on the animal is not limited to the exemplified knockout cell. Any carcinoma cell showing the characteristics of any carcinoma cell or cell line according to the invention may be implanted. Accordingly, the invention provides a non-human animal model, which is implanted with the knockout cells made according to any embodiment of the present invention for the purpose of drug screening and/or drug testing. In particular, the animal model of the invention may be used as negative control in order to screen, test or discover drugs which may show suppression of malignant phenotype, reversion of malignancy, increased sensitivity of cancer cells to apoptosis, and/or a reduction in the growth rate. The animal model of the invention may be a mammal, for example a mouse or rat.

According to another aspect, the invention provides a method for screening and/or identifying a candidate drug capable of inhibiting or reducing the activity of apoptosis-inducing factor (AIF) in cancer cells comprising: (a) administering a drug candidate to cancer cells, and (b) determining the ability
5 of the drug candidate to inhibit or reduce AIF activity in cancer cells. The method may further comprise (c) comparing the ability of the drug candidate to inhibit or reduce AIF activity in cancer cells to the reduced or inhibited AIF activity of the cell according to any embodiment of the present invention.

According to another aspect, the invention relates to a method for screening
10 and/or identifying a candidate drug capable of inhibiting or reducing the activity of apoptosis-inducing factor (AIF) in cancer cells comprising: (a) administering a drug candidate to cancer cells, and (b) determining the ability of the drug candidate to reduce the intracellular concentration of ROS. For example, the method comprises determining the ability of the drug candidate
15 to reduce the intracellular concentration of superoxide and/or H₂O₂, compared to the wild type cell. Further, the method may comprise (c) comparing the ability of the drug candidate to reduce the intracellular concentration of superoxide and/or H₂O₂ to the reduced intracellular concentration of the cell of any embodiment of the invention.

20 According to a further aspect, the invention relates to a method for screening and/or identifying a candidate drug capable of inhibiting or reducing the activity of apoptosis-inducing factor (AIF) in cancer cells comprising: (a) administering a drug candidate to cancer cells, and (b) determining the ability of the drug candidate to suppress the malignant phenotype, reverse the
25 malignancy and/or increase the sensitivity of cancer cells to apoptosis. The method may further comprise (c) comparing the ability of the drug candidate to suppress the malignant phenotype, reduce the growth rate, reverse the malignancy, increase the sensitivity of cancer cells to apoptosis to the level of

suppression, reversion and/or increased sensitivity of the cell of any embodiment of the invention.

According to another aspect, the invention relates to a method for screening and/or identifying a candidate drug capable of inhibiting or reducing the activity of apoptosis-inducing factor (AIF) in cancer cells comprising: (a) administering a drug candidate to cancer cells, and (b) determining the ability of the drug candidate to induce apoptosis.

According to another aspect, the invention relates to a method for screening and/or identifying a candidate drug capable of inhibiting or reducing the activity of apoptosis-inducing factor (AIF) in cancer cells comprising: (a) administering a drug candidate to cancer cells, and (b) determining the ability of the drug candidate to increase the sensitivity of the treated cancer cells to cytotoxic substances, including DNA modifying compounds, for example, etoposide, trichostatin A, camptothecin, t-butyl hydrogen peroxide and/or H_2O_2 .

According to a particular aspect, the effects of the drug candidate obtained with the step (b) are compared in the step (c) to a negative control represented by *aif* knockout and/or knockdown cells or on cells treated according to any embodiment of the invention for reducing the activity of AIF.

According to a more particular aspect, the negative control is represented by *aif* knockout cells or the cells treated according to the invention implanted on a non-human animal model for drug screening, as mentioned above. The *aif* knockout cell implanted on the animal may be colon, breast and/or lung carcinoma cell or cell line. For example, *aif* knockout HCT-116, DLD-1 and/or SW480 cell(s) or cell line(s). The non-human animal model may be a mammal, for example a mouse or rat.

According to a further aspect, the invention provides a pharmaceutical composition comprising one or more of the compound used for inhibiting AIF

in vitro or in vivo. For example, the invention provides a pharmaceutical composition comprising at least one of ROS scavengers including Magnanese superoxide dismutase (Mn-SOD) mimetics such as EUK-8 (Calbiochem) and EUK-134; manganese porphyrins such as MnTMPyP ($C_{44}H_{36}Cl_5MnN_8$),
5 MnTM-2-PyP ($C_{44}H_{36}Cl_5MnN_8$), MnTE-2-PyP ($C_{44}H_{36}Cl_5MnN_8$) (all examples of manganese porphyrins provided are from Calbiochem). The pharmaceutical composition optionally comprises a pharmaceutically acceptable excipient, diluent, and/or carrier, or a mixture thereof.

Further, the invention provides a pharmaceutical composition comprising at
10 least one nucleic acid molecule for inhibiting the *aif* gene and/or *aif* mRNA according to any embodiment of the invention. In particular, the invention provides a pharmaceutical composition comprising at least one nucleic acid molecule complementary or which hybridise to a nucleic acid molecule having the sequence of SEQ ID NO:1 or SEQ ID NO:3 or a fragment thereof (the
15 sequences SEQ ID NO:1 and SEQ ID NO:3 representing the mouse and human *aif* gene, respectively). In particular, the pharmaceutical composition comprises a nucleic acid molecule is 18-30, 18-25, or 19-23 nucleotides, complementary or which hybridise to a nucleic acid molecule having the sequence of SEQ ID NO:1 or SEQ ID NO:3 or a fragment thereof. In
20 particular, the pharmaceutical composition comprises at least one nucleic acid molecule which is complementary to the *aif* gene and has the sequence of SEQ ID NOS:13, and 15, and SEQ ID NOS:14 and 16.

Accordingly, the invention also relates to the use of at least one nucleic acid molecule of the invention for the preparation of a pharmaceutical composition
25 for use in therapy. The composition is for inhibiting or reducing the activity of AIF and/or for inhibiting, delaying or repressing the expression of *aif* gene or *aif* mRNA. More in particular, the use is for suppressing malignant phenotype, reversion of malignancy, increasing sensitivity of cancer cells to apoptosis, induction of apoptosis, partial or complete loss of tumorigenicity, sensitizing

the treated cancer cells to drug or stress-induced apoptosis, and/or a reduction in the growth rate.

According to a further aspect, the invention relates to a pair of cells comprising: a first cell; and a second cell, wherein the first cell and the second cell are isogenic but for an *aif* gene and a gene encoding a fluorescent protein; wherein the first cell comprises a gene that encodes a first fluorescent protein having a first absorption spectrum and a first emission spectrum; wherein the second cell comprises a gene that encodes a second fluorescent protein having a second absorption spectrum and a second emission spectrum; and wherein either: the first and second absorption spectra are not identical; and/or the first and second emission spectra are not identical. In particular, the first and second absorption spectra are not identical and the first and second emission spectra are not identical. The first and second cells may be mammalian cells, for example, human, mouse or rat cells. In particular, the first cell is a knockout and/or knockdown *aif* carcinoma cell according to the invention. Any carcinoma pair of cell can be used, in particular, colon, breast and lung cancer cells. For example, the pair of cells are HCT-116, DLD-1 cells or SW480. The first and second fluorescent proteins may be chosen from the group consisting of green fluorescent protein, red fluorescent protein, blue fluorescent protein, yellow fluorescent protein, and cyan fluorescent protein.

According to a further aspect, the invention provides a method of making a pair of cells, comprising the steps of: genetically modifying a first cell to make an *aif* knockout and/or knockdown cell, which is isogenic with a second cell but for the *aif* gene; transfecting the first cell with a first gene that encodes a first fluorescent protein having a first absorption spectrum and a first emission spectrum; and transfecting the second cell with a second gene that encodes a second fluorescent protein having a second absorption spectrum and a

second emission spectrum, wherein either the first and second absorption spectra are not identical and/or the first and second emission spectra are not identical. In particular, the first and second absorption spectra are not identical and wherein the first and second emission spectra are not identical. The first
5 cell according to the present method is a knockout and/or knockdown *aif* cancer cell prepared according to the invention. In particular, the cancer cells are from a continuous (immortal) mammalian cell line. For example, the continuous (immortal) mammalian cell line is DLD-1, HCT-116 or SW480.

10 According to another aspect, the invention provides a method of identifying a test compound as selectively affecting a gene of interest (which is the *aif* gene) or its expression protein (AIF) comprising the steps of: culturing a first and second cell, wherein the first and second cells are isogenic but for the *aif* gene and a gene encoding a fluorescent protein, wherein the first cell
15 comprises a first gene that encodes a first fluorescent protein having a first absorption spectrum and a first emission spectrum, and wherein the second cell comprises a second gene that encodes a second fluorescent protein having a second absorption spectrum and a second emission spectrum, wherein either the first and second absorption spectra are not identical and/or
20 the first and second emission spectra are not identical; contacting the first and second cells with a test compound; and identifying the test compound as selectively affecting the *aif* gene or its expression protein if the growth rate of the first cell is not altered with respect to the growth rate of the second cell. Alternatively, criteria for comparison are whether the test compound may
25 cause in the cancer cell suppression of malignant phenotype, reversion of malignancy, increased sensitivity of the cancer cells to apoptosis and/or induction to apoptosis. The first cell according to the present method is a knockout and/or knockdown *aif* cancer cell prepared according to the invention. The first and second cells may be co-cultured. In particular, an

equal number of the first and second cells may be cultured. In particular, the first and second absorption spectra are not identical and the first and second emission spectra are not identical. More in particular, the fluorescent proteins may be detected using fluorescence microscopy to assess growth rate.

5 According to a particular example, the fluorescent proteins are detected using high-throughput fluorescence spectroscopy to assess growth rate. However, other criteria as indicated below may also be used. The first and second fluorescent protein may be selected from the group consisting of green fluorescent protein, red fluorescent protein, blue fluorescent protein, yellow

10 fluorescent protein, and cyan fluorescent protein.

According to another aspect, the invention provides a method for screening carcinoma cells wherein the suppression of AIF and/or ROS increases the sensitivity of the treated cancer cells, shows suppression of malignant

15 phenotype, reversion of malignancy, increases the sensitivity of cancer cells to apoptosis, and/or induces apoptosis. Accordingly, the invention provides a method for screening carcinoma cells comprising administering to carcinoma cells a compound for reducing and/or inhibiting AIF and/or ROS, or inhibiting the *aif* gene and/or *aif* mRNA, and determining whether inhibition or reduction

20 of activity of AIF and/or reduction of ROS is capable of increasing the sensitivity of the treated cancer cells, showing suppression of malignant phenotype, causing reversion of malignancy, increasing the sensitivity of cancer cells to apoptosis, and/or inducing apoptosis, compared to the wild type cells and/or compared to a cell prepared according to any embodiment of

25 the invention as negative control.

Brief description of the figures

Figure 1: Design of replacement bipartite targeting vectors for *aif* gene

- Figure 2A: PCR Analysis of Gene Targeting in HCT-116^{aif-/y} subclones
- Figure 2B: Gene targeting of *aif* gene
- Figure 3: Growth curve for HCT-116 (Figure 3A) and DLD-1 (Figure 3B) cell lines. 10³ cells were cultured for 4 days. At each time point, cells were treated with MTS/PES solution (Promega Cell Titer Proliferation Assay) for 1 hr. Colour reaction absorbance was measured at 485nm. Fold increase in cell number was measured using OD_{Tn}/OD_{To}.
- Figure 4A-C: Morphological changes in HCT-116 knockout clones. Knockout clones detach and appear as aggregates when cultures are continuously cultured for 5-7 days post-confluency without changing media.
- Figure 4D-E: Wild-type (WT) and BL12 clone enlarged from Figure 4A and Figure 4B respectively.
- Figure 5A-D: Anchorage-independent culture of HCT-116 *aif* mutants
- Figure 6A-J: Cytotoxicity assays for HCT-116 cells using camptothecin, t-butyl hydrogen peroxide, staurosporine, etoposide, hydrogen peroxide, actinomycin, TRAIL, anti-FAS, doxorubicin and trichostatin A respectively.
- Figure 7A-I: Cytotoxicity assays for DLD-1 cells using camptothecin, t-butyl hydrogen peroxide, staurosporine, etoposide, hydrogen peroxide, actinomycin, TRAIL, anti-FAS and doxorubicin respectively.
- Figure 8A-D: Sytox/Hoechst Stain of Cells exposed to 60 μ M etoposide (48 hrs)
- Figure 9A: Soft agar assays for DLD-1 cell lines
- Figure 9B: Colony assays for DLD-1 cell lines
- Figure 10A: Soft agar assays for HCT-116 cell lines
- Figure 10B: Colony assays for HCT-116 cell lines
- Figure 11A: In vivo tumour formation of DLD-1 cell lines (5th week)

- Figure 11B: In vivo tumour formation of DLD-1 cell lines (8th week)
- Figure 12: In vivo tumour growth of HCT-116 cell lines
- Figure 13: In vivo tumour formation of DLD-1 cell lines
- Figure 14: Proposed AIF NADH Oxidase Activity (NADH Oxidase Activity of
5 mitochondrial AIF, Miramar D et. al., JBC 276:16391, 2001)
- Figure 15: Intracellular Superoxide Levels in Cell lines
- Figure 16: Nitroblue tetrazolium reduction assay for intracellular superoxide
levels. Cells were incubated with 2mM NBT for 30 minutes allow
formazan formation then lysed with 50% DMSO/1M KOH.
10 Absorbance was read at 650nm.
- Figure 17: Aconitase Assay. Superoxide inhibits aconitase hydratase
activity. Aconitase activity was measured by measuring NADPH
formation during citrate isomerization to isocitrate at 340nm.
- Figure 18: Relative superoxide levels using acotinase assay. Superoxide
15 inhibits aconitase hydratase activity. Aconitase activity was
measured by measuring the rate of NADPH formation during
citrate isomerization to isocitrate at 340nm. Relative superoxide
levels were computed by subtracting the increase in aconitase
activity relative to the wt cells and shown as a percentage of the
20 wt control cells.
- Figure 19: Intracellular ROS Levels in cell lines DLD-1 and HCT-116
- Figure 20: Catalase activity in cell lines. Assay was performed using the
Amplex Red Catalase assay kit (Molecular Probes).
- Figure 21: Transfection of NIH3T3 cells with AIF-GFP (Figure 21A is
25 +pEGFP and Figure 21B is +AIF-GFP)
- Figure 22: Foci formation in NIH3T3 cells transformed by AIF-GFP
- Figure 23: Foci formation in NIH3T3 cells transformed by AIF-GFP. This
figure shows a magnified field of Figure 22 (+AIF-GFP). Foci
formation in pre-neoplastic NIH3T3 cells is an indicator of
30 transformation.

Figure 24: Cytotoxicity assays for SW480 cells using etoposide (Figure 24A), t-butyl hydrogen peroxide (Figure 24B) and hydrogen peroxide (Figure 24C).

Figure 25A: Soft agar assays for SW480 cell lines

5 Figure 25B: Colony assays for SW480 cell lines

Detailed description of the invention

10 The present invention relates to a new mechanism of action and to new applications of the apoptosis-inducing factor (AIF).

The apoptosis-inducing factor (AIF) is a protein or polypeptide encoded by the *aif* gene (Lipton and Bossy-Wetzel, Cell, 111(2):147-50, October 2002; Hansen and Nagley, Sci. STKE, 193, page 31, July 2003; Penninger and
15 Kroemer, Nature Cell Biology, 5, 97-99, 2003; Cregan et al, Oncogene 23, 2785, 2004). The nucleic acid sequence of the mouse and human *aif* genes and the amino acid sequence of the corresponding AIFs have been characterised (Susin et al., Nature 397: 441- 446 (1999)). These sequences have also been reported in WO 03/089465. For ease of comprehension, the
20 *aif* gene and AIF sequences described by Susin et al. are also reported in the sequence listings of the present application. In particular, SEQ ID NO: 1 is the mouse *aif* gene nucleotide sequence; SEQ ID NO: 2 is the mouse AIF amino acid sequence; SEQ ID NO: 3 is the human *aif* gene nucleotide sequence; and SEQ ID NO: 4 is the human AIF amino acid sequence. AIF isoforms
25 have also been described in WO 00/31254. The AIF isoforms described in WO 00/31254 as well as further AIF isoforms or variants known in the art or AIF isoforms or variants which have not been discovered yet are within the scope of the present invention.

In order to investigate the function(s) of AIF in apoptosis and in cancer, the present inventors have prepared *aif* knockout and/or knockdown cell lines. In particular, the cancer cells are from a continuous (immortal) mammalian cell line. The present inventors employed human colon carcinoma cell lines, more
5 in particular the cell lines DLD-1 and HCT-116 (Source = HCT-116 from American Type Culture Collection (ATCC) ref. no. CCL-247; DLD-1 from ATCC ref. no. CCL-221; DLD-1 reference, Shirasawa et al, Science 260, 85, 1993; HCT-116 reference, Bunz et al, Science, 282, 1497, 1998 (and references therein)). The cells obtained from ATCC were the wild-type cells.
10 However, the present invention is not limited to colon carcinoma cell lines. Any cell line suitable for the purpose of the present invention can also be used. For example, breast cancer or lung cancer cell lines, but not limited to these cell types. The knockout and/or knockdown cell can be prepared according to any technology known in the art. In particular, the DLD-1 cell line
15 and the HCT-116 have been prepared by knocking out by homologous recombination (Sedivy et al., Science, 283, 9a, 1999; Sedivy and Dutriaux, Trends Genet., 15, 88, 1999) the X-linked *aif* gene in both these cell lines (Figure 1 and Figure 2A-B). Knockout and/or knockdown SW480 cells or cell lines are also within the scope of the present invention.

20

The *aif* knockout HCT-116 and DLD-1 cells were surprisingly more sensitive to three cancer drugs (etoposide, trichostatin A and camptothecin) and to two oxidising agents (t-butyl hydrogen peroxide and H₂O₂) compared to the parental cells harbouring AIF.

25

aif knockout HCT-116 and DLD-1 cells grew slightly slower than their wild-type counterparts in monolayer culture (Figures 3A and 3B), did not reach confluence without some clumping (Figure 4), and they failed to grow in suspension (anchorage-independent) culture. *aif* knockout cells either failed to

form multicellular spheroids (MCS) in suspension (DLD-1) or the formation of MCS was much delayed (HCT-116) as seen in Figures 5A-D. *aif* knockout DLD-1 cells completely failed to grow in soft agar (Figures 9A-B), while *aif* knockout HCT-116 cells grew very poorly in soft agar (Figure 10A-B). These results imply a severe loss of tumorigenicity of DLD-1 cells, and a strong partial loss of tumorigenicity and/or a reduction in the growth rate of HCT-116 cells. In order to investigate more comprehensively whether AIF-negative cells had lost tumour forming potential, the inventors examined the ability of wild-type and *aif* knockout cells to form tumours in the nude mouse model. Remarkably, DLD-1 *aif* knockout cells had almost completely lost their ability to grow subcutaneously as solid tumours in these animals, whereas the HCT-116 cells grew only very poorly. This can be seen in Figures 11A-B, 12 and 13. Thus, loss of AIF curtails or impairs the growth of colon carcinoma cells in vivo, consistent with several indicators of loss of the transformed phenotype in vitro. A summary of the results (Table 2) obtained can be found under the experimental section.

Thus, the experimental data demonstrates that AIF acts as an "Apoptosis Inhibiting Factor" (instead of "Apoptosis Inducing Factor") in colon carcinoma cell lines, although the mechanisms are unclear. This contrasts with published results of apoptosis studies in mouse *aif* knockout embryos and primary embryonic cells in which evidence was presented that AIF is pro-apoptotic (Joza et al., above). Further, AIF appears to contribute significantly to ROS production in two colon cancer lines. The enhanced sensitivity of the AIF-negative tumour cells to apoptosis and the loss of tumorigenicity of AIF-negative cells may due to a reduction in intracellular superoxide and/ or H_2O_2 as a consequence of the loss of AIF (Figure 19). Thus, the findings of the present inventors have a major potential application in colon cancer.

Accordingly, inhibiting or reducing the activity of AIF in colon carcinoma cells, for example by selective inhibition of AIF, is understood to have the same effect as knocking out the gene, that is, suppressing the malignant phenotype and sensitizing the tumour cells to apoptosis. Suppression of the transformed state leading to the reversion of malignancy may in itself be sufficient to eliminate the cancer. Therefore, inhibitors of AIF are anticipated to represent a completely novel approach to colon cancer therapy.

According to a first aspect, the present invention provides a method for the treatment of cancer comprising inhibiting or reducing the activity of AIF. The method therefore comprises administering in vivo or in vitro to cancer cells a drug, compound or substance inhibiting or reducing the activity of AIF. The inhibition or reduction of the activity of AIF can be carried out according to any suitable technology known in the art. For example, the treatment comprises selectively inhibiting the AIF. For “selectively inhibiting” it is herein understood to mean any drug, compound or substance capable of binding or interacting with the AIF and inhibiting, reducing or substantially inhibiting or reducing the activity of AIF. For example, by blocking the AIF or degrading AIF. Drugs, compounds or substances capable of inhibiting or reducing the activity of AIF can be selected among the drugs, compounds and substances known in the art or can be newly discovered or synthesised. Examples of such include general NADH oxidase inhibitors, such as Quinacrine; Diphenyleneiodonium chloride (DPI); Diamide; p-chloromercuriphenylsulfonate (pCMPS). The screening, selection of these types of drugs, compounds or substances will be described later.

The reduction or inhibition of activity of AIF can also be obtained by in vivo or in vitro inhibiting, delaying or repressing the expression of the *aif* gene encoding the AIF polypeptide. Accordingly, the invention provides a method which comprises inhibiting, delaying or repressing the expression of the *aif*

gene encoding AIF. As a particular example, the invention provides a method which comprises inhibiting, delaying or repressing the expression of *aif* mRNA. Accordingly, the method comprises administering to or transfecting in vivo or in vitro the cancer cells with a nucleic acid construct comprising a
5 nucleic acid molecule binding or hybridising to the *aif* gene and/or *aif* mRNA or to a portion thereof. The nucleic acid molecule is complementary or substantially complementary to the *aif* gene and/or *aif* mRNA or to a portion thereof.

Accordingly, the present invention extends to the preparation of antisense
10 nucleotides and ribozymes that may be used to interfere with the expression of the AIF proteins at the translational level. This approach involves antisense nucleic acid molecules and ribozymes to block translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or cleaving it with a ribozyme.

15 Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, Sci. Am., 262:40-46 (1990); Marcus-Sekura, Anal. Biochem., 172:289-295, 1988). In the cell, they hybridise to that mRNA, forming an untranslatable double-
20 stranded molecule. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Oligomers of about fifteen nucleotides and molecules that hybridise to the AUG initiation codon will be particularly efficient, since they are easy to synthesise and are likely to pose fewer problems than larger molecules when introducing them into AIF peptide-
25 producing cells. Antisense methods have been used to inhibit the expression of many genes in vitro [(Marcus-Sekura, 1988 supra; Hambor et al., J. Exp. Med., 168:1237-1245 (1988)].

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA molecules in a manner somewhat analogous to DNA restriction endonucleases. Ribozymes were discovered from the observation that certain mRNAs have the ability to excise their own introns.

5 By modifying the nucleotide sequence of these RNAs, researchers have been able to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, J. Am. Med. Assoc., 260:3030-3034, 1988). Because they are sequence-specific, only mRNAs with particular sequences are inactivated.

10

For example, the nucleic acid molecule is an antisense DNA and/or RNA molecule. The nucleic acid molecule may be an antisense single strand RNA (sRNA), double strand RNA (dsRNA), double strand DNA (dsDNA), double strand hybrid RNA/DNA (RNA/DNA), small interfering RNA (siRNA) and/or

15 ribozymes. The nucleic acid construct can be any suitable vector, phage, plasmid, a nucleic acid fragment or the like comprising the nucleic acid molecule.

There is no limit to the size of the nucleic acid construct and the nucleic acid molecule. In particular, the nucleic acid molecule is 15-40 nucleotides, in particular 18-30 nucleotides, more in particular 18-25 nucleotides, for example

20 19-23 nucleotides.

More in particular, the nucleic acid molecule may be complementary or hybridise to a nucleic acid molecule having the sequence of SEQ ID NO: 1 or SEQ ID NO: 3 or a fragment thereof. In particular, the nucleic acid molecule of

25 15-40, 18-30, 18-25 or 21-23 nucleotides may be a nucleotide sequence complementary or which hybridises to any region of SEQ ID NO: 1 or SEQ ID NO: 3 or a fragment thereof, wherein in the case of the mRNA the U in SEQ ID NO: 1 and SEQ ID NO: 3 is indicated as T. The isolated nucleic acid

molecule may also be that complementary to or which hybridises with any AIF isoform or variant known in the art. For example, those described in WO 00/0331254 (herein incorporated by reference). More in particular, the invention provides nucleic acid molecules which are complementary to the *aif* gene and have the sequence of SEQ ID NO:13 and/or SEQ ID NO:15, or complementary to the *aif* mRNA and have the sequence of SEQ ID NO:14 and/or SEQ ID NO:16. In particular, targeting nucleic acid molecules comprising the sequences of SEQ ID NOS: 13 and 15, and siRNA comprising the sequences of SEQ ID NOS:14 and 16 are suitable for the sRNAi technology for the inhibition of *aif* mRNA. The nucleic acid molecules may be introduced into a construct and/or a vector, comprising a promoter for RNA polymerase, and administered in vitro or in vivo to the cell. The RNA polymerase of the cell transcribes the siRNA molecules complementary to the *aif* mRNA, thereby inhibiting the AIF activity. According to one aspect, the invention provides a construct and/or vector comprising a RNA polymerase promoter and nucleic acid molecules having the sequence of SEQ ID NO:13 and SEQ ID NO:15. In alternative, RNA molecules comprising the sequence of SEQ ID NO:14 and SEQ ID NO:16, are prepared and administered in vitro or in vivo to the cell. Accordingly, the isolated nucleic acid molecule may be an antisense DNA and/or RNA molecule. For example, a single strand RNA (sRNA), double strand RNA (dsRNA), double strand DNA (dsDNA), double strand hybrid RNA/DNA (RNA/DNA), small interfering RNA (siRNA) and/or ribozymes.

A nucleic acid molecule is "hybridisable" to another nucleic acid molecule (in the present case, the *aif* gene or *aif* mRNA), when a single-stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (Sambrook and Russell, Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press, 2001). The conditions of temperature and ionic

strength determine the "stringency" of the hybridisation. Hybridisation requires the two nucleic acids to contain complementary sequences. Depending on the stringency of the hybridisation, mismatches between bases are possible. The appropriate stringency for hybridising nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridisation decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (Sambrook and Russell, 2001, as above). For hybridisation with shorter nucleic acids, i.e. oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (Sambrook and Russell, 2001, as above). Preferably a minimum length for a hybridisable nucleic acid is at least about 10 nucleotides; more preferably at least about 15 nucleotides; most preferably the length is at least about 18 nucleotides.

According to a particular aspect, the inhibition, delay or repression is carried out by the silencing interference RNA (siRNA) technology. RNA interference technology is well known and consists of a process in which a double stranded RNA (dsRNA) induces the postranscriptional degradation of homologous transcripts. RNAi can be initiated by exposing cells to dsRNA either via transfection or endogenous expression. According to the exemplified embodiment, DNA targeting sequences, specific for the inhibition of the *aif* mRNA, are selected and prepared according to standard technology, for example, the DNA targeting sequence are generated using Ambion siRNA target finder (http://www.ambion.com/techlib/misc/siRNA_finder.html). The DNA targeting sequences may be inserted into a construct and/or vector and used to transfect the cancer cell or cell lines in vitro or in vivo. The RNA

polymerase of the cell transcribes the siRNAs complementary to the *aif* mRNA or to a fragment thereof. These siRNAs form a complex known as the RNA-induced silencing complex or RISC (Bernstein et al., 2001a; Hammond et al., 2001) which functions in homologous target RNA destruction (in the present case in the *aif* mRNA destruction). In mammalian systems, the sequence-specific RNAi effect has been observed by the introduction of siRNAs either via transfection or endogenous expression of 19-23 base transcripts capable of forming duplexes, or via expression of short hairpin RNAs. The siRNA expression constructs and/or vectors may be constructed according to any method known in the art, for example by chemical synthesis, in vitro transcription, by digestion of long dsRNA by an RNase III family enzyme (e.g. Dicer, RNase III), by expression in cells from an siRNA expression plasmid or viral vector, and expression in cells from a PCR-derived siRNA expression cassette. The construct is directly transfected into mammalian cells resulting in functional expression of siRNAs. A further description regarding the siRNA technology can be obtained for example from Reynolds et al Nature Biotechnology 22, 326, 2004 or from the Ambion web page (<http://www.ambion.com>).

The method according to the invention can be applied on cancer cells suitable to be used for the purpose of the present invention. The cancer cells can be mammal cancer cells, for example mouse or human. The cancer can be colon, breast and/or lung carcinoma. However, the cancer cells are not limited to colon, breast and/or lung carcinoma cells. Any cancer cell showing or sharing the characteristics of the colon carcinoma cells exemplified in the present invention can be used. The cancer cells may be from a continuous (immortal) mammalian cell line. For example, cancer breast and lung cell lines. For example, HCT-116, DLD-1 and/or SW480. The present method comprising inhibition or reduction of activity of AIF increases the sensitivity of the treated cancer cells to cytotoxic substances, including DNA modifying

compounds, for example, etoposide, trichostatin A, camptothecin, t-butyl hydrogen peroxide and/or H_2O_2 , compared to the wild type cells. Accordingly, any cancer cells treated according to the method of the invention and sharing the same effect can be used for the purpose of the invention. Accordingly, the present invention also provides a method for screening cancer cells suitable in the method of the present invention comprising treating the cells to inhibit or reduce AIF and treating the cells with at least one cytotoxic substance selected from the group consisting of etoposide, trichostatin A, camptothecin, t-butyl hydrogen peroxide and/or H_2O_2 , and comparing the results with appropriate control cells (treated knockout and/or knockdown cells or untreated wild type AIF-containing cells). A further step includes comparing the obtained results with those obtained using colon carcinoma.

The cancer cells treated according to the method of the invention comprising the inhibition or reduction of activity of AIF results in suppression of malignant phenotype, reversion of malignancy, increased sensitivity of cancer cells to apoptosis, induction of apoptosis, a partial or complete loss of tumorigenicity and/or a reduction in the growth rate.

Further, the method of the invention is capable of sensitizing the treated cancer cells to drug or stress-induced apoptosis.

The present inventors have further investigated whether the absence of AIF led to a reduction in intracellular ROS based on the in vitro activity of AIF as a superoxide-generating enzyme.

Many tumour cells have significantly elevated levels of ROS (superoxide and H_2O_2 compared to normal untransformed cells, and there is a large body of evidence that this increase in ROS is required for the promotion, progression of and/or maintenance of the transformed state (Szatrowski and Nathan. Cancer Res. 51, 794, 1991; Suh Y. et. al. Nature 401, 79, 1999; Arnold RS.

et. al., PNAS 98, 5550, 2001; Arbiser et. al., PNAS 99, 715, 2002; Brar S. S et. al., Am. J. Physiol. Cell Physiol. 285, C353, 2003; Weydert C. et. al., Mol. Cancer Ther. 2, 361, 2003; Kondo et al, Free Rad. Biol. Med. 27, 401, 1999; Zhao et al, Cancer Res. 61, 6082, 2001; Church et al, PNAS, 90, 3113, 1993; 5 Cerutti PA, Science 227, 375, 1985; Dreher D & Junod AF, Eur. J. Cancer 32A, 30, 1996; Oberley TD, Am. J. Pathol. 160, 403, 2002). H₂O₂ can mediate cell growth, transformation and angiogenesis caused by overexpression of the mitogenic oxidase Nox1 (Suh Y. et. al., Nature 401, 79, 1999; Arnold RS. et. al., PNAS 98, 5550, 2001; Arbiser et al., PNAS 99, 715, 2002). On the other 10 hand, superoxide may play a role in the transformation or maintenance of the malignant state in certain models of carcinogenesis. Some tumour cells have higher endogenous levels of superoxide, which may be due to their lower expression of the enzyme MnSOD, a scavenger of superoxide (Zhao et. al., Cancer Res. 61, 6082, 2001; Cullen et. al., Cancer Res. 63, 1297, 2003; 15 Church SL et. al., PNAS 90, 3113, 1993; Weydert C. et. al., Mol. Cancer Ther. 2, 361, 2003).

Other than mitochondrial respiration (Figure 14), sources of ROS can be explained as follows. In addition to the prototype NADPH oxidase of 20 phagocytes (Babior BM, Curr. Opin. Immunol. 16, 42, 2004), there is a growing family of oxidases in addition to AIF including Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 & 2 that could represent enzymatic sources of ROS (Lambeth JD et. al., TIBS 25, 459, 2000; Bokoch GM and Knaus UG, TIBS 28, 502, 2003). Another oxidoreductase with the potential to regulate 25 superoxide is NADPH:quinone oxidoreductase (NQO1), which is overexpressed in some cancers, and has been implicated in carcinogenesis (Cullen et. al., Cancer Res. 63, 5513, 2003; Brar S. S. et. al., Am. J. Physiol. Cell Physiol. 280, C659, 2001), perhaps through its ability to interact with the tumour suppresser p53 (Asher G et. al., PNAS 98, 1188, 2001; Asher G. et. 30 al., PNAS 99, 3099, 2002). The link between NQO1 and carcinogenesis is,

however, very weak. It is important to recognize that in primary tumours, the enzymatic sources and targets of superoxide and H_2O_2 are unknown; there may be one or many sources.

5 Using assays that measures mainly superoxide or mainly H_2O_2 , the present inventors found that superoxide and/or H_2O_2 levels were significantly lower in both DLD-1 and HCT-116 cells (Figures 15-18). This is consistent with a role for AIF in superoxide and H_2O_2 generation, and is the first such demonstration in living cells.

10

The relationship between ROS levels and tumorigenicity was examined. Lower levels of superoxide and H_2O_2 correlate with absent or much reduced tumour growth in mice, as well as with the absent or much reduced growth of DLD-1 and HCT-116 cells in soft agar. Thus, in conclusion, ROS (both
15 superoxide and H_2O_2) produced by the action of AIF appears to influence the tumorigenicity of DLD-1 and HCT-116 cells, but the molecular targets of ROS are unknown.

Accordingly, the method according to the present invention for the treatment
20 of cancer comprising inhibiting or reducing the activity of AIF results in a reduction of the intracellular production of ROS in the cancer cells. In particular, the inhibition of the activity of AIF reduces the intracellular concentration of superoxide and/or H_2O_2 . The reduction of intracellular production of superoxide and/or H_2O_2 induces or sensitises the cancer cells to
25 apoptosis.

According to another aspect, the present invention provides a method for the treatment of cancer comprising reducing the intracellular concentration of ROS of the cancer cells. ROS is superoxide and/or H_2O_2 . The reduction of intracellular concentration of superoxide and/or H_2O_2 in cancer cells may

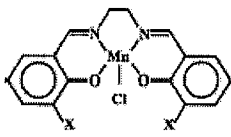
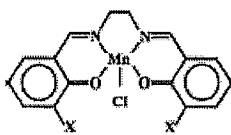
result in the suppression of malignant phenotype, reversion of malignancy, increased sensitivity of the cancer cells to apoptosis, and/or a reduction in the growth rate. In particular, the reduction of intracellular concentration of superoxide and/or H_2O_2 in cancer cells induces apoptosis. Further, as a
 5 consequence of the reduction of intracellular concentration of superoxide and/or H_2O_2 , the treated cancer cells may show a partial or complete loss of tumorigenicity and/or a reduction in the growth rate.

The invention also provides a method comprising reducing the intracellular concentration of superoxide and/or H_2O_2 in cancer cells by administering ROS
 10 scavengers including Mn-SOD mimetics such as EUK-8 ($C_{16}H_{14}ClMnN_2O_2$) (Calbiochem) and EUK-134 (Sharpe M.A. and others, 2002, Biochemical Society, page 98); manganese porphyrins such as MnTMPyP ($C_{44}H_{36}Cl_5MnN_8$), MnTM-2-PyP ($C_{44}H_{36}Cl_5MnN_8$), MnTE-2-PyP ($C_{44}H_{36}Cl_5MnN_8$) (examples of the manganese porphyrins obtained from
 15 Calbiochem). The structural information about the Mn-SOD mimetics and manganese porphyrins is shown in Table 1.

20

25

Table 1: Structural Information about Mn-SOD mimetics and manganese porphyrins

Commercial Name	Chemical Name/Structure	Molecular Weight	Reference
EUK-8	 X = H	374.7	Brandier C. et al., J. Vasc. Res., 1997, 34:49
EUK-134	 X = OCH ₃	-	Martyne A Sharpe et al., 2002, Biochem. J., 366:97-107
MnTMPyP	Mn(III)tetrakis(1-Methyl-4-pyridyl)porphyrin pentachloride	909.0	Izbicka E. et al., 1999, Cancer Res., 59:634
MnTM-2-PyP	Mn(III)meso-tetrakis(N-methyl-2-pyridyl)porphyrin pentachloride	909.0	Batinic-Haberle I., 2002, Methods Enzymol. 349:223
MnTE-2-PyP	Mn(III)meso-tetrakis(N-ethyl-2-pyridyl)porphyrin pentachloride	965.1	Batinic-Haberle I., 2002, Methods Enzymol. 349:223

The cancer cells may be treated either in vitro or in vivo. The cancer cells may be mammal cancer cells. For example, mouse or human cancer cells. In particular, the cancer is colon carcinoma.

According to another aspect, the invention provides an isolated apoptosis-inducing factor (AIF) knockout and/or knockdown carcinoma cell, wherein the knockout and/or knockdown cell shows suppression of malignant phenotype, reversion of malignancy and/or increased sensitivity of cancer cells to apoptosis compared to the wild type cell. The knockout cells may be prepared according to any standard technology, for example, by knocking out the *aif* gene by homologous recombination. Knockdown cells may also be prepared according to any standard technology, for example by inhibiting the expression of the *aif* mRNA by RNAi as described above. In particular, by administering a construct and/or vector comprising the sequences SEQ ID NOS:13 and 15, or by administering the sequences SEQ ID NO:14 and 16. The cell may be a mammal cell, like a mouse or human cell. In particular, the cell is a colon carcinoma cell.

The knockout and/or knockdown cell of the invention shows an increased sensitivity to some drugs, compounds and substances. Accordingly, the invention relates to one or more knockout and/or knockdown cells, in particular knockout and/or knockdown cancer cells like colon, breast and/or lung carcinoma cells, wherein the inhibition of AIF is capable of increasing the sensitivity of the treated cells to cytotoxic substances comprising etoposide, trichostatin A, camptothecin, t-butyl hydrogen peroxide and/or H₂O₂, compared to the wild type cell.

The knockout and/or knockdown cell may show an intracellular concentration of peroxide lower than the wild type cell. A non-limiting example of the knockout and/or knockdown cells according to the invention includes *aif* knockout and/or knockdown DLD-1, HCT-116 and/or SW480. In particular, the knockdown cell is *aif* knockdown in SW480.

The *aif* knockdown cell of SW480 is prepared by any standard technique known in the state of the art by a skilled person. In particular, it is prepared by siRNA.

5 The knockout and/or knockdown cell shows suppression of malignant phenotype, reversion of malignancy and/or increased sensitivity of cancer cells to apoptosis.

According to a further aspect, the invention provides a use for the knockout cells made according to the method of the present invention, wherein the knockout cells are implanted in an animal, for the purposes of drug testing and/or drug screening. For example, for the purpose of being used as negative control of drug testing and/or drug screening. Any knockout cells prepared according to the invention may be implanted on the animal. In particular, *aif* knockout carcinoma colon, breast, and lung cell or cell line. For example, *aif* knockout cell lines HCT-116, DLD-1, and/or SW480 prepared according to the invention can be used. However, the knockout cells to be implanted on the animal is not limited to the exemplified knockout cell. Any carcinoma cell showing the characteristics of any carcinoma cell or cell line according to the invention may be implanted. Accordingly, the invention provides a non-human animal model which is implanted with the knockout cells made according to any embodiment of the present invention for the purpose of drug screening and/or drug testing. In particular, the animal model of the invention may be used as negative control in order to screen, test or discover drugs which may show suppression of malignant phenotype, reversion of malignancy, increased sensitivity of cancer cells to apoptosis, and/or a reduction in the growth rate. The animal model of the invention may be a mammal, for example a mouse or rat. Further, the non-human animal model may also be implanted with a knockdown cell made according to any embodiment of the present invention.

The invention further provides a use of the knockout cells prepared by any method of the present invention. The knockout cells are implanted in a non-human animal, for the purpose of drug testing and/or drug screening. In particular, as negative control. This is carried out by any suitable method
5 known in the art. For example, as shown in Torrance C J et al., Nature Biotechnology, 2001, 19:940-945 (herein incorporated as reference) or described Torrance et al., in WO 03/027632 (herein incorporated by reference). The non-human animal may be a mammal, for example a rat or mouse. Potential drugs are tested by administering them to cancer cells and
10 then comparing the results with the knockout cells or animal model implanted with knockout cells prepared according to the invention. Non-limiting examples of *aif* knockout cells are HCT-116 and/or DLD-1. Accordingly, the invention also provides an animal model implanted with at least one of the *aif* knockout cells of the invention.

15 As mentioned above the method of the invention comprises treating cancer cells in vivo or in vitro comprising administering to the cancer cells a drug, a compound or a substance or selectively inhibiting or reducing the activity of AIF. Any drug, compound or substance known in the art and suitable for the present method may be used.

20 Accordingly, the invention is also addressed to the screening and/or identification of candidate drugs, compounds or substances capable to inhibit or reduce the activity of AIF.

According to one aspect, steps to generate and assay such a candidate drug, compound or substance comprises: the purification of recombinant AIF and
25 an in vitro assay for NADH oxidase activity; screen for or design a selective inhibitor of AIF; assay the candidate drug, compound or substance for its ability to induce apoptosis of colon carcinoma cells, block the formation of

tumour cell colonies in soft agar, and inhibit the growth of colon carcinoma cells.

The crystal structure of the AIF polypeptide has been described in WO
5 03/089465 (herein incorporated by reference). Accordingly, the design of a
candidate drug, compound or substance can be done by following the
instructions and information disclosed in WO 03/089465 and further selecting
or identifying a candidate drug, compound or substance capable of binding or
reacting with AIF, at least one AIF domain, or with a portion of AIF, and
10 capable of inhibiting or reducing the biological activity of AIF.

According to a further aspect, the drug, compound or substance candidate
can be selected from known drugs, compounds and substances. Accordingly,
the invention provides a method for screening and/or identifying a candidate
15 drug capable of inhibiting or reducing the activity of apoptosis-inducing factor
(AIF) in cancer cells comprising: (a) administering a drug candidate to cancer
cells, and (b) determining the ability of the drug candidate to inhibit or reduce
AIF activity in cancer cells. The method may further comprise (c) comparing
the ability of the drug candidate to inhibit or reduce AIF activity in cancer cells
20 to the reduced or inhibited AIF activity of the cell according to any
embodiment of the present invention.

According to another aspect, the invention relates to a method for screening
and/or identifying a candidate drug capable of inhibiting and/or reducing the
activity of apoptosis-inducing factor (AIF) in cancer cells comprising: (a)
25 administering a drug candidate to cancer cells, and (b) determining the ability
of the drug candidate to reduce the intracellular concentration of ROS. For
example, the method comprises determining the ability of the drug candidate
to reduce the intracellular concentration of superoxide and/or H₂O₂. Further,
the method may comprise (c) comparing the ability of the drug candidate to

reduce the intracellular concentration of superoxide and/or H_2O_2 to the reduced intracellular concentration of the cell of any embodiment of the invention.

According to a further aspect, the invention relates to a method for screening
5 and/or identifying a candidate drug capable of inhibiting or reducing the
activity of apoptosis-inducing factor (AIF) in cancer cells comprising: (a)
administering a drug candidate to cancer cells, and (b) determining the ability
of the drug candidate to suppress the malignant phenotype, reverse the
malignancy and/or increase the sensitivity of cancer cells to apoptosis. The
10 method may further comprise (c) comparing the ability of the drug candidate
to suppress the malignant phenotype, reverse the malignancy and/or increase
the sensitivity of cancer cells to apoptosis to the level of suppression,
reversion and/or increased sensitivity of the cell of any embodiment of the
invention.

15 According to another aspect, the invention relates to a method for screening
and/or identifying a candidate drug capable of inhibiting or reducing the
activity of apoptosis-inducing factor (AIF) in cancer cells comprising: (a)
administering a drug candidate to cancer cells, and (b) determining the ability
of the drug candidate to induce apoptosis.

20 According to another aspect, the invention relates to a method for screening
and/or identifying a candidate drug capable of inhibiting or reducing the
activity of apoptosis-inducing factor (AIF) in cancer cells comprising: (a)
administering a drug candidate to cancer cells, and (b) determining the ability
of the drug candidate to increases the sensitivity of the treated cancer cells to
25 cytotoxic substances comprising etoposide, trichostatin A, camptothecin, t-
butyl hydrogen peroxide and/or H_2O_2 .

According to a particular aspect, the effects of the drug candidate obtained
with the step (b) are compared in the step (c) to a negative control

- represented by *aif* knockout cells or on cells treated according to any embodiment of the invention for reducing the activity of AIF. According to a more particular aspect, the negative control is represented by *aif* knockout cells or the cells treated according to the invention implanted on a non-human animal model for drug screening, as mentioned above. The *aif* knockout cell
5 implanted on the animal may be colon, breast and/or lung carcinoma cell or cell line. For example, *aif* knock out HCT-116, DLD-1 and/or SW480 cell(s) or cell line(s). The non-human animal model may be a mammal, for example a mouse or rat.
- 10 The invention further provides a pharmaceutical composition comprising the nucleic acid molecule of the invention. The pharmaceutical composition is useful for inhibiting, delaying or repressing the expression of *aif* gene and/or *aif* mRNA or for inhibiting or reducing the biological activity of AIF. More in particular, the pharmaceutical composition is useful for treating cancer, for
15 example colon carcinoma, or any other cancer suitable in the method of the invention. Accordingly, a pharmaceutical composition comprising a drug, compound, or substance capable of inhibiting or reducing the biological activity of AIF is within the scope of the present invention. The drug, compound or substance can be screened, selected or identified as described
20 above. In particular, the invention provides a pharmaceutical composition comprising at least one nucleic acid molecule complementary or which hybridise to a nucleic acid molecule having the sequence of SEQ ID NO:1 or SEQ ID NO:3 or a fragment thereof (the sequences SEQ ID NO:1 and SEQ ID NO:3 representing the mouse and human *aif* gene, respectively). In
25 particular, the pharmaceutical composition comprises a nucleic acid molecule is 18-30, 18-25, or 19-23 nucleotides, complementary or which hybridise to a nucleic acid molecule having the sequence of SEQ ID NO:1 or SEQ ID NO:3 or a fragment thereof. In particular, the pharmaceutical composition comprises

at least one nucleic acid molecule which is complementary to the *aif* gene and have the sequence of SEQ ID NOS: 13, 14, 15 and/or 16.

The invention also relates to the use of the nucleic acid molecule of the invention for the preparation of a pharmaceutical composition for use in therapy. The composition is for inhibiting or reducing the activity of AIF and/or for inhibiting, delaying or repressing the expression of *aif* gene or *aif* mRNA. More in particular, the use is for suppressing malignant phenotype, reversion of malignancy, increased sensitivity of cancer cells to apoptosis, induction of apoptosis, partial or complete loss of tumorigenicity and/or sensitizing the treated cancer cells to drug or stress-induced apoptosis.

Another aspect of the invention is a method of administering a drug according to any embodiment of the present invention to a non-human animal to suppress cancer and to the use of that treated animal as an animal for drug testing and/or screening. The animal model may be a mammal, for example a rat or mouse.

Tissue culture cell-based screening for anti-cancer drugs has been used for decades. However, a persistent problem with such cell-based screening lies in the nature of the control cells. Many compounds are toxic to cancer cells, but most are also toxic to all growing cells. Normal cells corresponding to the cell types represented by common tumors are generally not available or do not exhibit growth properties comparable to those of the tumors. For these and other reasons, it is difficult to determine whether the drugs are targeting tumor-specific molecules. Many strategies have been employed to overcome this problem. A particular general methodology has been developed by Torrance et al., and described in WO 03/027632 (and corresponding US patent application No. US 2003/0069256, both of them herein incorporated by reference).

According to a further aspect, the invention relates to a pair of cells comprising: a first cell; and a second cell, wherein the first cell and the second cell are isogenic but for: a *aif* gene and a gene encoding a fluorescent protein; wherein the first cell comprises a gene that encodes a first fluorescent protein
5 having a first absorption spectrum and a first emission spectrum; wherein the second cell comprises a gene that encodes a second fluorescent protein having a second absorption spectrum and a second emission spectrum; and wherein either: the first and second absorption spectra are not identical; and/or the first and second emission spectra are not identical. In particular,
10 the first and second absorption spectra are not identical and the first and second emission spectra are not identical. In particular, the cancer cells are from a continuous (immortal) mammalian cell line. In particular, the continuous (immortal) mammalian cell line is DLD-1 and/or HCT-116.

The term "isogenic," as it is used here, refers to the cell's own endogenous
15 genome, wherein the first cell is distinguished by the second cell for the absence of the *aif* gene. Further, the term isogenic also includes a cell in which the activity of the *aif* gene is reduced by RNAi. In particular, the first cell is an *aif* knockout and/or knockdown cell or animal implanted with the *aif* knockout and/or knockdown cell. Any type of cancer mammalian tumor cells
20 that can be maintained in a culture can be transfected and used to generate a cell with specific genetic alterations.

The first and second cells may be mammalian cells, for example, human, mouse or rat cells. In particular, the first cell is an *aif* knockout and/or
25 knockdown carcinoma cell according to the invention. Any carcinoma pair of cell can be used, in particular, colon, breast and lung cancer cells. For example, the pair of cells are HCT-116, DLD-1 cells or SW480. The first and second fluorescent proteins may be chosen from the group consisting of

green fluorescent protein, red fluorescent protein, blue fluorescent protein, yellow fluorescent protein, and cyan fluorescent protein.

According to a further aspect, the invention provides a method of making a pair of cells, comprising the steps of: genetically modifying a first cell to make an *aif* knockout and/or knockdown cell, which is isogenic with a second cell but for the *aif* gene; transfecting the first cell with a first gene that encodes a first fluorescent protein having a first absorption spectrum and a first emission spectrum; and transfecting the second cell with a second gene that encodes a second fluorescent protein having a second absorption spectrum and a second emission spectrum, wherein either the first and second absorption spectra are not identical and/or the first and second emission spectra are not identical. In particular, the first and second absorption spectra are not identical and wherein the first and second emission spectra are not identical. The first cell according to the present method is an *aif* knockout and/or knockdown cancer cell prepared according to the invention.

According to another aspect, the invention provides a method of identifying a test compound as selectively affecting a gene of interest (which is the *aif* gene) or its expression protein (AIF) comprising the steps of: culturing a first and second cell, wherein the first and second cells are isogenic but for the *aif* gene and a gene encoding a fluorescent protein, wherein the first cell comprises a first gene that encodes a first fluorescent protein having a first absorption spectrum and a first emission spectrum, and wherein the second cell comprises a second gene that encodes a second fluorescent protein having a second absorption spectrum and a second emission spectrum, wherein either the first and second absorption spectra are not identical and/or the first and second emission spectra are not identical; contacting the first and second cells with a test compound; and identifying the test compound as selectively affecting the *aif* gene or its expression protein if the growth rate of

the first cell is not altered with respect to the growth rate of the second cell. Alternatively, criteria for comparison are whether the test compound may cause in the cancer cell suppression of malignant phenotype, reversion of malignancy, increased sensitivity of the cancer cells to apoptosis, induction to
5 apoptosis, and/or a reduction in the growth rate. The first cell according to the present method is a knockout and/or knockdown *aif* cancer cell prepared according to the invention. The first and second cells may be co-cultured. In particular, an equal number of the first and second cells may be cultured. In particular, the first and second absorption spectra are not identical and the
10 first and second emission spectra are not identical. More in particular, the fluorescent proteins may be detected using fluorescence microscopy to assess growth rate. According to a particular example, the fluorescent proteins are detected using high-throughput fluorescence spectroscopy to assess growth rate. However, other criteria as indicated below may also be
15 used. The first and second fluorescent protein may be selected from the group consisting of green fluorescent protein, red fluorescent protein, blue fluorescent protein, yellow fluorescent protein, and cyan fluorescent protein.

The pair of cells of the invention can be cultivated in a single vessel, or a
20 divided vessel, including, without limitation, a cell culture dish or flask, a multi-well cell culture plate.

The isogenic cells described above can contain a gene that encodes a fluorescent protein. The following have been described in WO 03/027632
25 (herein incorporated by reference). All proteins capable of detection by fluorescence techniques are contemplated, including variants of known fluorescent proteins such as mutants of green fluorescent protein that have been modified to alter the absorbance and emission spectra of the protein (see, e.g. Prasher et al, Gene, 111:229-33, 1992; Chalfie, Photochem.
30 Photobiol., 62:651-6, 1995; Miteli & Spector, Nature Biotechnol., 15:961,

1997; Heim, et al., *Nature* 373:663-4, 1995; Cubitt, et al., *Trends Biochem.*, 20:448-55, 1995. Examples of suitable fluorescent proteins include green fluorescent protein (395 nm absorption/509 nm emission), yellow fluorescent protein (530 nm absorption/590 nm emission), red fluorescent protein, cyan
5 fluorescent protein (433 nm absorption/475 nm emission) and blue fluorescent protein (390 nm absorption/510 nm emission) (Clontech Living Colors.RTM. User Manual, August 2000).

The gene may be integrated in the genome, or more preferably, on an
10 expression vector. Any expression vector encoding a protein capable of detection by fluorescence spectroscopy is contemplated. Useful fluorescent protein vectors include, but are not limited to pGFPtpz-cmv and pGFPsph-cmv (Packard Instrument Co.) as well as pGFP, pEGFP, pEBFP, pEYFP, pECFP, and pd2EGFP (Clontech Laboratories, Inc.). Expression vectors
15 may be further modified to include selectable markers, such as antibiotic resistance genes. Preferably progeny of a single transfection will exhibit uniform fluorescent protein expression. An appropriate expression vector contains the necessary elements for the transcription and translation of the inserted coding sequence in a given cell type. Methods which are well known
20 to those skilled in the art can be used to construct expression vectors containing sequences encoding fluorescent proteins and appropriate transcriptional and translational control elements. Such techniques are described, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor,
25 N.Y., 1989, and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y., 1989. Depending upon the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used. In mammalian cell systems, promoters from mammalian genes or from
30 mammalian viruses are preferable. A number of viral-based expression

systems can be used to express fluorescent proteins in mammalian host cells. For example, if an adenovirus is used as an expression vector, sequences encoding fluorescent proteins can be ligated into an adenovirus transcription/translation complex comprising the late promoter and tripartite
5 leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome can be used to obtain a viable virus which is capable of expressing fluorescent proteins in infected cells (Logan & Shenk, Proc. Natl. Acad. Sci., 81:3655-3659, 1984).

Any method known in the art for detecting a fluorescent protein is
10 contemplated. The detection method may be, though need not be, quantitative. Fluorescent proteins can be detected by flow cytometry and fluorimetric assays. Preferably, fluorescent proteins are detected using fluorescence microscopy or high throughput fluorescence spectroscopy. More preferably, relative growth of the cells is followed in real time using
15 fluorescence spectroscopy.

Cells can be co-cultured or cultured separately. When cells are co-cultured, each cell preferably contains a different fluorescent protein. To facilitate detection, the fluorescent proteins should have absorption and/or emission
20 spectra that allow the proteins to be distinguishable in the detection method selected. It should be possible to either selectively excite the fluorescent protein or to selectively detect the emission of the fluorescent protein. The fluorescent proteins should either have absorption spectra that are not identical or emission spectra that are not identical or both. Preferably, the
25 fluorescent proteins will have either absorption or emission spectra that are non-overlapping. More preferably, the fluorescent proteins will have non-overlapping absorption and emission spectra.

Cells that are isogenic except for the *aif* gene and a gene that encodes a fluorescent protein can be used to screen test compounds for the ability to specifically affect the gene of interest. The cells are contacted with a test compound and the growth rates of the cells are monitored both before contact
5 with the test compound and after contact with the test compound. A test compound that does not change the growth rate of the first cell relative to the second cell is identified as selectively affecting the gene of interest. Growth can be monitored using any fluorescence technique known in the art, as discussed above. A test compound may be supplied in any appropriate carrier
10 or solvent and may be added to the cell culture by any means known in the art. Suitable solvents include, but are not limited to, aqueous solvents and DMSO. The growth rate of each cell in the pair of cells is then monitored in the presence of the test compound. The concentration of test compound used will vary widely depending on the compound and the conditions of the assay.
15 Other criteria which can be used for comparison with reference to the cancer cells within the scope of the present invention are whether the test compound may cause in the cancer cell suppression of malignant phenotype, reversion of malignancy, increased sensitivity of the cancer cells to apoptosis, induction to apoptosis, and/or a reduction in the growth rate.
20
Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be
25 produced recombinantly, or synthesized by chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead
30 one-compound" library method, and synthetic library methods using affinity

chromatography selection. The biological library approach is typically used for polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds. See Lam, *Anticancer Drug Des.*, 12:145, 1997.

5

Test compounds may be screened for the ability to slow the growth of cells possessing defined, known genetic alterations, including oncogenic mutations. More particularly, cells that are isogenic except for the *aif* gene may be contacted with a test compound and the relative growth rates of the
10 cells determined. To identify a test compound as selectively affecting expression of a gene of interest a pair of cells may be individually cultured or co-cultured in a single vessel using any appropriate cell growth medium and any appropriate growth vessel. Preferably, cells are co-cultured to allow precise internal calibration of each drug assay, eliminating a variety of errors
15 encountered when screening cell pairs that are maintained in separate plates.

Using high throughput screening, many discrete test compounds can be tested in parallel so that large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96-well microtiter
20 plates. The wells of the microtiter plates typically require assay volumes that range from 50 to 500 μ l. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, and plate readers are commercially available to fit the 96-well format. Other formats can be used as are convenient.

25

According to another aspect, the invention provides a method for screening carcinoma cells wherein the suppression of AIF and/or ROS increases the sensitivity of the treated cancer cells, shows suppression of malignant phenotype, reversion of malignancy, increases the sensitivity of cancer cells
30 to apoptosis, and/or induces apoptosis. Accordingly, the invention provides a

method for screening carcinoma cells comprising administering to carcinoma cells a compound for reducing and/or inhibiting AIF and/or ROS, or inhibiting the *aif* gene and/or *aif* mRNA, and determining whether inhibition or reduction of activity of AIF and/or reduction of ROS is capable of increasing the sensitivity of the treated cancer cells, showing suppression of malignant phenotype, causing reversion of malignancy, increasing the sensitivity of cancer cells to apoptosis, inducing apoptosis, and/or a reducing the growth rate, compared to the wild type cells and/or compared to a cell prepared according to any embodiment of the invention as negative control.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

Examples

Example 1

Inactivation of aif gene by homologous recombination

Gene targeting in diploid male cell lines HCT-116 and DLD-1 via homologous recombination have been described (Waldman et. al., Nature 381, 713-716, 1996; Shirasawa et. al., Science, 260:85-88, 1993). The present inventors utilised this strategy to modify the single X-linked locus of the *aif* gene by adapting the design for promoter-trap vectors (Sedivy and Dutriaux, 1999) in construction of the targeting vector. Briefly, PCR amplification of a 0.6kB region upstream of Exon 1 and 5.2kB region downstream of Exon 1 from the *aif* locus of HCT-116 genomic DNA were cloned into pGEM-T vector (Promega). Exon1 was replaced by Hygromycin gene (with polyadenylation signal) exactly at the start codon of *aif* gene (Figure 1).

To further reduce background selection of hygromycin-resistant clones due to non-homologous recombination, the bipartite method (Jallepalli et. al, Cell 105, 445-457, 2001) was utilised. The conventional promoter-trap vector
 5 described above was digested with Mlu 1, SacII and Pst1 (New England Biolabs) restriction enzymes to generate vectors with overlapping regions of the truncated *hyg* gene. The vectors were gel purified and transfected at $1 \mu\text{g}/10^6$ HCT-116 or DLD-1 cells via electroporation using Bio-Rad Gene Pulser system (250v, 960 μF). Transfected cells were plated at 10^3 cells/well
 10 in 96-well plates and selected in McCoy's 5A medium (Bio-Industries) and 0.3mg/ml Hygromycin (Clontech) for 2-3 weeks. 96-well plates were screened for individual clone which were isolated and analyzed by PCR analysis of genomic DNA using primers,

15 PgAFA: 5'TTGGGTGGGAAAGAGCCGGCAACTGGTAAAAG-3' (SEQ ID 5)
 Hyg-Rev: 5'AGATTCTTCGCCCTCCGAGAGCTGCATCAGGT 3'. (SEQ ID 6)

Candidate *aif*^{-y} (the minus sign denotes loss of single *aif* allele, y denotes Y chromosome) clones were confirmed by additional PCR analysis for the loss
 20 of 900 bp wild type junctions:

Primer A: GAGTCTGCGTAATGTGCGTGTGAAGAGAGACTGG (SEQ ID 7)
 Primer B: TTGCCTGGAATGGGTCAGTCACCTGGGAG (SEQ ID 8)

25 To further validate gene targeting of *aif* gene, we analyzed clones using RT-PCR by preparing oligo-dT primed cDNA from clones and analyzed with mRNA specific PCR primers for AIF and GAPDH:

AFRTA: 5'CGGTCGCCGAAATGTTCCGGTGTGGAG3' (SEQ ID 9)
 30 AFRTB: 5'ACGCGGCCTTTTTCTGTTTCTGTTCTGG3' (SEQ ID 10)

GPDA: 5'CCAATATGATTCCACCCATGGC3' (SEQ ID 11)

GPDB: 5'TTCTCCATGGTGGTGAAGAC3' (SEQ ID 12)

Clones negative for half mRNA were analyzed for protein expression via
5 Western blot analysis using anti-AIF polyclonal antibody (N-1, 1:2000) (QED Biosciences) as seen in Figure 2B.

Morphological Assessment of AIF knockout cell lines

Cells in attached culture were regularly checked morphologically via phase-
10 contrast microscopy. Cells were also assessed for morphological changes when cultured at different conditions; sub-confluent, confluent and post-confluence.

Interestingly, the AIF knockout cell lines detach and appear as aggregates when cultures are continuously cultured for 5-7 days post-confluency without
15 changing media (Figure 4).

Multicellular Spheroid Formation Assay

Trypsinized cells were resuspended in supplemented McCoy's 5A media at
20 10^4 cells/ml and plated on top of solidified medium containing 1% noble agar. Spheroids were allowed to form for 48 hours and photographed using Nikon Coolpix 995 digital camera attached to a Zeiss Axiovert 25CFL microscope, see Figures 5A-D.

25 *Cell Growth and Cytotoxicity Assays*

5×10^3 cells were plated in 96-well plates and exposed to different concentrations of drugs; etoposide, camptothecin, staurosporine, doxorubicin, actinomycin D and trichostatin A; oxidative stress agents t-butyl hydrogen peroxide and hydrogen peroxide; and apoptosis inducers TRAIL

(Biogen) and anti-FAS (Roche). The cytotoxicity results obtained can be seen in Figure 6A-J and Figure 7A-I. Cells were incubated for 4 days at 37°C at 5% CO₂. Survival was measured by processing cells with MTS/PES solution (Promega Cell Titer Proliferation Assay) for 1 hour, measurement of color reaction absorbance 485nm and expressed as a fraction of untreated cells. For cell growth, 10³ cells were plated and processed with MTS/PES solution every 24 for hours for 4 days. Fold increase was measured as OD increases relative to day 1. The results obtained are shown in Figures 3A-B.

10 *Sytox- Hoechst stain for apoptotic cells:*

To detect apoptotic cells post-drug exposure, cells were stained with cell impermeable Sytox Green (Molecular Probes) and cell permeable, Hoechst 33342 (Sigma) dyes at a concentration of 500µg/ml for 10 minutes at 37°C (15 <http://www.probes.com/handbook>). Apoptotic cells are identified by condensed blue nuclei while necrotic cells stain green when viewed under a Fluorescence microscope, Zeiss Axiovert 25CFL.

In Figure 8A-D, AIF knockout cell lines are more sensitive to the cytotoxic drug, etoposide (60µM, 48 hrs) and undergo apoptosis readily compared to the wild type cells and vector control cell lines. (20

Colony Formation in Soft Agar

Cells were trypsinized and re-suspended in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100ug /ml streptomycin and 0.3% noble agar (Difco); 10³ cells per well were plated in six-well plates containing solidified medium with 1% noble agar. Plates were incubated at 37°C in 5% CO₂ and fed with media every 5 days. After 14-21 days, cells were incubated with 1mg/ml MTT (Sigma) for 1 hour and images (30

generated using a scanner and processed using Adobe Photoshop (Figure 9A-B and Figure 10A-B).

Tumorigenesis in athymic mice

- 5 Cells (5×10^6) were subcutaneously injected into the flanks of 4-5 weeks old athymic nude mice (and incubated for 3 weeks in an SPF environment. The results are shown in Figures 11A-B, 12 and 13. Tumours were excised, weighed and dimensions measured using calipers. Tumour volume (cm^3) was determined by using the standard formula $a^2 \times b/2$, where a is the width and b is the length of the horizontal tumour perimeter. 10-15 athymic nude mice were used for each cell line in two experiments, and the data were represented by the mean value as seen in Table 2.

Table 2: Summary of tumour size in DLD and HCT cell lines

Cell line	Sample No.	Weight (g)	Volume (cm^3)
DLD wt	15	0.5356 ± 0.2355	3.23
DLD vct	10	0.4356 ± 0.3210	2.35
DAF5	15	0.0432 ± 0.0321	0.210
DAF24	15	0.0356 ± 0.0125	0.132
HCTwt	15	1.2455 ± 0.3261	4.32
HCTvct	15	0.9456 ± 0.4532	3.65
BL12	15	0.1245 ± 0.0356	0.31
CL8	15	0.2365 ± 0.0521	0.25

Aconitase Assay

- Superoxide measurement using aconitase assay was adapted from Gardner et al., Superoxide radical and iron modulate aconitase activity in mammalian cells, J. Biol Chem, 270:13399-405, 1995. The protocol followed for this assay was that as described in Gardner et al. Briefly, 10^6 cells were lysed using M-PER (Pierce) solution and protein concentration measured using BioRad Protein Assay Solution. 30 μg protein samples were incubated with 50mM

Tris-Cl (pH 7.4), 0.6 mM MnCl_2 , 20 mM NADP^+ , 250 mM sodium citrate (pH 7.0) and 1U isocitrate dehydrogenase (Sigma) in a 200 μl reaction volume. Aconitase activity was quantified by measuring the rate of NADPH formation during citrate isomerization to isocitrate at 340nm using TECAN microplate reader. Relative superoxide levels was computed by subtracting the increased
5 in aconitase activity relative to the wt cells and shown as a percentage of the wild type control cell lines. The results are shown in the form of a graph in Figure 17.

10 *Lucigenin Assay*

Lucigenin based chemiluminescence measurement of intracellular superoxide has been described (Clement and Stamenkovic, Superoxide anion is a natural inhibitor of FAS-mediated cell death, EMBO J, 15:216-25, 1996). The protocol
15 in the above mentioned reference was followed. Briefly, 2×10^6 cells were lysed in 400 μl ATP releasing solution (Sigma) and pelleted at 12,000xg. Together with supernatant, 100 μl 850 μM Lucigenin (Sigma) was immediately added and chemiluminescence measured for 20s using a Turner Luminometer. Relative superoxide levels are expressed as a fraction of wild
20 type control cell lines. The results are shown in Figure 15.

Nitroblue Tetrazolium Reduction Assay

Cells (5×10^4) were incubated with 2 mM NBT for 30 minutes at 37°C at 5%
25 CO_2 to allow formazan formation then lysed with 50% DMSO/1M KOH. Absorbance was read at 650nm and relative superoxide levels are shown as a fraction of the wild type cell lines (J. Immunol. Methods 82:161, 1985.). The results are shown in Figure 16.

Catalase Activity

Catalase activity was assayed using Amplex Red Catalase assay kit (Molecular Probes) as per the manufacturer's protocol. The results obtained are shown in Figure 20. One of the observations in Harlequin mouse model (decreased AIF expression by 80%), is a significant increase in catalase activity which was hypothesized to be the decreased scavenging of superoxide leading to increased intracellular hydrogen peroxide in primary neuronal cells.

In contrast, our studies show that when AIF is diminished in colon cancer cell lines, catalase activity is reduced and this correlates with the lower levels of superoxide in the knockout cell lines. This is further supported by in vitro assays demonstrating that the major product of AIF oxidoreductase activity is superoxide. (Miramar et al., J. Biol. Chem. 276. 16391. 2001).

15 *Flow cytometric analysis of intracellular ROS*

Intracellular ROS levels were determined by staining with DCFH-DA (Molecular Probes), which is oxidized to DCF by ROS. Cells were exposed to 5 mM DCFH-DA at room temperature for 1 hour, and analyzed by flow cytometry using Becton Dickinson FACScan machine and analyzed using CellQuest program (Figure 19).

Transfection and Cell Sorting

PEGFP-C1 vector (Clontech) alone and vector containing *aif* coding region in frame with GFP cDNA were routinely transfected into 2×10^6 cells using Eugene 6 (Roche) transfection reagent according to manufacturer's instructions. 48 hours post-transfection cells were trypsinized and GFP positive cells sorted out using Becton Dickinson FACSVantage SE Cell Sorting System. Sorted cells were cultured for another 24-48 hours prior to

assays. The expected distribution of GFP is shown in figure 21A (nuclear /cytoplasmic) and that of AIF-GFP (mitochondrial/ cytoplasmic) is shown in figure 21B.

5 *Foci Formation in NIH3T3 cells*

To demonstrate the transforming properties of AIF, NIH3T3; a pre-neoplastic cell line, was transfected with AIF-GFP constructs and cell sorted as described above. Cells were continuously cultured for 3-6 weeks and
 10 monitored for foci formation. Foci formation shows lack of contact inhibition, one of the indicators of transformed cells (figures 22 and 23). NIH3T3 cells were transformed by AIF-GFP after 6 weeks of incubation (figures 22 and 23). However, compared to Nox1 (a mitogenic oxidase) (Nature 401, 79, 1999), which transform NIH3T3 cells in 2-3 weeks, the transforming potential of AIF
 15 is significantly less. Cells transformed with GFP alone failed to form any foci (figure 22).

Example 2

20 *AIF Knockdown via siRNA in SW480 colon carcinoma cells*

The experiment was conducted to determine whether knockdown of AIF via siRNA method in another colon carcinoma cell line (SW480) could replicate the observations in AIF knockout colon carcinoma cell lines, HCT-116 and
 25 DLD-1. Target sequences of SEQ ID NO: 13 and SEQ ID NO:15 were generated using Ambion siRNA target finder (http://www.ambion.com/techlib/misc/siRNA_finder.html). The sequences are as shown:

30 SW480 C3:

5' GCCGTACTGGCATCAGTCA 3' (SEQ ID NO:13)

SW480 A5:

5' GTTCCTCAAGACAAGGCGC 3' (SEQ ID NO:15)

5 Control Sequence SW480 D1:

5' CTGGTATCCGATCAGAGAG 3' (SEQ ID NO:17)

These DNA sequences were inserted in pSilencer 2.1-hyg (Ambion) vector using the protocol provided by the manufacturer comprising. Constructed
10 vectors comprising at least one of the DNA sequences SEQ ID NO:13 and 15, and a promoter for RNA polymerase were transfected into SW480 cells using Eugene Reagent (Roche) and selected with 3mg/ml Hygromycin (Clontech) for 2-3 weeks.

15 Once transfected into the SW480 cells, siRNA complementary to the *aif* mRNA were expressed by the cell RNA polymerase. The expressed siRNA comprised the sequence:

5' GCCGUACUGGCAUCAGUCA 3' (SEQ ID NO:14), and

5' GUUCCUCAAGACAAGGCGC 3' (SEQ ID NO:16).

20

Clones were isolated and evaluated for AIF levels of expression using Western blot analysis with anti-AIF polyclonal antibody (N-1, 1:2000) (QED Biosciences). Clones exhibiting greater than 50% reduction in AIF protein were analyzed for colony formation in soft agar and increased sensitivity to
25 cytotoxic agents: etoposide, hydrogen peroxide and t-butyl hydrogen peroxide. The cell growth and cytotoxicity assays, as well as the colony formation in soft agar were carried out in the same manner as in the Example 1.

The two target sequences of SEQ ID NO: 13 and SEQ ID NO:15 (and corresponding siRNA SEQ ID NO:14 and SEQ ID NO:16) significantly inhibited AIF expression. Cells transfected with a vector comprising the target sequence of SEQ ID NO:13 and/or SEQ ID NO:15 (or transfected directly by
5 administration of RNA sequences SEQ ID NO:14 and/or SEQ ID NO:16) reduced AIF expression by more than 50% compared to the SW480 wild type cells. As a control vector, cell line D1, which contained an ineffective target sequence (SEQ ID NO:17), was also isolated.

10 Cytotoxicity assays showed increased sensitivity to etoposide and oxidative stress inducers; hydrogen peroxide and t-butyl hydrogen peroxide in cell lines demonstrating reduced AIF expression (cell lines C3 and A5) but the same was not observed in the control cell line D1, as seen in Figures 24A-C.

15 The anchorage independent growth assay in soft agar also showed significant reduction in colony formation for the AIF knockdown cell lines (Figures 25A-B).

This experiment demonstrated that the inhibition of AIF expression via siRNA
20 in another colon cancer cell line (SW480) can reiterate the phenotype observed in the AIF knockout cell lines of HCT-116 and DLD-1.

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 Val Leu Val Leu Ile Val Gly Leu Ser Thr Ile Gly Ala Gly Ala Tyr
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Ser Ala Thr Glu Gly Gly Ser Val Pro Gln Ile Arg Ala Pro Ser His	
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Val Pro Phe Leu Leu Ile Gly Gly Gly Thr Ala Ala Phe Ala Ala Ala	
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Arg Ser Ile Arg Ala Arg Asp Pro Gly Ala Arg Val Leu Ile Val Ser	
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Glu Asp Pro Glu Leu Pro Tyr Met Arg Pro Pro Leu Ser Lys Glu Leu	
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Trp Phe Ser Asp Asp Pro Asn Val Thr Lys Thr Leu Gln Phe Arg Gln	
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Trp Asn Gly Lys Glu Arg Ser Ile Tyr Phe Gln Pro Pro Ser Phe Tyr	
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Val Ser Ala Gln Asp Leu Pro Asn Ile Glu Asn Gly Gly Val Ala Val	
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ctc act ggg aaa aag gta gta cat ctg gat gta aga ggc aac atg gtg	777
Leu Thr Gly Lys Lys Val Val His Leu Asp Val Arg Gly Asn Met Val	
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Lys Leu Asn Asp Gly Ser Gln Ile Thr Phe Glu Lys Cys Leu Ile Ala	
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acg gga ggc act cca aga agt ctg tct gcc atc gat agg gct gga gca	873
Thr Gly Gly Thr Pro Arg Ser Leu Ser Ala Ile Asp Arg Ala Gly Ala	
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Glu Val Lys Ser Arg Thr Thr Leu Phe Arg Lys Ile Gly Asp Phe Arg	
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Val Lys Arg Glu Gly Val Lys Val Met Pro Asn Ala Ile Val Gln Ser	
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Val Gly Val Ser Gly Gly Arg Leu Leu Ile Lys Leu Lys Asp Gly Arg	
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Lys Val Glu Thr Asp His Ile Val Thr Ala Val Gly Leu Glu Pro Asn	
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Gly Phe Arg Val Asn Ala Glu Leu Gln Ala Arg Ser Asn Ile Trp Val	
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Val Glu His His Asp His Ala Val Val Ser Gly Arg Leu Ala Gly Glu	
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Asn Met Thr Gly Ala Ala Lys Pro Tyr Trp His Gln Ser Met Phe Trp	
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Ser Asp Leu Gly Pro Asp Val Gly Tyr Glu Ala Ile Gly Leu Val Asp	
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Ser Ser Leu Pro Thr Val Gly Val Phe Ala Lys Ala Thr Ala Gln Asp	
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Pro	Ala	Val	Pro	Gln	Val	Pro	Val	Glu	Gly	Glu	Asp	Tyr	Gly	Lys	Gly		
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Val	Ile	Phe	Tyr	Leu	Arg	Asp	Lys	Val	Val	Val	Gly	Ile	Val	Leu	Trp		
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Glu	Gln	His	Glu	Asp	Leu	Asn	Glu	Val	Ala	Lys	Leu	Phe	Asn	Ile	His		
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Ser His Val Pro Phe Leu Leu Ile Gly Gly Gly Thr Ala Ala Phe Ala
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Ala Ala Arg Ser Ile Arg Ala Arg Asp Pro Gly Ala Arg Val Leu Ile
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Glu Leu Trp Phe Ser Asp Asp Pro Asn Val Thr Lys Thr Leu Gln Phe
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Arg Gln Trp Asn Gly Lys Glu Arg Ser Ile Tyr Phe Gln Pro Pro Ser
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Ala Val Leu Thr Gly Lys Lys Val Val His Leu Asp Val Arg Gly Asn
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Glu Lys Val Lys Arg Glu Gly Val Lys Val Met Pro Asn Ala Ile Val
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Gln Ser Val Gly Val Ser Gly Gly Arg Leu Leu Ile Lys Leu Lys Asp
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Pro Asn Val Glu Leu Ala Lys Thr Gly Gly Leu Glu Ile Asp Ser Asp
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Val Asp Ser Ser Leu Pro Thr Val Gly Val Phe Ala Lys Ala Thr Ala
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Gln Asp Asn Pro Lys Ser Ala Thr Glu Gln Ser Gly Thr Gly Ile Arg
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Lys Gly Val Ile Phe Tyr Leu Arg Asp Lys Val Val Val Gly Ile Val
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Leu Trp Asn Val Phe Asn Arg Met Pro Ile Ala Arg Lys Ile Ile Lys
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 Gly Gly Leu Ala Ala Gly Ala Leu Lys Gln Lys Leu Val Pro Leu Val
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Asn	Leu	Phe	Gln	Arg	Trp	His	Val	Pro	Leu	Glu	Leu	Gln	Met	Thr	Arg	
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caa	atg	gct	agc	tct	ggg	gca	tca	ggg	ggc	aaa	atc	gat	aat	tct	gtg	246
Gln	Met	Ala	Ser	Ser	Gly	Ala	Ser	Gly	Gly	Lys	Ile	Asp	Asn	Ser	Val	
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Leu	Val	Leu	Ile	Val	Gly	Leu	Ser	Thr	Val	Gly	Ala	Gly	Ala	Tyr	Ala	
	70					75				80						
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Tyr	Lys	Thr	Met	Lys	Glu	Asp	Glu	Lys	Arg	Tyr	Asn	Glu	Arg	Ile	Ser	
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Gly	Leu	Gly	Leu	Thr	Pro	Glu	Gln	Lys	Gln	Lys	Lys	Ala	Ala	Leu	Ser	
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Ala	Ser	Glu	Gly	Glu	Glu	Val	Pro	Gln	Asp	Lys	Ala	Pro	Ser	His	Val	
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Pro	Phe	Leu	Leu	Ile	Gly	Gly	Gly	Thr	Ala	Ala	Phe	Ala	Ala	Ala	Arg	
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Ser	Ile	Arg	Ala	Arg	Asp	Pro	Gly	Ala	Arg	Val	Leu	Ile	Val	Ser	Glu	
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Asp	Pro	Glu	Leu	Pro	Tyr	Met	Arg	Pro	Pro	Leu	Ser	Lys	Glu	Leu	Trp	
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Phe	Ser	Asp	Asp	Pro	Asn	Val	Thr	Lys	Thr	Leu	Arg	Phe	Lys	Gln	Trp	
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aat	gga	aaa	gag	aga	agc	ata	tat	ttc	cag	cca	cct	tct	ttc	tat	gtc	678
Asn	Gly	Lys	Glu	Arg	Ser	Ile	Tyr	Phe	Gln	Pro	Pro	Ser	Phe	Tyr	Val	
			200					205					210			
tct	gct	cag	gac	ctg	cct	cat	att	gag	aat	ggg	ggg	gtg	gct	gtc	ctc	726
Ser	Ala	Gln	Asp	Leu	Pro	His	Ile	Glu	Asn	Gly	Gly	Val	Ala	Val	Leu	
		215					220					225				
act	ggg	aag	aag	gta	gta	cag	ctg	gat	gtg	aga	gac	aac	atg	gtg	aaa	774
Thr	Gly	Lys	Lys	Val	Val	Gln	Leu	Asp	Val	Arg	Asp	Asn	Met	Val	Lys	
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Leu	Asn	Asp	Gly	Ser	Gln	Ile	Thr	Tyr	Glu	Lys	Cys	Leu	Ile	Ala	Thr	

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Gly Gly Thr Pro Arg Ser Leu Ser Ala Ile Asp Arg Ala Gly Ala Glu														
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Val Lys Ser Arg Thr Thr Leu Phe Arg Lys Ile Gly Asp Phe Arg Ser														
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Leu Glu Lys Ile Ser Arg Glu Val Lys Ser Ile Thr Ile Ile Gly Gly														
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Ala Leu Gly Thr Glu Val Ile Gln Leu Phe Pro Glu Lys Gly Asn Met														
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gga aag atc ctc ccc gaa tac ctc agc aac tgg acc atg gaa aaa gtc														1110
Gly Lys Ile Leu Pro Glu Tyr Leu Ser Asn Trp Thr Met Glu Lys Val														
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Arg Arg Glu Gly Val Lys Val Met Pro Asn Ala Ile Val Gln Ser Val														
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Phe Arg Val Asn Ala Glu Leu Gln Ala Arg Ser Asn Ile Trp Val Ala														
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Gly Asp Ala Ala Cys Phe Tyr Asp Ile Lys Leu Gly Arg Arg Arg Val														
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Glu His His Asp His Ala Val Val Ser Gly Arg Leu Ala Gly Glu Asn														
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Met Thr Gly Ala Ala Lys Pro Tyr Trp His Gln Ser Met Phe Trp Ser														
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Ile Phe Tyr Leu Arg Asp Lys Val Val Val Gly Ile Val Leu Trp Asn	
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Lys Glu Leu Trp Phe Ser Asp Asp Pro Asn Val Thr Lys Thr Leu Arg
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Phe Lys Gln Trp Asn Gly Lys Glu Arg Ser Ile Tyr Phe Gln Pro Pro
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Ser Phe Tyr Val Ser Ala Gln Asp Leu Pro His Ile Glu Asn Gly Gly
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Val Ala Val Leu Thr Gly Lys Lys Val Val Gln Leu Asp Val Arg Asp
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Asn Met Val Lys Leu Asn Asp Gly Ser Gln Ile Thr Tyr Glu Lys Cys
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Leu Ile Ala Thr Gly Gly Thr Pro Arg Ser Leu Ser Ala Ile Asp Arg
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Ala Gly Ala Glu Val Lys Ser Arg Thr Thr Leu Phe Arg Lys Ile Gly

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Asp Phe Arg Ser Leu Glu Lys Ile Ser Arg Glu Val Lys Ser Ile Thr 290 295 300		
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Asp Gly Arg Lys Val Glu Thr Asp His Ile Val Ala Ala Val Gly Leu 385 390 395 400		
Glu Pro Asn Val Glu Leu Ala Lys Thr Gly Gly Leu Glu Ile Asp Ser 405 410 415		
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Claims:

1. Use of a compound inhibiting or reducing the activity of AIF in the preparation of a medicament for the treatment of cancer, wherein the treatment comprises inhibiting or reducing the activity of apoptosis-inducing factor (AIF).
5
2. The use of claim 1, wherein the treatment comprises selectively inhibiting the AIF.
3. The use of claim 1, wherein the inhibition or reduction of the activity of AIF induces or sensitises cancer cells and/or tumours to apoptosis.
10
4. The use of claims 1-3, wherein the cancer is colon carcinoma.
5. The use of claims 1-4, wherein the treatment increases the sensitivity of the treated cancer cells to a cytotoxic substance comprising at least one DNA modifying compound.
- 15 6. The use of claim 5, wherein the cytotoxic substance is selected from the group consisting of: etoposide, trichostatin A, camptothecin, t-butyl hydrogen peroxide and H₂O₂.
7. The use of claims 1-6, wherein the cancer cells are from a continuous mammalian cell line, the cell line comprising, DCD-1 and/or HTC-116.
- 20 8. The use of claims 1-7, wherein the inhibition or reduction of activity of AIF reduces the intracellular concentration of ROS.
9. The use of claim 1 to 8, wherein the inhibition or reduction of activity of AIF reduces the intracellular concentration of superoxide and/or H₂O₂.
10. The use of claims 8 or 9, wherein the reduction of intracellular concentration of ROS induces or sensitizes the cancer cells to apoptosis.
25

11. The use of claims 1-10, wherein the inhibition or reduction of activity of AIF results in the suppression of malignant phenotype, reversion of malignancy and/or increased sensitivity of cancer cells to apoptosis.
- 5 12. The use of claims 1-11, wherein the treatment enhances the sensitivity of cancer cells to apoptosis.
13. The use of claims 1-12, wherein the treatment comprises inhibiting, delaying or repressing the expression of the *aif* gene encoding the AIF polypeptide.
- 10 14. The use of claims 1-13, wherein the treatment comprises inhibiting, delaying or repressing the expression of *aif* mRNA.
- 15 15. An isolated apoptosis-inducing factor (AIF) knockout carcinoma cell, wherein the knockout cell shows suppression of malignant phenotype, reversion of malignancy and/or increased sensitivity of cancer cells to apoptosis compared to the wild type cell.
16. The cell of claim 15, wherein the cell is implanted in a non-human animal model.
17. A non-human animal model, wherein the animal model is implanted with the cell of claim 15.
- 20 18. An isolated cell, wherein the apoptosis-inducing factor (AIF) activity has been inhibited, delayed or reduced and wherein the cell shows suppression of malignant phenotype, reversion of malignancy and/or increased sensitivity of cancer cells to apoptosis.
19. The cell of claim 18, wherein the cell is a mammal cell, human cell and/or colon carcinoma cell.

20. The cell of claims 18 or 19, wherein the cell shows an increased sensitivity to cytotoxic substances comprising DNA modifying compounds.
- 5 21. The cell of claim 20, wherein the cytotoxic substance is selected from the group consisting of: etoposide, trichostatin A, camptothecin, t-butyl hydrogen peroxide and/or H₂O₂.
22. The cell of claims 18-21, wherein the nucleic acid molecule complementary to *aif* mRNA comprises a sequence of SEQ ID NO:14 or SEQ ID NO:16.
- 10 23. The cell of claims 18-21, comprising a construct and/or vector comprising a DNA nucleic acid comprising a molecule having the sequence of SEQ ID NO:13 and/or SEQ ID NO:15, and a RNA polymerase promoter.
24. The cell of claims 19-23, wherein the cell is implanted in a non-human animal model.
- 15 25. A non-human animal model, wherein the animal model is implanted with the cell of claims 21-23.
26. A method for screening and/or identifying a candidate drug capable of inhibiting or reducing the activity of apoptosis-inducing factor (AIF) in cancer cells comprising the steps of:
- 20 (a) administering a drug candidate to cancer cells, and
- (b) determining the ability of the drug candidate to
- inhibit or reduce AIF activity in cancer cells; or
 - reduce the intracellular concentration of ROS; or
 - reduce the intracellular concentration of superoxide and/or H₂O₂; or

- suppress the malignant phenotype, reverse the malignancy and/or increase sensitivity of cancer cells to apoptosis;

or

- induce apoptosis or determining the ability of the drug candidate to increase the sensitivity of the treated cancer cells to cytotoxic substances comprising etoposide, trichostatin A, camptothecin, t-butyl hydrogen peroxide and/or H₂O₂.

27. An isolated nucleic acid molecule comprising 15-40 nucleotides complementary or which hybridise to the *aif* gene or *aif* mRNA or fragment thereof, wherein the nucleic acid is for use in the treatment of cancer.

28. The isolated nucleic acid sequence of claim 27, which is complementary or hybridises with the *aif* gene or *aif* mRNA having the sequence of SEQ ID NO: 1 or SEQ ID NO: 3, or a fragment thereof, wherein in case of the mRNA the U in SEQ ID NO: 1 and SEQ ID NO: 3 is indicated as T.

29. The isolated nucleic acid molecule according to claims 27 or 28, wherein the nucleic acid molecule comprises a nucleotide sequence of SEQ ID NO:13 and/or SEQ ID NO:15 which is complementary to the *aif* gene or to a portion thereof or the nucleic acid molecule comprises a nucleotide sequence of SEQ ID NO:14 and/or SEQ ID NO:16, which is complementary to the *aif* mRNA or to a portion thereof.

30. A construct and/or vector comprising a DNA nucleic acid comprising a molecule having the sequence of SEQ ID NO:13 and/or SEQ ID NO:15, and a RNA polymerase promoter, wherein the nucleic acid molecule is for use in the treatment of cancer.

31. A pharmaceutical composition comprising the nucleic acid molecule of claims 27-29, the construct and/or vector of claim 30.
32. Use of the nucleic acid molecule of claims 27 -29 for the preparation of a pharmaceutical composition for use in therapy.
- 5 33. The use of claim 32, wherein the composition is for inhibiting or reducing the activity of AIF.
34. The use of claims 32-33, wherein the use is for suppressing malignant phenotype, reversion of malignancy, increased sensitivity of cancer cells to apoptosis, induction of apoptosis, partial or complete loss of
10 tumorigenicity and/or sensitizing the treated cancer cells to drug or stress-induced apoptosis.