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(54) Title: ALTERNATIVE OXIDASE (AOX) PREVENTS SMOKING-ASSOCIATED LUNG DAMAGE

(57) Abstract: The present invention relates to a nucleic acid molecule encoding a polypeptide, the polypeptide having the activity of alternative oxidase (AOX), wherein said nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule encoding a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 1; (b) a nucleic acid molecule encoding a polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence similarity to the amino acid sequence of SEQ ID NO: 1; (c) a nucleic acid molecule comprising or consisting of the nucleotide sequence of SEQ ID NO: 2; (d) a nucleic acid molecule having at least 60% sequence similarity to the nucleic acid molecule of (c); and (e) a nucleic acid molecule degenerate with respect to the nucleic acid molecule of (c) or (d), for use in a method of treating or preventing a disease or condition associated caused by the inhalation of toxic smoke. Further, the present invention relates to a vector comprising said nucleic acid molecule for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke and to a polypeptide encoded by said nucleic acid molecule for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke. The present invention further relates to a non-toxic host cell carrying the nucleic acid molecule, the vector or the polypeptide of the invention for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke and to a product of the non-toxic host cell for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

Alternative oxidase (AOX) prevents smoking-associated lung damage

The present invention relates to a nucleic acid molecule encoding a polypeptide, the polypeptide having the activity of alternative oxidase (AOX), wherein said nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule encoding a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 1; (b) a nucleic acid molecule encoding a polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence similarity to the amino acid sequence of SEQ ID NO: 1; (c) a nucleic acid molecule comprising or consisting of the nucleotide sequence of SEQ ID NO: 2; (d) a nucleic acid molecule having at least 60% sequence similarity to the nucleic acid molecule of (c); and (e) a nucleic acid molecule degenerate with respect to the nucleic acid molecule of (c) or (d), for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke. Further, the present invention relates to a vector comprising said nucleic acid molecule for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke and to a polypeptide encoded by said nucleic acid molecule for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke. The present invention further relates to a non-toxic host cell carrying the nucleic acid molecule, the vector or the polypeptide for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke and to a product of the non-toxic host cell for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

In this specification, a number of documents including patent applications and manufacturer's manuals are cited. The disclosure of these documents, while not considered relevant for the patentability of this invention, is herewith incorporated by reference in its entirety. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

Millions of people die each year due to the chronic effects of tobacco smoking. This includes not only cancer and cardiovascular disease, but also smoking-related emphysema, which according to WHO estimates will be the third major cause of death in the industrialized

countries in the next decade. Smoking imposes an acute stress on the lung epithelium, primarily due to metabolic poisons in tobacco smoke that disable cellular energetic processes. A major poison in tobacco smoke that interferes with cell respiration is carbon monoxide (CO) which directly cripples cytochrome oxidase (COX), a major enzyme of the mitochondrial energy system. This inhibition disables the cells in the lung from performing their normal physiological functions: for example, the ciliated cells are unable to expel from the lung the foreign particles also present in cigarette smoke, and which contain the tobacco tar and other toxins that bring about long-term damage, including the genetic changes that lead to cancer. The smoke from a single cigarette contains sufficient toxins acting on the respiratory chain that cells in the lung are functionally disabled (Wang et al., 2012). In addition, DNA damage induced by cigarette smoke leads to cellular senescence and apoptosis, and thus to progressive structural changes in the tissue, resulting in a global loss of lung function (Adcock et al., 2011; Aoshiba and Nagai, 2009; Deslee et al., 2010; Karrasch et al., 2008; Vadhanam et al., 2012).

Similarly, fumes or smoke from other sources such as toxic fumes from chemical industries or domestic appliances, exhaust emissions from a car or smoke developed during a fire also contain toxins such as CO that impair the function of cell respiration.

Thus, inhibition of the respiratory chain e.g. at the level of COX plays a key role in the development of symptoms observed in acute CO poisoning, and in some diseases related to smoking associated with a dysfunctional respiratory chain (Alonso et al., 2003).

Lower organisms possess an additional mitochondrial enzyme, known in the art as alternative oxidase (AOX), which can at least partially replace COX and by-pass inhibition of the respiratory chain e.g. at complexes III or IV resulting from toxins, genetic mutations or high cellular energy levels. However, AOX is absent from more advanced animal species, including humans.

Hakkaart et al., 2006 and Dassa et al., 2009 showed that AOX from the marine invertebrate *Ciona intestinalis* can be expressed in human embryonic kidney (HEK293T) cells and in human fibroblasts, where it is efficiently targeted to mitochondria by the cell's own protein-sorting machinery, and enzymatically functional under conditions where the electron flow through COX is inhibited by cyanide or genetic mutations.

U.S. Pat. No. 7,572,616 discloses polypeptides having AOX activity and showing at least 95% sequence identity to the sequence of *Ciona intestinalis* AOX or to fragments thereof and

polynucleotides encoding said polypeptides. Further, pharmaceutical compositions comprising the AOX peptides or polynucleotides and cells transformed or transfected with said polynucleotides are also disclosed. Finally, it is described that polypeptides, polynucleotides of AOX or cells transformed or transfected with such polynucleotides can be used in treating disorders associated with mitochondrial oxidative phosphorylation dysfunction such as Leigh syndrome; MERRF syndrome; Parkinson's Disease and related conditions; mitochondrial encephalomyopathies, including progressive external ophthalmoplegia, Kearns-Sayre syndrome and MELAS syndrome; diverse, multisystem pediatric disorders affecting organs such as liver, kidney, the CNS, heart, skeletal muscle, or the endocrine and sensorineural systems; diseases the pathogenesis of which are known or believed to involve excessive production of reactive oxygen species in mitochondria, such as amyotrophic lateral sclerosis, Alzheimer's disease, Friedreich ataxia or forms of cardiovascular disease attributable to defects in antioxidant defenses; other ataxias or neurological conditions resulting from genetic defects in POLG, clOorG (Twinkle) or other components of the system of mitochondrial DNA maintenance; mitochondrial hearing impairment, both syndromic and nonsyndromic forms of diabetes mellitus attributable to defects of the mitochondrial oxidative phosphorylation (OXPHOS) system; side-effects of antiretroviral therapies that impact the mitochondrial OXPHOS system; obesity or other metabolic disorders resulting from disturbances in the mobilization of food resources; NARP syndrome; Alpers-Huttenlocher disease; sensorineural deafness; benign infantile myopathy; fatal infantile myopathy; pediatric myopathy; adult myopathy; rhabdomyolysis; Leber hereditary optic neuropathy; cardiomyopathy; Barth syndrome; Fanconi syndrome; mtDNA depletion syndrome; Pearson syndrome; diabetes mellitus and lactic acidemia.

However, no indication is given that AOX could be used in treating or preventing tissue-damage associated with smoking or smoke poisoning.

Smoking-related disease is an enormous health burden worldwide, including most cases of chronic obstructive pulmonary disease (COPD) and emphysema, as well as lung cancer and possibly up to 25% of all cancers. It is also a major cause of heart disease and a risk factor for mortality in many other conditions, including pneumonia, tuberculosis and various age-related degenerative diseases such as osteoporosis, kidney failure and stroke. Accordingly, there is a great need for improved treatment options and developments towards treatment options for acute CO poisoning as well as for smoking related diseases and conditions.

This need is addressed by the provision of the embodiments characterised in the claims.

Accordingly, the present invention relates to a nucleic acid molecule encoding a polypeptide, the polypeptide having the activity of alternative oxidase (AOX), wherein said nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule encoding a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 1; (b) a nucleic acid molecule encoding a polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence similarity to the amino acid sequence of SEQ ID NO: 1; (c) a nucleic acid molecule comprising or consisting of the nucleotide sequence of SEQ ID NO: 2; (d) a nucleic acid molecule having at least 60% sequence similarity to the nucleic acid molecule of (c); and (e) a nucleic acid molecule degenerate with respect to the nucleic acid molecule of (c) or (d), for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke.

As will be apparent from the description provided herein below, toxic smoke can cause a variety of diseases or may render a subject more susceptible to a specific condition or disease such as COPD. In accordance with the present invention, AOX is to be used only in treating the subgroup of diseases caused or exacerbated by toxic smoke. Accordingly, only the subgroup of subjects at risk of getting a specific disease or patients suffering from a specific disease who have been or will be exposed to toxic smoke are to be treated in accordance with the present invention. Especially preventive treatment is promising and envisioned herein. An example is smoke-induced COPD. Toxic smoke is causative in particular for diseases of the respiratory tract. In the context of these medical indications the term “respiration” refers to respiration on the level of an organism rather than on the molecular level.

Accordingly, in a related aspect the present invention provides a nucleic acid molecule encoding a polypeptide, the polypeptide having the activity of alternative oxidase (AOX), wherein said nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule encoding a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 1; (b) a nucleic acid molecule encoding a polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence similarity to the amino acid sequence of SEQ ID NO: 1; (c) a nucleic acid molecule comprising or consisting of the nucleotide sequence of SEQ ID NO: 2; (d) a nucleic acid molecule having at least 60% sequence similarity to the nucleic acid molecule of (c); and (e) a nucleic acid molecule degenerate with respect to the nucleic acid molecule of (c) or (d), for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

In accordance with this aspect as well as the other embodiments, the disease or condition caused by the inhalation of toxic smoke preferably is a disease of the respiratory tract. More preferably, the disease or condition caused by the inhalation of toxic smoke is chronic obstructive pulmonary disease (COPD), emphysema, lung cancer or pneumonia. Most preferably, the disease or condition caused by the inhalation of toxic smoke is COPD, herein also referred to as "smoke-induced COPD". The term "smoke-induced" not only refers to the disease being caused by smoke derived from cigarette smoking but also by smoke derived from other sources, such as, for example, form vehicle exhaust emissions, in particular from diesel vehicles, or from indoor pollution caused by biomass-combustion stoves.

The following terms are all known in the art and are used in accordance with the definitions provided there. The definitions provided in this specification are solely intended to reflect the understanding of these terms in the art. Where nevertheless a discrepancy arises, the definitions provided herein supersede.

In accordance with the present invention, the term "nucleic acid molecule" includes DNA, such as cDNA or genomic DNA, and RNA. Preferably, embodiments reciting "RNA" are directed to mRNA. Further included are nucleic acid mimicking molecules known in the art such as synthetic or semi-synthetic derivatives of DNA or RNA and mixed polymers. Such nucleic acid mimicking molecules or nucleic acid derivatives according to the invention include phosphorothioate nucleic acid, phosphoramidate nucleic acid, 2'-modified ribonucleic acid such as 2'-O-methoxyethyl ribonucleic acid, morpholino nucleic acid, hexitol nucleic acid (HNA) and locked nucleic acid (LNA) (Braasch and Corey, 2001). LNA is an RNA derivative in which the ribose ring is constrained by a methylene linkage between the 2'-oxygen and the 4'-carbon. They may contain additional non-natural or derivative nucleotide bases, as will be readily appreciated by those skilled in the art. The term "nucleic acid molecule" is used interchangeably with the term "polynucleotide" herein. Preferably, the nucleic acid molecule is DNA. It is also preferred that the nucleic acid molecule is in an expressible form, i.e. in a form that can lead to the expression of the encoded polypeptide.

The term "polypeptide", in accordance with the present invention, describes linear molecular chains of amino acids, including single chain polypeptides or their fragments, containing more than 30 amino acids. The term "fragment" in accordance with the present invention refers to a portion of the polypeptide comprising at least the amino acid residues necessary to maintain the biological activity of the polypeptide. Polypeptides may further form higher order structures, typically by non-covalent association, such as oligomers consisting of at least two

identical or different molecules. The corresponding higher order structures of such multimers are, correspondingly, termed homo- or heterodimers, homo- or heterotrimers etc. Furthermore, peptidomimetics of such proteins/polypeptides where amino acid(s) and/or peptide bond(s) have been replaced by functional analogues are also encompassed by the invention. Such functional analogues include all known amino acids other than the 20 gene-encoded amino acids, such as selenocysteine. The terms "polypeptide" and "protein" are used interchangeably herein and also refer to naturally modified polypeptides wherein the modification is effected e.g. by glycosylation, acetylation, phosphorylation and the like. Such modifications are well known in the art.

AOX is an abbreviation for "alternative oxidase" and both terms are used interchangeably herein. AOX is widely expressed in plants, many fungi and some protozoa as well as in some animal phyla, where it is targeted to the mitochondria by a mitochondrial targeting peptide (ranging from 50-80 residues in length) at the N-terminus of the peptide, but not in mammals (Dassa et al., 2009). Structural models and studies classify AOX as a di-iron protein (Siedow et al., 1995, Moore et al., 1995, Andersson and Nordlund, 1999, Berthold et al., 2002). The iron atoms are thought to be ligated by Glu-X-X-His motifs of AOX. However, the structural models as published differ with regard to exact positions of the residues that are involved in coordinating the iron atoms. Accordingly, the studies differ also in the assumed overall structure of AOX. In the original model proposed by Siedow et al. and Moore et al. AOX is suggested to comprise two transmembrane helices in addition to a C-terminal four-helix bundle containing the coupled binuclear iron centre. It is suggested that the ferric centre is coordinated by two histidines, two glutamates, one aspartate and two water molecules. Modification of that original hypothesis using additional sequence data and evolutionary considerations has generated a model of AOX as a di-iron carboxylate protein wherein a different Glu-X-X-His is proposed to play a role in ligating the iron centre. Since this motif is located in the intermembrane space according to the model proposed by Siedow et al., Andersson and Nordlund proposed an overall structure of AOX, wherein it interacts with one leaflet of the membrane bilayer as an interfacial integral membrane protein rather than a transmembrane protein (Andersson and Nordlund, 1999). The model of AOX being an integral membrane protein associated with the matrix side of the mitochondrial membrane is also supported by Albury et al. (Albury et al., 2002). In this study AOX from *Schizosaccharomyces pombe* and its mutants were analysed and it was found that mutation of Glu-217, Glu-270 and Tyr-275 led to a loss of activity of AOX. Accordingly, a potential role for Tyr-275 in providing a tyrosyl radical involved in electron transport was suggested. Electron paramagnetic resonance (EPR) studies on recombinantly expressed AOX1a from

Arabidopsis thaliana in combination with mutagenesis of putative iron-coordinating residues indicated that AOX contains a hydroxo-bridged di-iron centre and that the putative iron-coordinating residues Glu-222, His-225, Glu-273, and His-327 are required for AOX activity (Berthold et al., 2002).

Where expressed, AOX functions as an alternative to COX as the terminal oxidase of the electron transfer chain, accepting electrons from ubiquinol, with the concomitant reduction of molecular oxygen to water. Thus, AOX functions as an ubiquinol oxidase. The alternative oxidase branches the respiratory chain, which can be envisioned as chain of protein complexes. In case of genetic or pharmacologic inhibition of one complex, all up-stream complexes are functionally blocked. AOX circumvents this blockade, restores ATP production and decreases oxidative stress. However, electron transfer through AOX is not coupled to proton translocation, so two of the three sites of energy conservation of the conventional respiratory chain are bypassed and the free energy released is lost as heat. Unlike the cytochrome pathway, the alternative pathway through AOX does not function directly as proton pump and, therefore, does not contribute directly to oxidative phosphorylation, even though it might facilitate electron flow through complex I and thereby support phosphorylation. Because AOX has the potential to decrease the efficiency of cellular respiration it is tightly regulated. It is generally assumed that the AOX pathway can serve to protect an organism that expresses AOX, such as a plant, during periods of stress (Wagner, 1995; Robson et al., 2002). In contrast to the conventional cytochrome c oxidase, AOX is resistant to inhibitors that act at electron transfer complexes III (including myxothiazol and antimycin A) and IV (including cyanide or carbon monoxide).

As used herein the alternative oxidase activity or activity of AOX is the capability to confer the ability to respire and to consume oxygen in the presence of an inhibitor of the respiratory chain to a cell, such as e.g. a mouse 3T3 fibroblast or a HEK293T cell. In particular the alternative oxidase activity or activity of AOX is the capability to confer the ability to respire and to consume oxygen in the presence of cyanide, CO or antimycin A. Preferably, the alternative oxidase activity or activity of AOX is determined by polarographically measuring oxygen consumption in the presence and absence of an inhibitor of the respiratory chain, in particular in the presence or absence of cyanide, CO or antimycin A. More preferably, the alternative oxidase activity is assessed in the presence and absence of antimycin A. A similar rate of oxygen consumption in presence and absence of the inhibitor of the respiratory chain is indicative of a functional AOX. In other words, a rate of oxygen consumption in the presence of the inhibitor that corresponds to at least 50%, such as at least 60%, such as at

least 70%, more preferably at least 80% of the rate of oxygen consumption in the absence of the inhibitor is indicative of a functional AOX. Most preferably, the alternative oxidase activity or activity of AOX is the capability to confer the ability to respire and consume oxygen in the presence of antimycin A to a mouse 3T3 fibroblast as assessed by polarographically measuring oxygen consumption in the presence and absence of antimycin A, wherein a rate of oxygen consumption in the presence of antimycin A that is at least 70% of the rate of oxygen consumption in the absence of antimycin A is indicative of a functional AOX.

Preferably, in accordance with the present invention AOX is the alternative oxidase of *Ciona intestinalis*. The amino acid sequence of the alternative oxidase of *C. intestinalis* is represented in SEQ ID NO:1.

The polypeptide having the amino acid sequence of SEQ ID NO: 1 is for example encoded by the nucleic acid molecule having the sequence of SEQ ID NO: 2. The polypeptide may further be a polypeptide produced for example by a host cell carrying the nucleic acid molecule or the vector for use in accordance with the invention and isolated from the cell or the medium in which the cell is grown. Methods for making a host cell produce a polypeptide and for isolating the polypeptide from the cell or the medium in which the cell is grown are well known in the art. Suitable prokaryotic host cells for the expression of the polypeptide comprise e.g. bacteria of the species Escherichia, Bacillus, Streptomyces and Salmonella typhimurium. Suitable eukaryotic host cells are e.g. fungal cells, inter alia, yeasts such as *Saccharomyces cerevisiae* or *Pichia pastoris* or insect cells such as Drosophila S2 and Spodoptera Sf9 cells and plant cells as well as mammalian cells. Appropriate culture media and conditions for the above described host cells are known in the art.

Mammalian host cells that could be used include, human Hela, HEK293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, monkey Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells. Also within the scope of the present invention are primary mammalian cells such as mouse embryonic fibroblasts (MEF). Alternatively, the recombinant (poly)peptide can be expressed in stable cell lines that contain the gene construct integrated into a chromosome. It is understood that the host cell, if derived from a cell line, is to be preferably killed prior to administration.

In a more preferred embodiment, said cell is a primary cell or primary cell line. Primary cells are cells, which are directly obtained from an organism. Preferably, said primary cell or primary cell line is a primary mammalian cell or primary mammalian cell line. Suitable primary mammalian cells are, for example, mouse embryonic fibroblasts, mouse primary hepatocytes, cardiomyocytes and neuronal cells as well as mouse muscle stem cells (satellite cells) and stable, immortalized cell lines derived thereof.

Also encompassed by the present invention is a nucleic acid molecule encoding a polypeptide having at least 60% sequence similarity to the amino acid sequence of SEQ ID NO:1 at least over the entire length of the AOX domain, preferably over the length of the mature polypeptide as defined by SEQ ID NO:3 or over the entire length of the polypeptide. More preferably, the nucleic acid molecule encodes a polypeptide having at least 65% sequence similarity to the AOX-domain, the mature polypeptide (SEQ ID NO:3) or the amino acid sequence of SEQ ID NO:1, such as for example at least 70%, such as at least 80%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, more preferably at least 98%, such as at least 98.5%, such as at least 99% and most preferably at least 99.5% sequence similarity to the AOX-domain, the mature polypeptide (SEQ ID NO:3) or the amino acid sequence of SEQ ID NO:1.

Molecules falling under this definition may be isoforms, homologous molecules from other species, such as orthologs, or mutated sequences from the same species to mention some preferred examples.

In accordance with the present invention, the term "% sequence similarity" describes the number of matches ("hits") of identical or similar nucleotides/amino acids of two or more aligned nucleic acid or amino acid sequences as compared to the number of nucleotides or amino acid residues making up the overall length of the nucleic acid or amino acid sequences (or the overall compared part thereof). In other terms, using an alignment, for two or more sequences the percentage of amino acid residues or nucleotides that are the same or similar (e.g., 80% or 85% similarity) may be determined, when the sequences are compared and aligned for maximum correspondence over a window of comparison, or over a designated region as measured using a sequence comparison algorithm as known in the art, or when manually aligned and visually inspected. In accordance with the invention amino acids that are not identical are considered to be similar if they are functionally identical, i.e. if the replacement of one amino acid with the other does not lead to a loss of activity of the polypeptide. Amino acids are classified as being similar based on the relative physiochemical properties of the amino acid side-chain substituents, for example, their hydrophobicity, charge, size, and the like. Exemplary pairs of similar amino acids which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine. Preferred nucleic acid molecules/polypeptides in

accordance with the present invention are those having at least 60% sequence similarity over the entire length of the AOX-domain. It is preferred that these polypeptides also comprise a functional mitochondrial targeting sequence. The term "AOX-domain" refers to the part of the AOX-polypeptide that is required for its activity as defined below or to the part of the nucleic acid molecule that encodes that part of the AOX-polypeptide. The skilled person is able to identify the AOX domain either bioinformatically e.g. by alignment of SEQ ID NOs: 1 or 2 with alternative oxidases from other organisms or with known oxidase domains from other proteins or experimentally e.g. by assessing a series of deletion mutants comprising only a part of the polypeptide for their ability to act as an oxidase. Assays for determining the ability of AOX or its mutants are well known in the art and are also described elsewhere herein. It is understood that the term "ability" refers to the activity of AOX as defined herein above. More preferably, the polypeptides or nucleic acid molecules exhibit at least 60% sequence similarity to the polypeptide of SEQ ID NO:3 or the nucleic acid molecule of SEQ ID NO:4, i.e. over the entire length of the mature protein. In accordance with the invention, the mature protein is characterized on the amino acid level by SEQ ID NO:3 and on the DNA level by SEQ ID NO:4. A nucleic acid/polypeptide having at least 60% sequence similarity to SEQ ID NO:3 or the part of SEQ ID NO:2 encoding the sequence of SEQ ID NO:3 may have less than 60% sequence similarity to SEQ ID NO:1 or SEQ ID NO:2 over the entire length of the sequence. It is envisioned that a nucleic acid or polypeptide having at least 60% sequence similarity to the AOX domain may harbour additions, deletions or substitutions of amino acids within the mitochondrial targeting sequence, i.e. in positions 1 to 51 of SEQ ID NO:1 or in the nucleic acid sequence encoding these residues, especially if these alterations have a beneficial effect e.g. on AOX function or on decreasing the possibility of an immune response in a subject. This includes polypeptides in which the targeting sequence has been replaced by an entirely different sequence as long as the sequence is still suitable for targeting AOX to the mitochondria. Methods for determining, either bioinformatically or experimentally, whether a sequence is suitable for targeting AOX to the mitochondria are well known in the art. For example, the mitochondrial targeting peptide, i.e. positions 1 to 51 of SEQ ID NO:1, may be replaced by the targeting peptide of the ATP5B gene (SEQ ID NO:6) or by the targeting peptide the ACO2 gene (SEQ ID NO:7). Consequently, the nucleic acid molecule as defined herein above may comprise the nucleotides encoding these peptides (SEQ ID NO: 8 or 9) instead of the nucleotides encoding positions 1 to 51 of SEQ ID NO:1. In other words, nucleotides 1 to 153 of SEQ ID NO:2 may be replaced by the nucleotides of SEQ ID NO: 8 or 9. In addition, the nucleic acid molecule may comprise the natural 3' untranslated region (UTR) of AOX or, preferably, the 3' UTR of a human gene encoding a mitochondrially located protein, such as e.g. the 3' UTR of ATP5B (SEQ ID NO:10) or of ACO2 (SEQ ID NO:11). Also

preferred are nucleic acid molecules/polypeptides having at least 60% sequence similarity over the entire length of the SEQ ID NO:1 or SEQ ID NO:2. Those having skill in the art will know how to determine percent sequence similarity between/among sequences using, for example, algorithms such as those based on the NCBI BLAST algorithm (Altschul, et al., 1997), CLUSTALW computer program (Thompson, 1994) or FASTA (Pearson and Lipman, 1988), as known in the art.

The NCBI BLAST algorithm is preferably employed in accordance with this invention. The BLASTN program for nucleic acid sequences uses as default a word length (W) of 11, an expectation (E) of 10, M=5, N=4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as default a word length (W) of 3, and an expectation (E) of 10. The BLOSUM62 scoring matrix (Henikoff, Proc. Natl. Acad. Sci., 1989, 89:10915) uses alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands. Accordingly, all the nucleic acid molecules having the prescribed function and further having a sequence similarity of at least 60% as determined with the NCBI BLAST program fall under the scope of the invention.

It is preferred that the polypeptide having at least 60% sequence similarity to SEQ ID NO:1 (or any other level of sequence similarity as disclosed above) does not comprise amino acid additions, deletions or substitutions in the sequence and boundaries of any conserved functional domain(s) or subsequences in the sequence of SEQ ID NO: 1. Determination of such domains can be based, for example, on the published structural models of AOX (such as for example the ones referred to herein above). Further means and methods for determining such domains are well known in the art and include experimental and bioinformatic means. Experimental means include the systematic generation of deletion mutants and their assessment in assays for the biological activity of alternative oxidase. As used herein the term "domain" in particular refers to the AOX domain. Such assays for the determination of the biological activity of AOX are well known in the art and an exemplary assay is described in the appended example. Bioinformatic means include database searches. Suitable databases include protein sequence databases. In this case a multiple sequence alignment of significant hits is indicative of domain boundaries, wherein the domain(s) is/are comprised of the/those subsequences exhibiting an elevated level of sequence conservation as compared to the remainder of the sequence. Further suitable databases include databases of statistical models of conserved protein domains such as Pfam maintained by the Sanger Institute, UK (www.sanger.ac.uk/Software/Pfam). It is also understood that the polypeptide having at least 60% sequence similarity to SEQ ID NO:1 does not contain amino acid additions, deletions or non-conservative substitutions in positions that are essential for the activity of AOX. This

includes amino acid residues that have already been published to be essential for the activity of AOX as well as residues that are predicted to be essential structurally or functionally for example by bioinformatic means. It is further preferred that the polypeptide having at least 60% sequence similarity to SEQ ID NO:1 maintains the iron-coordinating residues, the residues involved in electron transport as well as a functional N-terminal sequence targeting the protein to mitochondria. The ability of an N-terminal sequence of targeting the protein to mitochondria can be predicted by bioinformatical means or can be assessed experimentally. MitoProt II (Claros and Vincens, 1996) predicts a cleavage site at position 52 with the amino acids MLSTGSKTFLFRPFLGSCHALQSGKLPCSNLHTTPTKITVKRYLVGYSWST (SEQ ID NO:5) relevant for mitochondrial import. Methods for determining the mitochondrial localisation of a protein (i.e. for ensuring experimentally that the N-terminal sequence is efficient in targeting the protein to the mitochondria) are well known in the art and include, for example, immunocytochemistry. Further, the activity of AOX (as assessed by activity assays such as the one described in the appended example) is indicative of the presence of a functional targeting sequence since the activity of AOX depends on its correct mitochondrial localisation. It is envisioned that a polypeptide having at least 60% sequence similarity to SEQ ID NO:1 as defined above may harbour additions, deletions or substitutions of amino acids within the targeting sequence, i.e. in positions 1 to 51 of SEQ ID NO:1, especially if these have a beneficial effect e.g. on AOX function or on decreasing the possibility of an immune response in a subject. However, it will be understood that the altered targeting sequence nevertheless needs to be capable of targeting AOX to the mitochondria. It is also preferred that the polypeptide having at least 60% sequence similarity to SEQ ID NO:1 does not contain amino acid additions, deletions or substitutions in residues corresponding to Glu-217, Glu-270 or Tyr-275 of the amino acid sequence of *Schizosaccharomyces pombe* or in residues corresponding to Glu-222, His-225, Glu-273, and His-327 of the amino acid sequence of AOX1a of *Arabidopsis thaliana*. It is understood that the AOX polypeptide comprises two essential elements, the mitochondrial targeting sequence and the AOX domain. Both need to be present or need to have functional equivalents in the polypeptide and need to be encoded by the polynucleotide in accordance with the present invention.

Alternatively, it is preferred that the polypeptide showing sequence similarity to SEQ ID NO:1 retains at least 80% similarity to the polypeptide of SEQ ID NO:1 in its tertiary structure. The tertiary structure of a polypeptide is the overall three-dimensional fold assumed by the polypeptide. Methods for determining the tertiary structure of a polypeptide are well known in the art and include for example X-ray crystallography or NMR spectroscopy. The similarity between tertiary structures can be determined by structural alignment. The difference between

the structures of two proteins may be expressed in terms of the root mean square deviation (RMSD). The RMSD is the root mean square distance (in Å) between corresponding atoms of the least-squares fitted protein structures and is a numerical measure for the difference between two structures. The smaller the RMSD, the more similar the compared protein structures.

In accordance with the present invention, it is required that the polypeptide having at least 60% sequence similarity has the activity of alternative oxidase, as defined herein above, especially with regard to conferring the ability of substrate oxidation in presence of carbon monoxide. Accordingly, it will be appreciated that sequence modifications of the polypeptide for use in accordance with the invention that lead to a total loss of this function are not encompassed by the present invention. Consequently, only forms of AOX retaining at least a minimal level of activity such as at least 1%, such as at least 10%, such as at least 20%, such as at least 30% or at least 40% of the activity of the polypeptide of SEQ ID NO:1 are encompassed. Polypeptides retaining only a minimal level of activity are not necessarily preferred. Preferably, polypeptides show at least 50%, preferably at least 60%, such as at least 70%, more preferably at least 80% and even more preferably at least 90%, such as at least 95% and most preferably at least 99% residual activity. It is preferred that the polypeptide having at least 60% sequence similarity has the same or even an increased alternative oxidase activity as compared to the polypeptide of SEQ ID NO:1. It is further preferred that a polypeptide having at least 60% sequence similarity has the ability to mediate an equal or higher rate of oxygen consumption as compared to the polypeptide of SEQ ID NO:1. It is well known in the art how to determine the activity of AOX and the rate of oxygen consumption. Suitable methods are also disclosed in the appended examples and comprise the ectopic expression of AOX in a host cell followed by assessing the ability to confer the capability to respire in the presence of the respiratory poison antimycin A to permeabilized cells. For this purpose, oxygen consumption can be measured polarographically after successive addition of substrates for and inhibitors of different complexes of the respiratory chain, such as rotenone (abolishing electron flow through complex I), succinate (enabling electron flow from complex II) and antimycin A (inhibiting complex III). A similar rate of oxygen consumption in presence and absence of an inhibitor of the respiratory chain such as, for example, antimycin A is indicative of a functional AOX. The term "toxins to the respiratory chain" refers to substances that reduce or block the flow of electrons through the mitochondrial respiratory chain. The mitochondrial respiratory chain comprises several mitochondrial protein complexes that mediate the flow of electrons from donors such as NADH and succinate to oxygen, which is reduced to water. In that chain an electron is passed

from each donor to a more electronegative acceptor, which in turn donates these electrons to another acceptor, a process that continues until electrons are eventually passed to oxygen, the most electronegative and terminal electron acceptor in the chain. Passage of electrons is coupled to the generation of a proton gradient across the mitochondrial membrane by actively "pumping" protons into the intermembrane space. This gradient is in turn used to drive ATP production. Whether a substance is a toxin to the respiratory chain can be assessed by methods well known in the art such as for example by polarographically measuring oxygen consumption e.g. as described in the appended example. A reduced consumption of oxygen in presence of a substance as compared to the consumption in absence of the substance is indicative of the substance being a toxin to the respiratory chain. Examples for toxins of the respiratory chain are carbon monoxide, nitric oxide, azide, cyanide, myxothiazole and antimycin A.

The term "degenerate" in accordance with the present invention refers to the redundancy of the genetic code. Degeneracy results from the fact that there are more codons than encodable amino acids. For example, if there were two bases per codon, then only 16 amino acids could be coded for (4^2=16). Because at least 21 codes are required (20 amino acids plus stop), and the next largest number of bases is three, then 4^3 gives 64 possible codons, meaning that some degeneracy must exist. As a result, some amino acids are encoded by more than one triplet, i.e. by up to six triplets. The degeneracy mostly arises from alterations in the third position in a triplet. This means that nucleic acid molecules having a different sequence than that specified above, but still encoding the same polypeptide lie within the scope of the present invention.

It is preferred that the nucleic acid molecule for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke is comprised in a pharmaceutical composition. In accordance with the present invention, the term "pharmaceutical composition" relates to a composition for administration to a patient, preferably a human patient. The pharmaceutical composition for use in accordance with the invention comprises one or more of the nucleic acids recited above as pharmaceutically active agents.

In a preferred embodiment said one or more nucleic acids are the only pharmaceutically active agents to be used in said method or comprised in said pharmaceutical composition.

Also envisioned is a pharmaceutical composition comprising said nucleic acids in combination

with one or more additional pharmaceutically active agents. Additional pharmaceutically active agents can, for example, be the active ingredients of established medications for the disease or condition to be treated. In case of chronic obstructive pulmonary disease this includes for example beta-agonists, muscarinic receptor antagonists or steroids or a combination thereof. The pharmaceutical composition may, optionally, also comprise pharmaceutically inactive substances such as carriers or fillers. Thus, for example, liposomes, nanoparticles or cell-penetrating peptides may be used to increase the uptake of the nucleic acid molecule into the cell. The composition may be in solid, liquid or gaseous form and may be, inter alia, in the form of (a) powder(s), (a) tablet(s), (a) solution(s) or (an) aerosol(s). Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions, organic solvents including DMSO etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. The therapeutically effective amount for a given situation will readily be determined by routine experimentation and is within the skills and judgement of the ordinary clinician or physician. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 5 g units per day. However, a more preferred dosage might be in the range of 0.01 mg to 100 mg, even more preferably 0.01 mg to 50 mg and most preferably 0.01 mg to 10 mg per day. The length of treatment needed to observe changes and the interval following treatment for responses to occur vary depending on the desired effect. The particular amounts may be determined by conventional tests, which are well known to the person skilled in the art.

Alternatively, the nucleic acid for use in accordance with the present invention may also be comprised in a food product or may be used as a food additive. As used herein the term "food product" includes all nutritional products for uptake via the oral cavity. This includes natural or processed food, functional food, food for young children, infant modified milk, premature infant modified milk, geriatric food, etc. The term "natural food" refers to food which has not been subjected to any type of processing and to which no ingredient has been added, whereas the term "processed food" indicates that the food was subject to processing, such as, for example, cooking, freezing, dehydration, milling or the addition of ingredients which did not previously occur in the food. The term "functional food" refers to natural or processed food

products in which a certain ingredient is enriched or which are enhanced by additives and which, in addition to the normal nutritional value, have a beneficial effect on promoting health or preventing or managing a disease. The term "food for young children" refers to a food product given to children up to about 6 years old. The term "geriatric food" refers to a food product treated to facilitate digestion and absorption when compared to untreated foods. The term "infant modified milk" refers to modified milk given to children up to about one year old. The term "premature infant modified milk" refers to modified milk given to premature infants until about 6 months after birth.

The food product may further comprise additives generally used for food and drink as for example sweetener, colorant, preservative, thickening stabilizer, antioxidant, colour former, bleach, gum base, bittering agent, enzyme, gloss agent, acidulant, seasoning, emulsifier, enhancement agent, natural or synthetic flavour and the like.

Further the nucleic acid for use in accordance with the present invention may also be comprised in a food supplement. The term "food supplement" or "food additive" in accordance with the present invention relates to a composition comprising the nucleic acid molecule for use in accordance with the invention that can be added to existing foods or food products such as drink, beverage water, yoghurt, jelly, milk beverage and the like. The food supplement may be provided in the form of, for example, a liquid, an emulsion, a jelly, a gum, a powder, granules, a sheet, a capsule, a tablet and the like.

As used herein the term "treating" refers to undertaking measures in order to counteract a disease. Specifically, it refers to relieving or eliminating the symptoms associated with a disease. Accordingly, in the present case "treating" refers to measures undertaken after the detrimental effects, especially the inhibition of COX, caused by the inhalation of toxic smoke have occurred. In this regard it is preferred that the toxic smoke is derived from chemical industries, domestic appliances, a fire or exhaust emissions from a car. It is further preferred in this regard that the nucleic acid of the present invention or a pharmaceutical composition thereof is to be administered as a single dose.

By contrast, the term "preventing" as used herein refers to measures undertaken in order to avoid the occurrence of a disease or symptoms thereof *ab initio*. Usually, preventive measures are therefore taken if a subsequent exposure to toxic smoke can be anticipated. It is understood that preventive measures can also be taken during the exposure to toxic smoke, i.e. at the same time. However, as long as the measures undertaken are directed toward keeping negative effects of the inhalation of toxic smoke such as the inhibition of COX or secondary effects thereof from occurring, the measures are considered preventive even if an

additional exposure to toxic smoke has taken place at a prior time. Preventive measures can also be taken subsequently to exposure to toxic smoke but before the detrimental effects of said exposure such as the inhibition of COX or secondary effects thereof have occurred. In this regard it is preferred that the toxic smoke is tobacco smoke. Further, is preferred in this regard that the nucleic acid for use in accordance with the present invention or a pharmaceutical composition comprising said nucleic acid to be administered regularly.

The term "to be administered regularly" as used herein refers to an administration that occurs more than once and preferably at intervals that are approximately the same. The interval between administrations will be determined mainly by the stability of the compound to be used in accordance with the present invention, i.e. as soon as the amount of functional AOX is no longer sufficient another dose is to be administered. The amount of functional AOX in a cell is considered to be sufficient as long as at least 10%, such at least 20%, such as at least 30%, such at least 40%, preferably at least 50%, such as at least 60 %, such as at least 70%, more preferably at least 80%, such as at least 90%, such as at least 95% of the initial activity of AOX remains. The activity of AOX can be determined by performing suitable activity assays. Such activity assays are well known in the art and are also described elsewhere herein. Alternatively, the amount of functional AOX in a cell can be considered sufficient as long as at least 10%, such at least 20%, such as at least 30%, such at least 40%, preferably at least 50%, such as at least 60 %, such as at least 70%, more preferably at least 80%, such as at least 90%, such as at least 95% of the initial amount of AOX remains. The amount of AOX can be determined on the transcriptional i.e. RNA or the translational i.e. amino acid level. Methods for determining the amount of a protein on the transcriptional or the translational level include e.g. northern blotting, PCR, RT-PCR or real time PCR and western blotting or polyacrylamide gel electrophoresis in conjunction with protein staining techniques such as Coomassie Brilliant blue or silver-staining, immunodetection, Enzyme-linked Immunosorbent Assay (ELISA), fluorescence activated cell sorting (FACS) or mass spectrometry. Detailed protocols for these methods are well known in the art. It will be appreciated that it is not necessary to determine before every administration whether sufficient AOX is present. Once the average stability of AOX, i.e. the time until an additional administration is necessary, has been determined, this interval can be used for all further administrations, even if they occur in a different subject. Alternatively, the interval between administrations can also depend on the natural lifetime of the target cells or their mitochondria. In case of lung cells this is expected to be around 10 to 30 days. Accordingly, regular administration could for example be bi-weekly or monthly administration. Further, the intervals between the administrations can vary depending on the amount administered in a single dose and on the therapeutic or preventative need.

The term "toxic smoke" as used herein refers to smoke, fume or vapour comprising a substance that has a detrimental effect on a subject such as an animal or a human. Most commonly smoke is derived from burning processes. Such burning processes include, for example, the burning of tobacco during smoking, burning of fuel, e.g. in a car, industrial processes, a fire e.g. of a building, or burning e.g. of gas in domestic appliances such as lights, cookers or heaters. "Smoking" as used herein refers to the practice of burning a substance, most commonly tobacco, and inhaling the resulting smoke. Common forms of smoking include the smoking of cigarettes, cigars, pipes or water pipes (Hookah). In accordance with the present invention it is preferred that the detrimental effect of a toxic substance contained in the smoke is at least partially due to an inhibition of the mitochondrial electron transport chain. More preferably the detrimental effect is due to an inhibition of the mitochondrial electron transport chain at complex III (cytochrome bc1 complex; EC 1.10.2.2) or IV (cytochrome c oxidase (COX); EC 1.9.3.1). It is also preferred that the toxic substance comprised in the smoke is carbon monoxide.

In accordance with the present invention the term "disease or condition associated with the inhalation of toxic smoke" refers to diseases or conditions caused by the inhalation of toxic smoke as well as to diseases or conditions that are aggravated by the inhalation of toxic smoke. The term also refers to diseases or conditions for which the inhalation of toxic smoke is not causative on its own but for which the inhalation of toxic smoke represents a risk factor. Further, the term refers to secondary diseases or conditions that are caused, aggravated or made more likely by diseases or conditions caused, aggravated or made more likely by the inhalation of toxic smoke.

Current treatments for smoke inhalation injury and especially CO-poisoning often include mechanical ventilation and in case of CO-poisoning the administration of 100% oxygen or hyperbaric oxygenation therapy (Rehberg et al., 2009). The latter measures are employed to encourage rapid displacement of CO from haemoglobin and from COX. However, as long as CO is present the inhibition of COX cannot be circumvented. Further, to the best of the inventor's knowledge, no countermeasures for treating or preventing diseases or conditions associated with regular inhalation of toxic smoke, as in the case of smoking, are established. Thus, the present invention provides not only an alternative but also an improved treatment opportunity for acute CO-poisoning as well as for diseases and conditions associated with the inhalation of toxic smoke.

The present invention further relates to a vector comprising the nucleic acid molecule for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke.

In a related aspect, the present invention further relates to a vector comprising the nucleic acid molecule for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

Preferably, the vector is a plasmid, cosmid, virus, bacteriophage or another vector conventionally used e.g. in genetic engineering. Incorporation of the nucleic acid into a vector offers the possibility of introducing the nucleic acid molecule efficiently into the cells and preferably the DNA of a recipient. The recipient may be a single cell such as a cell from a cell line. Such a measure renders it possible to express, when expression vectors are chosen, the respective nucleic acid molecule in the recipient. Thus, incorporation of the nucleic acid molecule into an expression vector opens up the way to a permanently elevated level of the encoded protein in any cell or a subset of selected cells of the recipient.

In a preferred embodiment, the recipient is a mammal. In a more preferred embodiment, the mammal is a human.

The nucleic acid molecule may be inserted into several commercially available vectors. Non-limiting examples include vectors compatible with an expression in mammalian cells like pREP (Invitrogen), pcDNA3 (Invitrogen), pCEP4 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2neo, pBPV-1, pdBPVMMTneo, pRSVgpt, pRSVneo, pSV2-dhfr, pIZD35, pLXIN, pSIR (Clontech), pIRES-EGFP (Clontech), pEAK-10 (Edge Biosystems) pTriEx-Hygro (Novagen), pCINeo (Promega), Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pRc/CMV, pcDNA1, pSPORT1 (GIBCO BRL), pGEMHE (Promega), pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) or pBC12MI (ATCC 67109).

The nucleic acid molecule referred to above may also be inserted into vectors such that a translational fusion with another nucleic acid molecule is generated. The vectors may also contain an additional expressible polynucleotide coding for one or more chaperones to facilitate correct protein folding.

For vector modification techniques, see e.g. Sambrook and Russell, 2001. Generally, vectors can contain one or more origin of replication (ori) and inheritance systems for cloning or

expression, one or more markers for selection in the host, e.g., antibiotic resistance, and one or more expression cassettes.

The coding sequences inserted in the vector can e.g. be synthesized by standard methods, or isolated from natural sources. Ligation of the coding sequences to transcriptional regulatory elements and/or to other amino acid encoding sequences can be carried out using established methods. Transcriptional regulatory elements (parts of an expression cassette) ensuring expression in eukaryotic cells are well known to those skilled in the art. These elements comprise regulatory sequences ensuring the initiation of the transcription (e. g. translation initiation codon, promoters, enhancers, and/or insulators), internal ribosomal entry sites (IRES) (Owens et al., 2001) and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally-associated or heterologous promoter regions. Preferably, the nucleic acid molecule is operatively linked to such expression control sequences allowing expression in eukaryotic cells. The vector may further comprise nucleotide sequences encoding secretion signals as further regulatory elements. Such sequences are well known to the person skilled in the art. Furthermore, depending on the expression system used, alternative or additional leader sequences capable of directing the expressed polypeptide to a cellular compartment may be added to the coding sequence of the polynucleotide to be used in accordance with the invention. Such leader sequences are well known in the art.

Possible examples for regulatory elements ensuring the initiation of transcription comprise the cytomegalovirus (CMV) promoter, SV40-promoter, RSV-promoter (Rous sarcome virus), the lacZ promoter, the gai10 promoter, human elongation factor 1a-promoter, CMV enhancer, CaM-kinase promoter, the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) polyhedral promoter or the SV40-enhancer. Examples for further regulatory elements in prokaryotes and eukaryotic cells comprise transcription termination signals, such as SV40-poly-A site or the tk-poly-A site or the SV40, lacZ and AcMNPV polyhedral polyadenylation signals, downstream of the polynucleotide. Moreover, elements such as origin of replication, drug resistance gene, regulators (as part of an inducible promoter) may also be included. Additional elements might include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from retroviruses, e.g., RSV, HTLVI, HIVI, and the early promoter of the

cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter).

The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells. The transfected nucleic acid can also be amplified to express large amounts of the encoded (poly)peptide. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., 1991; Bebbington et al., 1992). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. As indicated above, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture.

Methods for delivery of vectors comprise for example viral delivery. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the polynucleotides or vector into a targeted cell population.

It is preferred that the vector for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke is comprised in a pharmaceutical composition, in a food product or in a food supplement. The definitions of the terms "pharmaceutical composition", "food product" and "food supplement" as provided elsewhere herein apply *mutatis mutandis* also in this case.

In another embodiment, the present invention relates to a polypeptide encoded by the nucleic acid molecule for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke. In a related preferred embodiment the present invention relates to a polypeptide encoded by the nucleic acid molecule for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

The preferred embodiments and definitions of terms as provided herein above also apply *mutatis mutandis* to these embodiments. For example, it is preferred that the polypeptide for the use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke is comprised in a pharmaceutical composition. It is further preferred that the pharmaceutical composition contains additional compounds promoting the efficient

uptake of the protein into a cell, i.e. cellular import or cell penetration, as well as its delivery to mitochondria such as, for example, cell-penetrating peptides or nano particles.
Such cell-penetrating peptides and nano particles are well known in the art and are described, for example in Milletti, 2012 and Liu et al., 2012. The considerations regarding the components of a pharmaceutical composition, the therapeutic dosage and administration methods and intervals as described for the nucleic acid molecule *mutatis mutandis* also apply to this embodiment.

Alternatively, the polypeptide for use in accordance with the present invention may be comprised in a food product or may be used as a food additive.

The present invention further relates to a non-toxic host cell carrying the nucleic acid molecule, the vector or the polypeptide for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke. In a related aspect the invention further relates to a non-toxic host cell carrying the nucleic acid molecule, the vector or the polypeptide for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke. The host cell may be cultivated according to conventional methods. In accordance with the present invention the term "non-toxic" refers to the cell not being toxic or harmful, such as not being pathogenic, in particular non cancerogeneous, to a human. The host cell can be an isolated cell, which may also be part of a cell culture. In case the host cell is a cell naturally expressing AOX it is preferred that the amount of AOX expressed has been enhanced e.g. by introducing the nucleic acid molecule or the vector into the host cell. The non-toxic host cell could for example be a cell of a non-pathogenic intracellular bacterial strain or a recombinant non-pathogenic bacterium engineered for the delivery of DNA or protein into mammalian cells such as for example, recombinant invasive Escherichia coli. Methods for engineering suitable non-pathogenic invasive bacteria and for use of these bacteria in gene delivery are known in the art and are described e.g. in Castagliuolo et al., 2005. Alternatively, the non-toxic host cell is an eukaryotic cell such as a yeast, insect, plant or mammalian cell. Depending on the route of administration it is preferred that the non-toxic host cell is a human cell. Especially if the cell is to be administered via a route that would allow for a immune response to be elicited it is preferred that the non-toxic host cell is derived from a matched donor or more preferably from the recipient. It is also preferred that the host cell is a primary cell or a primary cell line. The host cell may for example be comprised in a pharmaceutical composition, a food product or a food supplement. It is preferred that the non-toxic host cell, if derived from a cell line, is killed prior to administration. Alternatively, the non-toxic host cell may form part of an organ or tissue, such as for example a lung or a heart, or a part thereof

that is to be transplanted to a subject. It is envisioned that a tissue or organ expressing AOX could be generated by introducing the nucleic acid molecule, the vector or the polypeptide for use in accordance with the invention into at least some of the cells present in a tissue or organ to be transplanted or by using a non-toxic host cell carrying the nucleic acid molecule, the vector or the polypeptide as a progenitor in growing an artificial organ or tissue in vitro which is to be used as a transplant. In this regard it is preferred that the non-toxic host cell is able to pass on the ability to express AOX to its daughter cells. As compared to a normal transplant, i.e. a transplant not expressing AOX, a transplant modified in the manner described above would have the advantage of being more resistant towards damage induced by the inhalation of toxic smoke.

Further, the present invention relates to a product of a non-toxic host cell for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke. In a related aspect, the invention further relates to a product of a non-toxic host cell for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

In accordance with the present invention the term "product of a non-toxic host cell" refers to a composition comprising a processed form of the non-toxic host cell as defined herein above. The product of a non-toxic cell may be produced e.g. by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Thus, the product does not contain the intact cell but instead comprises the non-toxic cell in a non-viable state obtained e.g. by mechanical, chemical or enzymatic lysis of the cell. The term is not to be understood to relate to compounds or fractions isolated from said host cell, unless they are or comprise the nucleic acid molecule, the vector or the polypeptide as defined herein above. Accordingly, the product of a non-toxic host cell can also be the nucleic acid molecule, the vector or the polypeptide for use in accordance with the present invention isolated from the host cell or fractions thereof. Methods for isolating nucleic acid molecules, vectors or polypeptides from a cell are well known in the art. Further, the product may contain additional compounds such as e.g. stabilizers, carriers, substances facilitating the uptake of the nucleic acid molecule, the vector or the polypeptide into a cell or nutrients and may be for example be a solution, a granulate, a dragee, a capsule or a lyophilisate.

In a preferred embodiment, said disease or condition associated with the inhalation of toxic smoke is selected from the group consisting of: chronic obstructive pulmonary disease

(COPD), emphysema, lung cancer, heart disease, pneumonia, tuberculosis, osteoporosis, kidney failure and stroke. In accordance with all aspects and embodiments of the present invention the most preferred disease to be treated is COPD. Further, as discussed herein above, only a subclass of COPD, i.e. smoke-induced COPD is to be treated in accordance with the invention.

With regard to the medical indications discussed herein below, respiration is the respiration of the organism rather than mitochondrial respiration on a molecular or cellular level.

COPD is an abbreviation of "chronic obstructive pulmonary disease", which is also known as chronic obstructive lung disease (COLD), chronic obstructive airway disease (COAD), chronic airflow limitation (CAL) and chronic obstructive respiratory disease (CORD). It is characterized by irreversible airflow limitation, frequently associated with airspace enlargement and pulmonary inflammation and causes shortness of breath (dyspnea). COPD is most often caused by noxious particles or gas, most commonly from tobacco smoking (Gosker et al., 2008). The diagnosis of COPD is usually established by X-ray of the chest, high-resolution computed tomography (CT) and lung function tests such as, for example, spirometry, a test that measures the forced expiratory volume in one second (FEV1) (for further information see the Global initiative for chronic lung disease at *www.goldcopd.org*). FEV1 is the greatest volume of air that can be breathed out in the first second of a large breath. Spirometry also measures the forced vital capacity (FVC), which is the greatest volume of air that can be breathed out in a whole large breath. Normally, the FEV1/FVC ratio is >70%. A FEV1/FVC ratio <70% in spirometry following bronchodilator treatment, proves irreversible bronchial obstruction and defines the patient as having COPD. An additional indicator of COPD is a FEV1 value lower than the normal value predicted based on a person's age, gender, height and weight.

The term "emphysema" relates to a chronic, irreversible and often progressive disease of the lungs that primarily causes shortness of breath. In people with emphysema, the tissues necessary to support the physical shape and function of the lungs are destroyed. Specifically, the destruction of the alveoli (small gas exchange units) reduces the surface area for gas exchange of these air sacs thereby limiting blood oxygenation, and in addition leads to loss of elastic recoil of the lung tissue leading to early small airway collapse during exhalation, severe bronchial obstruction and shortness of breath.Emphysema is most often caused by tobacco smoking and long-term exposure to air pollution (Forey et al., 2011; Taraseviciene-Stewart and Voelkel, 2008). The diagnosis and severity of emphysema is usually confirmed by

pulmonary function testing (e.g. body plethysmography). However, X-ray radiography may also aid in the diagnosis. Diffusing capacity of the lung for carbon monoxide (DLCO) may be tested to differentiate emphysema from other types of obstructive disorders such as chronic bronchitis and asthma. DLCO is a test that measures the ability of gases to diffuse across the alveolar-capillary membrane. DLCO will be decreased in emphysema, whereas it will be normal or increased in asthma and chronic bronchitis.

"Lung cancer" refers to a disease characterized by uncontrolled cell growth in tissues of the lung. Primary lung cancers are most often carcinomas that derive from epithelial cells. The main types of lung cancer are small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). Methods employed in diagnosing lung cancer include radiography of the chest, CT imaging and bronchoscopy or CT-guided biopsy. The most common cause of lung cancer is long-term exposure to tobacco smoke which causes 85–90% of lung cancers (Samet et al., 2009). The other cases are often attributed to a combination of genetic factors, radon gas, asbestos and air pollution, including second-hand smoke. In accordance with the present invention the use of the nucleic acid, the vector, the polypeptide, the non-toxic host cell or the product thereof in a method of treating or preventing of lung cancer is preferably only envisioned for those cases of lung cancer that are caused by smoking or by air pollution, such as second-hand smoke.

The term "heart disease" (also "cardiac disease") refers to a group of diseases affecting e.g. the coronary arteries the cardiac muscle or the valves of the heart. Accordingly the term refers to, for example, coronary heart disease (also ischaemic heart disease or coronary artery disease), cardiomyopathy, hypertensive heart disease, heart failure, cardiac dysrhythmias, endocarditis, inflammatory cardiomegaly, myocarditis and valvular heart disease. The exposure to air pollution and especially smoking or second-hand smoking are established risk factors e.g. for coronary heart disease, hypertensive heart disease and heart failure (Brook et al., 2004; Prasad et al., 2009). The diagnosis of heart disease depends on the subtype of disease. Methods for diagnosing the individual diseases are well known in the art and include analysis by electrocardiogram (EKG or ECG), chest X-ray, echocardiogram, electrophysiology test, CT scan, magnetic resonance imaging (MRI). Further, a myocardial biopsy, cardiac catheterization or pericardiocentesis may be required in establishing a diagnosis. In accordance with the invention the nucleic acid molecule, the vector,the polypeptide, the non-toxic host cell or the product thereof are preferably to be used in methods for treating or preventing the forms of heart disease that are caused, aggravated or made more likely by the inhalation of toxic smoke.

The term "pneumonia" describes an inflammatory condition of the lung and more specifically of the alveoli. Typically it is caused by infection with bacteria or viruses. Pneumonia is typically diagnosed based on a combination of physical signs such as productive cough, fever accompanied by shaking chills, shortness of breath, sharp or stabbing chest pain during deep breaths, and an increased respiratory rate and a chest X-ray. Since the inhalation of toxic smoke such as tobacco smoke does not usually cause pneumonia but merely represents a risk factor predisposing to pneumonia (Reynolds et al., 2010), it is appreciated that the compounds to be used in accordance with the present invention are preferably to be used primarily in preventing cases of pneumonia associated with inhalation of toxic smoke and in assisting the treatment of these cases, i.e. the compounds are to be used in therapeutic methods against pneumonia in combination with other therapeutics such as for example antibiotics.

The term "tuberculosis" refers to an infectious disease which is usually caused by *Mycobacterium tuberculosis* and which mainly affects the lungs. Most infections are asymptomatic and latent. Symptoms of active infections are, for example, a chronic cough with blood-tinged sputum, fever, night sweats, and weight loss. Diagnosis can involve chest X-rays, examination of bodily fluids such as blood or the Mantoux tuberculin skin test. Smokers, i.e. subjects regularly exposed to toxic smoke, are thought to have nearly twice the risk of tuberculosis than non-smokers (van Zyl Smit, et al, 2010). In accordance with the present invention, it is preferred that the nucleic acid, the vector, the polypeptide, the non-toxic host cell or the product thereof to be used in accordance with the present invention are primarily to be used in preventing tuberculosis.

"Osteoporosis" describes a condition wherein the bone mineral density is reduced, bone microarchitecture deteriorates, and the amount and variety of proteins in bone are altered and the risk of bone fractures is increased accordingly. Diagnosis is usually based on radiography and on measuring bone mineral density for example by dual-energy X-ray absorptiometry and can also include blood tests. Although many studies have associated smoking, i.e. the regular exposure to toxic smoke, to an increased risk of osteoporosis, the mechanism underlying this increase remains unclear. It has been suggested that tobacco smoking inhibits the activity of osteoblasts and increases the breakdown of endogenous estrogen. The resulting lack of estrogen is thought to increase bone resorption and to decrease the deposition of new bone that normally takes place in weight-bearing bones. As in the case of other diseases for which the inhalation of toxic smoke is not a direct cause on its own but merely a risk factor (Wong et al., 2007), it will be appreciated that the nucleic acid molecule, the vector, the polypeptide, the

non-toxic host cell or the product thereof for use in accordance with the present invention or pharmaceutical compositions thereof are to be used primarily in preventing osteoporosis. If used in treating osteoporosis, it is preferred that the compounds for use in accordance with the present invention are used in combination with other established therapeutics for osteoporosis such as, for example, raloxifene.

The term "kidney failure" or "renal failure" refers to medical conditions wherein the kidneys fail to adequately filter toxins and waste products from the blood. Kidney failure may be acute or chronic. Whereas acute kidney injury is associated with a rapid and progressive loss of renal function, which may however be reversible, chronic kidney disease typically leads to a progressive and irreversible loss in renal function over a period of months or years. Diagnosis can be based on a decreased urine output, increased serum creatinine levels, size of the kidneys as measured by abdominal ultrasound or on the analysis of kidney biopsies. Several studies have suggested that smoking represents a risk factor for developing kidney failure, especially chronic kidney disease (Murphree and Thelen, 2010).

"Stroke" refers to a disturbance in the blood supply to the brain leading to a rapid loss of brain function. Symptoms can include face weakness, the inability to move one or more limbs, usually on one side of the body, the inability to speak or to understand speech and the inability to see, especially on one side of the visual field. Observation of these symptoms may represent a first step in diagnosing stroke. Diagnosis can further be based on neurological examination, CT or MRI scans, Doppler ultrasound, and arteriography. Both active and passive smoking have been suggested to increase the risk of stroke (McClure et al., 2011). It is thus preferred that the compounds for use in accordance with the present invention are used in preventing stroke.

In a preferred embodiment, said toxic smoke is tobacco smoke or smoke derived from chemical industries, domestic appliances, a fire or exhaust emissions from cars.

In a preferred embodiment, said toxic smoke contains carbon monoxide (CO).

In a preferred embodiment, the present invention relates to the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of the non-toxic host cell, for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke, wherein the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host cell is to be administered as an aerosol or a liquid. In

other words, it is preferred that the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of the non-toxic host cell, for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke is formulated as an aerosol or as a liquid. In a related preferred embodiment the present invention relates to the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of the non-toxic host cell, for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke, wherein the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host cell is to be administered as an aerosol or a liquid. In other words, it is preferred that the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of the non-toxic host cell, for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke is formulated as an aerosol or as a liquid.

The term "aerosol" describes a suspension of fine solid particles or liquid droplets in a gas. Usually the gas is the vapour of a liquid with boiling point slightly lower than room temperature. In aerosol sprays, for example, hydrofluoroalkanes (HFA) such as HFA 134a (1,1,1,2,-tetrafluoroethane) or HFA 227 (1,1,1,2,3,3,3-heptafluoropropane) or combinations of the two can be used as propellants. Alternatively, a manual pump can be used as an alternative to a stored propellant. In that case the gas of the aerosol is air. Preferably the solid particles of the aerosol are pharmaceutical compositions of the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product thereof for use in accordance with the present invention. It will be appreciated that the application of the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product thereof for use in accordance with the invention as an aerosol is especially preferred if the nucleic acid molecule, the vector,the polypeptide, the non-toxic host cell or the product thereof is to be used in a method for treating conditions or diseases of the lung such as e.g. COPD, emphysema or lung cancer. On the other hand, in case of conditions or diseases or conditions such as heart disease or stroke the application as a liquid e.g. by local injection is preferred. The liquid may for example be a buffered solution to be administered, for example, via local injection or via a catheter, a mouthwash, a washing solution or a drink.

Further, the present invention in a preferred embodiment relates to the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of the non-toxic host cell for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke, wherein the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host cell is to be administered regularly, prior or

subsequently to the inhalation of toxic smoke. In other words, the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host cell as described herein above is preferably to be administered regularly, as defined herein above. This administration can occur either prior or subsequently to the inhalation of toxic smoke. In a related preferred embodiment the disease or condition to be treated is a disease or condition caused by the inhalation of toxic smoke. Further, the present invention, in a further preferred embodiment, relates to the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of the non-toxic host cell for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke, wherein the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host cell is to be administered regularly, simultaneously with the inhalation of toxic smoke.

In another preferred embodiment, the present invention relates to the nucleic acid molecule, the vector, the polypeptide the non-toxic host cell or the product thereof for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke, wherein the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host cell is to be administered as a single dose prior or subsequently to the inhalation of toxic smoke. In other words, it is preferred that the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host cell as described herein above is confectioned as a single dose for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke. In a related preferred embodiment, the disease or condition to be treated is a disease or condition caused by the inhalation of toxic smoke. Further, the single dose comprising the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host cell as defined herein above may be for administration simultaneously with the inhalation of toxic smoke. In other words, the present invention in a further preferred embodiment relates to the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of the non-toxic host cell for use in a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke, wherein the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host cell is to be administered as a single dose, simultaneously with the inhalation of toxic smoke.

Further, the present invention also relates to a method of treating or preventing a disease or condition associated with the inhalation of toxic smoke, comprising contacting the nucleic acid molecule, the vector, the polypeptide, the non-toxic host cell or the product of a non-toxic host

cell with a subject in need thereof. In a related aspect the disease or condition to be treated or prevented in the method of the invention is a disease or condition caused by the inhalation of toxic smoke.

The preferred embodiments and definitions of terms as provided herein above also apply *mutatis mutandis* to the method of the present invention.

The term "subject" in accordance with the invention refers to an animal. Preferably the animal is an animal not endogenously expressing AOX. More preferably the subject is a mammal and yet more preferably a rodent, a domestic animal or a pet animal such as horse, cattle, pig, sheep, goat, dog or cat, or a non-human primate. Most preferably the subject is a human.

The figures show:

Figure 1 Cigarette smoke extract (CSE) is obtained by passing the smoke of one cigarette through 10ml of cell culture medium (DMEM + 10% fetal bovine serum).

Figure 2 Oxygen consumption in permeabilized mouse 3T3 cells transfected with lentivector-AOX. Oxygen consumption is measured polarographically after the successive addition of cells (5 x 10^6), digitonin (Dig, 80 µg/ml, for permeabilization), other substrates followed by rotenone (Rot, 150 mM, to abolish electron flow through complex I), succinate (Succ, 10 mM, to enable electron flow from complex II), and finally antimycin A (Aa, 30 ng/ml, to inhibit complex III). Arrows indicate the addition of the different substances.

Figure 3 Phase contrast microscopy of cells after 48h of exposure to cigarette smoke extract (CSE). Control 3T3 fibroblasts (left) and AOX-3T3 cells (right) were exposed to 2.5% CSE for 48h and visualized by phase contrast microscopy. Control cells (left) have rounded up and failed to proliferate, whereas the AOX-3T3 cells (right) show normal morphology, spread out on the surface of the culture bottle and forming healthily proliferating cell colonies (right).

Figure 4 Immunocytochemistry with an antibody against 53BP1 (detects dsDNA breaks, left) or active caspase 3 (effector protease of apoptosis, centre) after 48 h of exposure to 2.5% CSE (bottom). Panel A: Control 3T3 fibroblasts are brightly stained an antibody against 53BP1 (left) or active caspase 3 (centre), and cells have failed to proliferate, compared with control cells not exposed to CSE (top), based on staining of nuclei with DAPI (right). Panel B:

In contrast to the control cells, AOX-3T3 cells give signals at or close to background as seen in unexposed cells (top) when stained with an antibody against 53BP1 (left) or active caspase 3 (centre) and cells have proliferated normally, based on staining of nuclei with DAPI (right).

Figure 5 (A) Western blot of total cell protein from NIH 3T3 cells transduced with pWPI-AOX, versus control cells as indicated, grown in normal DMEM or in DMEM containing 1% CSE. (B) Phase-contrast microscopy of AOX-transduced and control cells, grown in media containing the indicated sugar, with or without 2.5% CSE. White scale bars on black background denote 50 µm. (C, D) Immunocytochemistry of AOX-transduced and empty vector-transduced control cells, grown in DMEM, with or without 2.5% CSE, using antibodies as indicated. White scale bars on black background denote 25 µm.

Figure 6 Oxygen levels and extrapolated rates of oxygen consumption (traces indicated by bold arrows) of isolated heart mitochondria treated successively with (A) substrate mix (final concentrations 2 mM malate, 10 mM glutamate, 10 mM pyruvate), followed by two additions of ADP (to 75 µM then 2 mM final concentration), then carboxyatractylate (CAT, 125 µM) and FCCP (200 nM) in the presence of concentrations of CSE as shown or (B) three additions of 20 µl CSE, after addition of substrates and ADP, and giving final CSE concentrations as indicated. Note that ADP-stimulated respiration represents state 3, i.e. oxygen consumption coupled to ATP synthesis, whilst the addition of CAT, an inhibitor of the adenine nucleotide translocase, generates a value for state 4 respiration, i.e. driven only by proton leak across the inner mitochondrial membrane. FCCP acts as a protonophore, so permits respiration to resume, driven by a futile cycle of proton flow. CAT and FCCP are controls for the integrity of the mitochondria in these experiments.

Figure 7 (A) Western blot of total cell protein from HEK293T cells stably transfected with AOX (Hakkaart et al., 2006), versus control cells transduced with the pWPI vector, as indicated. (B) Immunocytochemistry of AOX-transduced and vector-transduced control cells, grown with or without 2.5% CSE, using antibodies as indicated. White scale bars on black background denote 25 µm. (C) Number of cells expressing β-galactosidase (per micrographic view of ~500 cells), in cultures of AOX-expressing and control cells grown in medium with or without 1% CSE, as indicated. Means ± SD, $p<0.0001$ by ANOVA, asterisk denotes significant difference from all other classes, Bonferroni test, $p < 0.05$).

Figure 8 Immunocytochemistry of AOX-transduced and control cells, grown in DMEM, with or without 100 µg/ml DEP extract, using antibodies as indicated. White scale bars on black

background denote 25 µm. Images were not manipulated, other than by optimization for brightness and contrast.

Figure 9 (A) Transgenic construct used to generate AOX^{R26} mice, showing 5′ and 3′ regions from the *Rosa26* locus used as probes in Southern blots to confirm integration, promoter sequences from the *Rosa26* locus, PGK and the synthetic CAG promoter (Kiwaki et al. 1996), selectable neomycin-resistance (Neo^R) marker followed by BGH (bovine growth hormone) poly(A) addition signal, and flanked by FRT recombination sites which enable removal by Flp recombinase following successful germline integration, *Ciona intestinalis* AOX coding sequence including natural stop codon followed by β-globin intron/splicing and poly(A) addition sites. (B) Northern blot of 5 µg total RNA from the tissues indicated (He – heart, Lu – lung, Li – liver, Br – brain, Ki – kidney, Sp – spleen, Ov – ovary, Sk – skeletal muscle), probed successively for AOX, Tfam, 12S and 18S rRNAs, as shown. (C) Western blots of 15 µg total cell protein from the indicated tissues (as in B, plus Cm – cerebrum, Ce – cerebellum, Pa – pancreas, Op – optic nerve, Sa – salivary gland, Fm – femoral muscle, Mm – masseter muscle), probed for the gene products as shown. (D) Immunohistochemistry of a section of lung tissue from an AOXR26 mouse (upper panel) and a wild-type mouse (lower panel). Shown is an overlay of the anti AOX-staining performed using a customized antibody against AOX (Fernandez-Ayala et al. 2009) and the counterstaining with DAPI. (E) Oxygen consumption of isolated heart mitochondria from AOX^{R26} transgenic mice, in the presence of a complex I-linked substrate mix (sub) containing 2 mM malate, 10 mM glutamate, 10 mM pyruvate, 300 µM CaCl2 and 2 mM ADP, to which was then added, successively, 1.5 µM rotenone + 10 mM succinate (R+S), to drive respiration purely from complex II, 125 µM carboxyatractylate (CAT) to inhibit respiration dependent on ADP, 1 µM antimycin (A), to block respiration through complexes III and IV, 2mM ascorbate + 500 µM TMPD (A+T), to drive electron transfer purely through complex IV and azide (az), to inhibit complex IV, all in the presence of increasing amounts of CSE, as shown.

Figure 10 Changes in pulmonary arterial pressure (ΔPAP) *ex vivo*, in lungs from wild-type and AOX^{R26} transgenic mice, exposed to (A) successive cycles of hypoxia (means + SD, n=12), followed by perfusion of vasoconstrictor U46619 (n=2) to check that lack of vasoconstriction is not due to tissue damage, (B) successive cycles of perfusion with 0, 5, 10 and 20% CSE (means + SD, n=4-5 for wild-type, n=3-4 for AOX^{R26} transgenic). Asterisks denote significant differences ($p<0.01$ in pairwise *t* tests). $p<0.05$ for ANOVA of whole experiment.

Figure 11 (A) Western blot of total protein. AOX and its unprocessed precursor are indicated on the Western blot. (B) Table showing the proportion of permeabilized cell respiration that was antimycin A resistant, for HEK293T cells transfected with different AOX-encoding constructs, as indicated. Note that control cells show no detectable antimycin-resistant respiration.

The examples illustrate the invention:

Example 1. Material and Methods

Generation of cigarette smoke extract (CSE)

Smoke from one cigarette is passed through 10ml of cell culture medium such as for example DMEM + 10% fetal bovine serum (Fig 1). This extract is then diluted into fresh medium for delivery to the cells.

Polarographic measurement of oxygen consumption

Changes in oxygen levels in time or oxygen consumption can be measured in a polarographic system. The measurement device is a Clark-type oxygen electrode coupled to a recording system. Traditionally data can be visualized by a flatbed strip chart recorder but also by computer-assisted data acquisition system. The method has been published previously, e.g. in Hakkaart et al., 2006.

Phase contrast microscopy

Phase contrast microscopy is an optical microscopy approach different from the so-called bright field microscopy. In principle, the phase contrast microscopy changes phase shifts in light passing through a transparent sample to brightness differences. Therefore, cellular structures become visible.

Immunocytochemistry

Immunocytochemistry is a method that uses antibodies targeted against specific proteins or peptides to visualize specific epitopes on cells. Usually a primary antibody is raised in a species different from the samples examined. Therefore, the primary antibody recognizes the epitope on the cells of interest while a secondary antibody recognizes the specific origin of the primary antibody. Labelling of the secondary antibody visualizes the epitopes. Labelled primary antibodies can also be used.

Transduction of mouse NIH 3T3 cells

Mouse NIH 3T3 cells were transduced with pWPI-AOX (Dassa et al. 2009b), using standard methods for virus production, transduction and cell culture (Dassa et al. 2009b and references therein).

Isolation of mouse heart mitochondria

Mouse heart mitochondria were prepared by differential and percoll-gradient centrifugation of homogenates, essentially as described in Gellerich et al. (1987).

Generation of HEK 293T cells expressing AOX

HEK293T cells expressing AOX were generated by retroviral transduction with pWPI-AOX, as described Dassa et al., 2009b.

Cellular senescence

Cellular senescence was analysed by histochemical staining of paraformaldehyde-fixed cells for β-galactosidase activity, using X-Gal, after which randomly chosen fields of approximately 500 cells were viewed for each culture and the number of β-galactosidase positive blue cells was assessed.

Immunohistochemistry of mouse lungs

For immunohistochemistry, mouse lungs were perfused transcardially with PBS, inflated to total lung capacity with 50% Optimal Cutting Temperature Medium (Leica Biosystems, Wetzlar, Germany), removed from the thorax, and frozen in isomethylbutane. Frozen tissue was cut into 10 µm cryosections and fixed with methanol/acetone (1:1) for 10 min at -20°C. Antibodies were diluted in histobuffer (3% BSA/PBS, 0.2% Triton X-100). After blocking with normal goat serum for 1 h, sections were sequentially reacted with AOX antibody (1:100) and goat anti-rabbit Alexa 488 antibody (Invitrogen, 1:500), and counter-stained with DAPI or Mitotracker Red 1:500 (Invitrogen). Images were acquired on a Zeiss LSM710 Laser Scanning Confocal Microscope.

Preparation of lungs for determination of pulmonary arterial pressure (PAP)

For determination of the pulmonary arterial pressure (PAP), lungs were removed from the chest under deep anaesthesia, and artificially ventilated and perfused as described previously (Weissmann et al., 2004; Weissmann et al., 2006).

Example 2

It was tested whether AOX could protect cells from the toxicity associated with the poisons in

tobacco smoke using a standard assay in which cells are exposed to the toxins from tobacco smoke. In this assay, control cells stop growing and eventually enter a cell suicide (apoptosis) programme. This is used as a test of possible agents that might protect cells from the acute toxicity of tobacco smoke. Whilst some inhibitors of the cell suicide programme can delay cell death in this assay, no previous agent tested has enabled cells to continue growing in the presence of the toxic cocktail from tobacco smoke. We implemented the cell-based assay described, to test whether the expression of *Ciona intestinalis* AOX in mouse 3T3 fibroblasts afforded any protection to the cells from the toxic effects of cigarette-smoke extract (CSE). 3T3 cells transfected with lentivector-AOX, in which AOX is expressed from a constitutive promoter, were first tested biochemically (Fig. 2) to verify the presence of functional AOX (conferring the ability of permeabilized cells to respire in the presence of the respiratory poison antimycin A). For this purpose, oxygen consumption was measured polarographically after the successive addition of cells (5×10^6), digitonin (Dig, 80 µg/ml, for permeabilization), other substrates followed by rotenone (Rot, 150 mM, to abolish electron flow through complex I), succinate (Succ, 10 mM, to enable electron flow from complex II), and finally antimycin A (Aa, 30 ng/ml, to inhibit complex III). In this experiment, the rate of oxygen consumption driven by succinate after the addition of antimycin A is similar to (~80%) of that prior to adding the inhibitor. This represents respiration that has been enabled through AOX instead of complex III. In control cells, antimycin A shuts down oxygen consumption almost completely.

AOX-3T3 cells were then exposed to medium containing 2.5% CSE (Fig. 3) for 2 days alongside control cells. Unlike non-AOX expressing control cells, exposure to CSE had almost no detectable effect on AOX-expressing cells, which grew almost as well as untreated cells in the presence of the toxic cocktail (Fig. 3). The few non-expressing control cells that remained after 2 days of CSE exposure were found to be positive for 2 markers of apoptosis, namely 53BP1, which detects double-strand breaks in nuclear DNA, and active caspase 3, which is one of the proteases effecting key steps in the suicide programme (Fig. 4A). AOX-expressing 3T3 cells were caspase-negative, and showed only minimal evidence of nuclear DNA damage, consistent with normal processes (Fig. 4B). These results are indicative of a protective effect of AOX against cigarette smoke. Thus, delivery of AOX for example in the form of cell-permeable polypeptides or by ectopic expression may be a novel approach in treating or preventing diseases or conditions associated with the inhalation of toxic smoke.

Example 3

Mouse NIH 3T3 cells were transduced with pWPI-AOX (Dassa et al. 2009b), using standard methods for virus production, transduction and cell culture (Dassa et al. 2009b and references

therein). Cigarette-smoke extract (CSE) was produced by combustion of three 100 mm Research Grade Cigarettes (University of Kentucky), using a variable speed pump (Fisher Scientific) or water jet filter-pump (Brand GmbH). The smoke was bubbled though 30 ml of serum-containing DMEM at a speed of 0.5 cigarette/min, which was then filtered through a 0.22 µm pore filter and used within 30 min. 5×10^5 cells were plated per well of a 6-well plate, serum starved for 24 h then treated overnight with CSE at the dilutions indicated in figure legends. Immunocytochemistry used antibodies against 53BP1 (Bethyl, 1:200) and activated caspase 3 (BD Pharmingen, 1:200), together with secondary antibodies (Invitrogen) Alexa 488 goat anti-rabbit and Alexa 594 goat anti-mouse, respectively, both at 1:1000.

Using Western blotting (Fig. 5A) it was verified that the AOX construct was efficiently expressed in the transduced cells, and that its expression was not affected by growth of the cells in medium containing sub-lethal concentration of CSE (1%), based on preliminary trials. Growth of control cells, transduced with the empty vector pWPI, was greatly impaired by 2.5% CSE in the growth medium (Fig. 5B), and this effect was exacerbated by growth of the cells on medium containing galactose in place of glucose, which dictates the use of mitochondrial respiration to generate ATP (Robinson et al, 1992). AOX-transduced cells grew normally under all conditions (Fig. 5B), i.e. were immune to the growth-inhibitory effects of CSE. Immunocytochemistry revealed that control cells grown in the presence of CSE for 24 h showed activation and nuclear translocation of caspase 3, the effector caspase of apoptosis, with induction of 53BP1, a marker of nuclear DNA damage (Fig. 5C). At higher magnification this was seen throughout the nucleus (Fig. 5D). AOX-expressing cells grown under these conditions showed no caspase 3 activation, and minimal nuclear signal from 53BP1 (Fig. 5C), which was seen in just a few nuclear foci in each cell at higher magnification (Fig 5D). Control cells grown without CSE showed no 53BP1 induction or caspase 3 activation.

AOX expression thus protects cells from apoptosis in presence of CSE, and limits the amount of DNA damage.

Example 4

Mouse heart mitochondria were prepared as described in Example 1 herein above. Oxygen consumption in the presence of various substrates, cofactor and inhibitors was measured using an Oroboros 2k oxygraph (2 ml chamber) at 30°C and heart mitochondria suspended at 60 µg protein/ml. CSE resulted in a substantial inhibition of respiration by isolated heart mitochondria (Fig. 6). At the dose range of 2-3% CSE, which was sufficient to produce

widespread growth inhibition, apoptosis and DNA damage (Example 3), oxygen consumption was decreased by 30-50%. The data confirm that the toxic effect on cells, which AOX was able to rescue in Example 3, is due to a substantial inhibition of mitochondrial respiration by the cocktail of poisons in CSE.

Example 5

HEK293T cells expressing AOX were generated as described in Example 1 herein above and cultured in media containing different levels of CSE. HEK293T cells expressing GFP only, obtained by transducing the pWPI-derived control virus, were used as a control in all experiments. AOX expression was verified by Western blot against whole cell protein, using a customized AOX antibody (Fernandez-Ayala et al., 2009). Activation of caspase 3 was tracked by immunocytochemistry, as in Example 3. Cellular senescence was analysed as described in Example 1 herein above and the number of β-galactosidase positive cells showing the expected blue coloration were plotted as a mean number per view. HEK293T cells were slightly more resistant to CSE than mouse NIH 3T3 cells. However, when cells were grown in galactose medium, 2.5% CSE again had a major inhibitory effect on cell growth and survival that was prevented by AOX expression. After confirming AOX expression by western blotting (Fig. 7A), the cells were analysed by immunocytochemistry for activated caspase 3, a marker of apoptosis. Control cells showed expression of caspase 3 when grown in CSE, but no such signal was seen cells grown in normal medium, nor in AOX-expressing cells irrespective of the presence of CSE (Fig. 7B). AOX expression also conferred a significant resistance against cellular senescence (Fig. 7C), based on expression of the biomarker β-galactosidase (Dimri et al., 1995). Replicative senescence has elsewhere been linked to mitochondrial dysfunction (Passos et al., 2006).

Our finding implies that this process is enhanced by the mitochondrial toxicity of CSE, but is blocked by AOX expression, indicating a further mechanism by which AOX limits the pathological effects of the damage/repair/remodelling cycle in the chronically smoke-exposed lung, instead promoting healthy tissue regeneration.

Example 6

Diesel Exhaust Particles (DEP, US National Institute for Standards and Technology, SRM 1650b) were dissolved in DMSO by vigorous vortexing, and the extract was added to cell cultures in place of CSE, as in Example 3. Control cultures were treated with the same amount of DMSO as added to the DEP-treated cells. All other methods were as in Example 3. All cells grew somewhat more slowly in 100 μg/ml DEP extract but, under these conditions,

control cells showed general activation of caspase 3 and induction of 53BP1 throughout the cell nucleus (Fig. 8), similar to the effect of 2.5% CSE (Fig. 5B). AOX-expressing cells grown in DEP extract showed no such markers, indicating a similar degree of protection as against cigarette smoke.

Example 7

AOX transgenic mice were established both by lentivector transduction of early mouse embryos (El-Khoury et al., 2013) and, using a broadly similar construct (Fig. 9A), by knockin to the *Rosa26* locus. These two lines of mice, here designated AOX^{lv} and AOX^{R26}, respectively, were then analyzed by Southern blotting and PCR (to confirm the nature of integrated genetic information), by Northern blotting and Q-RT-PCR (to verify expression in different tissues at the RNA level) and by Western blotting and immunohistochemistry (to document the extent of expression at the protein level). Methods used in regard to AOX^{lv} mice are described in El-Khoury et al., 2013. For AOX^{R26} mice, the transgenic construct shown in Fig. 9A was assembled by PCR-based cloning from existing modules, sequence-verified, then used to replace one copy of the endogenous *Rosa26* locus using standard ES-cell gene targeting technology. After verification by Southern blotting (5′ probe: mutant 5.8 kb, wild-type 15.6 kb; 3′ probe: mutant 9.9 kb, wild-type 6.7 kb), chimeric founder mice were bred to the C57Bl6 background over >6 generations, tracking germline transmission of the AOX transgene by Southern blotting and PCR. For expression analysis, Northern blots were probed overnight at 37 °C in (250 mM sodium phosphate buffer pH 7.2, 240 mM NaCl, 2 mM EDTA, 7% SDS, 25% formamide, 1% BSA), using oligonucleotide probes as follows, all 5′ to 3′.

AOX: CTTGACCCACTGTTTCTCATCTAGCCG (SEQ ID NO:12)
Tfam: CCCAATGACAACTCCGTCTT (SEQ ID NO:13)
12S rRNA: CATGGGCTACACCTTGACCT (SEQ ID NO:14)
18S rRNA: TCGAACCCTGATTCCCCGTCACCC (SEQ ID NO:15)

Western blots and immunohistochemistry used a customized rabbit anti-AOX antibody as previously described (Fernandez-Ayala et al., 2009), mouse monoclonal anti-NDUFS3 (Ab 14711 Abcam), mouse monoclonal anti-SDHA (Mol. Probes A11142), mouse monoclonal anti-UQCRFS1 (Ab 14746 Abcam), mouse monoclonal anti-MTCO1 (COX1, MitoSciences, MS404) and rabbit polyclonal anti-Tom40 (Santa Cruz, sc-11414), together with Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG and Anti-Mouse IgG (Jackson ImmunoResearch Laboratories).

Mouse lungs were prepared for immunohistochemistry as described in Example 1 herein above and pictures were acquired on a Zeiss LSM710 Laser Scanning Confocal Microscope. Oxygen consumption of isolated heart mitochondrial from AOX^{R26} mice was measured in an Oroboros 2k oxygraph as described above (Example 4).

Both lines of AOX transgenic mice were fully healthy, showing no significant difference from wild-type mice under non-stress conditions. AOX expression in AOX^{lv} transgenic mice was analyzed by a combination of Western blotting and immunocytochemistry (see Fig. 2 of El-Khoury et al., 2013). AOX protein was widely expressed in the tissues, including the lung. In AOX^{R26} mice, analyzed also by Northern blotting (Fig. 9B), AOX showed widespread expression both at the RNA and protein level, with high levels of AOX protein seen in heart, skeletal muscle, lung, liver and pancreas (Fig. 9C). In the lung, expression was ubiquitous, based on immunohistochemistry (Fig. 9D) with punctuate cytoplasmic signal as seen in previous studies (Fernandez-Ayala et al., 2009; El-Khoury et al., 2013), indicative of mitochondrial localization. Mitochondria from expressing tissues, e.g. heart (Fig. 9E) showed robust antimycin-resistant substrate oxidation, that was resistant to all but the highest doses of CSE when driven by the complex I-linked substrate pyruvate, and fully resistant to CSE when driven by the complex II-linked substrate succinate. Complex IV itself (COX), when driven directly by ascorbate + TMPD in the presence of antimycin, was progressively inhibited by increasing doses of CSE as expected from studies on control mouse heart (see Fig.6).

The results indicate that AOX expression in the AOX^{R26} mice protects mitochondrial substrate oxidation from the toxic effects of CSE.

Example 8

For determination of the pulmonary arterial pressure (PAP), lungs were prepared as described in Example 1 herein above. Pressures in the pulmonary artery, left atrium, and trachea, as well as lung weight were continuously registered. All lungs included in the study exhibited a homogeneous white appearance with no signs of hemostasis, edema, or atelectasis; revealed a constant mean pulmonary artery and peak ventilation pressures in the normal range; and were isogravimetric during the initial 15 min steady-state period. Because flow-rate of the perfusate was constant, changes in pulmonary artery pressure are proportional to pulmonary vascular resistance. PAP was measured after the initial steady-state period and again during hypoxic ventilation with a premixed gas (1% O_2, 5% CO_2, balanced with N_2), lasting 10 min. Three consecutive procedures with alternating normoxic and hypoxic ventilation were performed (Weissmann et al., 2004; Weissmann et al., 2006). Competence for vasoconstriction was verified by subsequent application with the

thromboxane analogue U46619 (Bolla et al., 2004). 66 ng of U46619 were dissolved in 1 ml of perfusate and applied via a bypass three times, each for 25 min. Changes in PAP were measured similarly, following successive perfusion by increasing concentrations of CSE (0, 5, 10, 20%) at 20 min intervals, interspersed with hydrostatic challenge to check capillary filtration coefficient. Wild-type mice in this experiment showed the expected acute response to hypoxia, namely an increase in pulmonary arterial pressure of approximately 2 mm of mercury. AOX transgenic mice, however, showed a greatly attenuated hypoxia response (Fig. 10A). The direct response to perfusion with CSE was also decreased, especially when CSE concentration was raised to 10 or 20% (Fig. 10B).

Example 9

As a further test of the hypothesis that AOX confers protection from lung damage due to inhaled toxic smoke the protective ability of AOX was tested *in vivo* in a transgenic mouse model already described. AOX-expressing male mice were exposed to cigarette smoke in a closed chamber (courtesy of Dr Robert Bals, Saarland University, Homburg), in an alternating regime of 87 min exposure to smoke (300 mg/m^3 TSP [tobacco smoke pollution], determined gravimetrically, CO concentration 400-500 ppm, generated by a Teague Enterprises robotic device) followed by 40 min of air, over a period of 5 hours each workday for 5 mo. Four groups of 7 mice were studied: AOX and wild-type (non-AOX) littermates (as a control for the still mixed genetic background), with and without smoke exposure. After sacrifice, mouse lungs were analyzed by stereological microscopy (Fehrenbach et al., 2008) and for parameters of lung function, using the flexiVent system (SCIREQ, Montreal, Canada). Vascular muscularization was profiled as described previously (Dahal et a., 2011). Some of the mice had agouti coat-colour indicating a continuing genetic contribution of the original ES cell strain background. The data for volume-weighted alveolar number and static compliance (elastic recoil pressure) obtained for these mice are suggestive of a protective effect.

Example 10

Four novel AOX constructs were generated as follows. All are based on the codon-optimized AOX cDNA used by El-Khoury et al 2013.

1 – pcDNA5-TO-AOXopt, containing the full, codon-optimized AOX coding sequence cloned into the vector pcDNA5 TO (Invitrogen), including its natural N-terminal peptide

2 – pcDNA5-TO-AOXopt-ATP5B(3´UTR), the same construct, but in which the AOX-encoding sequence is followed by the 3´UTR of the human ATP5B gene.

3 – pcDNA5-TO-ATP5B(MTS)-AOXopt-ATP5B(3´UTR), the same construct but with the sequence encoding the AOX putative mitochondrial targeting peptide

MLSTGSKTFLFRPFLGSCHALQSGKLPCSNLHTTPTKITVKRYLVGYSWST replaced by that encoding the targeting peptide of Atp5b.

4 – pcDNA5-TO-ACO2(MTS)-AOXopt-ACO2(3′UTR), the same construct but with the sequences encoding the ATP5B mitochondrial targeting peptide and 3′ UTR replaced with those from the ACO2 gene.

The constructs were transfected into HEK293T cells, which were cultured for 48 h then analysed for AOX protein by Western blotting (Fig. 11A) and for permeabilized cell respiration driven by succinate, in the absence and presence of antimycin, by polarography, all as described in Cannino et al., 2012 (Fig.11B). HEK293T cells, stably transduced with the original pWPI-AOX lentivirus, were used as a positive control. All four constructs gave substantial expression and antimycin-resistant respiration, comparable with or better than the control cells. *Ciona* AOX can thus be expressed and targeted to mitochondria where it is functional in respiration, using both the targeting pre-sequence and 3' UTR from a natural human gene encoding a mitochondrially localized protein, such as ATP5B or ACO2. Of the variants tested, the most efficient processing of the mitochondrially targeted protein (ratio of mature to precursor form) was achieved with the ACO2 pre-sequence peptide; more efficient even than the natural AOX pre-sequence. These refinements enable nucleic acid constructs encoding AOX to be used in therapeutic applications without risking an immune or inflammatory response. They also define the functional domain of the AOX protein, commencing with amino acid 52 of the encoded polypeptide.

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CLAIMS

1. A nucleic acid molecule encoding a polypeptide, the polypeptide having the activity of alternative oxidase (AOX), wherein said nucleic acid molecule is selected from the group consisting of

(a) a nucleic acid molecule encoding a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 1;

(b) a nucleic acid molecule encoding a polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence similarity to the amino acid sequence of SEQ ID NO: 1;

(c) a nucleic acid molecule comprising or consisting of the nucleotide sequence of SEQ ID NO: 2;

(d) a nucleic acid molecule having at least 60% sequence similarity to the nucleic acid molecule of (c); and

(e) a nucleic acid molecule degenerate with respect to the nucleic acid molecule of (c) or (d),

for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

2. A vector comprising the nucleic acid molecule as defined in claim 1 for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

3. A polypeptide encoded by the nucleic acid molecule as defined in claim 1 for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

4. A non-toxic host cell carrying

(i) the nucleic acid molecule as defined in claim 1,

(ii) the vector as defined in claim 2 or

(iii) the polypeptide as defined in claim 3

for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke.

5. A product of the non-toxic host cell as defined in claim 4 for use in a method of treating or preventing a disease or condition caused by the inhalation of toxic smoke, wherein

said product preferably comprises the nucleic acid as defined in claim 1, the vector as defined in claim 2 or the polypeptide as defined in claim 3.

6. The nucleic acid molecule for use according to claim 1, the vector for use according to claim 2, the polypeptide for use according to claim 3, the non-toxic host cell for use according to claim 4 or the product of a non-toxic host cell for use according to claim 5, wherein the disease or condition to be prevented or treated is selected from the group consisting of: chronic obstructive pulmonary disease (COPD), emphysema, lung cancer, heart disease, pneumonia, tuberculosis, osteoporosis, kidney failure and stroke.

7. The nucleic acid molecule for use according to claim 1 or 6, the vector for use according to claim 2 or 6, the polypeptide for use according to claim 3 or 6, the non-toxic host cell for use according to claim 4 or 6, or the product of a non-toxic host cell for use according to claim 5 or 6, wherein the toxic smoke is tobacco smoke, exhaust emissions from a car or smoke derived from chemical industries, domestic appliances or a fire.

8. The nucleic acid molecule for use according to any one of claims 1 or 6 to 7, the vector for use according to any one of claims 2 or 6 to 7, the polypeptide for use according to any one of claims 3 or 6 to 7, the non-toxic host cell for use according to any one of claims 4 or 6 to 7 or the product of a non-toxic host cell for use according to any one of claims 5 to 7, wherein the smoke contains carbon monoxide (CO).

9. The nucleic acid molecule for use according to any one of claims 1 or 6 to 8, the vector for use according to any one of claims 2 or 6 to 8, the polypeptide for use according to any one of claims 3 or 6 to 8, the non-toxic host cell for use according to any one of claims 4 or 6 to 8 or the product of a non-toxic host cell for use according to any one of claims 5 to 8, wherein the nucleic acid molecule of claim 1, the vector of claim 2, the polypeptide of claim 3, the non-toxic host cell of claim 4 or the product of a non-toxic host cell of claim 5 is to be administered as an aerosol or a liquid.

10. The nucleic acid molecule for use according to any one of claims 1 or 6 to 9, the vector for use according to any one of claims 2 or 6 to 9, the polypeptide for use according to any one of claims 3 or 6 to 9, the non-toxic host cell for use according to any one of claims 4 or 6 to 9, or the product of a non-toxic host cell for use according

to any one of claims 5 to 9, wherein the nucleic acid molecule of claim 1, the vector of claim 2, the polypeptide of claim 3, the non-toxic host cell of claim 4 or the product of a non-toxic host cell of claim 5 is to be administered regularly, prior or subsequently to the inhalation of toxic smoke.

11. The nucleic acid molecule for use according to any one of claims 1 or 6 to 9, the vector for use according to any one of claims 2 or 6 to 9, the polypeptide for use according to any one of claims 3 or 6 to 9, the non-toxic host cell for use according to any one of claims 4 or 6 to 9, or the product of a non-toxic host cell for use according to any one of claims 5 to 9, wherein the nucleic acid molecule as defined in claim 1, the vector as defined in claim 2, the polypeptide as defined in claim 3, the non-toxic host cell as defined in claim 4 or the product of a non-toxic host cell as defined in claim 5 is to be administered as a single dose prior or subsequently to the inhalation of toxic smoke.

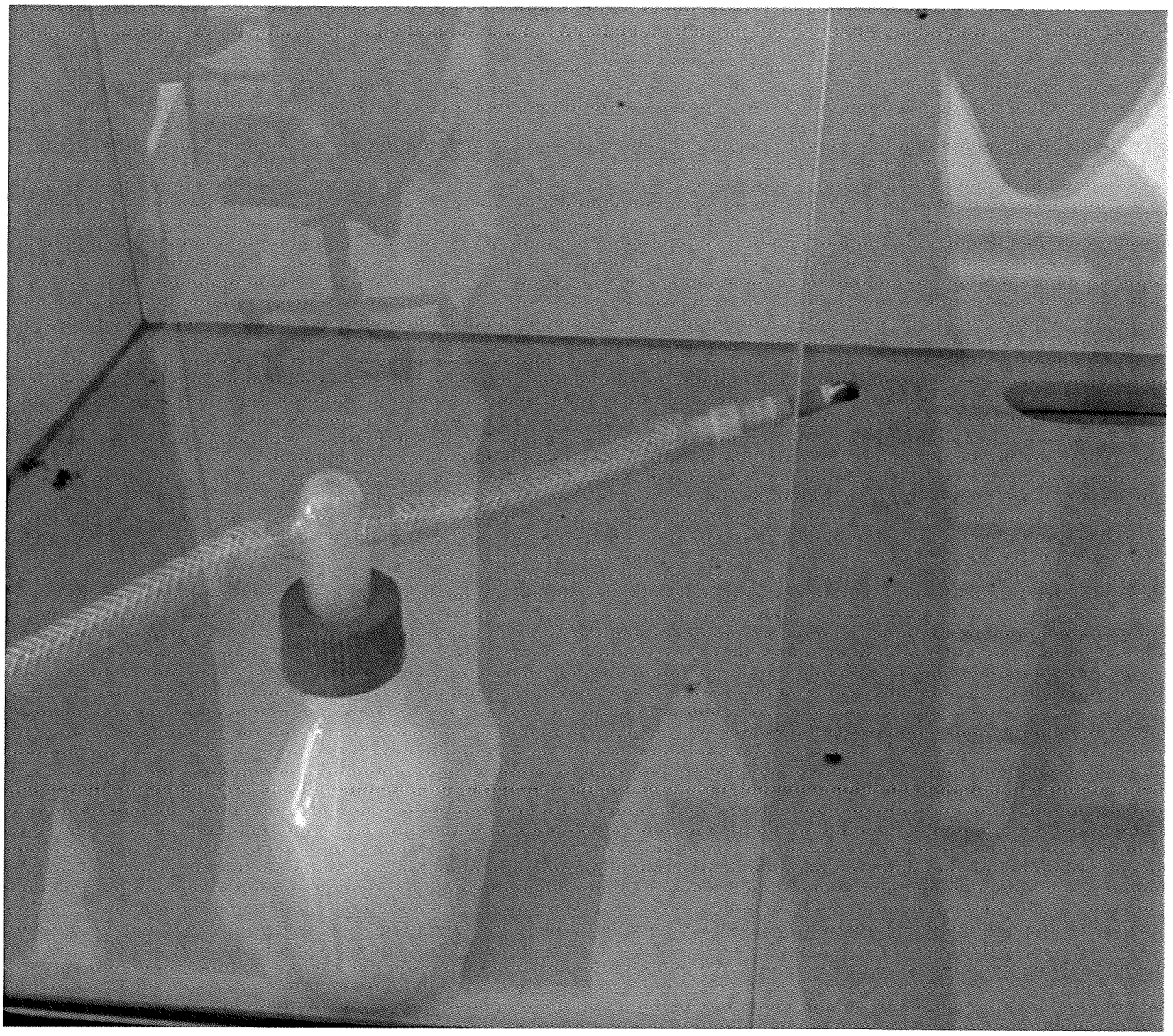


Figure 1

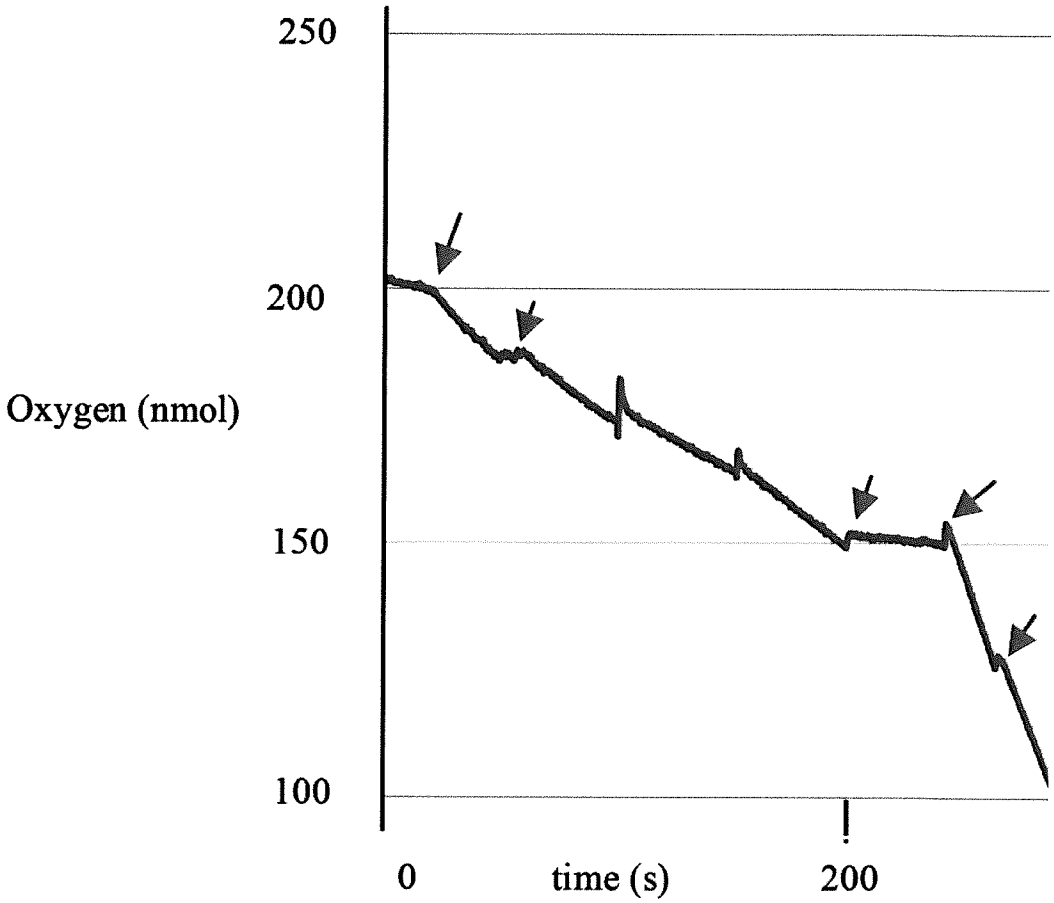
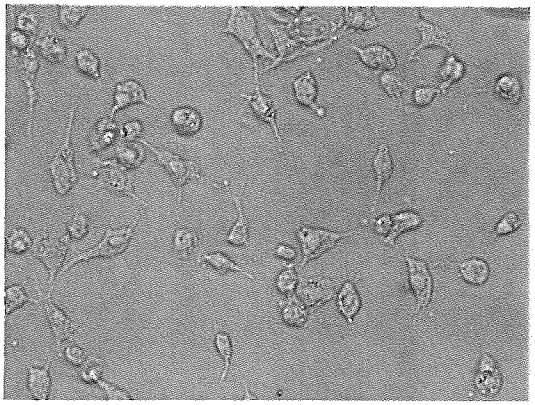


Figure 2

Control 3T3



AOX-3T3

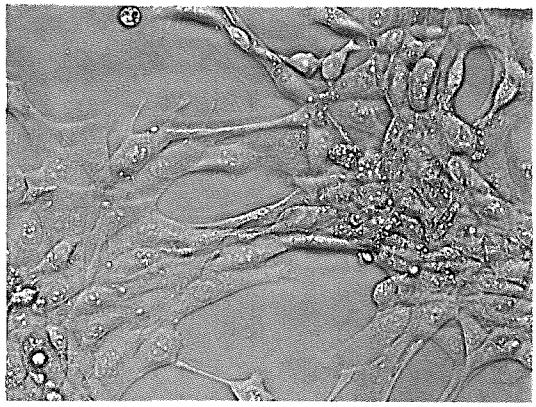


Figure 3

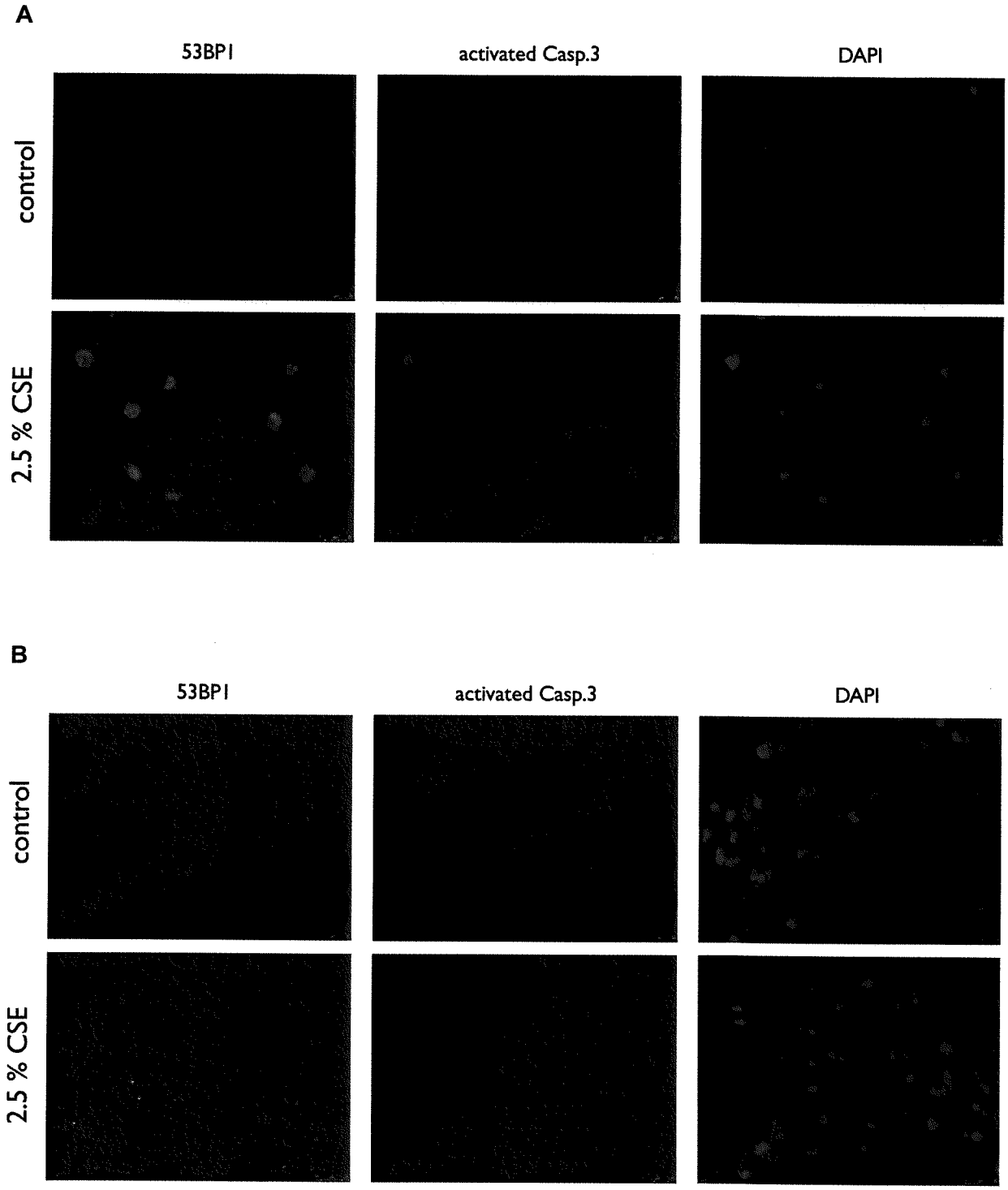

A
53BP1
activated Casp.3
DAPI
control
2.5 % CSE
B
53BP1
activated Casp.3
DAPI
control
2.5 % CSE

Figure 4

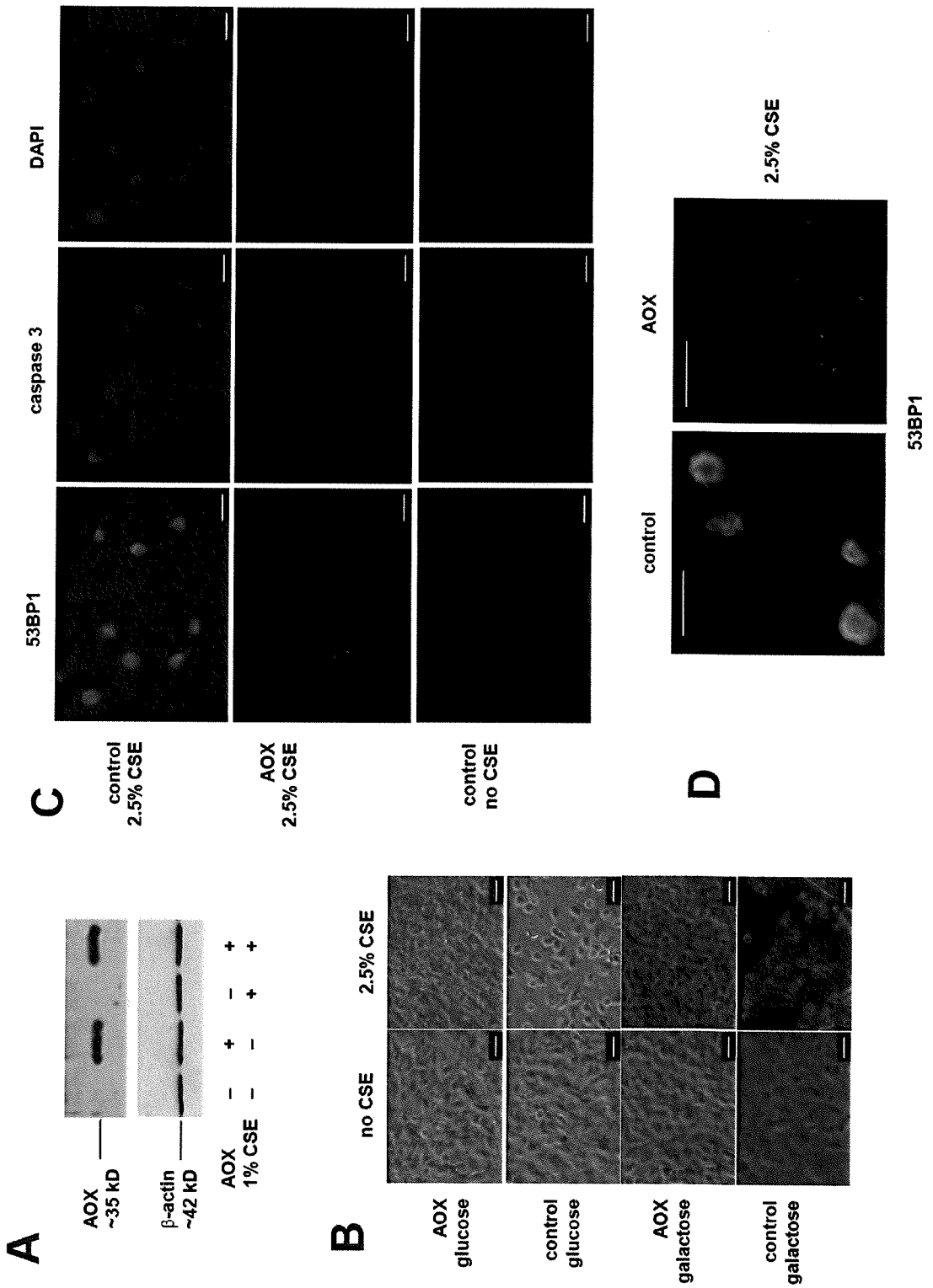

A
AOX ~35 kD
β-actin ~42 kD
AOX – + – +
1% CSE – – + +
B
no CSE
2.5% CSE
AOX glucose
control glucose
AOX galactose
control galactose
C
53BP1
caspase 3
DAPI
control 2.5% CSE
AOX 2.5% CSE
control no CSE
D
AOX
2.5% CSE
control
53BP1

Figure 5

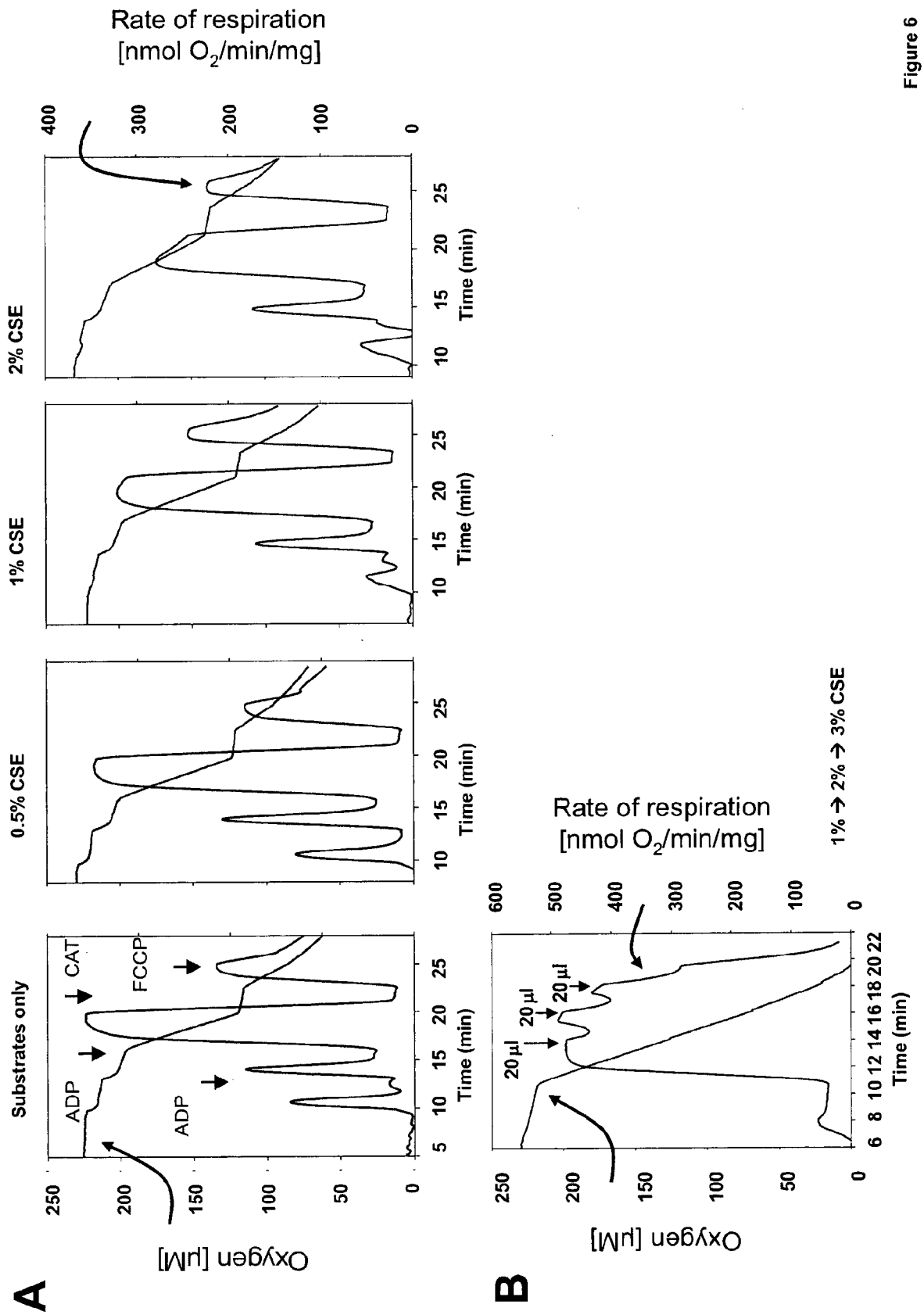

A
Substrates only
0.5% CSE
1% CSE
2% CSE
Rate of respiration
[nmol O2/min/mg]
Oxygen [µM]
Time (min)
ADP
CAT
FCCP
B
Rate of respiration
[nmol O2/min/mg]
1% → 2% → 3% CSE
20 µl
Oxygen [µM]
Time (min)

Figure 6

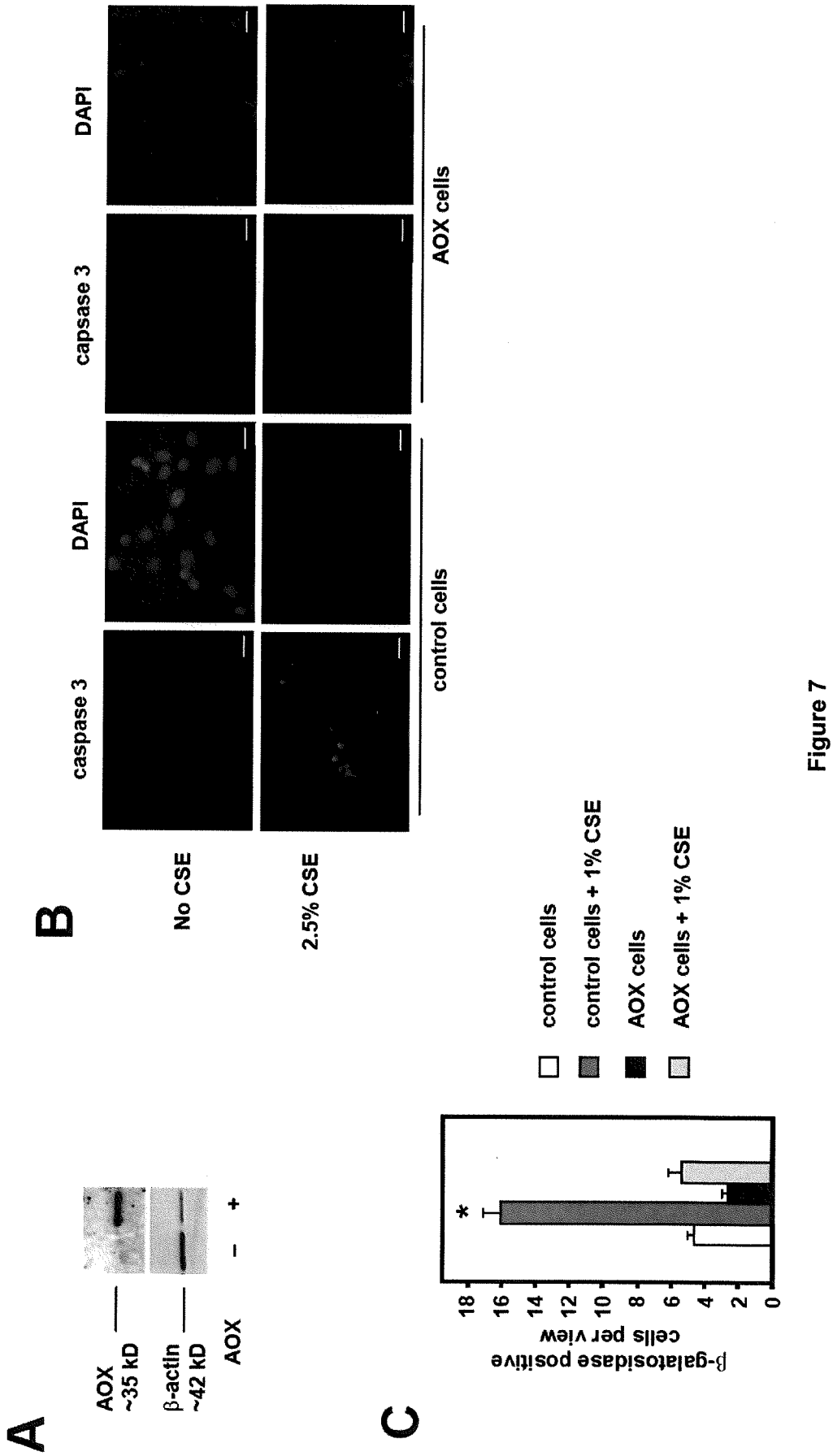

A
AOX
~35 kD
β-actin
~42 kD
AOX
–
+
B
caspase 3
DAPI
caspase 3
DAPI
control cells
AOX cells
No CSE
2.5% CSE
C
control cells
control cells + 1% CSE
AOX cells
AOX cells + 1% CSE
β-galatosidase positive
cells per view
0
2
4
6
8
10
12
14
16
18
*

Figure 7

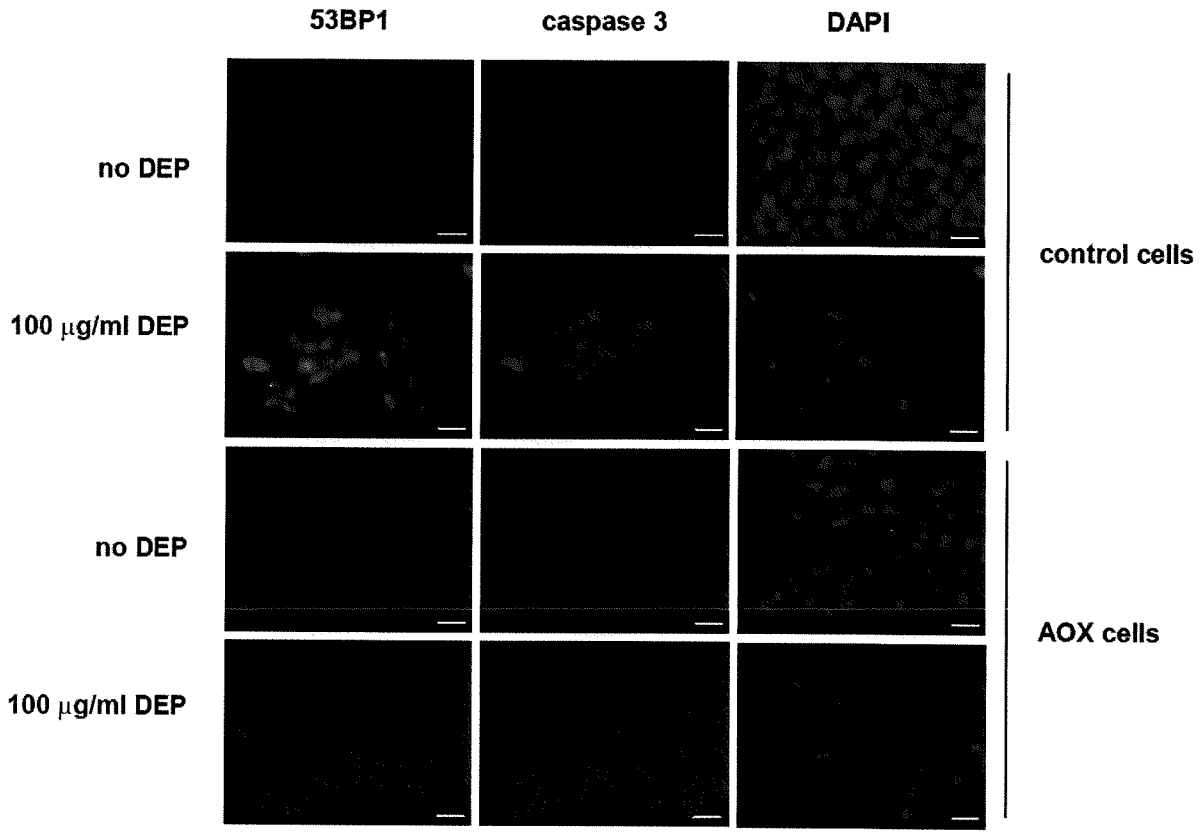

53BP1
caspase 3
DAPI
no DEP
100 μg/ml DEP
no DEP
100 μg/ml DEP
control cells
AOX cells

Figure 8

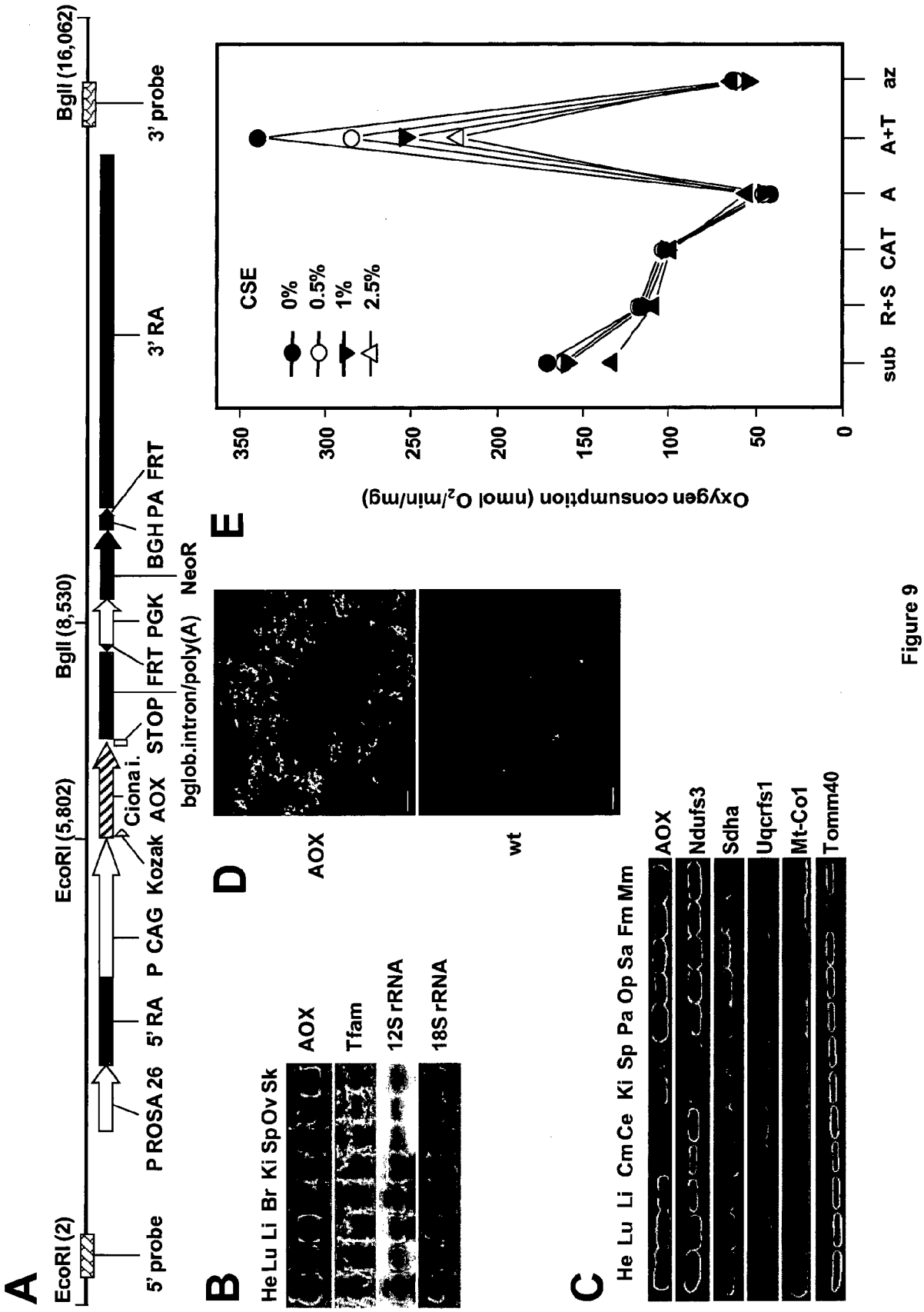

A
EcoRI (2)
5' probe
P ROSA 26
5' RA
P CAG
Kozak
AOX
Ciona i.
STOP
EcoRI (5,802)
BglI (8,530)
FRT
PGK
bglob.intron/poly(A)
NeoR
BGH PA
FRT
3' RA
BglI (16,062)
3' probe
B
He Lu Li Br Ki Sp Ov Sk
AOX
Tfam
12S rRNA
18S rRNA
C
He Lu Li Cm Ce Ki Sp Pa Op Sa Fm Mm
AOX
Ndufs3
Sdha
Uqcrfs1
Mt-Co1
Tomm40
D
AOX
wt
E
CSE
0%
0.5%
1%
2.5%
Oxygen consumption (nmol O2/min/mg)
350
300
250
200
150
100
50
0
sub
R+S
CAT
A
A+T
az

Figure 9

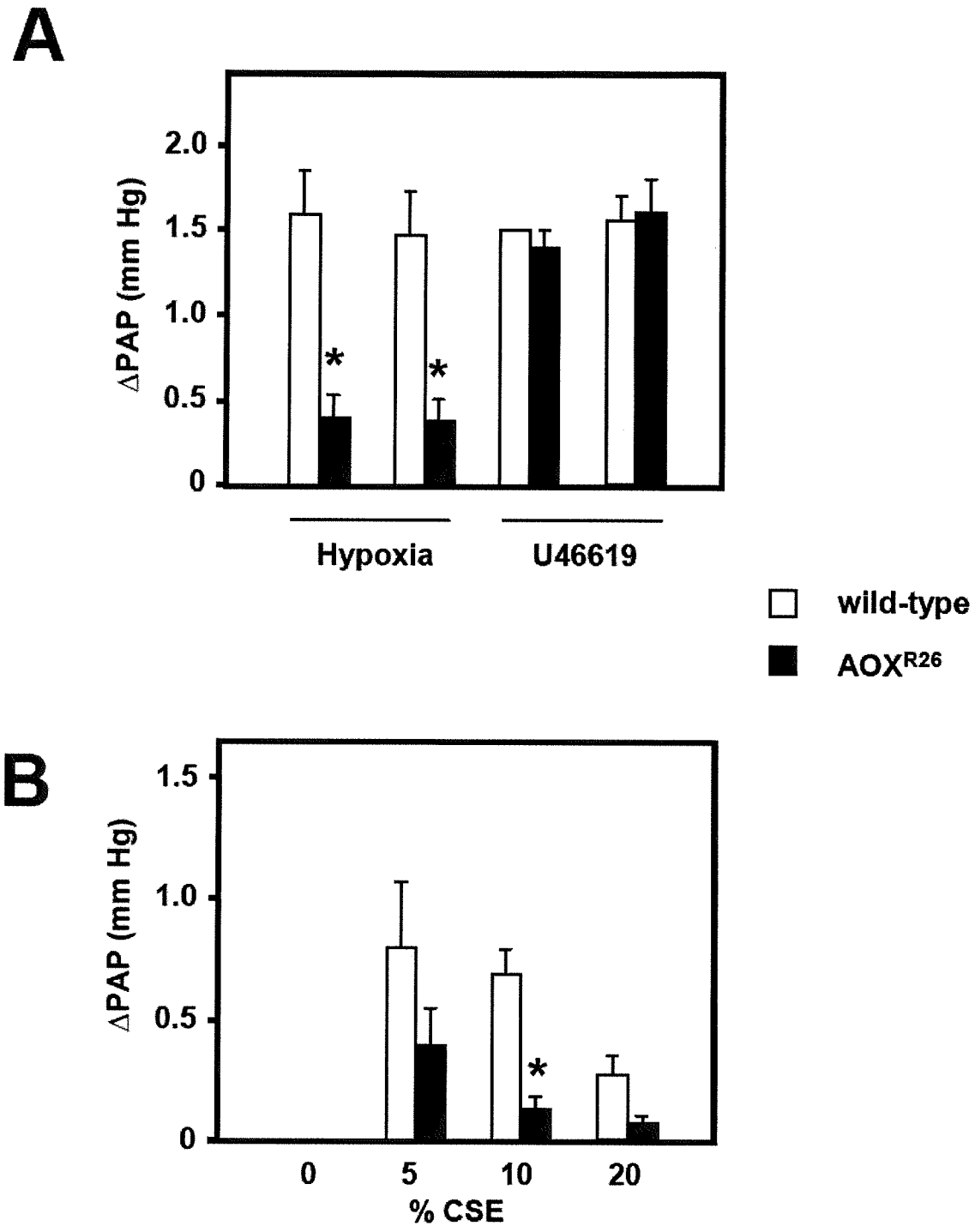
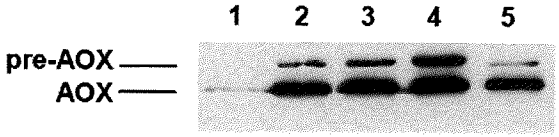

A
2.0
1.5
1.0
0.5
0
ΔPAP (mm Hg)
*
*
Hypoxia
U46619
wild-type
AOXR26
B
1.5
1.0
0.5
0
ΔPAP (mm Hg)
*
0
5
10
20
% CSE

Figure 10

A



1 = pWPI-AOX transfected HEK283T cells (positive control)
2 = pcDNA5-TO-AOXopt
3 = pcDNA5-TO-AOXopt-ATP5B(3´UTR)
4 = pcDNA5-TO-ATP5B(MTS)-AOXopt-ATP5B(3´UTR)
5 = pcDNA5-TO-ACO2(MTS)-AOXopt-ACO2(3´UTR)

B

Construct	% Antimycin-resistant respiration
pWPI-AOX transfected HEK283T cells (positive control)	39
pcDNA5-TO-AOXopt	47
pcDNA5-TO-AOXopt-ATP5B(3´UTR)	55
pcDNA5-TO-ATP5B(MTS)-AOXopt-ATP5B(3´UTR)	49
pcDNA5-TO-ACO2(MTS)-AOXopt-ACO2(3´UTR)	45

Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2013/077865

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/077865

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/44 C12N9/02 C12N15/63 A61P39/00 A61P39/06
A61P9/04 A61P9/10 A61P11/00 A61P19/10 A61P13/12
A61P31/06 A61K48/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, COMPENDEX, EMBASE, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/051898 A1 (LICENTIA OY [FI]; INST NAT SANTE RECH MED [FR]; RUSTIN PIERRE JEAN FRE) 10 May 2007 (2007-05-10) page 1, line 5 - line 11 page 12, line 16 - line 23 page 13, line 25 - page 14, line 11 page 22, line 30 - page 23, line 2 page 41, line 11 - page 42, line 26 page 47, line 13 - line 22 page 48, line 8 - line 11 -----	1-11
X	WO 2010/040901 A1 (RUSTIN PIERRE [FR]; DASSA EMMANUEL PHILIPPE [FR]; JACOBS HOWARD T [FI]) 15 April 2010 (2010-04-15) paragraphs [0002], [0065], [0273], [0275], [0276], [0309], [0321], [0323] -----	1-11

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 January 2014	11/02/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pilling, Stephen

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/077865

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2008/103088 A1 (FREDERIC RUSTIN PIERRE JEAN [FR] ET AL) 1 May 2008 (2008-05-01) paragraphs [0003], [0054], [0061], [0151], [0152], [0166], [0168]	1-11
X	MULLER-SCHWEINITZER E ET AL: "Reactive oxygen species mediate functional differences in human radial and internal thoracic arteries from smokers", JOURNAL OF VASCULAR SURGERY, C.V. MOSBY CO., ST. LOUIS, MO, US, vol. 51, no. 2, February 2010 (2010-02), pages 438-444, XP026876515, ISSN: 0741-5214 [retrieved on 2010-01-29] abstract	1-11
X	ZI-WEI ZHANG ET AL: "Direct determination of hydrogen cyanide in cigarette mainstream smoke by ion chromatography with pulsed amperometric detection", JOURNAL OF CHROMATOGRAPHY, ELSEVIER SCIENCE PUBLISHERS B.V, NL, vol. 1218, no. 7, 24 December 2010 (2010-12-24), pages 1016-1019, XP028128593, ISSN: 0021-9673, DOI: 10.1016/J.CHROMA.2010.12.100 [retrieved on 2011-01-04] abstract	1-11
X	JONES J ET AL: "Toxic smoke inhalation: Cyanide poisoning in fire victims", AMERICAN JOURNAL OF EMERGENCY MEDICINE, CENTRUM PHILADELPHIA, PA, US, vol. 5, no. 4, July 1987 (1987-07), pages 317-321, XP026126548, ISSN: 0735-6757, DOI: 10.1016/0735-6757(87)90360-3 [retrieved on 1987-07-01] abstract	1-11

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/077865

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2010022628 A1	28-01-2010