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REVIEW

Engineering the alternative oxidase gene to better understand and counteract mitochondrial defects: state of the art and perspectives

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Mitochondrial disorders are nowadays recognized as impinging on most areas of medicine. They include specific and widespread organ involvement, including both tissue degeneration and tumour formation. Despite the spectacular progresses made in the identification of their underlying molecular basis, effective therapy remains a distant goal. Our still rudimentary understanding of the pathophysiological mechanisms by which these diseases arise constitutes an obstacle to developing any rational treatments. In this context, the idea of using a heterologous gene, encoding a supplemental oxidase otherwise absent from mammals, potentially bypassing the defective portion of the respiratory chain, was proposed more than 10 years ago. The recent progress made in the expression of the alternative oxidase in a wide range of biological systems and disease conditions reveals great potential benefit, considering the broad impact of mitochondrial diseases. This review addresses the state of the art and the perspectives that can be now envisaged by using this strategy.

LINKED ARTICLES
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Abbreviations
AOX, alternative oxidase; COX, cytochrome oxidase; IMM, inner mitochondrial membrane; OXPHOS, oxidative phosphorylation; PHD, prolyl hydroxylase; RC, respiratory chain

Mitochondrial defects

Respiratory chain (RC) subunits only represent a subset of the more than a thousand components required for the biosynthesis and function of the mitochondria. Nevertheless, RC dysfunction, as narrowly defined, leads to a striking number of different diseases in humans (Munnich and Rustin, 2001). Primary genetic defects are presumably only the tip of the iceberg of diseases involving functional deficiency of the mitochondrial RC (Nunnari and Suomalainen, 2012). In addition, they almost certainly include major age-related cancers, neurodegenerative and cardiac diseases (Wallace, 2005). Depending on their nature and expression, RC defects can impair, to a variable extent, the many intra-mitochondrial catabolic and biosynthetic pathways, the handling of oxidative stress, signals governing cell death and the structural organization of the mitochondrial network. It is therefore not surprising that they can affect virtually any organ of the human body, either alone or in combination, and at any age. So far, while loss of mitochondrial function is increasingly recognized as an important underlying factor of human disease, with a significant number of genetic causes identified (Chinnery and Hudson, 2013), our current understanding of major features of even typical mitochondrial diseases remains poor. The reasons for their progressive nature, for their often unpredictable course and their tissue specificity are still largely mysterious (Briere *et al.*, 2004). As a result, except for a tiny minority of cases, we still lack any effective

treatments to cure or even stabilize such conditions (Dimauro and Rustin, 2008). So far, strategies envisaged to combat mitochondrial dysfunction have mainly focused on negating the effects of mutant material (nucleic acids or proteins) or on alleviating a supposed increased oxidative stress. However, these strategies have never been demonstrated to be successful, except in the case of a few specific animal models (Suomalainen, 2011). This was our major motivation for investigating systems used in nature to cope with mitochondrial blockade.

What has evolved in nature to cope with a mitochondrial blockade?

It remains the case that, in most situations, nature copes only poorly with mitochondrial blockade. Poisoning of mitochondrial function(s), especially the RC, is lethal under most conditions. Nevertheless, one situation of blockade is encountered every day in plants around us. Each morning, when solar energy radiates upon plant leaves, it causes photosynthesis to be turned on. This mobilizes most of the adenine nucleotide pool of the cell to the detriment of the mitochondria (Hampp *et al.*, 1982). The presence of high levels of cellular ATP, produced by photosynthesis, combined with the corresponding lack of ADP to drive ATP production in mitochondria, prevents the dissipation of the proton gradient ($\Delta\mu H^+$) across the inner mitochondrial membrane (IMM). In turn, respiratory electron flow and thus substrate oxidization are disabled, a necessary consequence of the tight coupling of respiration and oxidative phosphorylation (OXPHOS) dictated by the chemiosmotic mechanism (Figure 1). Under such conditions, intra-mitochondrial metabolism would be stopped almost in its entirety, due to the centrality of the tricarboxylic acid cycle, which is directly dependent upon respiration. Any reducing power entering the RC can escape only as potentially deleterious superoxides. However, mitochondrial metabolism must somehow proceed during the day because many vital biochemical reactions are carried out uniquely in the mitochondria of the plant cell. In fact, this happens without plants suffering any catastrophe due to excessive superoxide overproduction. This is due to the activation of a set of bioenergetic safety valves provided by two specific classes of electron-transporting protein (Rustin *et al.*, 1980). These safety valves allow electrons to be diverted from the standard RC to oxygen, despite the impedance of the proton gradient across the IMM. In plants, the 40 subunits of the rotenone-sensitive respiratory chain complex I can be bypassed by a single-subunit NADH dehydrogenase (e.g. nda1 of *Arabidopsis*, Michalecka *et al.*, 2003), which is resistant to rotenone, while the rest of the RC (from quinones to oxygen) can be bypassed by the activity of one other single protein, the so-called alternative oxidase (AOX, (Palmer, 1976). This entire non-proton motive electron transfer circuit (nda1 *plus* AOX), bypassing all proton-translocation sites of the RC, permits the necessary operation of mitochondrial metabolism during the day (Rustin and Queiroz, 1985), simultaneously avoiding mitochondrial overproduction of superoxides (Cvetkovska and Vanlerberghe, 2012). For several decades, the occurrence and operation of such a non-proton motive circuit (also detected in various fungi and microorganisms) was considered to be one of the major differences between the plant and animal kingdoms (Henry and Nyns,

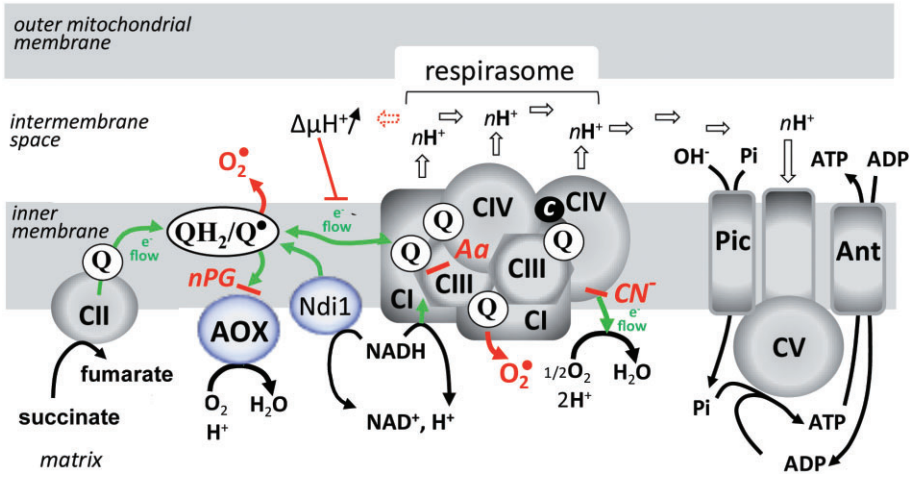

Figure 1

A schematized view of the mitochondrial respiratory chain of the animal kingdom. Electron flow (green line) through the chain is coupled to proton extrusion (open arrows) by the respirasome, which associates various proportions of respiratory complexes I, III and IV (CI, CIII, CIV). Protons released in the intermembrane space are subsequently used by complex V (CV; ATP synthase) to phosphorylate ADP (imported by the adenylate carrier; Ant) to ATP, making use of the inorganic phosphate imported by the phosphate carrier (Pic). Build-up of the proton gradient ($\Delta\mu H^+$) in case of impairment of ATP synthase function exerts a negative feedback control on electron flow in the respirasome. Under these conditions, quinones (Q) are essentially converted to reduced forms (QH_2), with formation of highly unstable semi-quinones (Q°) prone to react with oxygen to produce superoxides (O_2°). The scheme additionally features the two allotopic bypasses that have been successfully introduced in human cultured cells, *Drosophila*, and rodents, namely the internal NADH dehydrogenase from yeast (Ndi1; see Yagi *et al.*, 2006 for a review) and the AOX. Site of action (red dash) of inhibitors is indicated for nPG, Aa (antimycin A) and cyanide (CN^-).

1975). This belief was challenged by the observation, by Dr A MacDonald, of the occurrence of a gene encoding a protein similar to AOX in the genomes of a subset of lower animals (McDonald and Vanlerberghe, 2004; McDonald *et al.*, 2009).

Given the biological roles of their homologues in plants, this discovery provided an incentive to determine the functional properties of metazoan AOX-like proteins and test their potential to rescue RC impairment in different biological contexts (Rustin and Jacobs, 2009).

Nature and function of AOX

The nature of the AOX has been a matter of debate since the 1980s. At one point, it was even proposed that the oxidase did not really exist and that the corresponding cyanide-insensitive oxygen consumption resulted from underlying lipoperoxidation in the absence of any specific protein (Rustin *et al.*, 1983). The situation was clarified by the biochemical isolation of the AOX (Elthon and McIntosh, 1987), whose structure is now resolved (Moore *et al.*, 2013). The AOX is an integral membrane protein that is bound to the matrix side of the IMM. The enzyme was suggested to contain a coupled di-iron centre and it was shown by electron spin resonance that the *Arabidopsis thaliana* AOX AOX1a contains a hydroxo-bridged mixed-valent Fe(II)/Fe(III) binuclear iron centre. The catalytic cycle would make use of this di-iron centre and a tyrosine-residue free radical. Two major functions have been attributed to AOX in plants. First, under light conditions in the green parts of plants, AOX allows the mitochondria to work in parallel to photosynthesis (see above). Accordingly, the expression of the AOX was shown to be triggered by the greening of *A. thaliana* (Zhang *et al.*, 2010). Second, under numerous stress conditions, especially those resulting in decreased functioning of the standard cytochrome pathway, the AOX would avoid the conversion of the excess reducing power accumulated in the ubiquinone pool into deleterious superoxides (Figure 1) (Vanlerberghe, 2013). Because the AOX, similarly to nda1, is a non-proton motive oxidase, its permanent functioning might be problematic, as it would decrease the yield of ADP phosphorylation associated with substrate oxidation. However, due to a very low affinity for its substrate, ubiquinol, as compared with the first complex of the cytochrome pathway (complex III, also known as the bc_1 complex), AOX does not efficiently compete with the latter (Bahr and Bonner, 1973a,b). In other words, the AOX essentially works only when the cytochrome pathway does not. This unique property was predicted to make it innocuous when expressed in any cell with a functional cytochrome pathway (Rustin and Jacobs, 2009).

Allospecific expression

Based on the above considerations, Hakkaart *et al.* (2006) successfully expressed an AOX in cultured human cells. In order to maximize the chance of successful expression of an active protein, the AOX gene from an animal species endowed with AOX, the chordate *Ciona intestinalis* was chosen rather than the gene from plants. The gene was placed under the control of a tetracycline-inducible promoter so as to allow the cells to grow in case the AOX had an unsuspected deleterious effect (Hakkaart *et al.*, 2006). Subsequently, this was revealed to have been an unnecessary precaution because human cells expressing the AOX grew normally, without any sign of AOX-related toxicity. Cell growth rate, cell and mitochondrial morphology, cell respiration and mitochondrial substrate oxidation, RC activities and OXPHOS process were all found to be unaffected by the presence of the AOX protein. Indeed, just as predicted, none of the parameters tested suggested that the AOX was working in these cells as long as the cytochrome pathway operated freely. However, in the case of a chemical blockade of the cytochrome pathway triggered by cyanide (targeting complex IV, cytochrome *c* oxidase) or by antimycin (targeting complex III), the AOX was activated, allowing significant cell respiration to proceed in the presence of these inhibitors, which fully inhibited the respiration of the parental cells devoid of AOX. Accordingly, the AOX-endowed cells were able to grow in the presence of cyanide or antimycin, conditions under which control cells rapidly died. A few years subsequently, a plant AOX from *Arum concinnatum* was successfully expressed in HeLa cells, indicating that the plant enzyme and the gene that encodes it can also be functional in human cells (Kakizaki *et al.*, 2010; Kakizaki and Ito, 2013).

The *C. intestinalis* AOX was next engineered for conditional expression in *Drosophila melanogaster* (Fernandez-Ayala *et al.*, 2009). Ubiquitous AOX expression in *Drosophila* at the level of a typical abundant mRNA appeared to be benign. The protein was stable and correctly targeted to mitochondria, conferring substantial cyanide resistance to mitochondrial substrate oxidation *in vitro*. As in human cells, it appeared to be enzymatically inert in the absence of a cytochrome pathway inhibitor (Fernandez-Ayala *et al.*, 2009). Consistent with this, it had only a minimal effect on development with a slightly increased weight loss experienced by young adult flies, suggestive of only a minimal drop in the overall efficiency of catabolism. AOX-expressing flies were also fertile, indicative that AOX expression clearly does not compromise any somatic functions required for fertility in this model organism. A slight impairment in energetic efficiency might nevertheless be sufficient to account for the evolutionary loss of AOX in both vertebrates and arthropods, organisms where maximal muscle-force generation, dependent on aerobic ATP supply, may be needed to escape predators or catch prey. Among metazoans, AOX genes have so far been found only in phyla composed of slow-moving or sessile organisms (McDonald *et al.*, 2009).

The successful allotopic expression of the AOX was an incentive to revisit conditions in which superoxide overproduction has been suspected to be instrumental. In keeping with this, AOX expression in HEK cells was used to demonstrate that superoxides produced by the RC do not play an instrumental role in hypoxia-dependent HIF-1α stabilization triggered by RC inhibitors (Chua *et al.*, 2010). HIF-1α plays an essential role in cellular and systemic responses to hypoxia. In addition to transcriptional regulation, HIF-1α abundance depends on the coordinated activity of the oxygen-dependent superoxide-sensitive prolyl hydroxylases (PHDs), which maintain the appropriate balance of HIF-1α protein by targeting it for ubiquitination and proteasome-dependent

degradation. Accordingly, it has been hypothesized that superoxides are instrumental in controlling the activity of the PHDs. However, using AOX as a tool to decrease mitochondrial superoxide production, it was observed that this decrease did not affect the HIF-1α balance, suggesting that superoxides did not play a major role in this balance (Chua *et al*., 2010). It was rather found that an increase in cellular oxygen resulting from inhibition of the RC leads to activation of the PHDs. According to the free radical theory of ageing, organisms age because cells accumulate oxidative damage over time (Harman, 1956; 1972). In keeping with this, we used the AOX to investigate the role of mitochondrial superoxide overproduction in the aging of the flies, and established that mitochondrial superoxide production correlates with but does not directly regulate lifespan in *Drosophila*. According to this study (Sanz *et al*., 2010), mtDNA influences longevity in (female) flies, but does so independently of superoxide production which was maintained at a low level by the AOX.

More recently, the *C. intestinalis* AOX gene was successfully expressed in the mouse (El-Khoury *et al*., 2013). The gene was recoded to maximize its expression and introduced into early mouse embryos by germ line lentiviral transduction. It was placed under the control of the ubiquitously active, chimeric cytomegalovirus/β-actin/β-globin promoter, together with the Woodchuck hepatitis virus post-transcriptional regulatory element, in order to further enhance AOX transgene expression. This was the first demonstration that a functional AOX can be expressed in a mammal and transmitted between generations, conferring significant cyanide resistance to mitochondrial substrate oxidation and tissue respiration as well as the whole organism (El-Khoury *et al*., 2013). The enzyme was targeted to the mitochondria but did not interfere or compete significantly with the cytochrome pathway. The AOX in this mouse (MitAOX mouse) was enzymatically functional only upon blockade of the latter, when the pool of ubiquinone is expected to become highly reduced. Altogether, the expression of the AOX did not result in any deleterious consequence, while spectacularly increasing the survival of the mice in the presence of a lethal concentration of gaseous cyanide. Thus, the proposed protective mechanism provided by the AOX to organisms naturally harbouring the enzyme was preserved when the oxidase was expressed in a mammal (El-Khoury *et al*., 2013).

AOX expression in disease contexts

Human cultured cells

Based on the observation that AOX expression provided an efficient bypass of a chemically-induced blockade of the RC, the expression of AOX gene was next attempted in cultured skin fibroblasts derived from a patient presenting an early onset fatal hypertrophic cardiomyopathy due to a deleterious cytochrome oxidase (COX) 15 gene mutation (Dassa *et al*., 2009b). *COX15* encodes an enzyme required for synthesis of haem *a*, an essential prosthetic group of complex IV required for assembly of the functional complex (Petruzzella *et al*., 1998). The respiration of the COX15-mutant cells decreased by 30%, compared with control cells, and was fully cyanide sensitive. The cells were transduced by a lentiviral construct containing the *C. intestinalis* AOX coding sequence under the control of the EF1a promoter. The respiration of the cells was increased by 25–30% compared with parental COX15-mutant cells, and was >50% cyanide resistant. When cultured under respiratory restrictive conditions (low glucose), the growth of untransduced COX15-mutant cells was significantly impaired, but was restored to levels observed in high glucose by the expression of AOX. The study next focused on the ability of the AOX to protect RC-deficient cells from oxidative insults, taking advantage of the observation that oxidant sensitivity is greatly increased in cultured human fibroblasts rendered COX deficient by mutations in SURF1, COX10 or COX15 (Dassa *et al*., 2009b). Cells grown in non-restrictive medium were treated with antimycin, which causes endogenous oxidative insult, or with exogenously supplied H_2O_2. Measurements of cell viability confirmed that COX-deficient cells were significantly more sensitive to both types of oxidative insult than control cells. In particular, antimycin caused massive cell death under respiratory restrictive conditions, which was fully counteracted by AOX. The protection of cell growth by AOX expression was abolished if antimycin-treated cells were further treated with *n*-propyl gallate (*n*PG), a specific inhibitor of AOX (K_i of about 2 μM) (Siedow and Girvin, 1980). The protection afforded by AOX expression was attributable to avoidance of the excess superoxide production caused by antimycin inhibition of complex III. Next, HEK293-derived cells harbouring tetracycline-inducible AOX were made COX-deficient using shRNA targeted against COX10, another gene in the haem *a* biosynthetic pathway required for assembly of complex IV. The decreased respiration of these cells was largely compensated when AOX expression was boosted by tetracycline (Dassa *et al*., 2009b). The inhibitory effect of *n*PG on respiration further confirmed the involvement of AOX in the recovery of respiration. Finally, in a restrictive growth medium requiring competent respiration, the COX10-knockdown cells showed a significant growth defect that was fully eliminated upon AOX induction.

Drosophila

The innocuousness of AOX expression in otherwise wild-type *Drosophila* (Fernandez-Ayala *et al*., 2009) and the successful rescue of phenotypes resulting from COX deficiency in cultured cells (Dassa *et al*., 2009b) were an incentive to study the effect of AOX expression on mutant flies. The expression of AOX using the ubiquitous da-GAL4 driver was shown to complement the semi-lethality of a partial RNAi knockdown of either *cyclope* (encoding the COXVIc structural subunit of complex IV) or the complex IV assembly factor *Surf1*. AOX expression allowed hatching of flies knocked down for *cyclope* at 18°C, conditions that normally prohibit the completion of development. At a higher temperature (25°C), AOX prevented the developmental delay and small size of the eclosing progeny knocked down for *cyclope* (Fernandez-Ayala *et al*., 2009). *Surf1* was next targeted by RNAi technology using a mifepristone (RU486) inducible tub-GS driver. At a dose where *Surf1* knockdown was lethal or semi-lethal, concomitant AOX expression under the control of the same driver prevented the lethality (Fernandez-Ayala *et al*., 2009). In subsequent applications of this approach (K.K. Kemppainen

et al., unpublished observations), AOX was shown to be capable of preventing neurodegeneration resulting from a point mutation in the gene *levy*, encoding subunit COXVIa of complex IV (Liu *et al.*, 2007) or from the neuron-specific knockdown of essential complex IV subunits. It also corrected the late developmental lethality produced by knockdown of complex IV subunits in muscle.

In human cultured cells, AOX expression inhibits superoxide overproduction because it prevents over-reduction of the RC (Rustin and Jacobs, 2009). This effect was further investigated in the *Drosophila* mutant *dj-1β*, a model of Parkinson's disease associated with defects in ROS handling (Park *et al.*, 2005). The human homologue (DJ1) has been identified as the causative gene in some cases of familial Parkinson's disease (Tan and Skipper, 2007). The fly mutant exhibits progressive locomotor decline. The locomotor ability of *dj-1b* mutant flies was studied over a period of 4 weeks post-eclosion, with or without the concomitant expression of AOX (Fernandez-Ayala *et al.*, 2009). The presence of the AOX transgene conferred a partial rescue of locomotor decline, which was more obvious in the case of ubiquitous or nervous system-directed AOX expression. In AOX-expressing males, the *dj-1β* locomotor defect was almost completely abolished. Along with the improved phenotype, the increased production of mitochondrial ROS observed in the *dj-1b* mutant fly was decreased to wild-type levels by ubiquitous AOX expression. This work demonstrates that, at least in flies, phenotypes associated with partial COX deficiency, whatever its genetic cause, or with defects in ROS handling, can be successfully rescued by AOX.

Prospects

From data that have accumulated over the past few years, it appears that the predictions made on the potential use of AOX (Dassa *et al.*, 2009a; Rustin and Jacobs, 2009) have been verified. Firstly, the expression of AOX in human cultured cells, in the fly and in the mouse is essentially innocuous. Secondly, it renders human cells, flies and mice resistant to inhibitors of the cytochrome pathway, such as antimycin or cyanide. Thirdly, it prevents superoxide overproduction triggered by an excessive reduction of the RC. As a result, in accordance with our fourth prediction, it offers an efficient way to prevent the consequences of a wide set of genetic or environmentally determined lesions targeting the cytochrome pathway in model systems.

Based on this body of data, it is now possible to define additional perspectives, taking advantage of AOX expression (Figure 2). Firstly, it is tempting to use the MitAOX mouse to investigate the long list of pathological conditions where overproduction of superoxide by mitochondria has been postulated to be instrumental (Fernandez-Checa *et al.*, 2010), especially when already modelled in the mouse. This can be achieved by crossing selected mouse lines with transgenic mice either expressing AOX ubiquitously (El-Khoury *et al.*, 2013) or in a tissue-restricted fashion (under construction). As already done in *Drosophila*, its effects should be determined in physiological conditions where mitochondrial superoxide overproduction has thus far merely been suggested to be of aetiological or pathological significance (Brand *et al.*, 2013).

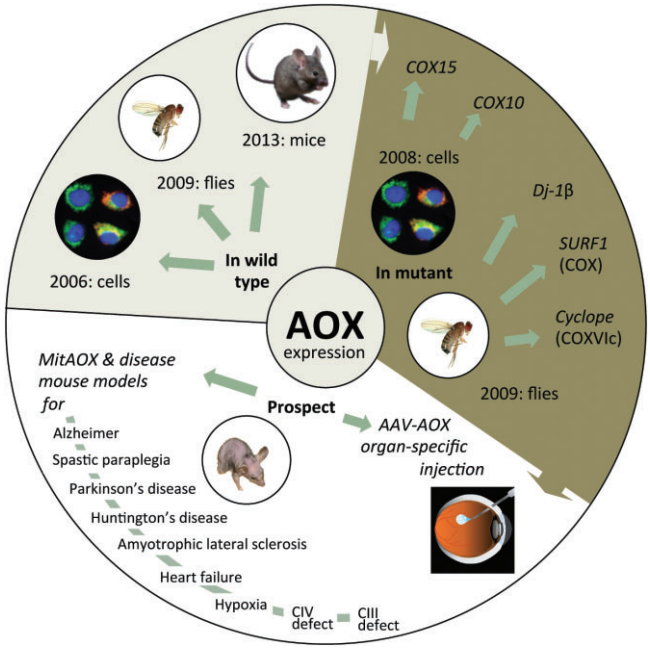

Figure 2

State of the art and prospect for allotopic AOX expression. Upper left sector: first successful expression of the AOX gene in human cultured cells (cells), *Drosophila melanogaster* (flies) and *Mus musculus* (CD-1/B6 MitAOX mice). Upper right sector: first complementation of respiratory chain defect in human COX15-mutant fibroblasts, with siRNA-down-regulated COX10 HEK cells, in *cyclope*, *Surf1* and *dj-1β* mutant *D. melanogaster*. Lower sector (left): a partial list of mouse models which should be informative to cross with the MitAOX mouse, either to establish/rule out the pathological involvement of mitochondrial superoxides, and/or of components from the mitochondrial cytochrome pathway, or to demonstrate the ability of AOX to complement genetic defects of the mitochondrial cytochrome pathway (CIV, CIII). One on the right-hand side: potential use of adeno-associated virus (AAV) constructs containing the AOX gene as a therapeutic strategy to target affected organs, for example, the eyes in cases of mutations affecting the cytochrome pathway.

This includes, for example, ageing, but also many types of physiological insult affecting mitochondrial function in diverse tissues. AOX-expressing mice should also be crossed with mouse lines in which a deficiency of complex IV or other cytochrome pathway components has been observed or engineered (Fukui *et al.*, 2007). AOX-expressing mice can also be used directly in other types of models, such as surgical or toxicological paradigms of human disease. In such cases, the mechanism of any eventual rescue brought about by AOX expression (restoration of metabolic homeostasis, energetic compensation, and/or alleviation of oxidative stress) will be of major interest: the consequences of RC deficiency are far from fully understood. Finally, as an alternative to the tissue-restricted AOX-expressing mouse, the use of injectable AOX, including its mitochondrial targeting sequence, delivered by a viral or similar vector, can also be considered. Recent progress made in ensuring the safety and longevity of viral vectors (Bouaita *et al.*, 2012) makes this approach particularly attractive, as it might allow the therapeutic use of the AOX gene.

The findings with *Drosophila* already suggest that AOX expression could be of benefit in a wide spectrum of OXPHOS disorders. In keeping with this, it is worth emphasizing that the single AOX gene can compensate for the impairment of any of at least 30 genes encoding structural components of RC complexes III or IV, as well as assembly factors required specifically for their biosynthesis, mutations that cause a wide spectrum of currently intractable diseases. However, even in the mouse, a number of questions remain to be answered before it is possible to conclude that AOX expression is a feasible therapeutic strategy. In particular, as the AOX gene we used so far derives from *C. intestinalis* which lives in highly oxygenated (intertidal zone) and cold water (<20°C), we have to consider the possibility that different temperature and oxygen tension in mammalian organs might affect the functionality of the AOX, and thus its capacity to counteract RC deficiencies, although the first data with regard to the brain are highly encouraging (El-Khoury *et al.*, 2013).

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Conflict of interest

None.

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