Beneficial effects of Resveratrol on respiratory chain defects in patients' fibroblasts involve estrogen receptor and estrogen-related receptor α signaling.

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ABSTRACT

Mitochondrial respiratory chain disorders are the most prevalent inborn metabolic diseases and remain without effective treatment to date. Up-regulation of residual enzyme activity has been proposed as a possible therapeutic approach in this group of disorders. Since resveratrol (RSV), a natural compound, was proposed to stimulate mitochondrial metabolism in rodents, we tested the effect of this compound on mitochondrial functions in control or in Complex I (CI)- or Complex IV (CIV)-deficient patients' fibroblasts. We show that RSV stimulates the expression of a panel of proteins representing structural subunits or assembly factors of the five RC complexes, in control fibroblasts. In moderate RC-deficient patients' cells, RSV treatment increases the amount of mutated proteins and stimulates residual enzyme activities. In these patients' cells, we establish that up-regulation of RC enzyme activities induced by RSV translates into increased cellular O_2 consumption rates and results in the correction of RC deficiencies. Importantly, RSV also prevents the accumulation of lactate that occurred in RC-deficient fibroblasts. Different complementary approaches demonstrate that RSV induces a mitochondrial biogenesis that might underlie the increase in mitochondrial capacities. Finally, we showed that, in human fibroblasts, RSV stimulated mitochondrial functions mainly in a SIRT1- and AMPK-independent manner, and that its effects rather involved the estrogen receptor (ER) and estrogen-related receptor alpha (ERRα) signaling pathways. These results represent the first demonstration that RSV could have a beneficial effect on inborn CI and CIV deficiencies from nuclear origin, in human fibroblasts and might be clinically relevant for the treatment of some RC deficiencies.

INTRODUCTION

Inborn respiratory chain (RC) deficiencies, first identified in the 80's, are now considered as the most common inborn metabolic disorder in humans, with an estimated incidence of 1:5000 to 1:8000 individuals (1). These diseases are characterized by an extraordinary diversity of phenotypes, possibly affecting almost any tissue, with any age of onset. Molecular analysis of these disorders initially identified disease-causing mutations in the mitochondrial DNA (mtDNA), and, more recently, in a growing number of nuclear genes. Despite advances in our understanding of the molecular and biochemical basis of these disorders, the development of effective therapies has been so far extremely limited (2). Accordingly, the vast majority of mitochondrial disorders still remain without treatment, to date. Until now, current pharmacological therapies essentially focused on vitamin or co-factors supplementation, administration of electron acceptors, or use of free radical scavengers. Agents susceptible to improve lactic acidosis, in particular dichloroacetate, which acts by inhibiting pyruvate dehydrogenase kinase (3), were also tested. These treatments are largely supportive, and aim at relieving the symptoms of the disease, rather than the cause, i.e. the RC deficiency. Overall, recent reviews of clinical trials concluded that none of these compounds were deemed beneficial for the therapy of mitochondrial disorders (2).

In recent years, new strategies started to emerge, based on a fully different rationale. Indeed, progress in the characterization of master regulators of mitochondrial oxidative metabolism stimulated the research of compounds capable to target these regulatory factors, in order to "boost" mitochondrial energy production. In line with this, pharmacological enhancement of mitochondrial function is now admitted to carry significant implications for the treatment of common metabolic or neurodegenerative diseases associated to mitochondrial dysfunction. This might also represent a promising approach for the therapy of inborn mitochondrial disorders. Indeed, in 2008, our group reported the first proof-of-concept that, in patient cells harboring CI

or CIV deficiency due to nuclear gene mutations, treatment by bezafibrate was able to stimulate residual enzyme activity of the deficient RC complex and led to restore normal oxygen consumption rates in some treated cells (4). Similar conclusions on the potential of bezafibrate were drawn from the study of transmitochondrial cybrids carrying common pathogenic mtDNA mutations (5). Interpretation of these data converges on the pivotal role of the nuclear co-activator PGC-1α, whose expression is strongly induced by bezafibrate, in mediating OXPHOS up-regulation. The importance of PGC-1α as a putative therapeutic target in mitochondrial disorders has also been shown in various models of mice with mitochondrial chain defects (6, 7). Induction of PGC-1α promotes the recruitment of the main transcription factors governing the expression of nuclear RC genes, such as Nuclear Respiratory Factors 1 and 2 (NRF1 and NRF2), which also control the expression of the mitochondrial transcription factor Tfam (8). Over the last decade, numerous studies demonstrated that the PGC-1α signaling cascade could mediate increases in respiratory rates and mitochondrial biogenesis, and serves critical regulatory functions to activate energy metabolism in tissues with high oxidative capacity, such as heart, slow-twitch skeletal muscle, or brown adipose tissue (8, 9).

Accordingly, delineating the signaling pathways involved in the activation of PGC-1α has focused much attention in recent years (10). It was thus shown that posttranslational modification played an essential role in up-regulating PGC-1α activity, in particular through deacetylation and phosphorylation. While the deacetylation is catalyzed by SIRT1, a member of the NAD-dependent histone deacetylases sirtuin family, the phosphorylation can be catalyzed by different protein kinases including AMPK (AMP activated protein kinase) (10). A number of small molecules, including resveratrol (RSV), were proposed to act as allosteric activators of SIRT1 (11), opening new prospects for a possible use of these compounds in the treatment of metabolic disorders. However, during the past years, the assertion that SIRT1 was a direct target of RSV has been challenged, it was recently proposed that RSV rather indirectly activated SIRT1. Indeed, Park et al reported that RSV might directly inhibit the cAMP specific phosphodiesterase, resulting in the activation of AMPK and in increased levels of NAD+, which, subsequently would

activate SIRT1 (12). Therefore, the precise sequence of events mediating PGC-1α activation in response to RSV is still debated (13).

However, long before attracting the attention as a putative SIRT1 activator, RSV was classified as a phytoestrogen capable to bind and activate estrogen receptors (14). Interestingly, in recent years, there has been increasing evidence that estrogens and estrogen receptors (ERs) might be important regulator of mitochondrial RC (15). ERs belong to the superfamily of nuclear receptors, which also include the estrogen-related receptors (ERRs). As their name implies ERRs share close structural homology with ERs but do not bind natural estrogens and are still considered as orphan receptors (16). Importantly, among the three existing ERR (α, β, and γ) isoforms, ERRα has been identified as a master regulator of mitochondrial energy metabolism and biogenesis. Thus, in mouse liver, ERRα occupies the extended promoter region of many genes encoding enzymes and proteins of the RC (16).

Accordingly, our study aimed at testing whether RSV could stimulate mitochondrial functions and biogenesis in a panel of patient cells harboring Complex I deficiency (CI), one of the commonest inborn mitochondrial RC disorder, or Complex IV deficiency (CIV). Furthermore, we analyzed the involvement of SIRT1, AMPK, ER, and ERRα in mediating the effects of RSV in primary human fibroblasts. There are yet limited data, especially in primary human cells, connecting these signaling pathways to the respiratory chain. This study establishes the proof of concept that RSV can correct moderate CI and CIV deficiencies, and provides evidence that the effects of RSV in this cell type are mainly SIRT1- and AMPK- independent and rather involve the ER and ERRα signaling pathways.

RESULTS

Resveratrol stimulates RC proteins expression in control and in moderate RC-deficient fibroblasts

The optimal conditions for cell treatment by RSV (75µM, 48 or 72h) were established from dose-response experiments (Fig.1), performed in control and in patient cells; 75µM was chosen as the most effective dose, without cell toxicity. Using these conditions, we then assessed the effects of RSV on the expression of a panel of proteins representing structural subunits or assembly factors of the five RC complexes, in control fibroblasts. As shown in Fig. 2, western-blot analysis revealed that, in control fibroblasts, the levels of the thirteen RC proteins considered were all significantly increased in response to RSV. Proteins induction by RSV ranged from +22% (NDUFS3) to +141% (core 2) and was observed for nuclear and mitochondrial-encoded (ND1, COX2) subunits.

We then evaluated the effects of RSV on the expression of mutated RC proteins, in CI- and in CIV-deficient fibroblasts. Six CI-deficient cell lines harboring distinct mutations of NDUFV1 gene were first compared (Fig. 3A). In the absence of treatment, five of them (Patients 2-6) exhibited a marked NDUFV1 protein deficiency, whereas Patient 1 was in the normal range. Cell treatment with RSV resulted in a significant increase in NDUFV1 protein levels in 3 out of 6 patients' cell lines, with inductions varying from +68% (Patient 1; $p<0,001$) to +117% (Patient 3; $p<0,001$).

The other CI-deficient cells came from patients harboring NDUFV2, NDUFS1, or NDUFS3 gene mutations (Fig. 3B, 3C, 3D). In these fibroblasts, the levels of the various mutant proteins were generally much lower than normal in the absence of treatment. Treatment with RSV stimulated the expression of mutant NDUFV2 protein in Patient 7 fibroblasts (+ 43%, $p<0,001$; Fig. 3B). By contrast, RSV had no effect on NDUFS1 protein levels in Patient 8 and 9 (Fig. 3C), or on NDUFS3 protein level in Patient 10 cells (Fig. 3D).

Three CIV-deficient cells were considered in this study, corresponding to two patients with distinct SURF1 gene mutant genotypes (Patients 15 and 16), and one patient with COX10 gene mutations (Patient 14). Patients 15 and 16 presented a profound SURF1 protein deficiency,

which was found unchanged after treatment by RSV (Fig. 3E). For the COX10-deficient patient, in the absence of commercially available COX10 antibody, we analyzed the expression of two CIV subunits, the mitochondrial-encoded COX2, and the nuclear-encoded COX4 subunits. As shown in Fig. 3F, the protein levels of both COX subunits were strongly reduced in untreated cells, and these patient fibroblasts exhibited a marked response to RSV, which induced a +121% and +84% increase in COX2 and COX4 protein levels, respectively.

Resveratrol increases CI and CIV enzyme activity and can correct RC-deficiencies

Under baseline conditions, variable levels of CI deficiency were found in our panel of patient fibroblasts (Fig. 4A). In control cells, treatment with RSV resulted in a marked increase in CI enzyme activity (+41%; p<0,001). In CI-deficient cells, treatment with RSV resulted in a significant (p<0,001) increase in CI enzyme activity in 5 patient cell lines, including 3 NDUFV1-deficient (Patients 1-3), one NDUFV2-deficient (Patient 7), and one cell line with unknown mutations (Patient 13). No significant changes in CI enzyme activity occurred in the other cell lines in response to RSV. In the RSV-responsive cells, the inductions of CI enzyme activity were at least +25% (Patient 2) and reached +90% in Patient 13, compared to untreated cells. Notably, cells from Patients 2 and 13 exhibited normal CI enzyme activity values after treatment with RSV. As observed for CI, CIV enzyme activity value was found significantly augmented (+60%; p<0,001) in control cells following treatment with RSV (Fig. 4B). Among the CIV-deficient patient fibroblasts, the two SURF1-deficient cells (Patients 15 and 16) did not respond to RSV. By contrast, treatment with RSV induced a significant increase (+ 33%, p<0,001) in CIV enzyme activity in COX10-deficient fibroblasts.

Further experiments were then performed to determine whether up-regulation of RC enzyme activities induced by RSV translated into changes in the cellular O_2 consumption rates. Indeed, measurements of oxygen consumption in patient cells provide a reliable index to evaluate the functional consequences of RC complex deficiencies on the respiratory chain activity i.e. to reveal oxidative phosphorylation deficiencies in living cells. We therefore used a new method

based on oxygen-sensitive fluorescent probes, which allowed measuring maximal respiration rates in fibroblasts. This method (17), which has already been used to measure cell respiration in suspensions of CHO cell line (18), in adherent HepG2 (19) and Caco2 cells (18) and in isolated mitochondria (20) was adapted to suspensions of primary human fibroblasts. Preliminary experiments were performed (Fig. 5) to check that the oxygen uptake rates (O.U.R.) were linear to the amount of cells (Fig. 5A), were measured at optimal concentrations of uncoupling agent (CCCP) (Fig. 5B), and were fully (>95%) rotenone-, and KCN-, sensitive (Fig. 5C).

We then analyzed the O.U.R. in control, CI, or CIV-deficient fibroblasts, and their variations in response to RSV (Fig. 6A and 6B). All the CI-deficient cells previously shown to respond to RSV were considered in these experiments (group 1; Patients 1, 2, 3, 7, 13), along with some non-responsive patient CI-deficient fibroblasts, taken as negative controls (group 2, Patients 5, 6, 8, 12). Under baseline conditions, O.U.R. values were around 70% of normal value in group 1 cells, versus 10-30% in group 2 cells (Fig. 6A). In this latter group, treatment with RSV induced no changes in oxygen consumption. By contrast, exposure to RSV had a marked stimulatory effect on O.U.R in control (+37%, $p<0.001$), and in group 1 fibroblasts. In these patients, O.U.R values were restored to the control range following treatment with RSV (Fig. 6A).

Finally, similar experiments were performed in the COX10-deficient cell line in which RSV was previously shown to increase COX enzyme activity. As seen in Fig. 6B, these patient cells exhibited basal O_2 consumption representing 75% of normal values, and responded to RSV by an increase in their O_2 consumption rate. Interestingly, and as observed in some CI-deficient cells, treatment by RSV was able to restore O_2 consumption value to the normal range in CIV-deficient patient 14 fibroblasts.

Resveratrol improves Lactate/Pyruvate ratio

Another informative index of whole cell energy metabolism in living cells is the lactate to pyruvate (L/P) ratio, reflecting the cellular NADH/NAD ratio, which we compared in CI-deficient or control cells (Fig. 6C). In OXPHOS-deficient patients, a severe lactic acidosis, which could be

fatal, is commonly encountered (21). In control cells under baseline conditions, the mean L/P ratio value was 33,1 with little inter-individual variation (±10%). As expected, CI deficiency was associated with high L/P ratio, varying from about 43 up to 78 in the patient cells studied. Treatment with RSV resulted in a decrease in the L/P value in control fibroblasts and remarkably, in these 5 CI-deficient cells in which RSV restored normal oxygen consumption rates, treatment by RSV resulted in the restoration of normal L/P values.

Resveratrol induces mitochondrial biogenesis

By use of different complementary approaches, we then addressed the question whether treatment by RSV could stimulate the mitochondrial biogenesis in human fibroblasts. Experiments using the Mitotracker green probe clearly revealed increases in staining intensity in both control and patients' fibroblasts upon exposure to RSV (Fig. 7A). Quantification of the data confirmed this, showing increased fluorescence in response to RSV in patients (+30-50%; $p<0,05$) and in control (+80%; $p<0,05$) fibroblasts.

Measurements of citrate synthase enzyme activity, commonly considered as a marker of mitochondrial density, were also performed in control cells, and in a panel of "responsive" and "non-responsive" fibroblasts. Interestingly, treatment with RSV was found to increase citrate synthase activity (+30 to +90%) both in control cells and in all the RC-deficient patient cells tested (Fig. 7B).

Last, we analyzed in the same panel of cells the protein expression of Tfam, the mitochondrial transcription factor A, which co-localizes with mtDNA in the mitochondrial nucleoids (22). Western-blot analysis revealed a general stimulation of Tfam protein expression in all the cell lines, in response to RSV (Fig. 7C).

Resveratrol effects on RC capacity in human fibroblasts are mainly SIRT1- and AMPK-independent

In order to analyze the molecular mechanisms underlying the effects of RSV on RC in our cell system, we first evaluated SIRT1 involvement. Knockdown of SIRT1 (siRNA from Dharmacon) was performed in control cells and in patient 3 fibroblasts, and western blots confirmed the total suppression of SIRT1 expression (Fig. 8A). Two readouts were chosen to study the effects of siSIRT1: the NDUFV1 protein levels and the oxygen consumption rates. In control fibroblasts, NDUFV1 protein levels were significantly increased in vehicle-treated cells transfected with siSIRT1, compared to non-target siRNA (Fig. 8A), suggesting that SIRT1 might have an inhibitory effect on basal NDUFV1 expression. However, in vehicle-treated patient cells, this difference in NDUFV1 levels between siSIRT1 and non-target siRNA was not found significant. Overall, in the absence of treatment by resveratrol, there was a trend towards higher oxygen consumption rates in fibroblasts transfected with siSIRT1, compared to the corresponding non-target siRNA groups (Fig. 8B). Our results clearly show that SIRT1 knockdown only partially abolished the response to resveratrol. Indeed, in control and patient 3 fibroblasts lacking SIRT1, there were still significant increases in NDUFV1 proteins after treatment with RSV (Fig. 8A) (+ 88% and +82%, respectively, versus +146% and 104% in the corresponding non-target siRNA fibroblasts). From a functional point of view, the changes in cellular respiration paralleled those observed in protein levels (Fig. 8B). Accordingly, silencing of SIRT1 led to reduce, but did not prevent, the increases in O.U.R in response to RSV. It should be mentioned that the same results were obtained after transfecting control and patient 3 cells with another siSIRT1 (from Sigma), ruling out possible off-target effects.

A recent study proposed that SIRT1 would be required to mediate the effects of low doses of RSV whereas the cellular response to high doses of RSV could be SIRT1-independent (13). However, in experiments using a lower RSV dose (20 μM), no significant increases in O.U.R were obtained in control or in patient 3 fibroblasts (data not shown).

Since some authors proposed that RSV effects could primarily be mediated through activation of AMPK (12), we tested this hypothesis and performed siAMPK experiments in control and in patient cells. Western-blot analysis and measurements of respiratory rates showed that robust silencing of AMPK did not prevent the increase in NDUFV1 protein levels (Fig. 8C) and oxygen consumption (Fig. 8D) in response to RSV. Finally, since there is an obvious crosstalk between SIRT1 and AMPK, and to rule out possible compensatory mechanisms between the two pathways, we also performed a double knockdown of SIRT1 and AMPK. Western blot analysis revealed that, the RSV-induced increases in NDUFV1 were still significant, and these data therefore confirmed that the RSV stimulatory effects on RC did not mainly require SIRT1 and AMPK (Fig. 8E).

ER and ERRα are likely involved in the effects of resveratrol on RC capacity in human fibroblasts.

Given the fact that RSV was identified as a phytoestrogen and binds ERs, and considering the proposed role of ERRα in regulating the expression of RC genes, we explored a possible role of ER and of ERRα in the cell response to resveratrol. We used the specific ER antagonist ICI182780, or the specific ERRα inverse agonist XCT790, in conjunction with RSV, and measured O.U.R to determine whether or not the increases triggered by RSV persisted in the presence of these inhibitors. As can be seen in Fig. 9A, in control and patients' fibroblasts incubated with ICI182780 or with XCT790, the inductions of O.U.R. by RSV treatment were fully abolished, and the values of oxygen consumption were not statistically different from those measured without RSV treatment. These data clearly indicate the involvement of both receptors in mediating the stimulatory effects of RSV on oxygen consumption in human fibroblasts.

We speculated from these results that RSV might regulate ERRα expression. Western-blot analysis showed detectable levels of ERRα protein in vehicle-treated cells that did not differ between control and patient cells. Interestingly, it appeared clearly that RSV treatment led to up-regulate, by about 50%, ERRα receptor expression in all treated fibroblasts (Fig. 9B).

Accordingly, our results indicate that resveratrol can positively modulate the levels of mitochondrial RC complexes and oxygen consumption, but also the expression of a key regulatory factor involved in its own signaling pathway, namely ERRα, which is known to represent a master regulator of mitochondrial RC and biogenesis.

Discussion

The present study shows that, in control human fibroblasts, treatment by RSV up-regulated the overall expression of the respiratory chain, as indicated by the coordinate increases in the levels of proteins representative of complexes I to V, together with the increase in oxygen consumption. In patients' cells with various nuclear gene mutations affecting complex I or IV, studies of oxygen consumption revealed RSV-induced increases in cell respiratory rates, with restoration to the control range in 6 out of 16 cell lines. In these fibroblasts, it can therefore be concluded that, enzyme activities were restored to a level allowing normal OXPHOS activity. Regarding CI defect, this was corroborated by analysis of lactate to pyruvate ratio (L/P), reflecting the cellular NADH/NAD ratio.

Improvement or correction of RC defect was only observed in CI deficient cell lines in which significant up-regulation of the mutant protein was achieved in response to resveratrol. This clearly suggests that mutations harbored in these cell lines did not induce marked protein instability, and did not greatly hamper its ability to functionally interact with the other CI subunits. Accordingly, these cells can be considered to exhibit moderate CI deficiencies. On the other hand, severe CI deficiencies exhibited very low mutant protein levels unchanged after treatment with RSV, which emphasizes a strong destabilizing effect of some of the mutations considered. Accordingly, the diversity of responses to RSV among the patient cell lines might, for a large part, be ascribed to the variable effects of the different mutations on protein stability, with highly unstable mutant proteins being no longer inducible by RSV. Altogether, these data show that RSV could correct moderate but not severe CI or CIV deficiency in human fibroblasts.

Several studies suggest that RSV stimulates energy metabolism via a stimulation of mitochondrial biogenesis (23, 24); however, this has hardly been documented in human cells (25). In the present study, analysis of several markers of mitochondrial density consistently indicated that RSV stimulated the mitochondrial biogenesis in control, and in all the RC-deficient

fibroblasts. Thus, in patients' cells in which a correction of RC defect was observed after treatment with RSV, mitochondrial biogenesis might underlie the increase in RC capacity. However, taking citrate synthase activity and Tfam abundance as indices it is noteworthy that a mitochondrial biogenesis was uniformly found in all the patients' cells, including those in which RSV did not improve the RC complex deficiency. This points out that the stimulation of mitochondrial biogenesis triggered by RSV is not, by itself, sufficient to induce improvement of CI or CIV deficiency in deficient cell models, and that up-regulation of residual enzyme activity is a pre-requisite.

The exact cascade of activation leading to the stimulation of mitochondrial energy metabolism is presently a matter of controversy (26). In particular, several authors attempted to identify the primary target of RSV, and proposed either that RSV directly activates SIRT1 that subsequently activates AMPK (13) or, alternatively, that RSV works primarily by activating AMPK (27) through indirect mechanisms, like inhibition of PDEs (12). In this latter hypothesis, activation of SIRT1 is proposed to be indirect via elevation of NAD+ level (28). However, whatever the molecular mechanism is in reality, there seems to be a consensus on the involvement of SIRT1 and AMPK to explain the beneficial effects of RSV on mitochondrial functions. We therefore used siRNA approaches to investigate the requirement of SIRT1 and AMPK in the observed effects. Concerning SIRT1, we repeatedly observed increased NDUFV1 expression and oxygen consumption values in untreated control fibroblasts after transfection with siSIRT1, indicating a potential repressor role of SIRT1 under basal conditions. Though surprising, these results are in agreement with a recent study showing that SIRT1 KO MEF cells exhibited higher mitochondrial cellular respiration than wild type MEFs (29). Concerning the involvement of SIRT1 in the response to RSV, silencing SIRT1 abolished only a part of RSV effects on protein levels and respiration. Accordingly, in human fibroblasts, RSV acts mainly via a SIRT1-independent signaling pathway to stimulate mitochondrial energy metabolism. Recent data suggest that the implication of SIRT1 in mediating the response to RSV might vary depending on the dose of RSV used for cell treatment. Indeed, in the rodent C2C12 cell line, a moderate dose of RSV (25μM)

elicited mitochondrial effects that were found entirely SIRT1-dependent, whereas at higher RSV doses (50μM), the effects appeared SIRT1 independent (13). This is in agreement with our observations and with the results of Csiszar et al who showed that in human endothelial cells, a mitochondrial biogenesis could be triggered by 10μM of RSV and that this effect was SIRT1-dependent (25). However, at this point, it should be recalled that the dose of 75 μM RSV was determined by dose-response experiments and was chosen as the most effective to stimulate CI enzyme activity in control and patient fibroblasts, without toxic effects. In our hands, fibroblasts usually require higher concentrations of active molecule than human myoblasts (30, 31). Nevertheless, we also performed experiments at 20μM RSV and showed that this concentration is not an efficient dose to stimulate mitochondrial respiration. Therefore, in our cell culture conditions, a stimulation of O.U.R in primary human fibroblasts is only achieved at high RSV concentrations. We next tested a potential involvement of AMPK in mediating the effects of RSV. Our data clearly indicate that in our cell models, AMPK is not required. Finally, and given the literature data clearly showing an interaction between SIRT1 and AMPK pathways, not only in response to RSV but also in situations like exercise and fasting, we silenced both proteins, and demonstrated that up-regulation of mitochondrial expression by RSV was still present. There are abundant data claiming the involvement of SIRT1 in the signaling cascade triggered by RSV that leads to stimulate energy metabolism, both *in vivo* and *in vitro*. However, it is worth mentioning that many of these studies support their conclusions by showing a concomitant increase in SIRT1 mRNA, SIRT1 protein or SIRT1 activity in response to RSV, which does not prove the actual implication of SIRT1 in the signaling cascade. Some authors used SIRT1 KO mice or SIRT1 silencing approaches to sustain their conclusions (13, 24, 32). In these cases, the apparent discrepancy with our own conclusions might be due to species differences between humans and rodents, to cell-specific action of RSV, since many of these results were obtained in C2C12 cells, or as already discussed, to a RSV-induced SIRT1 activation that could be dose-dependent.

These unexpected results led us to consider other targets in different signaling pathways, which might explain the beneficial effects of RSV on mitochondrial RC in our cell system. Our search revealed that RSV was known to bind and activate ERα and ERβ (33), which, interestingly, are present in the nucleus but also in the mitochondria in number of cell types and tissues (15). Moreover, in recent years, there has been increasing evidence showing that ERs regulate the mitochondrial RC expression and biogenesis (15). The regulation of mitochondrial RC genes by ERs probably involves direct and indirect mechanisms (Fig. 9C): - a direct regulation of mitochondrial genes by ERs that are localized in mitochondria in many cells and tissues and - an indirect regulation through induction of NRFs, TFAM and PGC-1α by ER. We therefore sought to identify the role of ERs in RSV-induced up-regulation of RC functions using the specific ERα and ERβ inhibitor ICI182780. The results obtained support the hypothesis that RSV effects on RC are mediated through its interaction with ER (Fig. 9D). ERβ very likely mediates these effects since its presence has been demonstrated in human skin fibroblasts whereas ERα is not expressed (34, 35). Interestingly, it has been established that RSV regulates the mitochondrial superoxide dismutase via its interaction with ERβ (36). Finally, estrogens have recently been shown to ameliorate mitochondrial functions in Friedreich ataxia (35) and in Leber's hereditary optic neuropathy (34).

The search of RSV's targets naturally led us to consider the possible involvement of ERRα because of its kinship with ERs and its recognized role in the regulation of a wide range of mitochondrial pathways, including RC (16). We investigated the effects of XCT790 and showed that the effects of RSV on oxygen consumption were abolished in the presence of this specific ERRα inverse agonist (Fig. 9E). XCT790 has been shown to block the expression of PGC-1α target genes (37) and is often used to demonstrate a role of ERRα on cellular respiration in various cells (16, 38). Therefore, both ER and ERRα appear to mediate the effects of RSV. We cannot completely rule out the possibility that RSV might act as an ERRα agonist. Indeed, ERRα is still considered to be an orphan receptor but Suetsugi et al have shown that some phytoestrogens were agonists of ERRα (39), even though RSV was not considered in their study.

Nevertheless, we rather favor an indirect effect of RSV on ERRα, mediated by ER. This hypothesis is supported by our data showing an up-regulation of ERRα expression after RSV treatment in human fibroblasts, and is strengthened by literature data showing that ERRα gene is a downstream target of ERs in breast cancer cells (40).

In conclusion, we provide, in this study, the first evidence that RSV could have a beneficial effect on inborn CI and CIV deficiencies from nuclear origin, in human fibroblasts. This natural product, with no known adverse effects, is presently tested in several clinical trials in various diseases. Importantly, in a recent paper, RSV (resVida) given to obese individuals for 30 days was shown to improve mitochondrial function in skeletal muscle (41). This supports the hypothesis that the stimulatory effects of RSV observed in the present study might be clinically relevant for the treatment of some RC deficiencies in humans. From a fundamental point of view, our study and many data of the literature indicate that RSV has wide-ranging effects on cell biology. Therefore, not surprisingly, numerous signaling pathways have been reported to underlie the effects of RSV. In our primary human fibroblasts, up-regulation of RC observed after treatment by RSV appears mainly SIRT1 and AMPK independent, but rather requires the ER and ERRα signaling pathways. Altogether, this study unravels new therapeutic targets for the correction of mitochondrial disorders.

Materials and Methods

Patients and control fibroblasts

The CI- and CIV-deficient human fibroblasts used in our study originate from skin biopsies performed from 15 patients that have been previously described and 1 patient for which disease-causing mutations are still unknown. Mutations data are summarized in Table 1.

Cell culture and treatments

Human skin fibroblasts from control and patients were cultured at 37°c, 5% CO_2 in RPMI with Glutamax™ (Gibco) supplemented with 10% (V/V) fetal bovine serum, 100U/ml penicillin, 0.1mg/ml streptomycin and 5µg/ml plasmocin (Invivogen). For treatment, the media were removed and cells were incubated with fresh media containing 75µM of *trans*-RSV (Cayman chemical) or RSV together with ICI182780 (Fulvestrant, Sigma) or with XCT790 (Sigma) or equivalent amounts of dimethylsulfoxide (0.04% DMSO,vehicle).

RNA interference

Small interfering RNAs (siRNAs) targeting SIRT1 sequence were obtained either from Dharmacon (ON-TARGET plus SMARTpool) or from Sigma, as well as control non target siRNAs. Fibroblasts were transfected with 30 nM siRNAs 48h prior to RSV treatment, using Lipofectamine® RNAiMAX Reagent according to the manufacturer's instructions.

Western Blot analysis

Western blots were performed as previously described (42). Proteins were detected with the following antibodies: NDUFV1 (Proteintech); NDUFS3, Ip, Core1, Core2, COX2, COX4, SURF1, mtTFA, ERRa (Abcam); NDUFV2 (Sigma); NDUFS1, SIRT1 (Santa Cruz); Fp (Molecular Probe); β-actin (Millipore); ND1 (Kindly provided by A.Lombes, France).

Oxygen consumption assay

Fibroblasts oxygen consumption was measured using Oxoplates®OP96U, 96-well microplates with integrated optical oxygen sensors (PreSens, Germany). Fibroblasts were harvested and Oxoplates® wells were filled with 180 000 to 200 000 cells (60 to 70µg) suspended in RPMI 1640 without glucose (Gibco) supplemented with 1.25µM of Carbonyl cyanide *3*-chlorophenyl hydrazone (CCCP). Cell suspensions were then covered with 200µl pre-warmed (37°c) heavy mineral oil (Sigma) to seal them from ambient oxygen. Finally, Oxoplates® were read out from the bottom side every 30 sec for 1h by a fluorescence intensity microplate reader (infinite®M200, Tecan). The kinetics of fluorescence intensities was analyzed with Microsoft Excel according to the manufacturer's instruction manual. In each well, Oxygen Uptake Rates (O.U.R) were assessed by determining the maximal slope of oxygen partial pressure decrease.

Determination of Lactate and Pyruvate

Fibroblasts were seeded in 24-well plates at 70 000 cells/well. 72h after treatment with RSV, the media was removed, wells were rinsed twice with PBS and cells were incubated for 4 hours with 200µl of Krebs Henseleit bicarbonate buffer pH=7.4 (20mM Hepes buffer pH=7.4, 26mM $NaHCO_3$, 124mM NaCl, 5mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 2.5mM $CaCl_2$) supplemented with 11mM Glucose. At the end of the incubation, supernatants were collected. Lactate and pyruvate contents were measured by fluorimetric assays according to Passoneau and Lowry (43). Results were normalized to the amount of proteins in each well.

Enzyme activity measurements

CI enzyme activity was measured using the CI Enzyme Activity Microplate Assay Kit (MitoSciences) from Abcam, following the manufacturer recommendations. CIV and citrate synthase enzyme activities were measured according to the methods described in (44) and (43), respectively.

MitoTracker staining

Fibroblasts were stained with 100nM of MitoTracker Green FM and the fluorescence intensity of the mitochondria relative to the cell volume was calculated using the ImageJ software.

Statistical analysis

Differences between groups were analyzed by one way ANOVA and the Fisher test or by the paired t test.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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LEGENDS TO FIGURES

Figure 1: Resveratrol induces a dose-dependent increase in Complex I enzyme activity.

One control and one CI-deficient (Patient 3) cell lines were treated at various concentrations of RSV for 72 h before determination of CI enzyme activity. Results are expressed relative to the value measured in vehicle-treated control fibroblasts, taken as reference. Each point represents the mean ± SD of at least triplicates. * P<0.05, **P<0.01, ***P<0.001 compared to vehicle-treated cells.

Figure 2. Resveratrol up-regulates respiratory chain constituents in human fibroblasts.

Changes in the levels of proteins representative of the five RC complexes in response to RSV treatment in control fibroblasts. Western-blot experiments were performed in fibroblasts grown for 48 h in the presence of vehicle (DMSO) or 75μM resveratrol. Representative Western-blots and histograms of protein amounts are shown. The results were expressed relative to the vehicle-treated control values, taken as reference. Values are means ± SD of at least two independent experiments. *P<0,001 compared to vehicle-treated cells.

Figure 3. Resveratrol up-regulates the expression of some mutated respiratory chain proteins.

Expression levels of mutated proteins: NDUFV1 (**A**), NDUFV2 (**B**), NDUFS1 (**C**), and NDUFS3 (**D**) in the various CI-deficient cells; SURF1 (**E**) and CIV subunits (**F**) in the CIV-deficient cells. Western-blot experiments were performed in fibroblasts grown for 48 h in the presence of vehicle (DMSO) or 75μM resveratrol. Representative Western-blots and histograms of protein amounts are shown. The results were expressed relative to the vehicle-treated control values. Values are means ± SD of at least two independent experiments.

*P< 0,001 compared to vehicle-treated cells.

Figure 4. Resveratrol increases CI and CIV enzyme activities.

(**A**) CI enzyme activity in control and complex I-deficient fibroblasts. (**B**) CIV enzyme activity in control and complex IV-deficient fibroblasts. Fibroblasts were treated 72h with vehicle or 75µM RSV. Each bar represents the means ± SD of at least three independent experiments. In each experiment, the assays were performed in duplicate or triplicate. *P<0,001 compared to vehicle-treated cells.

Figure 5. Control of optimal conditions for measurements of cell oxygen consumption using Oxoplate.

(A) Absolute values of Oxygen Uptake Rates (i.e. O.U.R.; changes in fluorescence intensity per unit of time) increase linearly between 25 and 90 µg of cell protein. (**B)** Titration of cell respiration by CCCP: maximal uncoupled respiration was reached at 1.25 µM CCCP. (**C)** Effect of rotenone or KCN: >90% inhibition of cell respiration was obtained with each compound. Each point or bar is means ± SD of at least three determinations in control fibroblasts.

Figure 6. Resveratrol can correct CI or CIV deficiency.

(**A**) Oxygen Uptake Rates (O.U.R.) per µg of cell protein in control and CI-deficient fibroblasts. (**B**) O.U.R. per µg of cell protein in control and CIV-deficient fibroblasts. (**C**) Lactate to pyruvate ratio in control and CI-deficient fibroblasts. Fibroblasts were treated 72h with vehicle or 75µM RSV. Each bar represents the means ± SD of at least three independent experiments. In each experiment, the assays were performed in duplicate or triplicate. *P<0,001 compared to vehicle-treated cells.

Figure 7. Resveratrol induces mitochondrial biogenesis in human fibroblasts.

(**A**) Mitotracker Green staining of mitochondria. Left panel: representative photographs. Right panel: quantification of Mitotracker fluorescence intensities per unit of cell volume. (**B**) Citrate synthase enzyme activity measurements. (**C**) Changes in Tfam protein levels. Fibroblasts were

treated 48h with vehicle or 75µM RSV. The results were expressed in fold-increase relative to the vehicle-treated cells. Bars are means ± SD of at least two different experiments. In each experiments, determinations were performed at least in triplicate. *P<0,001 compared to vehicle-treated cells.

Figure 8. Resveratrol stimulates mitochondrial functions mainly via a SIRT1- and AMPK-independent pathways.

(**A**) NDUFV1 protein levels in fibroblasts transfected with siNon-Target (NT) or siSIRT1 (n=5-10 independent experiments). (**B**) Oxygen uptake rates of fibroblasts transfected with siNT or siSIRT1 (n=3; in each experiments, determinations were performed at least in triplicate). (**C**) NDUFV1 protein levels in fibroblasts transfected with siNT or siAMPKa1 (n=3-5). (**D**) O.U.R. in fibroblasts transfected with siNT or siAMPKa1 (n=3). (**E**) NDUFV1 protein levels in fibroblasts transfected with siNT or siSIRT1+siAMPKa1 (n=3). Fibroblasts were transfected with siRNAs 48h prior to RSV treatment (75µM for 48h). Values are means ± SD *P< 0.05, **P<0.01, ***P<0.001 compared to vehicle-treated cells.

Figure 9. Resveratrol-induced up-regulation of RC capacity in human fibroblasts likely involves ER and ERRα.

(**A**) Oxygen Uptake Rates per µg of cell protein in control and CI-deficient fibroblasts treated 48h with vehicle, or RSV, or RSV+ ICI182780 (20µM) or RSV+XCT790 (5µM) (n=2-5; in each experiments, determinations were performed at least in triplicate). (**B**) Representative immunoblot for ERRα in fibroblasts treated 48h with vehicle or 75µM RSV. Values are means ± SD ***P<0.001 compared to vehicle-treated cells. (**C-E**) Proposed scheme for the effects of resveratrol involving ER and ERRα. (**C**) RSV has been shown to bind and activate ERs. The presence of ERs has been demonstrated in mitochondria where they might regulate mtDNA-encoded mitochondrial proteins. Estrogens/ER have been shown to up-regulate the expression

of NRF1, NRF2, ERRα and PGC-1α. (**D**) ICI182780, a specific ERs antagonist prevented the increase of mitochondrial RC activity triggered by RSV. (**E**) XCT790 has been shown to specifically inhibit ERRα activity without affecting ER signaling. In the presence of XCT790, expression of ERRα and ERRα/PGC-1α target genes is blocked.

Table 1 : Genotypes of the respiratory chain deficient patients

Patients	Mutated gene	Nucleotide change	Amino acid change	Nucleotide change	Amino acid change	References
1	**NDUFV1**	c.611A>G	Y204C	c.616T>G	C206G	(45)
2	**NDUFV1**	c.640G>A	E214K	c.1162+4A>C(IVS8)	ex 8 del	(45)
3	**NDUFV1**	c.1294G>C	A432P	c.988-89delTC	G388X	(45)
4	**NDUFV1**	c.1129G>A	E377K	c.1129G>A	E377K	(46)
5	**NDUFV1**	c.1157G>A	R386H	c.755C>G	P252R	(47)
6	**NDUFV1**	c.1156C>T	R386C	c.753delCCCC	S251fsX44	
7	**NDUFV2**	IVS2+5_+8delGTTA	ex 2 del	IVS2+5_+8delGTTA	ex 2 del	(48)
8	**NDUFS1**	del entire gene		c.2119A>G	M707V	(45)
9	**NDUFS1**	c.721C>T	R241W	c.1669C>T	R557X	(45)
10	**NDUFS3**	c.434C>T	T145I	c.595C>T	R199W	(49)
11	**NDUFS6**	unknown	ex 3 and 4 del	unknown	ex 3 and 4 del	(50)
12	**C8orf38**	c.296A>G	Q99R	c.296A>G	Q99R	(51)
13	**unknown**					
14	**COX 10**	c.612C>A	N204K	c.612C>A	N204K	(52)
15	**SURF1**	c.312_321del10insAT	L105X	c.312_321del10insAT	L105X	(53)
16	**SURF1**	c.539G>A	G180E	c.589-1G>A	IVS6-1G>C	(54)

Patients 1-13: CI-deficient patients

Patients 14-16: CIV-deficient patients

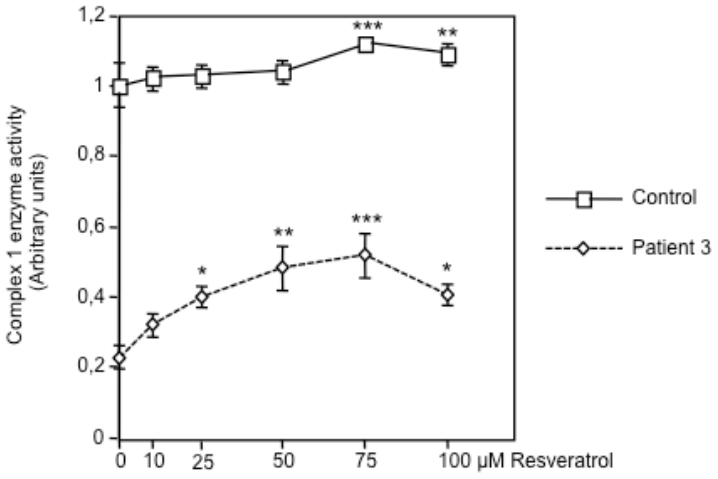
1,2
1
0,8
0,6
0,4
0,2
0
Complex 1 enzyme activity
(Arbitrary units)
0
10
25
50
75
100 µM Resveratrol
***
**
**
***
*
*
Control
Patient 3

Figure 1

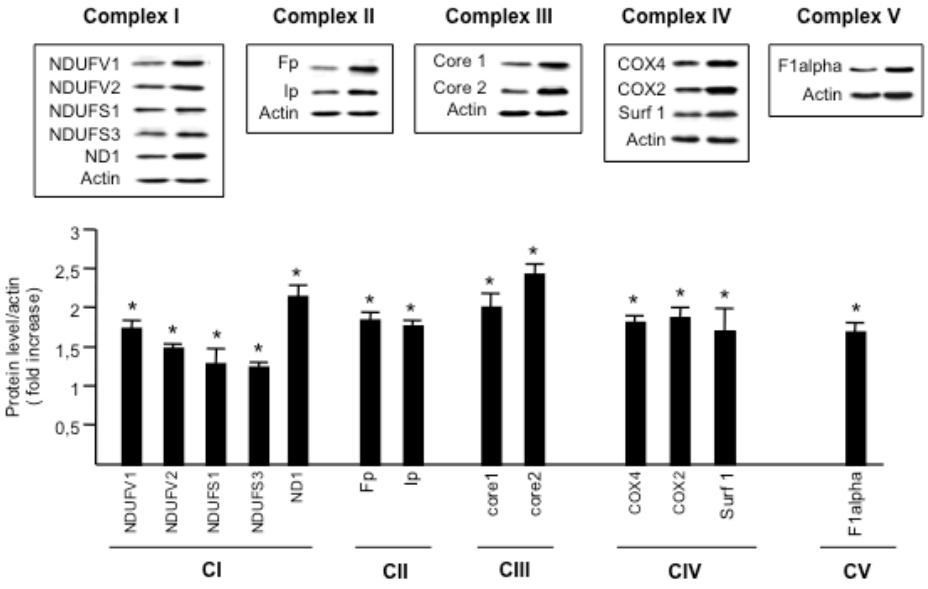
Complex I
Complex II
Complex III
Complex IV
Complex V
NDUFV1
NDUFV2
NDUFS1
NDUFS3
ND1
Actin
Fp
Ip
Actin
Core 1
Core 2
Actin
COX4
COX2
Surf 1
Actin
F1alpha
Actin
Protein level/actin
(fold increase)
3
2,5
2
1,5
1
0,5
NDUFV1
NDUFV2
NDUFS1
NDUFS3
ND1
Fp
Ip
core1
core2
COX4
COX2
Surf 1
F1alpha
CI
CII
CIII
CIV
CV

Figure 2

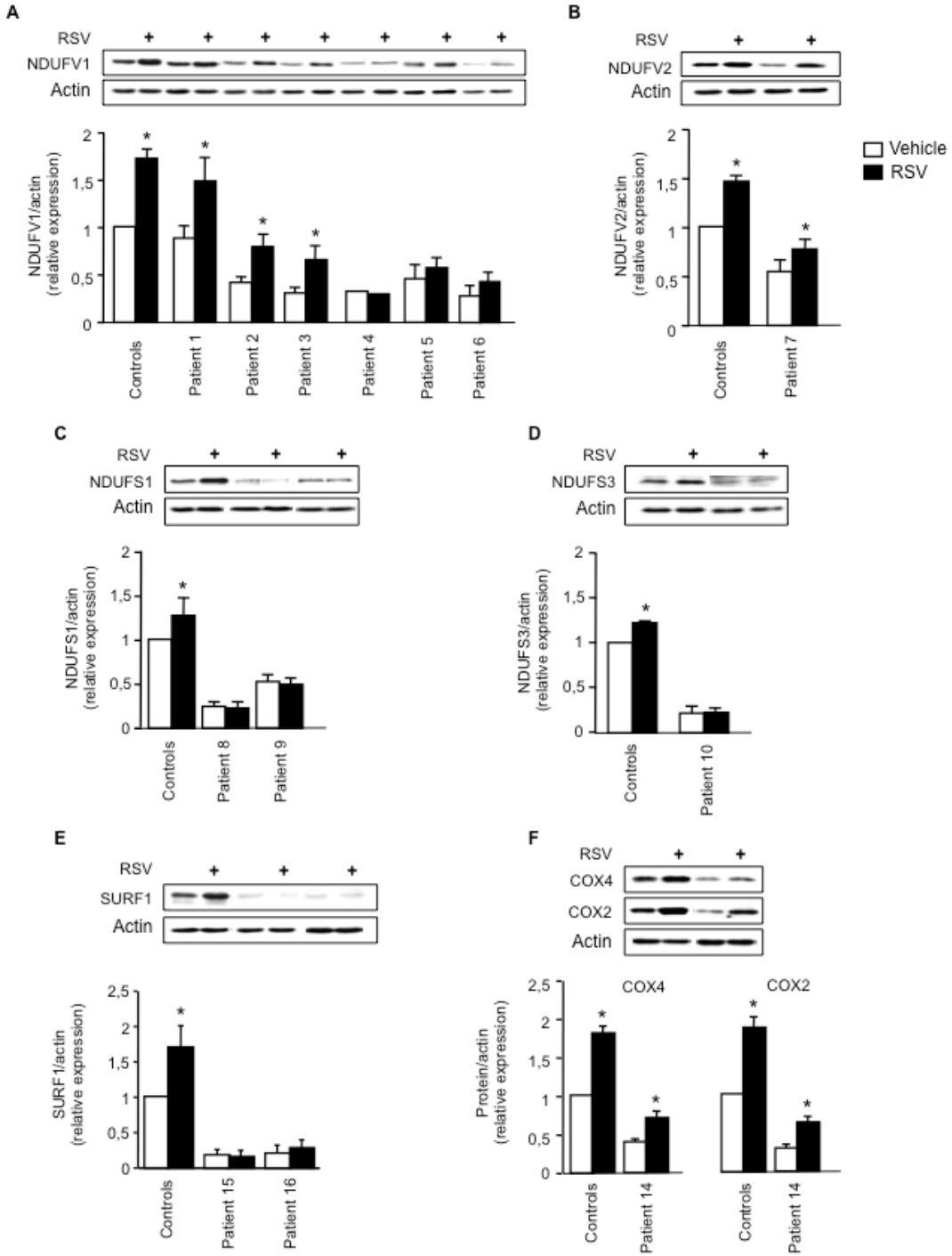
A
RSV
NDUFV1
Actin
NDUFV1/actin (relative expression)
Controls
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
B
RSV
NDUFV2
Actin
NDUFV2/actin (relative expression)
Controls
Patient 7
Vehicle
RSV
C
RSV
NDUFS1
Actin
NDUFS1/actin (relative expression)
Controls
Patient 8
Patient 9
D
RSV
NDUFS3
Actin
NDUFS3/actin (relative expression)
Controls
Patient 10
E
RSV
SURF1
Actin
SURF1/actin (relative expression)
Controls
Patient 15
Patient 16
F
RSV
COX4
COX2
Actin
Protein/actin (relative expression)
COX4
COX2
Controls
Patient 14
Controls
Patient 14

Figure 3

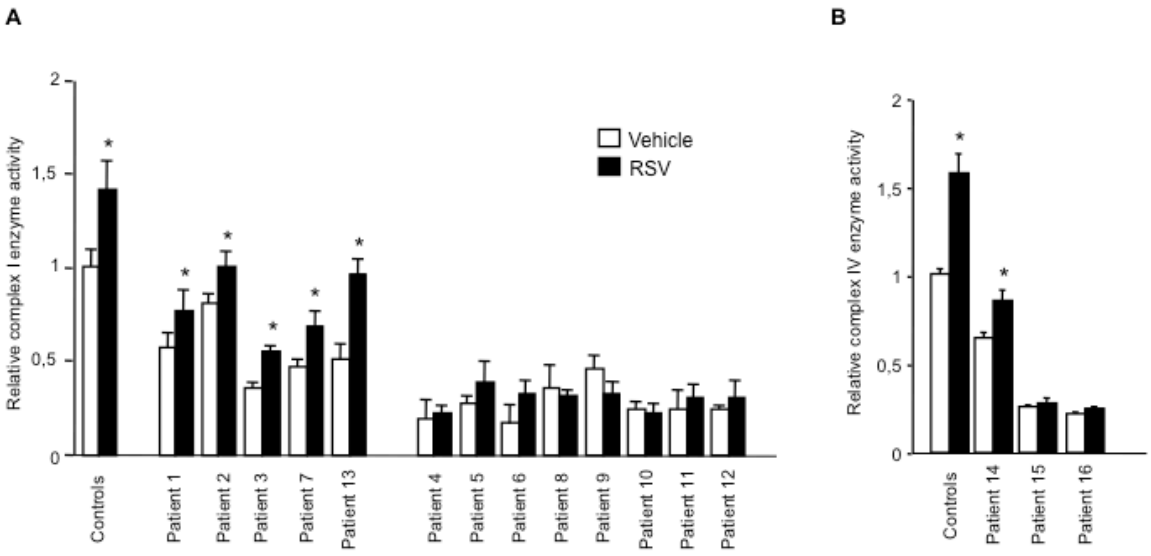
A
B
Relative complex I enzyme activity
Relative complex IV enzyme activity
Vehicle
RSV
0
0,5
1
1,5
2
Controls
Patient 1
Patient 2
Patient 3
Patient 7
Patient 13
Patient 4
Patient 5
Patient 6
Patient 8
Patient 9
Patient 10
Patient 11
Patient 12
Patient 14
Patient 15
Patient 16

Figure 4

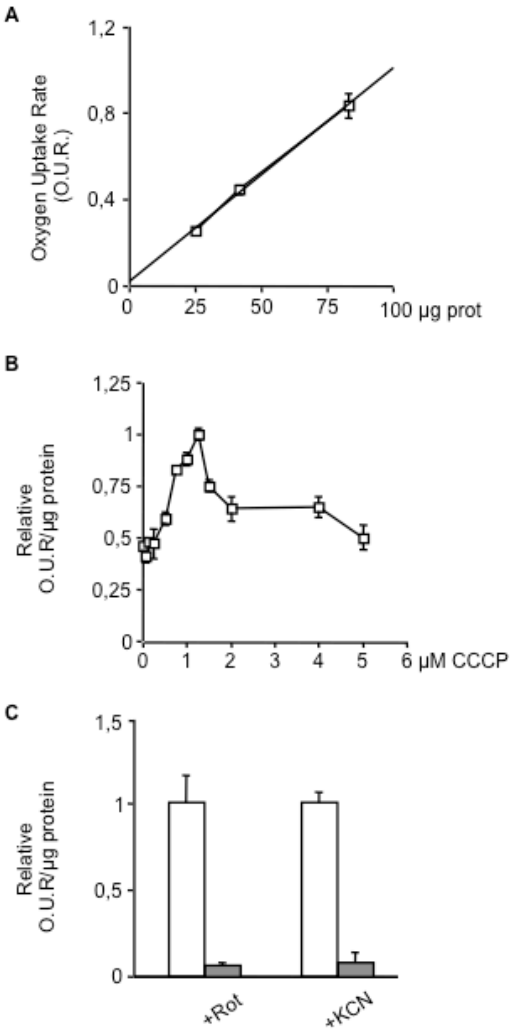
A
Oxygen Uptake Rate (O.U.R.)
1,2
0,8
0,4
0
0
25
50
75
100 µg prot
B
Relative O.U.R/µg protein
1,25
1
0,75
0,5
0,25
0
0
1
2
3
4
5
6 µM CCCP
C
Relative O.U.R/µg protein
1,5
1
0,5
0
+Rot
+KCN

Figure 5

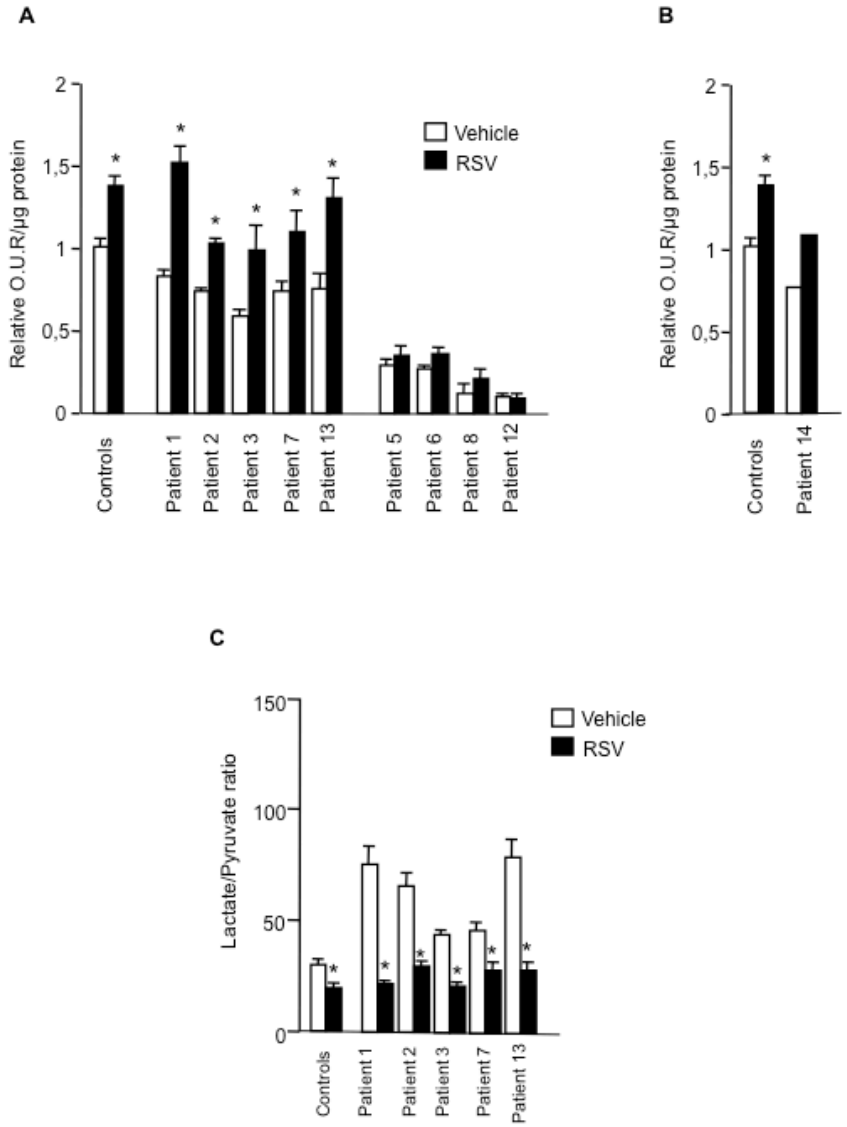
A
B
C
Relative O.U.R/µg protein
Vehicle
RSV
Controls
Patient 1
Patient 2
Patient 3
Patient 7
Patient 13
Patient 5
Patient 6
Patient 8
Patient 12
Patient 14
Lactate/Pyruvate ratio

Figure 6

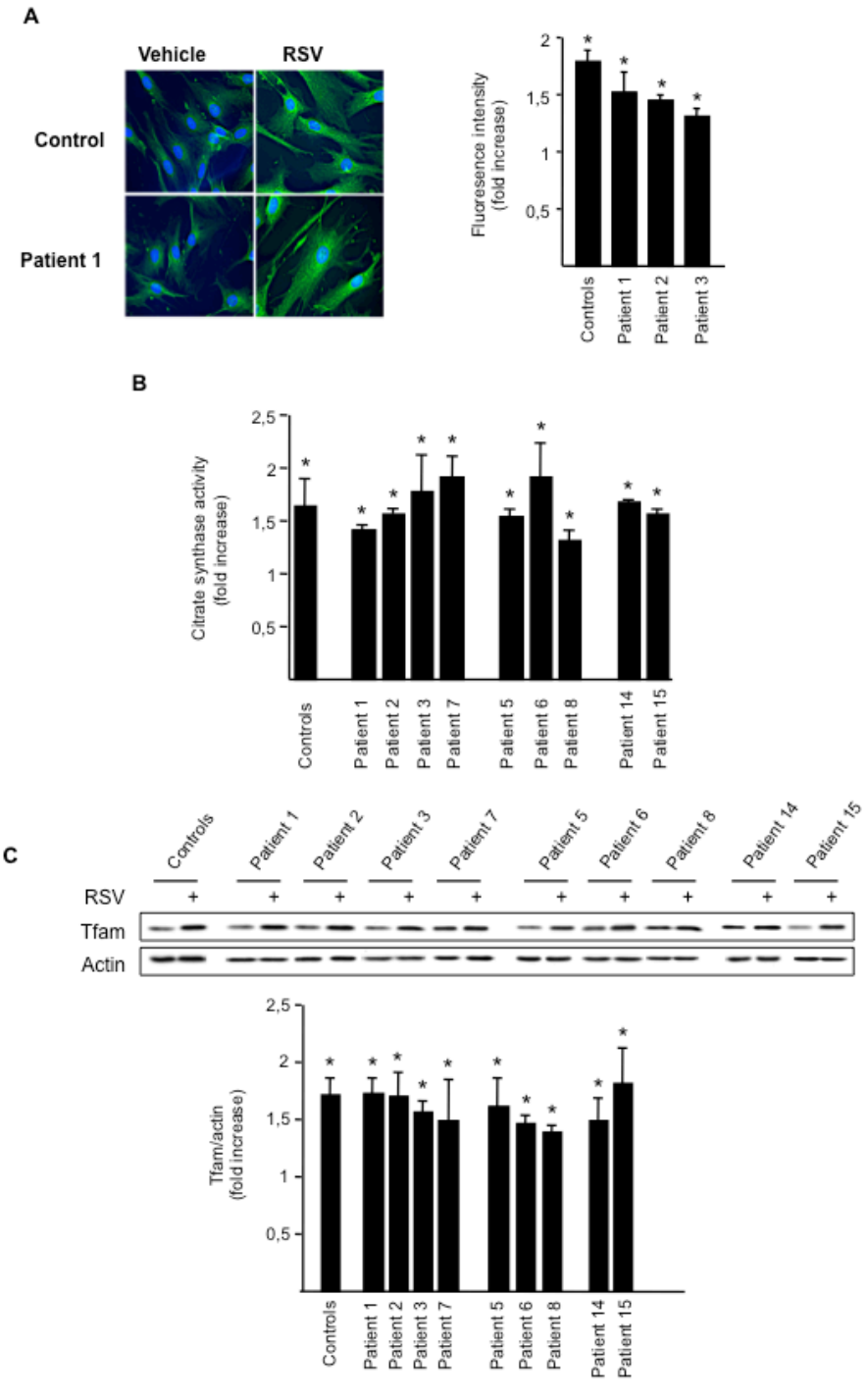
A
Vehicle
RSV
Control
Patient 1
Fluoresence intensity (fold increase)
2
1,5
1
0,5
Controls
Patient 1
Patient 2
Patient 3
B
Citrate synthase activity (fold increase)
2,5
2
1,5
1
0,5
Controls
Patient 1
Patient 2
Patient 3
Patient 7
Patient 5
Patient 6
Patient 8
Patient 14
Patient 15
C
Controls
Patient 1
Patient 2
Patient 3
Patient 7
Patient 5
Patient 6
Patient 8
Patient 14
Patient 15
RSV
Tfam
Actin
Tfam/actin (fold increase)
2,5
2
1,5
1
0,5

Figure 7

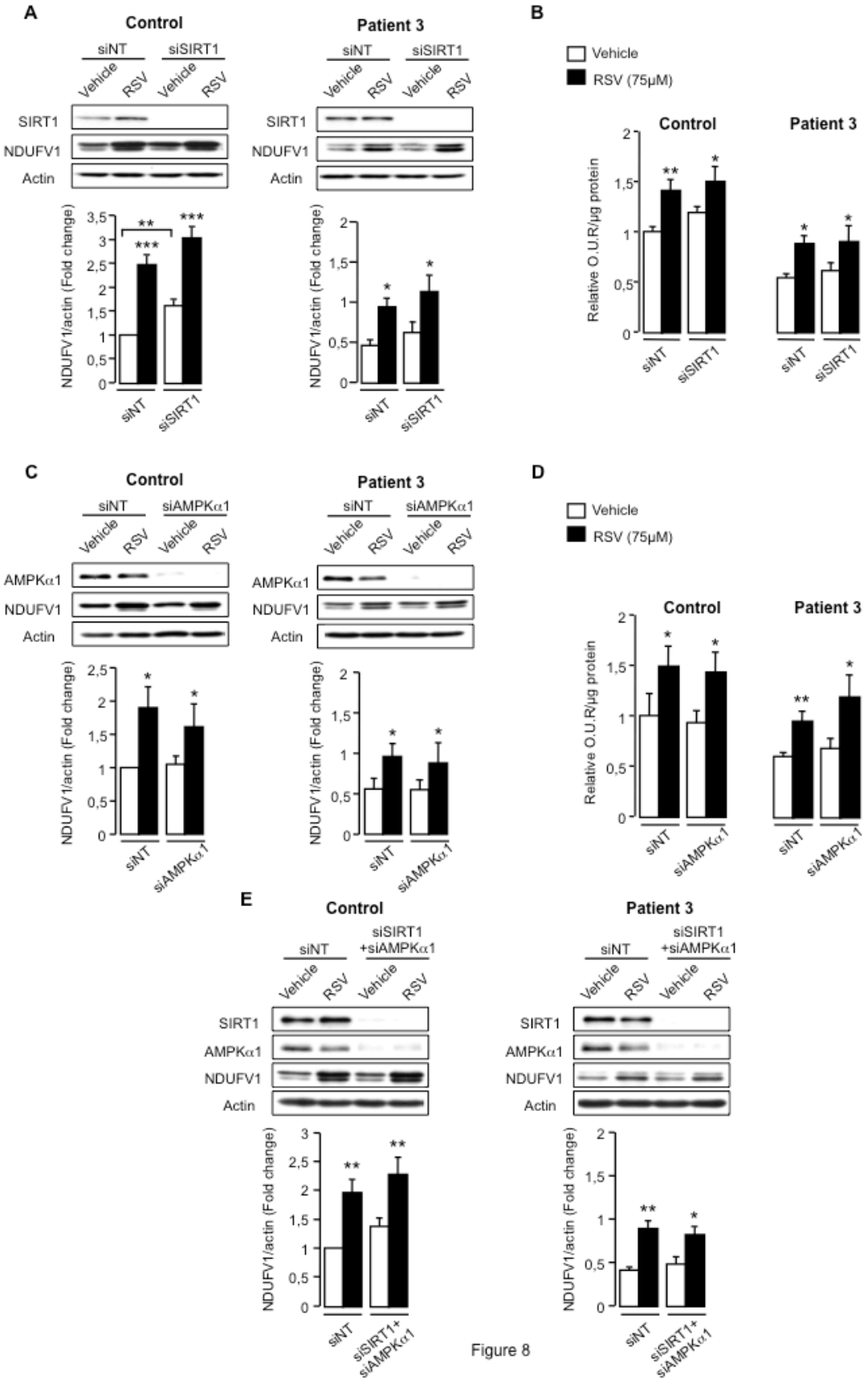
A
Control
Patient 3
siNT
siSIRT1
Vehicle
RSV
SIRT1
NDUFV1
Actin
NDUFV1/actin (Fold change)
B
Vehicle
RSV (75µM)
Relative O.U.R/µg protein
C
siAMPKα1
AMPKα1
D
E
siSIRT1 +siAMPKα1
siSIRT1+ siAMPKα1

Figure 8

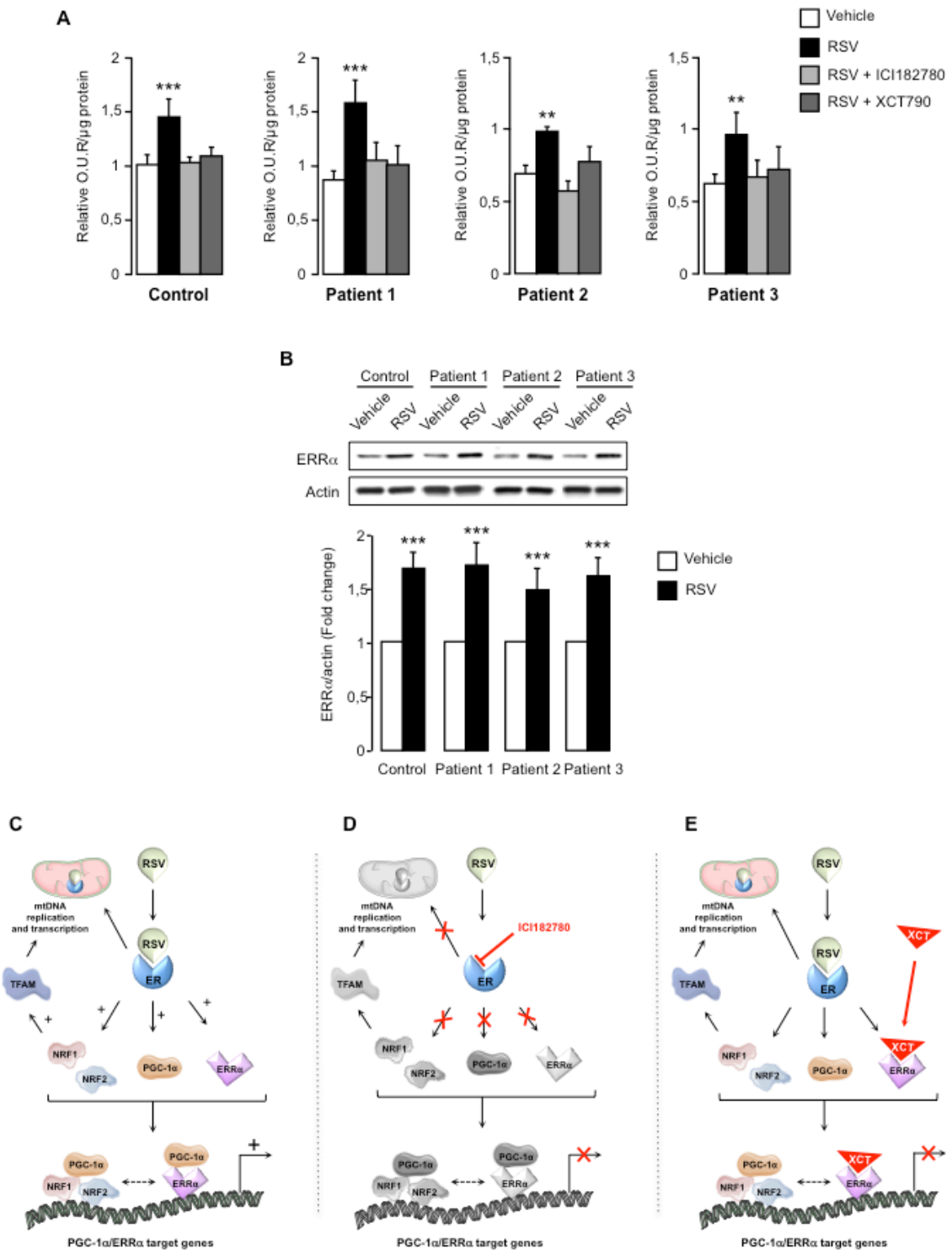
A
Vehicle
RSV
RSV + ICI182780
RSV + XCT790
Relative O.U.R/µg protein
Control
Patient 1
Patient 2
Patient 3
B
Control
Patient 1
Patient 2
Patient 3
Vehicle
RSV
ERRα
Actin
ERRα/actin (Fold change)
C
D
E
mtDNA replication and transcription
RSV
ER
TFAM
NRF1
NRF2
PGC-1α
ERRα
ICI182780
XCT
PGC-1α/ERRα target genes

Figure 9