

# Deficit of complex I activity in human skin fibroblasts with chromosome 21 trisomy and overproduction of reactive oxygen species by mitochondria: involvement of the cAMP/PKA signalling pathway

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DS (Down's syndrome) is the most common human aneuploidy associated with mental retardation and early neurodegeneration. Mitochondrial dysfunction has emerged as a crucial factor in the pathogenesis of numerous neurological disorders including DS, but the cause of mitochondrial damage remains elusive. In the present study, we identified new molecular events involved in mitochondrial dysfunction which could play a role in DS pathogenesis. We analysed mitochondrial respiratory chain function in DS-HSFs (Down's syndrome human foetal skin fibroblasts; human foetal skin fibroblasts with chromosome 21 trisomy) and found a selective deficit in the catalytic efficiency of mitochondrial complex I. The complex I deficit was associated with a decrease in cAMP-dependent phosphorylation of the 18 kDa subunit of the complex, due to a decrease in PKA (protein kinase A) activity related to reduced basal levels of cAMP. Consistently, exposure of DS-HSFs to db-cAMP (dibutyl-cAMP), a membrane-permeable cAMP analogue, stimulated

PKA activity and consequently rescued the deficit of both the cAMP-dependent phosphorylation and the catalytic activity of complex I; conversely H89, a specific PKA inhibitor, suppressed these cAMP-dependent activations. Furthermore, in the present paper we report a 3-fold increase in cellular levels of ROS (reactive oxygen species), in particular superoxide anion, mainly produced by DS-HSF mitochondria. ROS accumulation was prevented by db-cAMP-dependent activation of complex I, suggesting its involvement in ROS production. Taken together, the results of the present study suggest that the drastic decrease in basal cAMP levels observed in DS-HSFs participates in the complex I deficit and overproduction of ROS by DS-HSF mitochondria.

**Key words:** cAMP level, Down's syndrome, mitochondrion, mitochondrial complex I, protein kinase A (PKA)-mediated phosphorylation, reactive oxygen species.

## INTRODUCTION

Mammalian complex I (NADH:ubiquinone oxidoreductase; EC 1.6.5.3) is the largest protein assembly in the mitochondrial respiratory chain and forms the major entry-point for electrons into the OXPHOS (oxidative phosphorylation) system [1]. Several studies have shown that, in human and mammalian cell cultures, complex I activity is enhanced in the presence of cAMP and regulated by cAMP-dependent phosphorylation of complex I subunits, including the NDUFS4 18 kDa subunit [2–4]. The phosphoregulation of complex I, as well as of other proteins involved in OXPHOS, is believed to be governed by the activity of both cytosolic- and mitochondrial-localized protein kinases such as PKA (protein kinase A) [5,6]. In several pathological conditions, such as some types of hereditary inborn complex I dysfunctions, it has been reported that stimulation of cAMP-dependent phosphorylation of the complex by db-cAMP (dibutyl-cAMP), a derivative of cAMP, enhanced residual complex I activity [7].

Complex I deficiency is also intimately involved in common neurological diseases in adults, including Parkinson's disease [8], Alzheimer's disease [9] and Huntington's disease [10], as well as in aging [11]. Some evidence for complex I

deficiency has also been described in DS (Down's syndrome), a multifactorial disorder caused by trisomy of human chromosome 21 and associated with mental retardation, premature aging and neurodegeneration [12,13]. A selective defect in complex I-mediated respiration has been reported in isolated mitochondria from the brain of Ts16, a mouse model of DS [14], and a decrease in the protein levels of the 30 kDa subunit of complex I has been found in the cerebral cortex of DS brain [15]. However, no direct evidence of a deficit in complex I activity has been shown and there are no studies which have analysed alterations in mitochondrial respiratory chain complexes at a functional level in DS. Recently, we have described a decreased efficiency of the mitochondrial energy production apparatus in DS human skin fibroblasts [16], so it was of interest to examine whether defects in mitochondrial respiratory chain function also occurred in DS, with particular attention to complex I, which is well known to influence mitochondrial energy production [17].

It is well established that complex I impairment determines generation of ROS (reactive oxygen species) and thus oxidative stress [18,19]. There is clear evidence of oxidative stress in DS cells; markers of oxidative stress such as lipid peroxidation [20], protein modifications [21] and DNA damage [22] were found to be increased, as well as SOD1 (superoxide dismutase 1) expression

Abbreviations used: AA, antimycin; ASC, ascorbate; COX, cytochrome *c* oxidase; DCF, dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; db-cAMP, dibutyl-cAMP; DS, Down's syndrome; DS-HSF, Down's syndrome human foetal skin fibroblast; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone; GLU, glutamate; HSF, human foetal skin fibroblast; MAL, malate; NAO, nonyl Acridine Orange; N-HSF, normal human foetal skin fibroblast; OXPHOS, oxidative phosphorylation; PKA, protein kinase A; ROS, reactive oxygen species; ROT, rotenone; SOD1, superoxide dismutase 1; SUCC, succinate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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and activity [23]. However, the mechanism and the cellular site responsible for the overproduction of ROS in DS have not been elucidated. The up-regulation of the *SOD1* gene, located on chromosome 21, has been proposed as a potential culprit [23]. However, the TS1Cje mouse, an animal model of DS, carrying a subset of triplicated human chromosome 21 orthologues that lacks *Sod1*, shows oxidative stress and mitochondrial dysfunction [24]. We therefore hypothesized that a redox imbalance may arise from mitochondrial dysfunction; ROS are generated inside mitochondria by respiratory chain complexes I and III [18,19,25]; thus dysfunction of these complexes could result in an increase in ROS production.

In the present study, using DS-HSFs (DS human foetal skin fibroblasts; human foetal skin fibroblasts with chromosome 21 trisomy) as a cellular model system, we investigated the activity of mitochondrial complex I as well as of complexes II–IV, and explored whether abnormalities in mitochondrial complex activities might lead to ROS overproduction. Additionally, since we found a drastic decrease in intracellular cAMP levels in DS-HSFs, the effects of a permanent derivative of cAMP, db-cAMP, on cAMP-dependent PKA, complex I phosphorylation and activity as well as ROS production in DS-HSFs were also investigated.

We found that DS-HSFs show a selective drastic reduction in the catalytic efficiency of respiratory chain complex I, due to a decrease in its cAMP-dependent phosphorylation, rescued by exposure of DS-HSFs to db-cAMP. Measurements of cellular levels of ROS in DS-HSFs revealed enhanced  $O_2^-$  (superoxide anion) production by mitochondria abrogated by activation of complex I by db-cAMP, thus suggesting that the complex I deficit may be involved in the overproduction of ROS by DS-HSF mitochondria.

## EXPERIMENTAL

### Cell culture

Five normal (N-HSFs) and five DS human foetal skin fibroblast (DS-HSFs) cell lines were obtained from the Galliera Genetic Bank (Galliera Hospitals, Genova, Italy). The Galliera Genetic Bank operates in agreement with ethical guidelines stated in the TGB Network Charter with informed consent obtained from the patient or their guardians. The cell lines were established from fetuses spontaneously aborted at a gestational age between 14 and 19 weeks. DS and matched normal cell strains were processed and studied in parallel.

The cells were cultured at 37 °C in humidified 5%  $CO_2$ /95% air in RPMI 1640 medium (Gibco) supplemented with 15% heat-inactivated FBS (foetal bovine serum; Gibco), 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml). Cells were subjected to a 1:2 split every 6 days. A cell protein assay was carried out as described previously [26]. Comparison of the functional features of normal and DS fibroblasts was made with subconfluent cultures using a comparable number of culture passages (5–15), in which the growth rate of DS fibroblasts was comparable with that of normal cells; doubling time was approx. 18 h.

### Measurement of respiration rate and mitochondrial membrane potential in permeabilized HSFs

HSFs were trypsinized, washed with PBS and permeabilized with 0.01% digitonin as described previously [16].

To measure the respiration rate in permeabilized HSFs (1 mg of protein), cells were incubated at 37 °C in 1.5 ml of the

respiration medium [210 mM mannitol, 70 mM sucrose, 20 mM Tris/HCl, 5 mM  $KH_2PO_4/K_2HPO_4$  (pH 7.4), 3 mM  $MgCl_2$  and 5 mg/ml BSA], in a Gilson 5/6 oxygraph with a Clark electrode, and either GLU (glutamate; 5 mM) plus MAL (malate; 5 mM) or SUCC (succinate; 5 mM) plus ROT (rotenone; 3  $\mu$ M), or ASC (ascorbate; 5 mM) plus TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine; 0.2 mM) and AA (antimycin; 2.5  $\mu$ M) were added to the cells in the presence of the uncoupler FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 1.25  $\mu$ M) which allows the maximum activity of the respiratory chain [27].

Mitochondrial membrane potential ( $\Delta\psi$ ) was measured by monitoring fluorescence changes of the probe safranin O at an excitation wavelength of 520 nm and an emission wavelength of 570 nm, essentially as described previously [28]. Permeabilized HSFs (1 mg of protein) were incubated at 37 °C in 2 ml of a standard medium consisting of 0.3 M sucrose, 10 mM KCl, 1 mM  $MgCl_2$ , 20 mM Hepes/Tris (pH 7.2) plus 10  $\mu$ M safranin O in the presence of either 5 mM GLU plus 5 mM MAL, 5 mM SUCC plus 3  $\mu$ M ROT, or 5 mM ASC plus 0.2 mM TMPD in the presence of 2.5  $\mu$ M AA and 3  $\mu$ M MYXO (myxothiazol).

### Measurement of respiratory chain complex activities

Measurements of respiratory chain complex activities were carried out in mitochondrial membrane-enriched fractions from cultured HSFs. Aliquots of trypsinized HSFs were washed with ice-cold PBS, frozen in liquid nitrogen and kept at –80 °C until use. For isolation of mitochondrial membrane-enriched fractions, the pellets were thawed at 2–4 °C, suspended in 1 ml of 10 mM Tris/HCl (pH 7.5), supplemented with 1 mg/ml BSA, and exposed to ultrasound energy for 15 s at 0 °C. The ultrasound-treated cells were centrifuged (10 min at 600 *g* at 4 °C). The supernatant was centrifuged again (10 min at 14 000 *g* at 4 °C) and the resulting supernatant was carefully removed. The pellet was suspended in 0.3 ml of the respiration medium and subdivided to perform three assays, essentially as described previously [29], which relies on the sequential addition of reagents to measure the activities of: (i) NADH:ubiquinone oxidoreductase (complex I); (ii) succinate:ubiquinone oxidoreductase (complex II); and (iii) cytochrome *c* oxidase (complex IV) followed by cytochrome *c* oxidoreductase (complex III).

### Immunoblotting analysis

Cell extracts (0.05 mg of protein) were loaded on to SDS/PAGE (10% gels), separated and transferred on to a PVDF membrane which was probed with primary antibodies against the following proteins: the 20 kDa complex I subunit, the 30 kDa complex II subunit, core 2 complex III, COX (cytochrome *c* oxidase) I complex IV and complex V  $\alpha$  subunit of  $F_1$ ATPase (MitoProfile OXPHOS cocktail, 1:250 dilution; MitoSciences), the 30 kDa complex I subunit (NDUFS3, 1:250 dilution; Santa Cruz Biotechnology), ND1 complex I subunit (1:200 dilution; Santa Cruz Biotechnology), the 75 kDa complex I subunit (NDUFS1, 1:250 dilution; Santa Cruz Biotechnology); porin (1:1000 dilution; MitoSciences) and PKA $\alpha$  cat (B-5) subunit (1:250 dilution; Santa Cruz Biotechnology). Immunoblot analysis was performed essentially as described previously [30] using HRP (horseradish peroxidase)-conjugated anti-mouse or anti-rabbit antibodies and enhanced chemiluminescence Western blotting reagents (Amersham, Pharmacia Biotech). Protein levels were normalized using the constitutively expressed  $\beta$ -actin protein using an anti-( $\beta$ -actin) antibody (1:500 dilution, Sigma–Aldrich).

Densitometry values for immunoreactive bands were quantified, and protein levels were calculated as a percentage

of those in normal fibroblasts taken as 100 in arbitrary units after normalization on the basis on the amount of  $\beta$ -actin in each lane on the same filter.

### Measurement of cellular cAMP levels and PKA activity

Intracellular cAMP levels were measured in cell extracts with the cAMP EIA Kit from Stressgen, following the manufacturer's instructions.

The activity of PKA was measured in cell extracts with the cAMP-dependent PKA assay System (Stressgen), according to the manufacturer's protocol. PKA activity was stimulated 5–10-fold by incubating the cell extract with 5  $\mu$ M db-cAMP for 5 min at 37°C.

### Immunoprecipitation and immunodetection of phosphoserine-containing proteins

For immunoprecipitation of complex I, 500  $\mu$ g of mitochondrial membrane-enriched fraction, prepared as described above for measurements of respiratory chain complex activities, was incubated for 2 h at 4°C with 4  $\mu$ g of antibodies against the 30 kDa complex I subunit in the presence of 20  $\mu$ l of Protein A–Sepharose beads as described previously [31]. Following SDS/PAGE, proteins were transferred on to a PVDF membrane and probed with an anti-phosphoserine antibody (1:1000 dilution; Santa Cruz Biotechnology), and immunoblot analysis was performed as described above. The membrane was stripped and reprobed with both an anti-(complex I 75 kDa subunit) antibody (1:250 dilution; Santa Cruz Biotechnology) and an anti-(complex I 30 kDa subunit) antibody (1:250 dilution; Santa Cruz Biotechnology) to normalize the immunoprecipitated level of complex I.

### Intracellular ROS detection

Intracellular ROS production by cultured HSFs was evaluated using the following probes: MitoSOX<sup>TM</sup> (3  $\mu$ M, Molecular Probes), selective mitochondria-targeted specifically for superoxide anion [32]; or DCFH-DA [2',7'-DCF (dichlorofluorescein) diacetate; 5  $\mu$ M; Sigma–Aldrich], a non-fluorescent dye which is hydrolysed in cells and reacts with multiple types of ROS, mainly H<sub>2</sub>O<sub>2</sub>, to give the fluorescent product DCF [33]. In addition, MitoTracker Deep Red (5  $\mu$ M) and NAO (nonyl Acridine Orange, 1  $\mu$ M; Molecular Probes) were used as fluorescent mitochondrial indicators.

ROS were visualized in live cells by using laser-scanning confocal microscopy imaging. Cells were cultured at low density on fibronectin-coated 35-mm glass-bottomed dishes and incubated for 20 min at 37°C with the probes described above. After washing with PBS, stained cells were examined under a Leica TCS SP5 II microscope (images collected using a 60 $\times$  objective). The green fluorescence of oxidized DCF and NAO was analysed by exciting the sample with a Diode 405 laser (excitation wavelength of 488 nm); the red fluorescence of MitoSOX<sup>TM</sup> and MitoTracker Deep Red was analysed by exciting the sample with a HeNe laser 543 (excitation wavelength of 543 nm) and a HeNe laser 633 (excitation wavelength of 633 nm) respectively.

Quantitative analysis of ROS production was performed by means of an LS50 PerkinElmer spectrofluorimeter. Cells cultured for 2 days in 10 cm diameter dishes were incubated with either DCFH-DA or MitoSOX<sup>TM</sup>, trypsinized, resuspended in 0.5 ml of PBS, and the fluorescence of the samples was measured at an excitation wavelength of 488 nm and an emission wavelength of 520 nm for DCF, and an excitation wavelength of 510 nm and an emission wavelength of 580 nm for MitoSOX<sup>TM</sup>. The fluorescence

intensity, normalized to the protein content, was used to determine the relative ROS production.

### Statistical analysis

Statistical evaluation of the differential analysis was performed by one way ANOVA and Student's *t* test. The threshold for statistical significance was set at  $P < 0.01$ .

## RESULTS

### Analysis of mitochondrial respiration chain complexes in DS-HSFs: selective deficit of complex I

To investigate whether and how changes in mitochondrial respiratory chain function can occur in DS fibroblasts, we analysed certain functional parameters of mitochondria inside cells by comparing trisomic fibroblasts permeabilized with 0.01% digitonin (DS-HSF<sub>+dig</sub>) with respect to normal-permeabilized fibroblasts (N-HSF<sub>+dig</sub>).

We first checked whether changes in respiratory capacity can occur in DS-HSFs. To do this, we measured the respiratory complex-dependent substrate oxidation inside DS-HSF<sub>+dig</sub> compared with N-HSF<sub>+dig</sub> upon addition of GLU plus MAL, SUCC, or ASC plus TMPD.

The rate of complex I-mediated oxidation of the substrates GLU and MAL was found to be significantly lower in DS-HSF<sub>+dig</sub> (40  $\pm$  5%) compared with N-HSF<sub>+dig</sub>, as shown in a series of experiments carried out comparing five different DS cell lines with respect to normal cells (Figure 1A). Conversely, the rates of oxygen consumption obtained in the presence of the complex II or complex IV substrates (SUCC, or ASC plus TMPD respectively) were found to be comparable with those measured in N-HSF<sub>+dig</sub>.

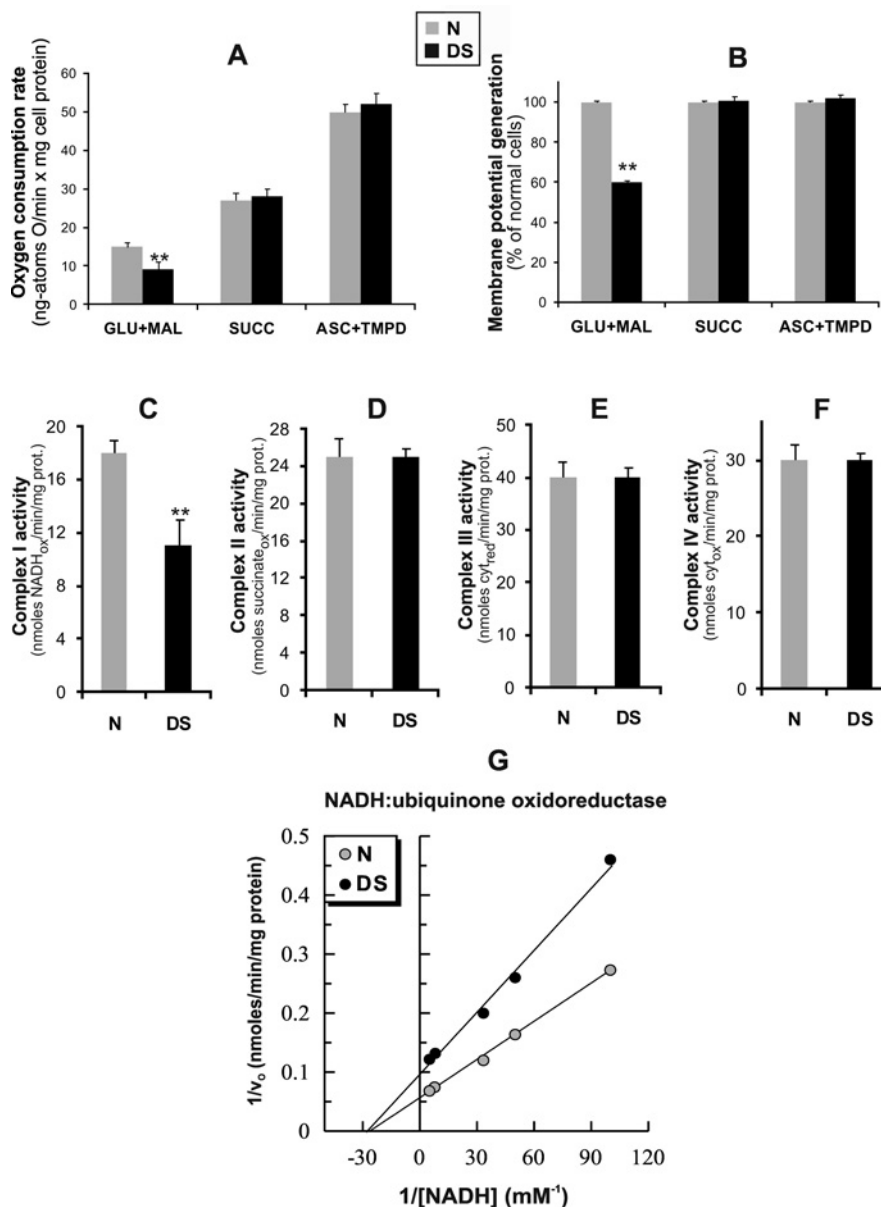
To determine whether the decrease in complex I-dependent respiration rate in DS-HSF<sub>+dig</sub> reduces mitochondrial energization, we measured the mitochondrial membrane potential ( $\Delta\psi$ ), by using safranin O as a  $\Delta\psi$  probe (see the Experimental section). Consistent with results obtained above, the rate of  $\Delta\psi$  generation measured when the complex I substrates GLU and MAL were added to DS-HSF<sub>+dig</sub> was significantly lower (40  $\pm$  5%) than in N-HSF<sub>+dig</sub>. Conversely, no statistically significant differences occurred in the rate of  $\Delta\psi$  generation following addition of SUCC, or ASC plus TMPD (Figure 1B).

These results show that in DS fibroblasts both respiration and generation of membrane potential due to complex I substrates are selectively reduced, as compared with normal cells.

To verify whether direct and specific alterations of complex I occurred in DS-HSFs, the activity of complex I, as well as the activity of the other complexes (II–IV) were carried out in mitochondrial membrane-enriched fractions from DS-HSFs and N-HSFs. Complex I (Figure 1C), but not complexes II (Figure 1D), III (Figure 1E) or IV (Figure 1F), exhibited a significant reduction in specific activity (40  $\pm$  2%) in DS-HSFs with respect to N-HSFs.

Kinetic analysis revealed a significant reduction in complex I activity in DS-HSFs (Figure 1G), with mean  $V_{\max}$  values of 10  $\pm$  3 nmol/min per mg of protein, as measured in five different DS cell lines, compared with 16  $\pm$  3 nmol/min per mg of protein measured in the respective N-HSFs, whereas no changes in the affinity of complex I for the substrate NADH were found (mean  $K_m$  values of 31  $\pm$  3  $\mu$ M in DS-HSFs compared with 36  $\pm$  2  $\mu$ M measured in N-HSFs).

To give some insight into the molecular basis of complex I deficit, and given that a decrease in protein levels of the 30 kDa



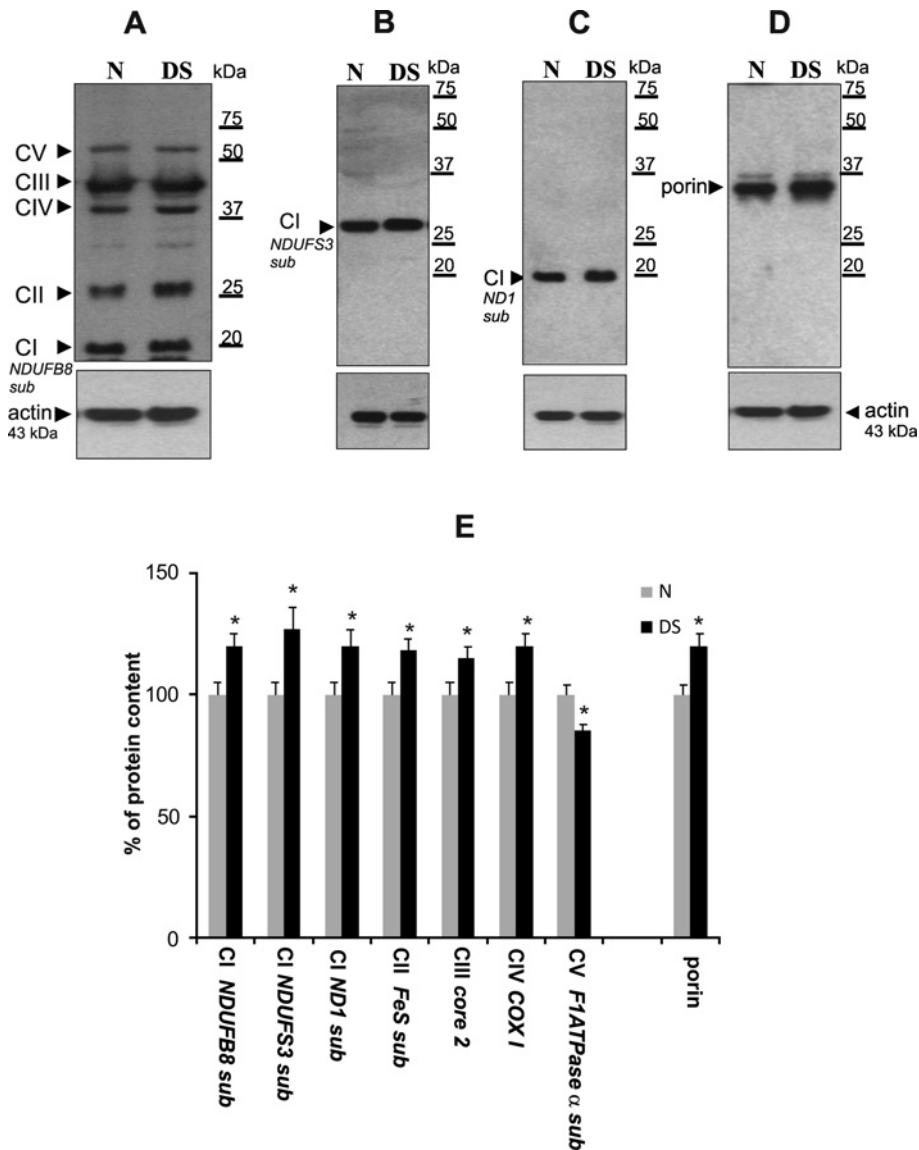
**Figure 1** Mitochondrial respiratory capacity, membrane potential generation and respiratory chain complex activities in DS-HSFs

(A) N-HSF<sub>+dig</sub> and DS-HSF<sub>+dig</sub> (each 1 mg of protein) were incubated at 37 °C in 1.5 ml of respiration medium and oxygen consumption was measured polarographically as a function of time. Where indicated, either 5 mM GLU plus 5 mM MAL or 5 mM SUCC, and 3  $\mu$ M ROT or 0.5 mM ASC plus 0.2 mM TMPD were added in the presence of 1.25  $\mu$ M FCCP. Values are means  $\pm$  S.E.M. for three different experiments carried out on five normal and the corresponding five trisomic cell lines. (B) N-HSF<sub>+dig</sub> and DS-HSF<sub>+dig</sub> (each 1 mg of protein) were incubated at 37 °C in 1.5 ml of standard medium plus 10  $\mu$ M safranin and the membrane potential generation was measured fluorimetrically. Where indicated, 5 mM GLU plus 5 mM MAL, or 5 mM SUCC and 3  $\mu$ M ROT, or 5 mM ASC plus 0.2 mM TMPD were added. Values are means  $\pm$  S.E.M. for experiments performed on five normal and five trisomic cell lines, expressed as a percentage of normal cells. The activities of complex I (NADH:ubiquinone oxidoreductase) (C), complex II (CII, succinate:ubiquinone oxidoreductase) (D), complex III (CIII, cytochrome *c* reductase) (E) and complex IV (CIV, COX) (F) were measured spectrophotometrically at 37 °C in 1 ml of respiratory medium on mitochondrial membrane-enriched fractions (0.1 mg of protein) as described in the Experimental section, and reported as the means  $\pm$  S.E.M. for three different experiments on five DS and normal samples. (G) Lineweaver–Burk analysis of complex I-mediated NADH:ubiquinone oxidoreductase activity in N-HSFs and DS-HSFs. NADH was added at the indicated concentrations to mitochondrial membrane-enriched fractions of N-HSFs and DS-HSFs respectively, and the inverse of the rate of NADH oxidation was plotted against the inverse of NADH concentration. Significant differences between normal and DS samples are indicated with asterisks (\*\* $P < 0.001$ ).

subunit of complex I has been found in the cerebral cortex of DS brain [15], we investigated the protein levels of certain protein subunits of complex I. Three different subunits from complex I were examined, i.e. the 20 kDa NDUFB8 subunit, the 30 kDa NDUF3 subunit and the mitochondrial-encoded ND1 subunit, compared with certain subunits from all other respiratory chain complexes (II–V). As shown in representative immunoblots from the proteins detected (Figures 2A–2D), and in statistical analysis of protein densitometric values (Figure 2E), there is a shared

increase in the level of the complex I subunits analysed, as well as of all mitochondrial proteins tested in DS samples, with the exception of the ATPase  $\alpha$  subunit which we had previously found to be selectively decreased [16]. However, on comparing the ratios of OXPHOS protein/porin for DS-HSFs and N-HSFs, no changes were obtained for complex I, nor for complexes II–IV.

No statistically significant changes in the mRNA levels of complex I subunits were found in DS-HSF samples compared with N-HSFs ( $P > 0.02$ ), as measured by real-time RT (reverse



**Figure 2** Protein levels of respiratory chain complexes in DS-HSFs

Protein levels of the 20 kDa NDUFB8 complex I subunit (A), the 30 kDa NDUFS3 complex I subunit (B) and the mitochondrial-encoded 20 kDa ND1 complex I subunit (C) compared with the 30 kDa subunit of complex II, core 2 protein of complex III, COX I of complex IV and  $\alpha$  subunit of F<sub>1</sub> ATPase (A), were measured by immunoblotting analysis of cell extracts (0.05 mg of protein) of DS-HSFs and N-HSFs using the respective antibodies; protein levels of porin (D) and  $\beta$ -actin (A–D) as mitochondrial and cytosolic protein markers respectively, were also measured. The molecular mass in kDa is indicated on the right-hand side of the gel. For each protein, values are reported, after densitometric analysis and normalization with  $\beta$ -actin, as the means  $\pm$  S.E.M. for experiments performed on five normal and five trisomic cell lines (E). For each cell line, the measurements were performed in triplicate. The data are expressed as a percentage of those in normal cells. Significant differences between normal and DS samples are indicated with asterisks (\* $P < 0.01$ ).

transcription)–PCR (results not shown), thus suggesting that a post-transcriptional and/or post-translational regulation of mitochondrial complex I occurs in DS-HSFs.

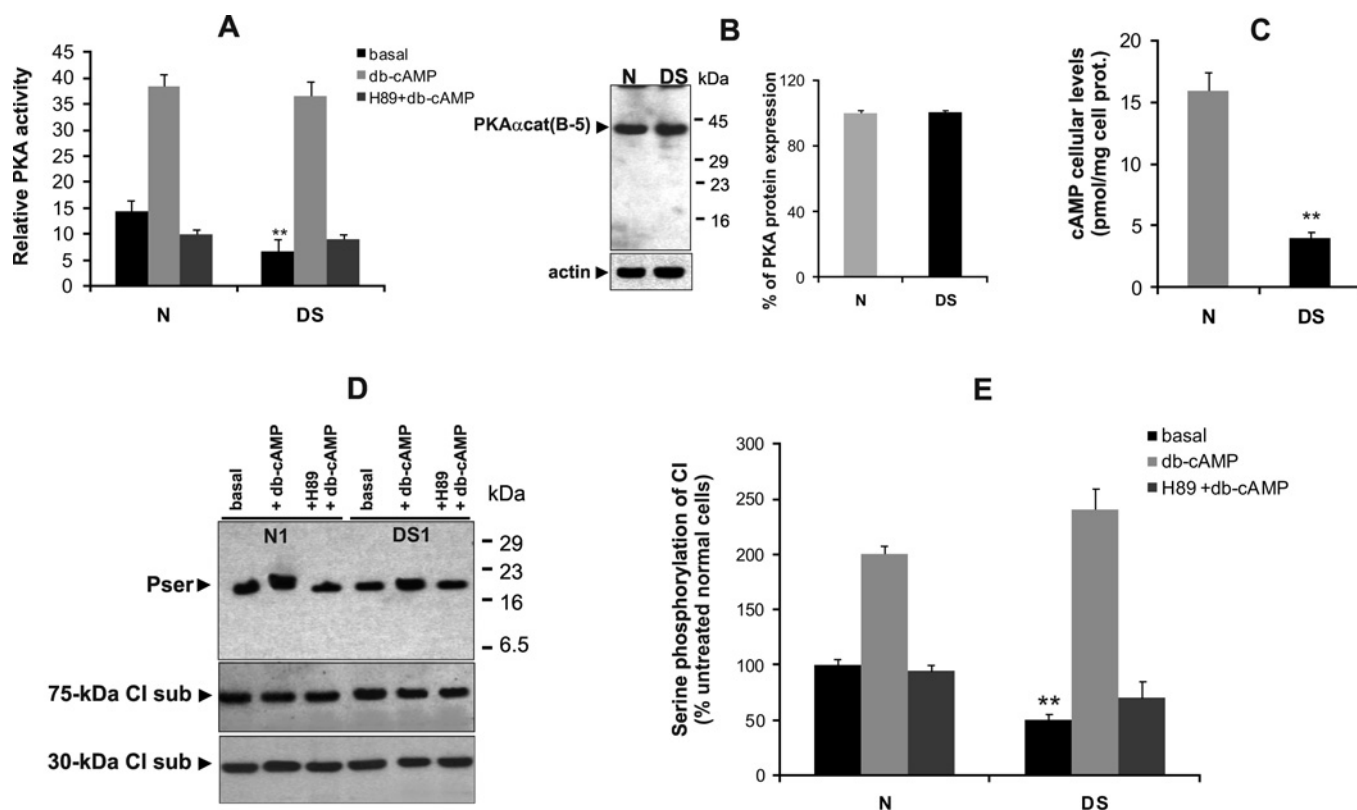
#### Decrease in cAMP-dependent phosphorylation of complex I

It has been shown that PKA regulates complex I activity through cAMP-dependent phosphorylation of the 18 kDa subunit of the complex [2]. To gain some insight into the mechanism of complex I impairment in DS-HSFs, we analysed the involvement of the cAMP/PKA pathway in DS complex I deficit.

When basal PKA activity was measured in DS cells, a significant decline was found in enzyme activity (approximately

1.5-fold less as compared with N-HSFs) (Figure 3A). Conversely, no changes in PKA protein levels were found in DS-HSFs compared with normal cells, as shown in a representative immunoblot and in statistical analysis of protein densitometric values of the catalytic subunit of PKA (Figure 3B). In addition, a drastic decrease in intracellular cAMP basal levels was found in DS cells (approximately 4-fold less as compared with N-HSFs) (Figure 3C).

To test the hypothesis that the decrease of basal PKA activity might be ascribed to decreased basal levels of cAMP, we exposed cells with a short-time incubation with db-cAMP, a membrane-permeable cAMP analogue which increases intracellular cAMP. We found that the cAMP-stimulated PKA activity was similar in both DS and normal cells, and H89, a specific inhibitor



**Figure 3** cAMP-dependent phosphorylation of complex I in DS-HSFs

(A) PKA activity was measured in cell lysates as described in the Experimental section and reported as relative PKA activity. Where indicated, N-HSFs and DS-HSFs were incubated for 2 h in the absence (basal) or in the presence of 100  $\mu$ M db-cAMP (db-cAMP); when present, cells were pre-incubated for 2 h with H89 (50  $\mu$ M) before adding db-cAMP (H89 + db-cAMP). (B) Protein levels of PKA $\alpha$  cat (B-5) were measured by immunoblot analysis of cell extracts (0.05 mg of protein) of DS-HSFs and N-HSFs using the respective antibody; protein levels of  $\beta$ -actin, as a cytosolic protein marker, were also measured. Values are reported, after densitometric analysis and normalization with  $\beta$ -actin, as means  $\pm$  S.E.M. for experiments performed on five normal and five trisomic cell lines. The data are expressed as a percentage of those in normal cells. The molecular mass in kDa is indicated on the right-hand side. (C) Cellular basal levels of cAMP were determined in N-HSFs and DS-HSFs as described in the Experimental section. (D) Immunodetection of phosphoserine-containing proteins in the immunoprecipitate of complex I in N1 and DS1 fibroblast pair samples. Solubilized proteins immunoprecipitated from mitochondrial membrane-enriched fractions with the antibody against the 30 kDa subunit of complex I were subjected to SDS/PAGE, Western blot analysis, and phosphoserine-containing proteins were detected by immunoblotting with a specific anti-phosphoserine (Pser) antibody. Protein levels of complex I subunits of 75 kDa (75-kDa CI sub) and 30 kDa (30-kDa CI sub) were also measured in immunoprecipitated samples. The molecular mass in kDa is indicated on the right-hand side. (E) Values of serine phosphorylation of complex I are reported, after densitometric analysis and normalization with both the 75 kDa and 30 kDa subunits of complex I, as a percentage of those in normal untreated cells. Significant differences between normal and DS samples are indicated with asterisks (\*\* $P < 0.001$ ).

of PKA [34], when incubated with the cells before adding db-cAMP, prevented this cAMP-dependent PKA activation (Figure 3A).

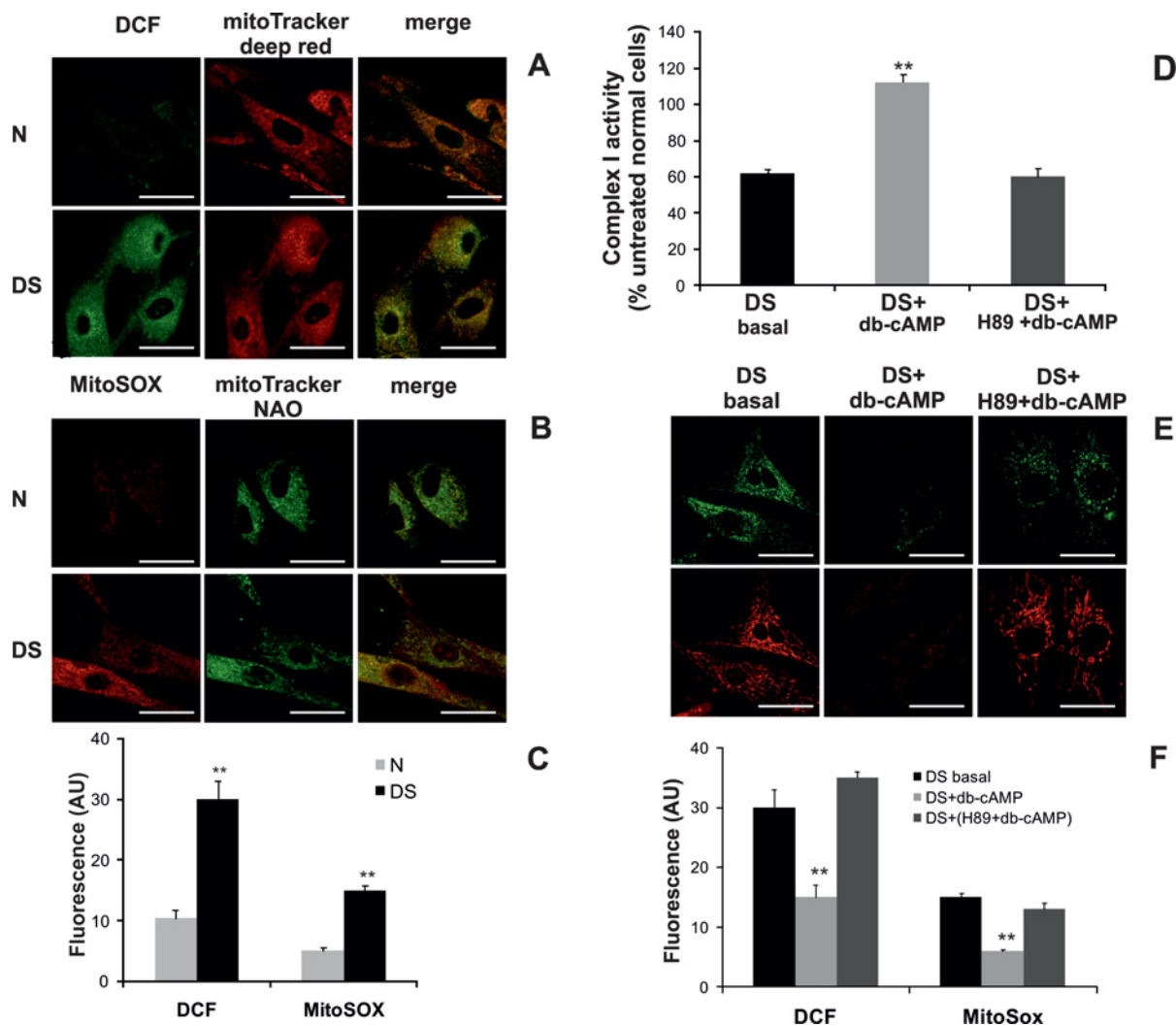
To investigate whether the decrease in basal PKA activity could determine changes in PKA-dependent phosphorylation of complex I in DS cells, complex I was immunoprecipitated using an antibody against the 30 kDa subunit of the complex, and the serine phosphorylation was detected by immunoblotting with an anti-phosphoserine antibody (Figure 3D). The only protein band detected with the anti-phosphoserine antibody ran at approximately 18 kDa, probably corresponding to the subunit of complex I which contains consensus phosphorylation sites for PKA [35,36]. A decrease of approximately 50% in serine phosphorylation of this complex I subunit was found in DS cells as compared with normal cells, as revealed by one representative immunoblot (Figure 3D) and by densitometric analysis carried out comparing five different DS and normal cell lines (Figure 3E). The amount of serine phosphorylation of complex I was measured after normalization of immunoprecipitated complex I levels with both the 75 kDa and 30 kDa complex I subunits. Four other representative immunoblots, performed with the other pairs of DS-HSFs and N-HSFs, are shown in Supplementary Figure S1 (at

<http://www.BiochemJ.org/bj/435/bj4350679add.htm>). Treatment with db-cAMP, found to stimulate PKA activity (Figure 3A), resulted in an enhancement of serine phosphorylation of complex I in DS as well as in normal cells (Figures 3D and 3E); H89, when incubated with the cells before adding db-cAMP, prevented the cAMP-dependent serine phosphorylation of the complex in both normal and DS cells, showing that the phosphorylation was mediated by PKA.

These results suggest that the reduced basal levels of cAMP, but neither expression nor catalytic alterations of PKA, could lead to a decrease in PKA activity and in turn a reduction in 18 kDa complex I serine phosphorylation in DS-HSFs.

#### Overproduction of ROS by DS-HSF mitochondria: involvement of complex I deficit

Since complex I is a well-established source of ROS [18,19], to test whether the altered cAMP-dependent phosphorylation of complex I is involved in complex I deficit and consequent ROS overproduction by mitochondria, we analysed the effect of db-cAMP on both complex I activity and ROS production.



**Figure 4** Involvement of complex I deficit in overproduction of ROS by DS-HSF mitochondria

(A and B) Imaging by confocal microscopy of ROS generation and subcellular localization in normal and DS fibroblasts. Cells were stained with 5  $\mu\text{M}$  DCF-DA (A) and 3  $\mu\text{M}$  MitoSOX<sup>TM</sup> (B), whereas fluorescence of DCF and of the mitochondria-selective O<sup>-</sup> probe MitoSOX<sup>TM</sup> was visualized in live fibroblasts by laser-scanning confocal microscopy, as described in the Experimental section. When DCF-DA was present, cells were co-stained with MitoTracker Deep Red (5  $\mu\text{M}$ ) (A); when MitoSOX<sup>TM</sup> was present, cells were co-stained with the MitoTracker NAO (1  $\mu\text{M}$ ) (B). Images are superimpositions of ten confocal Z-planes and represent typical examples. Scale bars are 50  $\mu\text{m}$ . (C) Quantification of intracellular ROS was reported as the means  $\pm$  S.E.M. of DCF and MitoSOX<sup>TM</sup> fluorescence arbitrary units (A.U.) measured by fluorimetric analysis for three different experiments on five normal and five DS cell lines. Significant differences between normal and DS are indicated with asterisks (\*\* $P < 0.001$ ). (D) Activity of complex I was assayed spectrophotometrically on mitochondrial membrane-enriched fractions (0.1 mg of protein), as described in the Experimental section, and expressed as a percentage of that measured in normal untreated cells. Where indicated, DS cells were incubated for 2 h in the absence (basal) or presence of 100  $\mu\text{M}$  db-cAMP (db-cAMP); when present, cells were incubated 2 h with H89 (50  $\mu\text{M}$ ) before adding db-cAMP (H89 + db-cAMP). Significant differences between treated and non-treated DS samples are indicated with asterisks (\*\* $P < 0.001$ ). Values are means  $\pm$  S.E.M. for experiments performed on five normal and five trisomic cell lines. For each cell line, the measurements were performed in triplicate. (E) Imaging by confocal microscopy of ROS generation in DS fibroblasts as described in (A) and (B). Cells were co-stained with the DCF-DA and the MitoSOX<sup>TM</sup> probes. (F) Quantification of intracellular ROS in DS-HSFs was reported as the means  $\pm$  S.E.M. of DCF and MitoSOX<sup>TM</sup> fluorescence arbitrary units (A.U.) measured by fluorimetric analysis in three different experiments on five DS cell lines. Significant differences between untreated and treated cells are indicated with asterisks (\*\* $P < 0.001$ ).

We first checked whether changes in ROS production occurred in DS-HSFs. Confocal microscopy analysis revealed a significant increase in ROS levels in DS cells compared with normal cells, with the DCF green fluorescence essentially merged with the red fluorescence of the MitoTracker Deep Red probe (Figure 4A), revealing production of ROS mainly from mitochondria. Staining with the MitoSOX<sup>TM</sup> probe (Figure 4B), which accumulates in mitochondria and reacts specifically with O<sup>-</sup> [32], revealed higher production of this radical molecule from mitochondria of DS cells than normal cells. Co-staining with the corresponding green MitoTracker NAO confirmed that MitoSOX<sup>TM</sup> was retained in mitochondria and detected localized rises in O<sup>-</sup> levels in DS cells. ROS levels were approximately 3-fold higher in

DS-HSFs compared with N-HSFs, as revealed by fluorimetric measurements (Figure 4C).

Interestingly, exposure of DS-HSFs to db-cAMP, which activates PKA-dependent phosphorylation of complex I (Figure 3E), completely prevented the deficit of complex I activity in DS-HSFs (Figure 4D), thus indicating that the deficit in complex I activity in DS cells occurred through a decrease in cAMP-dependent phosphorylation of the protein complex. The involvement of the PKA pathway in the cAMP-dependent activation of complex I was further demonstrated by the ability of H89 to prevent activation of complex I activity when incubated before adding db-cAMP (Figure 4D).

Consistent with the involvement of the complex I deficit in ROS overproduction, both confocal microscopy (Figure 4E) and fluorimetric analysis (Figure 4F), revealed that when complex I was activated by db-cAMP (Figure 4D), ROS accumulation was almost completely prevented, reproducing a condition comparable with that observed in normal cells; whereas H89, found to prevent the cAMP-dependent activation of complex I, also prevented the effect of db-cAMP on ROS production. These results strongly suggest that changes in cAMP-dependent phosphorylation of complex I leading to deficit of the complex activity are involved in the overproduction of ROS in DS cells.

## DISCUSSION

In the present paper we report a detailed functional study on the mitochondrial respiratory chain in DS cells which sheds light on how mitochondrial dysfunction could contribute to DS pathogenesis.

We show, for the first time in DS cells, a selective deficit of complex I which contributes to ROS overproduction by DS mitochondria, related to changes in the cAMP/PKA signalling pathway.

The selective defect in complex I-mediated respiration (Figure 1A) and mitochondrial membrane potential (Figure 1B) in DS cells suggest that, whereas mitochondria in DS-HSFs are intact and do not differ from those from normal cells in the electron flow from complex II to complex IV, either/both the generation of NADH from MAL/GLU or/and its utilization by complex I are defective. In principle, there are several steps that could be responsible for the observed behaviour, i.e. (i) changes in the selective permeability of mitochondria to the respiratory substrates, (ii) changes in the enzymes responsible for oxidation of GLU and MAL, or (iii) a lower catalytic efficiency of complex I. We addressed this last step directly by measuring the activity of complex I and we show that, unlike the other complexes, i.e. II–IV, its activity was significantly decreased in isolated DS mitochondria (Figures 1C–1F). We can, therefore, with some confidence, attribute the mitochondrial dysfunction in fibroblasts with chromosome 21 trisomy to a multifactorial impairment of OXPHOS which involves the respiratory chain complex I together with the molecular machinery for mitochondrial ATP synthesis (ATP synthase, ADP/ATP translocator and mitochondrial adenylate kinase) [16].

Kinetic analysis of NADH:ubiquinone oxidoreductase activity (Figure 1G) revealed a severe reduction in the catalytic efficiency of complex I despite unchanged affinity for its substrate NADH, giving an indication as to how complex I is impaired in DS-HSFs.

The deficit of complex I activity in DS-HSFs is particularly interesting because it occurred even though certain complex I subunits were not only not differentially expressed at the gene level (results not shown), but up-regulated at the protein level (Figure 2); thus we assume that the impairment of complex I activity could be caused by a post-translational modification. Note that in some tissues such as cerebral cortex of DS brain [15] and in the heart of DS fetuses [37], some complex I subunits are down-regulated. The up-regulation of complex I subunits we found in DS cultured cells, shared with many other mitochondrial proteins tested in DS samples, could be attributed to enhanced mitochondrial mass in proliferating DS-HSFs as a compensative mechanism partially offsetting the deficit of mitochondrial functions in DS cells [16].

In the present study we provide indications aiming to account for the deficit of complex I activity found in DS fibroblasts.

It is well known that complex I activity is regulated by cAMP-dependent phosphorylation of complex I subunits; in particular, the 18 kDa subunit of complex I has consensus phosphorylation sites for PKA, and the cAMP-dependent phosphorylation of this subunit promotes the activation of complex I [2,35]. Consistently, the complex I deficit in DS cells correlates with a decrease in the basal PKA-dependent phosphorylation of the 18 kDa subunit of the complex we found in DS-HSFs (Figures 3D and 3E). In agreement with the decrease in both PKA activity and cellular cAMP levels (Figures 3A and 3C), we showed that complex I activity was restored by short-term incubation with a permanent derivative of cAMP, db-cAMP, which strongly increases both PKA activity and complex I phosphorylation in DS-HSFs in a manner comparable with that observed in normal cells (Figures 3A and 3E); this cAMP-promoting effect is completely suppressed by the PKA inhibitor H89, thus further demonstrating that the deficit of complex I activity involves PKA. The fact that DS cells are equally sensitive to db-cAMP when compared with normal cells actually indicates that the PKA pathway is fully functional in DS; hence, to explain the lower complex I phosphorylation and activity, we propose that the drastic decrease in basal levels of cAMP found in DS-HSFs (Figure 3C) possibly participates in the decreased activities downstream of PKA which could lead to the deficit of complex I activity. A decrease in cAMP basal levels has also been reported in hippocampus of the Ts65Dn mouse model of DS due to impaired basal activity of adenylate cyclase [38,39]. Whether this also occurs in DS-HSFs is under investigation. However, we cannot rule out the possibility that alternative mechanism(s) could be accounting for reduced complex I phosphorylation and activity, such as alterations of AKAPs (PKA-anchoring proteins), which localize PKA to particular cellular compartments including mitochondria, enhancing the efficiency of cAMP signal-transducing pathways [5,40]. Further studies are required to verify such a hypothesis.

The present study and our previous results [16] give new indications as to the DS pathogenic mechanism, i.e. post-translational cAMP/PKA-mediated alteration of the catalytic activity of certain mitochondrial proteins involved in OXPHOS determined a mitochondrial energy deficit in DS cells which could promote a downward spiral of bioenergetic capacity that progressively contributes to disease progression.

There is general consensus that a disturbance in the balance of ROS might play a crucial role in DS pathogenesis. It is believed that overexpression of genes on chromosome 21, including SOD1 and amyloid- $\beta$  precursor protein [23,41], as well as a misbalance between SOD1 and glutathione peroxidase activity [42], may underlie the increased oxidative stress in DS. It is well known that ROS, and particularly the superoxide anion, can be produced in the mitochondria by a defective complex I and accumulated by antioxidant system imbalances [18,19].

We directly monitored the level and the source of ROS in our cell system by using specific fluorescence probes, showing increases in ROS in/around mitochondria and accumulation of superoxide anion into DS-HSF mitochondria, as compared with normal fibroblasts (Figures 4A–4C). The results of the present study strongly indicate mitochondria as the major cellular site responsible for the overproduction of ROS in DS-HSFs. The mitochondrial origin of ROS in DS fibroblasts is further substantiated by the fact that direct treatment of fibroblasts with db-cAMP caused stimulation of NADH:ubiquinone oxidoreductase activity (Figure 4D) and prevented ROS accumulation (Figures 4E and 4F); the level of ROS appeared to be inversely related to cAMP-dependent activation of complex I. Thus our results substantiate the view that complex I is the



major source of superoxide anion and ROS derivatives in human fibroblasts [18,19,43] and show, for the first time, that in DS cells ROS imbalance correlates with a deficit in the complex I activity due to its reduced cAMP-dependent phosphorylation.

Since in the present study we used foetal fibroblasts, we argue that all modifications of DS cells described above are inherent features of these cells themselves and are already established before birth.

In conclusion, the remarks emerging from the present study and from our previous results [16] provide new evidence for a critical role of mitochondrial dysfunction in the pathogenesis of DS: indeed, DS cells show a reduction in a series of mitochondrial functions such as respiratory capacity, membrane potential generation and ATP synthesis as a result of a deficit in OXPHOS machinery involving mitochondrial complex I, ATP synthase, ADP/ATP translocator and adenylate kinase activities, which might be ascribed to post-translational activities which appear to be modulated by cAMP-dependent events involving PKA. The defective complex I is involved in the production of ROS by mitochondria and therefore in oxidative stress associated with DS. The present observation may provide useful input to help in designing a new therapeutic strategy for some of the pathological conditions associated with DS.

#### AUTHOR CONTRIBUTION

Daniela Valenti and Rosa Anna Vacca designed this research, performed the experimental work, analysed the data, wrote the manuscript and co-ordinated the study. Rosa Anna Vacca conceived the study. Gabriella Manente performed the experiments of complex I phosphorylation; Laura Moro participated in the analysis and discussion of data concerning the PKA-dependent signalling pathway and performed a critical revision of the manuscript. Ersilia Marra participated in the co-ordination of the study and performed a critical revision of the manuscript.

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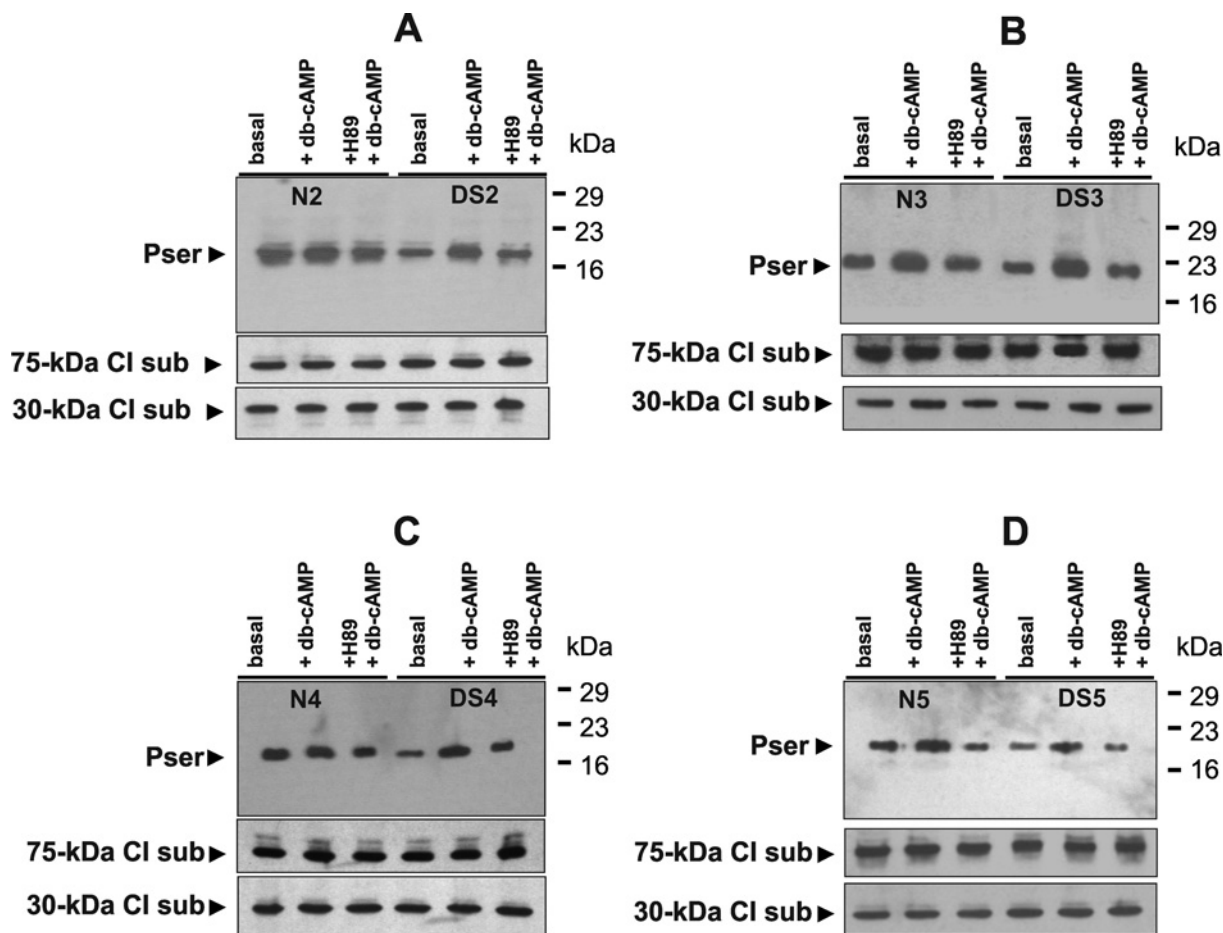
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SUPPLEMENTARY ONLINE DATA

**Deficit of complex I activity in human skin fibroblasts with chromosome 21 trisomy and overproduction of reactive oxygen species by mitochondria: involvement of the cAMP/PKA signalling pathway**

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**Figure S1 Immunodetection of phosphoserine containing proteins in immunoprecipitate of complex I**

N2- and DS2 (A), N3- and DS3 (B), N4- and DS4 (C) and N5- and DS5 (D) fibroblast pair samples were subjected to isolation of mitochondrial membranes and complex I was immunoprecipitated with the antibody against the 30 kDa subunit of complex I. Immunoprecipitated proteins were subjected to SDS/PAGE and phosphoserine-containing proteins were detected by immunoblotting with a specific anti-phosphoserine (Pser) antibody. Protein levels of complex I subunits of 75 kDa (75-kDa CI sub) and 30- kDa (30-kDa CI sub) were also measured in immunoprecipitated samples to normalize the immunoprecipitated level of complex I. The molecular mass in kDa is indicated on the right-hand side.

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