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

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Short title: Complex I deficit and ROS overproduction in Down's syndrome

Synopsis

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 this research, we identified new molecular events involved in mitochondrial dysfunction which could play a role in DS pathogenesis. We analyzed mitochondrial respiratory chain function in DS-HSF (human foetal skin fibroblasts with chromosome 21 trisomy) and found a selective deficit in the catalytic efficiency of mitochondrial complex I. Complex I deficit was associated with a decrease in cAMP-dependent phosphorylation of 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-HSF to db-cAMP (dibutyl-cAMP), a membrane-permeable cAMP analog, 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, here we report a three-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, these results suggest that the drastic decrease in basal cAMP levels observed in DS-HSF participates to complex I deficit and overproduction of ROS by DS-HSF mitochondria.

Keywords: Down syndrome, mitochondria, mitochondrial complex I, cAMP levels, PKA-mediated phosphorylation, reactive oxygen species.

Abbreviations: AA, antimycin A; ASC, ascorbate; DCF, dichlorofluorescein; db-cAMP, dibutyl-cAMP; DS, Down's syndrome; DS-HSF, human foetal skin fibroblasts with trisomic karyotype; DS-HSF_{+dig}, digitonin-permeabilized human skin fibroblasts with trisomic karyotype; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazine; GLU, glutamate; HSF, human foetal skin fibroblasts; MAL, malate; mGDH, mitochondrial glutamate dehydrogenase; MYXO, myxothiazol; NAO, nonyl acridine orange; N-HSF, human foetal skin fibroblasts with normal karyotype; N-HSF_{+dig}, digitonin-permeabilized human skin fibroblasts with normal karyotype; O⁻, superoxide

anion; OXPHOS, oxidative phosphorylation; PKA, protein kinase A; ROT, rotenone; SOD1, superoxide dismutase 1; SUCC, succinate; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

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 (dibutyryl-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 [8], Alzheimer's [9] and Huntington's [10] diseases, as well as in aging [11]. Some evidence for complex I deficiency has also been described in Down's syndrome (DS), 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 analyzed alterations in mitochondrial respiratory chain complexes at a functional level in DS. Recently, we 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 occur 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 increased, as well as SOD1 (superoxide dismutase 1) expression 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 orthologs that lacks SOD1, shows oxidative stress and mitochondrial dysfunctions [24]. We hypothesized therefore 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, dysfunctions of these complexes could result in an increase in ROS production.

In the present study, using DS-HSF (human foetal skin fibroblasts with chromosome 21 trisomy) as a cellular model system, we investigated the activity of mitochondrial complex I as well 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-HSF, the effects of a permanent derivative of cAMP, db-cAMP, on cAMP-dependent PKA (protein kinase A), complex I phosphorylation and activity as well as ROS production in DS-HSF were also investigated.

We found that DS-HSF show a selective drastic reduction in the catalytic efficiency of the respiratory chain complex I, due to a decrease in its cAMP-dependent phosphorylation, rescued by

exposure of DS-HSF to db-cAMP. Measurements of cellular levels of ROS in DS-HSF revealed enhanced $O_2^{\cdot -}$ (superoxide anion) production by mitochondria abrogated by activation of complex I by db-cAMP, thus suggesting that complex I deficit may be involved in the overproduction of ROS by DS-HSF mitochondria.

EXPERIMENTAL

Cell culture

Five normal and five DS human foetal skin fibroblast 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% CO₂/95% air in RPMI 1640 medium (GIBCO/BRL) supplemented with 15% heat-inactivated fetal bovine serum (GIBCO/BRL), 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml). Cells were subjected to a 1:2 split every 6 days. Cell protein assay was carried out according to [26]. Comparison of the functional features of normal and DS fibroblasts was made with sub-confluent 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 HSF (human foetal skin fibroblasts)

HSF were trypsinized, washed with PBS and permeabilized with 0.01 % digitonin (w/v), as described in [16].

To measure the respiration rate in permeabilized HSF (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 KH₂PO₄/K₂HPO₄ (pH 7.4), 3 mM MgCl₂, 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) and ROT (rotenone; 3 µM) or ASC (ascorbate; 5 mM) plus TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine; 0.2 mM) and AA (antimycin; 2.5 µM) were added to the cells in the presence of the uncoupler FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazine; 1.25 µM) which allows the maximum activity of the respiratory chain [27].

Mitochondrial membrane potential ($\Delta\psi$) was measured by monitoring fluorescence changes at 520 nm excitation and 570 nm emission of the probe safranin O, essentially as in [28]. Permeabilized HSF (1 mg protein each) were incubated at 37°C in 2 ml of a standard medium consisting of 0.3 M sucrose, 10 mM KCl, 1 mM MgCl₂, 20 mM HEPES-TRIS (pH 7.2) plus 10 µM safranin O in the presence of either 5 mM GLU plus 5 mM MAL or 5 mM SUCC plus 3 µM ROT or 5 mM ASC plus 0.2 mM TMPD in the presence of 2.5 µM AA and 3 µM MYXO (myxothiazol).

Measurement of respiratory chain complex activities

Measurements of respiratory chain complex activities were carried out in mitochondrial membrane-enriched fractions from cultured HSF. Aliquots of trypsinized HSF were washed with ice-cold phosphate-buffered saline, 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 1mg/ml BSA, and exposed to ultrasound energy for

15 s at 0°C. The ultrasound-treated cells were centrifuged (10 min at 600 *g* and 4°C). The supernatant was centrifuged again (10 min at 14000 *g* and 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 in [29], which rely on the sequential addition of reagents to measure the activities of: i) NADH:ubiquinone oxidoreductase (Complex I) ii) succinate:ubiquinone oxidoreductase (Complex II); iii) cytochrome *c* oxidase (Complex IV) followed by cytochrome *c* oxidoreductase (Complex III).

Immunoblotting analysis

Cell extracts (0.05 mg protein) were loaded on to a SDS polyacrylamide gel (10%), separated and transferred on to a PVDF membrane which was probed with primary antibodies against the following proteins: 20-kDa complex I subunit, 30-kDa complex II subunit, core 2 complex III, COX 1 complex IV and complex V α subunit of F_1 ATPase (MitoProfile OXPOS cocktail 1:250, MitoSciences); 30-kDa complex I subunit (*NDUFS3*, 1:250 dilution; Santa Cruz); *ND1* complex I subunit (1:200, Santa Cruz); 75-kDa complex I subunit (*NDUFS1*, 1:250 dilution; Santa Cruz); porin (1:1000, MitoSciences) and PKA (protein kinase A) α cat (B5) subunit (1: 1:250 dilution; Santa Cruz). Immunoblot analysis was performed essentially as described in [30] using 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 β -actin protein using anti- β -actin antibody (1:500, 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 β -actin in each lane on the same filter.

Measurements of cellular cAMP levels and PKA activity

Intracellular cAMP levels were measured in cell extracts with 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), as recommended by the manufacturer. PKA activity was 5-10-fold stimulated by incubating cell extract with 5 μ M db-cAMP for 5 min at 37°C.

Immunoprecipitation and immunodetection of phosphoserine-containing proteins

For immunoprecipitation of complex I, 500 μ g of mitochondrial membrane-enriched fraction, prepared as described above for measurements of respiratory chain complex activities, were incubated for 2 hours at 4 °C with 4 μ g of antibodies against 30-kDa complex I subunit in the presence of 20 μ l protein A-Sepharose beads as described in [31]. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to a PVDF membrane and probed with anti-phosphoserine antibody (1:1000, Santa Cruz) and immunoblot analysis was performed as described above. The membrane was stripped and reprobed with both anti-complex I 75-kDa subunit antibody (1:250, Santa Cruz) and anti-complex I 30-kDa subunit antibody (1:250, Santa Cruz) to normalize the immunoprecipitated level of complex I.

Intracellular ROS detection

Intracellular ROS production by cultured HSF was evaluated using the following probes: MitoSOXTM (3 μ M, Molecular Probes) selective mitochondria-targeted specifically for superoxide anion [32]; DCFH-DA (2',7'-dichlorofluorescein diacetate; 5 μ M; Sigma Aldrich) a non-fluorescent dye which is hydrolyzed in cells and reacts with multiple types of ROS, mainly H₂O₂, to

give the fluorescent product DCF (dichlorofluorescein) [33]. In addition MitoTracker Deep Red (5 μ M) and NAO (nonyl acridine orange; 1 μ 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-bottom dishes and incubated 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 60x objective). The green fluorescence of oxidized DCF and NAO was analyzed by exciting the sample with a Diode 405 laser (λ_{ex} 488 nm); the red fluorescence of MitoSOX and MitoTracker Deep Red was analyzed by exciting the sample with a HeNe laser 543 (λ_{ex} 543 nm) and HeNe laser 633 (λ_{ex} 633 nm), respectively.

Quantitative analysis of ROS production was performed by means of an LS50 Perkin Elmer spectrofluorimeter. Cells cultured for 2 days in 10-cm diameter dishes were incubated with either DCFH-DA or MitoSOX, trypsinized, resuspended in 0.5 ml of PBS and the fluorescence of the samples was measured at λ_{ex} 488 nm and λ_{em} 520 nm for DCF and λ_{ex} 510 nm and λ_{em} 580 nm for MitoSOX. 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-HSF: 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_{+dig}) with respective normal-permeabilized fibroblasts (N-HSF_{+dig}).

We first checked whether changes in respiratory capacity can occur in DS-HSF. To do this, we measured the respiratory complex-dependent substrate oxidation inside DS-HSF_{+dig} compared with N-HSF_{+dig} 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_{+dig} ($40 \pm 5\%$) compared to N-HSF_{+dig}, as shown in a series of experiments carried out comparing five different DS cell lines with the respective normal cells, 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 to those measured in N-HSF_{+dig} (Figure 1A).

To determine whether the decrease in complex I-dependent respiration rate in DS-HSF_{+dig} reduces mitochondrial energization, we measured the mitochondrial membrane potential ($\Delta\psi$), by using safranin O as a $\Delta\psi$ probe (see the Experimental section). Consistently with results obtained previously, the rate of $\Delta\psi$ generation measured when complex I substrates GLU and MAL were added to DS-HSF_{+dig} was significantly lower ($40 \pm 5\%$) than in N-HSF_{+dig}, 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 to normal cells.

To verify whether direct and specific alterations of complex I occurred in DS-HSF, 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-HSF and N-HSF. Complex I (Figure 1C), but not complexes II (Figure 1D), III (Figure 1E) and IV (Figure 1F), exhibited a significant reduction in specific activity ($40 \pm 2\%$) in DS-HSF with respect to N-HSF.

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

To give some insight into the molecular basis of complex I deficit, and given that a decrease in protein levels of the 30-kDa 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 *NDUFS3* 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 (Figure 2A-D), 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 α subunit which we had previously found to be selectively decreased [16]. However, on comparing the ratios OXPHOS protein/porin for DS-HSF and N-HSF, 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-HSF ($P > 0.02$), as measured by real-time RT-PCR (not shown), thus suggesting that a post-transcriptional and/or post-translational regulation of mitochondrial complex I occurs in DS-HSF.

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-HSF we analyzed the involvement of 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 (approx. 1.5-fold less as compared with N-HSF) (Figure 3A). Conversely, no changes in PKA protein levels were found in DS-HSF 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 (approx. four-fold less as compared with N-HSF) (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 short-time incubation with db-cAMP, a membrane-permeable cAMP analog 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 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 anti-phosphoserine antibody (Figure 3D). The only protein band detected with anti-phosphoserine antibody ran around 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 to 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 75-kDa and 30-kDa complex I subunits. Four other representative immunoblots, performed with the other pairs of DS- and N-HSF, are reported as Supplementary data. 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 (Figure 3D and E); H89, when incubated with the cells before adding db-cAMP, prevented the cAMP-dependent serine phosphorylation of the complex in both N 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 catalytical 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-HSF.

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.

We first checked whether changes in ROS production occurred in DS-HSF. 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 probe (Figure 4B) which accumulates in mitochondria and reacts specifically with $O^{\cdot -}$ [32] revealed higher production of this radical molecule from mitochondria of DS cells than normal cells. Co-staining with the corresponding green MitoTracker NAO confirms that MitoSOX was retained in mitochondria and detected localized rises in $O^{\cdot -}$ levels in DS cells. ROS levels were approximately 3-fold higher in DS samples compared to N-HSF, as revealed by fluorimetric measurements (Figure 4C).

Interestingly, exposure of DS-HSF to db-cAMP which activates PKA-dependent phosphorylation of complex I (Figure 3E), completely prevented the deficit of complex I activity in DS (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).

Consistently with the involvement of 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 to 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 deficit of the complex activity are involved in the overproduction of ROS in DS cells.

DISCUSSION

We report here a detailed functional study on the mitochondrial respiratory chain in DS cells which sheds light on how mitochondrial dysfunctions 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 cAMP/PKA signaling 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-HSF 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 malate/glutamate 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 glutamate and malate; 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 (Figure 1C-F). 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-HSF.

The deficit of complex I activity in DS fibroblasts is particularly interesting because it occurred even though certain complex I subunits were not only not differentially expressed at the gene level (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-HSF as a compensative mechanism partially offsetting the deficit of mitochondrial functions in DS cells [16].

Here 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, 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-HSF (Figure 3D and E). In agreement with the decrease in both PKA activity and cellular cAMP levels (Figure 3A and C) 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-HSF in a manner comparable to that observed in normal cells (Figure 3A and E); 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-HSF (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 adenylyl cyclase [38, 39]. Whether this occurs also in DS-HSF is under investigation. However, we cannot rule out the possibility that alternative mechanism(s) could be accounted for reduced complex I phosphorylation and activity, such alterations of A kinase anchor proteins (AKAPs), which localize PKA to particular cellular compartments including mitochondria, enhancing the efficiency of cAMP signal-transducing pathways [5, 40]. Further studies are required to verified such a hypothesis.

These 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 the mitochondrial proteins involved in OXPHOS, determined a mitochondrial energy deficit in DS cells that 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 over-expression 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 anti-oxidant 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 (Figure 4A-C). Our data strongly indicate mitochondria as the major cellular site responsible for the overproduction of ROS in DS-HSF. 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 (Figure 4E and F); the level of ROS appeared to be inversely related to cAMP-dependent activation of complex I. Thus, our data 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 this study we used foetal fibroblasts, we argue that all modifications of DS cells described above are inherent features of these cells per se and already established before birth.

In conclusion, the remarks emerging from these and from our previous results 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-dependending 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, analyzed 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 PKA-dependent signaling 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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Figure legends

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

(A) N-HSF_{+dig} and DS-HSF_{+dig} (1 mg protein each) 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 (glutamate) plus 5 mM MAL (malate) or 5 mM SUCC (succinate) and 3 μM ROT (rotenone) or 0.5 mM ASC (ascorbate) plus 0.2 mM TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine) were added in the presence of 1.25 μM FCCP. Data are reported as the means (± SEM) of three different experiments carried out on five normal and the corresponding five trisomic cell lines. (B) N-HSF_{+dig} and DS-HSF_{+dig} (1 mg of protein each) were incubated at 37°C in 1.5 ml of standard medium plus 10 μM safranin and membrane potential generation measured fluorimetrically. Where indicated, either, 5 mM GLU plus 5 mM MAL or 5 mM SUCC and 3 μM ROT or 5 mM ASC plus 0.2 mM TMPD were added. Data are reported as the mean values (± SEM) of experiments performed on five normal and five trisomic cell lines, expressed as percentage of normal cells. The activities of CI (complex I, NADH:ubiquinone oxidoreductase) (C), CII (complex II, succinate:ubiquinone oxidoreductase) (D), CIII (complex III, cytochrome *c* reductase) (E), and CIV (complex IV, cytochrome *c* oxidase) (F) were measured spectrophotometrically at 37°C in 1 ml of respiratory medium on mitochondrial membrane-enriched fractions (0.1 mg protein) as described in the Experimental section and reported as the mean values (± SEM) from three different experiments on five DS and N samples. (G) Lineweaver-Burk analysis of complex I-mediated NADH:ubiquinone oxidoreductase activity in N-HSF and DS-HSF. NADH was added at the indicated concentrations to mitochondrial membrane-enriched fractions of N-HSF and DS-HSF, respectively, and the inverse of the rate of NADH oxidation was plotted versus the inverse of NADH concentration. Significant differences between N and DS samples are indicated with asterisks (** = $P < 0.001$).

Figure 2 Protein levels of respiratory chain complexes in DS-HSF

Protein levels of 20-kDa *NDUFB8* complex I subunit (A), 30-kDa *NDUFS3* complex I subunit (B) and the mitochondrial-encoded 20-kDa ND1 complex I subunit (C) compared with 30-kDa subunit of complex II, core 2 protein of complex III, COX I of complex IV and α subunit of F₁ ATPase (A), were measured by immunoblotting analysis of cell extracts (0.05 mg protein) of DS- and N-HSF using the respective antibodies; protein levels of porin (D) and β-actin (A-D) as mitochondrial and cytosolic protein markers, respectively, were also measured. For each protein, values are reported, after densitometric analysis and normalization with β-actin, as the means (± SEM) of 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 N and DS samples are indicated with asterisks (* = $P < 0.01$).

Figure 3 cAMP-dependent phosphorylation of complex I in DS-HSF

(A) PKA activity was measured in cell lysate as described in Experimental section and reported as relative PKA activity. Where indicated, N- and DS-HSF were incubated for 2 h in the absence (basal) or in the presence of 100 μM db-cAMP (db-cAMP); when present, cells were pre-incubated 2h with H89 (50 μM) before adding db-cAMP (H89+db-cAMP). (B) Protein levels of PKAαcat (B-5) were measured by immunoblot analysis of cell extracts (0.05 mg protein) of DS- and N-HSF using the respective antibody; protein levels of β-actin, as cytosolic protein marker, were also measured. Values are reported, after densitometric analysis and normalization with β-actin, as the

means (\pm SEM) of experiments performed on five normal and five trisomic cell lines. The data are expressed as a percentage of those in normal cells. **(C)** Cellular basal levels of cAMP were determined in N- and DS-HSF as described in Experimental section. **(D)** Immunodetection of phosphoserine containing proteins in 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 polyacrilamide gel electrophoresis and phosphoserine-containing proteins were detected by immunoblotting with 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. **(E)** Values of serine phosphorylation of complex I are reported, after densitometric analysis and normalization with both 75-kDa and 30-kDa subunits of complex I, as a percentage of those in normal untreated cells. Significant differences between N and DS samples are indicated with asterisks (** = $P < 0.001$).

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 sub-cellular localization in N- and DS- fibroblasts. Cells were stained with 5 μ M DCF-DA **(A)** and 3 μ M MitoSOX **(B)** while fluorescence of DCF and of the mitochondria-selective $O_2^{\cdot -}$ probe MitoSOX was visualized in live fibroblasts by laser scanning confocal microscopy, as detailed under Experimental. When DCF-DA was present, cells were co-stained with the MitoTracker Deep Red (5 μ M) **(A)**; when MitoSOX was present, cells were co-stained with the MitoTracker Nonyl acridine orange (NAO) (1 μ M) **(B)**. Images are superimpositions of 10 confocal z-planes and represent typical examples. Bars, 50 μ m. **(C)** Quantification of intracellular ROS was reported as the means (\pm SEM) of DCF and MitoSOX fluorescence arbitrary units (A.U.) measured by fluorimetric analysis in three different experiments on five N and five DS cell lines. Significant differences between N 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 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 μ M db-cAMP (db-cAMP); when present, cells were incubated 2h with H89 (50 μ M) before adding db-cAMP (H89+db-cAMP). Significant differences between treated and non-treated DS samples are indicated with asterisks (** = $P < 0.001$). All data are reported as the means (\pm SEM) of 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 panels A and B. **(F)** Quantification of intracellular ROS in DS-HSF was reported as the means (\pm SEM) of DCF and MitoSOX 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 (** = $P < 0.001$).

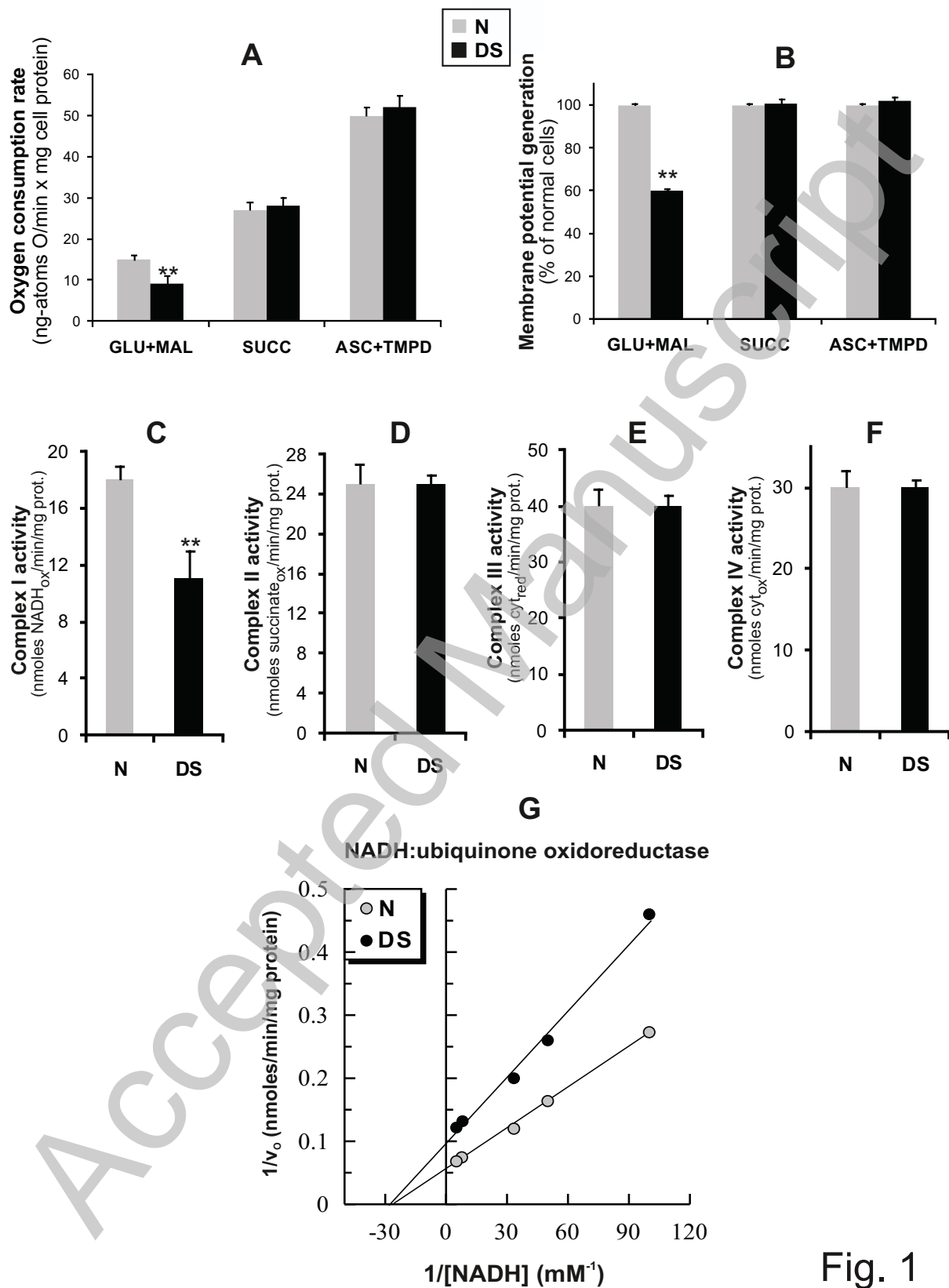


Fig. 1

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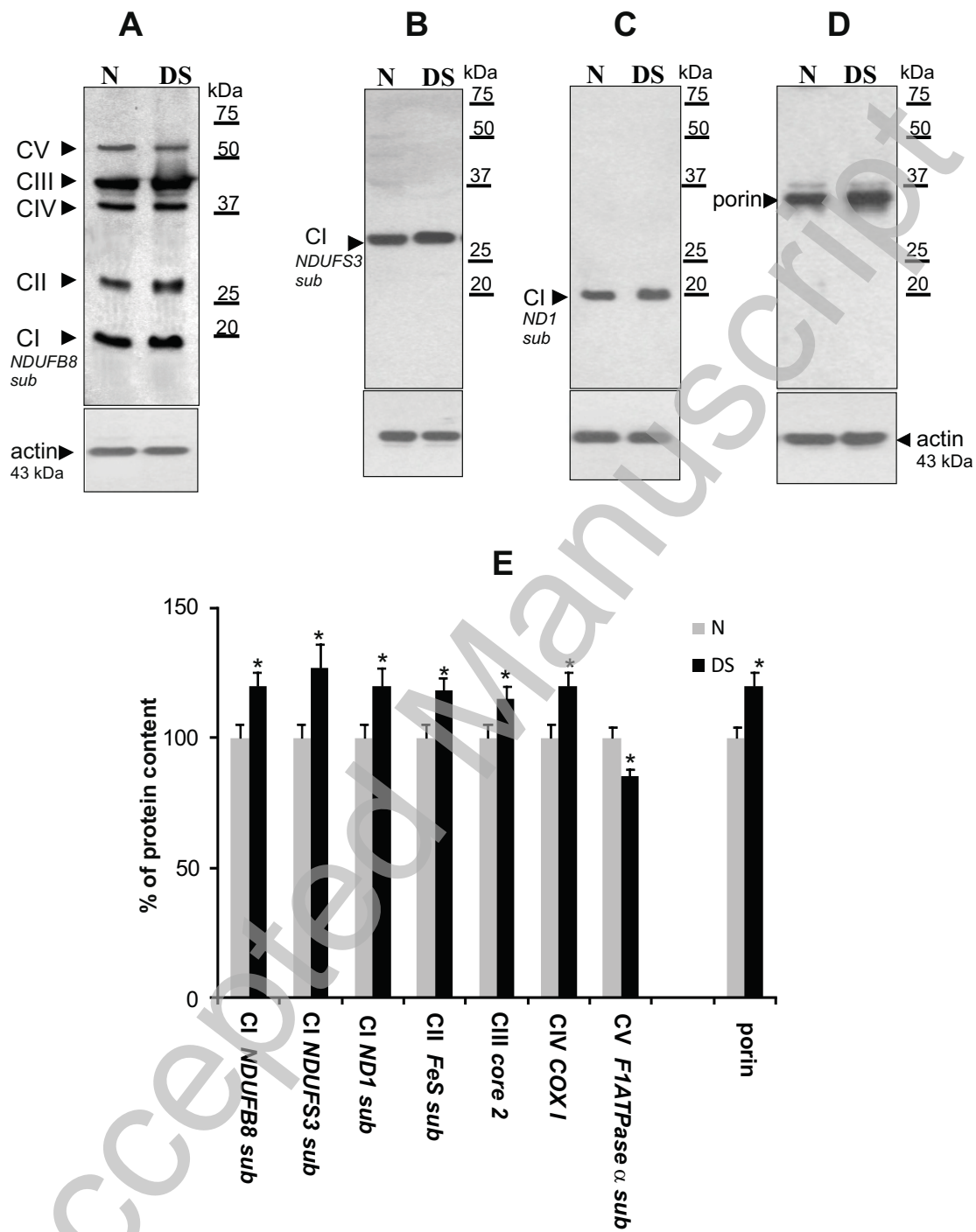


Fig. 2

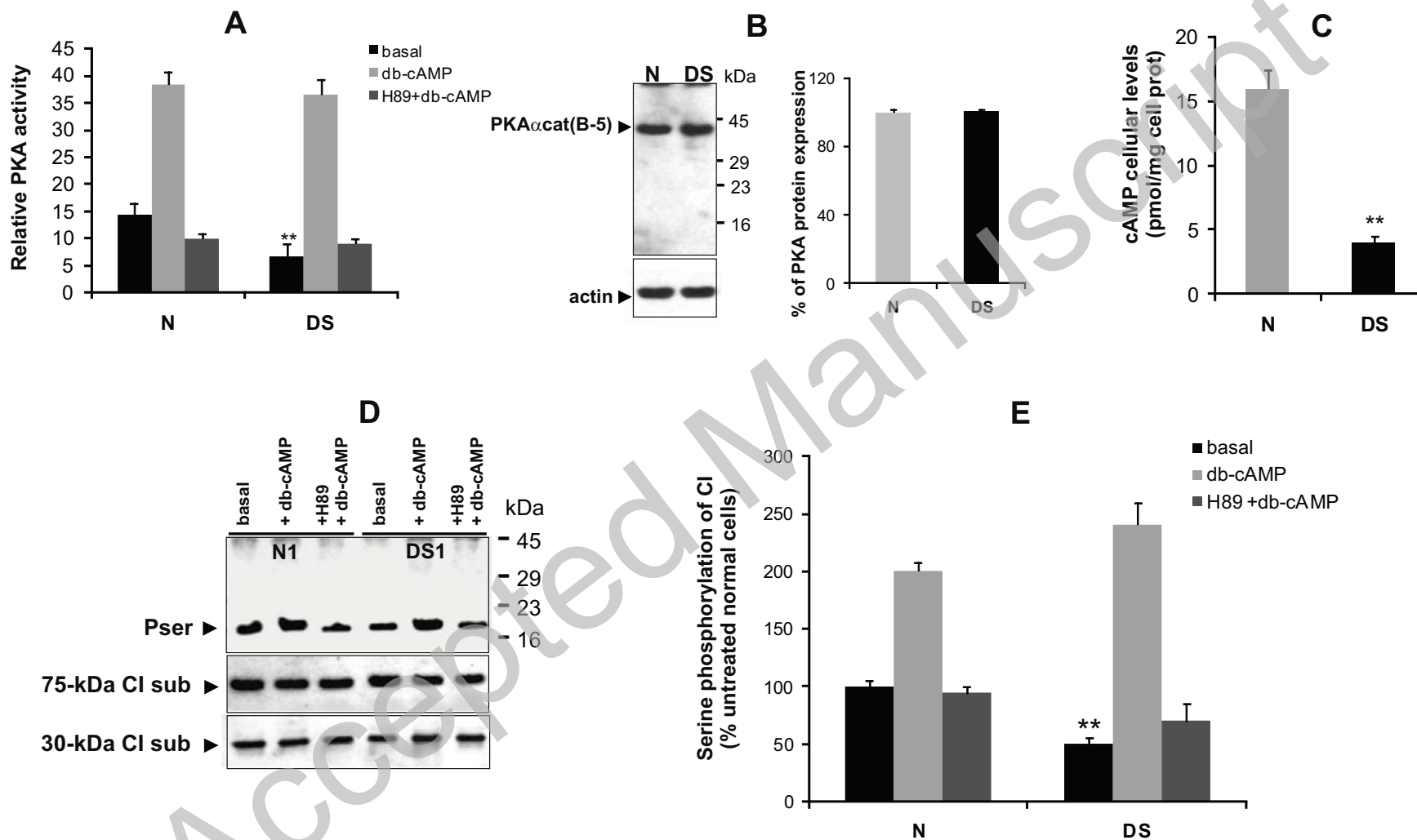


Fig. 3

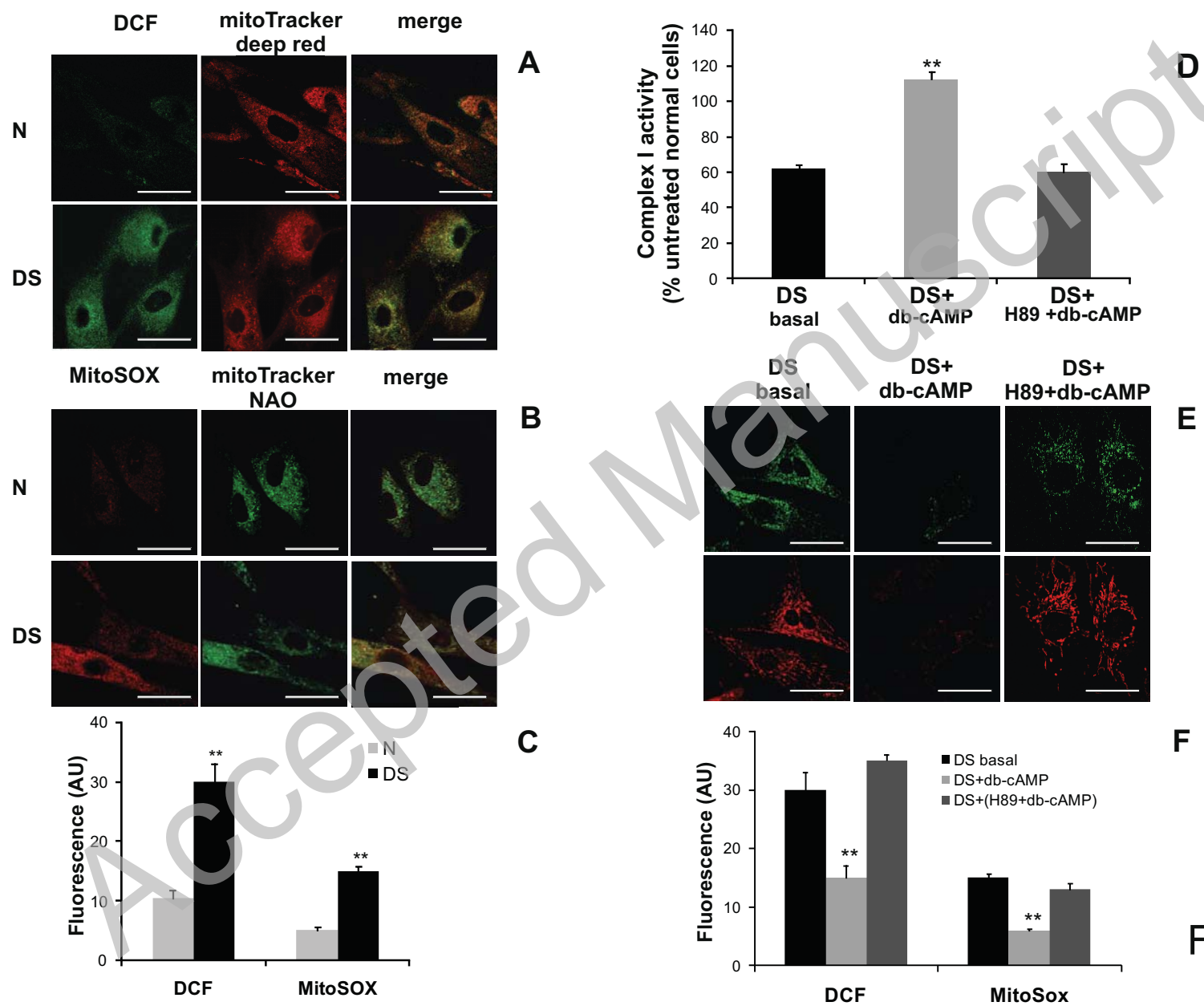


Fig. 4