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# Preservation of NADH ubiquinone-oxidoreductase activity by Src kinase-mediated phosphorylation of NDUFB10

Etienne Hebert-Chatelain [a,*,1], Caroline Jose [a,1], Nicolas Gutierrez Cortes [a], Jean-William Dupuy [b], Christophe Rocher [a], Jeanne Dachary-Prigent [a], Thierry Letellier [a]

[a] INSERM-U688 Physiopathologie mitochondriale, Université de Bordeaux, 146 rue Léo Saignat, Bordeaux 33076, France
[b] Pôle protéomique du Centre de Génomique Fonctionnelle, Université de Bordeaux, 146 rue Léo Saignat, Bordeaux 33076, France

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ABSTRACT

The tyrosine kinase Src is upregulated in several cancer cells. In such cells, there is a metabolic reprogramming elevating aerobic glycolysis that seems partly dependent on Src activation. Src kinase was recently shown to be targeted to mitochondria where it modulates mitochondrial bioenergetics in non-proliferative tissues and cells. The main goal of our study was to determine if increased Src kinase activity could also influence mitochondrial metabolism in cancer cells (143B and DU145 cells). We have shown that 143B and DU145 cells produce most of the ATP through glycolysis but also that the inhibition of OXPHOS led to a significant decrease in proliferation which was not due to a decrease in the total ATP levels. These results indicate that a more important role for mitochondria in cancer cells could be ensuring mitochondrial functions other than ATP production. This study is the first to show a putative influence of intramitochondrial Src kinase on oxidative phosphorylation in cancer cells. Indeed, we have shown that Src kinase inhibition led to a decrease in mitochondrial respiration via a specific decrease in complex I activities (NADH–ubiquinone oxidoreductase). This decrease is associated with a lower phosphorylation of the complex I subunit NDUFB10. These results suggest that the preservation of complex I function by mitochondrial Src kinase could be important in the development of the overall phenotype of cancer.



## 1. Introduction

While differentiated cells rely mainly on oxidative phosphorylation (OXPHOS) to produce ATP, cancer cells are generally seen as highly glycolytic cells because they produce a large amount of lactate regardless of oxygen availability [1,2]. Otto Warburg initially explained this high aerobic glycolysis by an OXPHOS defect [2,3]. It is now accepted that cancer cells may reprogram their metabolism in this way to sustain a high proliferative phenotype. For example, the stabilization of hypoxia-inducible factor 1-α (HIF1-α) induces a switch of energy metabolism from mitochondrial to glycolytic, including the up-regulation of glucose transporters and glycolytic enzymes as well as the inhibition of pyruvate dehydrogenase [4,5]. However, even if the mitochondria produce less ATP [6], they are still essential since most lipid, protein and nucleic acid biosynthetic machineries use precursors derived from the TCA cycle in proliferative cells [7]. Therefore, mitochondrial regulation pathways could be devoted to preserving mitochondrial function without relying too much on OXPHOS.

The tyrosine kinase Src, which is overexpressed or up-regulated in several cancer cells, is involved in several physiological processes such as increased cell survival, proliferation and metastatic properties [8–10]. Src activation can also induce the remodeling of energetic metabolism via the stabilization of HIF1-α and increased activities of several glycolytic enzymes [11,12]. On the other hand, recent papers have shown that Src kinase could be targeted to mitochondria where it modulates OXPHOS. Indeed, Src kinase phosphorylates cytochrome *c* oxidase (complex IV) subunit II in osteoclasts, resulting in the activation of the enzyme [13,14]. The Src and Lck-mediated phosphorylation of Y190 and Y194 in adenine nucleotide translocase was shown to be involved in its carrier function and in the respiration of yeast [10]. The enzymatic activity of ATP synthase (complex V) is decreased by Src-dependent phosphorylation, while the activity of complex IV is increased with Src activation in rat brain mitochondria [15,16]. However, the possible involvement of Src kinase in the regulation of mitochondrial metabolism in cancer cells still needs to be established.

Since Src kinase is highly activated in several cancer cells, the main goal of our study was to determine if Src kinase activity could influence mitochondrial metabolism in such cells. First, we showed that 143B and DU145 cells produce most of the ATP through glycolysis but also that the inhibition of OXPHOS led to a significant decrease in proliferation that was not due to a decrease in the total ATP level.

* Corresponding author. Tel.: +33 05 57 57 48 92; fax: +33 05 57 57 16 12.
*E-mail address:* etienne.hebert-chatelain@inserm.fr (E. Hebert-Chatelain).
[1] These authors share authorship.

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We then demonstrated an intramitochondrial localization of Src kinase in the 143B and DU145 cell lines. By using two different specific Src inhibitors, PP2 and SU6656, we showed that the inhibition of Src kinase leads to a specific decrease in mitochondrial respiration and complex I enzymatic activities, probably via the reduced phosphorylation of the complex I subunit NDUFB10. Our results suggest that Src kinase may be involved in the maintenance of mitochondrial functions that are important for the proliferative properties of cancer cells other than energy production.

## 2. Materials and methods

### 2.1. Chemicals

The mouse monoclonal antibody to phosphotyrosine (PY20), the mouse monoclonal antibody to c-Src, the mouse monoclonal antibody to LAMP-2, the rabbit polyclonal antibody to calregulin and the mouse monoclonal antibody to Bcl-2 were bought from Santa Cruz Biotechnology. Antibodies to phosphorylated Src species (PY419-Src) were bought from Cell Signaling.

The Src kinase inhibitors PP2 (4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine) and SU6656 were from Calbiochem. All antibodies against OXPHOS complexes (except NDUFB10 bought from Abcam) were bought from Mitosciences. Protease and phosphatase cocktail inhibitors were bought from Roche. All other chemicals were from Sigma.

### 2.2. Cell culture

The human osteosarcoma cell line 143B TK- and the human prostate cancer cell line DU145 were maintained in culture with high glucose ($4.5\ g\ l^{-1}$) DMEM (Dulbecco's modified Eagle's medium) supplemented with 2 mM glutamine, 1 mM pyruvate and 10% (v/v) fetal bovine serum. The 143B and DU145 cells were also grown in galactose medium, which consisted of DMEM without glucose, supplemented with 10 mM galactose, 2 mM glutamine, 1 mM sodium pyruvate and 10% fetal bovine serum. The cells grown in glucose DMEM will be referred to as 143B glu and DU145 glu whereas the cells grown in galactose will be referred to as 143B gal and DU145 gal. All of the cells were maintained in their respective medium for at least 5 passages before analysis. All of the cells were kept in a 5% $CO_2$ atmosphere at 37 °C and the medium was renewed every 2 to 3 days. Analysis and cell harvesting were always performed when confluency was about 80–90%.

### 2.3. Isolation of mitochondria

The cells were harvested, resuspended in isolation buffer (250 mM sucrose, 1 mM EDTA, 5 mM HEPES, pH 7.4) containing protease and phosphatase inhibitor cocktails and disrupted with 25 strokes using a 25-gage syringe. The cell debris and nuclei were removed by centrifugation at 400 *g* for 5 min (4 °C). The supernatant was kept and then centrifuged at 10,000 *g* for 10 min (4 °C). The pellet was then resuspended and the centrifugation cycle was repeated. Mitochondria were obtained from the last pellet. Cytosolic contamination was evaluated by measuring the lactate dehydrogenase (LDH) activity in the total cell lysate (TCL) and mitochondrial fractions. The LDH activity measurements revealed poor cytosolic contamination as the LDH activity in the mitochondrial fractions represented only $1.42 \pm 0.55\%$ of the TCL LDH activity ($n = 8$). Moreover, the immunodetection of α-tubulin among the total cell lysate and mitochondrial fractions showed no signal for the cytosolic protein in the mitochondrial fractions (data not shown). These preliminary experiments clearly minimized the possibility of cytosolic Src contamination in the mitochondrial fractions.

### 2.4. Oxygraphic measurements

Mitochondrial oxygen consumption assays were performed using the high-resolution respirometry system Oxygraph-2k (OROBOROS INSTRUMENTS, Austria). Cell respiration was measured at $5 * 10^5$ cells $ml^{-1}$ according to volume-specific flux at 37 °C in 2 ml chambers at a stirring rate of 750 rpm. Firstly, three different states of endogenous respiration with intact cells were measured: (i) basal respiration representing the endogenous physiological coupled state, (ii) respiration with oligomycin ($2\ \mu g\ ml^{-1}$) representing the non-coupled resting respiration, and (iii) maximal uncoupled respiration induced by CCCP (0.5 μM steps with 2 μM final concentration) providing a measure of the maximal capacity of ETS under conditions of physiological substrate supply in the intact cell.

Respiration was also measured in cells permeabilized with digitonin ($0.3\ \mu g/10^6$ cells) in respiration buffer (75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM Tris–phosphate, 10 mM Tris/HCl, 50 mM EDTA, pH 7.4). The use of this respiration buffer lowered the digitonin concentrations in order to ensure sufficient permeabilization and decreased the possibility of a loss in the integrity of the mitochondrial membrane. The digitonin amount was selected in order to obtain a maximal increase in respiration with the rotenone–succinate–ADP substrates without further stimulation by the addition of cytochrome *c* (data not shown). Notably, the basal $O_2$ consumption measured in the respiration buffer (143B glu: $34.56 \pm 15.06$ pmol $O_2\ s^{-1}\ 10^{-6}$ cells; DU145 glu: $57.22 \pm 30.96$ pmol $O_2\ s^{-1}\ 10^{-6}$ cells; 143B gal: $25.53 \pm 9.37$ pmol $O_2\ s^{-1}\ 10^{-6}$ cells; DU145 gal: $65.89 \pm 11.62$ pmol $O_2\ s^{-1}\ 10^{-6}$ cells) was not different from the respiration rate measured in DMEM ($p > 0.05$). Complex I-driven state III respiration (St3 PMD) was measured with pyruvate (5 mM), malate (2 mM) and ADP (5 mM). Complex II-driven state III respiration (St3 SD) was measured with rotenone (0.5 μM), succinate (10 mM) and ADP (5 mM).

### 2.5. Identification of the Src target proteins in complex I

Mitochondria were incubated with ATP without or with PP2 (20 μM), as in [15–17]. The treated samples were incubated overnight with complex I-agarose beads conjugated (Mitosciences, OR) then eluted with glycine buffer and the samples were finally processed for western blotting, as in [18]. Phosphotyrosines were then immunodetected using the PY20 antibody. The tyrosine-phosphorylated complex I subunit was identified by determining the peptides present in the phosphotyrosine signal. The spot of interest was digested by trypsin as indicated in [18]. The peptides were further analyzed by nano-liquid chromatography coupled to a MS/MS LTQ-Orbitrap XL mass spectrometer, as detailed in [16].

### 2.6. Measurements of mitochondrial membrane potential and ROS levels

After treatment, the cells were harvested. Then $5 * 10^5$ cells were incubated for 10 min at 37 °C with 50 nM tetramethylrhodamine methyl ester perchlorate (TMRM), 50 nM CM-$H_2$DCFDA or 50 nM MitoSox (Invitrogen, USA). The fluorescence of each probe was read as per the manufacturer's recommendation on a spectrofluorometer (SAFAS).

### 2.7. Gel electrophoresis and western blotting

The samples were subjected to SDS-PAGE (10 or 12.5%) and the separated proteins were transferred onto a nitrocellulose or PVDF membranes as already described in [17]. Primary antibodies were revealed with a horseradish peroxidase-conjugated $F(ab')_2$ fragment of anti-mouse, anti-rabbit or anti-goat IgG, $F(ab')_2$ fragment-specific (Jackson ImmunoResearch Laboratories, West Grove, PA). The blots were visualized using enhanced chemiluminescence (ECL) Plus from

Amersham. Labels were quantified by densitometric analysis using the Image J (NIH) software.

### 2.8. Statistical analysis

A Student's t-test was used to compare the means with the control values using the Excel software (12.2.8). Significance was assessed at the 0.05 (or lower) level for all tests.

## 3. Results

### 3.1. Mitochondria seem to play an important role in cancer cells in function(s) other than energy production

The glycolytic- and mitochondrial-dependent steady-state ATP levels were measured in the 143B and DU145 cells grown in glucose or galactose DMEM, as in [19]. When the 143B and DU145 cells were maintained in glucose DMEM, mitochondrial ATP production represented about 10% of the total ATP levels, while this mitochondrial ATP production was increased to approximately 50% for the 143B cells and 75% for the DU145 cells maintained in galactose medium (Fig. 1A).

The proliferation capacities of the 143B and DU145 cells grown in glucose DMEM supplemented with increasing doses of OXPHOS inhibitors during a 5 day period were then evaluated by trypan blue staining. As mitochondria produce as little as 10% of the total ATP in 143B and DU145 cells (Fig. 1A), we expected that the inhibition of mitochondrial ATP production would have little or no impact on proliferation. Interestingly, cells treated with rotenone or oligomycin for 5 days decreased proliferative capacity by more than 50% (Fig. 1B and C). As seen in Fig. 1C, the total ATP levels were not changed when OXPHOS was inhibited by rotenone for 5 days, showing that the reduction in proliferation was not due to a decline in ATP production. These first results suggest that a more important role for cancer-cell mitochondria could be to ensure other mitochondrial function(s) than preserving ATP production.

We then examined if Src kinase could help maintain such mitochondrial function(s) in cancer cells such as 143B and DU145.

### 3.2. A fraction of Src kinase locates to the mitochondria of 143B and DU145 cells

The fractionation of the DU145 cells on a sucrose gradient revealed that only Src kinase and mitochondria, as shown by the labeling of the 70 kDa subunit of succinate-coenzyme Q reductase (complex II), were present in fractions 6 and 7 (Fig. 2A), suggesting that this kinase could be located to the mitochondria. Src kinase was also present in fractions containing lysosome and endoplasmic reticulum. A similar Src kinase localization after a sucrose gradient was observed in the 143B cells (data not shown).

To examine the topological features of Src in mitochondria, we performed trypsin protection assays in the presence or absence of the detergent Triton X-100 using fractions 6 and 7 of both cell types. Indeed, any protein not imbedded within the organelle is available to trypsin cleavage while mitochondrial membranes protect intramitochondrial proteins from protease activity. As shown in Fig. 2B, the subunit I of Complex IV, which is located at the level of the mitochondrial inner membrane, is protected from trypsin digestion in the absence of detergent whereas Bcl 2, an antiapoptotic protein associated with the outer membrane, is completely degraded by

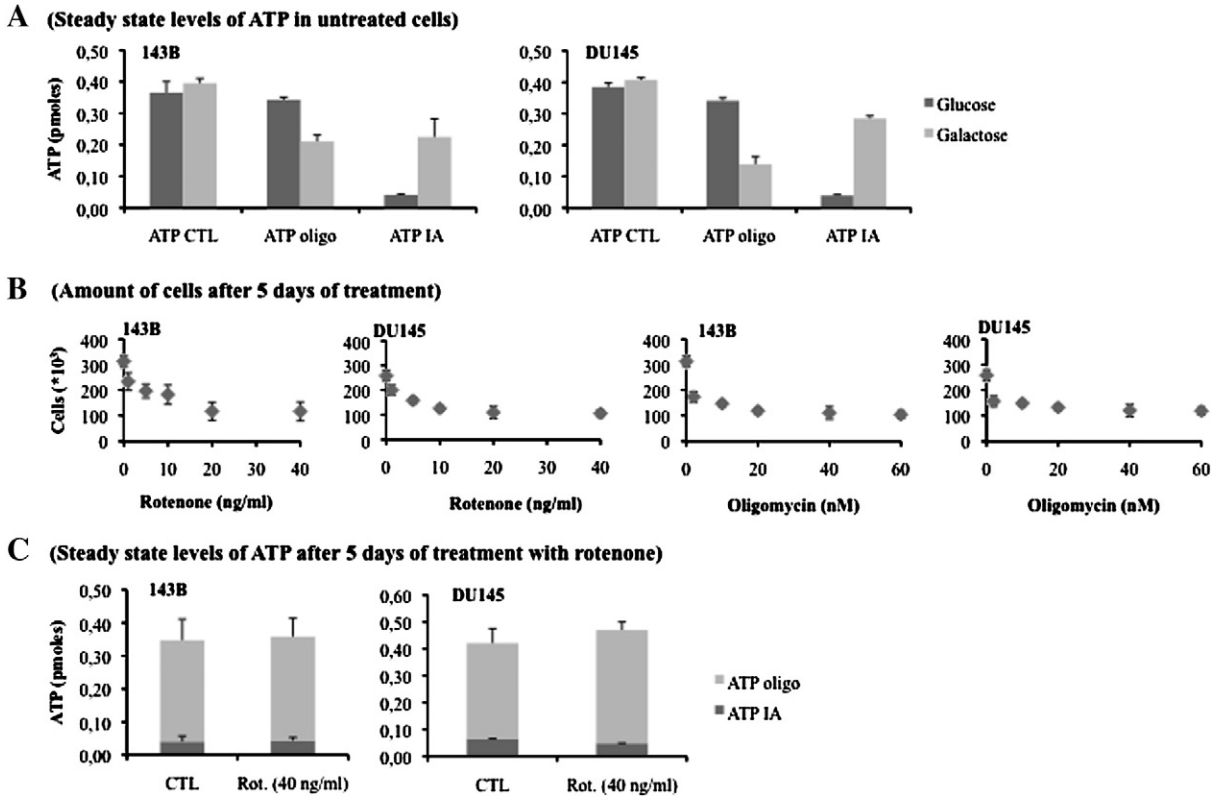


**Fig. 1.** Steady-state ATP level in the 143B and DU145 cells. (A) ATP steady-state levels (pmole) were measured with 100 000 cells incubated with vehicle, 2 μg ml$^{-1}$ oligomycin (indicative of glycolytic ATP production) or 500 μM iodoacetate (indicative of mitochondrial ATP production) for both cell lines in both culture conditions. Data (n = 5) are shown as means (S.D.). (B) Proliferation of 2000 cells grown in high glucose DMEM supplemented with different concentrations of rotenone (1, 2, 4, 10 and 40 ng ml$^{-1}$) and oligomycin (2, 10, 20, 40, 60 nM). Cells were counted by trypan blue staining after 5 days of treatment (n = 5). (C) Mitochondrial and glycolytic ATP levels were measured in 100 000 cells treated with high glucose DMEM supplemented with rotenone (40 ng/ml) for 5 days. No statistical difference was noted, according to a Student's t-test ($p > 0.05$). Data (n = 5) are shown as means (S.D.).

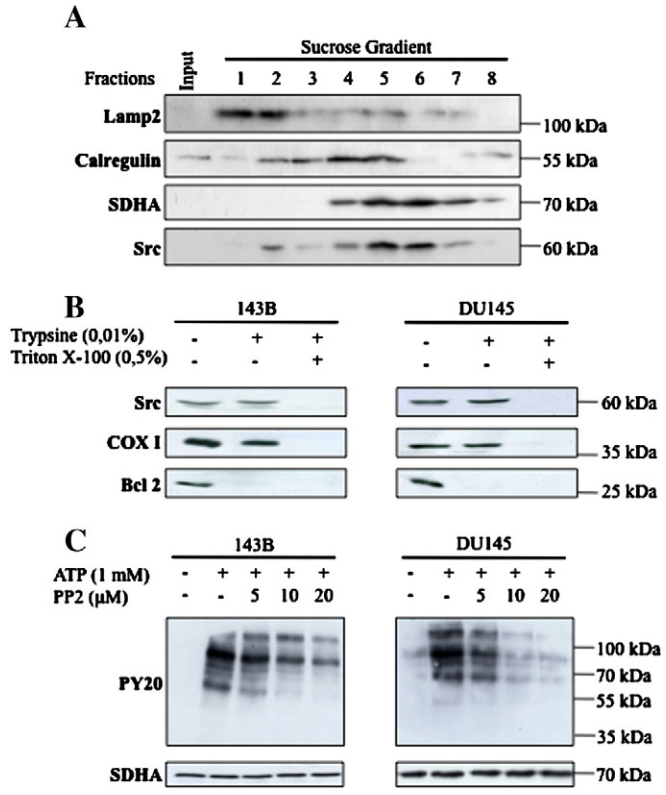


**Fig. 2.** Src kinase is located within the mitochondria of the 143B and DU145 cells. (A) Organelles were separated by ultracentrifugation (100000∗g for 4 h) on a discontinuous sucrose gradient (30–60%). Each fraction was then western blotted: lysosomes were immunodetected using the LAMP2 antibody, endoplasmic reticulum with the calregulin antibody, mitochondria with the succinate dehydrogenase A (SDHA) subunit antibody and Src kinase with the Src B12 antibody. (B) Fractions 6 and 7 (from panel A) were untreated or incubated with trypsin in the presence or absence of Triton X-100 and then western blotted for localization of Src kinase: COX I was used as marker of the intramitochondrial proteins while Bcl 2 was used as marker of the extramitochondrial proteins. (C) Isolated mitochondria were untreated or incubated with ATP, with DMSO (as a control) or with increasing concentrations of Src inhibitor PP2 and then western blotted. Phosphotyrosine was detected using the PY20 antibody while the SDHA antibody was used as a loading control.

trypsin regardless of the presence of Triton X-100. The resistance of Src to trypsin proteolysis in the absence of detergent suggests that Src kinase is located within the mitochondria of 143B and DU145 cells (Fig. 2B).

### 3.3. Impact of Src kinase activity on the mitochondrial bioenergetics of 143B and DU145 cells

The activity of Src kinase in the mitochondria was then studied by analyzing the tyrosine phosphorylation status of mitochondrial proteins in the presence of PP2, a specific Src inhibitor [16,17]. The incubation of mitochondria with ATP increased the level of tyrosine-phosphorylated proteins as compared to the non-treated mitochondria (Fig. 2C, lane 2). The intensity of the phosphotyrosine signal diminished with the increased inhibition of Src activity by PP2 (Fig. 2C, lanes 3–5), showing that Src kinase is an important player in the total mitochondrial tyrosine phosphorylation activity in 143B and DU145 cells.

To determine if the inhibition of Src kinase could influence mitochondrial metabolism, we incubated the 143B and DU145 cells with two specific and structurally non-related Src kinase inhibitors, PP2 [20] and SU6656 [21], for 4 h. The treatment of both cell lines with 5 μM PP2 or 10 μM SU6656 did not affect cell viability (143B control cells: 100.0 ± 11.0%; 143B PP2-treated cells: 98.5 ± 14.4%, $p > 0.05$; 143B SU6656-treated cells: 108.8 ± 6.2%, $p > 0.05$; DU145 control cells: 100.0 ± 9.5%; DU145 PP2-treated cells: 96.0 ± 28.8%, $p > 0.05$; DU145 SU6656-treated cells: 121.2 ± 18.9%, $p > 0.05$; $n = 5$) according to the neutral red assay [22].

After treatment with Src inhibitors, Src activation was evaluated by the immunodetection of phosphorylated Y419 residue of Src (PY419-Src). As shown in Fig. 3A, Src activation in mitochondria isolated from the 143B and DU145 cells was significantly reduced as compared to the untreated cells, without influencing the amount of total Src kinase.

Different steps of mitochondrial respiration (basal respiration, oligomycin respiration and uncoupled respiration) were measured in the intact cells. The basal and uncoupled respirations were significantly higher ($p < 0.05$) for the cells maintained in galactose DMEM as compared to the cells maintained in glucose medium (Table 1). Oligomycin respiration was similar ($p > 0.05$) between both culture conditions (Table 1). These results are consistent with the Crabtree effect observed in cancer cells: in high-glucose medium, a minimum amount of OXPHOS substrates enters into the mitochondria, explaining why mitochondrial ATP production and respiration are lower than in galactose medium. The Crabtree effect can be circumvented by using galactose DMEM. Galactose is not oxidized via glycolysis, thereby forcing cells to use glutamine and subsequent mitochondrial-dependent ATP production [22–24]. These results also demonstrate that mitochondria are not impaired per se in 143B and DU145 cells.

After the PP2 and SU6656 treatments, the basal and uncoupled respiration significantly decreased ($p < 0.05$) in each cell line (Fig. 3B and C). The same decrease in respiration was observed in both the glucose and galactose DMEM for the 143B and DU145 cells, indicating that Src influence on respiration does not depend on the bioenergetic phenotype. The reduction of $O_2$ consumption during uncoupled respiration suggests that Src kinase may modulate OXPHOS via one component of the ETS. To determine which ETS complexes were targeted by Src kinase, ADP-stimulated respiration was measured by using different respiratory substrates (pyruvate–malate or succinate) in permeabilized cells. Src inhibition induced a clear decrease in mitochondrial respiration when ETS was energized with complex I substrates but no effect was noted with the substrate of complex II (Fig. 4A and B). These results suggest that Src kinase mainly influences OXPHOS via complex I.

Complex I enzymatic activity was also strongly decreased in cells treated with the inhibitors (Fig. 4B). In contrast, no significant effect was observed for complex II, III and IV activities in the treated cells (Fig. 4B). Overall, these results show that Src kinase mainly regulates the bioenergetics of 143B and DU145 cells via complex I activity.

Citrate synthase activity, which is a reliable marker of mitochondrial content [25], was not changed in the treated cells (Fig. 4C), showing no change in mitochondrial content. The western blotting analysis of NDUFS2 and COXII as markers of complex I and complex IV, respectively, showed that the level of both complexes was not affected by the treatments (Fig. 4D). Moreover, the BN-PAGE analyses showed that the native complex I amount did not change after Src inhibition in both cell lines (data not shown), suggesting that the complex I assembly process was not impaired.

### 3.4. Identification of the Src-mediated phosphorylation site in complex I

The reduction in complex I activities could not be explained by the lower protein content, suggesting that its enzymatic activities were affected through post-translational modifications. In order to identify which subunit of complex I was differentially targeted by Src kinase among the treatments, complex I was immunoprecipitated from mitochondria of 143B and DU145 cells that were preincubated with ATP in the presence or absence of PP2, and analyzed by western blotting. As shown in Fig. 5, a tyrosine-phosphorylated band of about 20 kDa was observed after incubation with ATP and not (or to a lower intensity) when Src kinase was inhibited by PP2. Mass

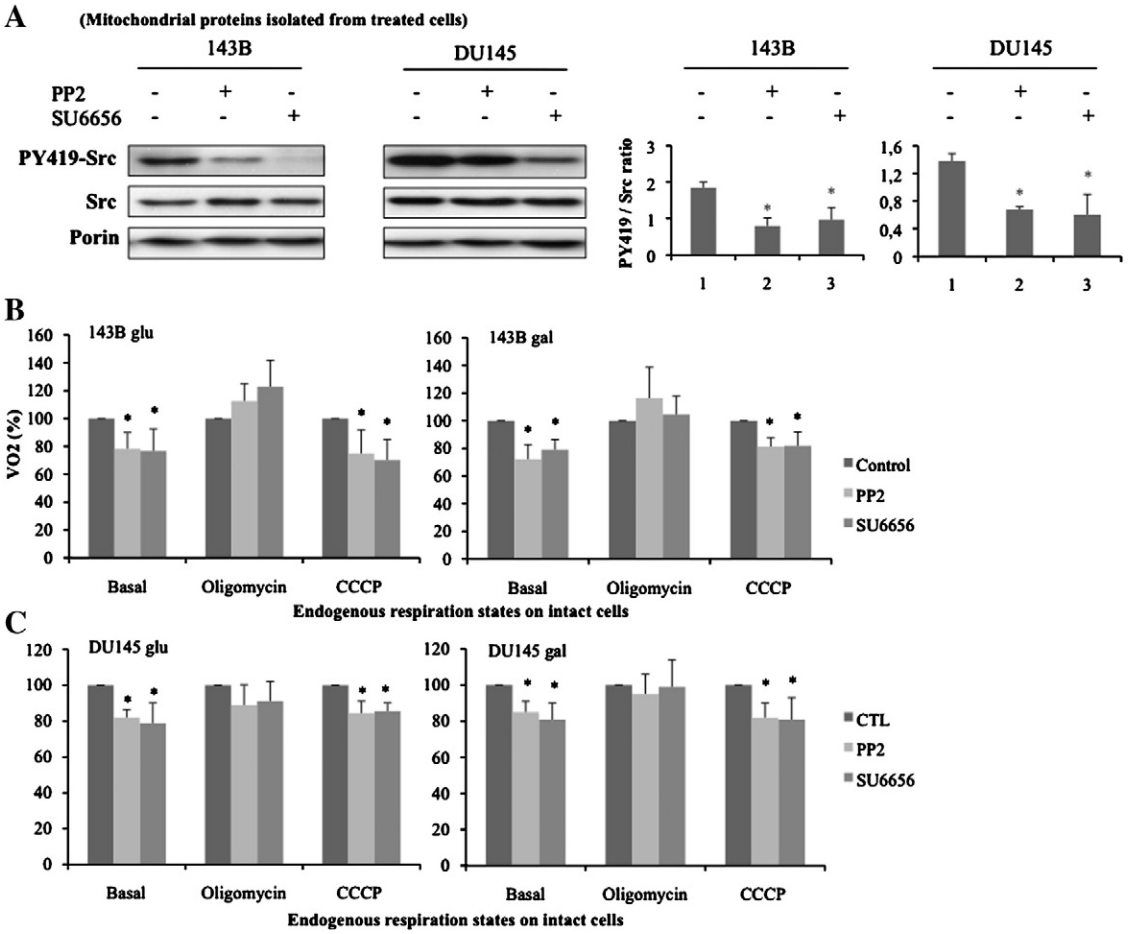


**Fig. 3.** Effect of Src inhibition on the bioenergetics of the 143B and DU145 cells. Cells were incubated for 4 h with DMEM supplemented with DMSO or Src inhibitors PP2 (5 μM) and SU6656 (10 μM). (A) After treatment, the mitochondria were isolated and immunoblotted with the PY419-Src and porin antibodies, stripped and reprobed with the Src B12 antibody. The intensity of the PY419-Src label among the treatments was normalized over the Src signal (n = 3). (B and C) After treatment, $O_2$ consumption was measured (n = 4–5) in the intact 143B and DU145 cells grown in glucose (glu) and galactose (gal) DMEM during the basal, oligomycin and CCCP respirations states. Means demarcated with an * are significantly different ($p<0.05$) from the control according to the Student's t-test.

spectrometry identified this band as NDUFB10 (Table 2) and the phosphorylated protein was further confirmed by immunoblotting with an antibody to NDUFB10 (Fig. 5). Therefore, Src kinase seems to sustain mitochondrial respiration by phosphorylating the NDUFB10 subunit of complex I.

### 3.5. Other mitochondrial parameters influenced by Src inhibition

Because mitochondrial ATP production does not seem to be the most important player in the proliferation of 143B and DU145 cells, the Src kinase-mediated maintenance of mitochondrial respiration should be related to other mitochondrial function(s). As complex I inhibition is known to increase ROS levels [26,27], we evaluated ROS levels after treatment with Src inhibitors using CM-$H_2$DCFDA and MitoSox probes (Fig. 6A and B). As no difference in these fluorescence probes was noted among the treatments, it seems that Src kinase did not influence ROS production, at least in our experimental conditions. Since the inhibition of complex I can also reduce the mitochondrial membrane potential [28,29], we then measured TMRM fluorescence. Accordingly with increased OXPHOS activity in galactose DMEM, the TMRM signal was higher in the galactose-grown control cells than in the glucose-grown control cells. This result could be in accordance with an increase in mitochondrial content [24] and is consistent with the increase of respiration in the galactose medium as compared to the glucose medium (see Table 1). Src inhibition treatments induced a clear decline of TMRM fluorescence in 143B gal and DU145 gal, but not in 143B glu and DU145 glu (Fig. 6C). These results clearly suggest that the Src kinase-mediated phosphorylation of NDUFB10 maintains the mitochondrial membrane potential ($\Delta\Psi$) when the cells are maintained in galactose medium.

**Table 1**
Mean (S.D.) values of the different respiration (pmol $O_2$ $s^{-1}$ $10^{-6}$ cells) steps for the intact and untreated 143B and DU145 cells grown in glucose or galactose DMEM (n = 4–5). * indicates that the respiration values are statistically different ($p \leq 0.05$) between the glucose and galactose DMEM.

| | 143B | | DU145 | |
|---|---|---|---|---|
| | Glucose | Galactose | Glucose | Galactose |
| Basal | 34.2 (3.5) | 52.3 (5.4)* | 37.1 (10.9) | 56.2 (1.6)* |
| Oligomycin | 11.6 (2.5) | 14.7 (3.2) | 17.4 (4.6) | 19.5 (3.5) |
| CCCP | 81.7 (12.7) | 119.5 (4.2)* | 65.4 (14.8) | 114.7 (13.5)* |

## 4. Discussion

Several cancer cells and tumors exhibit increased aerobic glycolysis, which is known as the Warburg effect [2,3]. Modifications of mitochondria have also been observed, for example: a decrease in mitochondrial biogenesis [30], the alteration of ETS complexes activities [31], a truncated TCA cycle with citrate efflux [7], an increase in the binding of hexokinase II to mitochondria [32,33], changes in mitochondria shape and size [34], and the accumulation of mutations in

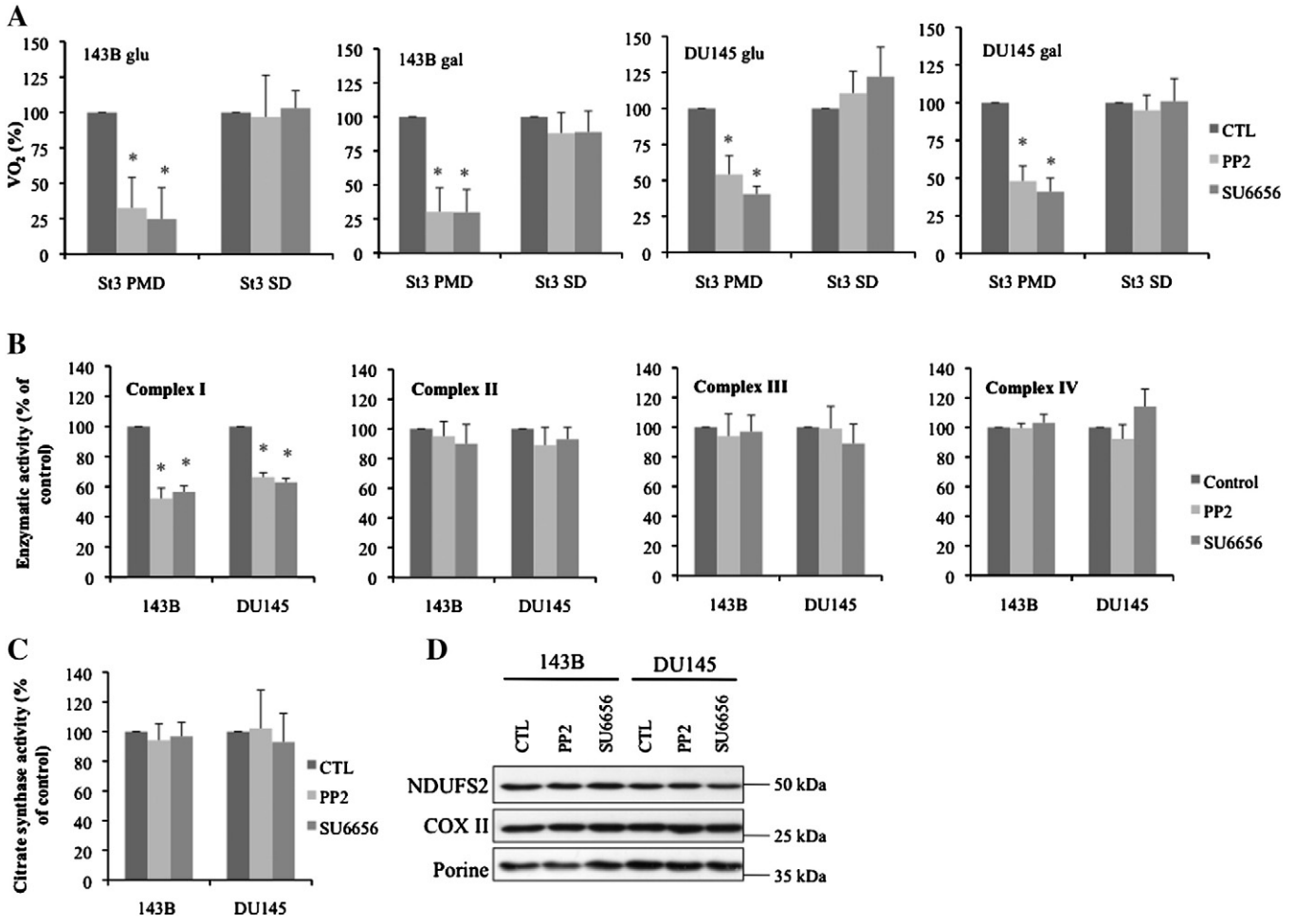


**Fig. 4.** Inhibition of Src kinase leads to a decrease in complex I activity in the 143B and DU145 cells. (A) After treating the 143B and DU145 cells grown in glucose (glu) and galactose (gal) DMEM with DMSO (as control), PP2 (5 μM) or SU6656 (10 μM) for 4 h, the $O_2$ consumption was measured (n = 4–5) in digitonin-permeabilized cells during basal respiration, pyruvate–malate–ADP driven respiration (St3 PMD) and rotenone–succinate–ADP driven respiration (St3 SD). (B and C) After treatment, the cells were harvested and complexes I, II, III and IV as well as citrate synthase enzymatic activities (n = 3) were measured as in [51]. (D) After the treatments, the cells were harvested and the mitochondrial fractions were immunoblotted with the NDUFS2 antibody (marker of complex I), the COX II antibody (marker of complex IV) and porine (as a loading control). All of the blots are representative of the three independent experiments. Means demarcated with an * are significantly different ($p<0.05$) from the control according to the Student's t-test.

mitochondrial DNA [for recent reviews, see 35,36]. All of these changes lead to increased glycolytic ATP production and lower mitochondrial ATP production, as we observed for the 143B and DU145 cells where ATP was mostly produced (approximately 90%) by glycolysis. Despite this low mitochondrial energy production in glycolytic cells, the inhibition of OXPHOS can lead to a significant decrease in proliferation, as we observed for the 143B and DU145 cells (Fig. 1). Therefore, mitochondrial metabolism seems to be important in maintaining the important proliferative phenotype of highly glycolytic cancer cells. Interestingly, most of the precursors that are used by biosynthesis pathways are produced by the TCA cycle. For example, most of the citrate is exported out of the mitochondria and used for de novo fatty acid synthesis in cancer cells. The increased glutamine

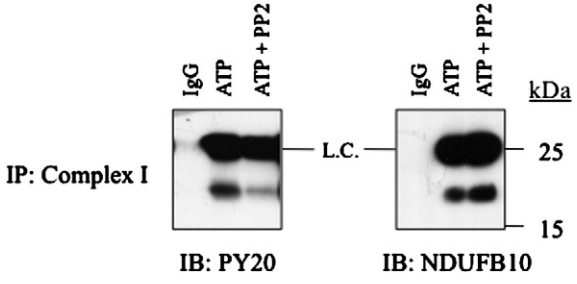


**Fig. 5.** Src kinase induces the tyrosine phosphorylation of the complex I subunit NDUFB10. The immunoprecipitation of complex I from 143B and DU145 was performed after the incubation of isolated mitochondria with ATP (1 mM) or ATP with PP2 (10 μM). The immunoprecipitates were then western blotted and phosphotyrosine was detected with the PY20 antibody. The identity of the phosphorylated subunit was first determined by mass spectrometry (as shown in Table 2) and further confirmed by labeling the NDUFB10 subunit. Immunoblots are only presented for complex I immunoprecipitated from the DU145 mitochondrial fractions although the same results were obtained from the 143B cells (L.C.: light chain).

**Table 2**
Proteins identified by LC–MS–MS in the tyrosine-phosphorylated band of complex I immunoprecipitates shown in Fig. 4.

| Accession number | Protein | MS/MS scans (*n*) | Peptides (*n*) | Protein sequence coverage of peptide (%) | Molecular weight (kDa) |
|---|---|---|---|---|---|
| P02768 | Serum albumin | 5 | 2 | 2.46 | 69.322 |
| P47985 | Complex III subunit Rieske | 3 | 3 | 15.69 | 29.649 |
| O96000 | Complex I beta subcomplex subunit 10 | 3 | 3 | 20.93 | 20.763 |
| P21291 | Cysteine and glycine-rich protein | 2 | 2 | 19.17 | 20.554 |
| P04083 | Annexin A1 | 2 | 2 | 7.80 | 38.690 |

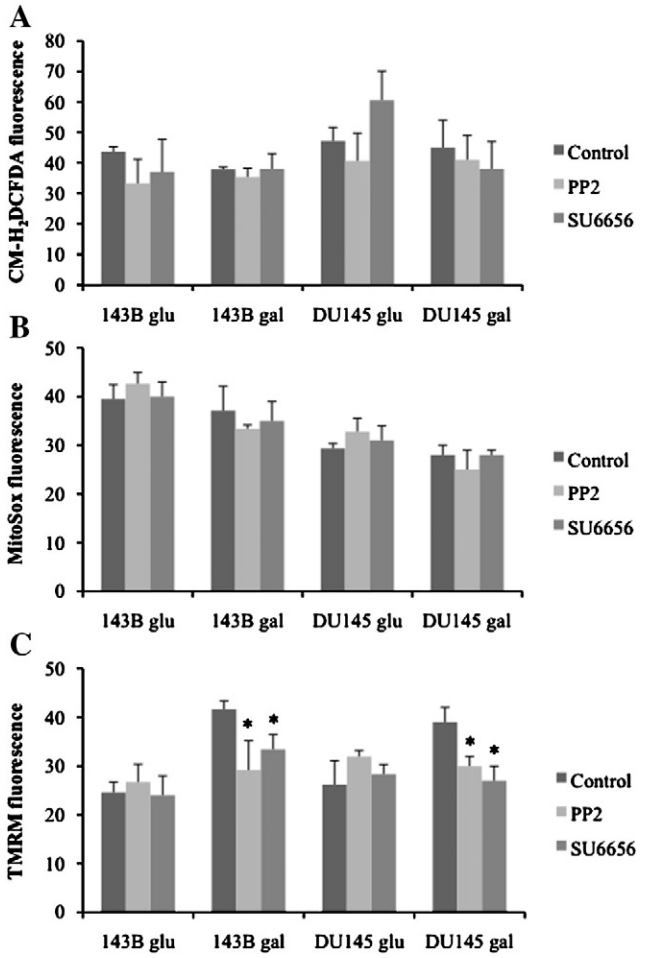


**Fig. 6.** The impact of Src kinase inhibition on mitochondrial membrane potential and ROS levels. After treating the 143B and DU145 cells grown in glucose (glu) or galactose (gal) DMEM with DMSO (as control), PP2 (5 μM) or SU6656 (10 μM) for 4 h, the ROS levels were evaluated by the fluorescence of (A) CM-$H_2$DCFDA (mainly indicative of cytosolic ROS) and (B) MitoSox (mainly indicative of mitochondrial ROS) probes while the mitochondrial membrane potential was evaluated by the (C) TMRM signal ($n=3$). Means demarcated with an * are significantly different ($p<0.05$) from the control according to the Student's t-test.

uptake and mitochondrial glutamine catabolism exhibited by cancer cells also allow higher biosynthesis rates as well as the production of TCA cycle intermediates in order to maintain the mitochondrial membrane potential [37,38]. Therefore, mitochondrial metabolism is essential to sustain the high proliferative phenotype of cancer cells, as we observed in the 143B and DU145 cells. Indeed, the depletion of mitochondrial DNA and OXPHOS in different cancer cell lines can induce lower tumorigenic properties and metastatic potential, both in vitro and in vivo [39,40].

Interestingly, the Src-dependent tyrosine phosphorylation of mitochondrial proteins increases concomitantly with stimuli triggering cell proliferation in neuroblastoma cells [41]. According to our results, we propose that the increase in Src kinase activity is important for the proliferative phenotype of cancer cells via its effect on complex I. Our study showed that Src kinase is present within the mitochondria of the 143B and DU145 cells where it plays a major role in in vitro tyrosine phosphorylation. The inhibition of Src kinase in the 143B and DU145 cells led to the inhibition of complex I-dependent respiration but no effect was observed with complex II substrates. This decrease in respiration was shown to be due to a specific decrease in the complex I enzymatic capacities (Fig. 3), which was probably explained by the reduced Src-dependent phosphorylation of the NDUFB10 subunit (Fig. 4). Src kinase inhibitors did not affect complex IV in 143B and DU145 cells, contrasting with the findings of Miyazaki and colleagues [13] who showed that Src kinase increased cytochrome *c* oxidase activity through phosphorylation of its subunit II in osteoclasts. The high energy demanding activity of bone resorption of osteoclasts seems to be highly dependent of mitochondrial energetic production. Indeed, the stimulation of oxidative metabolism by Src kinase-mediated phosphorylation of the complex IV subunit II was essential for maintaining the function of osteoclasts [13]. In contrast, mitochondrial ATP production does not seem important in 143B and DU145, at least for proliferation. Therefore, the absence of Src kinase impact on cytochrome *c* oxidase in 143B and DU145 cells could be explained by the different metabolic phenotype of cancer cells and osteoclasts.

The inhibition of Src kinase induced a clear decrease in the mitochondrial membrane potential in 143B and DU145 cells maintained in galactose medium (Fig. 6). In such conditions, Src kinase could preserve the mitochondrial membrane potential by inducing the phosphorylation of NDUFB10. Most mitochondrial functions, such as importing proteins into or across the mitochondrial inner membrane, require sufficient $\Delta\Psi$ [42–44], showing the possible importance of this regulation of $\Delta\Psi$ by Src kinase. However, it would be too hasty to definitively conclude about the impact of Src-mediated phosphorylation of NDUFB10 on mitochondrial membrane potential since Src-kinase inhibitors failed to induce a similar decrease in cells grown in glucose DMEM (Fig. 6).

Src kinase-mediated phosphorylation of NDUFB10 could participate in the establishment of the proliferative phenotype of cancer cells through different mechanisms. The increase in Src kinase activity could be important for maintaining a certain steady-state in terms of mitochondrial function in cancer cells. As the main role of the mitochondria is not ATP production in these cells, the Src kinase mediated increase of complex I activity and respiration should be related to other mitochondrial functions. For example, the modulation of complex I activity by Src kinase could be important in the control of mitochondrial redox potential and $NAD^+$/NADH ratio. By increasing complex I activity and NADH consumption, Src kinase could also ensure a sufficient flux through the TCA cycle, allowing an important production of TCA cycle intermediates used for biosynthesis pathways. The preservation of $\Delta\Psi$ via Src-dependent modulation of complex I activity could ensure the adequate importation of mitochondrial proteins (such as TCA cycle enzymes) into the mitochondria, enabling biosynthesis and proliferation. Further studies will be needed to test these hypotheses.

The structure of complex I is not completely understood but NDUFB10 seems to be located on the matrix side of subcomplex Iβ within the hydrophobic arm of complex I [45]. Four accessory subunits, including NDUFB10, contain cysteine-rich motifs and it has been proposed that these subunits might be involved in iron-cluster assembly [46,47]. The phosphorylation of NDUFB10 by Src kinase could therefore affect the transfer of electrons within complex I, and increase its enzymatic activity (Fig. 4). Complex I transfers electrons from NADH to ubiquinone and simultaneously pumps protons from the mitochondrial matrix to the intermembrane space. This translocation of protons is thought to occur in the membrane arm but the exact mechanism is unknown as several models still exist [45]. Therefore, NDUFB10 could also act as a regulator of complex I by modulating proton translocation through Src-mediated phosphorylation since Src inhibition led to a decreased mitochondrial membrane potential, at least for cells maintained in galactose medium (Fig. 6). Obviously, a more accurate understanding of the structure of complex I and of its proton translocation will be needed to confirm these mechanisms.

## 5. Conclusion

In conclusion, we propose that the Src-dependent phosphorylation of NDUFB10 could participate in the development of the proliferative phenotype of glycolytic cancer cells such as 143B and DU145 cells by preserving complex I activity. Src kinase is involved in several

properties of cancer cells such as increased survival, migration and proliferative capacities. While previous studies have shown that high Src kinase activity increases aerobic glycolysis (13–14), this study demonstrates that Src also participates in the maintenance of mitochondrial functions in cancer cells. The oncogene c-Myc is also well known for simultaneously stimulating aerobic glycolysis [48,49] and maintaining mitochondria function [37,50], suggesting that Src kinase should be also considered as a pinpoint control in the establishment of proliferative phenotypes.

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