p73 regulates primary cortical neurons metabolism: a global metabolic profile

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RUNNING TITLE: p73 regulates cortical neurons metabolism

Abbreviations: TAp73, Transcriptionally active p73; ΔNp73, amino truncated p73; DMED, Dulbecco minimal essential medium; FBS, foetal bovine serum; Ct, cycle; GC, Gas chromatography; MS, Mass Spectrometry; LC, Liquid chromatography; DMED, Dulbecco minimal essential medium; FBS, foetal bovine serum; DIV, day in vitro; ATP5A, Mitochondrial membrane ATP synthase F(1)F(0) ATP synthase or Complex V; FCCP, Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; UQCRC2, ubiquinol-cytochrome c reductase core protein II; MTCO1, mitochondrially encoded cytochrome c oxidase I; SDHB, succinate dehydrogenase complex, subunit B, iron sulphur; NDUFB8, NADH Dehydrogenase (Ubiquinone) 1 Beta Subcomplex, 8;

Acknowledgments

We would like to thank Dr Ivano Amelio for helpful comments and suggestions. This work has been supported by the Medical Research Council, UK; grants from Associazione Italiana per la Ricerca contro il Cancro (AIRC): AIRC 2014 IG15653 (to G.M.), AIRC 5xmille MCO9979 (to G.M.), Fondazione Roma malattie Non trasmissibili Cronico-Degenerative (NCDS-2013-00000334) Grant (to G.M.).

The authors declare that they have no conflict of interest.

Abstract (193 words)

The transcription factor p73 has been demonstrated to play a significant role in survival and differentiation of neuronal stem cells. In this report, by employing comprehensive metabolic profile and mitochondrial bioenergetics analysis, we have explored the metabolic alterations in cortical neurons isolated from p73 N-terminal isoforms specific knockout animals. We found that loss of the TAp73 or ΔNp73 triggers selective biochemical changes. In particular, p73 isoforms regulate sphingolipid and phospholipid biochemical pathways- signaling. Indeed, sphinganine and sphingosine levels were reduced in p73 null cortical neurons and decrease levels of several membrane phospholipids were also observed. Moreover, in line with the complexity associated with p73 functions, loss of the TAp73 seems to increase glycolysis while on the contrary loss ΔNp73 isoform reduces glucose metabolism, indicating an isoform-specific differential effect on glycolysis. These changes in glycolytic flux were not reflected by parallel alterations of mitochondrial respiration, as only a slight increase of mitochondrial maximal respiration was observed in p73 null cortical neurons. Overall, our findings reinforce the key role of p73 in regulating cellular metabolism and point out that p73 exerts its functions in neuronal biology at least partially through the regulation of metabolic pathways.

Keywords: p73, p53 family, metabolism, neurons, sphingolipids

Introduction

The transcription factor p73 [1-3], a member of the p53-family [4-6], [7,8] exerts its role through the regulation of several biological processes including cell survival [9-11], differentiation [12-14], cellular metabolism [15-20], and autophagy [21]. This pleiotropic role of p73 is mainly due to the fact that the *tp73* gene is expressed in numerous isoforms, including two main N-terminal isoforms: a full-length protein named TAp73, and a shorter, N-terminal truncated ΔNp73 isoform. It is generally accepted that the TA isoforms are transcriptionally active while the ΔN isoforms act as dominant negative and therefore transcriptionally inactive [1]. Notwithstanding these differences, mice generated by genetic deletion of the whole *tp73* gene (p73-/-) [22] or selective N-terminal isoforms (TAp73-/- and ΔNp73-/-) [23,24] all show postnatal alterations affecting the central nervous system (CNS). Indeed, the hippocampal dysgenesis observed in p73-/- and TAp73-/- mice manifests around 6 days of age, in agreement with the reported increase in TAp73 expression during neuronal maturation [25,26]. On the other hand, the neuronal loss and reduced cognitive and motor functions affecting ΔNp73-/- mice starts to be significantly evident in aged (26-27 months old) mice [24]. Overall, these findings indicate that p73 isoform are required for proper CNS development and neuronal homeostasis throughout the animal lifespan, although the downstream molecular pathways implicated appear complex and remain to be fully elucidated. Clearly, since p73 is a transcription factor, the phenotype observed in p73-/- mice is in part linked to regulation of gene expression. Indeed, several genes that are involved in neuronal biology, such as, SOX-2 [27], Hey-2 [28], TRIM32 [29] and p75NTR [30], are either directly or indirectly regulated by TAp73 [31-35]. In addition, TAp73 controls the expression of miR-34a, suggesting that microRNAs [36,26] also participate in the multifunctional role of p73 in neurons, such as differentiation [26]. Growing findings indicate that p73 proteins also regulate cellular metabolism [37-40], and we have recently shown that during neuronal differentiation, TAp73 promotes the expression of glutaminase 2 (GLS2), an enzyme that deaminates glutamine to produce glutamate, the most common excitatory neurotransmitter [41]. This latter discovery indicates that p73-mediated regulation of metabolism is an additional player in the regulation of neuronal function.

In this report, performing global metabolic profiling on cortical neurons isolated from isoform specific knockout mice, we widened our previous observations and investigated TAp73 and ΔNp73 regulation of cellular metabolism in neuronal cells. We describe significant changes in few, but key, metabolites that point to GABA, and ceramide signaling as potential targets regulated by both p73 isoforms. Moreover, consistent with the often intricate crosstalk between p73 isoforms, selective loss of the TAp73 and ΔNp73 leads to opposite outcomes on glycolysis and metabolism of neurotransmitters, but has a convergent effect on regulation of lipid signaling molecules.

Results

Global metabolic profile of cortical neurons derived from TAp73-/- and ΔNp73-/- mice

p73 proteins play a key role in neuronal differentiation and survival [42]. Recently we have also shown that p73 regulates cellular metabolism [37,15,18]. With regard to CNS, our previous data indicate that cortical neurons isolated from TAp73-/- mice appeared to have reduced levels of GABA, while levels of the inhibitory neurotransmitter glycine were reduced in ΔNp73-/- cells

[41]. To investigate whether these changes belonged to a wider metabolic rewiring caused by loss of p73 expression, a global metabolic profile was carried out [43], in cortical neurons derived from TAp73-/- and ΔNp73-/- mice after 7 days of in vitro culture (DIV7). 11 metabolites out of 217 were statistically ($p<0.05$) different in cortical neurons of both genotypes when compared to the control neurons (**Table 1 and 2**). Moreover, we found that levels of 14 and 28 metabolites were nearly-significant ($0.05<p< 0.10$) different in TAp73-/- and ΔNp73-/- respectively when compared to control cells. Overall, these findings suggest that p73 N-terminal isoforms regulate cellular metabolism in cortical neurons.

Bioenergetic ~~Glucose~~ metabolism is potentially affected by the loss of p73

Glycolysis is the main energetic source for neurons and is essential neuronal cells survival [44] [45] [46] (**Fig 1A**). Although not statistically significant, several biochemicals relating to glucose utilization, including the glycolytic intermediates 2-phosphoglycerate and phosphoenolpyruvate and the end product lactate, were increased in TAp73-/- cortical neurons. These increments were accompanied by elevated intracellular glucose levels, suggesting that glucose uptake may be increased in these cells (**Fig 1B**), and overall identifying a potential inhibitory role for TAp73 in the regulation of glycolysis. Conversely, glucose and glycolytic intermediates were slightly reduced in ΔNp73-/- neurons. Within the cell a second possible fate of glucose is fueling the Pentose Phosphate Pathway (PPP), leading to biosynthesis of the 5-carbon sugar ribose 5-phosphate (**Fig 2A**). As shown in **figure 2B,** levels of ribose 5-phosphate were statistically reduced in cortical neurons derived from ΔNp73-/- mice, while in TAp73-/- counterparts the reduction failed to reach the significance.

The changes observed in glucose metabolism prompted us to investigate whether the loss of p73 could affect bioenergetics metabolism. Then, because mitochondrial bioenergetics is compromised by the loss of TAp73 [18], we asked whether loss of p73 isoforms could also affect mitochondrial function in cortical neurons. To do so, we took advantage of the Seahorse Extracellular Flux Analyzer that allows measurement of the oxygen consumption rate (OCR), an indicator of mitochondrial activity, and performed a mitochondrial stress test (**Fig 3A**). We did not observe major alterations, although DIV7 cortical neurons derived from p73-/- mice show a slight, but significant, reduction in basal respiration when compared to cortical neurons derived from WT mice (**Fig 3B**). Moreover, the maximal respiratory capacity, measured following addition of the proton ionophore uncoupling agent FCCP was higher in p73-/- cortical neurons (Fig 3B). Although statistically not significant, the same trend was also observed in cortical neurons derived from both genotypes (Fig S1 and S2). However, no differences between p73-/- and WT cortical neurons were observed in the expression levels of the several subunits of the ETC, namely ATP5A, UQCRC2, MTCO1, SDHB and NDUF0B9, as assessed using western blotting (Fig 3C and Fig S3). Overall these data indicates that p73 has a minimal effect on the regulation of mitochondrial activity in neuronal cells.

Fatty acid synthesis and membrane remodelling in the absence of p73 isoforms

We have recently reported that, during differentiation of wild type (WT) cortical neurons, the levels of long chain fatty acids (LCFA) and lysolipids increase, whereas the intracellular content of medium chain fatty acids (MCFA) decreases [43]. This suggests that changes in lipid metabolism sustain the membrane remodeling that occurs during neuronal differentiation. Interestingly, our metabolic profile shows that in ΔNp73-/- cortical neurons at DIV 7 LCFA are decreased as compared to WT cells (**Fig 4 and table 2**). In addition, as shown in **figure 5**

membrane phospholipids, especially phosphocholine and phosphoethanolamine, were significantly reduced in ΔNp73-/- neurons. A trend towards reduced phospholipid levels was also observed in cortical neurons derived from TAp73-/-, although it failed to reach statistical significance (**Fig 5B**).

Overall, these results confirm previous findings showing that TAp73 is involved in the regulation of phospholipids biosynthesis [38].

Sphingolipid levels are reduced in p73 deficient cortical neurons

The sphingolipid metabolic pathway produces the cellular membrane component sphingomyelin and the signaling molecule ceramide (Fig 6A), which is necessary for the proper differentiation of neuronal cells al least in part, acting as signaling molecule downstream of the p75NTR in hippocampal neurons [47,48]. Sphinganine and sphingosine, the ceramide precursor and degradation products, respectively, are progressively elevated in differentiating cortical neurons, reflecting a sustained rate of sphingolipid metabolism.[43]. Notably, in the absence of either TAp73 or ΔNp73, these molecules were decreased, with a more dramatic effect in ΔNp73-/- cells (Fig 6B). Despite our analysis failed to directly detect ceramide, the changes in its metabolites might suggest that altered biosynthesis of these molecules could be partially responsible for the developmental defect and the aggravated neurodegeneration observed in the CNS of p73 deficient animals [49].

Discussion

The p53 family which is composed by the transcription factors p53 [50,51], p63 [52,53] and p73 play a key role in regulating cellular metabolism [54-56]. In particular, with a role in regulating gene expression, p73 has been demonstrated to play a significant role in neuronal cell biology, including survival and differentiation of stem cells [57]. To gain insight into the molecular mechanism by which p73 proteins regulate neuronal function a global metabolic profiling in cortical neurons was carried out. Here, we show that the steady-state levels of several metabolites are altered following the loss of p73 isoforms in DIV7 cortical neurons. Although the changes in metabolic profiles are subtle between genotypes, the data supports a role for p73 in regulating the differentiation of cortical neurons and points to GABA and ceramide signaling as potential targets regulated by p73 isoforms. Consistent with the complexity associated with p73 function, loss of the TAp73 and ΔNp73 isoforms have differential effects on glycolysis and neurotransmitter metabolism, but triggers similar effects on lipid signaling molecules.

Lipids are the most abundant compound present in the brain (REF). More importantly, changes in lipid composition are associated with several neurological and psychiatric disorders [58]. In particular, alterations of myelin biogenesis have widely documented links to structural and functional abnormalities, including axonal and neuronal degeneration, ataxia and tremor [59]. Interestingly, p73-/- mice show impairments in the peripheral nervous system with reduced thermal sensitivity, axon number, and myelin thickness [60]. Therefore, the decrease in sphinganine, sphingosine and steraoyl sphingomyelin is found in TAp73-/- and ΔNp73-/- cortical neurons may indicate that p73 isoforms regulate myelin biogenesis also in oligodendrocytes and Schwann cells. Even though this possibility should be experimentally tested, we would like to speculate that the reduction of myelin thickness observed in p73-/- mice could be in part explained by a reduction in the biosynthesis of myelin in oligodendrocytes and Schwann cells in absence of p73 isoforms. Indeed, we have recently shown that TAp73 induces biosynthesis of membrane

lipids in osteosarcoma cell line [38], supporting p73's role as regulator of lipids metabolism in different cellular context.

However, to determine whether these changes reflect persistent alterations of cellular metabolism, time course comparisons between WT and p73-/- neuronal cells at multiple time points to evaluate either global metabolism or selected metabolic pathways are warranted in future studies, supported by flux analysis of isotopic labelled compounds. In addition, it would be worth investigating the metabolic changes under pathological conditions, such as neurodegenerative disorders. Overall, the findings presented in this report strengthen the recent experimental evidences that p73 isoforms play an essential role in regulating cellular metabolism and contribute to our understanding of the phenotypes observed in knock-out mice.

Materials and Methods

Mice

Mice were bred and subjected to listed procedures under the Project Licence PPL 40/3442 released from the Home Office. p73 full KO mice (p73-/-) were generated and genotyped as previously described [22]. TAp73 KO mice (TAp73-/-) and ΔNp73 KO mice were generated and genotyped as previously described [23,24]. Wild type (WT), heterozygous (Het) and homozygous (-/-) mice for each genetic background were created by intercrossing Het mice. Offspring were genotyped by PCR analysis as previously described [22-24]

Cortical neurons preparation and treatment

Primary cortical neuronal were prepared from E17.5 embryos as previously described [43]. Briefly, cortices were harvested and cut into small pieces. Tissue was incubated with 1× Trypsin (Invitrogen, UK) for 10 minutes at 37 °C. After washing with Neurobasal medium (Invitrogen, UK) containing 10% FCS, penicillin (100U), streptomycin (100µg) (Invitrogen, UK), and 1X Glutamax (Invitrogen, UK), the tissue was resuspended by gentle pipetting. Cells were then counted using a~~n~~ hemocytometer, diluted to the appropriate concentration and then plated in aforementioned medium on poly-D-lysine–coated plates. After 1 h, medium was replaced with growth medium (Neurobasal medium (Invitrogen, UK) containing B27 supplement (Invitrogen, UK), penicillin (100U), streptomycin (100µg) (Invitrogen), and 1X Glutamax (Invitrogen, UK)) and maintained in culture for 7 days before being subjected to experimental procedures.

Global metabolic profiling

Cortical neurons were harvested at DIV7 and were immediately stored at -80°C. Samples were extracted and prepared for analysis using Metabolon's standard solvent extraction method (Metabolon, Inc. NC, USA). The sample preparation process was carried out as previously described [15]. Briefly, the protein fraction was removed by using a proprietary series of organic and aqueous extraction solutions. Then, the resulting extract was divided into two fractions; one for analysis by liquid chromatography (LC) and one for analysis by gas chromatography (GC). Each sample was first frozen and dried under vacuum. Samples were then processed for the appropriate instrument, either LC/MS or GC/MS. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument

standards) present in 100% of the Client Matrix samples, which are technical replicates of pooled samples. Following normalization to total protein determined by Bradford assay, log transformation and imputation with minimum observed values was performed for each compound.

Extracellular flux (XF) analysis.

Cortical neurons were seeded in XF 24-well cell culture microplate (Seahorse Bioscience, USA) in quintuplicate at $3x10^5$ cells/well in 1 ml growth medium and then incubated at 37°C in 5% CO_2. Assays were initiated, by removing the growth medium from each well and replacing it with fresh assay medium pre-warmed to 37°C. Oxygen Consumption Rate (OCR) was measured simultaneously for 3–5 min to establish a baseline rate. After the baseline measurement, 75–90µl of a testing agent (Oligomycin, FCCP and Rotenone) was injected into each well to reach the desired final working concentration. Generally, two to three baseline rate and two or more response rate (after compound addition) measurements were taken, and the average of two baseline rate or response rate readings was used for data analysis. The values of OCR levels were normalized to protein content.

Western Blot

Proteins were extracted from DIV7 cortical neurons with RIPA buffer containing protease inhibitor cocktail (Roche, UK). Protein concentration was determined using a Bradford dye-based spectrophotometry assay (Biorad, UK). Total protein was subjected to SDS–PAGE followed by immunoblotting with the indicated antibodies at the recommended dilutions. The following antibodies were used: MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (mouse, 1:250, Abcam, UK), β-Tubulin (rabbit, 1:1000, Santa Cruz, UK).

Statistical Analysis

All results are expressed as means ± s.d. $p<0.05$ was considered significant. In the metabolic profiling, Welch's two-sample t-tests were used to identify biochemicals that differed significantly between experimental groups. Statistical analysis was performed using GraphPad Prism 6.

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Table legends

Table 1. Statistical summary of biochemicals statistically or nearly statistically altered in TAp73-/- and ΔNp73-/- cortical neurons.
Table 2. Heat map of the global metabolic profile.

Figure legends

Figure 1: Glycolytic pathway in DIV7 cortical neurons derived from TAp73-/- and ΔNp73-/- mice. A) Schematic representation of the glycolytic pathway. **B)** DIV7 cortical neurons were harvested and levels of the indicated metabolites were assessed as described in material and methods section. Welch's Two Sample *t*-tests were used to identify biochemicals that differed significantly between experimental groups (n = 5-6 for each group). To note the robust increase in intracellular levels of glucose in TAp73-/- mice and the opposite decrease in ΔNp73-/- cells.

Figure 2: Pentose Phosphate Pathway (PPP) in DIV7 cortical neurons derived from TAp73-/- and ΔNp73-/- mice. A) Schematic representation of the PPP. The PPP is one of the main source of reductive NADPH in cells and the final product ribose-5-phosphate provides the sugar moiety in the biosynthesis of nucleotides. **B)** DIV7 cortical neurons were harvested and levels of the indicated metabolites were assessed as described in material and methods section. Welch's Two Sample *t*-tests were used to identify biochemicals that differed significantly between experimental groups (n = 5-6 for each group). Ribose-5-phopshate levels were significantly reduced in ΔNp73-/- cortical neurons.

Figure 3: Mitochondrial bioenergetics analysis of cortical neurons derived from WT and p73-/- mice. A) Real-time analysis of Oxygen Consumption Rate (OCR) in cortical neurons isolated from p73-/- mice and heterozygous controls (Het). ATP synthase inhibitor oligomicyn, mitochondrial uncoupler FCCP and mitochondrial complex I inhibitor rotenone were injected sequentially at the indicated time points into each well after baseline rate measurement. A representative experiment of 4 independent is shown. (**B**) Basal respiration and Maximal respiration (*right*). Each group is shown as a % of baseline (measurement before oligomycin injection). Values are mean ± SD (WT n=1, p73-/- n=2; Each point, n=5 technical replicates). (**C**) Western blot analysis showing expression levels of protein markers of the different complexes of the electron transport chain in DIV7 cortical neurons from WT and p73-/- mice. Assay was performed with MitoProfile Total OXPHOS and tubulin was used as loading control. Right panel, densitometric analysis of the indicated subunit of the different complexes of the electron transport chain (WT/Het n=4 and p73-/- n=4).

Figure 4: Cortical neurons derived from ΔNp73-/- mice show a reduced fatty acid synthesis. A) DIV7 cortical neurons were harvested and levels of the indicated fatty acids were measured in the described metabolomics profiling. Note the decreased levels of LCFA in ΔNp73-/- neurons. Welch's Two Sample *t*-tests were used to identify biochemicals that differed significantly between experimental groups (n = 5-6 for each group). EFA= Essential Fatty Acid; MCFA= Medium Chain Fatty Acid; LCFA= Long Chain Fatty Acid

Figure 5: Membrane phospholipid metabolism. A) Schematic representation of membrane phospholipid pathway. **B)** A decrease in several phospholipids was observed in both TAp73-/- and ΔNp73-/- DIV7 cortical neurons. Welch's Two Sample *t*-tests were used to identify biochemicals that differed significantly between experimental groups (n = 5-6 for each group).

Figure 6: Sphingosine and ceramide metabolism. A) Schematic representation of the sphingolipid pathway, which leads to the biosynthesis of ceramide and sphingomyelin. **B)** DIV7 cortical neurons isolated from TAp73-/- and ΔNp73-/- mice showed decrease levels of metabolites

belonging to the sphingolipid pathway, suggested altered biosynthesis of ceramide and sphingomyelin.. Welch's Two Sample *t*-tests were used to identify significantly different biochemicals (*n* = 5-6 for each group).

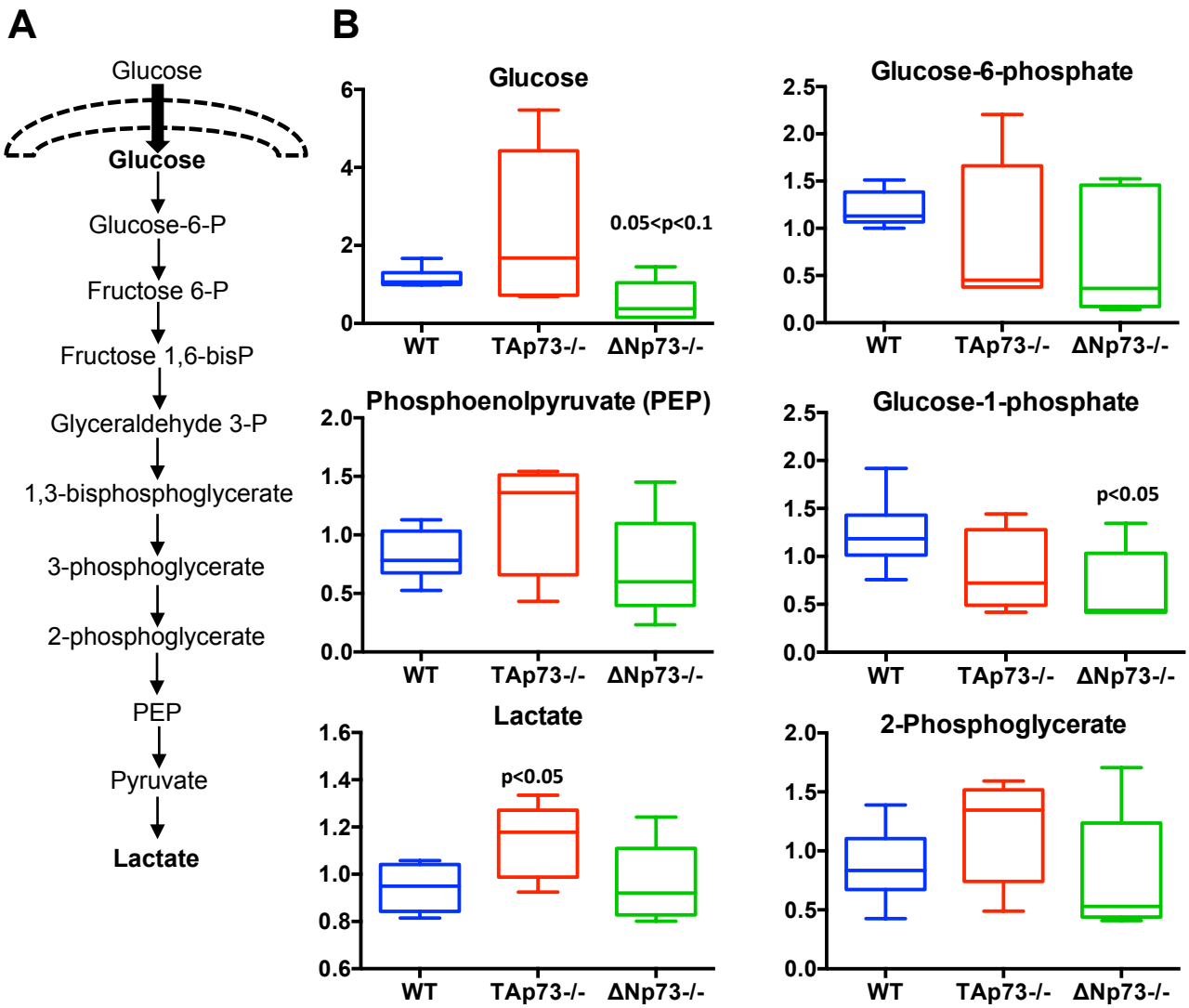
Supplementary Figure 1: Mitochondrial respiration analysis of cortical neurons derived from WT and TAp73-/- mice. A) Real-time analysis of Oxygen Consumption Rate (OCR) in cortical neurons. ATP synthase inhibitor oligomicyn, mitochondrial uncoupler FCCP and mitochondrial complex I inhibitor rotenone were injected sequentially at the indicated time points into each well after baseline rate measurement. A representative experiment of 2 independent is shown. (**B**) Basal respiration and Maximal respiration (*right*). Each group is shown as a % of baseline (measurement before oligomycin injection). Values are mean ± SD (WT n=1 TAp73-/- n=2; Each point, n=5 technical replicates).

Supplementary Figure 2: Mitochondrial respiration analysis of cortical neurons derived from control (Het) and ΔNp73-/- mice. A) Real-time analysis of Oxygen Consumption Rate (OCR) in cortical neurons. ATP synthase inhibitor oligomicyn, mitochondrial uncoupler FCCP and mitochondrial complex I inhibitor rotenone were injected sequentially at the indicated time points into each well after baseline rate measurement. A representative experiment of 2 independent is shown. (**B**) Basal respiration and Maximal respiration (*right*). Each group is shown as a % of baseline (measurement before oligomycin injection). Values are mean ± SD (Het n=1 ΔNp73-/- n=2; Each point, n=5 technical replicates).

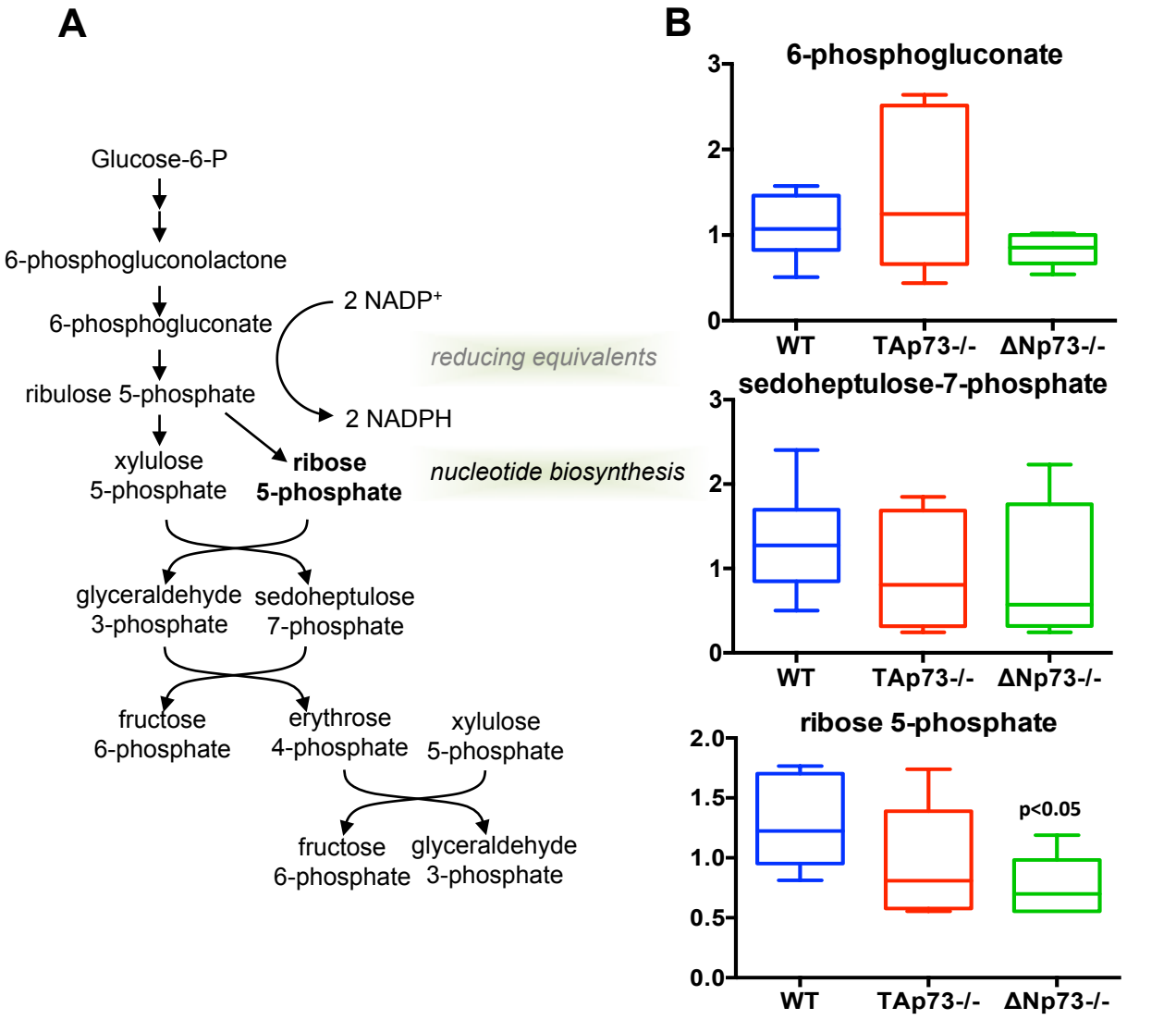
Supplementary Figure 3: Western blot analysis showing expression levels of protein markers of the different complexes of the electron transport chain in DIV7 cortical neurons from WT and p73-/- mice. Assay was performed with MitoProfile Total OXPHOS and tubulin was used as loading control.

	Total number of biochemicals with $p \leq 0.05$	Biochemicals (↑|↓) $p \leq 0.05$	Total number of biochemicals with $0.05 < p < 0.10$	Biochemicals (↑|↓) $0.05 < p < 0.10$
TAp73-/- / WT	11	3|8	14	2|12
ΔNp73-/- / WT	11	1|10	28	1|27

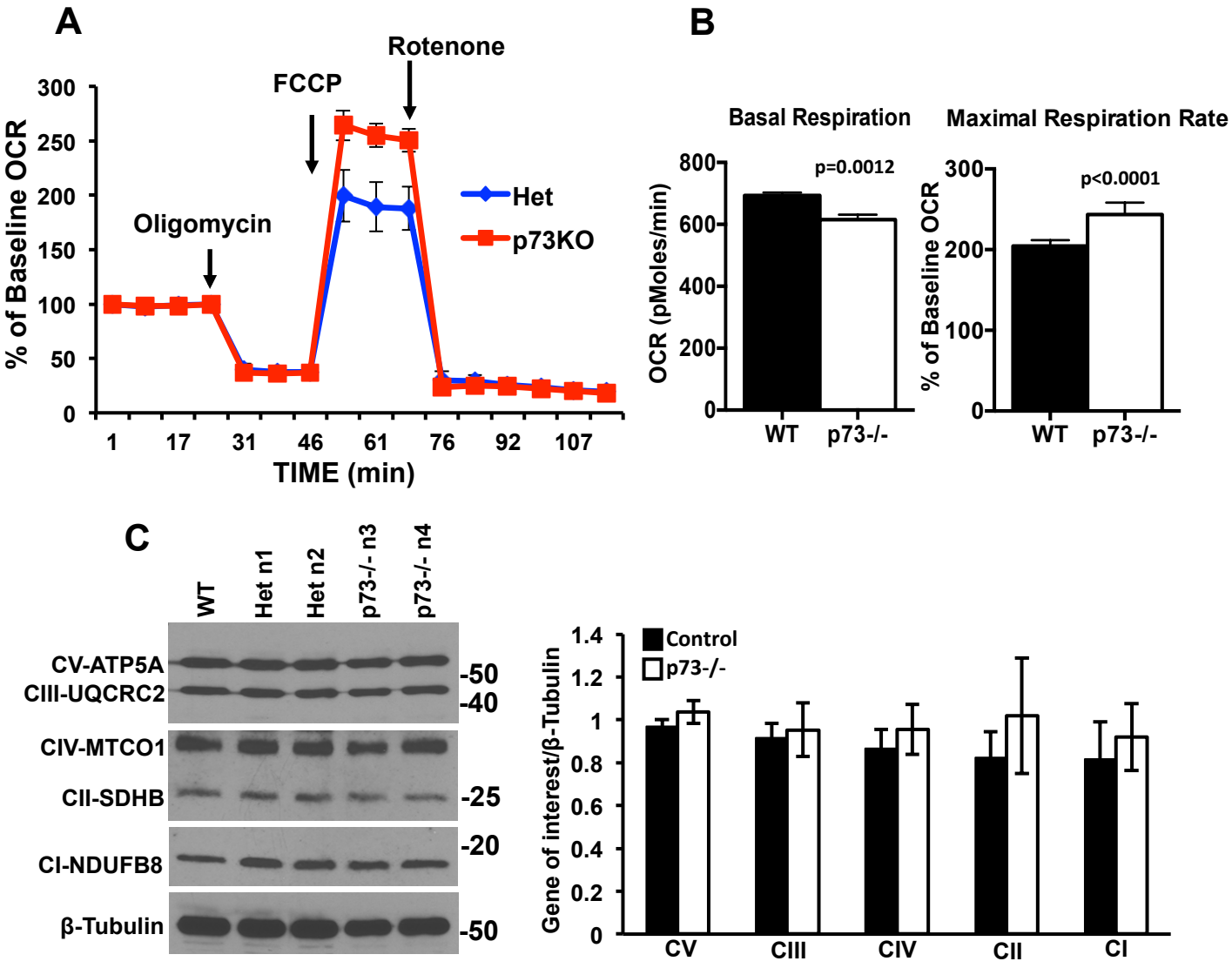
Table 1. Altered biochemical in TAp73-/- and DNp73-/- cortical neurons. The total number of significant (p-values ≤ 0.05) or nearly-significant ($0.05 < p < 0.10$) altered metabolites are reported. Red indicates up-regulated biochemicals and green indicates down-regulated compounds.



Agostini et al Figure 1



Agostini et al Figure 2



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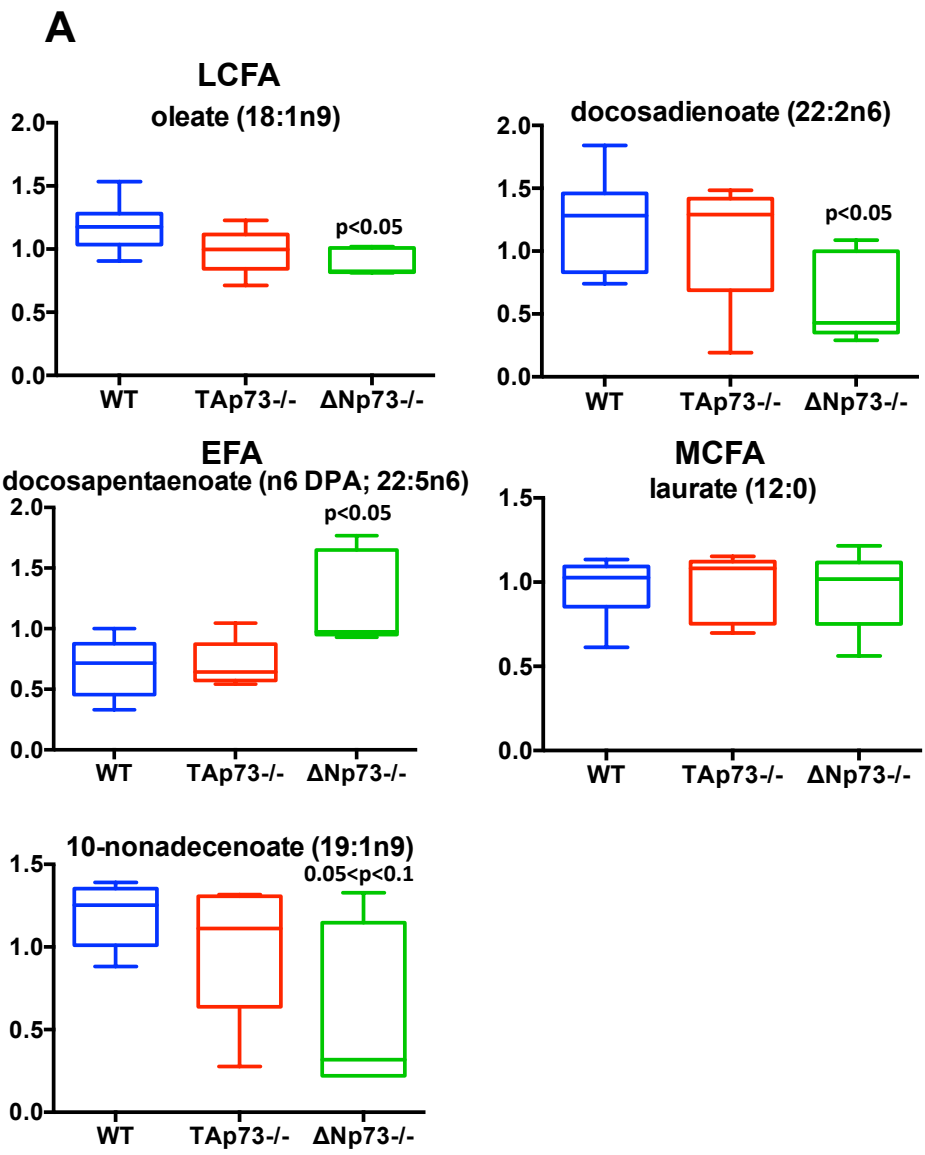

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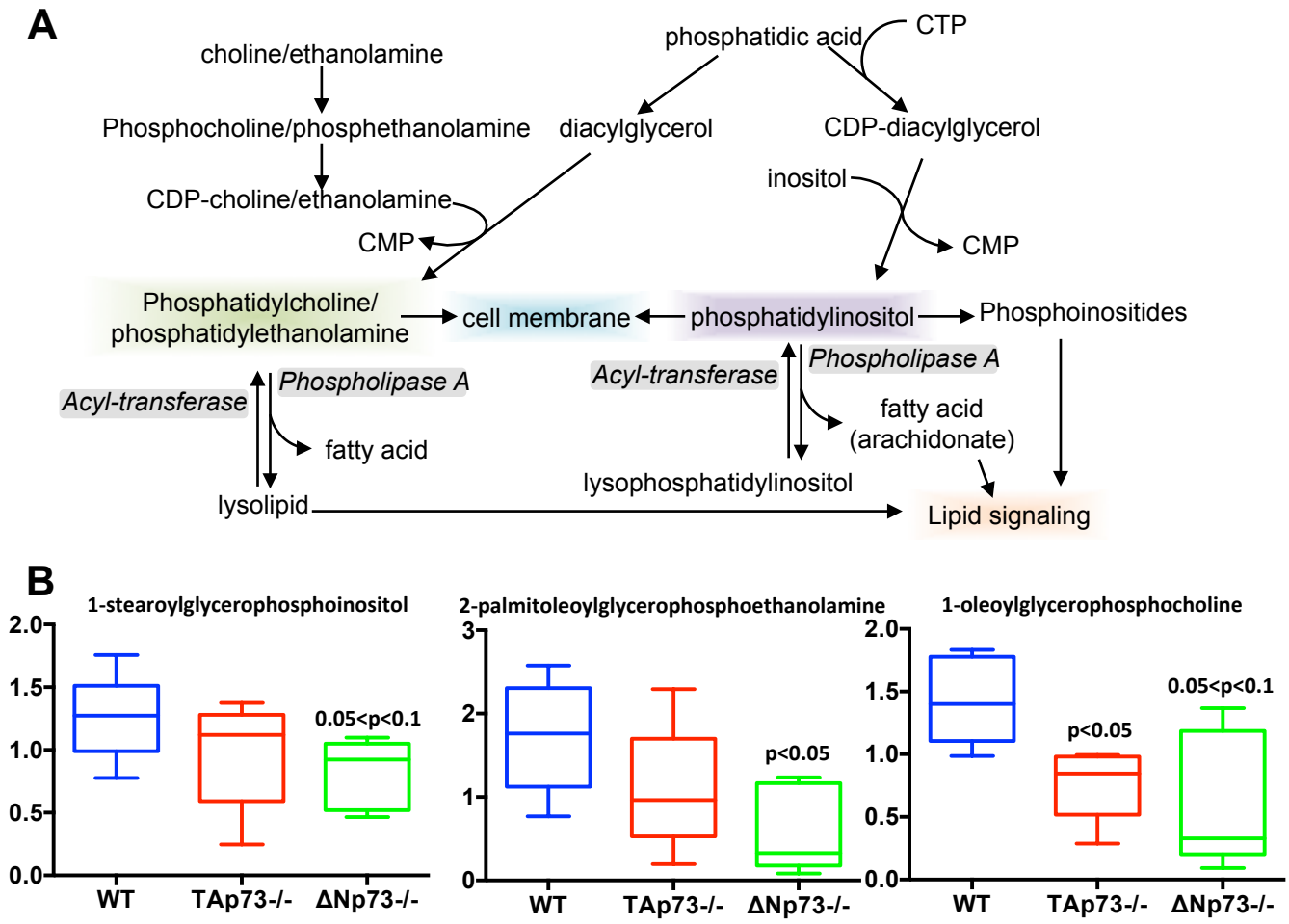

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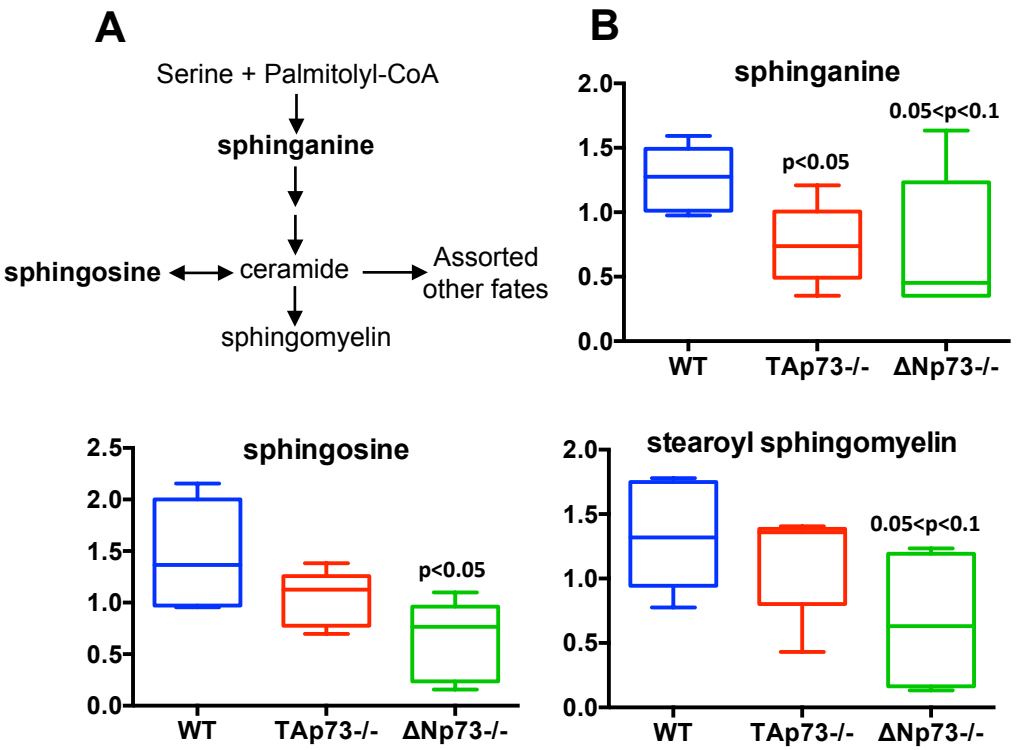

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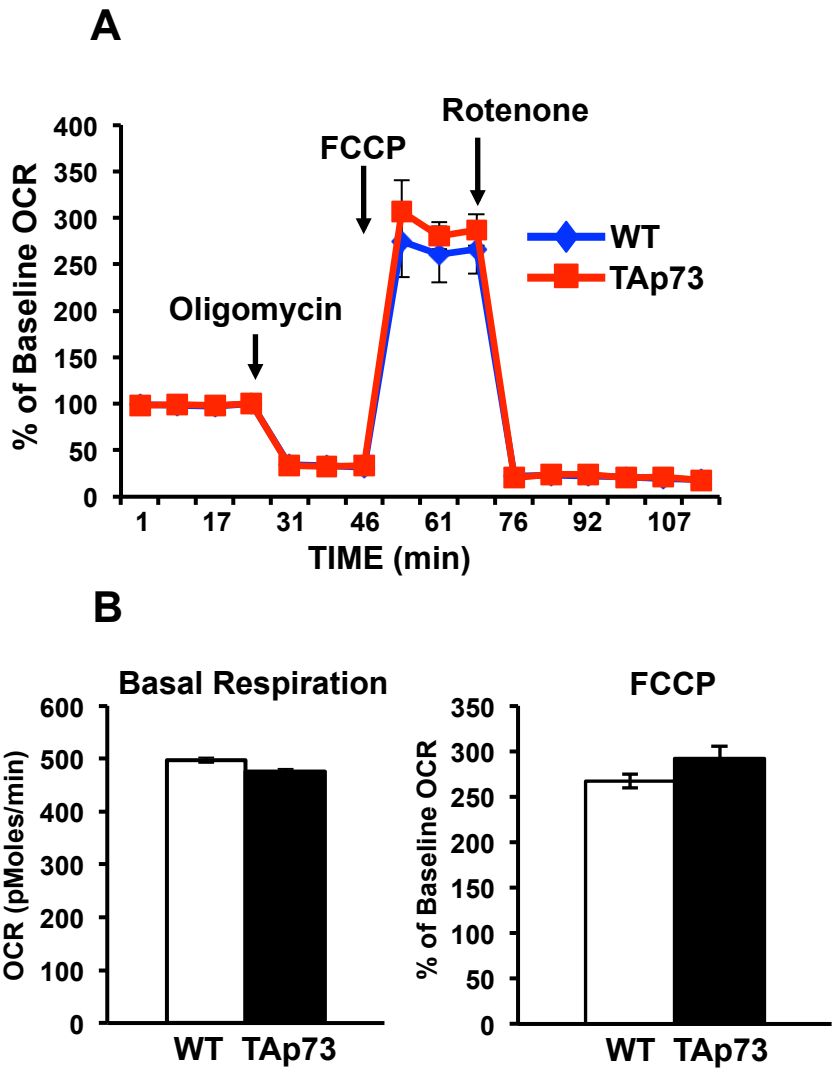
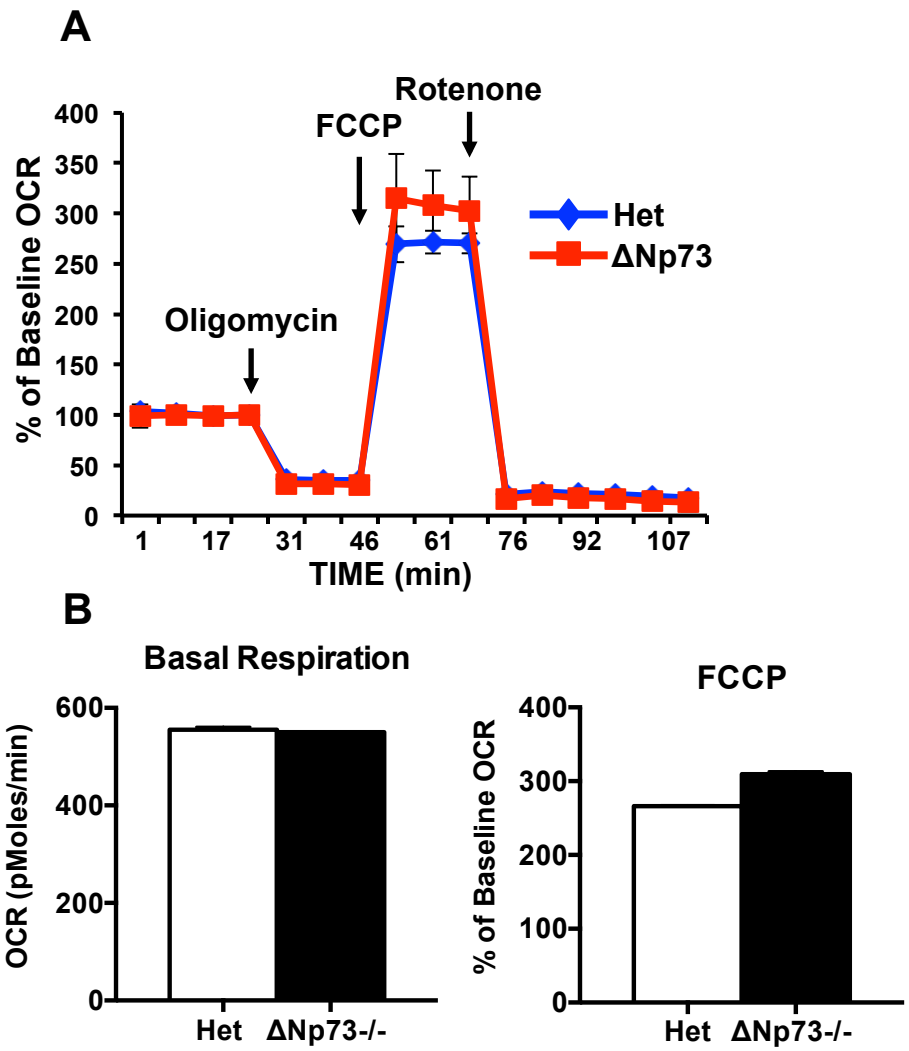


Agostini et al Figure S1



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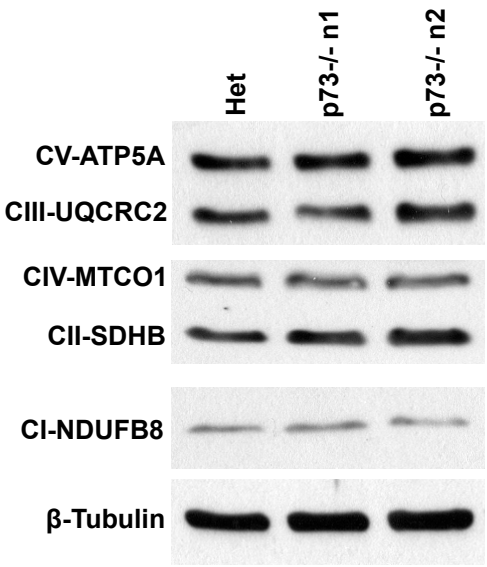

Agostini et al Figure S3