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**THE INTRA-MITOCHONDRIAL O-GLCNACYLATION SYSTEM ACUTELY
REGULATES OXPHOS CAPACITY AND ROS DYNAMICS IN THE HEART.**

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1 **ABSTRACT**

2
3 Protein O-GlcNAcylation is increasingly recognized as an important cellular regulatory
4 mechanism, in multiple organs including the heart. However, the mechanisms leading to O-
5 GlcNAcylation in mitochondria and the consequences on their function remain poorly understood.
6 In this study, we used an *in vitro* reconstitution assay to characterize the intra-mitochondrial O-
7 GlcNAc system without potential cytoplasmic confounding effects. We compared the O-
8 GlcNAcylation of isolated cardiac mitochondria with that of mitochondria acutely exposed to
9 NButGT, a specific O-GlcNAcylation inducer. Amongst the 409 O-GlcNAcylated mitochondrial
10 proteins identified, 191 displayed increased O-GlcNAcylation in response to NButGT. This was
11 associated with enhanced Complex I (CI) activity, increased maximal respiration in presence of CI
12 substrates, and a striking reduction of mitochondrial ROS release, which could be related to O-
13 GlcNAcylation of subunits within the NADH dehydrogenase module of CI. In conclusion, our
14 work underlines the existence of a dynamic mitochondrial O-GlcNAcylation system capable of
15 rapidly modifying mitochondrial function.

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17 **DATA ACCESSIBILITY TO THE REVIEWERS:**

18 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium
19 via the PRIDE partner repository with the following dataset identifier.

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1. INTRODUCTION

O-linked N-acetylglucosamination (O-GlcNAcylation) is a dynamic post-translational modification of proteins characterized by the addition of a single acetylated hexosamine moiety to certain Ser/Thr residues through O-linkage on their hydroxyl (Bond 2015, Mailleux 2016). Protein O-GlcNAcylation requires uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) as substrate, which is mainly derived from glucose through the hexosamine biosynthesis pathway (HBP), driven by its rate limiting enzyme the glutamine:fructose-6-phosphate amidotransferase (GFAT). O-GlcNAcylation is regulated by the uridine diphospho-N-acetylglucosamine transferase (OGT), and the O-GlcNAcase (OGA), which respectively add and remove O-GlcNAc moieties (Bond 2015, Mailleux 2016). These highly conserved and ubiquitously expressed enzymes are present in various cellular locations where they regulate several protein properties such as their activation state, localization, stability and/or degradation (Joiner 2019). Over the past several years, protein O-GlcNAcylation has emerged as both pathogenic factor (Nie 2019) and important mechanism involved in physiological processes such as development and protection against cellular stress (Ong 2018, Jensen 2019). Such dual action can be found in the heart. Indeed, chronic elevation of protein O-GlcNAcylation is believed to participate in the development of metabolic and contractile dysfunctions associated with diabetes (Ma 2016) and cardiac hypertrophy (Mailleux 2016, Gelinas 2018). Conversely, acute hyper O-GlcNAcylation is known to confer protection against ischemic damage (Jensen 2019) and sepsis-induced contractile dysfunction (Ferron 2019, Silva 2019), which has contributed to position protein O-GlcNAcylation as a potential therapeutic target for the management of both chronic and acute cardiovascular conditions.

While OGT and OGA are mainly localized to the nuclear and cytosolic compartments, several studies have shown that mitochondria are major targets for O-GlcNAcylation (Bond 2015, Zhao 2016). Furthermore, growing evidence suggest that mitochondrial protein O-GlcNAcylation in fact

1 plays a role not only in the development of diabetic cardiomyopathy (Hu 2009, Banerjee 2015),
2 but also in the cardio-protective effect of acute hyper O-GlcNAcylation (Ngoh 2011, Jensen 2019).
3 This notion has been recently reinforced by results showing the presence of an UDP-GlcNAc
4 carrier along with OGA and a 103 kDa isoform of OGT (mOGT) in the mitochondrial compartment
5 (Hanover 2003, Love 2003, Banerjee 2015). Some controversies nevertheless exist regarding the
6 presence of these enzymes in the mitochondrial compartment (Trapannone 2016). More
7 importantly, since mitochondrial O-GlcNAcylation has mainly been investigated in cell culture
8 models or *in vivo*, the direct effects of the putative mitochondrial O-GlcNAc cycling system have
9 been difficult to distinguish from the indirect effects mediated by the nucleocytoplasmic O-
10 GlcNAcylation system.

11 In this study, we therefore took advantage of an *in vitro* reconstitution assay to characterize
12 the intra-mitochondrial O-GlcNAcylation system in isolated cardiac mitochondria. Our results
13 confirm the presence of a fully functional and dynamic O-GlcNAc cycling system in these
14 organelles. Using comparative O-GlcNAc proteomics (O-GlcNAcyloomics), we provide evidence
15 that the local mitochondrial O-GlcNAcylation system can trigger broad and rapid changes in
16 protein O-GlcNAcylation, which are highly reminiscent of the mitochondrial O-GlcNAcylation
17 profile observed *in vivo*. Importantly, we also reveal that acute hyper-O-GlcNAcylation increases
18 maximal respiratory capacity, and drastically reduces ROS release through a complex-I mediated
19 mechanism, illustrating the capacity of this system to rapidly modify mitochondrial function.

2. RESULTS

2.1. *In vitro* reconstitution assay allows to target the intra-mitochondrial O-GlcNAcylation system.

In order to characterize the mitochondrial O-GlcNAcylation system without the potential confounding effects of the O-GlcNAcylation in other cellular compartments, we devised an *in vitro* reconstitution assay in which isolated cardiac mitochondria were acutely exposed (*i.e.* 30 min) to the OGT substrate UDP-GlcNAc in presence or absence of the OGA inhibitor NButGT, with the goal of inducing rapid changes in protein O-GlcNAcylation levels.

As some controversy exists regarding the expression of the mOGT isoform in murine tissues (Trapannone 2016), and the presence of sufficient OGA in the mitochondrial compartment (Banerjee 2015), the presence of these enzymes was first verified by immunoblotting in whole lysates from crude and Percoll-purified mitochondria (Fig 1A). Specific protein markers were firstly assessed to confirm the purity of the different purified fractions (Fig 1B). As expected, the mitochondrial marker TOM20 was highly enriched in the crude and Percoll-purified mitochondrial fractions, while histone H3 and alpha tubulin were mostly recovered in the nuclear and cytosolic fractions, respectively. Small amounts of histone H3 and alpha-tubulin remained present in the crude mitochondrial preparation, but were largely removed by the Percoll purification step. As represented in the Fig 1A, immunoblotting of crude mitochondrial fractions with anti-OGT antibody revealed a predominant band at 103 kDa, which corresponds to the expected molecular weight of the mitochondrial isoform mOGT (Hanover 2003). A band was also observed at 116 kDa, consistent with the presence of the nucleocytoplasmic isoform (ncOGT). However, this band was absent in Percoll-purified mitochondria, indicating that mOGT is the predominant, if not the sole, isoform present in mitochondria. For OGA, a single band, running at 75 kDa was observed in the mitochondrial fraction, which corresponds to the expected molecular weight of the short OGA

isoform (sOGA), also expressed in the nucleus (Comtesse 2001). Conversely, the full length OGA (fOGA) running at 130 kDa was absent from the mitochondrial fraction. (Fig 1A).

Figure 1: Characterization of the *in vitro* reconstitution assay used to investigate the mitochondrial O-GlcNAcylation cycling system. **a)** Representative western-blot of OGA and OGT protein content measured in the whole heart homogenate, nuclear, cytosolic, crude and Percoll-purified mitochondrial fractions (Pure Mito). Note that the image shown for the pure mitochondrial fraction is derived from a separate blot. **b)** Representative western-blot of the relative expression of mitochondrial (TOM20), nuclear (Histone H3), and cytosolic (Tubulin) marker proteins in the various cellular fractions. **c)** Overview of the *in vitro* reconstitution assay used to induce and detect mitochondrial protein O-GlcNAcylation in isolated mitochondria. Crude cardiac mitochondrial fractions isolated from rat hearts were incubated at room temperature in presence of UDP-GlcNAc in the absence or presence of the OGA inhibitor NButGT. After 30 min, proteins were denatured and O-GlcNAc-modified proteins were incubated with UDP-GalNAz in presence of the enzyme Y289L GalT. The GalNAz adducts obtained were functionalized with TAMRA, and O-GlcNAc-modified proteins were recovered by immunoprecipitation using an anti-TAMRA antibody. Immunoprecipitates were resolved by electrophoresis and revealed by fluorescence at the wavelength of TAMRA (570 nm). Following densitometric quantification of band intensity (shown in red and blue), gels were cut in 7 pieces of equal size and further processed for proteomics analysis.

To assess whether protein O-GlcNAcylation was increased in our reconstitution assay, crude mitochondrial fractions were lysed after exposure to UDP-GlcNAc in absence or presence of the O-GlcNAc inducer NButGT. Following denaturation, O-GlcNAc-modified proteins were stabilized and labeled with the fluorescence probe TAMRA using Click-iT chemistry (Fig 1C).

Following TAMRA-mediated O-GlcNAc specific immunoprecipitation, proteins were separated by gel electrophoresis and visualized by fluorescence. Multiple bands were observed in immunoprecipitates from control mitochondria indicating a baseline level of protein O-GlcNAcylation (Fig 1C). Importantly, staining intensity was consistently increased in NButGT-treated mitochondria indicating a broad and rapid rise in protein O-GlcNAcylation levels.

2.2. Acute stimulation of O-GlcNAcylation triggers rapid changes in the mitochondrial O-GlcNAcylome.

To gain knowledge on the repertoire of proteins modified by the mitochondrial O-GlcNAcylation system, gels were cut in seven bands of equal size and processed for tandem mass spectrometry (MS/MS) analysis. To maximize stringency, only proteins reliably detected in all experimental replicates from control and NButGT-treated mitochondria were considered. Using this selection criteria, a total of 842 proteins were identified (Fig 2A). Of these, 50% (409) had a known (339) or predicted (70) mitochondrial status in the Mitominer database (Smith 2016), while the remaining were non-mitochondrial (322), or had an unspecified status (111), which can be expected given that crude mitochondrial fractions were used for this analysis. Since these likely contained residual amounts of ncOGT and fOGA outside mitochondria, we sought to determine whether the impact of NButGT on protein O-GlcNAcylation varied according to the localization of these proteins. As shown in Fig 2B, treatment with NButGT predominantly increased O-GlcNAcylation of mitochondrial proteins (*i.e.* known + predicted mitochondrial status) compared to non-mitochondrial proteins, indicating that the reconstitution assay was effective at targeting the intra-mitochondrial O-GlcNAcylation system. Consistent with this notion, treatment with NButGT significantly increased O-GlcNAcylation of 191 ($q < 0.05$) to 246 ($q < 0.1$) mitochondrial proteins (Fig 2C), while none of the non-mitochondrial proteins were significantly affected (Fig S1).

1 Because mOGT was reported as preferentially associated with the mitochondrial inner membrane
2 (Banerjee 2015), we looked at the sublocalization of mitochondrial proteins. This analysis indicated
3 that a large proportion of the 409 O-GlcNAcylated mitochondrial proteins originated from the
4 matrix (126) and inner-membrane (124), with only a minor proportion coming from the
5 intermembrane space or outer membrane (Fig 2D). However, the effect of NButGT on O-
6 GlcNAcylation level did not differ significantly across submitochondrial compartments (Fig 2E).

Figure 2: Impact of NButGT treatment on protein O-GlcNAcylation according to their subcellular localization. **a)** Relative distribution of proteins detected by MS/MS according to their status on the MitoMiner database. Only proteins reliably detected in all experimental replicates from control and NButGT-treated mitochondria were considered. **b)** Fold change in the abundance of mitochondrial and non-mitochondrial O-GlcNAc-modified proteins between control and NButGT-treated mitochondria. **c)** Volcano plot analysis showing the impact of NButGT O-GlcNAcylation for proteins with a mitochondrial status on the Mitominer database. Red ($q < 0.05$) and orange ($q < 0.1$) dots represent proteins that were significantly affected in response to NButGT. Number of significantly affected proteins are indicated along with the adjusted p value (q) threshold. Statistical analysis was assessed using a linear regression model (empirical Bayes methods) followed by the Benjamini-Hochberg FDR procedure. **d)** Fold change in the abundance of O-GlcNAc-modified proteins between control and NButGT-treated mitochondria according to their sub-mitochondrial localization. **e)** Relative distribution of mitochondrial proteins (known + predicted) according to their sub-mitochondrial localization. Proteins were ascribed to a particular sub-mitochondrial compartment based on annotations available in the Uniprot and GO databases. For panel **b** and **e**, individual values for each protein identified as well as mean $\pm$ sem ($n=3-4$ biological replicates) are shown.

1 Based on these results, the mitochondrial processes targeted by acute mitochondrial O-
2 GlcNAcylation were examined. Pathway enrichment analysis and protein network clustering
3 revealed that proteins related to oxidative phosphorylation, tricarboxylic acid (TCA) cycle and
4 pyruvate and fatty acid metabolism were the top enriched pathways (Fig 3A-B). Among the multi-
5 proteins complexes of the oxidative phosphorylation machinery, complex I (23 subunits, 60%), V
6 (8 subunits, 50%), III (6 subunits, 75%), and II (3 subunits, 75%), were predominantly affected
7 with 50-75% of their subunits being significantly more O-GlcNAcylated in response to NButGT
8 compared to only 25% (4 subunits) for complex IV (Fig 4). TCA cycle enzymes and pyruvate
9 metabolism proteins displaying increased O-GlcNAcylation levels included isocitrate
10 dehydrogenase (IDH2, IDH3A, IDH3B, IDH3G), aconitase (ACO2), succinyl-CoA ligase
11 (SUCLG1, SUCLG2, SUCLA2), several subunits of the pyruvate dehydrogenase complex (
12 PDHB, PDHX, PDHA1L1, DLAT, DLD) pyruvate carboxylase (PC), and both isoforms of the
13 mitochondrial pyruvate carrier (MPC1-2). Besides, increased O-GlcNAcylated proteins of fatty
14 acid metabolism included the carnitine-palmitoyl transferases and carnitine transporter (CPT1-2,
15 SLC25A20), multiple β -oxidation enzymes (ACSL1, ACADS, ACADM, ACADL, ACADVL,
16 DECR, ECHS1, ECI1, HADHA), and electron transferring flavoproteins (ETFA, ETFB).

Figure 3: Characterization of the pathways over-enriched in response to NButGT in isolated mitochondria. Functional enrichment analysis of over-represented KEGG (a) and REACTOME (b) pathways in NButGT-treated vs control mitochondria performed using g:Profiler. Proteins were input in g:Profiler in order of decreasing q value with a threshold set at $q < 0.1$ (Ordered Query). Maximum size of functional categories was set at 250 to filter out large annotations that provide limited interpretative value. The g:SCS algorithm was used for multiple hypothesis testing corrections using a default alpha threshold of 0.05 for significance. Enrichment is expressed as a rich factor, which represents the ratio of the number of proteins observed for a given pathway term to the total number of proteins for this term. Circle size reflects the number of proteins per pathway, while color indicates the level of significance.

Beyond energy metabolism, smaller clusters of proteins related to several other mitochondrial functions displayed increased O-GlcNAcylation levels in response to NButGT (Fig 4). This included proteins related to: i) mitochondrial protein translation, such as proteins associated with the mitochondrial ribosomes (MRPS11, MRPS22, MRPS23, MRPS25, MRPL22, MRPL38), ii) protein processing such as proteases (CLPP, TRAP1), chaperones (HSPA9), and protein import channel subunits (TIMM21, TIMM44, SAMM50), iii) proteins involved in the regulation of

1 mitochondrial permeability transition such as cyclophilin-D (PPIF), voltage gated anion channel,
2 and ATP/ADP exchanger isoforms (DAC1-2, SLC25A5, SLC25A31) and iv) ROS detoxifying
3 systems including superoxide dismutase (SOD1-2), peroxiredoxins (PRX3, PRX5), thioredoxin
4 (TXN1) and thioredoxin reductase (TXNRD2).

5 **Figure 4: Characterization of the mitochondrial proteins displaying increased O-GlcNAcylation in**
6 **response to NButGT in isolated mitochondria.** High confidence (interaction score > 0.7 based on default
7 active interaction sources) STRING network of mitochondrial proteins displaying increased O-
8 GlcNAcylation in response to NButGT. Clustering was performed with the Markov Cluster (MCL)
9 algorithm using with a granular parameter set at 4. The Auto-annotate function of Cytoscape was used to
10 identify pathways/processes corresponding to these clusters based on Stringdb description and GO
11 annotations. Proteins were color coded according to the q values smaller than 0.05 (blue) or 0.1 (red).

2.3. The *in vitro* protein O-GlcNAcylation profile is reminiscent of protein O-GlcNAcylation observed following *in vivo* treatment with OGA inhibitors.

To gain insights on the contribution of the intra-mitochondrial O-GlcNAcylation system to protein O-GlcNAcylation in the heart, we sought to compare these results with a methodologically comparable (*i.e.* identical Click-iT labelling, IP and MS/MS protocol) cardiac O-GlcNAcylation dataset derived from mice that were subjected to NButGT or vehicle treatment 6 hours prior to sacrifice (Fig 5A). Of the 409 mitochondrial proteins detected in the *in vitro* reconstitution assay, 85% were also identified as being O-GlcNAcylated *in vivo*, and among them 122 displayed enhanced O-GlcNAcylation in the two data sets (FC >1.2, Table S1).

Comparison was also made with data from a previous study performed by Ma *and colleagues* in which O-GlcNAc sites on isolated cardiac mitochondria were mapped 12h after *in vivo* administration of the OGA inhibitor Thiamet G using a BEMAD labelling method (Ma 2015). Of the 88 O-GlcNAc-modified proteins identified by Ma and colleagues, 62 were found to display increased O-GlcNAcylation in our *in vitro* reconstitution assay, representing a highly significant over-enrichment (Fig 5B). The majority of shared proteins across the two datasets were components of the oxidative phosphorylation system, TCA cycle, and fatty acid oxidation pathway (Fig 5C), with a few noticeable proteins related to ROS metabolism (SOD2, PRX3) and permeability transition pore (mPTP)/apoptosis (VDAC1, SLC25A4, ENDOG). Within the OXPHOS system, overlap between the two datasets was observed for subunits located in the NADH dehydrogenase (N) and ubiquinone reductase (Q) modules of complex I (NDUFS1, NDUFAA7, NDUFA9), the F1 sector of complex V (ATP5O, ATP5B, ATP5A1), the hydrophilic head of complex II protruding in the matrix (SDHA, SDHB) and UQCRC2, a matrix facing subunit of complex III (Fig 5D and S2).

Figure 5: Comparison of mitochondrial protein O-GlcNAcylation profile following *in vitro* or *in vivo* OGA inhibition. **a)** The dataset obtained following *in vitro* exposure of isolated mitochondria to NButGT (#1) was compared to a methodologically comparable (i.e. same Click-iT labeling, IP and MS/MS workflow) cardiac O-glcnAcyloomic dataset derived from mice that were treated with NButGT or vehicle 6 h prior to sacrifice (#2). The *in vitro* reconstitution dataset and the *in vivo* dataset contained 409 and 350 mitochondrial proteins, respectively. The circular chart indicates the proportion of proteins that were shared or unique to a dataset. Hatched bar indicates shared proteins displaying sensitivity to NButGT. **b)** Venn diagram showing the overlap between the *in vitro* reconstitution assay, and data from the Ma et al. (Ma et al., 2015) study in which O-GlcNAc sites on isolated cardiac mitochondria were mapped 12 hours after *in vivo* administration of the OGA inhibitor Thiamet G. Fold enrichment in the actual vs expected number of shared hyper-O-GlcNAcyated proteins between both datasets is shown along with the hypergeometric p value. For this test, the number of shared hyper-GlcNAcyated protein was compared to the total number of mitochondrial proteins identified in both datasets. **c)** Number of shared proteins belonging to specific mitochondrial pathways/processes. **d)** Overview of OXPHOS and TCA cycle proteins found to be hyper O-GlcNAcyated in the *in vitro* reconstitution assay and the Ma et al. dataset.

1 To validate our O-GlcNAcyomic data, immunoprecipitation of O-GlcNAcylated proteins was
 2 performed on lysates from control and NButGT-treated mitochondria using an anti-O-GlcNAc
 3 antibody, and the resulting immunoprecipitates were probed with antibodies directed against
 4 several identified proteins including NDUFS1, ATP5A1, UQCRC2, MTCO1, and SDHB. As
 5 shown in Fig 6A, exposure to NButGT induced a strong increase in global mitochondrial O-
 6 GlcNAcylation, still conserved after immunoprecipitation. Furthermore, probing with the
 7 NDUFS1 antibody revealed a drastic increase in immunoreactivity following exposure to NButGT,
 8 without any changes in protein abundance (Fig 6B). Similarly, O-GlcNAc staining of ATP5A1,
 9 UQCRC2, MTCO1, and SDHB (using the antibody mix OXPHOS) was increased following
 10 immunoprecipitation with the anti-O-GlcNAc antibody. Similar results were obtained using
 11 cardiac lysates from mice injected with NButGT or vehicle 6 hours prior to sacrifice (Fig 6 A-B).

12 **Figure 6: Validation of mitochondrial protein O-GlcNAcylation following *in vitro* or *in vivo* OGA**
 13 **inhibition.** Mitochondrial and whole heart lysates used for experiment #1 and #2 depicted in Fig 5.A were
 14 submitted to immunoprecipitation using an anti-O-GlcNAc antibody. **a)** Immunoprecipitations were confirmed
 15 using an anti-O-GlcNAc antibody. **b)** Immunoprecipitate were then immunoblotted with anti-NDUFS1 or anti-
 16 OXPHOS set of antibodies.

2.4. Acute stimulation of O-GlcNAcylation enhances maximal electron flux through a complex I-driven mechanism.

To determine whether such acute stimulation of O-GlcNAcylation had a functional impact, mitochondria were pre-incubated with NButGT or vehicle during 30 min before monitoring basal, ADP stimulated and CCCP-uncoupled respiration (for the reader's convenience, the different substrates and inhibitors with action sites is represented in Fig 7A). As shown in Fig 7B-D, exposure to NButGT increased maximal ADP-stimulated respiration in presence of substrates feeding complex I (pyruvate – malate). A similar effect was observed when phosphorylation was uncoupled from respiration using CCCP (Fig 7E-G), which indicated that activation of the electron transport chain (ETC), rather than stimulation of the ATP synthase was responsible for the rise in maximal respiration observed with NButGT. Interestingly, NButGT had no significant effect on ADP-stimulated respiration when mitochondria were energized with complex II substrate (succinate in presence of rotenone), suggesting that the stimulatory effect of NButGT was linked to complex I (Fig 7H). This was directly confirmed by measuring the activity of respiratory chain complexes in mitochondrial lysates. Following exposure to NButGT, the activity of complex I was increased by ~ 50%, while those of complex II, complex IV and the TCA cycle enzyme citrate synthase were unchanged (Fig 7I-J). Of note, respirometry experiments were also performed in presence of the other O-GlcNAc inducer Thiamet G (also an OGA inhibitor), and yielded comparable results (Fig S3). Altogether, these data provided evidence that O-GlcNAcylation increases maximal electron flux through complex I. Furthermore, since the complex I assay measures electron transport from NADH to ubiquinone via the FMN and Fe-S redox centers (Rodenburg 2016), these results provided evidence that the putative O-GlcNAcylation sites underlying this stimulatory effect were located in the N module of complex I.

Figure 7: Impact of acute stimulation of mitochondrial O-GlcNAcylation on respiratory function. a) Schematic illustration of the different substrates and inhibitors with their action sites. Following pre-incubation with UDP-GlcNAc in absence or presence of NButGT, mitochondria were transferred to respirometry chambers for the recording of baseline state 2 (St. 2), maximal ADP-stimulated (ADP), and CCCP uncoupled respiration in presence of complex I (Pyruvate-Malate [Pyr-Mal]) or complex II (Succinate in presence of the complex I inhibitor rotenone [Succ-Rot]) substrates. For all experiments, control and NButGT-treated mitochondria were tested in parallel, allowing pairwise comparisons. Panels **b** and **e** show representative respirometry tracings. Panels **c**, **f** and **h** show the calculated means $\pm$ sem for each respiratory state in the two experimental groups. Panels **d** and **g** illustrate the effect of NButGT on ADP stimulated (D) or CCCP uncoupled respiration (G) for each of the paired incubations (3 biological replicates with 2-3 technical replicates per group). **i)** Enzyme activity of complex I, II, IV (CI, CII, and CIV) and citrate synthase (CS) measured in spectrophotometrically in whole mitochondrial lysates. Data is represented as means $\pm$ sem (3 biological replicates, 2 technical replicates). **j)** Effect of NButGT on complex I activity for each of the paired incubations performed. Statistical comparisons were made using paired two-sided t tests. *: $p < 0.05$, **: $p < 0.01$.

2.5. Acute stimulation of O-GlcNAcylation attenuates mitochondrial ROS release

To determine whether this had an impact on ROS release, H₂O₂ production was determined in mitochondria that were pre-incubated with NButGT, Thiamet G or vehicle. H₂O₂ release was first measured at baseline in presence of pyruvate and malate. As represented in Fig 8A, under this condition electron flux through the ETC is in the forward direction, and ROS mainly originate from the N module of complex I (*i.e.* the FMN and Fe-S clusters of complex I (IF site); for extensive review see (Brand 2010)). H₂O₂ release under this condition was decreased by more than 40-50% in NButGT (Fig 8B-C) or Thiamet G-treated (Fig S4) mitochondria compared to controls.

The complex II substrate succinate was next added to elicit a dual influx of electron in the ETC, and to stimulate reverse electron transport (RET) from complex II to complex I. Under this condition, a large proportion of ROS originates from the Q module of complex I (IQ) as a result of RET (Fig 8A) (Brand 2010). To a lesser extent, ROS release through the IF site within the N module can also contribute, as RET in presence of pyruvate-malate increases the reduction state of FMN clusters and promotes electron leakage (Goncalves 2015). Interestingly, no difference in H₂O₂ release was observed between control and NButGT (Fig 8B-C) or Thiamet G-treated (Fig S4) mitochondria under this condition, which altogether suggested that the stimulation of O-GlcNAcylation attenuates ROS release mainly at the IF site but has limited effects on the IQ site.

To test this further, mitochondria were progressively depolarized with the uncoupler CCCP since RET, and therefore ROS release by the IQ site, is exquisitely sensitive to the electrochemical gradient (Goncalves 2015). As expected, abolishing RET through uncoupling caused a drastic reduction of H₂O₂ release from the IQ site, and restored the 40-50% difference in H₂O₂ release observed between control and NButGT- or Thiamet G-treated mitochondria in absence of RET (*i.e.* when pyruvate-malate drive ROS release at the IF site) (Fig 8B-C and S3).

Figure 8: Impact of acute stimulation of mitochondrial O-GlcNAcylation on ROS release and sensitivity to Ca²⁺-induced permeability transition. **a)** Schematic illustration of the different substrates and inhibitors with the electron flux. Following pre-incubation with UDP-GlcNAc in absence or presence of NButGT, mitochondrial H₂O₂ release was measured following sequential addition of the complex I substrates pyruvate-malate (Pyr-Mal), the complex II substrate succinate (Succ), the uncoupler CCCP, and the complex III inhibitor Antimycin-A (Ant-A). For all experiments, control and NButGT-treated mitochondria were tested in parallel, allowing pairwise comparisons **b)** Representative Amplex Red fluorescence tracing of control and NButGT treated mitochondria. **c)** Rate of H₂O₂ emission calculated under the various respiratory states in two experimental groups. Data are represented as means ± sem (4 biological replicates with 2 technical replicates per group). **d)** Sensitivity to Ca²⁺-induced permeability transition pore opening was measured by measured swelling at 540 nm. A t=0 sec CaCl₂ (150 μM) was added to mitochondria energized with pyruvate and malate and absorbance changes were monitored during 60 min. Data is represented as means ± sem (3 biological replicate with 2-3 experimental replicates per experimental group). Multiple t-tests were used to establish statistical significance. *: p<0.05, corresponding to q value of <0.1.

The electron-transferring flavoprotein ubiquinone reductase (ETFQOR) site within complex III represents another major site of ROS production in the ETC. In isolated mitochondria, this site produces superoxides at high rates only when electron transfer is blocked with complex III inhibitors (Fig 8A) (Goncalves 2015). For this reason, antimycin-A was next added to fully uncoupled mitochondria in order to assess the impact of NButGT on ROS release from complex III. As expected, addition of antimycin-A caused a drastic rise in H₂O₂ release reflecting superoxide release from complex III in all groups (Fig 8B-C). In NButGT-treated mitochondria, H₂O₂ release was reduced by 30% compared to controls suggesting reduced release of ROS from the ETFQOR site (Fig 8B-C). However, this effect was not observed in presence of Thiamet G (Fig S4), which suggests that the main site of action of acute hyper O-GlcNAcylation on ROS production remains complex I.

Of note, previous studies reported that increased cellular O-GlcNAcylation protects from mitochondrial permeability transition pore (mPTP) opening (Ngoh 2011, Ma 2015). Mitochondrial swelling assays were therefore performed to determine whether sensitivity to Ca²⁺-induced mPTP opening was altered following acute exposure to NButGT. As shown in Fig 8D, although proteins associated with the regulation of the mPTP were found to be O-GlcNAcylated (see Fig. 8D), acute inhibition of mitochondrial OGA had no effect on the sensitivity to permeability transition.

3. DISCUSSION

O-GlcNAcylation of mitochondrial proteins was previously shown to be responsive to inhibition of O-GlcNAc cycling enzymes (OGT/OGA) (Banerjee 2015, Tan 2017) as well as to altered intracellular UDP-GlcNAc levels (Zhao 2014). However, controversy existed regarding the precise mechanism leading to mitochondrial protein O-GlcNAcylation. Moreover, the repertoire of proteins affected as well as the functional impact remained ill-defined. Taking advantage of our

1 *in vitro* reconstitution assay, which allows to isolate mitochondria from non-mitochondrial O-
2 GlcNAc cycling systems, our study shows that O-GlcNAc cycling enzymes are present in
3 functionally relevant amounts in the mitochondrial compartment and can trigger broad and rapid
4 changes in protein O-GlcNAcylation which are highly reminiscent of the mitochondrial O-
5 GlcNAcylation profile observed *in vivo*. Our proteomic workflow confirms previously reported O-
6 GlcNAc-modified proteins, and identifies several novel targets related to energy metabolism, and
7 multiple other facets of mitochondrial biology. Importantly, we show that acute hyper-O-
8 GlcNAcylation increases maximal respiratory capacity, and drastically reduces ROS likely through
9 a complex I-mediated mechanism.

10 The OGT gene encodes three splice variants whose products vary only in the number of N-
11 terminal tetratricopeptide repeat (TPR) domains known to be involved in protein-protein
12 interaction (Bond 2015). The longest splice variant encodes the 116 kDa nucleocytoplasmic
13 ncOGT isoform which is the most abundantly expressed, while the shortest 78 kDa isoform is
14 curiously derived from a longer transcript. In addition, Hanover's group identified a unique start
15 site in the fourth intron of the OGT gene that generates a 103 kDa isoform which was found to be
16 enriched in the mitochondrial fraction of Hela cells (Hanover 2003, Love 2003) and rat heart
17 (Banerjee 2015). However, the existence of this mitochondrial isoform in mammalian tissues has
18 been recently questioned (Trapannone 2016). In this study, endogenous mOGT was reported to be
19 undetectable in several human cell lines and mouse tissues, including heart. Genomic sequence
20 alignments also suggested that the predicted start site for mOGT was likely lacking in most species
21 analyzed except some primates. Based on these data, the authors concluded that the small amounts
22 of ncOGT detected in the crude mitochondrial fraction was likely sufficient for O-GlcNAcylation
23 of mitochondrial proteins. These results are however in contrast with our data and previous studies
24 from Hanover's group. Our data clearly indicate that the 103 kDa mOGT is the main isoform found

1 in Percoll-purified cardiac mitochondria. Comparison between crude and Percoll-purified
2 mitochondrial preparations in fact suggests that the small amount of ncOGT found in the crude
3 fraction is an extra-mitochondrial contaminant. Beside mOGT, our results also establish that the
4 nuclear-predicted sOGA isoform (Comtesse 2001) is the sole OGA isoform present in
5 mitochondria. The fact that inhibition of OGA with NButGT specifically increased O-
6 GlcNAcylation of mitochondrial proteins, without affecting non-mitochondrial proteins remaining
7 in the reconstitution assay, provides further support for the mitochondrial localization of sOGA.

8 Isolated mitochondrial preparations have been used previously to study UDP-GlcNAc uptake
9 kinetics, which has led to the identification of the pyrimidine nucleotide carrier (SLC25A33) as the
10 main transporter for UDP-GlcNAc (Banerjee 2015). However, the functional coupling between the
11 uptake of UDP-GlcNAc and the intramitochondrial O-GlcNAc cycling enzymes has never been
12 examined. Our data suggest that UDP-GlcNAc is transported in mitochondria in sufficient amounts
13 to allow changes in protein O-GlcNAcylation level by NButGT treatment. Using our reconstitution
14 assay, we were able to map a large proportion of previously identified O-GlcNAcylated proteins
15 (Ma 2015) involved in energy metabolism. Collectively these results confirm that proteins of the
16 OXPHOS machinery, TCA cycle, and fatty acid metabolism pathways are prominent targets for
17 O-GlcNAcylation. In addition, we identified several other proteins, including proteins involved in
18 mitochondrial protein translation, protein import/chaperoning, and ROS/Redox homeostasis.
19 Future studies may reveal a role for these proteins in the cardioprotective effect of hyper-O-
20 GlcNAcylation in the context of ischemic injury (Jensen 2019) and sepsis (Ferron 2019, Silva
21 2019).

22 Although O-GlcNAcylation of mitochondrial proteins has been associated with changes in
23 function such as respiration, ROS release, and Ca^{2+} -induced permeability transition (Zhao 2016),
24 the relationship between these changes and O-GlcNAcylation of specific proteins remains elusive.

1 By combining *in vitro* functional assays and O-GlcNAcyomic studies, we have obtained evidence
2 that O-GlcNAcylation of the N module in the peripheral arm of complex I may play an important
3 regulatory role. This module consists of several core catalytic subunits containing the FMN
4 prosthetic groups, the iron–sulfur clusters, as well as several accessory subunits which altogether
5 oxidize NADH and transfer electrons to the Q and P modules to support proton pumping. Our O-
6 GlcNAcyomic data revealed that subunits within this module displayed greater increases in O-
7 GlcNAcylation compared to other complex I subunits. Similarly, in the Ma *et al.* (Ma 2015) dataset,
8 subunits displaying the highest fold change following *in vivo* treatment, with Thiamet G were
9 located in the N module and two in particular were common to our dataset, namely NDUFS1 and
10 NDUFA7. It is interesting to note that NDUFS1 harbors an NADH-ubiquinone oxidoreductase
11 domain and Fe-S clusters, in addition to containing a number of phosphorylation (Gowthami 2019)
12 and acetylation sites, which makes it a potentially important candidate for post-translational
13 regulation of complex I activity. As for NDUFA7, recent data indicate that its ablation in the heart
14 increases mitochondrial ROS release, and triggers pathological cardiac hypertrophy (Shi 2020),
15 although it is still unclear whether this is linked to failed assembly of complex I or to altered
16 activity. It should also be noted that phosphorylation of four other subunits within the N module
17 (NDUFS4, NDUFV1, NFUFV3 and NDUFA12), including two that displayed sensitivity to
18 NButGT, were previously shown to regulate complex I activity and ROS release (Papa 2001, Lund
19 2008, Wang 2014). Further studies will clearly be required to fully elucidate the mechanisms by
20 which acute O-GlcNAcylation stimulates complex I.

21 Although *in vitro* reconstitution assays are well suited for mechanistic studies, they pose
22 obvious limitations as they do not fully mimic the complex conditions encountered *in vivo*. To
23 tackle this issue, we also compared our *in vitro* data with a methodologically comparable cardiac
24 O-GlcNAcyomic dataset derived from mice injected with NButGT for 6 h before cardiac isolation.

Our results reveal a strikingly high degree of overlap (85%) in the O-GlcNAc modified mitochondrial protein identified in the two datasets. Importantly, 122 of these proteins also displayed sensitivity to NButGT. Besides, highlighting the relevance of our reconstitution assay for mechanistic studies, these results highly suggest that the intra-mitochondria O-GlcNAc cycling system is the main mechanism through which mitochondrial proteins become O-GlcNAcylated *in vivo*.

4. METHODS

4.1. Animal care

For *in vitro* studies, all experiments on animals were approved by the University of Ottawa Institutional Animal Care Committee and conducted according to the directives of the Canadian Council on Animal Care. Rats were euthanized by thoracotomy following ketamine-xylazine anesthesia. For *in vivo* dataset, experiments were approved by the Animal Research Committee of the Université catholique de Louvain and conformed to the American Heart Association Guidelines for Use of Animal in Research. All animals, housed with a 12 h/12 h light/dark cycle, had free access to water and standard chow.

4.2. Preparation of isolated cardiac mitochondria

Heart mitochondria were prepared as described previously (Marcil 2006). Hearts were rapidly excised and immersed into ice-cold isolation medium (buffer A, in mM: 300 sucrose, 10 Tris-HCl, 1 EGTA, pH 7.3) and weighed. Ventricular tissue was minced with scissors in 5 ml of buffer A supplemented with 0.2% fatty acid free bovine serum albumin (BSA) and homogenized using a Polytron tissue tearer (~ 3 s at a setting of 3). The homogenate was then incubated with the protease Nagarse (1.5 mg/g) for 5 min and further homogenized at the same settings. The homogenate

1 volume was completed to 30 ml with Buffer A+ 0.2% BSA and centrifuged at 800×g for 10 min.
2 The pellet was discarded and the supernatant was decanted and centrifuged at 10 000×g for 10 min.
3 The pellet obtained was re-suspended in buffer B (in mM: 300 sucrose, 0.05 EGTA, 10 Tris-HCl,
4 pH 7.3) and centrifuged at 10,000×g for 10 min. After repeating this washing step twice, the final
5 mitochondrial pellet was re-suspended in 0.3 ml of buffer B to a protein concentration of ~ 20
6 mg/ml. All procedures were carried out at 4°C. Protein determinations were performed using the
7 bicinchonic acid method (Pierce, Rockford, IL, USA), with bovine serum albumin as a standard.

8 9 **4.3. *In vitro* reconstitution assay**

10 Mitochondria (2 mg/mL) were incubated at room temperature during 30 min in buffer C (in
11 mM: 250 sucrose, 10 MOPS, 0.005 EGTA, 2 KH₂PO₄, 0.2 MgCl₂, pH 7.2) containing UDP-
12 GlcNAC (2 mM) in absence or presence of the OGA inhibitor NButGT (10 μM). In some
13 experiments NButGT was replaced by Thiamet G (5 μM). After 30 min, mitochondrial suspensions
14 were used for functional analyses. Alternately, samples were centrifuged at 10,000×g for 10 min.
15 20 μL of supernatant was left and concentrated mitochondrial pellets were immediately frozen in
16 liquid nitrogen for MS/MS analysis.

17 18 **4.4. *Respirometry***

19 Mitochondria (0.5 mg of protein) were incubated at room temperature in 1 ml of buffer C.
20 After recording baseline state oxygen consumption, ADP-restricted state 2 respiration was
21 measured in presence of substrates for complex I (Pyruvate-Malate 5: 2.5 mM) or complex II
22 (Succinate + Rotenone 5 mM: 1μM). ADP (1mM) or CCCP (0.2 and 1 μM) was added to elicit
23 maximal ADP-stimulated or uncoupled respiration respectively (Marcil 2006).

1 **4.5. Mitochondrial H₂O₂ release**

2 Net H₂O₂ release by respiring mitochondria was measured fluorimetrically using the H₂O₂
3 sensitive probe Amplex red (excitation-emission: 563-887 nm) as previously described (Ascah
4 2011). Mitochondria (0.5 mg/mL) were incubated in 600 µL of buffer Z at 37°C (in mM: 110 K-
5 Mes, 35 KCl, 1 EGTA, 5 K₂HPO₄, 3 MgCl₂·6H₂O and 0.5 mg mL⁻¹ BSA, pH 7.3 at 4°C) containing
6 KH₂PO₄ (2 mM), MgCl₂ (0.2 mM), HRP (1.2 U/mL), Amplex Red (5 µM). Baseline fluorescence
7 readings were taken in the absence of any exogenous respiratory substrates. The following
8 additions were then made sequentially: Pyruvate-malate (5:2.5 mM), succinate (5 mM), CCCP
9 (0.2, 0.4, 0.6, 0.8, 1.0 µM) and Antimycin-A (8 µM). Rates of H₂O₂ release were calculated by
10 measured the slopes of change in Amplex red fluorescence and reported in arbitrary fluorescence
11 units.

13 **4.6. Enzyme activities**

14 Activities of complex I (NADH-CoQ reductase), complex II (succinate dehydrogenase),
15 complex IV (cytochrome oxidase), and citrate synthase were measured spectrophotometrically in
16 a plate reader using standard coupled enzyme assays adapted from (Marcil 2006). Activities were
17 expressed in mU.min⁻¹.mg mitochondrial prot⁻¹.

19 **4.7. In vivo NButGT treatment**

20 Twelve week-old mice (C57BL/6 N, male) from Janvier Labs were treated with NButGT
21 (50 mg/kg) by intraperitoneal injection 6 hours before sacrifice. Mice were anesthetized with an
22 intraperitoneal injection of a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg) and hearts
23 were washed in PBS before being freeze-clamped in liquid nitrogen and stored at -80°C.

4.8. O-GlcNAc immunoprecipitation & immunoblotting

Lysate supernatants (20 µg of heart homogenate, nuclear fraction, cytosolic fraction and crude mitochondria; 40 µg of purified mitochondria) were loaded on SDS-PAGE gel and transferred onto polyvinylidenedifluoride (PVDF) membrane. After blocking in BSA 5% TBS-Tween 20 0.1%, membranes were then probed with appropriate antibodies to assess total protein level. The appropriate secondary antibody conjugated to HRP and the BM chemiluminescence blotting system (Roche Molecular Systems, Bale, Switzerland) were used for detection. Antibodies used for these experiments are as follows: OGT (Cell Signaling Technology Inc. Danvers, Massachusetts, United States), OGA (Santa Cruz Biotechnology, Dallas, Texas, United States), Alpha tubulin (ThermoFisher Scientific Inc., Waltham, Massachusetts, United States), TOM20 (ThermoFisher), Histone 3 (Cell Signaling), OXPHOS (Abcam), NDUFS1 (Proteintech, Rosemont, Illinois, United States) and O-GlcNAc-HRP (Abcam). The uncropped version of all the blots presented in main figures can be found in Supplementary Figs S5-6.

For the immunoblotting of specific mitochondrial proteins after O-GlcNAc immunoprecipitation, 250 µg (isolated mitochondria) or 500 µg (total heart homogenate) of protein samples were immunoprecipitated with anti-O-GlcNAc RL2 antibody (1 µg, Abcam) overnight at 4°C following pre-clearing of the lysate with pre-washed protein G Sepharose beads. After three washing with TBS (50 mM Tris-Cl, 150 mM NaCl at pH 7.6), immunoprecipitated O-GlcNAc proteins were eluted with Laemmli buffer and boiled 10 min at 100°C. Protein separation was performed by SDS-PAGE and immunoblotting was realised as mentioned above with NDUFS1 or OXPHOS antibodies.

4.9. Mass spectrometry and protein identification

Preparation of proteins – Isolated mitochondria pellets or 20 mg of freeze-clamped hearts were homogenized in 200 μ L of RIPA lysis buffer (25 mM Tris HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS at pH 7.6) supplemented with a protease/phosphatase inhibitor cocktail (ThermoFisher) and 1 μ M of O-GlcNAc cycling enzyme inhibitors (Sigma-Aldrich, Saint-Louis, Missouri, United-States). 250 μ g of proteins from the lysate were then precipitated using chloroform/methanol (MeOH) precipitation method as follows. 600 μ L of MeOH were added to the 200 μ L sample, followed by 150 μ L of chloroform and 400 μ L of 18 megaOhm water. Samples were then vortexed briefly and centrifuged for 5 min at 13,000 $\times$ g. The upper aqueous phase was carefully removed and discarded. Additional 450 μ L MeOH were added to pellet the protein after brief vortex and centrifugation for 5 min at 13,000 $\times$ g. The supernatant was then removed, and the pellet air dried for 5 min. Finally, proteins were resuspended in 40 μ L of 1% SDS in 20 mM HEPES pH 7.9 and heated 5-10 min at 90°C to assure completely resuspension of proteins.

Enzymatic labelling and purification of O-GlcNAcylated proteins – O-GlcNAc groups from proteins were labelled with tetramethylrhodamine azide (TAMRA) using the Click-iT® O-GlcNAc enzymatic labelling system kit (C33368) followed by the Click-iT® protein analysis detection kit (C33370) from Invitrogen according to the manufacturer's instructions. SDS was then quenched with NEFTD buffer (100 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 6% NP-40 at pH 7.4). Before immunoprecipitation of TAMRA labelled proteins, lysate was precleared with washed protein G sepharose beads to avoid non-specific binding of proteins on the beads. Afterwards, supernatant was incubated with pre-washed protein G sepharose beads (10 μ L) coupled with anti-TAMRA antibody (10 μ g, A6397, Invitrogen) for 1.5 h at 4°C. Following centrifugation (500 $\times$ g, 1 min), the beads were washed once with NEFTD buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM

1 EDTA, 6% NP-40) and three times with NEFT buffer (NEFTD without NP-40). The beads were
2 then boiled 5 min in Laemmli buffer (2 mM EDTA, 4% SDS, 20% Glycerol, 0.004% bromophenol
3 blue, 50 mM DTT and 100 mM Tris at pH 6.8) to elute O-GlcNAc proteins. Proteins were then
4 separated on 1 mm on SDS-PAGE gel and stained with Coomassie blue (Sigma-Aldrich).

5 *In-Gel Digestion and identification of captured O-GlcNAc Proteins* – Gels were cut in seven
6 bands of equal size and in-gel digested with trypsin. Peptides separation was performed using a
7 C18 reversed-phase analytical column (Thermo Scientific) on an Ultimate 3000-nLC RSLC
8 system. The peptides were subjected to Nano-Spray-Ionization source followed by tandem MS/MS
9 in a tribrid Fusion Lumos Orbitap analyser coupled online to the nano-LC. Spectra were acquired
10 by a data dependent scan routine with ion precursor detection in the Orbitrap and daughter ions in
11 the Iontrap. The resulting MS/MS data were processed using Sequest HT search engine within
12 Proteome Discoverer 2.4 against a rat protein database obtained from Uniprot (29 953 entries).
13 Trypsin was specified as cleavage enzyme allowing up to 2 missed cleavages, 4 modifications per
14 peptide and up to 5 charges. Mass error was set to 10 ppm for precursor ions and 0.1 Da for
15 fragment ions. Oxidation on methionine, carbamidomethyl on cysteine were considered as variable
16 modifications. False discovery rate (FDR) was assessed using Percolator and thresholds for protein,
17 peptide and modification site were specified at 1%. The filtered Sequest HT output files for each
18 peptide were grouped according to the protein from which they were derived and abundance was
19 evaluated by label-free quantification within Proteome Discoverer from area under the curve of
20 MS1 intensities. Following such procedure, we were able to identify 2534 putative O-
21 GlcNAcylated proteins.

22 *LC-MS/MS analysis* – The analysis was conducted in R (R Core Team, 2020). The expression
23 values were log2 transformed but not subjected to normalization given the data acquisition
24 technique involving an enrichment of O-GlcNAc proteins prior to MS analysis. To maximize

stringency, only proteins reliably detected in all experimental replicates (n=4) from control and NButGT-treated mitochondria were considered. Of the 842 proteins identified, mitochondrial proteins (409) were selected according to their mitochondrial status from the Mitominer database. Prior to differential expression analysis and based on exploratory analyses, NButGT-treated replicate n°3 was removed as it did not respond to treatment (assessed by O-GlcNAc immunoblotting). The differential expression between control and NButGT-treated groups was statistically assessed through linear models using empirical Bayes methods for variance modelling, as implemented in the R/Bioconductor limma package (Ritchie et al., 2015; Phipson et al., 2016) where the group effect was the only one included in the models. P-values for the group effect were adjusted for multiple testing with the Benjamini-Hochberg FDR procedure (Benjamini & Hochberg, 1995). Proteins having a group effect with an adjusted p-value (q) < 0.1 and $q < 0.05$ were arbitrary considered differentially expressed between control and NButGT-treated mitochondria with either high or very high confidence score, respectively.

4.10. Pathway analysis and bioinformatics

To identify the pathway annotations of the mitochondrial O-GlcNAcylome, the list of proteins displaying significantly increased O-GlcNAcylation was ranked according to q values and uploaded into the g:Profiler (Reimand 2007) platform, and an ordered query was performed using the *Rattus norvegicus* database. KEGG and REACTOME pathway terms annotating 350 proteins or less were considered in order to filter out large annotations that provide limited interpretative value (Reimand 2019). The g:SCS (shortest common superstring) algorithm was used for multiple hypothesis testing corrections using a default *alpha* threshold of 0.05 (Reimand 2007). The ggplot2 R package was used to generate bubble plots in which pathway enrichments were expressed as Rich Factors, which represents the ratio of the number of proteins observed for a given pathway

term to the total number of proteins for this term. The Cytoscape (version 3.8.0) stringApp plugin was used to import protein-protein interaction data from STRING (Doncheva 2019). For this analysis, an interaction score of 0.7 (high confidence based on default active interaction sources) was set as minimum. Clustering of the STRING network was performed with the Markov Cluster (MCL) algorithm with a granular parameter set at 4. The Auto-annotate plugin (Kucera 2016) of Cytoscape was used to identify pathways/processes corresponding to these protein clusters based on Stringdb description and GO annotations. To compare overlap between distinct O-GlcNAcylic datasets, Venn diagrams were generated using the Eulerr R Package. Hypergeometric tests were used to determine the statistical significance of the actual vs expected number of shared hyper-O-GlcNAcylic proteins between datasets.

4.11. Statistics and reproducibility

For functional analyses, values are reported as mean $\pm$ sem for a minimum of 3 biological replicates, with 2-3 experimental replicates per experimental groups. Data are graphically represented as histograms. Paired two-sided t tests (GraphPad Prism 8.4.3) were used to determine statistical difference when two means were compared, with a significance threshold set at $p < 0.05$.

For proteomics, values reported were obtained from 3-4 biological replicates. Data are depicted as volcano plots or histograms of mean Log2 FC $\pm$ sem with values for individual proteins represented by dots. Difference in the abundance of individual proteins between groups was determined by linear models using empirical Bayes methods for variance modelling. For each p value obtained, a corresponding FDR was calculated according to the Benjamini and Hochberg method. Proteins having an adjusted p-value (q) < 0.1 and $q < 0.05$ were arbitrary considered differentially expressed between control and NButGT-treated mitochondria with either high or very high confidence score, respectively (Supplemental table 1).

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6. CONFLICTS OF INTEREST/COMPETING INTERESTS

The authors declare no competing interests.

7. AVAILABILITY OF DATA AND MATERIAL

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier reviewer_pxd026495@ebi.ac.uk. Requests for access to other data should be addressed to senior authors: Yan Burelle (yburell2@uottawa.ca) and Luc Bertrand (luc.bertrand@uclouvain.be). All requests will need to specify how the data will be used and will require approval by co-investigators.

8. AUTHORS' CONTRIBUTIONS

YB and LB had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the analyses; Concept and design: LuB, LB, YB; Acquisition, analysis,

or interpretation of data: JD, LBU, DV, AB, FD, AC, RR; Drafting of the manuscript: JD, LBU, LB, YB; Critical revision of the manuscript for important intellectual content: SH, CB, BL, LG, DV; Statistical analysis: AB, FD, RR, JD, MM, LG; Obtained funding: SH, CB, BL, LB, YB. All the authors have read the manuscript and approved its submission.

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10. SUPPLEMENTALS FIGURES

Figure S1: Investigation of non-mitochondrial protein O-GlcNAcylation following OGA inhibition. Volcano plot analysis showing the impact of NButGT O-GlcNAcylation for proteins with a non-mitochondrial status on the Mitominer database. Statistical analysis was assessed using a linear regression model (empirical Bayes methods) followed by the Benjamini-Hochberg FDR procedure.

a)

b)

Figure S2: Investigation of mitochondrial protein O-GlcNAcylation following OGA inhibition. a) Fold change in the abundance of O-GlcNAc-modified proteins between control and NButGT-treated mitochondria according to their localization in the different nodules of the Complex I. **b)** STRING network of mitochondrial proteins displaying increased O-GlcNAcylation in response to NButGT in the present study and in the study by Ma *et al.* (Ma *et al.*, 2015). Clustering was performed with the Markov Cluster (MCL) algorithm with a granular parameter set at 4. The Auto-annotate function of Cytoscape was used to identify pathways/processes corresponding to these clusters based on Stringdb description and GO annotations. Nodes were color coded according to the q values observed in the present study, while borders were color coded assorting to FC in O-GlcNAcylation observed in the Ma *et al.* study.

Figure S3: Impact of Thiamet G-induced mitochondrial O-GlcNAcylation on respiratory function.

Following pre-incubation with UDP-GlcNAc in absence or presence of Thiamet G (5 μ M), mitochondria were transferred to respirometry chambers for the recording of baseline state 2, maximal ADP-stimulated (ADP), and CCCP uncoupled respiration in presence of complex I (Pyruvate-Malate [Pyr-Mal]) or complex II (Succinate in presence of the complex I inhibitor rotenone [Succ-Rot]) substrates. For all experiments, control and Thiamet G-treated mitochondria were tested in parallel, allowing pairwise comparisons. Panels **a** and **d** show representative respirometry tracings. Panels **b**, **e** and **g** show the calculated means $\pm$ sem for each respiratory state in the two experimental groups. Panels **c**, **f** and **h** illustrate the effect of Thiamet G on ADP stimulated (F-H) or CCCP uncoupled respiration (H) for each of the paired incubations (3 biological replicates with 1-3 technical replicates per group).

Figure S4: Impact of acute Thiamet G-induced mitochondrial hyper O-GlcNAcylation on mitochondrial ROS release. Following pre-incubation with UDP-GlcNAc in absence or presence of Thiamet G, mitochondrial H₂O₂ release was measured following sequential addition of the complex I substrates pyruvate-malate (Pyr-Mal), the complex II substrate succinate (Succ), the uncoupler CCCP, and the complex III inhibitor Antimycin-A (Ant-A). For all experiments, control and Thiamet G-treated mitochondria were tested in parallel, allowing pairwise comparisons. **a)** Representative Amplex Red fluorescence tracing of control and Thiamet G treated mitochondria. **b)** Rate of H₂O₂ emission calculated under the various respiratory states in two experimental groups. Data are represented as means $\pm$ sem (4 biological replicates and 2 technical replicates per group). Multiple t test were used to establish statistical significance. *: p<0.05, corresponding to q value of <0.1.

Figure S5: Unprocessed images of membranes used for immunoblots presented in Fig 1A.

Figure S6: Unprocessed images of membranes used for immunoblots presented in Fig 6A-B.

Table S1 : Comparative analysis of O-GlcNAcylated proteins following in vitro or in vivo NButGT treatment. List of O-GlcNAcylated proteins identified obtained following in vitro exposure of isolated mitochondria to NButGT (#1) compared to cardiac O-glcNAcylomic dataset derived from mice treated with NButGT (#2). Dark blue cells highlight proteins with FC>1,5 (Log2FC>0,58); medium FC>1,2 (Log2FC>0,26) and light blue FC>1 (Log2FC>0).

#1 (in vitro)			#2 (in vivo)
Gene	AdjPval	Log2FC	Log2FC
Mlycd	0,036219	5,0542383	-0,22302367
Mrpl38	0,037828	4,5266909	-0,05239726
Tst	0,037828	4,4831217	-0,48521952
Gpx1	0,037828	4,3583643	0,03593602
Rtn4ip1	0,037828	4,3249218	0,51133591
Abcb8	0,037828	4,3111377	0,01807561
Nudt8	0,037828	4,1995844	-0,29416556
Ndufaf6	0,036219	4,1436254	0,31476548
Clybl	0,037828	3,9171971	0,00199168
Vwa8	0,037828	3,9128006	1,11277804
Mrpl15	0,036219	3,9010537	0,83132887
Fahd2a	0,036219	3,8808771	NA
Cyb5r3	0,037828	3,7295167	0,27592267
Oxa1l	0,037828	3,6297092	0,38995933
Clpp	0,037828	3,597414	0,14744399
Kat3	0,037828	3,4817683	NA
Nlrx1	0,037828	3,4501217	0,41551219
Rpl4	0,077144	3,3302439	-0,13896196
Higd1a	0,037828	3,3194459	1,06498434
Tmem65	0,037828	3,316931	0,53950296
Nit2	0,037828	3,3030395	0,03877761
Grpel1	0,037828	3,2851366	0,03618385
Timm50	0,037828	3,2645057	0,19863208
Letm1	0,037828	3,2629061	0,59281113
Aldh7a1	0,037828	3,2553931	NA
Ndufa8	0,041421	3,2462646	0,31652351

Lars2	0,054815	3,2389534	0,28823996
Auh	0,037828	3,2363892	0,62162374
Ppa2	0,037828	3,2139048	-0,01256295
Timm44	0,037828	3,2081809	0,0156414
Coq7	0,037828	3,1155229	0,05132324
Gstk1	0,037828	3,1023771	-0,13063292
Hsd17b8	0,037828	3,0912617	-0,09032497
Pitrm1	0,037828	3,0655927	1,15560495
Sardh	0,037828	3,058349	NA
Aldh5a1	0,038248	3,0179449	0,19133749
L2hgdh	0,037828	3,009186	0,17858421
Mrps23	0,037828	2,9945447	0,25893335
Endog	0,037828	2,981828	-0,03212739
Mut	0,037828	2,976578	NA
Atp5s	0,037828	2,9663696	NA
Acad8	0,037828	2,9357018	-0,1873788
Eci1	0,037828	2,9244637	0,17686736
Fh	0,037828	2,9209375	0,46170735
Slc25a4	0,037828	2,911997	0,27092103
Pdp1	0,049901	2,9056392	0,05172957
Pc	0,037828	2,8987469	NA
lars2	0,037828	2,8971452	0,06725274
Mecr	0,050603	2,8961787	-0,13667286
Etfa	0,037828	2,8958899	0,12607806
Hadh	0,037828	2,8954573	0,3014456
Atp5f1	0,037828	2,8937595	0,22458568
Cox20	0,037828	2,8880063	-0,47764041
Pdk1	0,037828	2,884855	0,39079188

Acad9	0,037828	2,8827262	0,41086873
Atpaf2	0,037828	2,8768181	-0,36720423
Mpc1	0,037828	2,8688056	0,01603131
Slc25a3	0,037828	2,8623411	0,63611025
Acadvl	0,037828	2,854331	0,47709402
Ndufv2	0,037828	2,8420339	-0,17590218
Samm50	0,038248	2,8376604	0,35005203
Hagh	0,039989	2,8347035	0,13422073
Acot2	0,037828	2,8298966	0,23839452
Echs1	0,037828	2,8266167	0,10699377
Coq6	0,038248	2,8187113	0,1784542
Mtch2	0,037828	2,808325	0,31751727
Immt	0,037828	2,7990808	0,58477071
Mrps11	0,037828	2,7918282	NA
Apoo	0,037828	2,7886388	0,14772638
Ech1	0,037828	2,7816691	0,10129361
Opa1	0,037828	2,7675834	0,1445723
Ghitm	0,037828	2,7654367	-1,60828548
Nnt	0,037828	2,7628583	0,24674829
Hibch	0,037828	2,7505282	0,11236017
Mpc2	0,037828	2,7376082	0,63947703
Acsl1	0,037828	2,7342343	0,38325043
Coq9	0,037828	2,7290267	0,45296988
Etfb	0,037828	2,7286495	-0,02287712
Bckdhb	0,037828	2,7274874	0,21908694
Pdpr	0,037828	2,7263795	-0,85281616
Ndufs1	0,037828	2,7218487	0,89224158
Slc25a11	0,037828	2,7179829	0,52532758
Trap1	0,037828	2,715586	0,45402647
Ak3	0,041732	2,7147469	-0,15852255
Pdhh	0,037828	2,7054367	0,20862285
Bcl2l13	0,037828	2,6962728	0,33733687
Ppif	0,037828	2,6911446	0,1198819

Surf1	0,037828	2,6901814	NA
Hibadh	0,037828	2,6882363	-0,05041585
Txnrd2	0,038248	2,6870513	-0,2355221
Slc25a5	0,037828	2,6823906	0,31639927
Mtif2	0,041732	2,6815279	-0,21269366
Sod2	0,037828	2,6749182	0,00976056
Bcat2	0,038248	2,6568079	0,00804277
Bphl	0,041732	2,6560808	-0,09641509
Mrps22	0,044297	2,6519941	0,32721033
Nln	0,037828	2,6501502	-0,16074895
Mccc1	0,037828	2,6400616	0,25181383
Cpt2	0,037828	2,633844	0,18325402
Aldh6a1	0,037828	2,6282189	0,32464817
Acss1	0,037828	2,6274513	0,29624419
Sucg2	0,047975	2,6255502	0,09860447
Rhot1	0,037828	2,6178301	1,13652091
Ndufs8	0,037828	2,6154271	-0,18259464
Sfxn1	0,037828	2,6077182	NA
Hadha	0,037828	2,5933258	0,35682994
Decr1	0,037828	2,5900095	0,15330168
Cpt1b	0,037828	2,5895803	0,29122348
Dbt	0,041732	2,5887541	0,16063568
Coq3	0,037828	2,5883947	0,37208171
Idh3a	0,038248	2,5876519	-0,07228971
Cyb5r1	0,041732	2,587069	-0,17730635
Hk1	0,037828	2,5834625	0,21251284
Hsdl2	0,037828	2,5756094	0,3522163
Hspa9	0,037828	2,5718361	0,40142143
Ak2	0,038695	2,5661968	-0,11756827
Ckmt2	0,040097	2,5467931	0,18855786
Mgst3	0,037828	2,5442059	0,30337081
Coq5	0,037828	2,5427428	-0,02768279
Slc25a31	0,047975	2,5333906	0,28761551

Cyc1	0,037828	2,5197744	0,26347057
Pcca	0,037828	2,5150739	0,66340991
Gfm1	0,037828	2,5105962	0,40625412
Mcee	0,037828	2,5099253	0,83080365
Gcdh	0,041732	2,509225	0,45885963
Etfdh	0,037828	2,4974547	0,58959335
Aifm1	0,037828	2,4850375	0,93064635
Got2	0,039598	2,4801307	0,22565213
Atp5h	0,041732	2,4676246	-0,13152819
Gatd3a	0,037828	2,46485	NA
Mtx2	0,041732	2,4635693	0,051137
Pdhx	0,037828	2,4557953	0,49045684
Sdhc	0,041732	2,4545357	0,02139544
Dnaja3	0,04552	2,4460635	0,0697668
Mrps25	0,037828	2,4458793	NA
Uqcrc1	0,041104	2,4435604	0,10731751
Aco2	0,037828	2,4389872	0,26036869
Cox6c	0,054815	2,4350818	-0,16707584
Pdha1l1	0,041104	2,4229818	0,23488777
Idh3g	0,044297	2,4148195	0,30119423
Sdhb	0,038248	2,4077221	-0,15465072
Acaa2	0,041732	2,4061614	0,1056513
Ogdh	0,037828	2,4038714	0,24839054
Oxct1	0,041104	2,4002236	0,29295506
Slc25a12	0,038248	2,3888083	0,07517014
Mrpl58	0,039598	2,3718849	NA
Sucla2	0,041732	2,366532	0,0643007
Slc25a13	0,041732	2,3651175	0,14124044
Dlat	0,037828	2,3631739	0,29052914
Cox15	0,093831	2,3493059	-0,23037647
Tomm20	0,04102	2,3486184	0,65501152
Uqcrrf1	0,041732	2,3449079	0,21166798
Acat1	0,041732	2,3197278	0,12786179

Idh2	0,041732	2,3187883	0,24986006
Ndufc2	0,037828	2,304323	0,85115054
Glrx5	0,041732	2,3038867	NA
Ndufa5	0,041732	2,301498	0,23481069
Ndufa10	0,042443	2,3014062	0,27033212
Atp5f1a	0,042265	2,2911764	0,4171115
Dld	0,041732	2,28971	0,4167506
Acads	0,041732	2,2848622	0,16549508
Vdac1	0,040699	2,2845899	0,0024684
Pmpca	0,071467	2,2807939	0,44809247
Ndufb9	0,038695	2,2803266	0,18187443
Timm29	0,054815	2,2728878	-0,55238273
Ndufa11	0,041732	2,2639729	0,55154816
Eci2	0,049843	2,2639217	0,35790967
Uqcrh	0,081746	2,2626072	NA
Aldh2	0,041732	2,2595511	0,04998952
Ndufb5	0,039989	2,2582696	0,54116387
Mrpl28	0,05463	2,2562575	0,69232263
Acadm	0,042717	2,2500718	0,18328941
Atp5j2	0,041623	2,2482512	0,58941599
Mpst	0,05802	2,2356737	1,48322926
Sdha	0,041732	2,2226005	0,30111375
Mrpl22	0,041732	2,2209564	0,60004664
Sdr39u1	0,041732	2,2130011	-0,07179587
Suc1g1	0,044297	2,2122551	0,25203841
Slc25a20	0,041732	2,2110913	0,29244073
Atp5me	0,048428	2,2096749	1,03973947
Ndufb10	0,044297	2,2048793	0,10287517
Mtnd1	0,041932	2,186787	0,90557082
Mtco2	0,041732	2,1852467	-0,26468431
Dhrs4	0,056845	2,1707468	-0,00812237
Vdac2	0,041732	2,1500778	1,08210663
ATP6	0,044297	2,1484145	0,40081305

Acot13	0,040769	2,13823	0,27044663
Tmem256	0,040014	2,1350548	-0,24058718
Ndufv1	0,053886	2,1339514	0,24384278
Hint2	0,041732	2,1314132	0,80723543
Prdx3	0,056845	2,1238232	-0,05107579
Tufm	0,061548	2,1150426	0,11488558
Ndufs2	0,05463	2,111338	0,21991198
C1qbp	0,077144	2,1052803	-0,10425796
Ndufs3	0,047975	2,1002756	-0,01965294
Acadl	0,049879	2,0994742	0,23963326
Nipsnap2	0,048428	2,0838596	-0,08071942
Vdac3	0,049901	2,0776962	0,28015942
Mtnd5	0,049843	2,0670821	0,05824992
Ndufs5	0,042398	2,0586188	0,62519793
Ndufa9	0,049706	2,0585522	1,26823594
Pyroxd2	0,067373	2,0555037	NA
Uqcrc2	0,059171	2,047706	0,00274392
Hadhb	0,056845	2,044118	-0,01067902
Slirp	0,051908	2,0054882	NA
Ndufb11	0,05728	2,0046953	0,43682147
Ndufb7	0,054815	1,9999401	1,46251693
Acsf3	0,080354	1,9914499	0,47113302
Coq10a	0,048428	1,9685649	0,02207303
Rrbp1	0,049901	1,9646757	0,22766867
Mrps24	0,053115	1,9614362	0,09247896
Ak4	0,169745	1,9540206	1,18006167
Abcb7	0,062066	1,9527762	-0,12456679
Timm21	0,049901	1,9515541	NA
Cisd1	0,057276	1,9496498	1,3892039
Lrprrc	0,073798	1,9429428	0,25534361
Hsd17b10	0,073798	1,9359112	0,04472029
Acsf2	0,059171	1,9309549	0,10074094

Atp5o	0,053101	1,9129802	-0,19233187
Pnpt1	0,074926	1,8902753	-0,20168422
Mtnd4	0,061548	1,8843288	0,35727065
Mt-Cyb	0,061054	1,8666031	NA
Uqcr10	0,089656	1,8445038	0,46524642
Ndufa7	0,061548	1,8271921	0,71831609
Ptges2	0,375967	1,8127957	-0,63314425
Cox4i1	0,079529	1,8084924	0,38431826
Prdx5	0,064383	1,8014474	0,51658331
Acad10	0,184539	1,7760286	NA
Pdk2	0,169449	1,7711309	0,51844296
Mtco3	0,074822	1,7641934	-0,11929586
Naxe	0,172205	1,7241928	NA
Tmem70	0,079219	1,7209715	NA
Cox6b1	0,077144	1,7202419	-1,88268291
Fam162a	0,074762	1,7107557	0,33906577
Coq8a	0,141677	1,6941973	0,60963251
LOC100912599	0,079529	1,6905654	NA
Ndufa4	0,093831	1,6877707	0,60129457
Mtco1	0,093831	1,676077	NA
Ndufb4	0,081552	1,6615315	0,05989266
Atp5f1b	0,081213	1,6594224	0,69495158
Idh3B	0,123628	1,6590926	0,23812006
Ndufab1	0,115825	1,6482976	0,46687922
Bckdha	0,13573	1,6409483	0,42210959
Apool	0,128537	1,6244624	0,3641231
Oxnad1	0,075663	1,6223195	-0,04738238
Ndufb8	0,096144	1,6084916	0,33514254
Mdh2	0,122126	1,581564	0,03562464
Slc25a42	0,21108	1,5711377	0,25243384
Gfm2	0,106798	1,546498	0,190473
Crat	0,113961	1,5309961	0,24179731

Mrpl21	0,11652	1,5202367	0,38833153
Dlst	0,123628	1,5160175	0,29093977
Mrpl27	0,119674	1,4872799	NA
Atp5f1e	0,120837	1,477325	0,37386784
Phb	0,153635	1,4730678	-0,25705859
Cox5b	0,150076	1,469482	0,26995404
Ndutfaf3	0,131832	1,4670782	0,64708666
Ndutfaf6	0,141161	1,4637284	0,5347326
Aldh9a1	0,208633	1,4617295	0,39107334
Cox7a2l	0,153957	1,4270863	NA
Ndutfaf2	0,118982	1,4259823	-0,29656537
Me3	0,303356	1,4218338	0,28866087
Tbrg4	0,246897	1,4187769	1,07749441
Rps14	0,230567	1,414642	NA
Glud1	0,204253	1,4061199	1,39987719
Ndutfaf7	0,204253	1,3877684	0,81023037
Timm22	0,142482	1,3845505	-0,35411841
Atp5l	0,172205	1,3694716	0,42857388
Pdf	0,138864	1,3649293	NA
Afg3l2	0,1673	1,3466654	0,03669781
Cmc1	0,280763	1,3442023	NA
Ivd	0,174029	1,3092634	-0,06561046
Isca2	0,169449	1,3065557	NA
Phb2	0,254371	1,2737454	0,27043876
Slc27a1	0,179403	1,2641414	0,83421505
Cox5a	0,192714	1,2611296	0,37937444
Cs	0,243672	1,1500017	0,13066695
Ndutfaf13	0,281546	1,1097151	0,19672459
Tomm40	0,39889	1,0848112	0,09116678
Tars2	0,280763	1,0523493	NA
Acadsb	0,599893	1,0262952	0,28414242
Fdx1	0,280763	1,0248786	NA
Sirt4	0,312715	0,9988482	NA

D2hgdh	0,495782	0,978324	NA
Ndutfaf4	0,293629	0,9781724	-0,15679461
Hspd1	0,330098	0,9400601	0,09673283
Mrpl45	0,320231	0,9347227	-0,07607517
Fabp3	0,315465	0,9346028	0,45051427
Atp5f1d	0,386904	0,905328	0,32742396
Ndutfaf12	0,51557	0,8268013	-0,34724204
Trak1	0,521078	0,7772172	0,24176825
Rab10	0,669586	0,7478115	0,54054898
Cox7a2	0,572851	0,7287986	1,48535044
Vars2	0,46779	0,7170921	0,18752902
Cat	0,507477	0,7042931	0,06050866
Ctsd	0,483149	0,6851963	-0,71921424
Ldhd	0,651223	0,6034217	NA
Myh6	0,543886	0,6002338	0,65952931
Pccb	0,572119	0,5971594	0,46811597
Cycs	0,527987	0,5761049	NA
Ywhae	0,739618	0,5098574	0,04206988
Ighm	0,621741	0,4917554	NA
Myom2	0,713452	0,4904926	0,28203171
Bdh1	0,651223	0,4593037	-0,44704946
Mrpl13	0,625807	0,4443854	-0,10851629
Atp5c1	0,669586	0,3927405	5,00E-05
Mrps36	0,741422	0,3836084	NA
Fis1	0,783607	0,3317174	0,51324202
Park7	0,81759	0,3117905	0,00757396
Prelid2	0,741422	0,3055199	-0,16274532
Lamc1	0,758729	0,2844704	0,5287584
Hsp90b1	0,849105	0,223191	NA
Shmt2	0,857363	0,1840997	0,75436682
Cryab	0,837829	0,1808333	-0,04780059
Acot9	0,837829	0,1803713	-0,11838661
Tomm22	0,868433	0,1446585	-0,03067709

Hsdl1	0,938639	0,1058461	-0,10596952
Clpx	0,947706	0,0805865	-0,13803961
Cps1	0,947706	0,0719743	NA
Mavs	0,950311	0,0577702	1,78901757
Hsp90ab1	0,95949	0,0568117	0,65099374
Rab1b	0,973458	0,0297501	NA
Calu	0,958106	-0,04574	0,78978854
Qdpr	0,950311	-0,063209	1,28916063
Hspa5	0,849105	-0,195341	0,2538068
Atic	0,906106	-0,228271	0,582148
Lonp1	0,809575	-0,244226	0,59322458
Rap1b	0,807952	-0,269749	NA
Mccc2	0,740326	-0,334722	0,34839469
Ldhb	0,788729	-0,339047	-0,00510539
Cyct	0,669878	-0,370647	NA
Myl6	0,768844	-0,398296	0,89692218
Ide	0,758278	-0,398695	NA
Nom1	0,64276	-0,425926	NA
Rplp0	0,741422	-0,436152	0,1623961
Hk2	0,625807	-0,455845	1,06809997
Cltc	0,741422	-0,511063	0,1092956
Rpl31	0,757776	-0,520502	NA
Maoa	0,669586	-0,522841	NA
Cad	0,621741	-0,541099	NA
Vim	0,605651	-0,552159	NA
Mrpl53	0,568544	-0,555685	NA
P4hb	0,70246	-0,560872	0,20643326
Arg1	0,543886	-0,578855	0,88536123
Rab5c	0,666482	-0,587382	0,1605778
Rpl23	0,559825	-0,63848	0,68798409
Rpl13	0,618003	-0,662365	NA
Ywhah	0,546491	-0,670496	0,37843263

Arf3	0,512525	-0,698098	0,21611898
Ociad1	0,456425	-0,753924	-0,13430152
Rpl10	0,568544	-0,817489	0,18956423
Rpl11	0,524636	-0,820118	NA
Nme2	0,507477	-0,843389	0,23322439
Rpl3	0,479182	-0,877657	-0,03571881
Idh1	0,349619	-0,899532	0,48332894
Rack1	0,470727	-0,914929	-0,00084766
Krt5	0,356878	-0,928035	0,71336642
Rps9	0,582765	-0,968437	NA
Rps3	0,46779	-0,972133	0,25167775
Sco1	0,434748	-0,998335	-0,11906997
Anxa6	0,543886	-0,998921	0,34358554
Gpd1	0,337105	-1,056118	0,3297055
Anxa2	0,285807	-1,099335	0,48278248
Rps8	0,427363	-1,110656	0,52016359
Rab7a	0,301549	-1,132676	0,01378458
Actb	0,284354	-1,155985	0,22653392
Rps16	0,391848	-1,16498	0,6083409
Hspe1	0,293808	-1,177697	0,39147887
Aldoa	0,339296	-1,19661	0,54190472
Ilf3	0,426508	-1,219273	1,11843993
Hspa1a	0,260232	-1,243412	0,61265345
Hnrnpk	0,25663	-1,249187	1,51277185
Prdx2	0,204253	-1,278542	-0,18003118
Mthfd1	0,425021	-1,279054	-0,30197773
Prdx6	0,369916	-1,283611	-0,25639062
Ssr4	0,315335	-1,284315	NA
Ddx3x	0,211487	-1,337232	-0,56753031
Myh9	0,315764	-1,376728	1,03921503
Rpl6	0,364051	-1,387759	1,0084159
Impa2	0,226315	-1,432246	0,69497908
Rps13	0,315764	-1,442536	0,64702276

Actn1	0,27735	-1,463038	1,33873402
Ppib	0,232388	-1,488362	0,37587076
Gapdh	0,2188	-1,572532	0,07458655
Pkm	0,184539	-1,607108	0,74248285
Dbi	0,142482	-1,641836	NA
Rplp2	0,120837	-1,659815	0,63175823
Uba1	0,309285	-1,761006	0,24042777
Hsp90aa1	0,204747	-1,785144	0,73855696
Tuba1a	0,238373	-1,802108	0,80797706
Rpl34	0,24184	-1,807517	0,04139266
Ldha	0,146727	-1,81486	0,64038507
Fasn	0,148832	-1,867677	0,68425597
Eef2	0,203742	-1,913778	0,8329657
Rpl7	0,202523	-1,929082	0,0272542
Rpl18	0,169745	-1,99812	0,09079739
Ucp1	0,292054	-2,051982	1,83480565
Pdia3	0,099963	-2,097317	0,13519511
Rab11b	0,122126	-2,149091	NA
Txn1	0,059171	-2,157831	0,2367291
Calm3	0,040805	-2,289742	NA
Sod1	0,042717	-2,320084	NA
Anxa1	0,09283	-2,387749	-0,62050168
Pgk1	0,073798	-2,512086	0,09797089
Ywhaz	0,049843	-2,678315	-0,42179595
Eef1g	0,056845	-2,78065	0,59717076
Hspb1	0,055337	-2,822628	0,04329966
Tubb5	0,103418	-3,087091	1,45741391
Bak1	0,069571	-3,247829	NA