

Intrinsic bioenergetic adaptations compensate for reduced mitochondrial content in HER2-driven mammary tumors

Sara M Frangos , Henver S Brunetta, Dongdong Wang, Maria Joy Therese Jabile, David WL Ma, William J Muller, Cezar M Khursigara, Kelsey H Fisher-Wellman, Gregory R Steinberg, Graham P Holloway 

Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Canada • Centre for Metabolism, Obesity and Diabetes Research, McMaster University, Hamilton, Canada • Division of Endocrinology and Metabolism, Department of Medicine, McMaster University, Hamilton, Canada • Department of Biochemistry, McGill University, Rosalind and Morris Goodman Cancer Research, Montreal, Canada • Department of Molecular and Cellular Biology, University of Guelph, Guelph, Canada • Department of Cancer Biology, Medical Center Boulevard, Wake Forest University School of Medicine, Winston-Salem, United States • Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Canada

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eLife Assessment

This **useful** study uses the MMTV-Neu-YD5 mouse model for HER2-dependent breast cancer to generate transcriptomic and proteomic datasets from extracted primary tumour samples. The data sets generated appear to be **solid** and will be of interest to the community. However, mechanistic studies to support the conclusion that mitochondrial function is increased in the tumours remain **incomplete** and would benefit from experiments that would directly interrogate aspects such as cellular heterogeneity, and signalling.

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Abstract

It is now recognized that mitochondria play a crucial role in tumorigenesis, however, it has become clear that tumor metabolism varies significantly between cancer types. The failure of recent clinical trials attempting to directly target tumor respiration with inhibitors of oxidative phosphorylation has highlighted the critical need for additional studies comprehensively assessing mitochondrial bioenergetics. Therefore, we systematically assessed the bulk tumor and mitochondrial metabolic phenotype between murine HER2-driven mammary cancer tumors and paired benign mammary tissue. Transcriptomic and proteomic profiling revealed that HER2-driven mammary tumors are characterized by a downregulation of mitochondrial genes/proteins compared to benign mammary tissue, including a general downregulation of OXPHOS subunits comprising Complexes I-IV. Despite

this observation, mitochondrial respiration supported by both carbohydrate-derived substrates (pyruvate) and lipids (palmitoyl-carnitine) was several-fold higher in HER2-driven tumors which persisted regardless of normalization method (i.e. wet weight, total protein content and when corrected for mitochondrial content). This upregulated respiratory capacity could not be explained by OXPHOS uncoupling; however, several subunits/regulators of Complex V function were not downregulated in the tumors, suggesting possible compensatory effects may contribute to high respiratory rates. Furthermore, tumor mitochondria displayed a smaller and more punctate morphology, aligning with a general reduction in mitochondrial fusion and increase in mitochondrial fission markers, which could contribute to improved OXPHOS efficiency. Together, this data highlights that the typical correlation of mitochondrial content and respiratory capacity may not apply to all tumor types and implicates the activation of mitochondrial respiration supporting tumorigenesis in this model.

Introduction

The field of cancer metabolism has rapidly evolved in the last decade. A specific focus of many researchers has been on understanding the role of mitochondria in tumorigenesis with the goal to potentially target aspects of mitochondrial metabolism to reduce proliferation^{1,2}. It was initially hypothesized that a universal characteristic of cancer cells was dysfunctional oxidative phosphorylation (OXPHOS), as proposed by Otto Warburg in the 1920s^{3,4}. This theory spurred interest in studying mitochondria in various cancers, highlighting the diverse mitochondrial phenotypes in this context. While instrumental for OXPHOS, mitochondria are now recognized for their multifaceted roles in regulating cellular homeostasis, including supporting macromolecule biosynthesis⁵, maintaining redox balance⁶ and generating metabolites that regulate gene transcription⁷. Given their pleiotropic effects within the cell, there is a growing recognition that mitochondria play an essential role in cancer initiation, progression, and metastasis¹, and an appreciation that interrogating mitochondrial bioenergetics in diverse cancers may identify novel therapeutic strategies.

Reductions in the content/activity of mitochondrial enzymes within the tricarboxylic acid (TCA) cycle and electron transport chain (ETC) impairs mitochondrial respiratory capacity and coincides with activating transcriptional programs driving tumorigenesis^{8–10}. Indeed, inhibition of succinate dehydrogenase (Complex II) in osteosarcoma cells⁸, and renal cell carcinoma⁹ enhances cancer growth despite OXPHOS inhibition. Furthermore, in colorectal cancer, age-related Complex I deficiency coincides with reduced respiratory capacity but enhanced serine synthesis and subsequent growth rates¹⁰. Despite this evidence suggesting that disruptions in mitochondrial bioenergetics contribute to tumor progression, several elegant studies using genetic and pharmacological approaches have solidified that a functional ETC is required for cancer cell growth^{6,11,12}. Furthermore, the use of whole-genome sequencing specifically interrogating mtDNA phenotypes across several human cancers has revealed that loss of function mutations in mtDNA genes are often under negative selection¹³, suggesting the maintenance of mitochondrial function may support tumorigenesis in some cancers and impairments in mitochondrial metabolism are not a ubiquitous finding. However, mitochondrial content represents one dimension of bioenergetic regulation, and may not fully reflect mitochondrial function *in vivo* where substrate delivery/sensitivity and mitochondrial coupling can be altered independently of mitochondrial content¹⁴. Therefore, a comprehensive assessment of mitochondrial function, particularly in the context of the mitochondrial proteome, is essential to provide deeper insights into the bioenergetic phenotypes observed in different cancers.

Within this framework, there has been considerable interest in investigating mitochondrial bioenergetics and dynamics in breast cancer subtypes due to the limited therapeutic options for triple-negative breast cancer (TNBC) and therapeutic resistance in breast cancers driven by

estrogen (ER), progesterone (PR) and human epidermal growth factor (HER2) receptor amplification^{15–17}. Indeed, increases in mitochondrial respiration or “OXPHOS reliance” is associated with poor outcomes in treatment naïve TNBC¹⁸, metastatic ER+ tumors¹⁹, and HER2-driven breast cancers²⁰. While inhibiting OXPHOS in this context^{18,19} (i.e. with Complex I inhibitor IACS-010759) has shown some pre-clinical success, systemic toxicity has prevented the translation of this strategy to the clinic²¹. Furthermore, it is unclear whether increases in mitochondrial respiration arise from increases in mitochondrial content or intrinsic enhancements in mitochondrial function (i.e. coupling efficiency, substrate delivery/sensitivity) – information that is critical to develop successful mitochondrial-targeted therapies with a wider therapeutic window. Thus, in the current study, we comprehensively assessed the bulk tumor and mitochondrial phenotype between murine HER2-driven mammary cancer tumors and benign mammary tissue in the context of the underlying transcriptome and proteome. Respiratory capacity supported by both pyruvate and lipids was enhanced in HER2-driven tumors even when corrected for mitochondrial content, suggesting intrinsic bioenergetic adaptations may exist to maintain OXPHOS during tumorigenesis in this model.

Results

Mammary tumors from MMTV/neu^{ndl}-YD5 mice display canonical HER2 pathway activation

Cell lines are commonly used to study isolated mechanisms driving tumorigenesis, however, they neglect the variety of cell types in the tissue of origin and the influence of the tumor microenvironment, which is a key regulator of cancer growth^{23,24}. To this end, we have utilized tumor and benign mammary tissues from MMTV/neu^{ndl}-YD5 transgenic mice²⁵ to investigate HER2-driven tumor biology (**Fig. 1A**). Signaling events linked to HER2 activation rely on the autophosphorylation of the tyrosine kinase domain^{25–27} and MMTV/neu^{ndl}-YD5 mice possess an add-back mutation at the Y1226/7 (YD) site, activating downstream signaling events linked to SHC/GRB2 with similar transforming potential as the wild-type protein^{27,28} (**Fig. 1B**). While the proximal signaling events linked to HER2 activation in this model have been described²⁷, to validate our bulk tissue-based model, we assessed the mRNA (transcriptomics) and protein (quantitative label-free proteomics and western blotting) expression of canonical HER2 pathway members (RAS/MAPK and PI3K/AKT/mTOR pathways) that are well-characterized in various HER2-overexpressing mammary cancer cell lines (**Fig. 1B**)^{29–31}. *Her2* transcript levels were increased ~175-fold (**Fig. 1C**) and transcripts belonging to the Ras/MAPK (*Hras*, *Mapk1*, *Mapk3*) and PI3K/AKT/mTOR (*Pik3cg*, *Pik3c2a*, *Pik3c2b*, *Pikrcg*, *Pik3c3*, *Akt3*) pathways were also upregulated in the tumors compared to the benign mammary tissue (**Fig. 1D**). At the protein level, HER2 was readily detectable in mammary tumors by quantitative label-free proteomics but undetectable in paired benign tissues (**Fig. 1E**) suggesting enhanced HER2 transcription also manifests as an increase in receptor content in the tumors. Downstream of HER2 activation, the total protein content of p38 MAPK, ERK1/2 and eEF2 were significantly increased in tumors compared to benign tissue detected by western blot (**Fig. 1F,G**). When normalized to total protein, p38 MAPK and eEF2 phosphorylation were not higher in tumors due to proportional increases in total protein abundance, while in contrast, phosphorylated ERK1/2 and mTOR were significantly upregulated suggesting both Ras/MAPK and the PI3K/AKT/mTOR pathways were activated in tumors (**Fig. 1H**, **S-Fig. 1**). Overall, these data demonstrate that mammary tumors display classical hallmarks of HER2-driven cancer cell signaling, allowing for the subsequent investigation of HER2-driven tumor biology and metabolism in this model.

Assessment of the HER2-driven mammary tumor transcriptome

To understand the biology and metabolism of HER2-driven mammary tumors, we assessed their transcriptomes using RNA sequencing. Principal component analysis (PCA) using the entire gene expression profile showed distinct separation between groups (**Fig.2A**). Visualizing all differentially expressed genes (DEGs) using a volcano plot (**Fig.2B**) and the top 3000 DEGs using a heatmap (**Fig.2C**) revealed several differentially upregulated and downregulated genes between groups where genes involved in MAPK- and AKT-linked signaling, ECM remodelling and cellular stress responses (*Etv1*, *Dusp6*, *Papln*, *Ier3*) were among the most upregulated genes in the tumors and genes involved in lipid metabolism (*Dgat2*, *Lipe*, *Cd36*) were among the most downregulated. Enrichment analysis of the top 3000 upregulated DEGs (**Fig.2D**) identified pathways characteristic of HER2-driven signaling (e.g. tyrosine kinase signaling, GTPase signaling) and gene ontology (GO) analysis identified processes indicative of active transcription, translation, and protein turnover characteristic of a rapidly proliferating tissue (**Fig.2E**). Interestingly, enrichment analysis of the top 3000 downregulated genes in the tumors consistently identified that mitochondrial metabolism, and in particular components of the oxidative phosphorylation (OXPHOS) system were downregulated in tumors compared to benign mammary tissue (**Fig.2F,G**). Altogether, these data further support that this whole tissue model aligns with canonical HER2-driven cell signaling events and highlights that HER2-driven tumors are characterized by reductions in mitochondrial gene expression.

Assessment of HER2-driven mammary tumor proteome

We further validated our transcriptomic findings at the protein level using label-free quantitative proteomics and detected 2589 proteins in the benign mammary tissue and 2448 proteins in the HER2-driven tumors; 1848 of which were found in both groups (**Fig.3A**). This initial analysis revealed 741 and 600 uniquely expressed (i.e. only detected in one tissue) proteins in benign mammary tissue and tumors respectively (**S-Fig. 2A**). Interestingly, OXPHOS and aerobic respiration proteins were identified among the top pathways in benign tissue, but not in tumors (**S-Fig. 2B-G**), further supporting the notion of downregulated mitochondrial content within tumors. Reflecting the PCA for the transcriptomic data, analysis of all overlapping proteins between benign and tumor samples revealed distinct separation (**Fig.3B**). Of the 1848 proteins found in both the tumors and benign tissue, 420 were significantly different (p-adj. <0.1, **Fig 3A,C,D**). However, the majority of the differentially expressed proteins were downregulated (n=411) in tumors compared to benign mammary tissue. Upregulated proteins (n=9) included those involved in canonical HER2 signaling, protein synthesis and processing, and glycolytic metabolism (**S-Table 1**). Supporting transcriptomic data, which indicated a reduction in mitochondrial content and OXPHOS, enrichment analysis revealed that 8 of the top 10 downregulated pathways (**Fig. 3E**) and all top 10 downregulated GO (**Fig. 3F**) were related to mitochondrial biogenesis and/or metabolism, and OXPHOS was identified in the top 10 downregulated KEGG pathways (**Fig.3G**). Together, these proteomic data support our transcriptomic analysis suggesting HER2-driven tumors are characterized by lower mitochondrial content than benign mammary tissue.

Integrated assessment of the HER2-driven tumor transcriptome and proteome

Considering both transcriptomic and proteomic data suggest that mitochondrial metabolism/OXPHOS are downregulated in mammary tumors, we also analyzed the 144 overlapping downregulated targets between these analyses (**Fig.4A**). KEGG enrichment analysis of the downregulated targets identified OXPHOS among the top 10 pathways, as well as carbon metabolism, BCAA degradation and fatty acid metabolism which encompass numerous

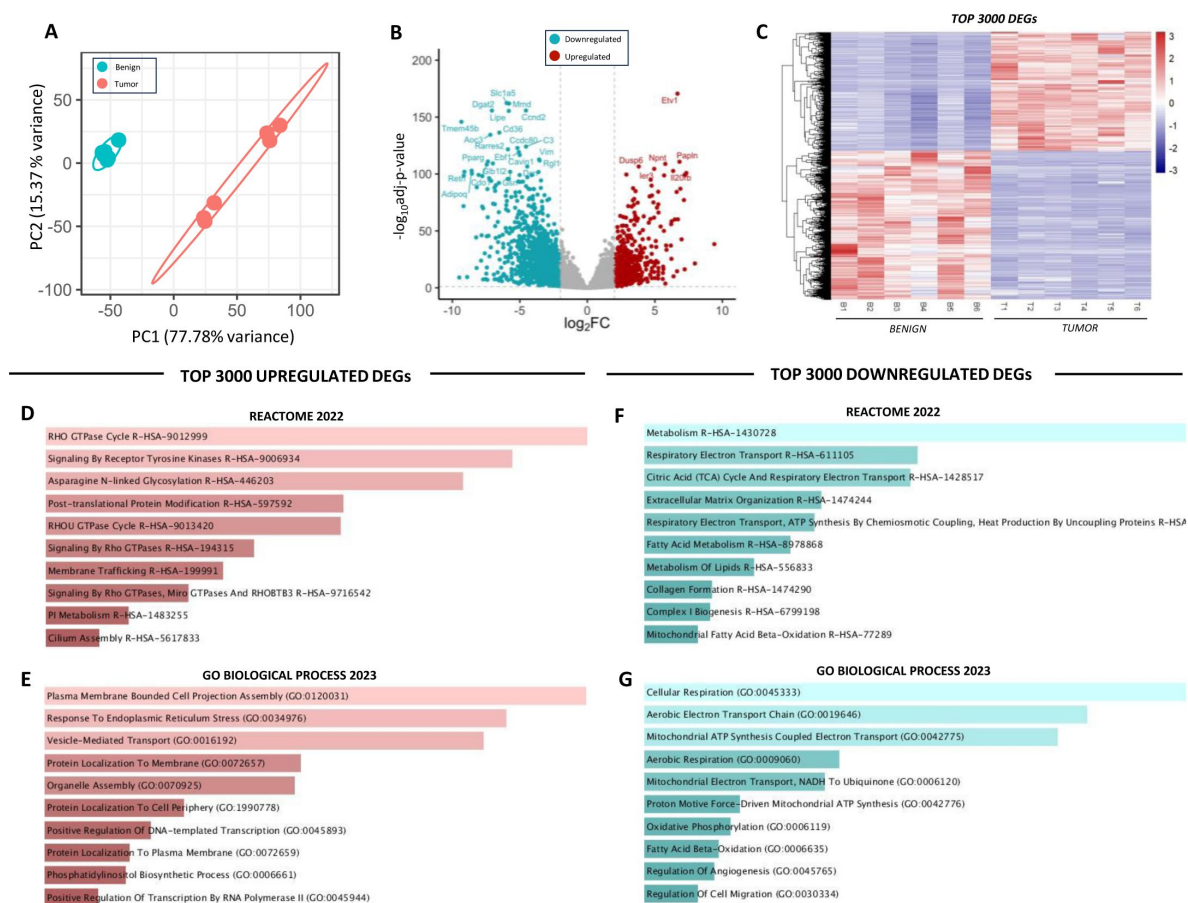


Figure 2.

Assessment of HER2-driven mammary tumor and benign mammary tissue transcriptomes.

A) PCA of benign mammary tissue and mammary tumors, **B)** Volcano plot showing differentially expressed genes (DEGs) identified between mammary tissue and mammary tumors. **C)** Heatmap of top 3000 DEGs, **D)** Top 10 upregulated pathways (Reactome 2022) and **E)** gene ontologies (GO Biological Process 2023) in tumors compared to benign mammary tissue, **F)** Top 10 downregulated pathways (Reactome 2022) and **G)** gene ontologies (GO Biological Process 2023) in tumors compared to benign mammary tissue. Panels **D,E** assess the top 3000 upregulated DEGs ($\log_2\text{fc} > 1$), panels **F,G** assess the top 3000 downregulated DEGs ($\log_2\text{fc} < -1$). An adjusted p-value of < 0.1 was considered statistically significant across all analyses. The Enrichr open access analysis web tool was used to generate panels **D-G** which were sorted by p-value.

mitochondrial targets (**Fig.4B** [↗](#)). The top 10 common downregulated pathways (**Fig.4C** [↗](#)) and GO (**Fig.4D** [↗](#)) all pertained to mitochondrial substrate metabolism/catabolism and OXPHOS. Together, transcriptomic and proteomic analyses strongly support that HER2-driven tumors are characterized by downregulated mitochondrial content.

Assessment of the mitochondrial transcriptome and proteome

Our initial transcriptomic and proteomic analyses encompassed all differentially expressed genes/proteins between tissues (including mitochondrial targets) and clearly identified reductions in mitochondrial content in the tumors. This emphasized the robust nature of mitochondrial adaptations in this model, considering mitochondrial proteins (identified using the MitoCarta 3.0 database) represented a small fraction of total cellular protein in both tumors and benign mammary tissue (**S-Fig. 3** [↗](#)). By filtering our proteomic dataset using MitoCarta, we identified 125 mitochondrial proteins that were commonly expressed between the tumors and benign mammary tissue (**Fig.5A** [↗](#)). When visualizing the 125 proteins with a heatmap, the majority of the mitochondrial proteome was downregulated in the tumors compared to the benign mammary tissue (**Fig.5B** [↗](#)). The summed absolute abundance of all detected mitochondrial proteins in each group was also lower in tumors (**Fig.5C** [↗](#)), again supporting our transcriptomic and proteomic analyses suggesting a reduction in mitochondrial content exists in the tumors.

While mitochondrial biogenesis is a coordinated process simultaneously upregulating several mitochondrial proteins, the total mitochondrial transcriptome/proteome does not necessarily change proportionally with OXPHOS complex and/or TCA cycle/dehydrogenase content, especially in cancerous tissues. Indeed, reductions in the content of specific respiratory complexes seem to be cancer specific, which can have functional repercussions on mitochondrial respiration rates and/or efficiency^{10,32} [↗](#). Within this context, we assessed the gene expression profile of all OXPHOS complex subunits between groups, which revealed relatively ubiquitous reductions in transcript count (transcripts per million) across subunits of Complexes I-IV (**Fig. 5D-H** [↗](#)). Interestingly, while the majority of Complex V subunits were also downregulated, this complex had the highest proportion of subunits, displaying no differences between groups (**Fig.5D,I** [↗](#)). The TCA cycle transcriptome also reflected a general downregulation of transcript abundance, apart from alpha-ketoglutarate dehydrogenase (*Ogdhl*) and succinyl-CoA synthetase GDP forming subunit-β (*Suc1g2*), which had a higher transcript abundance in some tumor samples (**Fig.5J** [↗](#)). While we focused on transcriptomic data for this analysis due to lower detection of certain OXPHOS subunits using label-free quantitative proteomics (**S-Fig. 4A-E** [↗](#)), the summed abundance of all OXPHOS subunits detected per complex expressed relative to the underlying mitochondrial proteome (**S-Fig. 4F** [↗](#)), and relative to the total proteome (**S-Fig. 4G** [↗](#)) displayed a similar pattern. Overall, these data further suggest that the mitochondrial proteome and OXPHOS complexes I-IV are downregulated in HER2-driven tumors compared to benign mammary tissue.

Mitochondrial respiratory capacity is increased in HER2-driven mammary tumors

Considering our transcriptomic and proteomic data strongly suggest HER2-driven mammary tumors display reductions in mitochondrial content, we next assessed whether this would manifest as a reduction in respiratory capacity by measuring mitochondrial respiratory flux rates. In permeabilized tumors and benign mammary tissue, we assessed basal/endogenous respiration and respiration with saturating carbohydrate (pyruvate/malate) and lipid (palmitoyl-carnitine) substrates. For each protocol, several biological replicates (individual tumors) were utilized, spanning a continuum of whole tumor wet weights/volumes (**S-Fig. 5A,B** [↗](#)). Since no strong correlations were found between tumor wet weight or volume and basal or maximal respiration supported by carbohydrate-or lipid-linked substrates (**S-Fig. 5C-J** [↗](#)), all data points were pooled. When normalizing respiration to tissue wet weight (**Fig. 6A** [↗](#)), tumors displayed significantly higher basal/endogenous respiration and higher respiration with subsequent additions of

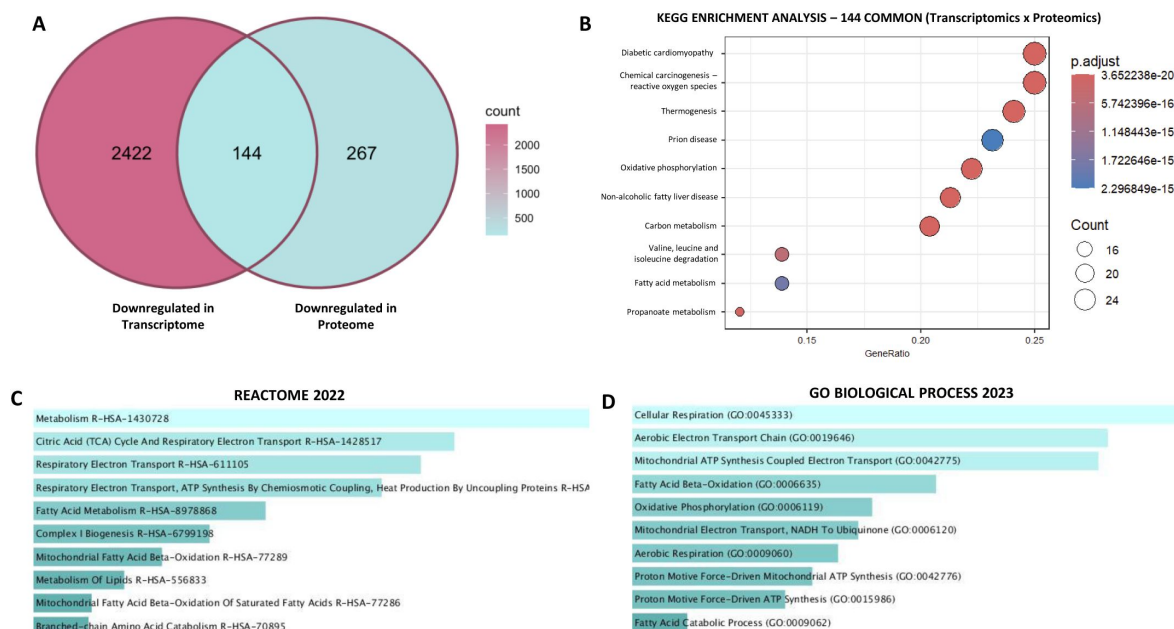


Figure 4.

Overlapping downregulated genes and proteins between HER2-driven mammary tumor transcriptome and proteome.

A) Venn diagram showing 144 overlapping downregulated targets between the assessed HER2-driven tumor and benign mammary tissue transcriptomes and proteomes. **B)** KEGG analysis for common downregulated targets. Top 10 downregulated **C)** pathways (Reactome 2022) and **D)** gene ontologies (GO Biological Process 2023). An adjusted p value of <0.1 was utilized for all analyses. The Enrichr open access analysis web tool was used to generate panels **C,D** which were sorted by p-value.

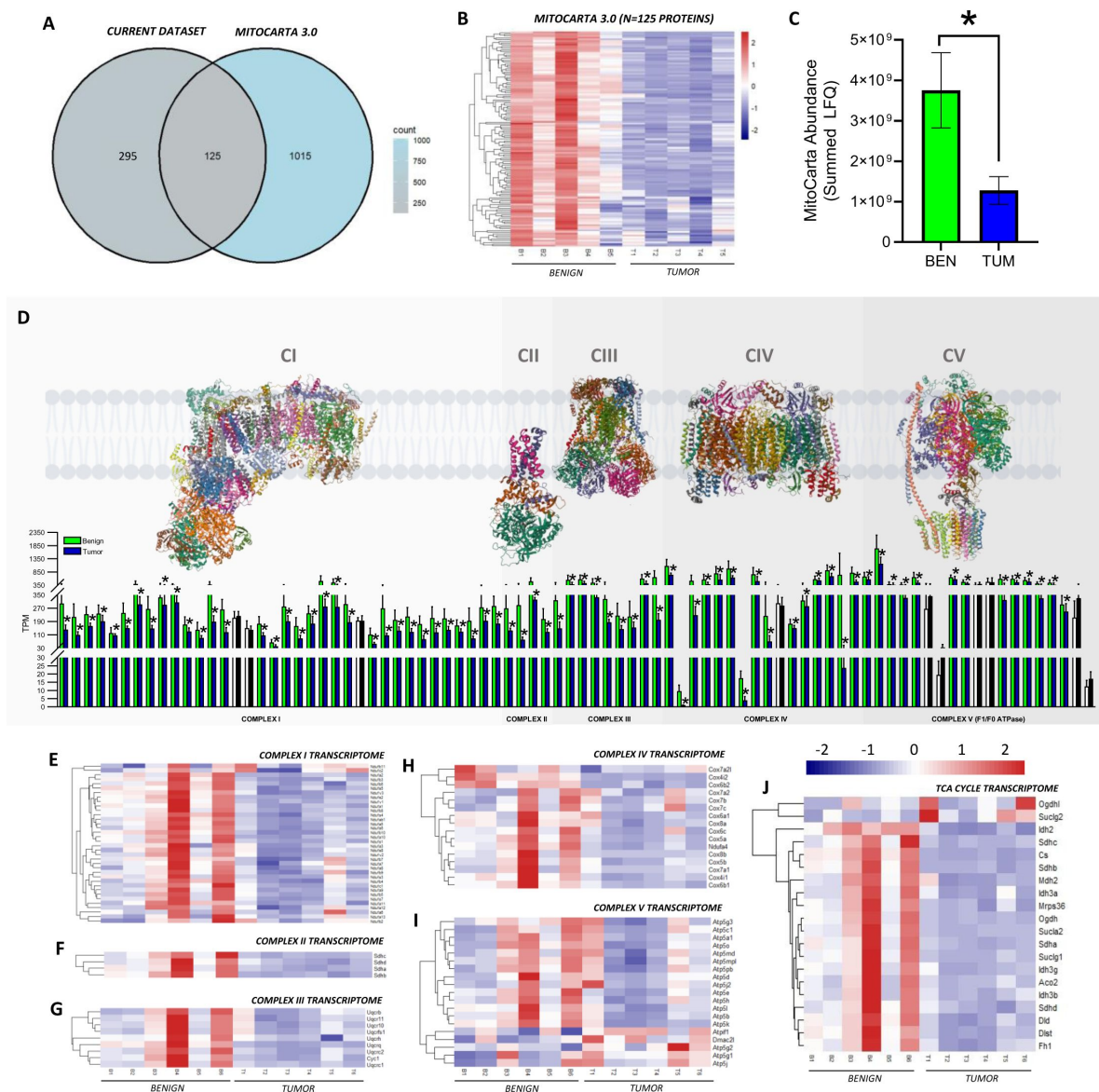


Figure 5.

Assessment of the mitochondrial transcriptome and proteome.

A) Venn diagram showing mitochondrial proteins detected in the current dataset when cross-referenced with the Mouse MitoCarta 3.0 database. **B**) Heatmap of 125 mitochondrial proteins identified in benign mammary tissue and HER2-driven tumors (n=5/group). **C**) Absolute abundance of all mitochondrial proteins detected in benign and tumor tissues. **D**) Gene expression (detected by transcriptomics; transcripts per million) of all detected OXPHOS subunits separated per respiratory complex, black and white bars represent subunits with no significant differences between groups. **E-J**) Heatmaps of **E**) Complex I, **F**) Complex II, **G**) Complex III, **H**) Complex IV, **I**) Complex V (ATP synthase) and **J**) TCA cycle transcriptomes. All proteomic analyses (panels A-C) were conducted on n=5/group. All transcriptomic analyses (panels D-J) were conducted on n=6/group. Panels B,C were analyzed using an unpaired two tailed Student's t-test (*p<0.05). In Panel D, * and coloured (blue and green bars) represent significant differences between benign and tumor samples based on an adjusted p value of <0.1. OXPHOS complex schematics in panel D were obtained from the open access RCSB Protein Data Bank (Complex I = TW1V, Complex II = 2A06, Complex III = 3SFD, Complex IV = 6NMP, Complex V = 5FIL). Bars in panel D are listed in the following order: Complex I: Ndufa1, Ndufa10, Ndufa11, Ndufa12, Ndufa13, Ndufa2, Ndufa3, Ndufa5, Ndufa6, Ndufa7, Ndufa8, Ndufa9, Ndufab1, Ndufb10, Ndufb11, Ndufb2, Ndufb3, Ndufb4, Ndufb5, Ndufb6, Ndufb7, Ndufb8, Ndufb9, Ndufc1, Ndufc2, Ndufs1, Ndufs2, Ndufs3, Ndufs4, Ndufs5, Ndufs6, Ndufs7, Ndufs8, Ndufv1, Ndufv2, Ndufv3; Complex II: Sdha, Sdhb, Sdhc, Sdhb; Complex III: Cyc1, Uqcr10, Uqcr11, Uqcrb, Uqcrc1, Uqcrc2, Uqcrcf1, Uqcrh, Uqcrg; Complex IV: Cox4i1, Cox4i2, Cox5a, Cox5b, Cox6a1, Cox6b1, Cox6b2, Cox6c, Cox7a1, Cox7a2, Cox7a2l, Cox7b, Cox7c, Cox8a, Cox8b, Ndufa4; Complex V: Atp5a1, Atp5b, Atp5c1, Atp5d, Atp5e, Atp5g1, Atp5g2, Atp5g3, Atp5h, Atp5j, Atp5j2, Atp5k, Atp5l, Atp5md, Atp5mpl, Atp5o, Atp5pb, Atpif1, Dmac2l.

pyruvate, malate (CI-linked respiration), ADP (State III respiration), glutamate (CI-linked respiration also supported by alpha-ketoglutarate anaplerosis), and succinate (to maximize CII-linked respiration) (**Fig. 6B** [↗](#)). Equally, tumors displayed significantly higher respiration rates supported by lipids (palmitoyl-carnitine, **Fig. 6C** [↗](#)), suggesting that HER2-driven tumors are capable of relying on both substrate sources, perhaps to maximize aerobic metabolism. This initial analysis of respiratory rates was normalized to tissue wet weight, which can be influenced by differences in total and mitochondrial protein content in a given tissue volume. To address this, we first normalized respiratory flux rates to total cellular protein (**Fig. 6D,E** [↗](#)), which minimizes differences in factors such as tissue density and hydration that are not directly contributing to mitochondrial oxygen consumption. The third normalization method employed was normalizing respiratory flux to a mitochondrial enrichment factor (MEF) (**Fig. 6F,G** [↗](#)) which is the ratio of mitochondrial proteins to the underlying total proteome (**S-Fig. 3A,B** [↗](#)). This approach controls for differences in mitochondrial content between tissues to determine whether increases in respiration are due to increases in mitochondrial content or intrinsic changes in mitochondrial function. Interestingly, the fundamental observation of increased respiratory flux rates in tumors compared to benign tissues was conserved independent of the normalization method, suggesting that HER2-driven mammary tumors are characterized by high respiratory flux rates independent of total cellular protein or reduced mitochondrial protein content. While greater intrinsic OXPHOS was observed with all substrates, lipid supported respiration was stimulated to a greater extent in the tumors, resulting in comparable carbohydrate (pyruvate, malate, ADP) and lipid (palmitoyl-carnitine, malate, ADP) supported respiration (**Fig. 7A** [↗](#)). This effect occurred despite many of the genes and proteins involved in lipid metabolism at the plasma membrane and in the cytosol (e.g. *Acs11*, *Adipoq*, *Dgat1/2*, *Lipe*, *Fabp4*, *Cd36*, *Pparg*) and mitochondria (e.g. *EtfA*, *EtfB*, *EtfDh*, *Hadh*, *Hadhb*, *Cpt1b*, *Cpt2*) being downregulated in tumors compared to benign mammary tissue (**Fig. 7B-E** [↗](#)). The intrinsic activation of respiration was not a ubiquitous finding for all processes linked to OXPHOS, as reactive oxygen species (**S-Fig. 6A** [↗](#)) emission rates were higher in tumors compared to benign tissue when normalized to wet weight (**S-Fig. 6B** [↗](#)), lower when normalized to total protein (**S-Fig. 6C** [↗](#)) and not different when normalized to mitochondrial content (**S-Fig. 6D** [↗](#)).

High respiratory flux rates cannot be explained by OXPHOS uncoupling

Typically, respiratory capacity correlates with mitochondrial content in fully differentiated tissues³² [↗](#). However, given the apparent discrepancy between mitochondrial/OXPHOS protein content and respiratory flux rates in HER2-driven tumors, we investigated alternative explanations for this observation. One possible explanation for the observed enhanced respiratory rates could be due to the uncoupling of OXPHOS from ATP synthesis, especially when ROS emission is not proportional to respiratory flux. To address this possibility, we utilized oligomycin (OMY) to inhibit the F₀ subunit of ATP synthase (Complex V) and examine its inhibitory potency between groups (**Fig. 7F** [↗](#)). While OMY inhibited respiration in benign mammary tissue by ~55%, respiration in tumors was inhibited by ~80% (**Fig. 7G** [↗](#)). Subsequent additions of antimycin A (AMA) to inhibit Complex III and rotenone (ROT) to inhibit Complex I, fully inhibited respiration in both tissues (**Fig. 7G** [↗](#)). Since respiration was inhibited to a greater extent in the tumors in response to OMY, this protocol strongly suggests that HER2-driven tumor mitochondria are highly coupled.

Tumor mitochondria display altered morphology

When assessing the subcellular ultrastructure of HER2-driven mammary tumors using transmission electron microscopy (TEM), we observed smaller, more punctate mitochondria in tumors compared to benign tissue (**Fig. 8A** [↗](#)). To determine whether ultrastructural observations aligned with the expression of genes and proteins involved in mitochondrial turnover, we filtered our transcriptomic and proteomic dataset using MitoCarta, specifically investigating mitochondrial proteins involved in mitochondrial dynamics and mitophagy. This approach

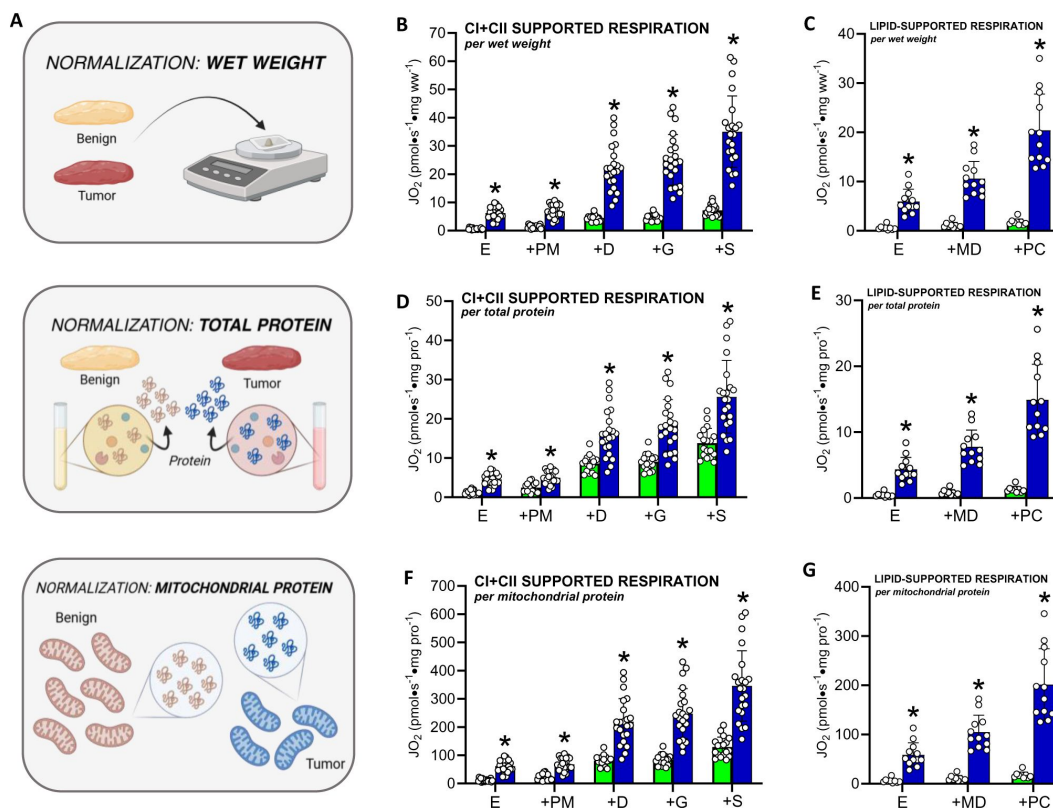


Figure 6.

Assessment of lipid and carbohydrate linked mitochondrial respiration and OXPHOS coupling in benign mammary tissue and HER2-driven mammary tumors.

A) Schematic depicting methods of normalization for respiration rates (*per wet weight*, *total protein*, *mitochondrial protein/enrichment*). **B)** Complex I and complex II-supported respiration normalized to tissue wet weight, **C)** lipid-supported respiration normalized to tissue wet weight, **D)** Complex I and complex II-supported respiration normalized to total protein content, **E)** lipid-supported respiration normalized to total protein content, **F)** Complex I and complex II-supported respiration normalized to mitochondrial protein (mitochondrial enrichment factor, MEF). **G)** Lipid supported respiration normalized to mitochondrial protein. All individual values represent biological replicates ($n=9-22$ /group depending on the protocol). Data are presented as means $\pm$ SD, statistical analyses were conducted using an unpaired, two-tailed Student's *t*-test ($*p<0.05$). Abbrev: E = Endogenous, PM = pyruvate (5 mM), malate (2 mM), D = ADP (5 mM), G = glutamate (10 mM), S = succinate (10 mM), PC = palmitoyl-carnitine (20 μ M), CI+II = maximal CI+II-supported respiration (PMDGS).

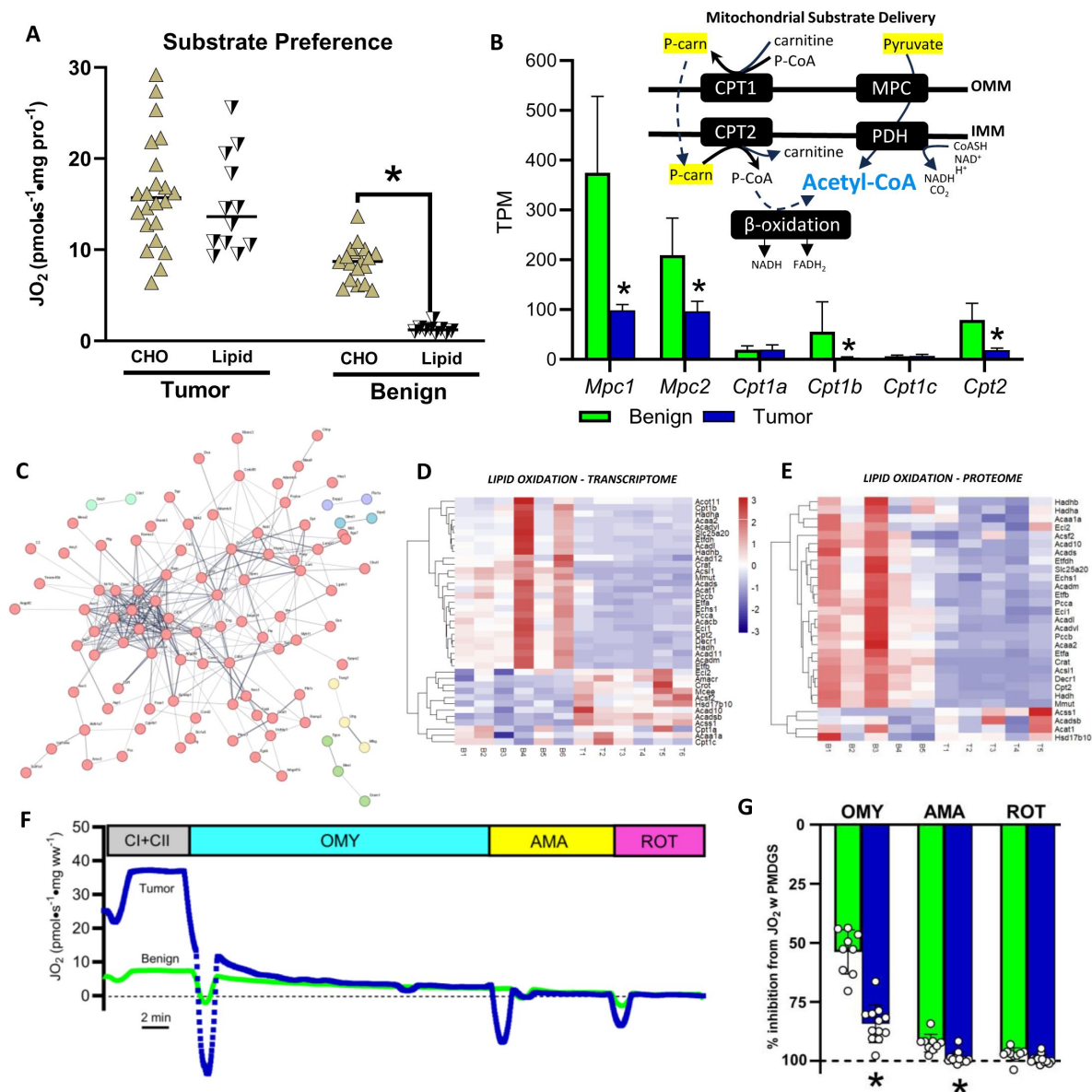


Figure 7.

Lipid and carbohydrate linked respiration and lipid metabolism transcriptome and proteome.

A) Maximal rates of carbohydrate (pyruvate, malate, ADP) and lipid (palmitoyl-carnitine, malate, ADP) supported respiration in tumors and benign mammary tissue. **B**) Gene expression (transcripts per million) of mitochondrial pyruvate carrier and carnitine palmitoyltransferase isoforms mediating mitochondrial pyruvate and long chain fatty acid transport respectively. **C**) Network interaction showing downregulated (unsupervised analysis) gene clustering in the HER2-driven tumor transcriptome, **D**) Heatmap of differentially expressed genes involved in lipid oxidation. **E**) Heatmap of differentially expressed proteins involved in lipid metabolism. **F**) Representative trace of protocol used to assess mitochondrial coupling. Following maximal rates of complex I and II-supported respiration, 1 μM oligomycin (OMY) was added to inhibit Complex V (ATP synthase), followed by 2.5 μM antimycin-A (AMA) and 1 μM rotenone (ROT) to inhibit complexes III and I respectively. **G**) Group data for experiment depicted in **(F)** depicted as relative inhibition from maximal complex I and II supported respiration (PMDGS) in each group. In panels A, B, F and G data are presented as means ± SD and statistical analyses were conducted using an unpaired, two-tailed Student's t-test (*p<0.05).

revealed the upregulation of several genes and proteins involved in mitochondrial fission and generally a downregulation of genes and proteins involved in mitochondrial fusion (**Fig.8B-F** [↗](#)). Together, these data show clear morphological differences in tumor versus benign tissue mitochondria, which could potentially influence OXPHOS efficiency.

Discussion

The aim of the current study was to investigate the metabolic characteristics of a mouse model of HER2-driven mammary cancer compared to benign mammary tissue. Specifically, we focused on the bioenergetic adaptations that have occurred with HER2-driven tumorigenesis both with respect to mitochondrial function (respiration and ROS) and mitochondrial content (total mitochondrial proteome and OXPHOS complexes). This investigation revealed several findings: 1) HER2-driven mammary tumors display high respiratory rates compared to benign mammary tissue, 2) tumors are capable of oxidizing lipids and pyruvate to a similar extent despite the downregulation of several genes involved in lipid catabolism, and 3) high tumor respiratory rates cannot be explained by higher mitochondrial content. These data highlight that HER2-driven mammary tumors have the capacity to maintain high OXPHOS rates despite lower mitochondrial content, challenging the typical bioenergetic relationship in fully differentiated tissues.

Relationship between mitochondrial content and function in breast cancer

Studies simultaneously assessing mitochondrial content and mitochondrial respiration and the contribution of these factors to breast cancer malignant transformation are scarce. While reductions in ETC subunit content/activity have been described in mitochondria isolated from human mammary carcinoma cultures compared to mammary epithelial cells³³ [↗](#), it has been assumed that a reduction in ETC subunit content would manifest as a reduction in respiratory capacity. However, this is at odds with several reports that classify breast cancer cells as OXPHOS reliant due to increases in mitochondrial respiration correlating with advanced disease²⁰ [↗](#),³⁴ [↗](#),³⁵ [↗](#). Indeed, across many cancers it is generally assumed that mitochondrial content and respiratory function increase or decrease concomitantly, however, recent work has challenged this dogma, demonstrating that high rates of respiration normalized to tissue weight can be negated when corrected for mitochondrial mass,¹⁴ [↗](#),³⁶ [↗](#) revealing intrinsic alterations in cancer cell bioenergetics. In the current study, utilizing this same approach to control for differences in mitochondrial content, we found that respiratory capacity supported by pyruvate and lipids in HER2-driven mammary tumors was significantly higher (~2-4 fold) compared to benign mammary tissue despite lower mitochondrial enrichment (total mitochondrial proteome) and lower abundance of several respiratory complex subunits, suggesting intrinsic changes in mitochondrial function/efficiency may explain the acquisition of increased mitochondrial respiratory capacity during HER2-driven tumorigenesis. Several mechanisms could enhance respiration in a mitochondrial-content independent manner, including increased coupling of electron transfer to ATP synthesis (coupling efficiency), changes in the delivery of substrates across the mitochondrial membranes, intrinsic changes in the activity of ETC complexes and/or ATP synthase, and alterations in mitochondrial morphology/dynamics.

OXPHOS efficiency: mitochondrial content-independent mechanisms supporting enhanced respiration rates

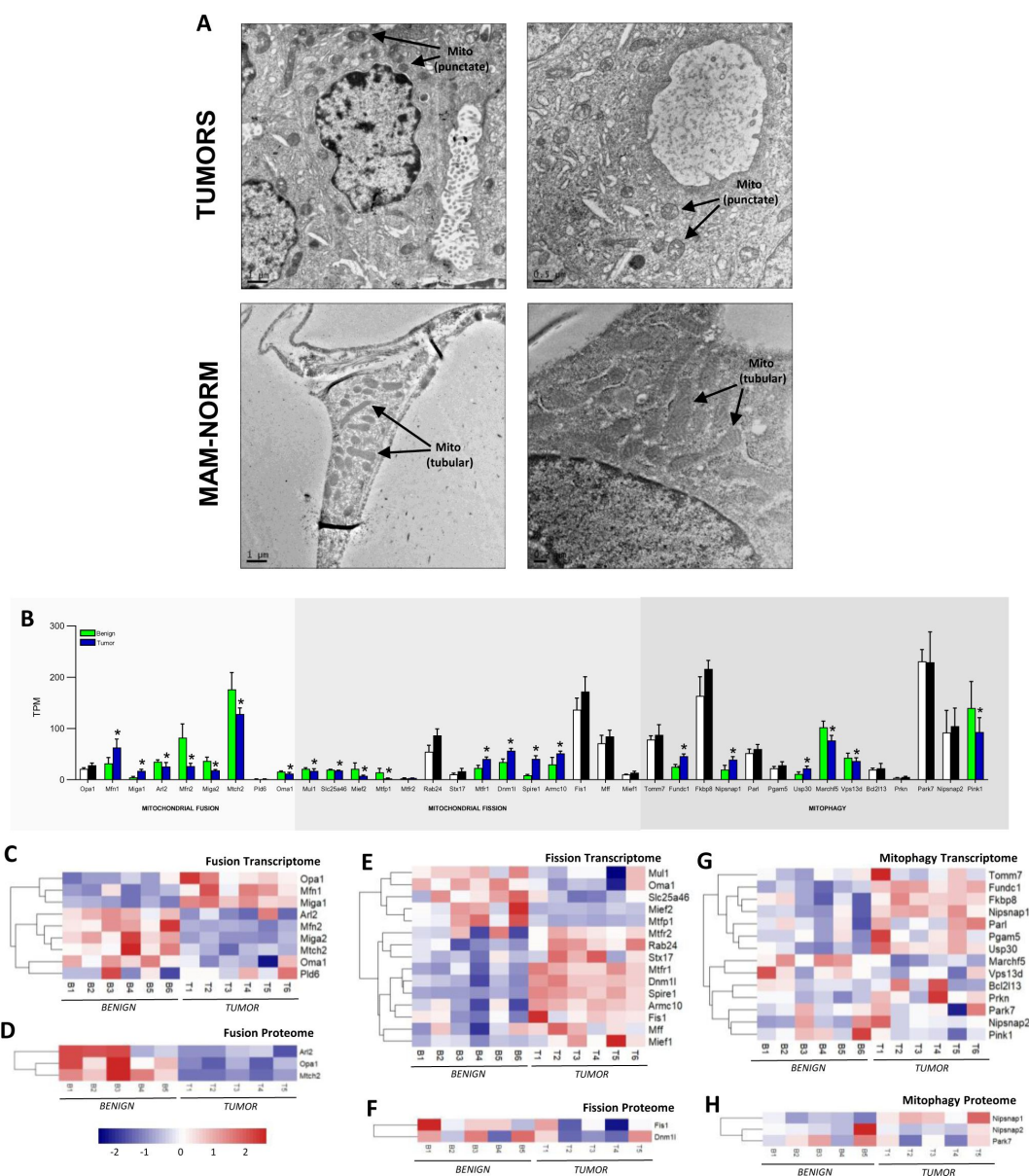


Figure 8.

Mitochondrial morphology and assessment of mitochondrial fusion, mitochondrial fission and mitophagy transcriptomes and proteomes in benign mammary tissue and HER2-driven tumors.

A) Transmission electron microscopy (TEM) images of tumor and benign samples showing punctate (tumor) versus elongated (benign) mitochondria. **B)** Gene expression (detected by transcriptomics; transcripts per million) of fusion, fission and mitophagy related genes separated per process filtered by MitoCarta, coloured bars and * represent genes with an adjusted p value of <0.1. Black and white bars represent genes with no significant differences between groups. **C)** Heatmap of fusion transcriptome, **D)** Heatmap of fusion proteome, **E)** Heatmap of fission transcriptome, **F)** Heatmap of fission proteome, **G)** Heatmap of mitophagy transcriptome, **H)** Heatmap of mitophagy proteome. TEM images are from independent tumor and benign tissue samples. Heatmaps depict all detected proteins (n=5/group) and transcripts (n=6/group).

Coupling Efficiency

Several studies have suggested that a key function of mitochondrial respiration in cancer cells is the regeneration of NAD^+ , FAD and ubiquinone to provide the necessary metabolites for biomass production, rather than ATP synthesis^{5,6,37}. From a bioenergetic perspective, this could be achieved by inducing uncoupled respiration, which could manifest in an apparent increase in intrinsic mitochondrial respiration, given that measures of respiratory flux quantify oxygen consumption rather than ATP synthesis. However, in the present study the Complex V inhibitor oligomycin inhibited respiration to a greater extent in HER2-driven tumors, suggesting that uncoupling cannot explain the enhanced respiration rates observed, and if anything, tumors are more coupled than benign mammary tissue. This interpretation aligns with work in MCF7 cells where mitochondrial ATP represents the majority of cellular ATP production³⁸, suggesting the ATP synthetic function of OXPHOS in breast cancer is likely intact. Indeed, enhanced mitochondrial ATP provision and OXPHOS-dependent biomass expansion are not mutually exclusive processes and increased coupled respiration would satisfy both the bioenergetic and biosynthetic requirements of rapid cell proliferation during tumorigenesis.

Substrate Delivery and Preference

Recent work has highlighted the dependency of HER2+ tumor cells on both lipid (long chain fatty acids) and carbohydrate-derived (pyruvate) substrate for optimal proliferative capacity⁴⁰. In addition to pyruvate, HER2-driven tumors may be particularly poised to utilize lipids, given the proximity of cancer cells to adipocytes in the mammary tumor microenvironment⁴¹, which has spurred interest in inhibiting lipid use/fat oxidation as an adjunct therapy in HER2-driven cancer⁴⁰. Indeed, the use of endogenous substrate may be preferred as solid tumors expand and factors such as increased interstitial fluid pressure and extracellular matrix remodelling impact tumor vessel quality⁴², possibly limiting exogenous substrate delivery. However, at the transcript and protein level, enzymes involved in mitochondrial lipid uptake (CPT1, CPT2) and pyruvate uptake (MPC) were largely downregulated compared to benign tissue which would limit rather than enhance mitochondrial substrate delivery. Despite this lower abundance of enzymes supporting mitochondrial substrate delivery, mitochondrial respiration supported by pyruvate and lipids were significantly higher in tumors. Considering lipid and carbohydrate oxidation converge within the mitochondrial matrix, both providing acetyl-CoA to the TCA cycle, it is possible that enhanced OXPHOS efficiency lies at a distal site, implicating intrinsic OXPHOS adaptations as a possible explanation for enhanced respiration in HER2-driven tumors.

Intrinsic OXPHOS Adaptations

Several cancers are characterized by the altered expression of individual ETC subunits and/or ATP synthase which can have functional repercussions on respiratory function^{10,14}. In the current study, despite a consistent downregulation of subunits comprising Complexes I-IV in HER2-driven tumors, a subset of Complex V genes were not downregulated (specifically two subunits of the c-ring of F0 domain (*Atp5g1*, *Atp5g2*), two subunits of the F6 domain stalk connecting the F0 and F1 domains (*Atp5j1*, *Atp5j2*), and regulatory proteins ATPase inhibitory factor 1 (*Atpif1*) and distal membrane assembly component 2 (*Dmac2l*). This shift essentially increases the ratio of ATP synthase/ETC and raises the possibility that a maintenance of these subunits may enhance OXPHOS rates in the face of downregulated CI-IV subunit content since maximal coupled respiration is usually limited by proton flux through ATP synthase. Indeed, despite mtDNA lesions in proton pumping complexes (I, III and IV) being common in many cancers, presumably reducing the content/function of these complexes, ATP synthase mutations are less common, suggesting positive selection may exist to maintain coupled respiration and ATP production^{10,43,44}. Thus, while speculative, it is possible that higher respiration rates in HER2-driven tumors may partially reflect maintained ATP synthase content/function. Alternatively, while bioenergetic efficiency is typically related to intrinsic changes in OXPHOS stoichiometry, mitochondrial

morphology can indirectly affect efficiency. We observed smaller, punctate mitochondria in HER2-driven mammary tumors when visualized by TEM, generally aligning with reductions in mitochondrial fusion and increases in mitochondrial fission-related targets. While the direct relationship between mitochondrial morphology and bioenergetics remains contentious, evidence suggests smaller mitochondria (due to enhanced fission or reduced fusion) may be associated with enhanced bioenergetics. Smaller mitochondria tend to have a higher surface-area-to-volume ratio which has been correlated with upregulated OXPHOS in the liver⁴⁵, and low rates of respiration is associated with the inhibition of mitochondrial fission⁴⁶. Furthermore, rare genetic mitochondrial diseases are often characterized by hyper-fused mitochondria which is associated with OXPHOS limitations⁴⁷. Indeed, morphological differences in breast cancer mitochondria are well described^{17,48–50} and targeting mitochondrial dynamics for therapy is an active area of study^{51–53}, however, there is a clear need to ascertain the relationship between mitochondrial dynamics and bioenergetics in tumorigenesis.

Conclusion

We provide evidence that HER2-driven mammary tumors display enhanced respiratory capacity despite lower mitochondrial content. This increased respiratory capacity could not be explained by changes in OXPHOS coupling, however, differences in mitochondrial morphology and the maintained expression of several subunits of ATP synthase suggest HER2-driven mammary tumors may undergo intrinsic adaptations in mitochondrial function during tumorigenesis.

Materials and methods

Animals and Breeding

All rodent experiments were conducted in accordance with institutional guidelines approved by the Animal Care Committee at the University of Guelph (AUP#5071). MMTV/*neu*^{ndl}-YD5 mice on an FVB background were obtained from Dr. William Muller (McGill University) and Dr. David Ma (University of Guelph). Female MMTV/*neu*^{ndl}-YD5 mice were obtained by breeding male heterozygous MMTV/*neu*^{ndl}-YD5 mice with female FVB mice to yield WT or heterozygous transgenics. Mice were group housed in ventilated cages at room temperature (22°C) and kept on a 12:12 light-dark cycle with *ad libitum* access to a chow diet and water. Starting at 10 weeks, mice were monitored for tumor development on a biweekly basis for ~10 additional weeks. Mice with tumors exceeding 17 mm in length/width or a volume over 5000 mm³ were euthanized before the 20-week time point.

Tumor and Benign Mammary Tissue Collection

Mice were anesthetized using sodium pentobarbital (60 mg/kg body weight) and checked for depth of anaesthesia by leg retraction after tail pinch. Once under the surgical plane, a ventral incision was made up the midline and the skin separated bilaterally from the underlying fascia to allow for the excision of the intact mammary tumors. Depending on the number of tumors present (typically 1-5), tumors were allocated for mitochondrial function (placed fresh in BIOPS preservation buffer), transmission electron microscopy (fixed in 2.5% glutaraldehyde/1% paraformaldehyde) or snap frozen in liquid nitrogen for further analysis (western blotting, transcriptomics, quantitative label-free proteomics) immediately after whole tumor wet weight was recorded. Paired mammary tissue with no visible tumors, furthest from the site of tumor excision (i.e. if tumors localized in cervical region benign tissue was harvested from inguinal region) were used as an internal comparison, denoted as benign mammary tissue.

RNA Sequencing and Transcriptomic Analyses

Frozen tissues were homogenized and lysed in TRIzol reagent. After centrifuging, the supernatant (aqueous phase) was applied to the RNeasy kit (Qiagen, 74106) for subsequent total RNA extraction and purification in accordance with the manufacturer's protocol. RNA samples were sent to the McMaster Genomics Facility where an RNA quality check, poly A enrichment, library prep and quality check were performed as previously described⁵⁴. These samples were then run in an Illumina Nextseq P2, 2×50bp sequencing run. FastQC and MultiQC were used for quality control of raw data. Salmon's transcript-level quantification⁵⁵ and DESeq2⁵⁶ was used to detect DEGs with the threshold of adjusted p-value<0.1. PCA analysis was performed by using variance stabilizing transformation (VST) data through DESeq2 using the pcaExplorer package⁵⁷ in R (version 4.2.1). Functional enrichment analysis was performed by GO enrichment analysis⁵⁸ and Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping using the GOSTATS, KEGG.db, and Bioconductor packages. Heatmaps and volcano plots were generated with ggplot2 package using all detected genes or genes sorted by an adjusted p-value<0.1 by false discovery rate method. Detailed descriptions for each analysis are included in the figure descriptions.

Western Blotting

Tumor and benign mammary tissues were homogenized in lysis buffer, diluted to equal protein concentration (1 µg/µL), separated by SDS-PAGE, transferred onto PVDF membranes and detected using enhanced chemiluminescence (ChemiGenius2 Bioimaging System, SynGene, Cambridge, UK) as previously described^{59,60}. Commercially available primary antibodies were used to detect total and phosphorylated targets (p38 MAPK (CS9212S, 1:1000), ERK1/2 (CS9102S, 1:1000), Akt (CS4691S, 1:1000), mTOR (CS2972S, 1:1000), p70s6k1 (CS9202S, 1:1000), eEF2 (CS2332S, 1:1000), p-p38 MAPK (CS9211S, 1:1000), p-ERK1/2 (CS9101S, 1:1000), p-AKT (CS9271S, 1:1000), p-mTOR (CS2971S, 1:1000), p-p70s6k1 (CS9234S, 1:1000), p-eEF2 (CS2331S, 1:1000). Due to rapid tissue/protein turnover in tumor tissues, Ponceau staining was an inappropriate loading control, however, trends in total protein targets between groups aligned with quantitative label-free proteomic analyses as a secondary method measuring protein content, validating targets detected by western blotting.

Protein Extraction and Purification for LC-MS

A Bradford assay was used to determine the protein concentration in the samples. Twenty-five µg of protein per lysate was resolubilized with denaturation buffer (6M urea/2M thiourea) and concurrently reduced with 10 mM DTT and alkylated with 20 mM iodoacetamide at RT for 60 min. Samples were precipitated by adding 6:1 v/v cold acetone and kept at -80°C for 60 minutes and the pellet was collected after centrifugation at 10,000g for 10 min at 4°C. Samples were resuspended with 50 mM ammonium bicarbonate and MD-grade trypsin (Thermo Fisher Scientific, Cat#90057) was added at a ratio of 1:50 protease to protein. Digestion occurred overnight at 37°C. After digestion, samples were dried by vacuum centrifugation and purified using Pierce C18 Spin columns (Thermo Fisher Scientific, cat#89873).

LC-MS

The Vanquish Neo UHPLC system was coupled with Orbitrap Exploris 240 mass-spectrometer using the Easy-Spray source for nanoLC-MS protein identification. The Vanquish Neo UHPLC system was configured for trap and elute analysis. Peptides were first trapped and washed on a Pepmap Neo C18 trap column (5 micron, 300 micron x5 mm) then separated on EASY-Spray columns 75 µm I.D. × 50 cm with the maximum pressure of 1200 bar. The nanoLC-MS system was controlled with Standard Instrument Integration (SII) for Xcalibur software. All hardware and data acquisition software were from Thermo Fisher Scientific. The mobile phase A and weak wash liquid was water with 0.1% FA, and the mobile phase B and strong wash liquid was 80% acetonitrile with 0.1% FA. The gradient was as follows 4-19% B over 72 minutes, 19-29% B over 28

minutes, 29–45% B over 20 minutes and a 14.5 min wash at 100% B with a flow rate of 300 nL/min. The autosampler temperature was 7 °C, and the column temperature was 45 °C. The sample was injected with Fast Loading set to ‘Enabled’ with Pressure Control at 500 bar. The column Fast Equilibration function was set to ‘Enabled’ with Pressure control at 800 bar, and the equilibration factor was set to 3. Vial bottom detection was set to ‘Enabled’.

DDA MS Method

The Orbitrap Exploris 240 MS was operated in DDA mode using a full scan with m/z range 375–1500, Orbitrap resolution of 60 000, normalized AGC target value 300%, and maximum injection time set to Auto. The intensity threshold for precursor was set to 1×10^4 . MS/MS spectra starting from 120 m/z were acquired in data-dependent acquisition (DDA) mode with a cycle time of 2 s, where the precursors were isolated in a window of 1.6 Da and subsequently fragmented with HCD using a normalized collision energy of 30%. Orbitrap resolution was set to 15 000 for MS2. The normalized AGC target was standard, and the maximum injection time was set to Auto.

Proteomics data analysis

Analysis of the tissue proteome was performed in R (version 4.2.1) and R Studio (version 2024.04.2+764). Principal component analysis was performed using the Vegan package. Volcano plots were created using log2 transformed fold change comparing cancer/benign tissue and $-\log_{10}$ adjusted p-value. P-values of differentially regulated proteins between each group were corrected with p.adjust (method = “fdr”) within each comparison. Depending on the analysis, heatmaps and volcano plots were generated using all detected proteins or proteins sorted by p.adjust <0.1, detailed descriptions for each analysis are included in the figure descriptions. Gene set enrichment was performed with the Kyoto Encyclopedia of genes and genomes (KEGG)⁶¹ and EnrichR⁶².

Mitochondrial Respiration

Mitochondrial respiration experiments were performed using the Oroboros Oxygraph-2k systems at 37°C in MiR05 respiration buffer (0.5 mM EGTA, 3 mM MgCl₂·H₂O, 60 mM potassium lactobionate, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 20 mM taurine, and 1 g/L fatty acid free BSA; pH 7.1) with constant stirring at room air saturation (starting at 180 μM O₂). Two–5 mg of permeabilized tumor tissue or 15–20 mg of permeabilized (both permeabilized with 20 μg/μL saponin) mammary tissue were used per 2 mL chamber and each protocol was conducted as at least two technical replicates (duplicate) per tumor or benign sample. Prior to the addition of substrates for any protocol, endogenous (basal) respiration was assessed with no substrates added. Complex I and Complex II supported respiration was assessed with the addition of 5 mM pyruvate and 2 mM malate, 5 mM ADP, 10 mM glutamate and 10 mM succinate. In a subset of these experiments, subsequent additions of oligomycin (OMY, 1 μM), antimycin A (AMA, 2.5 μM) and rotenone (ROT, 1 μM) were added to assess OXPHOS coupling. Lipid-supported respiration was assessed with 20 μM palmitoyl-carnitine in the presence of 2 mM malate and 5 mM ADP.

Reactive Oxygen Species Emission

Mitochondrial hydrogen peroxide (H₂O₂) emission was determined fluorometrically (Lumina, Thermo Scientific, Waltham, MA) in permeabilized mammary tissue and mammary tumors as previously described⁵⁹ with minor modifications. In brief, 2–5 mg of minced tissue was loaded into a cuvette containing Amplex Red (Invitrogen, Waltham, MA, USA), horseradish peroxidase (5U/mL), saponin (20 μg/μL), superoxide dismutase (40 U/mL) in Buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K₂HPO₄, 5 mM MgCl₂, 5 μM glutamate, 5 μM malate, 0.5% BSA, pH 7.4). Emission rates were assessed at 37°C in the presence of 10 mM succinate and calculated using a standard curve generated with known concentrations of H₂O₂.

Data Normalization

Mitochondrial respiration rates and H₂O₂ emission were normalized to individual sample wet weight, group total protein content and group mitochondrial content (mitochondrial enrichment factor, MEF). Total protein content was calculated as a group average (n=6/group) based on a Bradford assay and back-calculated total protein in a given wet weight of starting tissue. Data was normalized to mitochondrial protein by calculating the MEF for each group (n=g/group) based on all detected mitochondrial proteins by quantitative label-free proteomics (Mouse MitoCarta 3.0 database) normalized to total protein abundance per sample as previously described by others^{32,36}.

Transmission Electron Microscopy

Immediately following tissue dissection, tumor and benign mammary tissues were fixed in 2.5% glutaraldehyde/1% paraformaldehyde overnight at 4°C. The following day, tissues were washed 3x in 100 mM HEPES buffer in preparation for embedding. Tissue was stained with 1% osmium tetroxide for 3h, washed 3x in HEPES buffer and further stained with 1% uranyl acetate for 3h before washing 2x in HEPES buffer and once in ddH₂O. Stained tissue was dehydrated with a graded ethanol series followed by infiltration with LR White resin. Blocks were polymerized at 60°C for 18 hours before ultramicrotomy. Sections were post-stained with uranyl acetate and lead citrate. Sections were allowed to fully dry before imaging on the FEI Tecnai G2 F20 transmission electron microscope operated under normal conditions at 120 kVe.

Statistical Analysis

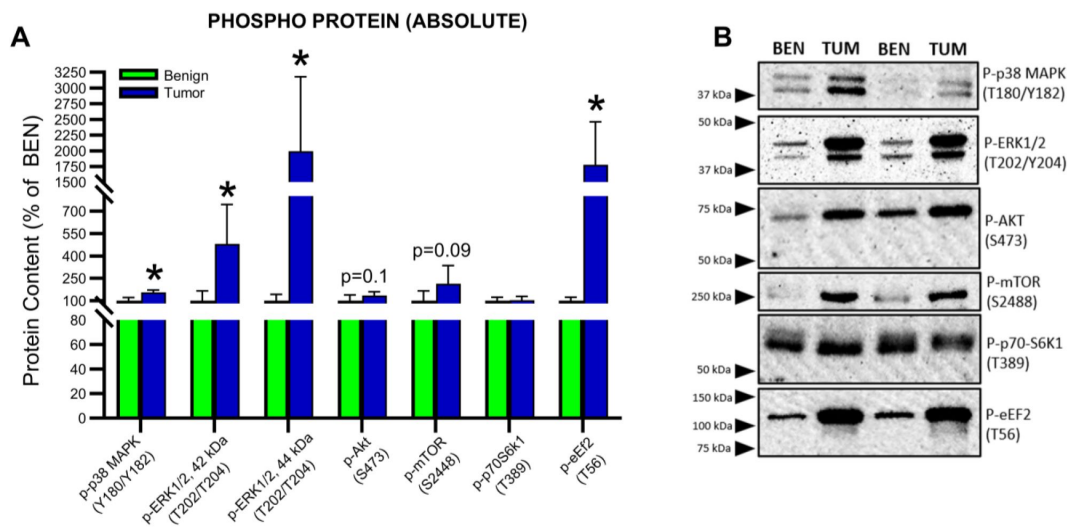
Statistical analysis and data visualization was performed using Microsoft Excel, GraphPad Prism (version 10.2.3), R (version 4.2.1) and R Studio (version 2024.04.2+764). All data are presented as mean ± SD and data was considered statistically significant if p<0.05. For transcriptomic and proteomic analyses, an adjusted p-value of <0.1 was utilized. Details regarding specific statistical analyses are included in the Results section and within each figure legend.

Data availability

Data is available from corresponding offers upon request.

Supplementary figures and table

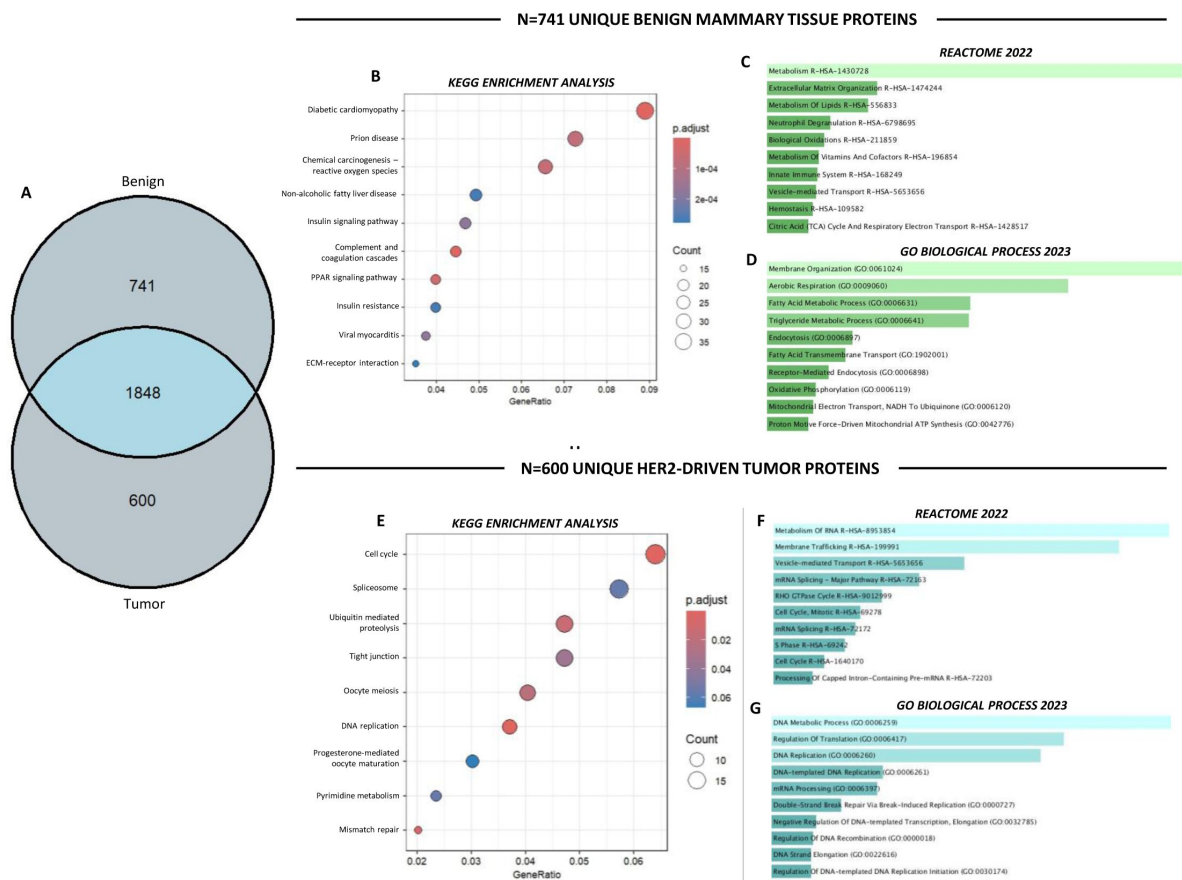
CANONICAL HER2 SIGNALING - PROTEIN



Supplementary Figure 1.

Absolute phosphorylated protein targets downstream of HER2 activation.

A) Quantified western blots for phosphorylated canonical HER2 signaling proteins (n=5-6/group). Phosphorylation sites are indicated for each target. **B)** Representative blot for each phosphorylated target. Data are presented as mean $\pm$ SD and analyzed with an unpaired, two tailed Student's t-test (*p<0.05).



Supplementary Figure 2.

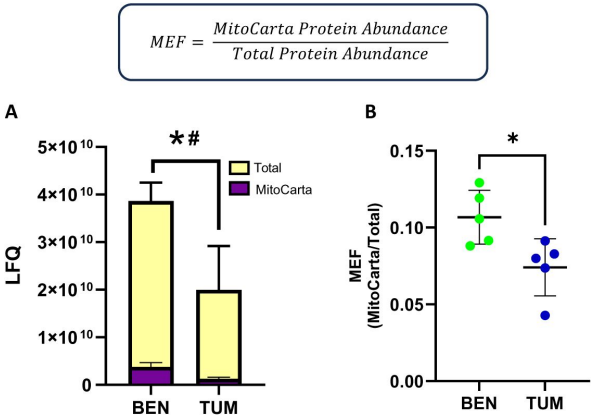
Unique HER2-driven tumor and benign mammary tissue proteins.

A) Venn diagram depicting 741 and 600 unique proteins identified in benign mammary tissue and HER2-driven tumors respectively, **B**) KEGG analysis of all detected unique benign mammary tissue proteins, **C**) Top 10 pathways (Reactome 2022), **D**) Top 10 gene ontologies (GO Biological Process) in benign mammary tissue, **E**) KEGG analysis of unique HER2-driven tumor proteins, **F**) Top 10 pathways (Reactome 2022), **G**) Top 10 gene ontologies (GO Biological Process) in HER2-driven tumors. KEGG, pathway and GO analyses were conducted on all mammary and tumor proteins detected by quantitative label-free proteomics. The Enrichr open access analysis web tool was used to generate panels C,D,F and G which were sorted by p-value.

Supplementary Figure 3.

Mitochondrial enrichment in benign mammary tissue and HER2-driven mammary tumors.

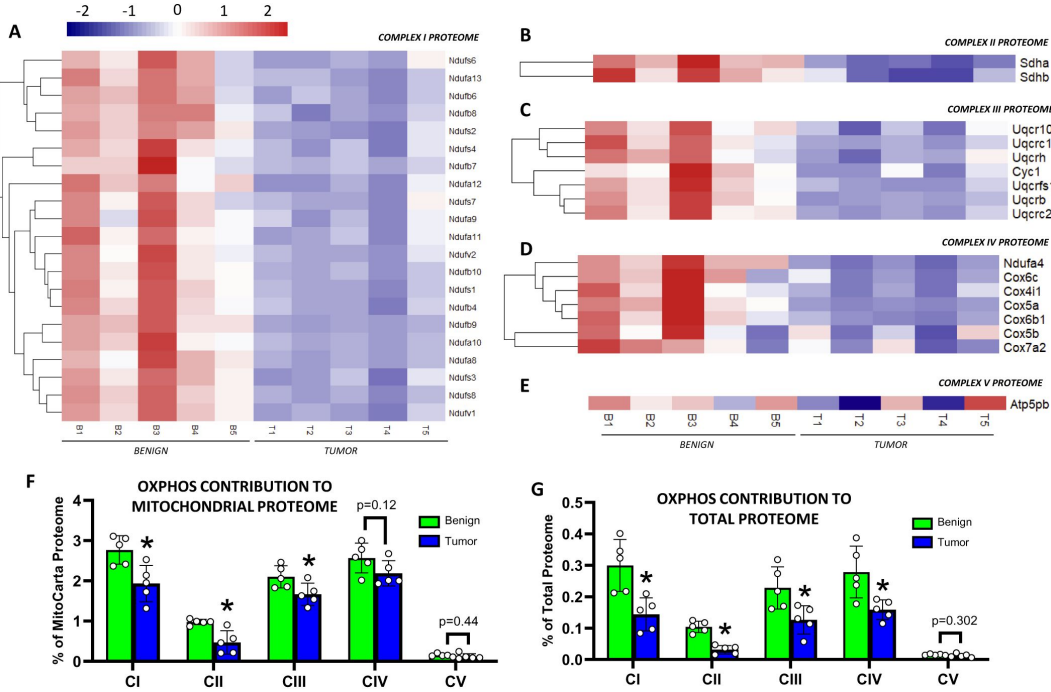
A) Total and mitochondrial (Mouse MitoCarta 3.0) absolute protein abundance assessed by quantitative label-free proteomics. **B)** Mitochondrial enrichment factor (MEF) calculated as the ratio of all detected MitoCarta proteins relative to the underlying total proteome per sample (n=5/group). Statistical analysis was conducted using an unpaired two tailed Student's t-test (*p<0.05). In panel A, ** denotes significant differences between both total and MitoCarta proteomes between groups.



Supplementary Figure 4.

Proteomic analysis of detected OXPHOS subunits.

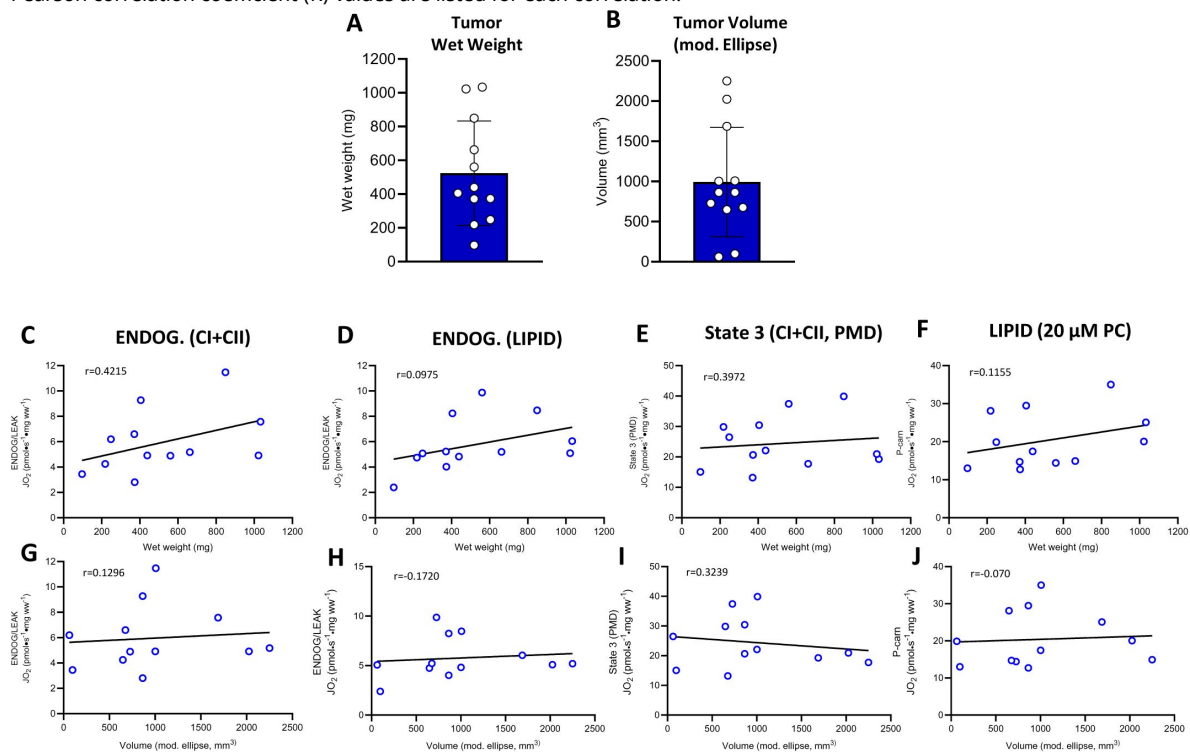
Heatmaps showing subunits detected for **A)** Complex I, **B)** Complex II, **C)** Complex III, **D)** Complex IV, **E)** Complex V (ATP synthase) using quantified label-free proteomics. **F)** OXPHOS complex I-V absolute abundance as a percentage of the mitochondrial proteome (n=5/group). **G)** OXPHOS complex I-V absolute abundance as a percentage of the total detected proteome (n=5/group). Data in panels F,G are presented as means ± SD and analyzed with an unpaired, two tailed Student's t-test (*p<0.05).



Supplementary Figure 5.

Correlation of tumor size and volume with maximal carbohydrate and lipid-supported respiration rates.

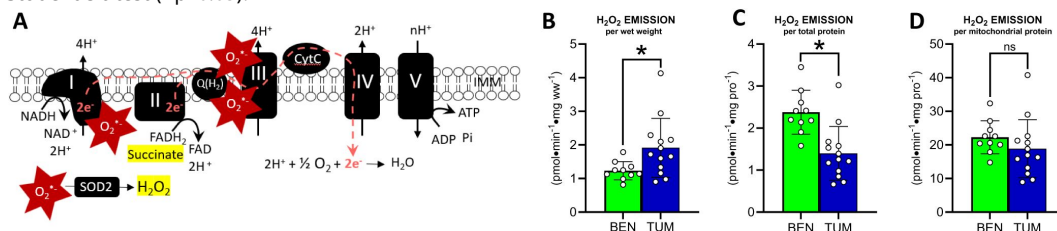
A) Tumor wet weight (whole tumor post excision), **B)** Tumor volume calculated as a modified ellipse ($V = 1/2(\text{length} \times \text{width}^2)$). **C-F)** Endogenous (no substrate, CI+CII protocol), Endogenous (no substrate, lipid protocol), State 3 (5 mM pyruvate, 2 mM malate, 5 mM ADP) and maximal lipid-supported (2 mM malate, 5 mM ADP, 20 μM palmitoyl-carnitine) respiration rates correlated with tissue wet weight. **G-J)** Same respiration data as **(C-F)** but correlated with tumor volume. The Pearson correlation coefficient (R) values are listed for each correlation.



Supplementary Figure 6.

Reactive oxygen species emission in benign mammary tissue and HER2-driven mammary tumors.

A) Schematic depicting major sites of superoxide emission from respiratory complexes I and III. Superoxide dismutase 2 (SOD2) subsequently catalyzes the dismutation of superoxide into hydrogen peroxide (H_2O_2) which can be measured fluorometrically. **B)** H_2O_2 emission per wet tissue weight, **C)** H_2O_2 emission per total protein, **D)** H_2O_2 emission per mitochondrial protein (MEF, mitochondrial enrichment factor). Statistical analyses were conducted using an unpaired, two-tailed Student's t-test (* $p < 0.05$).



Abbrev.	Protein	Pathway/Function	Fc (vs BEN)	p.adj
PGK1	Phosphoglycerate kinase	Glycolysis	2.48	0.068
KPYM	Pyruvate kinase	Glycolysis	3.17	0.048
MP2K4	Mitogen activated protein kinase kinase 4	MAPK Pathway	3.65	0.012
RL38	Large ribosomal subunit protein eL38	Protein synthesis/translation	4.11	0.078
PGTA	Geranylgeranyl transferase type-2 subunit alpha	Cysteine prenylation of Rab proteins	4.28	0.067
RI23	Large ribosomal subunit protein uL14	Protein synthesis/translation	4.41	0.042
CADH1	Cadherin-1	Cell adhesion and motility, epithelial cell marker	12.75	0.084
LMCD1	LIM and cysteine rich domains protein 1	Transcriptional cofactor for GATA6 involved in cell proliferation and metastasis (MMP, EGFR)	15.26	0.077
MPRI	Cation independent mannose 6 phosphate receptor	Lysosomal/Golgi trafficking, associates with IGFR2	22.3	0.093

Supplementary Table 1.

List of 9 significantly upregulated proteins between tumor and benign samples, sorted in order of fold change from benign (smallest to largest fold change).

General pathways or functions are listed per protein based on ID mapping from the Uniprot protein sequence database. Adjusted p-values are listed per target.

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Author information

Sara M Frangos

Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Canada
ORCID iD: [0000-0002-1590-8826](https://orcid.org/0000-0002-1590-8826)

For correspondence: sfrangos@uoguelph.ca

Henver S Brunetta

Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Canada

Dongdong Wang

Centre for Metabolism, Obesity and Diabetes Research, McMaster University, Hamilton, Canada, Division of Endocrinology and Metabolism, Department of Medicine, McMaster University, Hamilton, Canada

Maria Joy Therese Jabile

Centre for Metabolism, Obesity and Diabetes Research, McMaster University, Hamilton, Canada, Division of Endocrinology and Metabolism, Department of Medicine, McMaster University, Hamilton, Canada

David WL Ma

Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Canada

William J Muller

Department of Biochemistry, McGill University, Rosalind and Morris Goodman Cancer Research, Montreal, Canada

Cezar M Khursigara

Department of Molecular and Cellular Biology, University of Guelph, Guelph, Canada

Kelsey H Fisher-Wellman

Department of Cancer Biology, Medical Center Boulevard, Wake Forest University School of Medicine, Winston-Salem, United States

Gregory R Steinberg

Centre for Metabolism, Obesity and Diabetes Research, McMaster University, Hamilton, Canada, Division of Endocrinology and Metabolism, Department of Medicine, McMaster University, Hamilton, Canada, Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Canada

Graham P Holloway

Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Canada

For correspondence: ghollowa@uoguelph.ca

Editors

Reviewing Editor

Lynne-Marie Postovit

Queens University, Kingston, Canada

Senior Editor

Lynne-Marie Postovit

Queens University, Kingston, Canada

Reviewer #1 (Public review):

Summary:

In this manuscript, Frangos et al. used a transcriptomic and proteomic approach to characterise changes in HER2-driven mammary tumours compared to healthy mammary tissue in mice. They observed that mitochondrial genes, including OXPHOS regulators, were among the most down-regulated genes and proteins in their datasets. Surprisingly, these were associated with higher mitochondrial respiration, in response to a variety of carbon sources. In addition, there seems to be a reduction in mitochondrial fusion and an increase in fission in tumours compared to healthy tissues.

Strengths:

The data are clearly presented and described.

The author reported very similar trends in proteomic and transcriptomic data. Such approaches are essential to have a better understanding of the changes in cancer cell metabolism associated with tumourigenesis

Weaknesses:

This study, despite being a useful resource (assuming all the data will be publicly available and not only upon request) is mainly descriptive and correlative and lacks mechanistic links.

It would be important to determine the cellular composition of the tumour and healthy tissue used. Do the changes described here apply to cancer cells only or do other cell types contribute to this?

Are the changes in metabolic gene expression a consequence of HER2 signalling activation? Ex-vivo experiments could be performed to perturb this pathway and determine cause-effects.

The data of fission/fusion seem quite preliminary and the gene/protein expression changes are not so clear cut to be a convincing explanation that this is the main reason for the increased mitochondria respiration in tumours.

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Reviewer #2 (Public review):

Frangos et al present a set of studies aiming to determine mechanisms underlying initiation and tumour progression. Overall, this work provides some useful insights into the involvement of mitochondrial dysfunction during the cellular transformation process. This

body of work could be improved in several possible directions to establish more mechanistic connections.

(1) The interesting point of the paper: the contrast between suppressed ETC components and activated OXPHOS function is perplexing and should be resolved. It is still unclear if activated mitochondrial function triggers gene down-regulation vs compensatory functional changes (as the title suggests). Have the authors considered reversing the HER2-derived signals e.g. with PI3K-AKT-MTOR or ERK inhibitors to potentially separate the expression vs. functional phenotypes? The root of the OXPHOS component down-regulation should also be traced further, e.g. by probing into levels of core mitochondrial biogenesis factors. Are transcript levels of factors encoded by mtDNA also decreased?

(2) The second interesting aspect of this study is the implication of mitochondrial activation in tumours, despite the downregulation of expression signatures, suggestive of a positive role for mitochondria in this tumour model. To address if this is correlative or causal, have the authors considered testing an OXPHOS inhibitor for suppression of tumorigenesis?

(3) A number of issues concerning animal/ tumour variability and further pathway dissection could be explored with in vitro approaches. Have the authors considered deriving tumour-derived cell cultures, which could enable further confirmations, mechanistic drug studies and additional imaging approaches? Culture systems would allow alternative assessment of mitochondrial function such as Seahorse or flow cytometry (mitochondrial potential and ROS levels).

(4) The study could be greatly improved with further confirmatory studies, eg immunoblotting for mitochondrial components with parallel blots for phospho-signalling in the same samples. It would be interesting if trends could be maintained in tumour-derived cell cultures. It is notable that OXPHOS protein/transcript changes are more consistent (Figure 5, Supplementary Figure 4) than mitochondrial dynamics /mitophagy factors (Figure 8). Core regulatory factors in these pathways should be confirmed by conventional immunoblotting.

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