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Article

Comparison of Deeply versus Mildly Invasive Mesothelioma Proteomes Identifies Potential Links between Mitochondrial *Acadvl* Expression and Biomarkers of Aggressiveness

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Simple Summary: We aimed to assess the role of mitochondrial protein changes in the emergence of aggressive properties in the most invasive subtype of malignant mesothelioma. Our results demonstrate that the evolution from a mildly invasive to a deeply invasive form in experimental tumor models was associated with a rise in the level of the very long-chain specific acyl-CoA dehydrogenase. We also point to strong links between this mitochondrial event and parallel quantitative modifications of key proteins in the extracellular matrix, immune cell components and other subcellular compartments.

Abstract: Recent reports suggest that dysregulation of lipid metabolism is a key feature of the most invasive cancers. To identify potential biomarkers of tumor aggressiveness, we compared the proteomes of two experimental models of malignant mesothelioma in rats exhibiting different invasive properties. Quantitative changes between the most invasive, M5-T1, versus the least invasive, F4-T2, first led to a list of 424 proteins. A second step, cross-comparing this list with 433 proteins distinguishing invasive vs non-invasive tumors, led to identifying 88 proteins that specifically increased and 157 that decreased, respectively, characterizing the most aggressive M5-T1 tumor. Among the 15 mitochondrial proteins found in these lists, the very long-chain specific acyl-CoA dehydrogenase, encoded by the *Acadvl* gene and involved in fatty acid beta oxidation, appeared to play an important role in the metabolic reprogramming of the tumor microenvironment. Immunohistochemical staining of tumor sections confirmed increased expression of *Acadvl* in the M5-T1 tumor. Finally, the dramatic increase and decrease, observed in 25 and 17 proteins, respectively, suggested the existence of a strong link between mitochondrial events and modifications of the extracellular matrix, immune cell components or other subcellular compartments. These findings highlight some important aspects of the tumor microenvironment changes linked to aggressiveness.

Keywords: Cancer; aggressiveness; mitochondria; *Acadvl*; fatty acid beta oxidation; biomarker; proteomics; malignant mesothelioma

1. Introduction

According to the National Cancer Institute dictionary of cancer terms, the term “aggressive” describes “a tumor that forms, grows, or spreads quickly”. For malignant mesothelioma, this term is frequently preferred to “invasive”, which defines cancers that “spread beyond the layer of tissue in which it developed”, especially for cases associated with the poorest clinical outcomes [1]. For aggressive tumors, investigations of their molecular alterations are useful tools that may improve clinical decisions and patient outcomes [2]. Previous studies have shown the usefulness of proteome studies as considerable discordance can be found between genomic mutations and protein expression levels, due to post-transcriptional regulation [3]. This approach recently led to identifying biomarkers that characterized highly aggressive tumor behavior [4]. Applied to the study of a highly invasive

experimental tumor model, it also provided lists of candidate biomarkers exhibiting abundance changes associated with liver metastatic colonization, thus representing potential therapeutic targets [5].

In this field, numerous insights suggest that dysregulation of lipid metabolism is a major characteristic of highly invasive cancers, given its involvement in energy, changes in the composition of membranes, and signaling pathways [6]. This process is also associated with the epithelial-to-mesenchymal transition (EMT), Warburg effect, and extracellular matrix remodeling that frequently characterize these cancers [7].

Among the different metabolic pathways, alterations in fatty acid metabolism have been reported to contribute to aggressiveness in melanoma [8]. The role of cancer-associated adipocytes (CAAs), a new player in EMT in tumor microenvironment changes, was also emphasized [9]. Even naïve adipocytes were reported to be a key contributor to melanoma progression at a distance by providing extracellular vesicles that transfer both protein machinery and fatty acid substrates to cancer cells [10]. Given these recent insights into the role of mitochondria in tumor aggressiveness, in this study we first aimed to identify mitochondrial proteins whose abundance changes differentiate a deeply invasive from a mildly invasive experimental peritoneal mesothelioma designed in an immunocompetent rat strain. Among the fifteen mitochondrial proteins found, only one enzyme was involved in fatty acid beta oxidation (FAO). The marked increase and decrease, observed in 25 and 17 other candidate biomarkers of aggressiveness, respectively, led us to identify potential links between this mitochondrial enzyme and parallel changes in other subcellular compartments and the tumor microenvironment.

2. Materials and Methods

2.1. Rat Tumor Tissues for Proteomic Analyses

The formalin-fixed paraffin embedded (FFPE) tissue samples used in this study were taken from the same groups of Fisher F344 rats with four different experimental mesotheliomas of increasing stages of invasiveness, as previously described [11]. To generate the tumors, the experimental procedures used for *in vivo* manipulations in the UTE-IRS UN (Unité Thérapeutique Expérimentale de l'Institut de Recherche en Santé de l'Université de Nantes) between 2011 and 2015 followed the European Union guidelines for the care and use of laboratory animals in research protocols (approval #01257.03 of the French Ministry of Higher Education and Research (MESR)). The rats were purchased from Charles River Laboratories (L'Arbresle, 69210, France) and the experiments approved by the ethics committee for animal experiments (CEEAA) of the Pays de la Loire Region under the recorded number 2011.38. The non-invasive M5-T2, mildly invasive F4-T2, moderately invasive F5-T1 and deeply invasive M5-T1 tumors were collected after intraperitoneal injection of 3×10^6 cells of the corresponding cell lines (<https://technology-offers.inserm-transfert.com/offer/>, recorded as RT00418, RT00419, RT00421 and RT00417, respectively) to syngeneic rats.

2.2. Proteomic Analyses

For each sample analyzed, four or five 20 μm -thick sections of tumor tissue were scratched with a scalpel and collected in a 1.5-mL Eppendorf® microtube, and all the material collected deparaffinized in three successive xylene washes, then rehydrated in 100%, 95%, 70% and 50% ethanol solutions. The pellets were vacuum-dried, and the dried tissues resuspended in 200 μL of Rapigest SF (Waters, Milford, MA, USA). Dithiothreitol (AppliChem, Darmstadt, Germany) was then added (5 mM final concentration), and the samples incubated in a thermo shaker at 95°C for one hour, and then sonicated twice (ultrasonic processor 75,185, Bioblock Scientific, Illkirch, 67400, France). Cystein residues were alkylated by adding 200 mM of S-Methyl methanethiosulfonate at 37°C (10 mM final concentration). Sequencing-grade trypsin was added in a ratio $\geq 2 \mu\text{g mm}^{-3}$ tissue (at 37°C overnight), the reaction was stopped with formic acid (9% final concentration, incubation at 37°C for one hour), and the acid-treated samples were centrifuged at 16,000 g for ten minutes. After removing the salts from the supernatant, the peptides were collected in a new Eppendorf® microtube using C18 STAGE tips, and their concentration finally determined using the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, St Herblain, 44800, France). The rat spectral library, SWATH-MS analysis, peptide identification, and relative quantification were performed as previously described [11]. The statistical analysis of the SWATH data set, peak extraction output data matrix from

PeakView was imported into MarkerView (v.2, AB Sciex Pte, Ltd., Framingham, MA, USA) for data normalization, and relative protein quantification. Proteins with a statistical p -value < 0.05 estimated by MarkerView were considered to be differentially expressed under different conditions.

2.3. Histology and Immunohistochemical Analyses

The FFPE blocs were cut with a Leica RM2255 microtome (Leica Biosystems, Nussloch, Germany). Areas of interest for both proteomic and histological analyses were selected based on examination of sections of all samples stained with hematoxylin phloxine saffron (HPS), scanned on a Nanozoomer 2.0 HT Hamamatsu. For immunohistochemistry, tumor tissue sections were stained with anti-ACADVL antibody (NBP1-89287, BIO-TECHNE, Chatillon sur Seiche, 35092, France).

3. Results

3.1. Biomarkers of Deep Invasiveness and Immunosuppression

The procedure we used to identify mitochondrial biomarkers specifically related to the most aggressive tumor phenotype included four successive steps. First, we established a list of 433 proteins associated with the acquisition of invasive properties by comparing the proteome of the three invasive tumor models (F4-T2, F5-T1 and M5-T1) with that of the non-invasive tumor M5-T2 (Figure 1, step 1). In a second step, the comparison of the proteomes of the most invasive tumor, M5-T1 vs the least invasive, F4-T2, led to 424 proteins showing significant abundance changes (Figure 1, step 2). Next, we cross-referenced the two listings, defining two main subgroups within these 424 proteins. The first subgroup of 163 proteins that was common to the two listings was excluded from our study (supplementary Table S1). The second subgroup of 245 proteins showing abundance changes related only to deeply invasive properties and immunosuppression (Figure 1, steps 2 and 3), was analyzed in more detail in this study. The last 16 remaining proteins, which corresponded to tendencies towards an increase or decrease (with $0.05 < p < 0.085$, supplementary Table S2), were also excluded from the study. Interestingly, however, this list contained 4 proteins for which p -values were < 0.001 in the comparison between M5-T1 and F4-T2 tumors, including a cytoskeletal protein (encoded by *Fscn1*), two proteins involved in transcription (encoded by *Hmg1* and *Wt1*), which all increased, and a protein secreted to blood (encoded by *Cp*), which decreased.

The second subgroup was further divided into 88 proteins showing increased levels (Table 1), and 157 proteins with decreased levels (Table 2).

3.2. Mitochondrial Proteins Specifically Changed in the Deeply Invasive M5-T1 Tumor

Next, we recorded the subcellular/extracellular locations of these proteins on <https://www.proteinatlas.org> (accessed on February 9, 2023), and 15 proteins exclusively located in mitochondria were identified, 7 showing an increase and 8 a decrease (Figure 1, step 4).

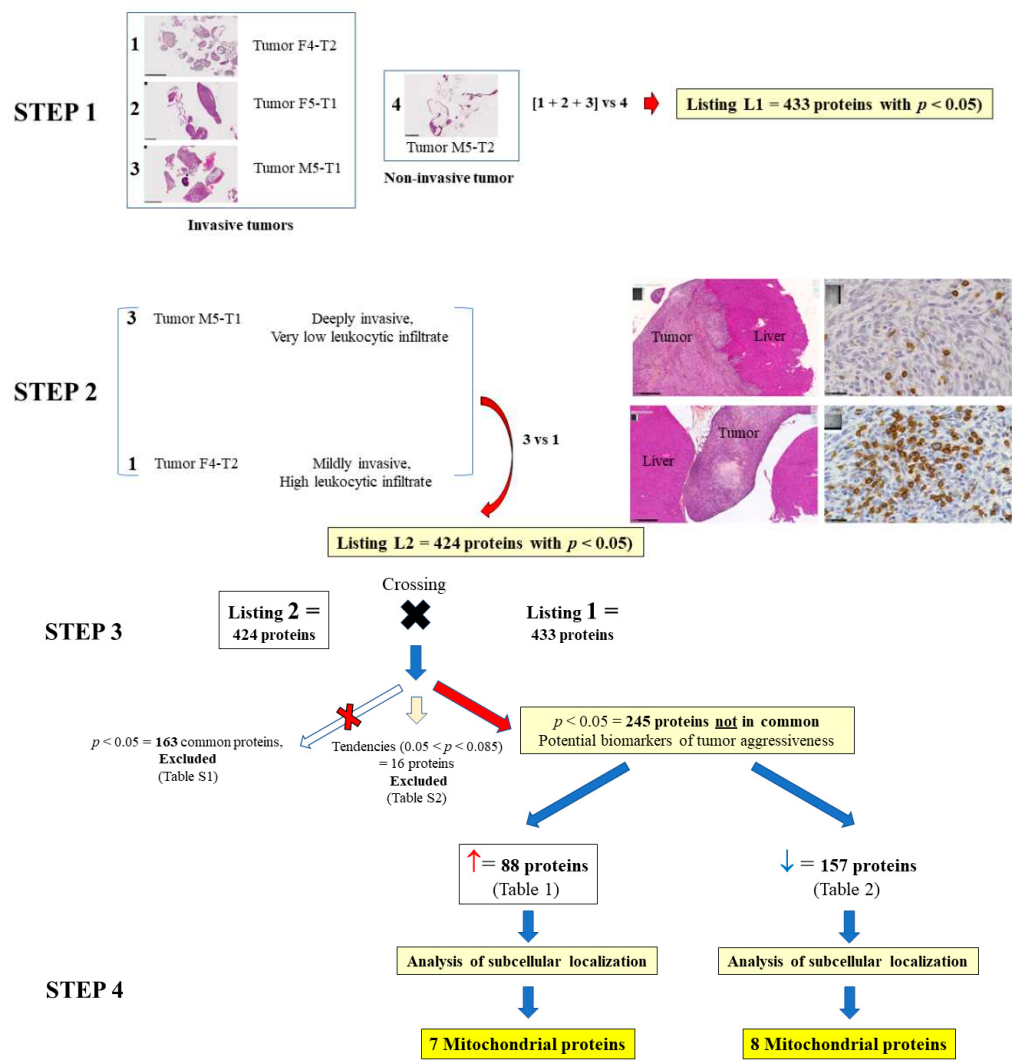


Figure 1. Methodological approach used to identify mitochondrial biomarkers of aggressiveness.

Table 1. List of tumor tissue proteins exhibiting significant abundance increases with maximum aggressiveness. Comparison M5-T1 vs F4-T2 (p -values < 0.05). Gene and full protein names are given for *Rattus norvegicus* according to www.uniprot.org.

Gene	Full name
<i>Ywhag</i>	14-3-3 protein gamma
<i>Hibadh</i>	3-hydroxyisobutyrate dehydrogenase, mitochondrial
<i>Got1</i>	Aspartate aminotransferase, cytoplasmic
<i>Acadvl</i>	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial
<i>Acsl6</i>	Long-chain-fatty-acid--CoA ligase 6
<i>Actn1</i>	Alpha-actinin-1
<i>Aebp1</i>	Adipocyte enhancer-binding protein 1
<i>Aldh4a1</i>	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial
<i>Ampd2</i>	AMP deaminase 2
<i>Amy2</i>	Pancreatic alpha-amylase
<i>Anxa3</i>	Annexin A3

<i>Bzw2</i>	Basic leucine zipper and W2 domain-containing protein 2
<i>Cc2d1b</i>	Coiled-coil and C2 domain-containing protein 1B
<i>Cpa1</i>	Carboxypeptidase A1
<i>Ccdc93</i>	REVERSED Coiled-coil domain-containing protein 93
<i>Ccs</i>	Copper chaperone for superoxide dismutase
<i>Cdc5l</i>	Cell division cycle 5-like protein
<i>Cel</i>	Bile salt-activated lipase
<i>Cma1</i>	Chymase
<i>Cnn3</i>	Calponin-3
<i>/</i>	UPF0449 protein C19orf25 homolog
<i>Ctbp2</i>	C-terminal-binding protein
<i>Ctnnb1</i>	Catenin beta-1
<i>Ctrb1</i>	Chymotrypsinogen B
<i>Dync1li1</i>	Cytoplasmic dynein 1 light intermediate chain
<i>Urod</i>	Uroporphyrinogen decarboxylase
<i>Dync1h1</i>	Cytoplasmic dynein 1 heavy chain 1
<i>Ecm1</i>	Extracellular matrix protein
<i>Eef1d</i>	Elongation factor 1-delta
<i>Eno2</i>	Gamma-enolas
<i>Fam65a</i>	Protein FAM65A
<i>Fmod</i>	Fibromodulin
<i>Fxr1</i>	Fragile X mental retardation syndrome-related protein 1
<i>Gdi1</i>	Rab GDP dissociation inhibitor alpha
<i>Gnaq</i>	Guanine nucleotide-binding protein G(q) subunit alph
<i>Hnrnpk</i>	Heterogeneous nuclear ribonucleoprotein K
<i>Hspa1b</i>	Heat shock 70 kDa protein 1B
<i>Hspb8</i>	Heat shock protein beta-8
<i>Irf2bpl</i>	Interferon regulatory factor 2-binding protein-like
<i>Ifitm3</i>	Interferon-induced transmembrane protein 3
<i>Itih3</i>	Inter-alpha-trypsin inhibitor heavy chain H3
<i>Krt8</i>	Keratin, type II cytoskeletal 8
<i>Lnpep</i>	Leucyl-cystinyl aminopeptidase
<i>Letm1</i>	LETM1 and EF-hand domain-containing protein 1, mitochondrial
<i>Pnlip</i>	Pancreatic triacylglycerol lipase
<i>Lman1</i>	Protein ERGIC-53
<i>Lrrc7</i>	Leucine-rich repeat-containing protein 7
<i>Lox</i>	Protein-lysine 6-oxidase
<i>Map1b</i>	Microtubule-associated protein 1B
<i>Mlec</i>	Malectin
<i>Mxra8</i>	Matrix-remodeling-associated protein 8
<i>Myh4</i>	Myosin-4
<i>Myh8</i>	Myosin-8

<i>Ppp1r12a</i>	Protein phosphatase 1 regulatory subunit 12A
<i>Nedd4</i>	E3 ubiquitin-protein ligase NEDD4
<i>Nid2</i>	Nidogen-2
<i>Nmes1</i>	Normal mucosa of esophagus-specific gene 1 protein
<i>Smpd3</i>	Sphingomyelin phosphodiesterase 3
<i>Pdha1</i>	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial
<i>Ddost</i>	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit
<i>Pycrl</i>	Pyrroline-5-carboxylate reductase 3
<i>Pcca</i>	Propionyl-CoA carboxylase alpha chain, mitochondrial
<i>Pdcd6ip</i>	Programmed cell death 6-interacting protein
<i>Pdlim4</i>	PDZ and LIM domain protein 4
<i>Pfkl</i>	ATP-dependent 6-phosphofructokinase, liver type
<i>Phldb1</i>	Pleckstrin homology-like domain family B member 1
<i>Plcb3</i>	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-3
<i>Plcd1</i>	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-1
<i>Plg</i>	Plasminogen
<i>Plod2</i>	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2
<i>Psmc6</i>	Proteasome subunit alpha type-6
<i>Rab18</i>	Ras-related protein Rab-18
<i>Ric8a</i>	Synembryn
<i>Rpn1</i>	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1
<i>Rps28</i>	40S ribosomal protein S28
<i>P3h4</i>	Synaptonemal complex protein SC65
<i>Sfxn1</i>	Sideroflexin-1
<i>Shc1</i>	SHC-transforming protein 1
<i>Sh3glb1</i>	Endophilin-B1
<i>Smad3</i>	Mothers against decapentaplegic homolog 3
<i>Stmn1</i>	Stathmin
<i>Dars</i>	Aspartate--tRNA ligase, cytoplasmic
<i>Synpo</i>	Synaptopodin
<i>Tubb3</i>	Tubulin beta-3 chain
<i>Prss1</i>	Anionic trypsin-1
<i>Try3</i>	Cationic trypsin-3
<i>Tsg101</i>	Tumor susceptibility gene 101 protein
<i>Vcl</i>	Vinculin
<i>Vkorc1l1</i>	Vitamin K epoxide reductase complex subunit 1-like protein 1

Table 2. List of tumor tissue proteins exhibiting significant abundance decrease with maximum aggressiveness. Comparison M5-T1 vs F4-T2 (p-values < 0.05). Gene and full protein names are given for *Rattus norvegicus* according to www.uniprot.org.

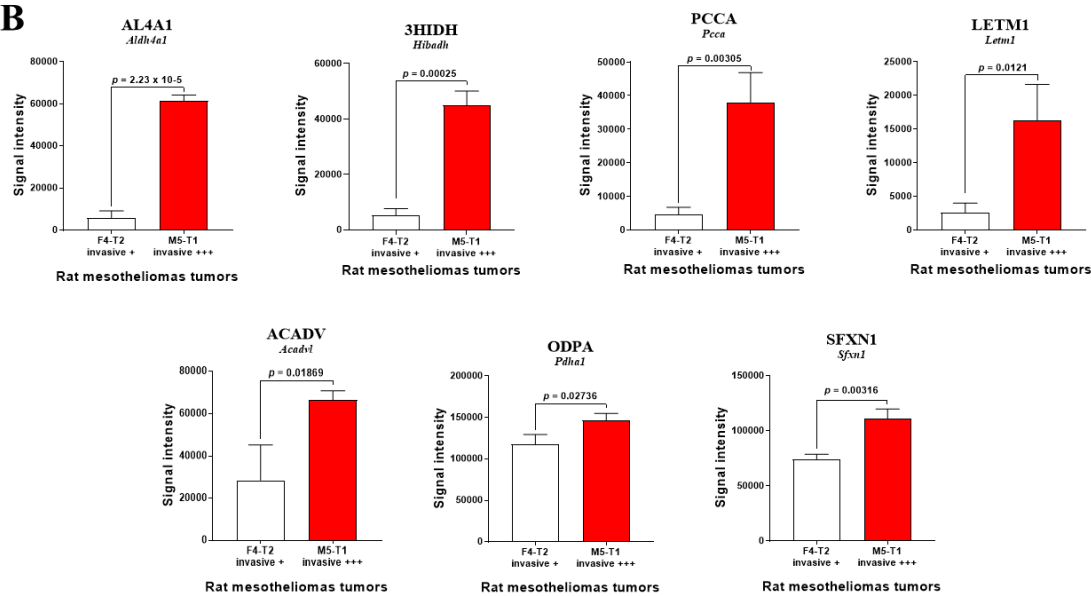
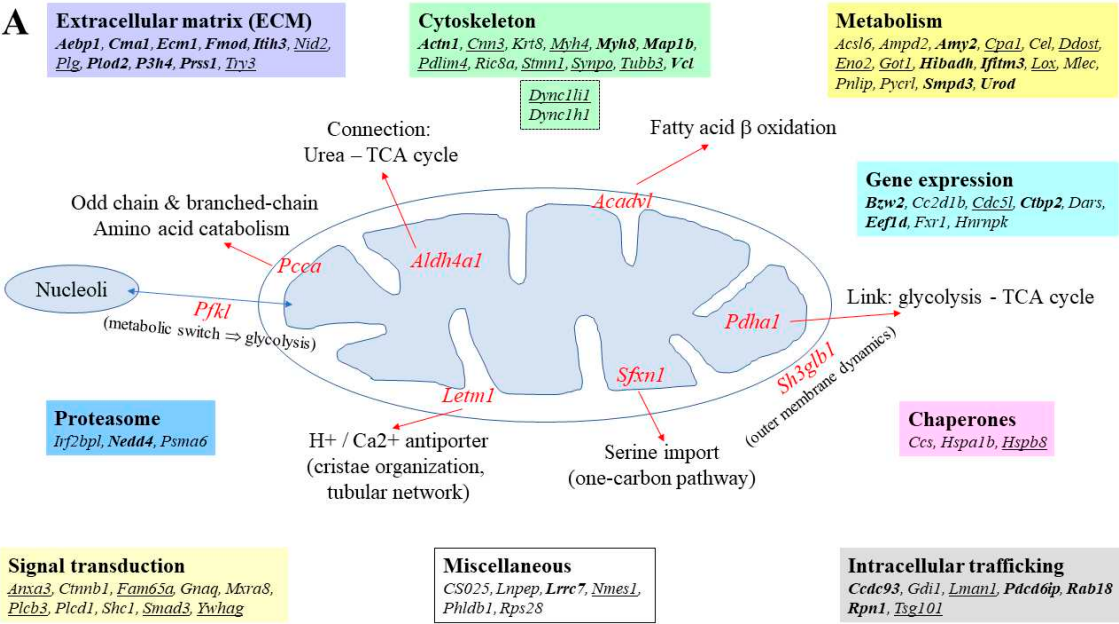
Gene name	Full protein name
<i>Ywhaq</i>	14-3-3 protein theta
<i>Ywhaz</i>	14-3-3 protein zeta/delta
<i>Slc3a2</i>	4F2 cell-surface antigen heavy chain
<i>Pgd</i>	6-phosphogluconate dehydrogenase, decarboxylating
<i>Xdh</i>	Xanthine dehydrogenase/oxidase
<i>Yap1</i>	Transcriptional coactivator YAP1
<i>Acta2</i>	Actin, aortic smooth muscle
<i>Actg1</i>	Actin, cytoplasmic 2
<i>Acta1</i>	Actin, alpha skeletal muscle
<i>Ada</i>	Adenosine deaminase
<i>Slc25a5</i>	ADP/ATP translocase 2
<i>Aif1</i>	Allograft inflammatory factor 1
<i>Akr1a1</i>	Alcohol dehydrogenase [NADP(+)]
<i>Alox5ap</i>	Arachidonate 5-lipoxygenase-activating protein
<i>Aldh9a1</i>	4-trimethylaminobutyraldehyde dehydrogenase
<i>Akr1b7</i>	Aldose reductase-related protein 1
<i>Aldh2</i>	Aldehyde dehydrogenase, mitochondrial
<i>Akr1b1</i>	Aldose reductase
<i>Rnpep</i>	Aminopeptidase B
<i>Lap3</i>	Cytosol aminopeptidase
<i>Anxa6</i>	Annexin A6
<i>Ap1b1</i>	AP-1 complex subunit beta-1
<i>Ap2a2</i>	AP-2 complex subunit alpha-2
<i>Aprt</i>	Adenine phosphoribosyltransferase
<i>Arpc1b</i>	Actin-related protein 2/3 complex subunit 1B
<i>Actr2</i>	Actin-related protein 2
<i>Actr3</i>	Actin-related protein 3
<i>Arpc2</i>	Actin-related protein 2/3 complex subunit 2
<i>B2m</i>	Beta-2-microglobulin
<i>C1qb</i>	Complement C1q subcomponent subunit B
<i>Canx</i>	Calnexin
<i>Cand1</i>	Cullin-associated NEDD8-dissociated protein 1
<i>Cap1</i>	Adenylyl cyclase-associated protein 1
<i>Capzb</i>	F-actin-capping protein subunit beta
<i>Casp1</i>	Caspase-1
<i>Ctsz</i>	Cathepsin Z
<i>Capza2</i>	F-actin-capping protein subunit alpha-2
<i>Ccdc50</i>	Coiled-coil domain-containing protein 50
<i>Cltc</i>	Clathrin heavy chain 1

<i>Cndp2</i>	Cytosolic non-specific dipeptidase
<i>Cfl1</i>	Cofilin-1
<i>Copb2</i>	Coatomer subunit beta'
<i>Coro1a</i>	Coronin-1A
<i>Coro1b</i>	Coronin-1B
<i>Coro7</i>	Coronin-7
<i>Cotl1</i>	Coactosin-like protein
<i>Cox4i1</i>	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial
<i>Csk</i>	Tyrosine-protein kinase CSK
<i>Csrp2</i>	Cysteine and glycine-rich protein 2
<i>Cux1</i>	Homeobox protein cut-like 1
<i>Dab2</i>	Disabled homolog 2
<i>Dbnl</i>	Drebrin-like protein
<i>Dld</i>	Dihydrolipoyl dehydrogenase, mitochondrial
<i>Emc2</i>	ER membrane protein complex subunit 2
<i>Exoc4</i>	Exocyst complex component 4
<i>F13a1</i>	Coagulation factor XIII A chain
<i>Fabp5</i>	Fatty acid-binding protein, epidermal
<i>Fat2</i>	Protocadherin Fat 2
<i>Fcerg1</i>	High affinity immunoglobulin epsilon receptor subunit gamma
<i>Fga</i>	Fibrinogen alpha chain
<i>Fgb</i>	Fibrinogen beta chain
<i>Fgg</i>	Fibrinogen gamma chain
<i>Fkbp9</i>	Peptidyl-prolyl cis-trans isomerase FKBP9
<i>Fnta</i>	Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha
<i>Fth1</i>	Ferritin heavy chain
<i>Fh</i>	Fumarate hydratase, mitochondrial
<i>Galm</i>	Aldose 1-epimerase
<i>Gdi2</i>	Rab GDP dissociation inhibitor beta
<i>Arhgdia</i>	Rho GDP-dissociation inhibitor 1
<i>Gphn</i>	Gephyrin
<i>Gimap4</i>	GTPase IMAF family member 4
<i>Gmfg</i>	Glia maturation factor gamma
<i>Gnai2</i>	Guanine nucleotide-binding protein G(i) subunit alpha-2
<i>Gpsm3</i>	G-protein-signaling modulator 3
<i>Hspa5</i>	78 kDa glucose-regulated protein
<i>Grpel1</i>	GrpE protein homolog 1, mitochondrial
<i>Gda</i>	Guanine deaminase
<i>Hist1h4b</i>	Histone H4
<i>/</i>	RT1 class I histocompatibility antigen, AA alpha chain
<i>Hexb</i>	Beta-hexosaminidase subunit beta
<i>Hmgn5</i>	High mobility group nucleosome-binding domain-containing protein 5

<i>Eif2s3</i>	Eukaryotic translation initiation factor 2 subunit 3, X-linked
<i>Ilkap</i>	Integrin-linked kinase-associated serine/threonine phosphatase 2C
<i>Irgm</i>	Immunity-related GTPase family M protein
<i>Ckb</i>	Creatine kinase B-type
<i>Lasp1</i>	LIM and SH3 domain protein 1
<i>Lta4h</i>	Leukotriene A-4 hydrolase
<i>Lrrc15</i>	Leucine-rich repeat-containing protein 15
<i>Lum</i>	Lumican
<i>Gaa</i>	Lysosomal alpha-glucosidase
<i>Lyn</i>	Tyrosine-protein kinase Lyn
<i>Mical1</i>	[F-actin]-methionine sulfoxide oxidase MICAL1
<i>Mpdz</i>	Multiple PDZ domain protein
<i>Mx1</i>	Interferon-induced GTP-binding protein Mx1
<i>Nqo1</i>	NAD(P)H dehydrogenase [quinone] 1
<i>Ncl</i>	Nucleolin
<i>Pdcd4</i>	Programmed cell death protein 4
<i>Pdia3</i>	Protein disulfide-isomerase A3
<i>Pepd</i>	Xaa-Pro dipeptidase
<i>Ptgs1</i>	Prostaglandin G/H synthase 1
<i>Pip4k2a</i>	Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha
<i>Srd5a3</i>	Polyprenol reductase
<i>Tmem123</i>	Porimin
<i>Ppp2ca</i>	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform
<i>Ppia</i>	Peptidyl-prolyl cis-trans isomerase
<i>Pfn1</i>	Profilin-1
<i>Psmc5</i>	26S protease regulatory subunit 8
<i>Psmb1</i>	Proteasome subunit beta type-1
<i>Ptbp3</i>	Polypyrimidine tract-binding protein 3
<i>Ptfn6</i>	Tyrosine-protein phosphatase non-receptor type 6
<i>Ptprc</i>	Receptor-type tyrosine-protein phosphatase C
<i>Ppat</i>	Amidophosphoribosyltransferase
<i>Paics</i>	Multifunctional protein ADE2
<i>Rab1A</i>	Ras-related protein Rab-1A
<i>Rab7a</i>	Ras-related protein Rab-7a
<i>Rap1b</i>	Ras-related protein Rap-1b
<i>Renbp</i>	N-acylglucosamine 2-epimerase
<i>Rhoa</i>	Transforming protein RhoA
<i>Rpl11</i>	60S ribosomal protein L11
<i>Rpl13a</i>	60S ribosomal protein L13a
<i>Rpl23a</i>	60S ribosomal protein L23a
<i>Rpl3</i>	60S ribosomal protein L3
<i>Rpl7</i>	60S ribosomal protein L7

<i>Rpl7a</i>	60S ribosomal protein L7a
<i>Rpl8</i>	60S ribosomal protein L8
<i>Hnrnpa1</i>	Heterogeneous nuclear ribonucleoprotein A1
<i>Rps2</i>	40S ribosomal protein S2
<i>Rps27a</i>	Ubiquitin-40S ribosomal protein S27a
<i>Rps29</i>	40S ribosomal protein S29
<i>Ahcy</i>	Adenosylhomocysteinase
<i>Ahcyl1</i>	Adenosylhomocysteinase 2
<i>Sec11a</i>	Signal peptidase complex catalytic subunit SEC11A
<i>Sdha</i>	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
<i>Sept9</i>	Septin-9
<i>Skap2</i>	Src kinase-associated phosphoprotein 2
<i>Smc1a</i>	Structural maintenance of chromosomes protein 1A
<i>Snap23</i>	Synaptosomal-associated protein 23
<i>Snx1</i>	Sorting nexin-1
<i>Snx3</i>	Sorting nexin-3
<i>Sod2</i>	Superoxide dismutase [Mn], mitochondrial
<i>Sparc</i>	SPARC
<i>Srgap2</i>	SLIT-ROBO Rho GTPase-activating protein 2
<i>Stk10</i>	Serine/threonine-protein kinase 10
<i>Wars</i>	Tryptophan--tRNA ligase, cytoplasmic
<i>Tap2</i>	Antigen peptide transporter 2
<i>Tuba1c</i>	Tubulin alpha-1C chain
<i>Tubb2a</i>	Tubulin beta-2A chain
<i>Tubb5</i>	Tubulin beta-5 chain
<i>Cct2</i>	T-complex protein 1 subunit beta
<i>Cct4</i>	T-complex protein 1 subunit delta
<i>Txn</i>	Thioredoxin
<i>Thy1</i>	Thy-1 membrane glycoprotein
<i>Tkt</i>	Transketolase
<i>Tmed7</i>	Transmembrane emp24 domain-containing protein 7
<i>Tf</i>	Serotransferrin
<i>Txnl1</i>	Thioredoxin-like protein 1
<i>Ube2n</i>	Ubiquitin-conjugating enzyme E2 N
<i>Ubxn1</i>	UBX domain-containing protein 1
<i>Ugt1a6</i>	UDP-glucuronosyltransferase 1-6
<i>Ufl1</i>	E3 UFM1-protein ligase 1

The role of the seven increased mitochondrial proteins (encoded by *Aldh4a1*, *Hibadh*, *Pcca*, *Letm1*, *Acadvl*, *Pdha1*, and *Sfxn1*) is summarized in Figure 2A and their abundance changes illustrated in Figure 2B. The main functions of the eight decreased mitochondrial proteins (encoded by *Slc25a5*, *Aldh2*, *Cox4i1*, *Dld*, *Fh*, *Grpel1*, *Sdha*, and *Sod2*) are summarized in Figure 2C and their abundance changes illustrated in Figure 2D.



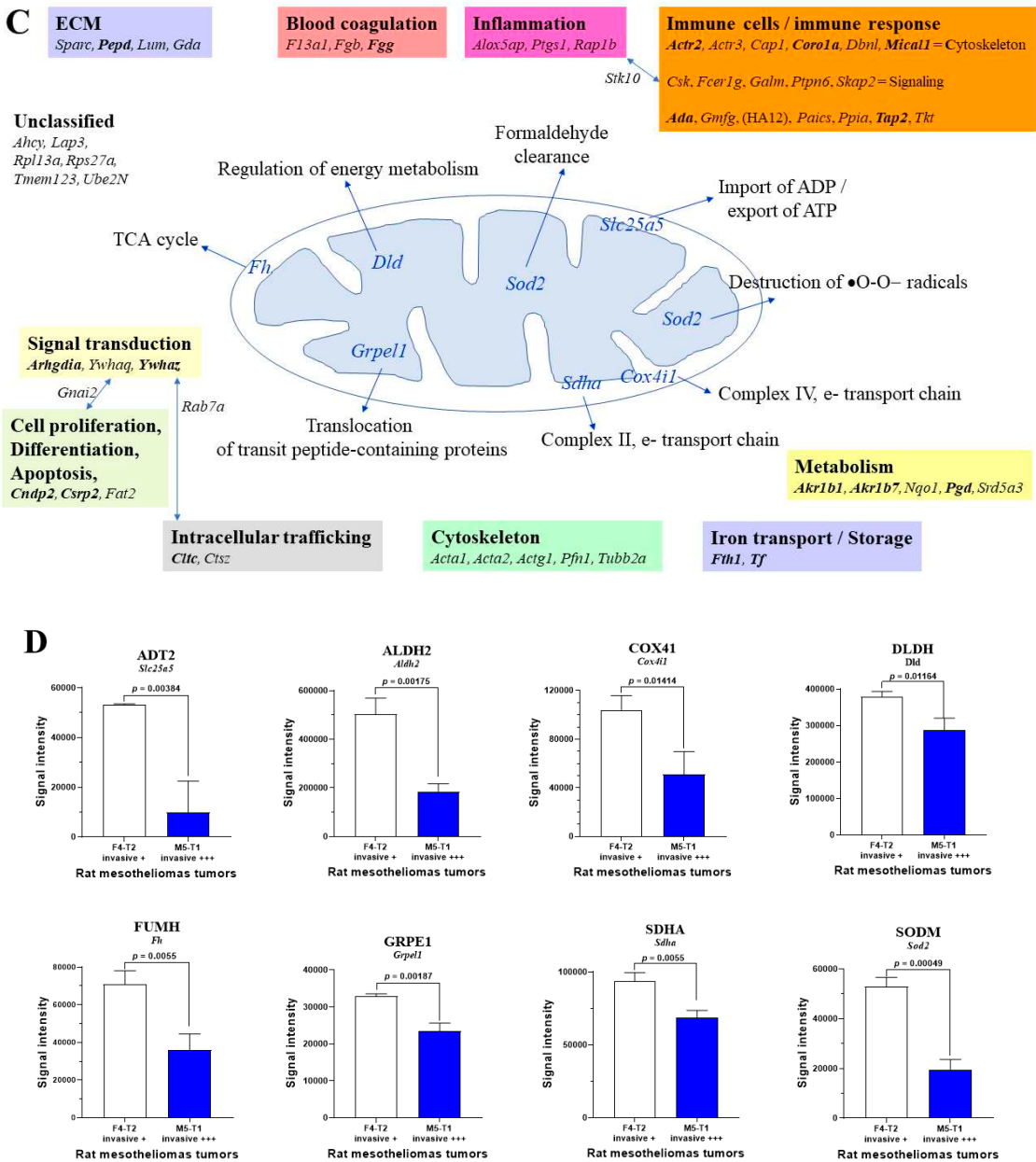
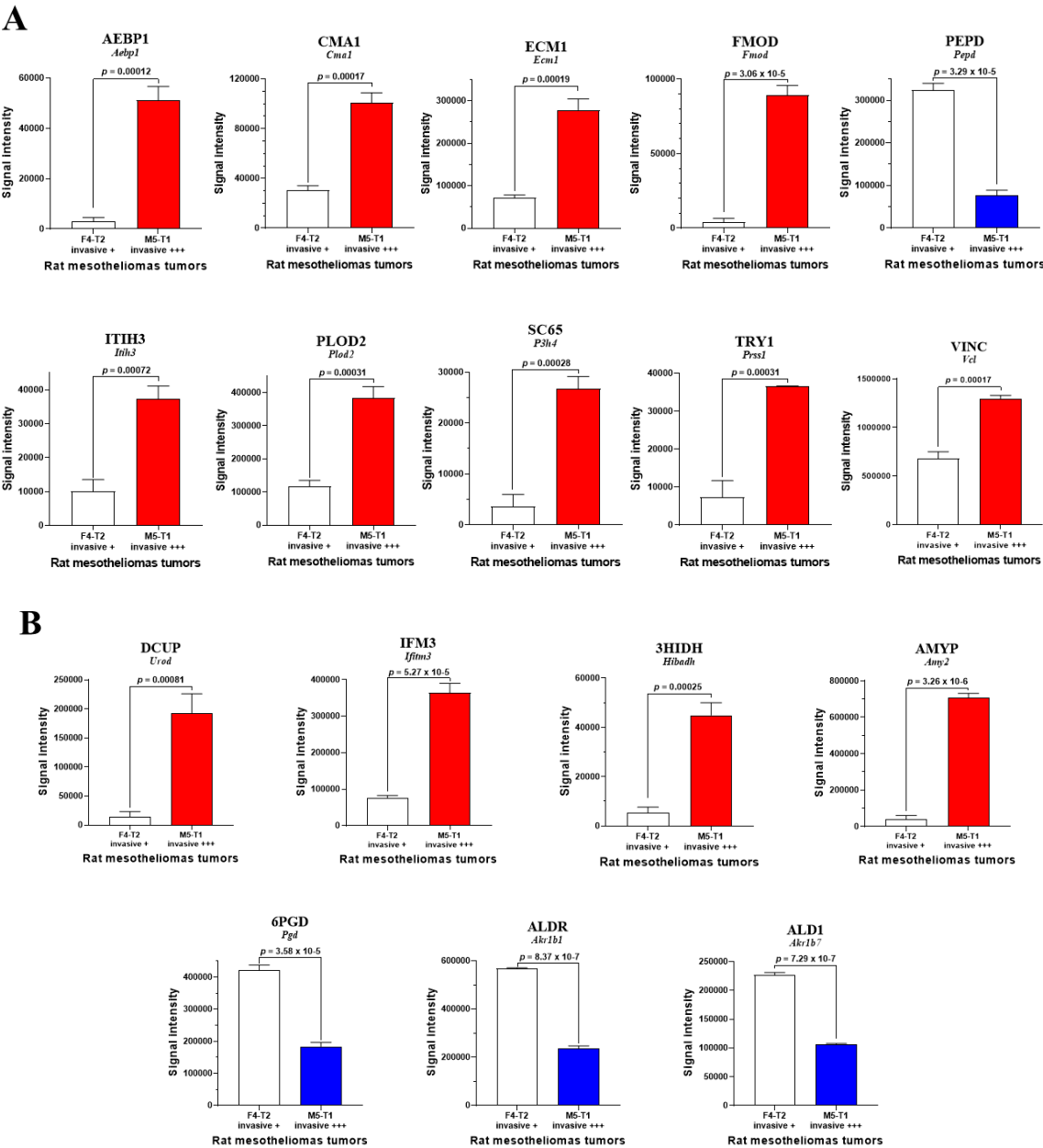


Figure 2. Subcellular localization of proteins showing abundance changes associated with tumor aggressiveness. A., Mitochondrial proteins that increased (encoding genes indicated in red). For other localizations, gene names indicated in bold correspond to proteins for which $p < 0.001$ (changes illustrated in Figure 3). Underlined gene names correspond to proteins for which $0.001 < p < 0.01$ (changes not shown for clarity). B., Abundance changes of mitochondrial proteins between M5-T1 and F4-T2 tumors. C., Mitochondrial proteins that decreased (encoding genes indicated in blue). For other localizations, gene names indicated in bold correspond to proteins for which $p < 1.00 \times 10^{-5}$ (changes illustrated in Figures 3 and 4). D., Abundance changes of mitochondrial proteins between M5-T1 and F4-T2 tumors.

3.3. Main Abundance Changes in Proteins Representing Potential co-Biomarkers of Aggressiveness

In parallel to mitochondrial events, dramatic abundance changes were also observed for other protein localizations that represented potential co-biomarkers of aggressiveness. A first important category corresponded to proteins in the extracellular matrix (ECM), nine being increased and one decreased (Figure 3A). A second group was represented by enzymes involved in different metabolic pathways, four being increased and three decreased (Figure 3B). A third group was involved in intracellular trafficking, four being increased and one decreased (Figure 3C). Of the proteins that only increased, three cytoskeletal, one proteasomal, one scaffold protein and three involved in the

regulation of gene expression were found (Figure 3D-F). Regarding proteins that only decreased, five corresponded mostly to immune cell components (Figure 4A), one in the coagulation system (Figure 4B), two involved in cell differentiation (Figure 4C), two in signal transduction (Figure 4D), and two in iron storage and transport (Figure 4E).



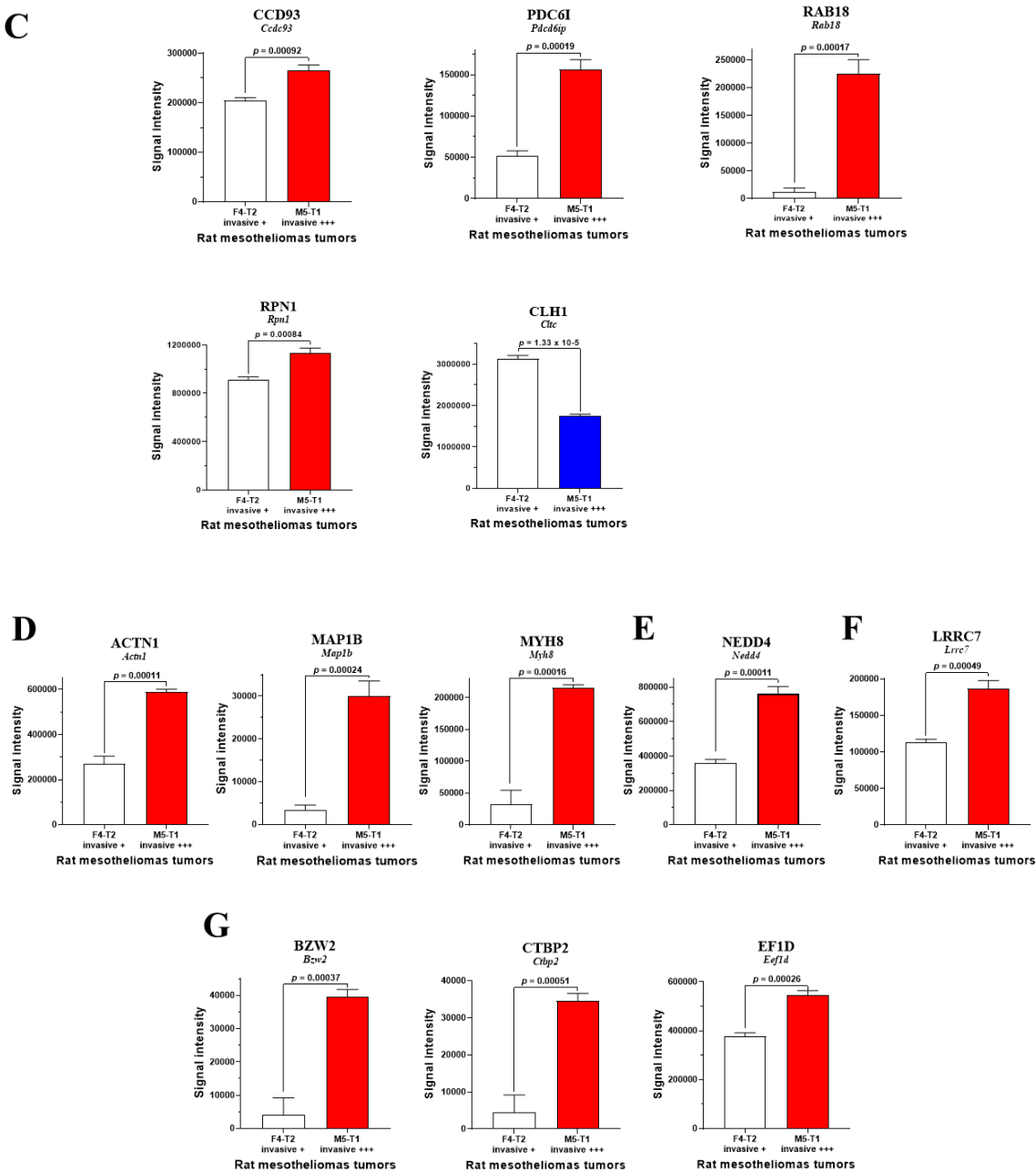


Figure 3. Abundance changes involved in tumor aggressiveness (part I). A, Extracellular matrix proteins. B, Metabolism. C, Intracellular trafficking. D, Cytoskeleton. E, Proteasome. F, Scaffolding proteins. G, Regulation of gene expression. .

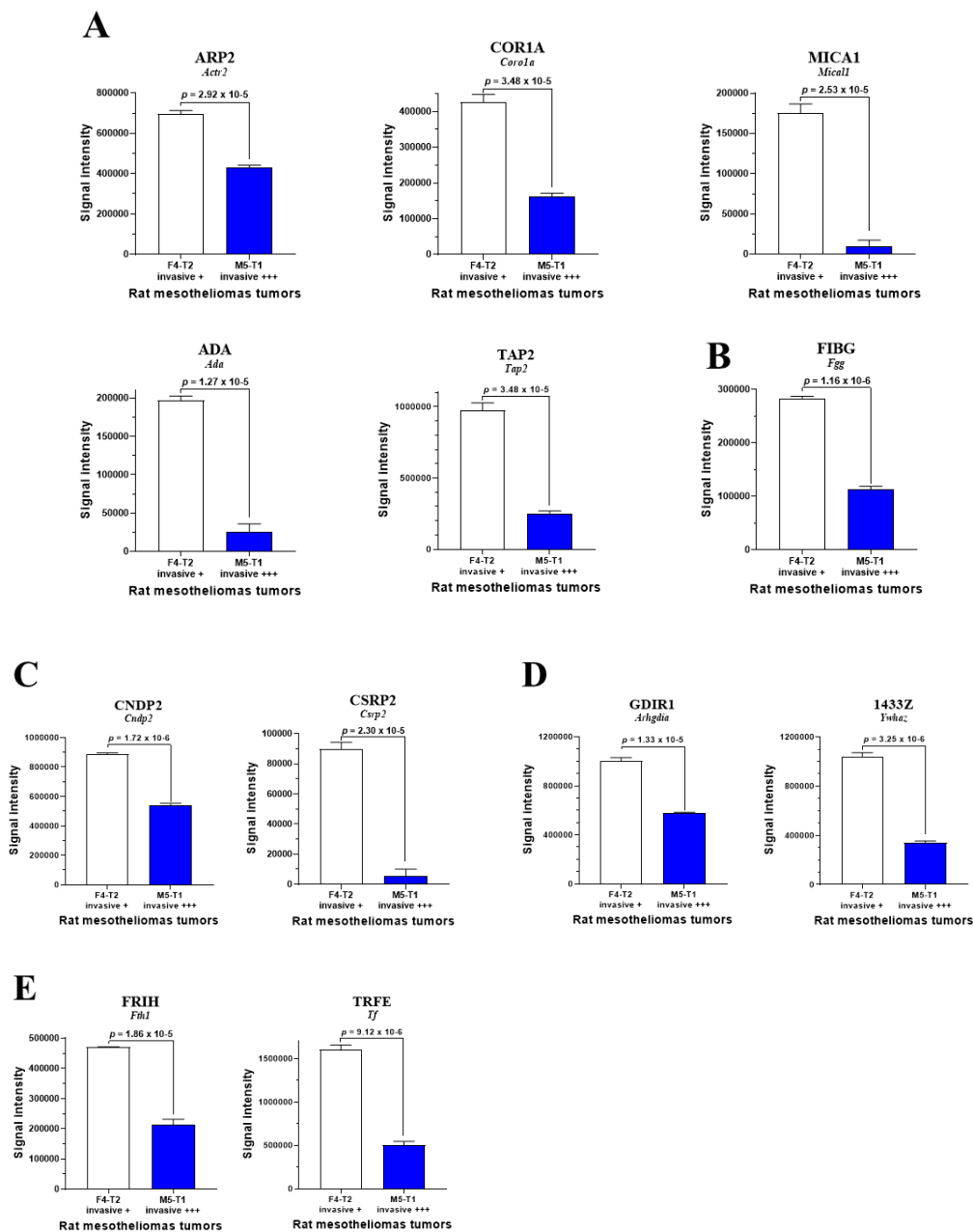


Figure 4. Additional abundance decreases involved in tumor aggressiveness (part II). A, Immune response. B, Coagulation. C, Cell differentiation. D, Signal transduction. E, Iron storage and transport.

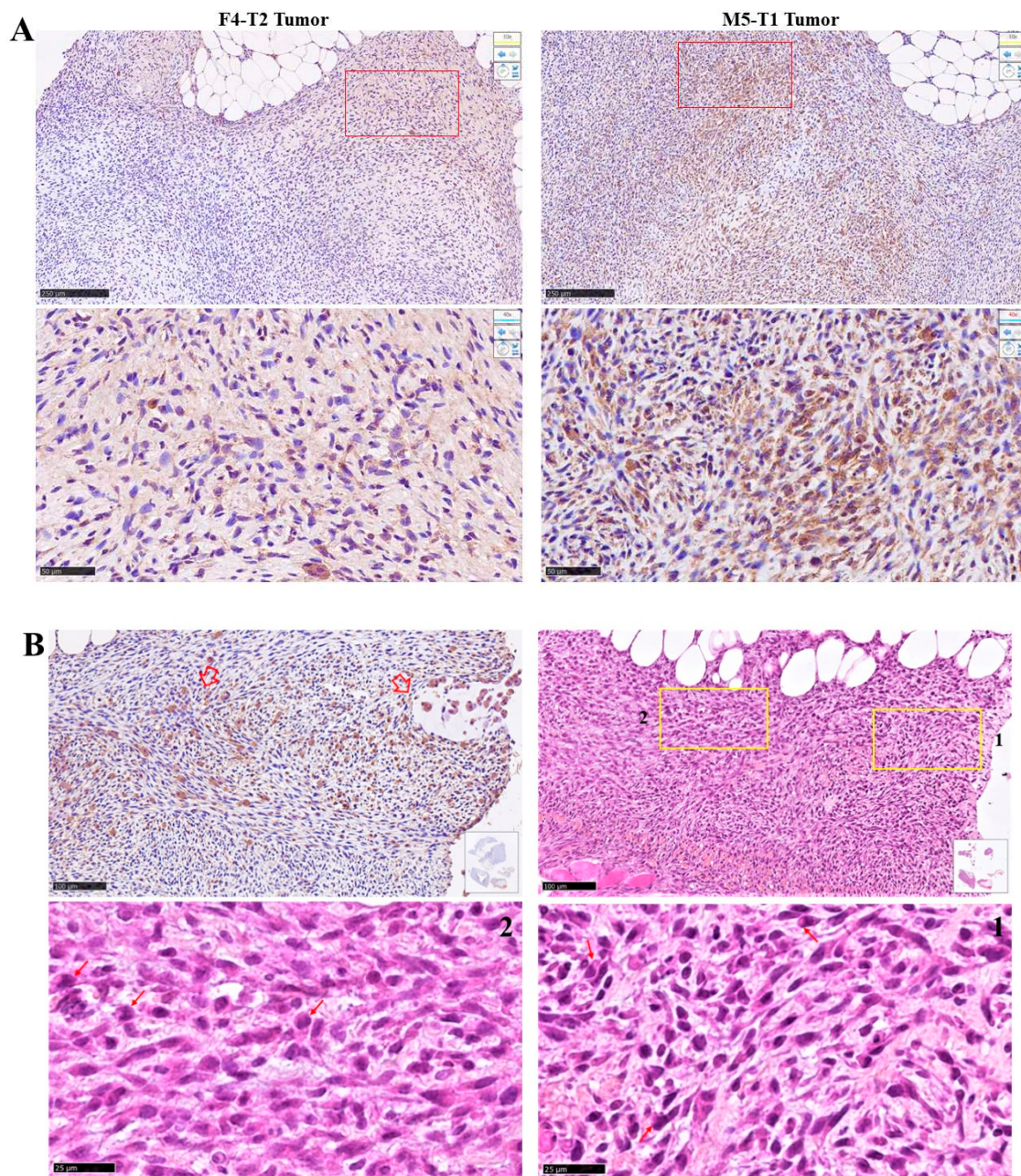


Figure 5. Distribution of ACADVL in tumor tissues. A, Comparison deeply invasive (M5-T1, top right) vs mildly invasive (F4-T2, top left) tumors, immunohistochemical staining with anti-Acadvl antibody, scale bars represent 250 μm . Magnifications of the areas delimited by the red rectangles (bottom), scale bars represent 50 μm . B, Areas of the M5-T1 tumor exhibiting clusters of positively stained cells (top left, open red arrows), the top right image showing the corresponding HPS stained slide, scale bars represent 100 μm . Areas delimited by the yellow rectangles are magnified above (bottom), with red arrows showing typical macrophage morphology, scale bars represent 25 μm .

4. Discussion

In previous reports, we identified biomarkers of increasing invasive properties in experimental models of malignant mesothelioma tumors growing in an immunocompetent rat strain [11]. However, in this study, proteins of interest exhibiting significant abundance changes were defined on the only basis of comparison between invasive vs non-invasive tumors. Herein we pursued these investigations with the aim of focusing this time on biomarkers of aggressiveness by comparing deeply vs mildly invasive tumors. The involvement of mitochondrial events in tumor aggressiveness and immunodeficiency has recently received considerable attention through their role in FAO. Evidence has confirmed the primary importance of this energetic pathway in tumor-infiltrating

immune cells [12]. In particular, the immune-metabolic reprogramming of mitochondria in tumor-associated macrophages (TAMs) was reported as playing a crucial role in aggressive tumor progression [13]. In our study, the comparison of two models led us to identify an increased level of the mitochondrial FAO enzyme encoded by *Acadl* in the most aggressive tumor. Furthermore, this observation was associated with the presence of higher amounts of TAMs positively stained with an antibody recognizing this enzyme in areas of active tumor stroma reorganization within the tumor tissue. Our data were consistent with the regulation of the immune response by mitochondria, and the peculiar role that FAO plays in the production of anti-inflammatory responses by M2 macrophages [14]. Linked to all these findings, Zhang et al. reported that FAO played a key role in the upregulated secretion of IL-1b by M2 macrophages to promote macrophage-mediated tumor cell migration [15]. In addition to mitochondrial proteins, another interesting finding observed among the numerous candidate biomarkers showing decreased abundances in the M5-T1 vs F4-T2 tumors, concerned FABP5. Hou et al. recently indicated that FABP5 deficiency led to reprogramming of metabolic pathways in macrophages by enhancing FAO in favor of M2 polarization [16].

Early investigations on ACADVL in experimental tumors revealed it was a rate-limiting enzyme in long-chain FAO, as FAO activity in the mitochondria toward palmitic acid and stearic acid was enhanced more than 3 times in the rat hepatoma cell line H4 [17]. Interestingly, a first insight into the role of this enzyme in tumor aggressiveness came with the observation that a profound transcriptional repression of four genes involved in mitochondrial energy metabolism, including *ACADVL*, was associated with a complete long-term remission of a patient with metastatic malignant melanoma [18]. Subsequently, Pan et al. reported that both *ACADVL* and *IFITM1* were overexpressed in women with squamous cell carcinoma of the cervix, these genes representing 2 out of 5 genes making it possible to distinguish it from normal tissue [19]. Interestingly, our study revealed the parallel increase in *ACADVL* and another protein in this family, *IFITM3*, two findings that agreed with the observations that both *IFITM1* and *IFITM3* were overexpressed in tumor tissues, and correlated with several poorly differentiated cancers [20]. Since then, three main studies have published additional insights consistent with our data showing the involvement of this FAO enzyme in both tumor aggressiveness and immune-suppressive properties. First, Wheeler et al. showed *ACADVL* is a driver of anoikis escape [21]. A second finding was the involvement of *ACADVL* overexpression in leukemia cell mitochondrial metabolism described by Tchong et al. [22]. Finally, Micheva et al. demonstrated that the upregulation of *ACADVL*, with some other genes involved in the mitochondrial removal of long-chain fatty acyls, led to dampened oxylipin levels with harmful consequences on leukocyte activation [23].

Besides *ACADVL*, many other potential candidate biomarkers of aggressiveness exhibited pronounced abundance changes. Some of them have already been discussed in two previous studies, including those encoded by *Ifitm3*, *Ecm1*, *Fmod*, *Itih3*, *Prss1*, *Map1b*, *Myh8*, [5], and *Cnn3*, *Coro1a*, *Smad3*, *Rpn1*, and *Hspa1b* [24]. Among the mitochondrial proteins exhibiting an increase, the case of ODP (encoded by *Pdha1*) is of great interest as it is an enzyme involved in the Warburg effect [25]. Linked with our findings, three reports recently highlighted the crucial role of this mitochondrial enzyme. First, Deng et al. revealed that it could represent a prognostic and immune-associated biomarker in multiple cancers, its expression being linked to DNA damage, cell invasion, and angiogenesis [26]. A second study by Olou et al. pointed to its involvement in adipocyte maintenance and secretion of the cancer-promoting cytokine IL-6 [27]. Finally, Tian et al. showed that silencing this gene suppressed proliferation, migration, and invasion in neuroblastoma [28]. Within mitochondrial proteins exhibiting a decrease, the two enzymes of the TCA cycle, succinate dehydrogenase (encoded by *SdhA*) and fumarate hydratase (encoded by *Fh*), which represent potential tumor suppressors, were frequently reported to be downregulated in cancers [29,30]. In good agreement with our data, a recent bioinformatic investigation also showed that the mitochondrial solute carrier family 25 (encoded by *Slc25a5*) had a prognostic value in patients with colon cancer, its upregulation being related to better overall survival [31]. Although controversial results have been reported on the role of SOD2 in cancers, its parallel decreased abundance found here agrees with early findings reporting that its suppression may create mitochondrial injury leading to increased ROS and glycolysis [32]. This observation is also consistent with the parallel decrease in the nucleotide exchange factor encoded by *Grpel1*, through its known role as a modulator of organelle function under stress conditions [33].

ECM proteins represented the category most concerned with marked abundance increase in the M5-T1 tumor. In addition to proteins encoded by the *Ecm1*, *Fmod*, *Itih3*, and *Prss1* genes, previously discussed [5], four other members, encoded by *Aebp1*, *Cma1*, *Plod2*, and *P3h4* played an important role in the acquisition of aggressive tumor properties. Linked with ACADVL, adipocyte metabolism and the proinflammation process in macrophages, AEBP1 was initially shown to promote EMT through activation of the NF- κ B pathway in gastric cancer cells [34]. Subsequently, these observations were confirmed in the context of promotion of colon adenocarcinoma [35] and glioblastoma [36] cell growth. Finally, while AEBP1 progressively became a prognostic biomarker, its knockdown suppresses tumor proliferation and invasion in glioma [37] and oral cancer [38]. Regarding *CMA1*, Fenger et al. demonstrated that enforced miR-9 expression in canine mast cell tumors upregulated its expression in addition to *IFITM3* mentioned above, leading to the development of an invasive phenotype [39]. The last two ECM proteins are involved in the stability of collagen intermolecular crosslinks. The expression of *P3h4*, which encodes a protein catalyzing the hydroxylation of lysine residues in collagen alpha chains, was reported to be a predictor of poor overall survival in both bladder [40] and lung cancer [41]. PLOD2, which provides attachment sites for carbohydrates in collagen, was also initially reviewed as a potential prognostic factor [42]. Recent clinicopathological studies have highlighted an inversed relationship between its high expression and overall survival in patients with esophageal squamous cell carcinoma [43], glioblastoma [44], laryngeal cancer [45], or osteosarcoma [46]. Moreover, an inverse relationship was also observed with immune cell infiltration [46].

Another major category concerned by abundance increase was proteins in the cytoskeleton. Of them, actinin-1 and vinculin appear to play an important role. High actinin-1 levels were recently associated with poor prognosis in oral squamous cell carcinoma [47]. The same association with poor prognosis was observed in gastric cancer through its involvement in the promotion of EMT via the AKT/GSK3 β / β -catenin pathway [48]. Moreover, Cao et al. showed that the promotion of the metastatic properties it provided in breast cancer appeared to involve CAF activation [49]. Concerning vinculin, one decade ago Goldmann et al. highlighted the role it played in the transmission of mechanical forces and in the orchestration of mechanical signaling events in the context of cancer cell migration [50]. Among new insights, interestingly, Li et al. revealed the impact of high levels of vinculin on the promotion of EMT and poor prognosis in gastric cancer [51].

Among the biomarkers that specifically characterized M5-T1 tumor aggressiveness, many proteins in the tumor microenvironment involved in the immune response conversely exhibited a dramatic decrease. One of them, encoded by *Coro1a*, presented common features with the decrease already observed in the process of liver metastatic colonization by M5-T1 tumor cells [5]. As previously discussed, induction of T-cell anergy could explain this fact [52]. The decrease in COR1A in the M5-T1 tumor is also consistent with its low leukocytic infiltrate [24], and with the inverse situation found in immune-rich breast tumors in patients [53]. In good agreement with our findings, *Ada* is another gene which, like *Coro1a*, when affected, is associated with severe combined immunodeficiency [54]. Moreover, Zucchetti et al. recently showed the crucial role of another protein, ARP2 encoded by *Actr2* which belongs to the Arp2/3 complex, in the protrusions developed by T-cells in immune surveillance [55]. Finally, Lee et al discovered that the protein MICA1, encoded by the gene *Mical1*, was involved in the regulation of actin via methionine-R-sulfoxidation, an important process in macrophage function [56]. In our study, this protein presented the most impressive drop within the decreased biomarkers, linked with tumor aggressiveness. Intriguingly, another comparable evolution was observed with CSRP2, while conflicting data were reported for both proteins regarding their link with cancer progression. For example, its overexpression was correlated with resistance to chemotherapy in leukemia [57], and this protein was presented as an invadopodial actin bundling protein in breast cancer promotion by hypoxia [58]. However, its decreased expression in colorectal cancer relative to normal tissues was indicative of poor prognosis in patients [59], and Kaplan-Meier analysis indicated that cohorts with low expression levels displayed worse relapse-free survival [60]. For MICAL1 too, Haikazian et al. have reported the existence of a potential relationship between *MICAL1* overexpression and increased tumor invasiveness [61], while conversely Gu et al. showed it played an inhibitory role in the migration and growth of colorectal cancer cells [62]. One possible explanation for these discrepancies could be the multiple roles reported for these proteins,

including cell differentiation, as CSRP2 has been documented as stimulating the smooth muscle differentiation program in normal tissues [63].

5. Conclusions

This study showed that the comparison of two experimental models of malignant mesothelioma emphasized the role of the mitochondrial FAO enzyme ADAVL in the emergence of both aggressive and immunosuppressive properties. Moreover, the observations made on quantitative changes in a set of key proteins in the extracellular matrix, immune cell components and other subcellular compartments, suggest the existence of connected events. This cancer is still a major public health problem, reinforcing the need to find innovative solutions to deal with the treatment of its most aggressive forms. Besides the specific case of malignant mesothelioma, these insights also have the potential to pave the way for interesting future basic research studies and therapeutic prospects for other types of aggressive cancers.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Supplementary Table S1; Supplementary Table S2.

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