



**ROMANIAN ACADEMY
INSTITUTE OF BIOCHEMISTRY**

Ph.D. THESIS SUMMARY

**Insights into functional interaction proteomics of
endoplasmic reticulum associated degradation
(ERAD) and antigen presentation in melanoma
using mass spectrometry**

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AIMS OF THESE STUDIES

Melanoma, the cancer of melanocytes - the pigment containing cells of the skin - has reached epidemic proportions and is associated with a high risk of mortality (Radovic-Kovacevic et al., 1997). Immunotherapy is a promising alternative to find new solutions to this problem as the Major Histocompatibility Complex I (MHC-I) pathway is not compromised in all melanoma cells and these are still able to present peptides to the cell surface, which in turn can trigger recognition by the immune system and consequently its response in killing the tumor. With increasing reports suggesting immunotherapy as a promising therapeutic intervention for this disease (Cheever et al., 2009), the search for new immunogenic sequences and understanding the mechanism by which they result is of considerable interest from both, tumor biology and clinical perspective.

One source of peptides is the endoplasmic reticulum (ER) proteins, which following their degradation by the ubiquitin proteasome pathway in Endoplasmic Reticulum Associated Degradation (ERAD) generate peptides amenable to be presented at the cell surface.

The aim of this study was to understand the molecular pathways of protein degradation by studying functional interactions in ERAD which lead to protein degradation with an emphasis on EDEM 2, an ER protein that triggers the degradation of misfolded proteins by a mechanism not yet completely understood. More specifically, the goals of my work were three-folded:

- Identify the proteins associated with EDEM 2 in melanoma
- Characterize EDEM 2 functional complexes in ERAD
- Analyze tyrosinase and EDEM 2 following the treatment using processing and traffic inhibitors

For these investigations, I used melanoma and human embryonic kidney cells in which I studied the proteins that associate with EDEM 2, but also the degradation of several canonical ERAD substrates. Among these was also tyrosinase, a copper-dependent enzyme involved in the reactions of melanin formation. Tyrosinase is a glycoprotein and previous studies in our lab have shown that its intracellular traffic is tightly regulated by its correct glycosylation and N-glycan processing into ER – depending on which it either continues its journey in the secretory pathway or it is switched to ERAD

pathway. Hence in the second part of the work I concentrated on using tyrosinase as a model to study its glycosylation pattern in melanoma and to further characterize tyrosinase-derived peptides with potential clinical application in immunotherapy. Here my specific goals were to:

- Analyze the glycosylation pattern of human tyrosinase in melanoma
- Characterize the tyrosinase epitope 369-377 using liquid chromatography and mass spectrometry in melanoma cells
- Identify in a discovery approach potential new tyrosinase-derived antigens

Technically, mass spectrometry and liquid chromatography were combined here with a large panel of biochemical methods and basic molecular and cellular biology techniques. For the analysis of MS results presented in this work I also have employed R scripting.

INTRODUCTION

Introduction: from ER protein biosynthesis to destruction

It is estimated that almost one third of the synthesized proteins of eukaryotic mammalian cells are sent to degradation (Schubert et al., 2000). Proteins destined for export through the secretory pathway are synthesized on ribosomes found on the endoplasmic reticulum (ER) (Walter and Blobel, 1980). The proteins are co-transnationally N-glycosylated by the *en bloc* transfer of the high mannose sequence GlcNAc₂Man₉Glc₃ (G₃M₉) to the selected asparagine (Asn) residues found in the consensus sequence Asn-X-Ser/Thr (where X can be any amino acid except Pro) (Aebi, 2013). Then, the two outermost glucose units are removed by Glucosidase I (Glc I) and Glucosidase II (Glc II) and the remaining single glucose unit triggers the subsequently binding of the protein by the calnexin and calreticulin chaperons (CNX/CRT) (Thomson and Williams, 2005, Kapoor et al., 2004, Zapun et al., 1997, Hubbard and Ivatt, 1981). This initiates the process of chaperone-assisted folding of the newly synthesized protein. An endoplasmic reticulum quality control (ERQC) system is proposed to act at this level by selecting folded proteins for export along the secretory pathway, while misfolded proteins either are sent for another CNX/CRT refolding attempt by the UGGT dependent reglucosylation of the high mannose core, or are sent for degradation by the Endoplasmic Reticulum Associated Degradation (ERAD) (Ellgaard and Helenius, 2003).

Human EDEM 2 – does the mannosidase tell the full story?

Similar to the folding process, a glycan-dependent mechanism has been proposed to act for the degradation of misfolded proteins and thus the concept of glycoprotein ERAD (gpERAD) has emerged. Much of the focus has been centered on proteins able to transform G₀M₉ to G₀M₈, by removing one of the mannose units, as reglucosylation of G₀M₈ was not shown to be possible, this suggesting that G₀M₈ is the signal that targets the selected substrate for degradation (Tannous et al., 2015, Hirao et al., 2006, Olivari and Molinari, 2007). Initially, ER Man I was proposed to be the initiating mannosidase in gpERAD by hydrolyzing the removal of one of the mannose residues from the

sequence G_0M_9 (Gonzalez et al., 1999, Tremblay and Herscovics, 1999), but later evidence revealed that the specificity is time and concentration-dependent with the possibility of resulting G_0M_5 - G_0M_6 -type structures (Herscovics et al., 2002). Recent studies have credited this role to EDEM 2 (Endoplasmic reticulum degradation-enhancing alpha-mannosidase-like protein 2), one of the members of the EDEM protein family (Ninagawa et al., 2014). Moreover, it was proposed that EDEM 2 acts by removing one mannose from the B branch of the glycan, thus targeting the selected substrate for gpERAD.

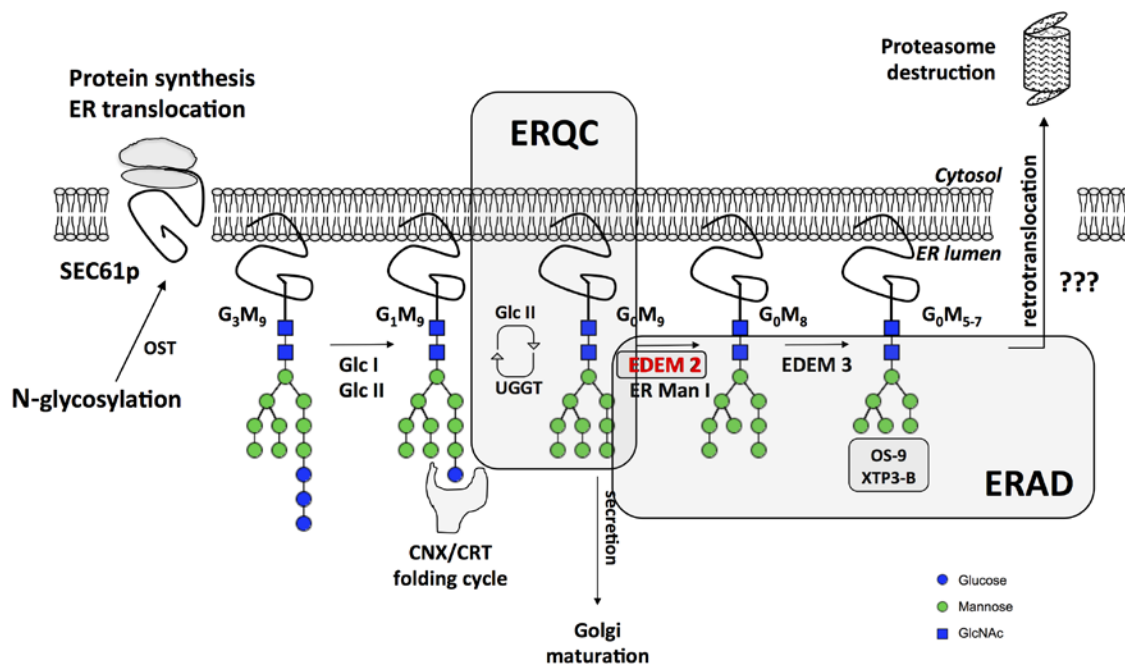


Figure 1. Schematic representation of the folding, ERQC and ERAD processes.

Following the translation on the ER-associated ribosome, the polypeptide chain is glycosylated by the transfer of the high mannose glycan. Glc I and Glc II start the trimming process of the glucose units from the glycan, which signal then entry in CNX/CRT cycle for protein folding. An ERQC system subsequently sorts folded vs. incompletely or misfolded proteins. Terminally misfolded proteins are subsequent sent to degradation via ERAD pathway.

EDEM 2 was almost concomitantly discovered and described by two independent groups, based on database analysis (Mast et al., 2005, Olivari et al., 2005). Human EDEM 2 is a 578 amino acid protein (~ 65 kDa) with an N-terminus signal sequence. Two isoforms are described in UniProt (UniProt, 2015) created by alternative splicing, one of them missing the amino acids between positions 36 and 72. Human EDEM 2 is described as a soluble protein with 4 potential N-glycosylation sites (N90, N112, N289 and N450), which seems to be occupied, as Endo H digestion of the overexpressed protein in HEK293 cells resulted in an electrophoretic mobility shift (Olivari et al.,

2005). However, until now, the glycosylation occupancy and its potential role in ERAD were not investigated so far. Sequence comparison with the other 2 homologues from this subfamily (EDEM1 and EDEM 3), showed a similarity restricted only to the mannosidase-like domain. Using northern blot analysis Mast et al., revealed that the protein is expressed in almost all tissues, with slightly higher levels in the intestine and peripheral blood leukocytes (Mast et al., 2005). Similar to the other homologues, EDEM 2 is also up regulated in tunicamycin-induced stress (Olivari et al., 2005). Using immunofluorescence microscopy it was found that EDEM 2 resides in ER and by removing the C-terminus amino acids beyond the mannosidase-like domain the protein is also found in the extracellular medium (Mast et al., 2005), although the protein it does not have a conventional ER retention signal (KDEL). This points to the hypothesis that this region of amino acids is important for the subcellular localization of human EDEM 2. Moreover, the truncated version of EDEM 2, obtained by removing the amino acids beyond the mannosidase-like domain did not result in an enhanced degradation of unfolded proteins (Mast et al., 2005), suggesting that ER location is critical for the interaction between EDEM2 and the substrates (probably because most of the ERAD substrates are also localized in ER). Initial *in vitro* experiments performed with pyridylamine tagged substrates of G₀M₉-G₀M₅ did not suggest any α -1,2 mannosidase activity, although the authors found that human EDEM 2 overexpressed in HEK293 cells does interact with several canonical misfolded ERAD substrates (Mast et al., 2005). Even more, the authors reported that EDEM 2 is able to induce the degradation of these proteins, but not of non-glycosylated substrates (Mast et al., 2005, Olivari et al., 2005). Others studies showed that EDEM 2 is involved in a SEC61p independent mechanism of retrotranslocation for ricin, a toxic glycoprotein endocytosed, which is partially transported to Golgi and subsequently retrograde to ER (Slominska-Wojewodzka et al., 2014). The authors proposed the notion of substrate specificity since they observed that an increased amount of ricin interacts with EDEM 2 compared with EDEM 1 (Slominska-Wojewodzka et al., 2014). In a different approach Tang et al. showed that EDEM 2 is required for the degradation of both glycosylated and nonglycosylated versions of sonic hedgehog (SHH), a protein involved in organogenesis (Lewis and Eisen, 2001), that is self-cleaved in ER resulting a C-terminus fragment which is an ERAD substrate (Tang et al., 2014). The authors also suggested that the degradation signal resides within the polypeptide backbone rather

than the glycan structure, arguing in favor of a non-glycan dependent ERAD possible mediated by EDEM 2. This report also revealed an interaction of EDEM 2 with other members of ERQC and ERAD. More specific the authors demonstrated that overexpressed EDEM 2 interacts with calnexin and SEL1L in HEK293 cells, and only the interaction with SEL1L is mediated by the glycan-binding domain of EDEM 2 (Tang et al., 2014). A major finding regarding the role of EDEM 2 in ERAD was an experiment performed by Ninagawa et al., in which following HPLC separation and fluorescence labeling of total N-glycans extracted from EDEM 2 gene knock-out (KO) cell lines, they detected an accumulation of G₀M₉ structures (Ninagawa et al., 2014). The authors also revealed that transiently expressed EDEM 2 in HCT116, a cell line derived from human colon colorectal carcinoma, does not interact with SEL1L (Hosokawa et al., 2006, Ninagawa et al., 2014), raising controversy about this interaction. Jansen et al. have showed that EDEM 2 could interact with several proteins involved in protein folding: calnexin, calreticulin, endoplasmic reticulum chaperone protein 5, PDIA3, PDIA4, PDIA6 or Thioredoxin domain-containing protein 5 (Jansen et al., 2012). However no data regarding interactions with other ERAD members is currently available and therefore an interaction map of EDEM 2 would be of great interest considering its currently accepted role in ERAD as the enzyme that performs the first mannose trimming.

Melanoma and immunotherapy

Cutaneous malignant melanoma was found to be the sixth most common type of cancer in USA (Erdei and Torres, 2010, Jemal et al., 2006). An important aspect of melanoma is the high level of deaths from patients that develop malignant skin tumors (Radovic-Kovacevic et al., 1997), as it is estimated that each hour one person dies from melanoma in USA (Erdei and Torres, 2010). Cell culture studies using monoclonal antibodies revealed the identification of a high number of antigenic systems on melanoma cells (Herlyn and Koprowski, 1988, Kath and Herlyn, 1989). Similar to a pathogen infection, cancer cells signal the malignant transformation to the immune system by tumor-associated antigens (TAAs), which subsequently activate the immune system and provides the ability to kill the malignant cell. The observation that immunodeficient mice develop an increased number of tumors and that tumors escape the immune system by down-regulating the Major Histocompatibility Complex (MHC),

provided the basis for what today is called immunotherapy (Dunn et al., 2002, Ahmad et al., 2004). In humans, MHC encodes the Human Leucocyte Antigens, which are found in two classes according to the type of peptides they present: HLA class I and HLA class II. Class I presents peptides resulted from intracellular protein degradation, while class II presents peptides resulted from the degradation of extracellular proteins (Krensky, 1997). Peptides presented by the HLA I system are derived from proteasomal degradation of the proteins. The observation that melanoma prevalence is higher in light skin individuals (Erdei and Torres, 2010), suggests a possible link between skin pigmentation and the disease. Melanin is responsible for skin pigmentation in humans and is produced in melanosomes (the lysosome-like structures of melanocytes) as a response to ultraviolet exposure (Lin and Fisher, 2007).

Tyrosinase as a source of HLA class I peptides

The biosynthesis of melanin is catalyzed by several enzymes like tyrosinase and tyrosinase related proteins 1 and 2 (Slominski et al., 2004, Jimenez-Cervantes et al., 1994). Tyrosinase (a copper dependent enzyme) catalyzes the rate-limiting step in melanin production by converting tyrosine to dihydroxyphenylalanine (DOPA) and oxidizing DOPA to DOPA quinone (Figure 2). The biosynthesis of tyrosinase proceeds in ER where it is N-glycosylated in a reaction catalyzed by N-oligosaccharyl transferase (OST) in which the high mannose glycan is transferred from dolichol-pyrophosphate to the polypeptide chain (Branza-Nichita et al., 2000b, Aebi, 2013). Subsequently, the protein is subject to ER chaperone assisted protein folding in the CNX/CRT cycle, and is further sorted by the ERQC system (Branza-Nichita et al., 1999). Thus, misfolded or unfolded molecules are targeted for ERAD while the native folded ones follow the secretory pathway via trans Golgi, to melanosome (Petrescu et al., 2000, Ostankovitch et al., 2005, Jimbow et al., 2000). The observation that tyrosinase is frequently misfolded and subjected to ERAD in melanoma, suggested that the protein could be a great source of HLA I peptides (Ostankovitch et al., 2005, Slingluff, 1996). Indeed, in a recent prioritization study of cancer antigens, tyrosinase ranked among the top 20 out of 75 analyzed antigens with potential clinical efficacy (Cheever et al., 2009).

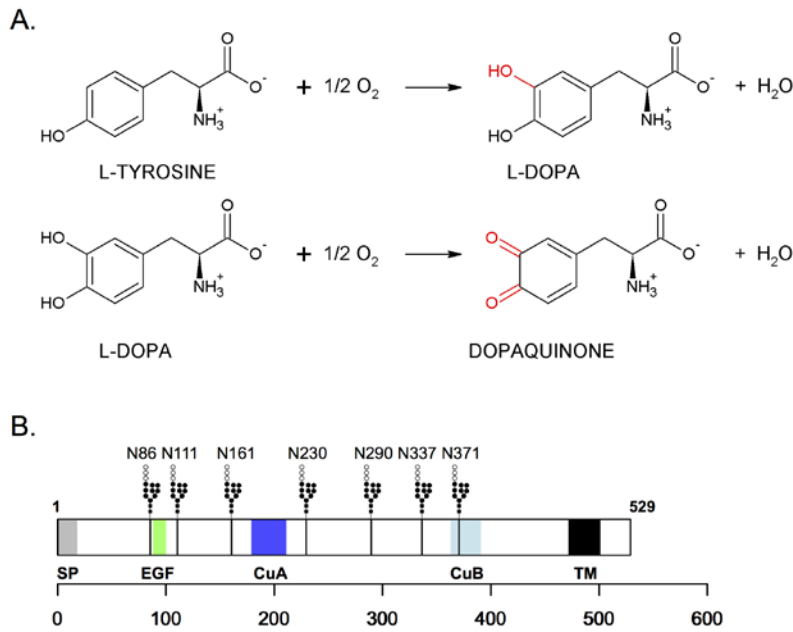


Figure 2. Schematic representation of the reactions catalyzed by tyrosinase (A) and the main structural features of tyrosinase (B).

A. Tyrosinase catalyzes the transformation of L-Tyrosine to L-DOPA and the oxidation reaction of the resulting product. B. Human tyrosinase contains a transmembrane domain, a cytosolic C-terminal portion and two copper binding sites.

One of the most abundant HLA presented peptides derived from tyrosinase was found to be the HLA-A*02 restricted epitope YMDGTMSQV (YMD peptide), which is estimated to be present as thousands of copies on melanoma cells (Michaeli et al., 2009). The peptide was discovered by mass spectrometry analysis of peptides extracted from melanoma cells (Cox et al., 1994, Engelhard et al., 1993), and it was described as one the first tyrosinase peptides to be presented as an altered sequence than the one deduced from the gene (Skipper et al., 1996), which corresponds to the sequence YMNGTMSQV. Later, it was shown that the deamidation of the Asn is a consequence of Peptide:N-Glycosidase F (PNGase F) deglycosylation of tyrosinase in the cytosol (Mosse et al., 1998). It was also found that melanoma patients positive for this peptide do present CTLs, which recognize this sequence.

RESULTS AND DISCUSSIONS

Human EDEM 2 associates with several protein members of the ER folding machinery and ERQC system

C-terminus HA-tagged EDEM 2 was overexpressed in A375 melanoma cells and subjected to affinity purification coupled with mass spectrometry detection in order to identify co-immunoprecipitated proteins. The protocol was optimized with respect to cell lysis buffer, antibody and elution buffer used for the retrieval of immunoenriched protein complexes etc. Besides this, several steps of the sample preparation for mass spectrometry analysis were also optimized for an improved GeLC-MS/MS workflow used for sample preparation (Figure 3). Since all the mass spectrometry experiments were performed on a high-resolution Orbitrap Velos Pro instrument (Thermo Fisher Scientific) equipped with ETD, the workflow was verified in terms of mass accuracy and peptide fractionation. Figure 4 presents a hierarchical clustering of the peptides identified in the full lane in one of the samples analyzed using this workflow. It can be observed that in most cases, each of the peptides identified in the full lane can be attributed back to a single fraction, which indicates an efficient fractionation.

The samples were prepared and analyzed in biological triplicates alongside a negative control (cells transfected with an empty plasmid). A data-dependent acquisition method was used, in which the peptides separated using low flow HPLC from a reversed-phase C18 column were detected in an Orbitrap survey scan (60 000 resolution at m/z 400) followed by their Collision Induced Dissociation (CID) fragmentation and subsequent detection of the ion fragments in the linear trap. The resulting data was further analyzed using SEQUEST for peptide identification and protein group assembly. At 1 % False Discovery Rate (FDR) approx. 3283 protein groups were identified in total.

The results were exported and reformatted, using a custom R script developed by the author, for subsequent SAINTexpress analysis.

Based on SAINTexpress scoring, 45 proteins were found as potential proteins associated with EDEM 2 in A375 melanoma cells. To increase the confidence of the results, a second strategy was considered for the analysis of the same dataset, in which the Andromeda search algorithm, integrated in MaxQuant (Cox and Mann, 2008) was used.

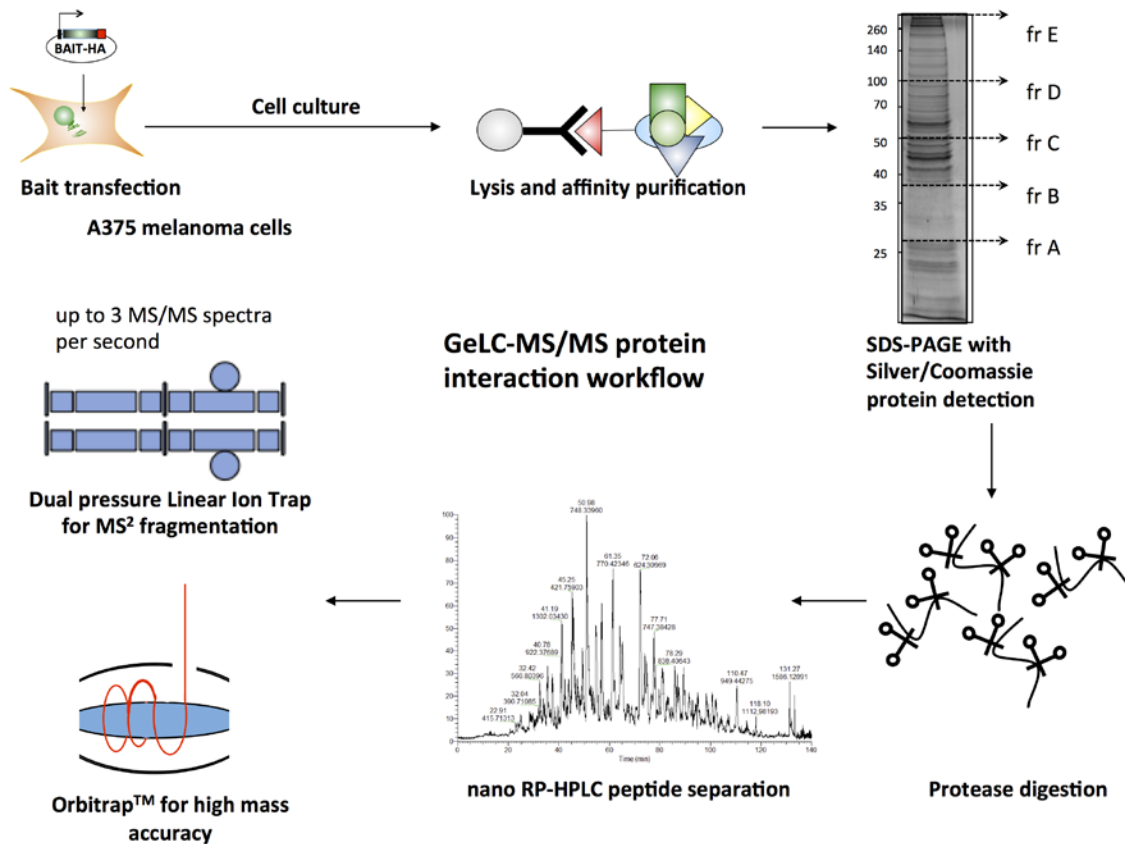


Figure 3. GeLC-MS/MS based protein interaction workflow for EDEM 2.

After bait transfection cells were expanded to a > 90 % confluence of a T75 cm flask (~ 10x10⁶ cells/replicate sample). The cell lysis and the elution of protein complexes from the agarose beads were optimized. Proteins were separated via SDS-PAGE detected with Coomassie or Silver staining and subject to manual fractionation. Each fraction was in gel digested with trypsin. The resulting peptides were extracted, separated by nanoflow RP-C18 HPLC and detected by high resolution (60 000) Orbitrap scanning MS and He Collision Induced Dissociation (CID) MS/MS with Linear Trap Quadrupole (LTQ) scanning. The workflow was developed empirically and based on already published guidelines (Kalli et al., 2013, Piersma et al., 2013, Shevchenko et al., 2006).

The maxLFQ quantification module (Cox et al., 2014) was activated for the calculation of the LFQ values.

These were further used for dataset analysis in which only proteins with values in each replicate group were further kept in the analysis. For protein scoring a two-sample t-test corrected by permutation-based FDR was used. Thus, 97 proteins were found to be potential associated with EDEM 2 using this method. It is interestingly to note that using these two data analysis strategies 28 proteins were found in the final list of both methods, reflecting the high probability that these proteins are indeed associated with EDEM 2 in A375 melanoma cells.

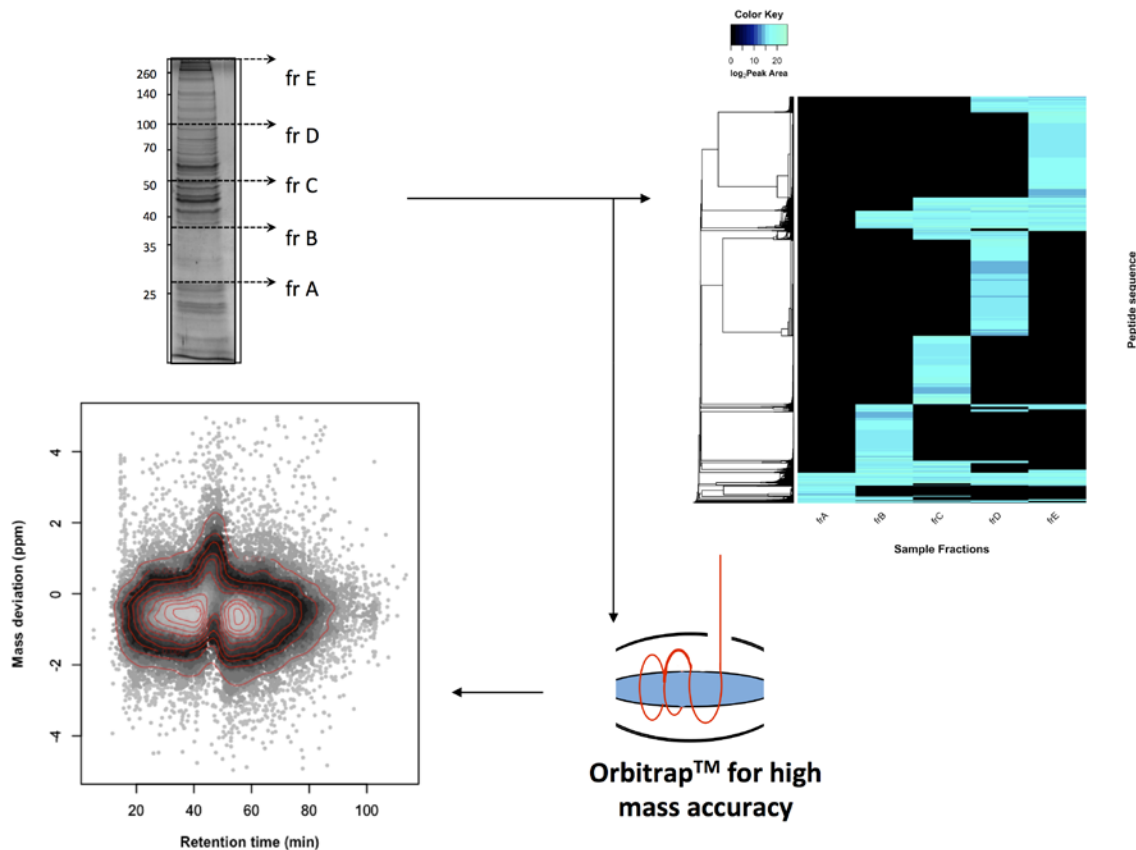


Figure 4. Workflow fractionation efficiency and mass accuracy.

The XIC area values of the peptides identified in a gel lane using the GeLC-MS/MS protein interaction workflow were clustered and represented as a color-coding heatmap (black - 0 and light green – max. value log scale). Shown is also a density scatter plot of peptide mass accuracies.

Among these, several members of the ER folding machinery and ERQC system were found, which were not previously shown to be associated with EDEM 2. However this experiment also confirmed previous reported interactions with proteins involved in folding and ERQC like PDIA4 or calnexin (Jansen et al., 2012, Tang et al., 2014). Some of these interactions were further confirmed using biochemical methods.

Besides the investigations of the proteins associated with EDEM 2 the functionality of the overexpressed protein was also tested. For this, human EDEM 2 was co-transfected in A375 melanoma cells or HEK293T cells alongside several canonical misfolded proteins, ERAD substrates and an empty plasmid used as a negative control. The steady-state level of misfolded proteins was investigated by immunoblotting, which revealed that the level of some ERAD substrates like NHK or BACE476 is indeed lower following EDEM 2 overexpression, further confirming previous findings that EDEM 2 is a protein involved in ERAD (Mast et al., 2005, Olivari et al., 2005). This

also confirmed that EDEM 2 forms functional complexes in both A375 melanoma cells and HEK293T cells and the interactions identified in this work are related to its major role is in ERAD.

Analysis of tyrosinase glycosylation pattern in A375 melanoma cells

To investigate the occupancy of the glycosylation sites in tyrosinase, A375 melanoma cells stably overexpressing WT human tyrosinase were lysed and subject to affinity purification using a mouse monoclonal antibody (T311) raised against recombinant tyrosinase (Chen et al., 1995). In order to characterize all potential N-glycosylation sites a two-sided workflow was implemented. The elution obtained from affinity purification was digested either using PNGase F or using Endoglycosidase H (Endo H). Endo H is a glycosidase that cleaves within the chitobiose core of high mannose oligosaccharides, leaving a HexNAc residue on the polypeptide chain (Maley et al., 1989, Robbins et al., 1984). After protease digestion, this residue can be used as a mass shift for glycopeptide detection, indicating the exact position of the N-linked high mannose glycan core. PNGase F digestion of N-glycoproteins results in the conversion of the occupied Asn residue in Asp, by the cleavage of the Asn-linked GlcNAc (Maley et al., 1989). Thus PNGase F is an amidase that besides cleaving the full glycan structure, it also catalyzes the hydrolysis of the amide bond from the side-chain of the Asn residue. This results in a mass increase of the polypeptide with 0.98 Da for each glycosylation site and can be used to track the position of the high mannose glycan, by searching peptides containing this modification after protease digestion. The proteins from Endo H samples were separated by SDS-PAGE and subsequent digested using trypsin. For PNGase F, the samples were first separated using the same method and then in gel digested using chymotrypsin. Peptides from both pools of samples were extracted and analyzed by LC-MS/MS. The MS sample analysis strategy used multiple fragmentation techniques in order to extract the maximum possible information from each sample. The Endo H samples, which should contain a HexNAc linked to the glycosylated Asn residue of the peptides, were first analyzed by a product ion ETD triggered method (Figure 5). Since it is well known that CID fragmentation of glycopeptides can result in the formation of the HexNAc oxonium ion at m/z 204.09 or HexNAc oxonium ion fragments at m/z 138.06, 168.07 or 186.08 (Singh et al., 2012), the method involved the fragmentation of

each +2, +3 or higher charge state ion using beam-type HCD (Olsen et al., 2007) and Orbitrap detection of the fragments ions. The presence of any one of these ions resulted in an ETD repeated fragmentation scan in the linear trap of the same precursor for peptide fragmentation.

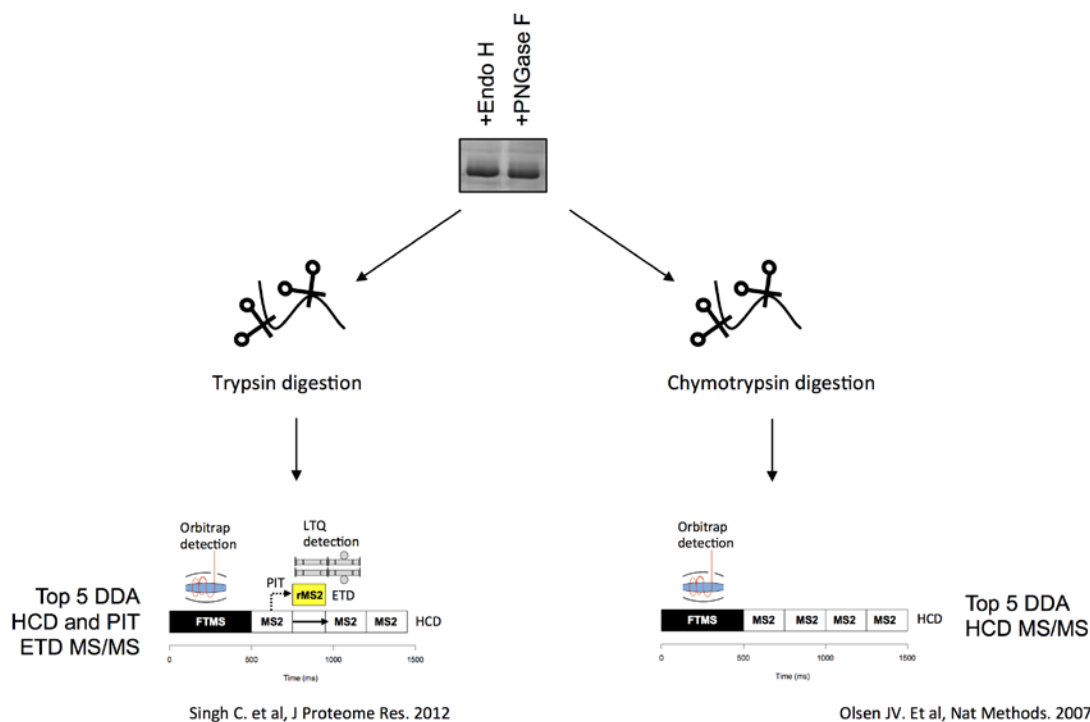


Figure 5. The MS acquisition strategy used for tyrosinase glycosylation analysis.

Human tyrosinase was immunopurified from A375 melanoma cells stably expressing the protein. The elution was split in half and subsequent treated either with PNGase F or with Endo H for glycan removal. PNGase F samples were further digested in gel with chymotrypsin for N161 and N371 detection and Endo H samples were digested with trypsin for the remaining five-glycosylation sites characterization. Accordingly, a HCD fragmentation method was used for the first set of samples and a HCD Product Ion Triggered (PIT) ETD method for Endo H samples.

The default fragmentation was set to HCD, since in linear ion trap CID the oxonium ion and oxonium fragment ions are not usually detected because of the low m/z cutoff (Schwartz et al., 2002, Yang et al., 2009). Nevertheless, the analysis was repeated using linear trap based detection of CID fragment ions as default fragmentation technique, combined with a second event of ETD fragmentation and detection of the resulting fragment ions in the linear ion trap. The chymotryptic peptides were analyzed using both, CID and HCD. No ETD fragmentation was used for these peptides, as only a mass shift increase would be expected for the glycosylation sites, which is readily detected by both methods. Beside, N-glycosylation site assignment, the data analysis involved an

extracted ion current-based quantification of the peptides, in order to establish the occupancy of each glycosylation site. In this analysis, multiple forms of each peptide were taken into consideration. For Endo H treated samples besides HexNAc modified peptides the non-modified sequences were also considered and the Asp modified sequences. For PNGase F samples only Asn or Asp sequences were considered. The Peptide Spectrum Matches (PSM) were filtered at an estimated False Discovery Rate (FDR) < 5 %. However, in order to characterize all seven glycosylation sites two additional PSMs were considered, although were scored lower than the selected score for 5 % FDR. The PSM corresponding to HexNAc modified peptide AAN*FSFR (where * and bold designates the modified residue) was manually analyzed and validated (Figure 6).

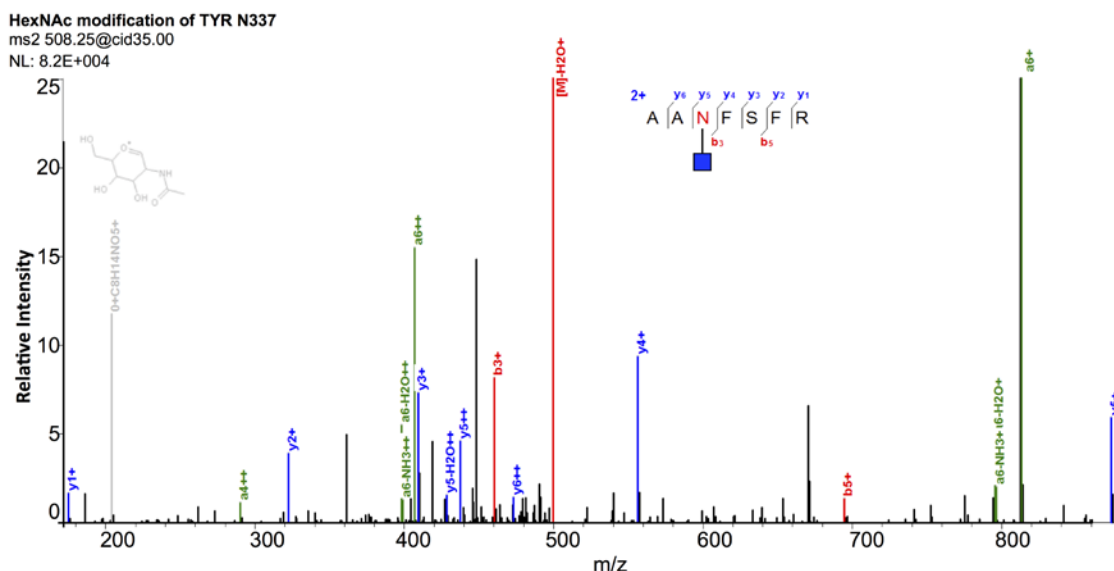


Figure 6. CID MS/MS fragmentation of the HexNAc modified peptide AANFSFR corresponding to N337 glycosylation site of human tyrosinase.

Almost all the y^+/y^{2+} ions could be detected as well as a and b ions. The diagnostic ion at m/z 208, corresponding to the oxonium ion of the HexNAc confirmed the modified sequence.

For this peptide almost all the y ions series were identified. Also, the precursor ion mass was in agreement with the predicted value within ppm range. An important observation confirming the presumed modified sequence was the identification of the oxonium ion of HexNAc at m/z 208, a diagnostic ion for fragmentation of glycosylated peptides. Finally, based on all these factors this PSM was considered in the final dataset. A possible explanation for the lower scoring of this PSM could be the length of the peptide, as it is known that in SEQUEST scoring scheme shorter sequences are

penalized (Eng et al., 1994). It is interesting to note that this could also be a possible explanation for the identification of the HexNAc oxonium ion in the CID spectrum, as in this type of fragmentation oxonium ions are not usually observed due to the low m/z cutoff (Yang et al., 2009). The PSM corresponding to the deamidated peptide ESWPSVFYN*R (where * and bold designates the deamidation site) was also considered in the final dataset (Figure 7) as the mass accuracy of the precursor and fragments corresponded to the instrument mass accuracy and almost all y ions confirmed the sequence. Also five consecutive b ions were identified.

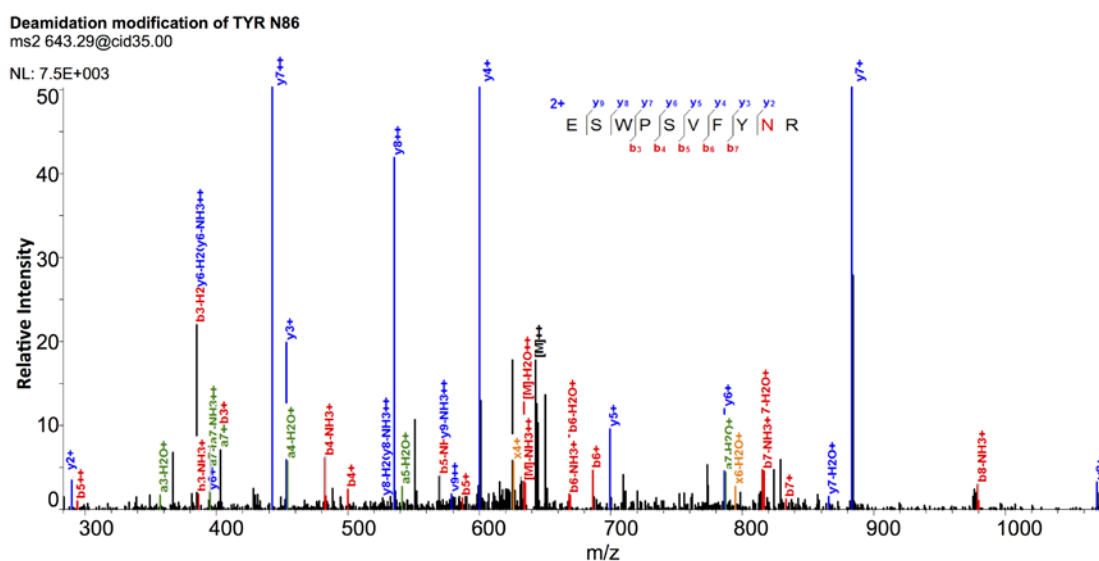


Figure 7. CID MS/MS fragmentation spectrum of the deamidated peptide ESWPSVFYNR, corresponding the first site of glycosylation in human tyrosinase. Almost all y ions can be observed, as well as the lower intensity b ions.

Both of the identified peptides were isolated and fragmented using CID with detection in the linear ion trap portion of the instrument. Analysis of the Endo H tryptic peptides revealed that tyrosinase is indeed glycosylated in A375 melanoma cells at N86, N111, N230, N290 and N337 (Figure 8). No tryptic peptides were detected for N161 and N371 sites. However PNGase F treated samples revealed the identification of the chymotryptic peptides containing N161 and N371. For both positions, Asp containing peptides were identified, suggesting that these sites are indeed glycosylated (Figure 8).

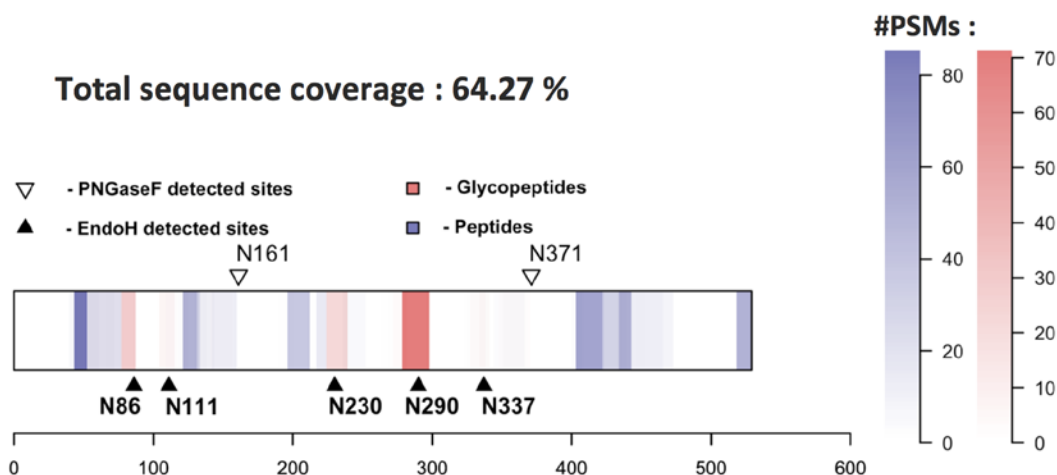


Figure 8. Human tyrosinase N-glycosylation analysis in melanoma cells using mass spectrometry.

A375 melanoma cells stably expressing the full-length human tyrosinase were lysed and subject to immunoaffinity enrichment. The resulting sample was digested using distinct endoglycosidases, and the proteins separated by SDS-PAGE. In gel digestion was performed using trypsin or chymotrypsin in order to identify peptides containing all the glycosylation sites. It can be observed that most of the N-glycosylation sites were identified by Endo H and trypsin digestion. Spectral counts distribution is similar for peptides and glycopeptides.

The analysis of all the samples revealed a sequence coverage of 64 %, with peptides being identified from most of the major regions of the protein sequence.

However, peptides from the N-terminal cleavable sequence and the transmembrane domain were not identified. Spectral counts distribution of the peptides and glycopeptides derived from tyrosinase did not reveal any bias toward the identification of any class (Figure 8).

By taking into consideration the area from the XIC of each peptide form, the prediction results and previous data available in the literature, the occupancy of each N-glycosylation site was estimated (Table 1). It was found that the third and the fifth site are partial glycosylated as suggested by the predictions using NetNGlyc server and N86, N111, N230, N337, N371 are occupied.

These results are in agreement with previous data suggesting no or partial occupancy for the third site (N161) and full glycosylation for N86, N230, N337 and N371. Although it was found that N111 is unoccupied in mouse tyrosinase (Branza-Nichita et al., 2000a), recent results have suggested that in the human homolog this site is indeed occupied (Cioaca et al., 2011).

Table 1. LC-MS occupancy analysis of human tyrosinase in A375 melanoma cells.

Immunoaffinity purified tyrosinase was subjected to Endo H digestion, separated by SDS-PAGE and in gel digested with trypsin. The peptides were extracted and analyzed using LC-MS/MS. The extracted ion current of the nonmodified peptides, HexNAc modified peptides and deamidated forms were used for occupancy estimation of each site. For N161 and N371, immunoaffinity enriched tyrosinase was digested with PNGase F, separated using SDS-PAGE and in gel digested using chymotrypsin. The assignment of the occupancy of these sites was based on the XIC of the deamidated form, but the prediction results from NetNGlyc server (<http://www.cbs.dtu.dk/services/NetNGlyc>) were also taken into consideration. Two of the sites (N161 and N290), were found as partial glycosylated, as this was also suggested by the prediction analysis.

Position	Predicted	Experimental	Peptide sequence	Type of peptide
N86	++	Glycosylated	ESWPSVFY N R	Tryptic
N111	++	Glycosylated	FGFWGPN C TER	Tryptic
N161	-	Partial	GQMKN G STPMFNDINIY	Chymotryptic
N230	++	Glycosylated	LTGDEN F TIPYWDWR	Tryptic
N290	---	Partial	LEEYN S HQSLC N GTPEGPLR	Tryptic
N337	+	Glycosylated	AAN F SFR	Tryptic
N371	++	Glycosylated	M N GTMSQVQGSANDPIF	Chymotryptic

Legend:

Glycosylated sites:

- + Potential > 0.5
- ++ Potential > 0.5 AND Jury agreement (9/9) OR Potential > 0.75
- +++ Potential > 0.75 AND Jury agreement
- ++++ Potential > 0.90 AND Jury agreement

Non-glycosylated sites:

- Potential < 0.5
- Potential < 0.5 AND Jury agreement (all nine < 0.5)
- Potential < 0.32 AND Jury agreement

Recombinant tyrosinase and new tyrosinase-derived peptides MS identification with potential applications in cancer vaccines

Mass spectrometry identification of recombinant mouse tyrosinase purified fractions

One of the first applications of the GeLC-MS/MS workflow was for the analysis of fractions collected from affinity purification of mouse tyrosinase. The protein was expressed in *E. coli*, then purified and further characterized by mass spectrometry for a subsequent potential use as antigen in melanoma immunization. Several fractions from the purification were analyzed using a data-dependent method. The SEQUEST analysis of these fractions revealed the co-purification of the chaperon GroEL. However, the mouse tyrosinase was identified in five out of 6 analyzed fractions with a sequence coverage that varied between 40.78 % and 70.00 %. The purification method and the mass spectrometry results were subsequent published (Chiritoiu et al., 2015).

Mass spectrometry analysis of potential new tyrosinase epitopes in melanoma

Analysis of the acidic cell surface elution of A375 melanoma cells stably expressing human tyrosinase revealed the identification of the peptide EEYNSHQSL, corresponding to the amino acids between 280 and 288 of human tyrosinase. In order to confirm the presence of the peptide a synthetic standard was analyzed in the same conditions. For the positive identification, the retention time, m/z, charge and CID MS/MS fragmentation were compared between the standard peptide and the biological sample. These properties of the ion in the acidic elution were identical with the ones found from the synthetic version. The parent cell line A375 did not revealed any ion with the same m/z in the analyzed retention time window. Analysis of cell surface mild acidic elution (MAE) and affinity-purified samples using W6/32 antibody revealed the identification of other tyrosinase-derived peptides like LEEYNSHQSL and LLMEKEDYHSL. Some of these sequences were also described in a recent study using several melanoma cell lines (Gloger et al., 2016). Future experiments will establish if these sequences are indeed immunogenic and could find subsequent applications in immunotherapy.

CONCLUSIONS

In conclusion, this work aims in its first part to identify and analyze a number of functional protein complexes of ERAD in melanoma with implications in Endoplasmic Reticulum (ER) protein quality control and protein degradation. In the second part I further focus on tyrosinase - a classic model glycoprotein used in our lab to investigate ER related events - by analyzing its glycosylation pattern in melanoma and identifying potential new tyrosinase-derived antigens.

Method implementation and development

In order to achieve the above mentioned objectives a significant effort of this work was devoted to develop and implement new MassSpec oriented workflows that include complex complementary techniques from molecular biology, analytical and preparative biochemistry, computational and statistical analysis; workflows which were critical for conditioning and analysis of different parts of cell proteome, interactome or glycome.

A part of the results obtained in this methodological work were published or are still under review such as those focused on: identification of oligosaccharides and small molecules (P5 – P11), identification of the proteins *E. coli* affinity chromatography purifications using bottom-up strategy from (P2, P4), analysis of glycosylation and MHC class I peptides and protein-protein interactions (P1, P3).

Results related to ERAD functional complexes

The main results obtained on this core subject of investigation might be summarized as follows:

- In order to identify the proteins associated with EDEM 2 in the ERAD of melanoma cells, I optimized several key-aspects of the AP-MS workflow. Moreover, to increase the level of results confidence – I combined two separate mass spectrometry data analysis platforms: SAINTexpress using spectral counts probability distribution and QUBIC using a protein intensity-derived analysis workflow.

- Data analysis performed as above, suggest that EDEM 2 associates with several ER proteins involved in protein folding and ERQC, but also with members of the ERAD pathway, which positions EDEM 2 at the cross-road of the ER sorting machinery.
- Several of these associations were further validated using biochemical methods alongside positive controls and for the first time an interaction map is proposed for EDEM 2 in melanoma.
- Association of EDEM 2 with other ERAD members results in an efficient disposal of misfolded proteins as revealed by the functional experiments, which also suggest a cross-regulation between ERAD components.
- EDEM 2 could be subjected to degradation using the same pathway as blocking the proteasome increases the intracellular level of EDEM 2 in melanoma cells.

Results related to Tyrosinase glycosylation and epitope analysis

The main findings in the second part of my work are summarized below:

- In A375 melanoma cells human tyrosinase N-glycosylation site occupancy is not uniform. Five out of seven sites are fully occupied, while the third and the fifth sites are only partial occupied. These results were further confirmed by MG132 experiments in which intracellular PNGase F can transform Asn to Asp completely in fully occupied sites. This is the first report revealing the site occupancy of human tyrosinase in a melanoma cell line using mass spectrometry.
- MS experiments demonstrate that both Methionine residues (M₃₇₁ and M₃₇₄) of the Tyr₃₆₉₋₃₇₇ epitope can be oxidized and this could have a major impact for peptide recognition by the immune system.

- Further kinetic HPLC analysis, revealed that the monosulfoxide forms are the most stable species of the Tyr₃₆₉₋₃₇₇ epitope. This observation resulted in the elaboration of an appropriate method in order to analyze the peptide in several melanoma cell lines.
- The MS analysis of an HLA class I associated tyrosinase-derived peptide in several melanoma cell lines is described, which could have potential applications in immunotherapy. Also, other tyrosinase-derived peptides are reported and the MS analysis of purified fractions of tyrosinase, which could find subsequent use in melanoma vaccination.

LIST OF PUBLICATIONS

PEER-REVIEWD JOURNAL PUBLICATIONS (P):

P1:

Chirițoiu GN*, Jandus C*, **Munteanu CVA***, Ghenea S, Gannon PO, Romero P, Petrescu SM, “*Epitope located N-glycans impair the MHC-I epitope generation and presentation.*” ***Electrophoresis*** 37(11):1448-60 (2016)

DOI: 10.1002/elps.201500449

AI = 0.6, IF = 3.028, WoS citations = 0

* authors with equal contribution

P2:

Chirițoiu GN*, **Munteanu CVA***, Mitrea N, “*Combining heterologous bacterial expression system with affinity chromatography purification to obtain native mouse tyrosinase.*” ***Farmacia*** 63(2):254-61 (2015)

AI < 0.1, IF = 1.003, WoS citations = 0

* authors with equal contribution

P3:

Popa IL, Milac AL, Sima LE, Alexandru PR, Pastrama F, Munteanu CVA, Negroiu G, “*Cross-talk between DopaChromTautomerase and Caveolin-1 is Melanoma Cell Phenotype Specific and Potentially Involved in Tumor Progression.*” ***Journal of Biological Chemistry*** 291(24):12481-500 (2016)

DOI: 10.1074/jbc.M116.714733

AI = 1.7, IF = 4.573, WoS citations = 0

P4:

Petrareanu G, Balasu MC, Vacaru AM, **Munteanu CVA**, Ionescu AE, Matei I, Szedlacsek SE, “*Phosphoketolases from Lactococcus lactis, Leuconostoc mesenteroides and Pseudomonas aeruginosa Dissimilar sequences, similar substrates but distinct enzymatic characteristics.*” ***Applied Microbiology and Biotechnology*** 98(18):7855-67 (2014)

AI = 1.0, IF = 3.337, WoS citations = 3

DOI: 10.1007/s00253-014-5723-6

P5:

Sârbu M, **Munteanu CVA**, Dehelean L, Petrescu AJ, Peter-Katalinic J, Zamfir AD, “*Identification and structural characterization of novel O- and N-glycoforms in the urine of a Schindler disease patient by Orbitrap mass spectrometry*”. ***Journal of Mass Spectrometry*** 50(9): 1044-56 (2015)

AI = 0.8, IF = 2.379, WoS citations = 0

DOI: 10.1002/jms.3616

P6:

Robu AC, Popescu L, **Munteanu CVA**, Seidler DG, Zamfir AD, “*Orbitrap mass spectrometry characterization of hybrid chondroitin/dermatan sulfate hexasaccharide domains expressed in brain.*” ***Analytical Biochemistry*** 485:122-31 (2015)

AI = 0.7, IF = 2.219, WoS citations = 0

DOI: 10.1016/j.ab.2015.06.028

P7:

Flangea C, Petrescu AJ, Seidler DG, **Munteanu CVA**, Zamfir AD, “*Identification of an unusually sulfated tetrasaccharide chondroitin/dermatan motif in mouse brain by combining chip-nanoelectrospray multistage MS2 -MS4 and high resolution MS.*” ***Electrophoresis*** 34(11):1581-92 (2013)

AI = 0.6, IF = 3.028, WoS citations = 5

DOI: 10.1002/elps.201200704

P8:

Tănase CI, Draghici C, Shova S, Cojocaru A, Maganu M, **Munteanu CVA**, Cocu F, “*Regioselective reactions on a 1,3-disubstituted dihydroxymethyl or dicarboxyl hexahydropentalene skeleton.*” ***Tetrahedron*** 71(38):6852-59 (2015)

AI = 0.6, IF = 2.641, WoS citations = 0

DOI: 10.1016/j.tet.2015.07.021

P9:

Tănase CI, Draghici C, Caproiu, MT, Shova, S, Cojocaru A, **Munteanu CVA**, “MCPB treatment of (\pm) -(1 α ,3 α ,3 $\alpha\beta$,6 $\alpha\beta$)-1,2,3,3a,4,6a-hexahydro-1,3-pentalenedimethanol and its O-acyl-protected derivatives; X-ray crystallography.” *Tetrahedron* 71(24):4154-62 (2015)

AI = 0.6, IF = 2.641, WoS citations = 1

DOI: 10.1016/j.tet.2015.04.096

P10:

Tănase C, Neguț CC, Udeanu DI, Ungureanu EM, Hrubaru M, **Munteanu CVA**, Voicu, SP, Cocu F, Ioniță AC, “New Oleamide Analogues with Potential Foodintake Regulator Effect.” *Revista de Chimie* -Bucharest- Original Edition 65(7):768-73 (2014)

AI < 0.1, IF = 0.810, WoS citations = 5

DOI: 10.13140/2.1.3270.2085

P11:

Tănase C, Neguț CC, Udeanu DI, Ungureanu EM, Hrubaru M, **Munteanu CVA**, Cocu F, Van Staden RIS, “New Oleamide Analogues with Potential Food - intake Regulator Effect. II.” *Revista de Chimie* -Bucharest- Original Edition 67(2):282-88 (2016)

AI < 0.1, IF = 0.810, WoS citations = 0

P12:

Pastramă F, **Munteanu CVA**, Chirițoiu GN, Petrescu SM, Petrescu AJ, “Interaction of tyrosinase and its soluble mutant with biological partners in melanoma cells.” *Rom. J. Biochem.*, 52(1):51–60 (2015).

AI = 0, IF = 0, WoS citations = 0

P13:

Butnaru CM, **Munteanu CVA**, Chirițoiu GN, Ghenea S, Petrescu AJ, Petrescu SM, “Comparison of protein extraction conditions for EDEM3 interactors in melanoma cells.” *Rom. J. Biochem.*, 52(1):31-38 (2015).

AI = 0, IF = 0, WoS citations = 0

P14:

Mentel M, Iancu I, Kibédi-Szabó CZ, **Munteanu CVA**, Szedlacsek SE, “Co-expression of human Wdr1 gene with a chaperone increases its protein solubility.” *Rom. J. Biochem.*, 50(1):39–51 (2013)

AI = 0, IF = 0, Citations = 1

AI – Article Influence Score (EIGENFACTOR.org)

IF – Impact Factor (JCR 2014)

WoS – Web of Science

PATENTS (PA):

Munteanu CVA, Chirițoiu GN, Petrescu SM, Jandus C, Ghenea S, Romero P, “*Novel tyrosinase antigenic peptides and uses thereof*”, International Patent to be filed (07/2016)

ORAL PRESENTATIONS (OP):

Munteanu CVA, Chirițoiu GN, Petrescu SM, Petrescu AJ, “*Mass Spectrometry interaction proteomics of EDEM2 in a melanoma cell line.*” International Conference of the Romanian Society of Biochemistry and Molecular Biology, 17-18 September 2015, Bucharest, Romania

SELECTED POSTERS (SP):

SP1:

Munteanu CVA, Chirițoiu GN, Petrescu SM, “*Mass spectrometry evaluation of a T cell presented epitope of tyrosinase.*” The Annual International Conference of the SRBMB & Workshop “Viral hepatitis from cell culture to clinic”, 5-6 June 2014, Băile Felix, Oradea, Romania – *best poster award*

SP2:

Chirițoiu GN, **Munteanu CVA**, Jandus C, Ghenea S, Romero P, Petrescu SM, “*Antigen glycosylation and cytotoxic T cells activation: case study of tyrosinase*

*mutants expressing HLA-A*02 restricted YMD epitope.” Annual International Symposium N. Cajal, 1-4 April 2015, Bucharest, Romania*

SP3:

Pastramă F*, **Munteanu CVA***, Popa IL, Negroiu G, “*Identification of interacting proteins with melanoma antigen dopachrome tautomerase by mass spectrometry analysis.*” International Conference of the Romanian Society of Biochemistry and Molecular Biology, 17-18 September 2015, Bucharest, Romania

* equal contribution authors

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Contract no.: POSDRU/159/1.5/S/135760

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