

## RESEARCH ARTICLE

# The Asia Oceania Human Proteome Organisation Membrane Proteomics Initiative. Preparation and characterisation of the carbonate-washed membrane standard

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The Asia Oceania Human Proteome Organisation (AOHUPO) has embarked on a Membrane Proteomics Initiative with goals of systematic comparison of strategies for analysis of membrane proteomes and discovery of membrane proteins. This multilaboratory project is based on the analysis of a subcellular fraction from mouse liver that contains endoplasmic reticulum and other organelles. In this study, we present the strategy used for the preparation and initial characterization of the membrane sample, including validation that the carbonate-washing step enriches for integral and lipid-anchored membrane proteins. Analysis of 17 independent data sets from five types of proteomic workflows is in progress.

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## 1 Introduction

Membrane systems, including membrane proteins, are essential constituents of cellular life. The topography of proteins in membranes is intimately related to their func-

tions. The transmembrane protein architecture includes proteins that span the membrane *via* transmembrane domains (TMDs) either as  $\beta$ -barrel structures or through relatively hydrophobic transmembrane helices [1]. Other proteins are selectively associated with one face of their membrane, including by binding through the hydrophobic peptide domains or lipid anchors. Technical challenges in the analysis of membrane proteins and membrane proteomes are well known, ranging from difficulties in detection of receptors and other proteins present at abundances less than 10 000 copies *per* cell to difficulties handling and detecting proteins that contain a high proportion

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**Abbreviations:** AOHUPO, Asia Oceania Human Proteome Organisation; ER, endoplasmic reticulum; JPSSL, Joint Proteomics Laboratory; MICR, microsomal; MPIS, Membrane Proteomics Initiative Standard; TMD, transmembrane domain

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of hydrophobic transmembrane regions. LC-MS/MS of proteolytic peptides is established for proteomic analysis of membrane fractions [2], including large-scale experiments relevant to our current study, which list hundreds of proteins from normal mouse liver [3–8]. Before these studies, and related shotgun MS/MS of other membrane systems, membrane proteins were poorly represented in proteomic reports.

As previously reported, the Asia Oceania Human Proteome Organisation (AOHUPO; www.aohupo.org) has embarked on a Membrane Proteomics Initiative with the goals of systematic comparison of strategies for the analysis of membrane proteomes and discovery of membrane proteins [9, 10]. This multilaboratory project is based on the analysis of a mouse liver membrane preparation (the Membrane Proteomics Initiative Standard, (MPIS)). Liver was chosen because of its relevance to several of the participating laboratories including those involved in the HUPO Liver Proteome Project [11], and because of extensively published knowledge about liver biochemistry, subcellular biology, and pathology, which can be used for the functional analysis of membrane proteins. The distributed MPIS was prepared by differential centrifugal pelleting of a liver homogenate to yield a microsomal (MICR) pellet. The resuspended pellet was subsequently washed with an alkaline carbonate solution [12, 13] to enrich for membrane proteins. We have in progress analysis of the MPIS using a range of workflows (Table 1) [9, 10]. In this study, we present the strategy used for preparation and initial characterization of the MPIS, based on LC-MS/MS of tryptic digests of the MPIS and precursor microsomes.

## 2 Materials and methods

### 2.1 Preparation of membrane fractions

C57BL/6J male mice from the Animal Resources Center (Murdoch, Australia) were shipped to New Zealand at age 5–6 wk and maintained in germ-free conditions at the Malaghan Institute of Medical Research (Wellington, New Zealand). Ethical approval was obtained from the Victoria University of Wellington Animal Ethics Committee. The mice were killed at age 10–11 wk using carbon dioxide anaesthesia and the livers were excised, and immersed in ice-cold homogenization medium containing 0.25 M sucrose, 5 mM Tris-HCl pH 7.4, 1 mM tetrasodium EGTA, 1 mM sodium orthovanadate, 2 mM sodium fluoride, and 1% v/v protease inhibitor cocktail (Sigma-Aldrich, MO, P8340). The livers were minced with scissors, washed twice with fresh medium, and suspended again in 4 volumes of fresh ice-cold homogenization medium. The sample was then homogenized at 4°C using a Polytron PT 10/35 (KINEMATICA, Switzerland) with a PT-20 probe at speed 5 for 40–60 s at 10 s intervals. Subcellular fractionation was carried out using differential centrifugation [14] at 4°C. The homogenate was centrifuged at  $12\,000 \times g_{av}$  for

15 min in a Sorvall SuperLite™ GSA rotor (Thermo Scientific, USA) and the resulting supernatant was centrifuged at  $100\,000 \times g_{av}$  for 1 h in a Beckman 45 Ti rotor (Beckman Instruments, USA) to produce a MICR pellet that was then washed twice by resuspension in fresh medium and centrifugation at  $100\,000 \times g_{av}$  for 1 h. The washed MICR pellet was then resuspended in 20 volumes of ice-cold 0.1 M sodium carbonate pH 11.5 containing 1% protease inhibitors. The suspension was slowly agitated on a shaker for 1 h at 4°C and then centrifuged again at  $100\,000 \times g_{av}$  for 1 h to produce a pellet which is called MPIS. The MPIS pellet was resuspended in 5 volumes of HPLC grade water and the aliquots were stored at –80°C. Aliquots of the stored MPIS were distributed to the participating laboratories using courier transfer of samples packed with at least 10 kg of dry ice. Transport was carried out by Logical Freight Solutions NZ, who ensured frozen transfer of the samples from despatch to delivery.

For the work described in this study, aliquots of the homogenate, MICR and MPIS fractions, and  $100\,000 \times g$  supernatant were processed by precipitation of protein using a Calbiochem Protein Precipitation kit (Calbiochem, Germany). Protein concentrations were measured using a 2-D Quant kit (GE Healthcare, Bucks, UK). Samples from all the fractions were reconstituted in 40 mM Tris, 7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.5% w/v aminosulfobetaine-14, and 1% protease inhibitor cocktail at a protein concentration of 5 mg/mL for proteomic analysis.

Aliquots of 20 µg protein from each of the above samples were mixed with Invitrogen NuPAGE lithium dodecyl sulfate sample buffer and electrophoresed on 4–12% precast polyacrylamide Bis-Tris NuPage gels using MOPS SDS electrophoresis, as described previously [15]. After electrophoresis, the gels were stained with colloidal Coomassie Brilliant Blue G-250 and imaged using a Molecular

**Table 1.** Summary of the five general workflows used by the participating laboratories for analysis of the MPIS

Workflows	No. of independent data sets
In-solution or in-gel digestion, followed by LC-MS/MS of proteolytic peptides	4
In-solution digestion followed by IPG-IEF separation of peptides, then by LC-MS/MS of proteolytic peptides	1
Protein separation by SDS-PAGE, followed by in-gel digestion, then by LC-MS/MS of proteolytic peptides	7
Chromatographic separation of proteins, followed by in-solution digestion, then LC-MS/MS of proteolytic peptides	4
Gel-based separation of proteins using 2-D Blue Native PAGE	1

The characterization of the MPIS reported in this manuscript used in-solution digestion with trypsin followed by LC-MS/MS of proteolytic peptides.

Dynamics Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA).

## 2.2 LC-MS/MS of tryptic digests

The reported results are based on replicate ( $n=6$  for each sample) LC-MS/MS analyses of MICR or MPIS samples containing 20  $\mu\text{g}$  protein, dissolved in 8 M urea, 100 mM Tris-HCl pH 8.5, to a final volume 50  $\mu\text{L}$ . Protein disulfide bonds were reduced with 10 mM DTT for 30 min at 56°C, followed by alkylation with 55 mM iodoacetamide for 40 min at room temperature in the dark. The reduced and alkylated samples were diluted threefold with 100 mM Tris-HCl pH 8.5 and digested with trypsin (Roche, modified sequencing grade) at an enzyme-to-substrate ratio of 1:50 w/w in the presence of 1 mM  $\text{CaCl}_2$  overnight at 37°C. After digestion, 90% formic acid was added to 4% concentration.

The resulting tryptic peptides were purified using 100  $\mu\text{L}$  OMIX C18 tips according to the manufacturer's instructions (Varian, CA). Peptides in each sample were eluted in 20  $\mu\text{L}$  of 70% ACN and 0.1% formic acid, and were further diluted with 0.1% formic acid. LC-MS/MS was carried out using a Dionex UltiMate™ 3000 LC system (LC Packings, The Netherlands) and a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray ion source (Thermo Electron, USA), as described previously [15]. Peptides were separated in a 75  $\mu\text{m} \times 15$  cm PepMap C18 analytical column (3  $\mu\text{m}$ , 300 Å Dionex) at a constant flow rate of 200 nL/min, using a gradient constructed from (A) 0.1% formic acid and (B) 0.1% formic acid in 80% ACN: 0% B for 17 min; 0–15% B for 30 min; 15–30% B for 133 min; 30–55% B for 70 min; 55–100% B for 30 min; 100% B for 5 min; and 100–0% B for 5 min. The LTQ was operated in data-dependent MS/MS mode, where the five most abundant precursor ions detected in a single MS scan from 400 to 2000  $m/z$  were dynamically selected for subsequent MS/MS scans with collision energy set to 35%, simultaneously incorporating dynamic exclusion option with 5 s exclusion duration to prevent reacquisition of MS/MS spectra of the same peptides.

## 2.3 Bioinformatics

In the initial phase of analysis, peak lists were extracted from the raw MS files, and peptide and protein lists were generated by groups at the Joint Proteomics Laboratory (JPSL) in Melbourne, Rockefeller University, Yonsei University, and the Beijing Proteome Research Centre. Subsequently, all extraction and processing of data from the raw files for the purpose of this manuscript was carried out at JPSL. Peak lists were extracted using *extract\_msn* (BioWorks 3.3.1 Thermo Scientific), using the following parameters: minimum mass 700; maximum mass 5000; no

merging of scans; 10 peaks minimum and total ion current 100. The *PeakListExtractor* program (JPSL, inhouse software) was used for generating optimized MASCOT generic files (MGF format) from the DTA files. This program removes duplicate peak lists for +2 and +3 spectra that are automatically generated by *extract\_msn* because of the low resolution survey scan of the LTQ instrument. For these spectra, a global “CHARGE = 2+, 3+” was written to the header of each MGF file, instructing the MASCOT search algorithm to search these spectra as doubly and triply charged, but retain only the highest scoring peptide. All the other spectra (*i.e.* not +2 or +3) were searched using the specified local “CHARGE = x” parameter determined by *extract\_msn*. MASCOT server (version 2.2.04, Matrix Science, UK) was used to identify peptides using the uninterpreted MS/MS ions search mode. Peak lists were searched against the International Protein Index mouse protein sequence database (version 3.36, 51 326 entries). The search parameters were as follows: carboxyamidomethylation of cysteine as a fixed modification (+57 Da) as well as variable modification consisting of  $\text{NH}_2$ -terminal acetylation (+42 Da) and oxidation of methionine (+16 Da), and allowance for up to two missed tryptic cleavage sites (trypsin/P). Precursor and fragment ion mass tolerance values were  $\pm 3$  and 0.8 Da, respectively.

MASCOT result files were loaded into the program MSPro (JPSL, inhouse software). For MSPro, peptide spectral matches with MASCOT ion scores  $\geq 15$  were retained and classified as either discrete (*i.e.* matching a unique protein record) or degenerate (*i.e.* matching multiple protein records). Peptide spectral matches, from six replicate LC-MS/MS analyses for each of the MPIS and precursor microsomes, were classified as significant when the ion score  $\geq$  the reported homology score or identity score (if the homology score was not present). Protein scores (a slightly modified MASCOT MudPIT score) were computed, as previously described [16]. To estimate the levels of false-positive protein identifications (*i.e.* false discovery rate), MS/MS spectra were also searched separately against the corresponding reversed-sequence (decoy) database. A 1% false discovery rate at the protein level was equated to a protein score of 55. Spectral counts (the number of MS/MS assigned to each protein) of significant peptide spectral matches were recorded and used to calculate the spectral count ratios to estimate the fold changes between MPIS and MICR. Spectral count ratio values ( $\log_2$ ) were calculated according to Beissbarth *et al.* [17] with a correction factor of 1.25 in accordance with Old *et al.* [18]. The sum of spectral counts for all inferred proteins (protein score  $> 55$ ) differed by  $< 7\%$  between MPIS and MICR. International Protein Index accession numbers of inferred proteins in MPIS and MICR were sent to the GOFact server (<http://61.50.138.118/gofact/>) for the analysis of Gene Ontology categories of the data sets. TMHMM (<http://www.cbs.dtu.dk/services/TMHMM>) was used for the TMD prediction. The MASCOT search result files and

experimental information were converted to PRIDE XML using PRIDE Converter (version 2.1.2). These data are available from PRIDE [19] <http://www.ebi.ac.uk/pride/> accession numbers 10632–10633.

### 3 Results and discussion

#### 3.1 Preparation and characterization of the MPIS

Our goal was to prepare a subcellular fraction that was enriched for integral membrane proteins in sufficient quantity for the participating laboratories to analyze using a range of proteomic techniques. We used the alkaline carbonate washing method that Fujiki *et al.* employed to strip the excess protein from endoplasmic reticulum (ER) and other organelle membranes in rat liver subcellular fractions [12, 13]. Although the alkaline carbonate method is frequently used in membrane isolation protocols, this is often carried out without systematic characterization of proteins that are depleted or retained after carbonate washing. We therefore examined the extent to which the integral membrane proteins and lipid-anchored membrane proteins were detected in the carbonate-washed MPIS and precursor microsomes MICR.

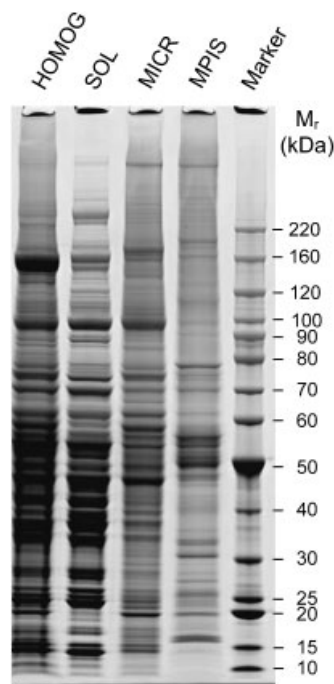
Fractionation of the mouse liver homogenate using differential centrifugation resulted in 100 000 × *g* sediment (MICR) and soluble supernatant fractions that differed in protein composition from each other and from the original homogenate. Approximately, one-tenth of the homogenate total protein amount separated in the MICR fraction, as is typically achieved for the preparation of an MICR fraction by differential centrifugation of homogenates [14, 15]. Further processing of the MICR fraction by washing with alkaline carbonate removed approximately 70% of the protein with a recovery of 2.7% of the original homogenate protein in the MPIS. The protein profiles detected by the Coomassie staining of SDS-PAGE separations showed selective retention and depletion of protein bands in the MPIS compared with MICR and total homogenate (Fig. 1).

The protein compositions of MICR and MPIS were examined by MS/MS of in-solution tryptic digests of the two fractions. A total of 628 proteins were identified (Supporting Information Tables 1 and 2) of which 294 were detected in both MICR and MPIS, 134 were found only in MPIS, and 200 only in MICR. Analysis of Gene Ontology annotations for Cell Component (Fig. 2A) showed the presence of proteins from ER, plasma membrane, mitochondrion, Golgi apparatus, endosome, vacuole, lysosome, nucleus, and peroxisome plus cytosol proteins in the precursor MICR as was expected from the differential centrifugation protocol. Comparison of Cell Component annotations for the carbonate-washed MPIS showed enhancement of annotations for membrane, plasma membrane, ER, endosome, vacuole, and lysosome; plus depletion of proteins derived from cytosol and nucleus in the MPIS. Accordingly, Molecular Function

annotations for nuclear and cytoplasmic proteins associated with nucleic acid binding were depleted in the MPIS, and there was enrichment of annotations associated with membrane activities, including receptor and transporter activities (Fig. 2B). The conclusions that membrane proteins were enriched in the MPIS, and that soluble proteins were depleted, were supported by the analysis of a number of proteins containing predicted TMDs. Forty-one per cent of the 428 MPIS proteins contained predicted TMDs, including 111 with a single predicted TMD, and 63 with predicted multiple TMDs (Fig. 2C).

#### 3.2 Membrane proteins are enriched in the MPIS

We next considered the extent to which the membrane proteins were enriched in the MPIS and whether the carbonate washing procedure was compromised by loss of membrane proteins. Proteins that were annotated for membrane locations (for all detected organelle proteins, and either containing predicted TMDs or membrane anchors), were located preferentially in the MPIS: numbering 88 in the MPIS alone, plus 72 in both MPIS and MICR (Fig. 3A). Two proteins with membrane annotations, each identified by three significant peptides, were detected only in the precursor MICR. One of these proteins, Picalm, is a phosphatidylinositol-binding clathrin assembly protein with no predicted TMDs whose membrane annotation reflects ability to associate with the



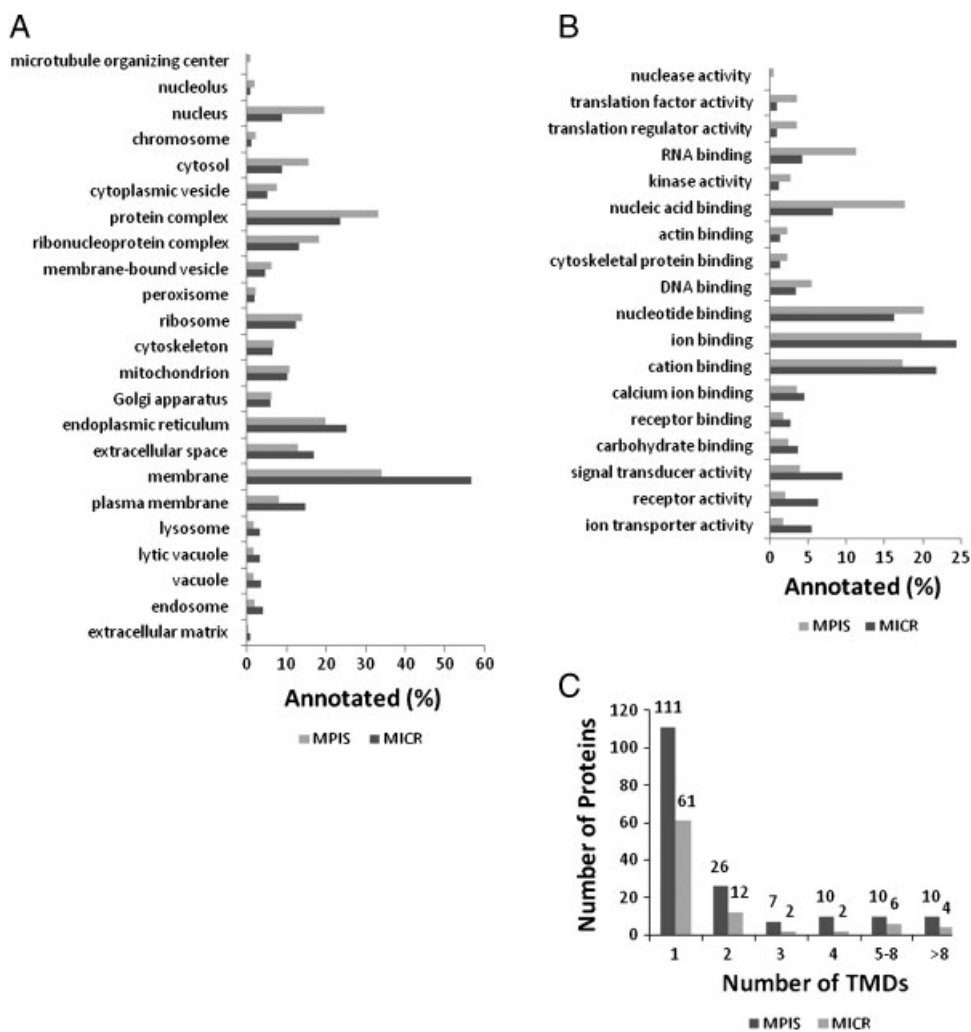
**Figure 1.** SDS-PAGE of subcellular fractions. Liver homogenate (HOMOG), post-MICR supernatant (SOL), MICR, MPIS, and marker proteins were electrophoresed and the gels were stained with Coomassie Blue G-250.

membrane phospholipids. The second protein, Dnaja1, is a potentially farnesylated protein that would associate with proteins through the farnesyl lipid anchor. It is possible that Dnaja1 was detected as the unmodified polypeptide.

Further support for the use of the carbonate wash to enrich membrane proteins was provided by the analysis of specific proteins that distributed between MPIS and precursor MICR. Of the 200 proteins detected only in MICR, approximately half were from the cytoplasm, including proteins with a dual cytoplasmic organelle location. The most numerous proteins with dual cytoplasmic organelle locations were those also with annotated nucleus, and components of the intracellular vesicular transport systems including some clathrin and coatamer subunits. Figure 3B illustrates the distributions of groups of proteins between MICR and MPIS. The effects of the carbonate wash included depletion of many cytosolic proteins. ER lumen proteins, including carboxylesterases (Supporting Information Table 3), were also depleted in the MPIS. These results confirm the action of the 0.1 M pH 11.5 carbonate solution for removal of soluble proteins and some membrane-asso-

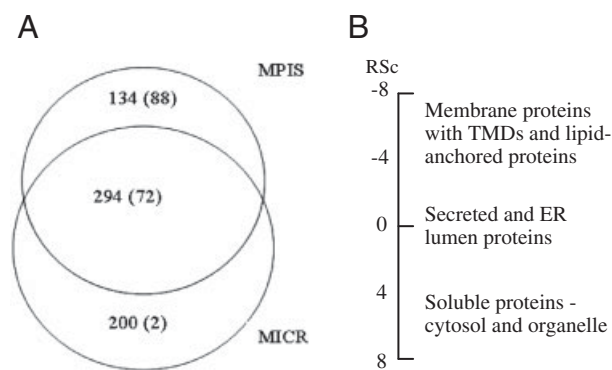
ciated proteins through: (i) disruption of membrane-bound vesicles, including ER-derived microsomes [12, 13, 20], and (ii) competing charge interactions between membranes and loosely bound soluble and cytoskeletal proteins.

Analysis of the distribution of individual proteins between MPIS and MICR indicated that the relative abundance of membrane proteins with TMDs or lipid anchors increased in the MPIS. Membrane proteins that were detected included 25 cytochrome P450 proteins, the enzyme NADPH-cytochrome P450 reductase plus 9 members of the UDP-glucuronosyltransferase family (Supporting Information Table 4). All of these proteins were detected in MPIS, but eleven of the P450s and three of the glucuronosyltransferases were barely detectable or not detectable in the MICR (0–5 spectral counts). The cytochrome P450 mixed function oxidase enzymes are variously distributed in ER or mitochondrial membranes and have roles in the metabolism of a wide range of endogenous and exogenous substrates [21]. Sutton *et al.* [22] reported 26 P450s, using MS/MS of the gel slices from mouse liver microsomes separated by SDS-PAGE, and of these 17 matched, the P450s we reported.



**Figure 2.** Comparison of MICR and MPIS. Gene Ontology (GO) annotations for Cell Component (A) and Molecular Function (B) indicate enrichment of membrane proteins and depletion of soluble proteins in MPIS compared with MICR. MPIS is also enriched for proteins containing predicted TMDs (C). Dark bars MPIS, light bars MICR.





**Figure 3.** Membrane proteins are enriched in the MPIS. The Venn diagram (A) shows the total number of proteins (number with membrane annotation in brackets) in MICR and MPIS. The second panel (B) illustrates the relative abundance of groups of proteins in the MPIS compared with the MICR. Spectral count ratios (RSc): negative values are enriched in the MPIS, and positive values are depleted in the MPIS with respect to MICR. The extreme values represent the most enriched or depleted groups of proteins. Proteins with similar distributions in MPIS and MICR were in the range  $-0.4$  to  $1.3$ . Ranges of RSc for specific groups of membrane proteins include cytochrome P450s  $-1.1$  to  $-6.2$ , membrane receptors and transporters  $-0.9$  to  $-7.6$ , lipid-anchored proteins, including Rabs  $-1.4$  to  $-7.8$ . For nonmembrane proteins the ranges were cytosolic  $0.2$ – $7.4$ , cytoskeletal  $-0.2$  to  $-0.8$ , ER lumen  $-0.1$  to  $3.7$ , histones  $1.3$ – $5.1$ , ribosome subunits  $-2.8$  to  $6.8$ , and secreted proteins  $-2.2$  to  $2.3$ .

Supporting Information Table 5 summarizes the membrane receptor and transport proteins, all of which were detected in the MPIS but only some in the MICR. The list of receptors includes proteins from the sinusoidal, basolateral, and canalicular domains [23] of the plasma membrane of liver parenchymal cells (hepatocytes). Detection of the sinusoidal, basolateral, and canalicular proteins indicates recovery of plasma membrane-derived vesicles in the MICR fraction which is consistent with the homogenization and centrifugation conditions that were used [14, 24]. Lipid-anchored proteins also preferentially distributed in MPIS, including two transferrin receptor isoforms and Rab monomeric G-protein subunits 1A, 1B, 2A, 5B, 5C, 6A, 7A, 8A, 10, 11B, 14, and 18 involved in intracellular membrane traffic. Gilchrist *et al.* reported the detection of 32 Rabs from a series of subcellular fractions of rat liver, of which 1A, 1B, 7, 2A, 6, 14, 10, and 18 were the most abundant in rough and smooth microsomes, Golgi, and COP1 vesicles [25], consistent with our results.

#### 4 Concluding remarks

In summary, our strategy for the AOHUPO Membrane Proteomics Initiative was to prepare a subcellular fraction enriched for membrane proteins that could be used to examine the extent to which membrane proteins can be detected by a range of proteomic workflows. We therefore

prepared a mixed organelle MICR pellet and used the carbonate washing procedure to enrich for membrane proteins. The analysis presented here demonstrates that membrane proteins containing TMDs or lipid anchors were captured and enriched in the MPIS. Enhanced detection of membrane proteins in the MPIS can be attributed to the greater concentration of membrane proteins that was achieved by depletion of approximately 70% of the total amount of MICR protein by carbonate washing (Fig. 1). The depleted components were largely soluble proteins from the cytoplasm and from the organelle matrix and lumen compartments. We have therefore established that treatment with carbonate enhanced detection of membrane proteins through the depletion of soluble proteins, including those from the membrane-enclosed compartments of the ER and other organelles, with retention of proteins with TMDs, lipid anchors, or other membrane-binding domains.

Work in progress (Table 1) from the laboratories that are represented by the authors of this manuscript indicates that up to two times as many membrane proteins can be detected as those reported here. Our subsequent manuscripts will describe the extent to which membrane proteins can be analyzed using the various workflows, plus characterization of the ER and plasma membrane proteins and proteomes captured in the MPIS.

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#### 5 References

- [1] Tan, S., Tan, H. T., Chung, M. C. M., Membrane proteins and membrane proteomics. *Proteomics* 2008, 8, 3924–3932.
- [2] Wu, C. C., MacCoss, M. J., Howell, K. E., Yates, J. R., III, A method for the comprehensive proteomic analysis of membrane proteins. *Nat. Biotechnol.* 2003, 21, 532–538.
- [3] Kislinger, K., Rahman, K., Radulovic, D., Cox, B. *et al.*, PRISM, a generic large scale proteomic investigation strategy for mammals. *Mol. Cell. Proteomics* 2003, 2, 96–106.
- [4] Zhang, L., Xie, J., Wang, X., Liu, X. *et al.*, Proteomic analysis of mouse liver plasma membrane: use of differential extraction to enrich hydrophobic membrane proteins. *Proteomics* 2005, 5, 4510–4524.
- [5] Shi, R., Kumar, C., Zougman, A., Zhang, Y. *et al.*, Analysis of the mouse liver proteome using advanced mass spectrometry. *J. Proteome Res.* 2007, 6, 2963–2972.
- [6] Lai, K. K., Kolippakkam, D., Beretta, L., Comprehensive and quantitative proteome profiling of the mouse liver and plasma. *Hepatology* 2008, 47, 1043–1051.

- [7] Wiśniewski, J. R., Zougman, A., Nagaraj, N., Mann, M., Universal sample preparation method for proteome analysis. *Nat. Methods* 2009, 6, 359–362.
- [8] Zgoda, V. G., Moshkovskii, S. A., Ponomarenko, E. A., Andreewski, T. V. *et al.*, Proteomics of mouse liver microsomes: performance of different protein separation workflows for LC-MS/MS. *Proteomics* 2009, 9, 4102–4105.
- [9] Chung, M., Nakamura, K., Jordan, T. W., The AOHUPO Membrane Proteomics Initiative, Fourth Workshop 22 June 2008, Cairns, Australia. *Proteomics* 2008, 8, 3920–3923.
- [10] Cottingham, K., AOHUPO takes on membrane proteins. *J. Proteome Res.* 2009, 8, 1109.
- [11] He, F., Human liver proteome project: plan, progress, and perspectives. *Mol. Cell. Proteomics* 2005, 4, 1841–1848.
- [12] Fujiki, Y., Hubbard, A. L., Fowler, S., Lazarow, P. B., Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 1982, 93, 97–102.
- [13] Fujiki, Y., Fowler, S., Shio, H., Hubbard, A. L., Lazarow, P. B., Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: Comparison with endoplasmic reticulum and mitochondrial membranes. *J. Cell Biol.* 1982, 93, 103–110.
- [14] Lim, W. C., Jordan, T. W., Subcellular distribution of hepatic bile acid-conjugating enzymes. *Biochem. J.* 1981, 197, 611–618.
- [15] Peng, L., Rawson, P., McLauchlan, D., Lehnert, K. *et al.*, Proteomic analysis of microsomes from lactating bovine mammary gland. *J. Proteome Res.* 2008, 7, 1427–1432.
- [16] Greening, D. W., Glenister, K. M., Kapp, E. A., Moritz, R. L. *et al.*, Comparison of human platelet membrane-cytoskeletal proteins with the plasma proteome: Towards understanding the platelet-plasma nexus. *Proteomics Clin. Appl.* 2008, 2, 63–77.
- [17] Beissbarth, T., Hyde, L., Smyth, G. K., Job, C. *et al.*, Statistical modeling of sequencing errors in SAGE libraries. *Bioinformatics* 2004, 20, i31–i39.
- [18] Old, W. M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K. G. *et al.*, Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol. Cell. Proteomics* 2005, 4, 1487–1502.
- [19] Vizcaíno, J. A., Côté, R., Reisinger, F., Foster, J. M. *et al.*, A guide to the Proteomics Identifications Database proteomics data repository. *Proteomics* 2009, 9, 4276–4283.
- [20] Blackler, A. R., Speers, A. E., Ladinsky, M. S., Wu, C. C., A shotgun proteomic method for the identification of membrane-embedded proteins and peptides. *J. Proteome Res.* 2008, 7, 3028–3034.
- [21] Porter, T. D., Coon, M. J., Cytochrome P-450 multiplicity of isoforms, substrates and catalytic and regulatory mechanisms. *J. Biol. Chem.* 1991, 266, 13469–13472.
- [22] Sutton, C.W., Sutherland, M., Shnyder, S., Patterson, L. H., Improved preparation and detection of cytochrome P450 isoforms using mass spectrometric methods. *Proteomics* 2010, 10, 327–331.
- [23] Hubbard, A. L., Wall, D. A., Ma, A., Isolation of rat hepatocyte plasma membranes. I. Presence of the three major domains. *J. Cell Biol.* 1983, 96, 217–229.
- [24] Wisher, M. H., Evans, M. H., Functional polarity of the rat hepatocyte surface membrane. Isolation and characterization of plasma-membrane subfractions from the blood-sinusoidal, bile-canalicular and contiguous surfaces of the hepatocyte. *Biochem. J.* 1975, 146, 375–388.
- [25] Gilchrist, A., Au, C. E., Hiding, J., Bell, A. W. *et al.*, Quantitative proteomics analysis of the secretory pathway. *Cell* 2006, 127, 1265–1281.

## 6 Addendum

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