MEDICAL UNIVERSITY OF VIENNA

Introducing two strategies for the reproducible detection of whole membrane and surface proteomes

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for obtaining the academic degree

Doctor of Philosophy

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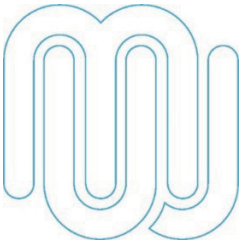

Declaration

All work described in this thesis has been performed by the author at the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences. All chapters of this thesis have been written by the author. Feedback to the writing of this thesis has been provided by Giulio Superti-Furga, Leonhard X. Heinz and Enrico Girardi.

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All experiments described in chapter 2.2 were performed by the author of this thesis with André C. Müller operating the mass spectrometers and Leonhard X. Heinz and Richard Kumaran Kandasamy contributing experimental and data analysis advice. Experimental design and analysis parameters were jointly determined by the author of this thesis, Giulio Superti-Furga, Jacques Colinge and Keiryn L. Bennett.

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Table of contents

List of figures

List of tables

Abstract

Membrane proteins are structurally and functionally highly diverse and changes in their expression pattern are among the first events taking place in pathological conditions. Thus, they are a rich source of biomarkers as well as therapeutic targets. Their large-scale analysis using proteomic strategies has, however, been challenging. Several protocols comprising either all membrane proteins expressed in a cell at a given condition and time or tailored towards proteins located at the plasma membrane have been developed. Still, comparative analyses are only scarcely available. This thesis provides a systematic comparison of different plasma membrane isolation strategies for subsequent analysis by one-dimensional gel-free liquid chromatography mass spectrometry. Moreover, the sulfo-NHS-SS-biotinylation procedure, which overall performed best for the monitored criteria, was simplified by a competitive biotin elution strategy that proved to be fast, cost-effective and robust empowering the routine evaluation of plasma membrane proteomes on a larger scale. Intriguingly, computational analysis using different databases and prediction tools indicated a total of over 90 % of the proteins purified with the modified sulfo-NHS-SS-biotinylation protocol to be associated with the plasma membrane, mostly as interactors. In addition, the cell surface proteomic procedure developed within this thesis could be successfully employed to determine genetic and drug-induced alterations of the cellular plasma membrane composition.

At the same time, Triton X-114 phase separation coupled to filter-aided sample preparation was established as an equally reproducible and robust protocol for the complement of all membrane proteins.

In summary, the work presented herein not only enables a more routine evaluation of membrane and surface proteomes relevant to the functional correlation of transport, signaling and drug response properties. The combination of both technologies allows to dissect them, ultimately increasing the resolution available for these key sub proteomes.

Zusammenfassung

Membranproteine sind in ihrer Struktur und Funktion höchst unterschiedlich und Veränderungen in ihrem Expressionsmuster gehören zu den ersten Ereignissen in pathologischen Situationen. Sie sind daher sowohl als Biomarker, als auch als therapeutische Ansatzpunkte interessant. Sie im großen Maßstab mittels Proteomik zu analysieren, war jedoch bisher eine schwierige Aufgabe. Mehrere Protokolle, die entweder alle Membranproteine umfassen, die unter definierten Bedingungen und zu einem bestimmten Zeitpunkt in einer Zelle exprimiert werden, oder auf Proteine an der Plasmamembran zugeschnitten sind, wurden entwickelt. Dennoch stehen vergleichende Analysen kaum zur Verfügung. Die vorliegende Arbeit bietet einen systematischen Vergleich verschiedener Plasmamembranisolierungsstrategien zur nachfolgenden Analyse durch eindimensionale gel-freie Flüssigchromatographie-Massenspektrometrie. Darüber hinaus wurde das Sulfo-NHS-SS-Biotinylierungsverfahren, das innerhalb der getesteten Kriterien insgesamt am besten abschnitt, durch eine kompetitive Biotin Eluierungsstrategie vereinfacht, die sich als schnell, kosteneffektiv und robust erwies und dadurch eine routinemäßige Evaluierung von Plasmamembran Proteomen in einem größeren Maßstab ermöglicht. Interessanterweise ergab die computergestützte Auswertung verschiedener Datenbanken und Programme zur Vorhersage zellulärer Lokalisationen, dass insgesamt über 90 % der mit der modifizierten Sulfo-NHS-SS-Biotinylierungsmethode identifizierten Proteine mit der Plasmamembran assoziiert sind, überwiegend als Interaktionspartner. Des Weiteren konnte das im Rahmen dieser Arbeit entwickelte Zelloberflächenproteomikverfahren erfolgreich zur Bestimmung von genetischen oder durch Medikamente bedingten Veränderungen in der zellulären Plasmamembranzusammensetzung angewendet werden.

Gleichzeitig wurde Triton X-114 Phasentrennung in Verbindung mit filtergestützter Probenvorbereitung als gleichermaßen reproduzierbares und robustes Protokoll zur ergänzenden Analyse aller Membranproteine etabliert.

Insgesamt ermöglichen die hier vorgestellten Ergebnisse nicht nur eine verstärkte routinemäßige Evaluierung von Membran- und Zelloberflächenproteomen, die für den funktionellen Zusammenhang von Transport- und Signalwegen sowie für das Verständnis

der Wirkungsweise von Medikamenten relevant sind. Vielmehr erlaubt die Kombination beider Technologien deren gegenseitige Abgrenzung und erhöht so die verfügbare Auflösung dieser Schlüsselproteome.

Abbreviations

ATP	adenosine triphosphate
ABC	ATP-binding cassette
ACN	acetonitrile
CAM	carbamidomethylation
CID	collision-induced dissociation
COP	coat protein
Da	Dalton
dNSAF	distributed normalized spectral abundance factors
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
emPAI	exponentially modified protein abundance index
ER	endoplasmic reticulum
FA	formic acid
FASP	filter-aided sample preparation
FCS	fetal calf serum
FDR	false discovery rate
GO.CC	Gene Ontology cellular component
GPCRs	G protein coupled receptors
GPI	glycosylphosphatidylinositol
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
IAA	iodoacetamide
IMDM	Iscove's modified Dulbecco's medium
IPA	isopropanol
LC	liquid chromatography
LTQ	linear trap quadrupole
m/z	mass-to-charge ratio
MBS	MES-buffered saline

MeOH	methanol
MES	2-(*N*-morpholino)ethanesulfonic acid
MS	mass spectrometry
MudPIT	multidimensional protein identification technology
NBDs	nucleotide-binding domains
OST	oligosaccharyl transferase
PAA	polyacrylic acid
PAI	protein abundance index
PBS	phosphate-buffered saline
PM	plasma membrane
PNGase	peptide-*N4*-(*N*-acetyl-beta-glucosaminyl)asparagine amidase
PPI	protein-protein interaction
PTM	post-translational modification
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLC	solute carrier
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SPE	solid phase extraction
SRP	signal recognition particle
STB	stage tip buffer
TBS	tris-buffered saline
TEAB	triethylammonium bicarbonate
TFA	trifluoroacetic acid
TGN	*trans* Golgi network
TRAM	translocating-chain-associated membrane protein
Tris	tris(hydroxymethyl)aminomethane
TX114	Triton X-114
wt	wild type

Acknowledgments

Pursuing my PhD at CeMM has been a unique experience in many ways. I have hugely profited from the welcoming and collaborative spirit one encounters throughout the institute and CeMM is definitely the place that has shaped my scientific abilities the most. Moreover, I got introduced to a full range of principles, starting with the 3 Ps (**p**rofessional, **p**ersistent, **p**olite) and working towards the 5 Ps (**p**rior **p**reparation **p**revents **p**oor **p**erformance) and learned a lesson that some CeMMies are still stuck in: ”Good morning from my side” is not a correct English sentence.

I am very grateful to every single CeMMie for all the support, help and advice I have received throughout my journey. Still there are people this thesis would not have been possible without. Thus, I am particularly thankful to my former supervisor Jacques Colinge, who taught me a lot about programming and data analysis; Keiryn Bennett for her constant support, especially regarding any proteomic sample analyses; and Giulio Superti-Furga for putting his trust in me right from the start, being my adoptive supervisor and contributing fantastic input and perspectives. Your enthusiasm for science is outstanding!

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Having to change supervisor halfway through my PhD was a challenge. Yet, the support and warm welcome I received from all members of the Superti-Furga lab made the transition so much easier and the experience quite rewarding all in all. Hence, I owe gratitude to every single one of my colleagues. Being welcomed and enjoying work as much as I have done with you, cannot be taken for granted and I am very grateful for all the great ideas and suggestions you have put forward and the unique moments and laughter I was able to share with you. A special thanks in that connection to Marielle, the right side of the lab, my adoptive brother and tandem language partner in crime Adrian

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The road towards a PhD degree can be a quite bouncy ride and I have found that “non-scientific” travel companions tremendously help to keep things in perspective. Therefore, I am particularly thankful to all my friends and family outside of work for all their love, support and understanding and the fantastically fun and relaxing times. You are what life is truly about!

1. Introduction

1.1 Cellular membranes

Membranes protect and separate cells. While the plasma membrane (PM) as outer boundary is a universal feature of all cells, eukaryotic cells – unlike prokaryotes – additionally contain internal membranes compartmentalizing their organelles (Tan et al, 2008). The fundamental phospholipid bilayer determines the structure of cellular membranes assembling spontaneously with the aliphatic chains facing each other. Glycerophospholipids are the main structural lipids in eukaryotic membranes. Like sphingolipids, another major class of structural lipids, variable polar head groups and lipidic fatty acid tails attached to their respective backbone, together with the structurally different class of sterols, enable the existence of more than 1,000 different lipid species in any eukaryotic cell. Furthermore, a cell's lipid repertoire can adopt defined phases allowing the cell to alter membrane properties such as fluidity, thickness, curvature and thermal stability (Sud et al, 2007; van Meer et al, 2008). Recently, their sphere of influence by functional crosstalk was even extended to major cellular processes (Atilla-Gokcumen et al, 2014; Köberlin et al, 2016; Köberlin et al, 2015).

Biological membranes are typically composed of 50 % lipids by mass. The second half is made up of proteins that can be divided into topological or functional subgroups. Lipid and protein composition of cellular membranes as well as their ratio vary between distinct cell types and organelles and are reciprocally modulated (Almen et al, 2009; Dobson & Kell, 2008; Laganowsky et al, 2014; Marsh, 2008; Tan et al, 2008).

Initially, cellular membranes were described using the "fluid mosaic model" with both, proteins and lipids, freely diffusing within the plane of the membrane (Singer & Nicolson, 1972). It then became clear, however, that membranes are rather compartmentalized into different types of subdomains with distinct characteristics and function (Helms & Zurzolo, 2004). One key discovery were micro domains enriched in specific lipid species and proteins, so-called "lipid rafts", that are now viewed as platforms for signaling, trafficking and transport (Simons & Toomre, 2000; Simons & van Meer, 1988; Tan et al, 2008).

1.2 Membrane proteins

1.2.1 Structure of membrane proteins

Membrane proteins are structurally and functionally highly diverse. They extend well beyond proteins displaying at least one transmembrane α-helix. In fact, pore-forming β-barrels consisting of alternating polar and non-polar amino acids represent another sub-type of integral or transmembrane proteins. Moreover, peripheral membrane proteins bound to the membrane to various extents and by different moieties belong to these common types of proteins (Tan et al, 2008) (Figure 1).

Integral membrane proteins are further categorized according to the localization of their N-terminus and their number of transmembrane domains, respectively. Peripheral membrane proteins, on the other hand, can either be lipid-anchored, *i.e.* bound to the membrane by direct interactions with phospholipids mediated by a glycosyl-phosphatidylinositol (GPI), a hydrocarbon or a fatty acyl moiety, or be associated to the membrane by interactions with lipids or integral membrane proteins (Alberts B, 2002; Tan et al, 2008) (Figure 1).

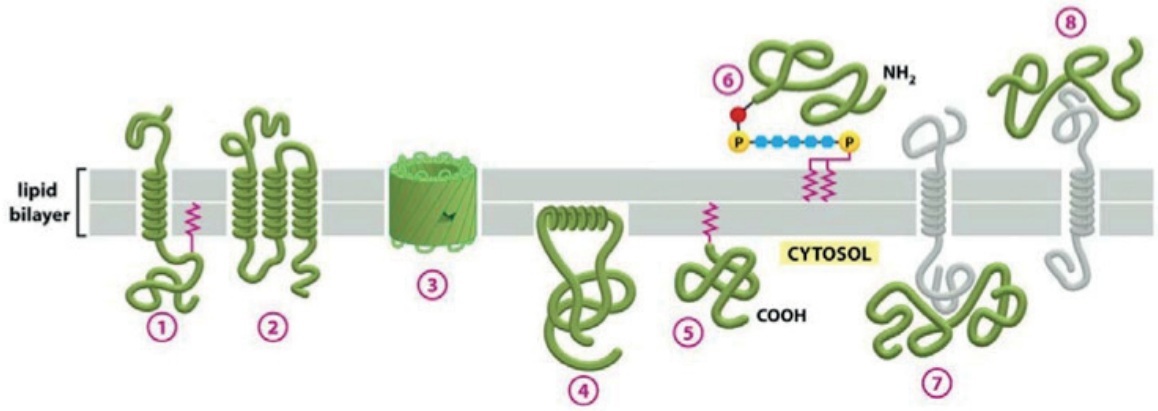

Figure 1: Types of membrane proteins

Schematic representation taken from (Alberts B, 2002). Copyright 2002 Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter. Integral membrane proteins either extend across the lipid bilayer as a single α-helix (1), as multiple α-helices (2), or as β-barrels (3). Peripheral membrane proteins are either lipid-anchored (5, 6) or attached to the membrane by interactions with lipids (4) or integral membrane proteins (7, 8).

1.2.2 Biosynthesis of membrane proteins

The biosynthesis of membrane proteins starts in the cytoplasm, where a nascent chain of amino acids emerges from the ribosome. Like secretory proteins, they carry a signal sequence that is bound by the signal recognition particle (SRP). By binding to its receptor on the surface of the endoplasmatic reticulum (ER), the ribosome-nascent-chain complex gets transferred to the translocon. In eukaryotes, the translocon consists of the Sec61 heterotrimer, which forms the protein conducting channel and several other proteins, *e.g.* the translocating-chain-associated membrane protein (TRAM) and the oligosaccharyl transferase (OST) (Conti et al, 2015; Dudek et al, 2015). While the exact role of TRAM remains controversial (Do et al, 1996; Heinrich et al, 2000; Mothes et al, 1997), OST was found to add N-linked sugars to the nascent polypeptide chain (Evans et al, 1986; Kelleher & Gilmore, 2006; Kelleher et al, 1992). There are different models on how membrane proteins are then integrated into the lipid bilayer and structural features play a role in that, too (Andersson & von Heijne, 1994; Hessa et al, 2005; Hessa et al, 2007). Specifically, the first potential transmembrane helix within the nascent precursor polypeptide chain that emerges at the ribosomal tunnel exit can either be inserted into the Sec61 complex in a “loop-like” or “head-on” fashion resulting in opposite localizations of the N-terminus. Moreover, a subsequent “flip turn” of “head-on” insertions can reverse protein orientations yet again (Dudek et al, 2015). Still, membrane proteins with the N-terminus located on the luminal side as well as oppositely-oriented membrane proteins are integrated into the lipid bilayer through a lateral gate in the protein-conducting channel (Do et al, 1996; Martoglio et al, 1995; Zimmermann et al, 2011) (Figure 2a).

The biosynthesis of multi-transmembrane proteins is less well understood. Mostly, they are currently viewed to have multiple hydrophobic signal sequences in the appropriate order (Dudek et al, 2015). Yet, this would require a “sewing-like” mechanism, sequentially integrating every single transmembrane domain into the ER membrane (High & Laird, 1997). In addition, complex multi-transmembrane proteins, for example many seven transmembrane G protein coupled receptors (GPCRs) lack N-terminal signal peptides for membrane insertion and hence do not fit this model (Wirtz, 2013). Some mitochondrial membrane proteins with less hydrophobic transmembrane segments even escape ER targeting mechanisms altogether (Miyazaki et al, 2005). At the same time, experimental

data suggests certain transmembrane segments to be independently integrated into the membrane, while the efficient insertion of others relies on cooperative effects between neighboring loops and/or transmembrane segments (Enquist et al, 2009; Tu et al, 2000). In essence, how important signal sequences are for the biosynthesis of multi-transmembrane proteins and how the integration of multiple sequential transmembrane regions into the lipid bilayer is coordinated, seems to be highly variable and diverse.

Even though most membrane proteins are co-translationally translocated, post-translational translocation represents an alternative Sec61-independent route (Dudek et al, 2015; Kutay et al, 1993; Steel et al, 2002; Yabal et al, 2003).
One of the first groups of membrane proteins associated with post-translational insertion were tail-anchored proteins with a single hydrophobic transmembrane region located at or near the C-terminus of the polypeptide. As a result, these polypeptides are released from the ribosome before their ER targeting signal gets recognized by cytosolic factors. The mechanism of post-translational insertion was suggested to be closely related to the co-translational mode, including SRP and its receptor as key players (Abell et al, 2004; Leznicki et al, 2010). At the same time, adenosine triphosphate (ATP) was observed to stimulate the integration of at least some TA proteins, seemingly contradicting the role of guanosine triphosphate (GTP)-dependent SRP (Abell et al, 2004; Kim et al, 1997; Kutay et al, 1995). Recently, the TRC40/GET pathway, identified in crosslinking experiments with several tail-anchored model proteins, received much attention (Favaloro et al, 2008; Shao & Hegde, 2011; Stefanovic & Hegde, 2007; Wang et al, 2014). Still, it is becoming increasingly clear that there is not one distinct post-translational insertion pathway, but multiple individual mechanisms, whose detailed specificities still need to be determined (Denic, 2012; Johnson et al, 2013; Ott & Lingappa, 2002; Rabu et al, 2009) (Figure 2b).

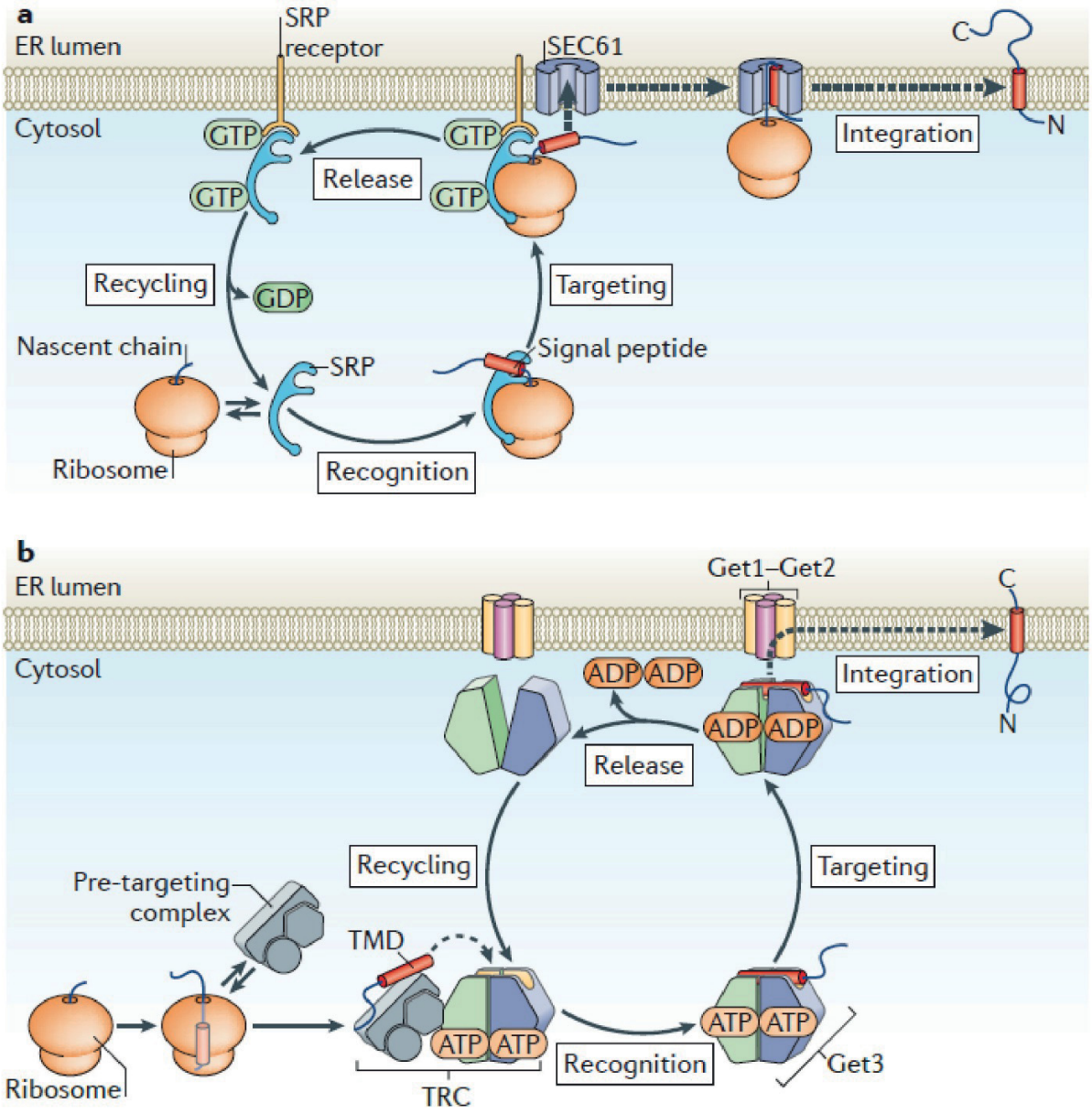

Figure 2: Co- and post-translational membrane protein biosynthesis

Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Hegde & Keenan, 2011), copyright 2011. a) In the co-translational pathway, a signal recognition particle (SRP) mediates transport of the ribosome-protein complex to the endoplasmic reticulum (ER). The Sec61 complexes then moves the nascent protein through the ER membrane while in the post-translational pathway, membrane proteins synthesized on free ribosomes are inserted into the ER membrane by specialized insertion receptors after translation has finished (b).

1.2.3 Trafficking of membrane proteins

The distinct membrane-enclosed organelles found in eukaryotic cells require specific and regulated transport mechanisms for membrane proteins, following their synthesis and insertion into the ER. Conventionally, trafficking starts with the sorting of proteins and the formation of vesicular or tubular carriers from donor membranes. Budding vesicles then move along microtubules and actin filaments in a well mapped-out way until fusion with the acceptor compartment occurs. Conventional exocytosis or secretion refers to membrane-bound cargo *en route* to the cell surface via sequential vesicular membrane transport through the ER, the Golgi apparatus and the *trans* Golgi network (TGN). In detail, proteins exit the ER by sequestration into coat protein II (COPII) coated vesicles, often having acquired a core *N*-linked glycosylation. These *N*-linked carbohydrate groups are then modified by glycosyltransferases, while traversing the Golgi and TGN. A couple of biochemically different recognition principles such as specific amino acid determinants, saturated fatty acid moieties and carbohydrates serve as hierarchical sorting signals marking proteins for transport. In addition, dynamic lipid domains play an important role in sorting certain membrane-associated proteins (Simons & Ikonen, 1997). Later, passage through the Golgi via the conventional exocytic pathway also requires the ADP-ribosylation factor GTPase-activating protein 1 and the COPI complex. On the other hand, certain integral membrane proteins have been shown to use different, less well characterized routes of secretion that seem to be independent of COPII-mediated ER budding and bypass the Golgi apparatus entirely (Chua et al, 2012; Schotman et al, 2008; Yoo et al, 2002).

Having budded off, vesicles are propelled along the filamentous structures of the cytoskeleton by ATP-dependent motor proteins until the transport vesicle and its target membrane fuse after mutual recognition (Figure 3).

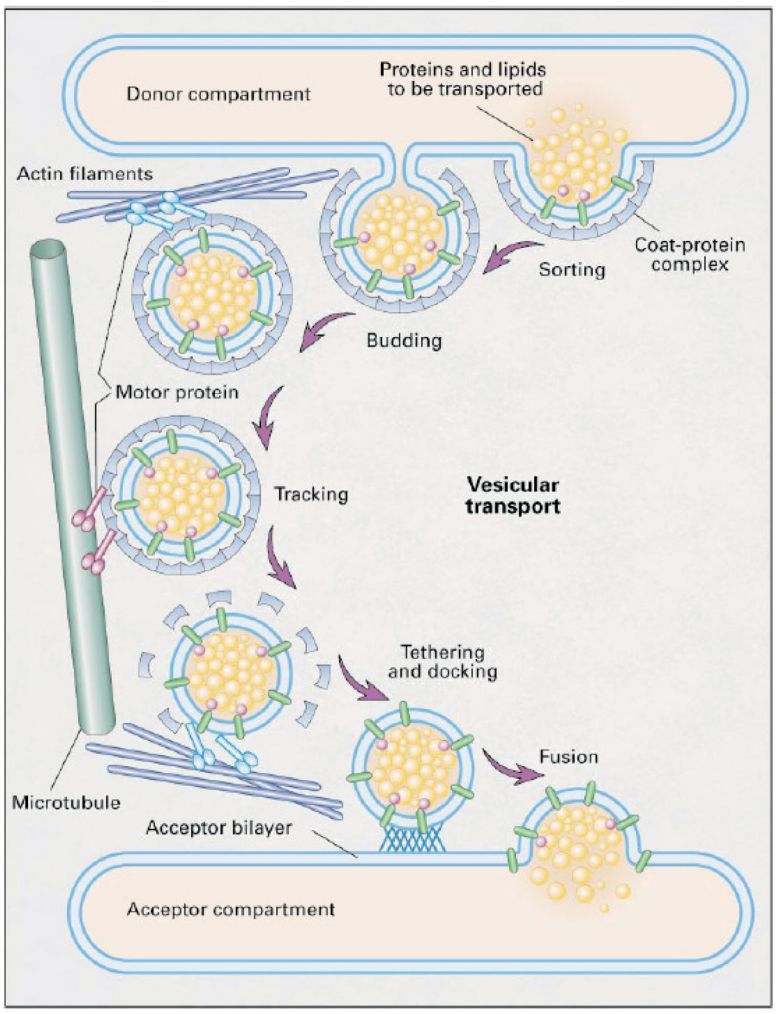

Figure 3: Vesicular transport Reproduced with permission from (Olkkonen & Ikonen, 2000), Copyright Massachusetts Medical Society; showing the vesicular transport of membrane proteins from a donor to an acceptor compartment.

The initial interaction between vesicles and their target membrane is known as tethering and so-called tethering factors have proven to be essential in linking the two membranes together and coordinating the correct assembly of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (Chia & Gleeson, 2014). Rab proteins are another set of key regulators in vesicle tethering and fusion. They are peripheral membrane proteins themselves, reversibly associated with the cytoplasmic face via hydrophobic geranylgeranyl groups and their function is tightly connected to the likewise membrane-anchored SNARE proteins. Rab proteins are GTPases alternating between two conformational states, GDP-bound “off” and GTP-bound “on”, and shift between cellular membranes and the cytoplasm with the help of the Rab GDP-

dissociation inhibitor (GDI) and the Rab escort protein (REP). In humans, more than 60 members are localized to distinct membranes. Their crosstalk to each other, coat components, motor proteins and SNAREs allows the multifaceted organization and spatiotemporal regulation of almost any membrane trafficking in eukaryotic cells (Olkkonen & Ikonen, 2000; Stenmark, 2009) (Figure 4).

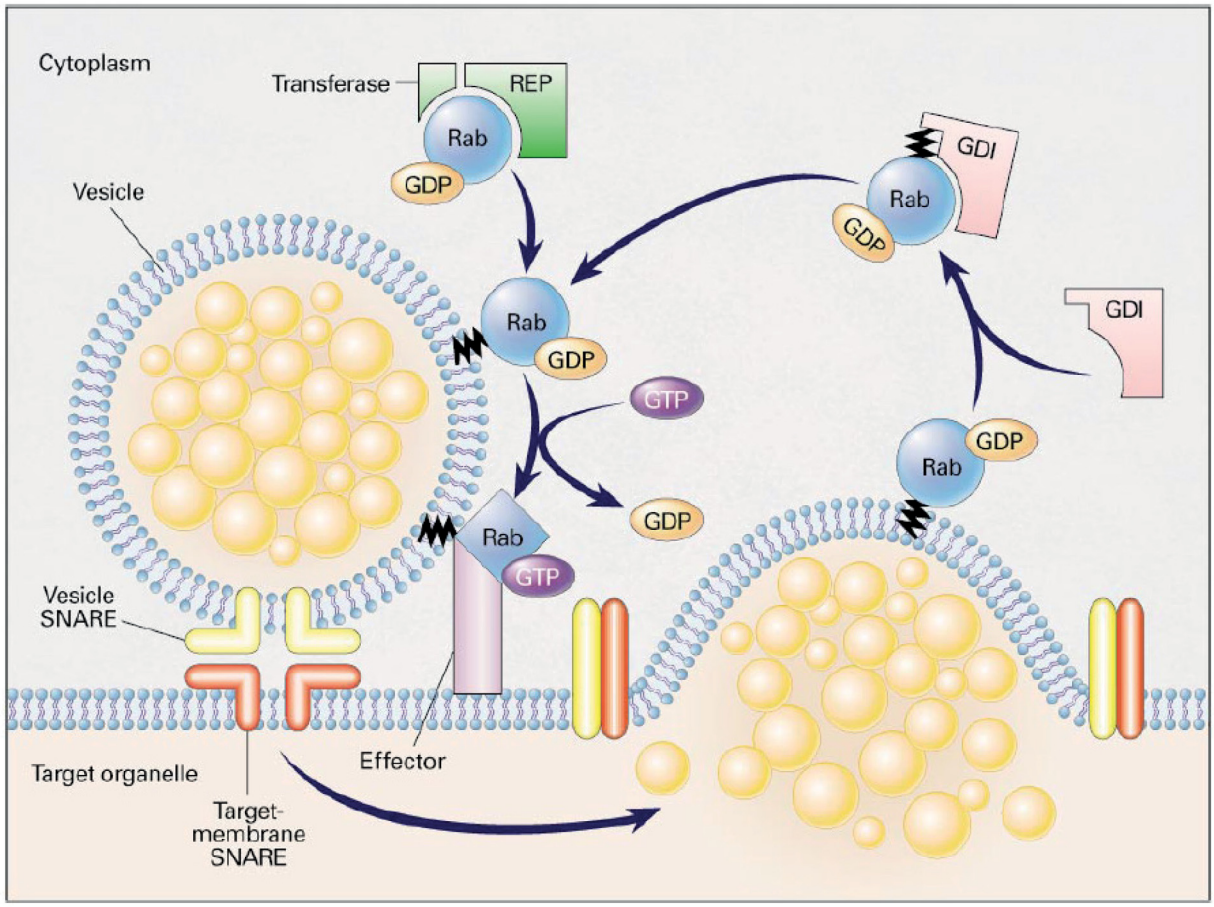

Figure 4: Membrane tethering

Reproduced with permission from (Olkkonen & Ikonen, 2000), Copyright Massachusetts Medical Society. Schema of the key regulatory processes and players involved in tethering and fusion of vesicles to their target membrane.

1.2.4 Functional roles of membrane proteins

Mining the human proteome using prediction tools for α-helices led to the allocation of most membrane proteins into three functional categories: receptors, transporters or enzymes (Almen et al, 2009) (Figure 5).

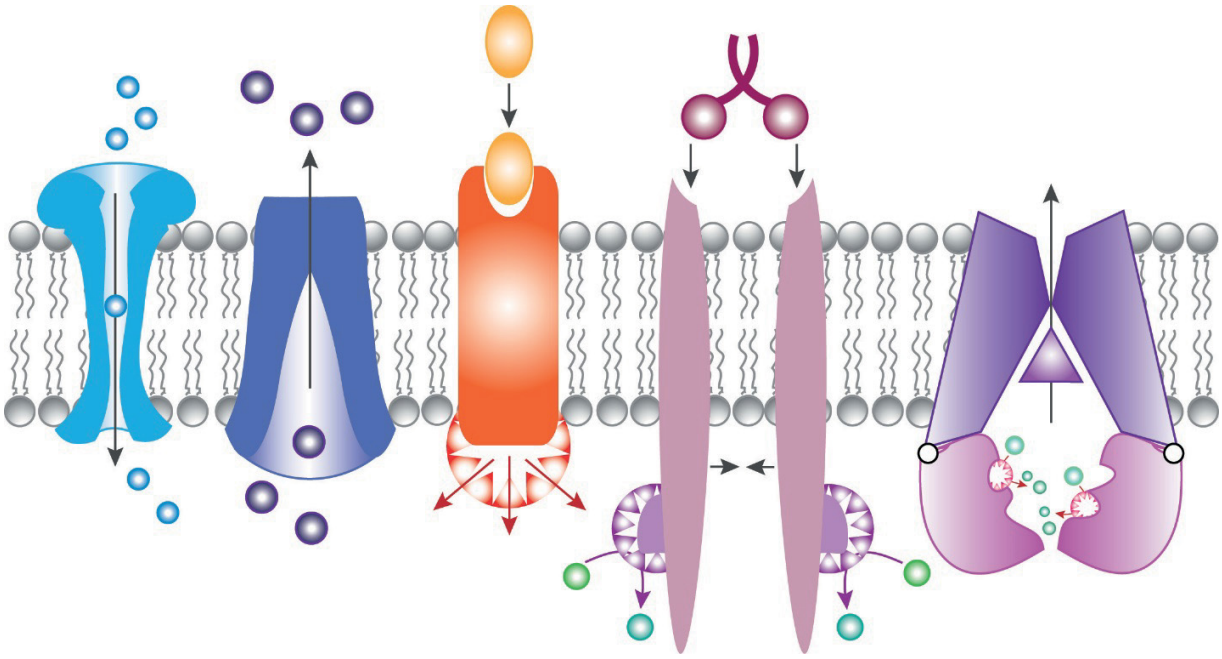

Figure 5: Functional roles of membrane proteins

Schematic representation of the three main categories of membrane proteins: transporters (blue), receptors (red) and enzymes (violet).

Features required by the specific subcellular localization contribute to determine the function of any membrane protein. Some proteins located at the PM, for example, sense a cell´s environment allowing it to respond to external conditions and stimuli. Equally important, other proteins at the cell membrane serve as structural anchors for cytoskeletal and extracellular matrix proteins. Likewise, proteins of the inner mitochondrial membrane are involved in energy production or form part of the apoptotic cascade (Tan et al, 2008). Due to their location at the interface of cells or organelles, membrane proteins have attracted massive therapeutic interest and currently account for more than two thirds of

all known drug targets (Josic et al, 2008; Rucevic et al, 2011; Yildirim et al, 2007). Furthermore, identifying differences in membrane proteins between healthy and non-healthy cells has led to the discovery of biomarker candidates, which prove to be a major source of information and prognosis in a variety of cancers (Grimm et al, 2011; Varady et al, 2013). Intriguingly, these differences can extend beyond the level of protein sequence. Notably, specific glycosylation patterns are recognized as biomarkers in tumors (Drake et al, 2010; Meany & Chan, 2011; Pinho & Reis, 2015; Reis et al, 2010) and certain diseases caused by parasites, for example malaria (Gilson et al, 2006). Differences can manifest on the expression level, too. For instance, specific ATP-binding cassette (ABC) transporters, responsible for the efflux of drugs from cells are overexpressed in various cancers contributing to chemotherapy resistance (Fletcher et al, 2010).
To convey an impression of the processes membrane proteins are implicated in, the following section describes selected examples and their roles and implications in diseases and therapeutics for each of the three main functional categories.

Receptors

Membrane receptors are involved in signaling allowing the cell to communicate with the outside world. Many are transmembrane proteins and vary in their membrane localization distribution. With over 800 members, GPCRs represent the largest family of membrane proteins in the human genome and a unique source for therapeutic approaches (Sheng Li, 2015). Despite their broad diversity in ligands and ligand binding domains, the same G protein can be activated by different receptors suggesting similar structural changes in the receptors upon activation. For rhodopsin and the β_2 adrenoreceptor, movements in distinct transmembrane domains were found to be such common activation patterns (Kobilka, 2007). Structural studies of the μ-opioid receptor that got compared to the active β_2 adrenoreceptor recently pinpointed packing rearrangements in three conserved amino acids to be conformationally linked to the ligand-binding pocket together with an extensive network bridging the pocket and the cytoplasmic domains as common signal propagation features (Huang et al, 2015).

Ligand-induced oligomerization has been thought to mediate signal transduction for some receptors, including kinase and cytokine receptors (Heldin, 1995; Maruyama, 2015; Schlessinger, 2002). Still, various representatives of these receptors exist in dimeric form prior to ligand binding, suggesting an alternative activation mechanism (Livnah et al, 1999; Moriki et al, 2001; Pang & Zhou, 2013). Emerging data indicates that receptor rearrangements might trigger activation in these cases, too (Dawson et al, 2005; Latz et al, 2007; Martin-Fernandez et al, 2002; Maruyama, 2015). Understanding these conformational changes in more detail might thus open up dramatically different drug design opportunities.

Membrane receptors can also serve as adhesion receptors binding components of the extracellular matrix and thereby provide traction for cell motility and invasion. The integrin family of cell adhesion receptors are obligate heterodimers. In vertebrates, 18 α and 8 β subunits get combined into 24 distinct integrins with specific ligand recognition patterns (Luo et al, 2007).

Integrins play a critical role in immunity serving as both, direct signaling molecules and indirect accessory molecules for the maintenance of cell contacts (Smith, 2008). Hence, mutations affecting their expression or function can cause profound disruptions in the immune system. Namely, several leukocyte adhesion deficiencies have been reported and drugs antagonizing integrins are approved for psoriasis and multiple sclerosis (Dunehoo et al, 2006).

Moreover, integrins control remodeling of the extracellular matrix and proliferation (Assoian & Klein, 2008). Their role in regulating several cell types that influence tumor progression has made integrins an appealing target for cancer therapy (Mizejewski, 1999; Seguin et al, 2015), however, the ways they affect tumor cell survival depend, amongst others, on their ligation state and are often contradictory (Desgrosellier & Cheresh, 2010). Additionally, their sphere of influence is not restricted to tumor cells, but extends to many cell types present in the tumor microenvironment (Avraamides et al, 2008). Crosstalk to growth factors and oncogenes in both, tumor cells and tumor-associated cells, represents yet another way of their critical implication in cancer (Desgrosellier & Cheresh, 2010). Phase II clinical trials with the integrin antagonist cilengitide had initially shown promising

results in glioblastoma patients, improving their progression-free and overall survival. Conversely, a subsequent study with over 500 patients did not confirm improved overall survival of newly diagnosed glioblastoma patients when cilengitide was added to the standard care regime (Merck, 2013).
Alternative approaches do not try to modulate integrins by agonists or antagonists, but rather aim to use integrin targeting to deliver other pharmaceutical compounds. For example, mutant RAF1 and doxorubicin were successfully delivered to the tumor vasculature by anti-integrin nanoparticles (Hood et al, 2002; Murphy et al, 2008).

Transporters

Membrane transport proteins tend to be multi-pass transmembrane proteins that either facilitate diffusion or mediate active transport. Correspondingly, they are referred to as channels or carriers, respectively. In their open state, channel proteins allow the rapid passage of specific molecules. Voltage-gated or ligand-gated ion channels, for instance, are crucial to the proper function of neurons and muscles transmitting excitatory or inhibitory synaptic and action potentials. Henceforth, they are targeted by drug development efforts for psychiatric disorders and diseases of the central nervous system such as epilepsy, Alzheimer´s disease and depression (Ryback, 2001).
Carrier proteins, in contrast, have binding sites through which they interact directly with their specific cargo. Upon binding, they undergo a series of conformational changes to transfer the bound molecule to the other side of the membrane. With a current count of 456 members in 52 subfamilies, solute carrier proteins (SLCs) are the largest group of membrane transporters and the second-largest family of membrane proteins in the human genome (Hediger et al, 2013; Hediger et al, 2004; Hoglund et al, 2011; Schlessinger et al, 2010; Schlessinger et al, 2013).
About 190 SLCs currently have an identified disease link. Yet, only a small fraction thereof also has an associated therapeutic compound (Cesar-Razquin et al, 2015; Williams et al, 2012).

At the same time, SLCs have a proven record in being both, drug targets themselves and mediators of drug disposition. Serotonin uptake inhibitors target one of the most studied SLCs, namely SLC6A4, while dipyridamole is specifically directed against SLC29A1 and part of a combination therapy for the secondary prevention of stroke (Leonardi-Bee et al, 2005).
The transport and preferential distribution of statins, in contrast, is mediated by SLCO1B1 and lately SLC35F2 was found to be the main determinant of sensitivity to the clinically evaluated anticancer compound YM155 (Winter et al, 2014).
Furthermore, SLC-mediated transport can influence pharmacokinetic characteristics by modulating drug-drug and nutrient-drug interactions. Specifically, naringin from citrus fruits was shown to inhibit the enterohepatic transporter SLCO1A2, potentially reducing the bioavailability of drugs depending on that transporter like the antihistamine agent fexofenadine (Bailey, 2010). Likewise, the clinical development of the JAK2 inhibitor fedratinib was stopped after reports of Wernicke's encephalopathy in myelofibrosis patients. The thiamine deficiency was traced back to the individual human thiamine transporter SLC19A2 being inhibited by fedratinib and highlighted the need to evaluate potential nutrient-drug interactions during drug development (Zhang et al, 2014).
A recent publication revealed SLCs to be the most neglected group of genes in the human genome (Cesar-Razquin et al, 2015). Provided that the vast majority of these carriers are potentially druggable and highly disease relevant makes it reasonable to expect SLCs to ultimately expand the group of therapeutically relevant membrane proteins.

Enzymes

Enzymatic membrane proteins are, in most cases, enzyme-linked receptors or transporters and therefore strongly intertwined with the two preceding functional categories.
Coupling to an enzymatic reaction can, for instance, be required if transport is executed against a chemical gradient. In the case of ABC transporters, ATP hydrolysis generates the driving force to pump their respective substrates across the membrane. Notably, in

eukaryotes, this process is almost exclusively unidirectional, *i.e.* ABC transporters serve as exporters only (Rees et al, 2009; Wilkens, 2015). Canonically, they consist of two nucleotide-binding domains (NBDs) and two transmembrane domains. Highly conserved motifs in the ATP-binding domains mediate ATP hydrolysis activity, while the lack of sequence conservation in the transmembrane domains reflects the wide spectrum of translocated substrates, ranging from small inorganic molecules to large organic compounds (Wilkens, 2015), with the multidrug transporter P-glycoprotein even displaying “poly-specificity” (Aller et al, 2009; Loo et al, 2003). Crystal structures of isolated NBDs bound to ATP and the fact that nucleotide-free NBDs crystallized as monomers or non-physiological dimers in most cases, established nucleotide-dependent dimerization of the NBDs to at least partly be driving subsequent conformational changes in the transmembrane domains required to complete translocation (Chen et al, 2003; Wilkens, 2015). Several models detailing ABC transporter mechanisms based on accepted key steps have been developed and it is well conceivable that most of them hold true, given the diverse nature of ABC transporters and the currently little evidence of them functioning by one sole mechanism (Wilkens, 2015).

Several genetically-encoded defects in ABC transporters are known and cystic fibrosis is probably the most prominent manifestation thereof (Cant et al, 2014; Wilkens, 2015). The role of ABC transporters and particularly ABCB1, ABCC1, and ABCG2 in chemotherapy resistance was mentioned before. Naturally, their inhibition by selective compounds is highly desirable. However, despite many inhibitors being identified (Ivnitski-Steele et al, 2008; Tarasova et al, 2005), none has found broad application in clinical cancer treatment so far (Sharom, 2008; Wilkens, 2015).

1.3 Proteomic analysis of membrane proteins

Although one third of the human genome is currently estimated to encode for membrane proteins (Ahram et al, 2006; Almen et al, 2009; Fagerberg et al, 2010), they are underrepresented in most proteomic studies (Rabilloud, 2009; Santoni et al, 2000). Challenges in their large-scale analysis include their heterogeneous, poorly soluble and low abundant nature. Factors contributing to heterogeneity are, for example, the number of transmembrane domains and the ratio of hydrophobic transmembrane domains to hydrophilic extracellular loops, respectively. Moreover, ratios of proteins to lipids, as well as lipid rafts are variable and together with the wide range and degree of post-translational modifications (PTMs) further increase the diversity of membrane proteins. In the past years, several approaches have been developed that take these characteristics into consideration. In particular, various strategies improving enrichment, solubilization and subsequent protein detection and identification by mass spectrometry (MS) have been suggested.

While shotgun proteomics, referring to the gel-free enzymatic digestion of proteins into more complex peptide mixtures prior to analysis by liquid chromatography coupled to mass spectrometry (LCMS), has emerged as the most common procedure on the MS side, enrichment and solubilization, but also digestion strategies remain more diverse. Furthermore, they can either be tailored to yield the membrane proteome, *i.e.* the entire complement of membrane proteins expressed in a cell at a given condition and time, or focus on the PM proteome.

1.3.1 Enrichment of membrane proteins

High pH conditions disrupt sealed membrane compartments, yet maintain their native topology favoring the formation of so-called “membrane sheets” with free edges. On the other hand, proteinase K cleaves exposed hydrophilic domains of membrane proteins (Chandramouli & Qian, 2009). Hence, the combination of high pH and proteinase K together with the multidimensional protein identification technology (MudPIT) was

described as an optimized global analysis strategy for membrane proteins (Wu et al, 2003).

Another way of obtaining crude membrane preparations is by exploiting their poor solubility performing repeated extractions of non-membrane proteins from a cell or tissue homogenate (Nagaraj et al, 2008; Nielsen et al, 2005). Alternatively, zonal centrifugation using different gradient media like Sucrose, Ficoll or Nycodenz has been applied (Cao et al, 2006; Stasyk & Huber, 2004) and delipidation of proteins from the membrane bilayer by chloroform extraction was presented as an entirely detergent-free method (Mirza et al, 2007) (Figure 6).

All of these enrichment strategies are, however, multistep protocols that eventually need to be combined to achieve sufficient power, ultimately entailing sample loss and reduced robustness. Two-phase partitioning systems and specifically Triton X-114 (TX114) phase separation have proven to be simple, yet highly efficient means for routine enrichment and purification of membrane proteins (English et al, 2012; Qoronfleh et al, 2003). The decisive unique feature of TX114 herein is that it not only solubilizes membrane proteins, but also separates them from hydrophilic proteins via phase partitioning at a physiological temperature (Bordier, 1981; Qoronfleh et al, 2003). Still, TX114 is a detergent that, even in small concentrations, impairs subsequent enzymatic protein digestion, chromatographic resolution and mass spectrometry analysis. At the same time, the strength of detergents in membrane solubilization is unmet, not least by reducing non-specific protein-protein interactions (PPIs) and by preventing protein loss due to surface adsorptions (Yeung & Stanley, 2010). For this reason, the development of filter-aided sample preparation (FASP), which allows the complete removal of detergents from solubilized samples represented a tremendous improvement digesting membrane proteins with an efficiency similar to that of soluble proteins (Manza et al, 2005; Wisniewski et al, 2009b).

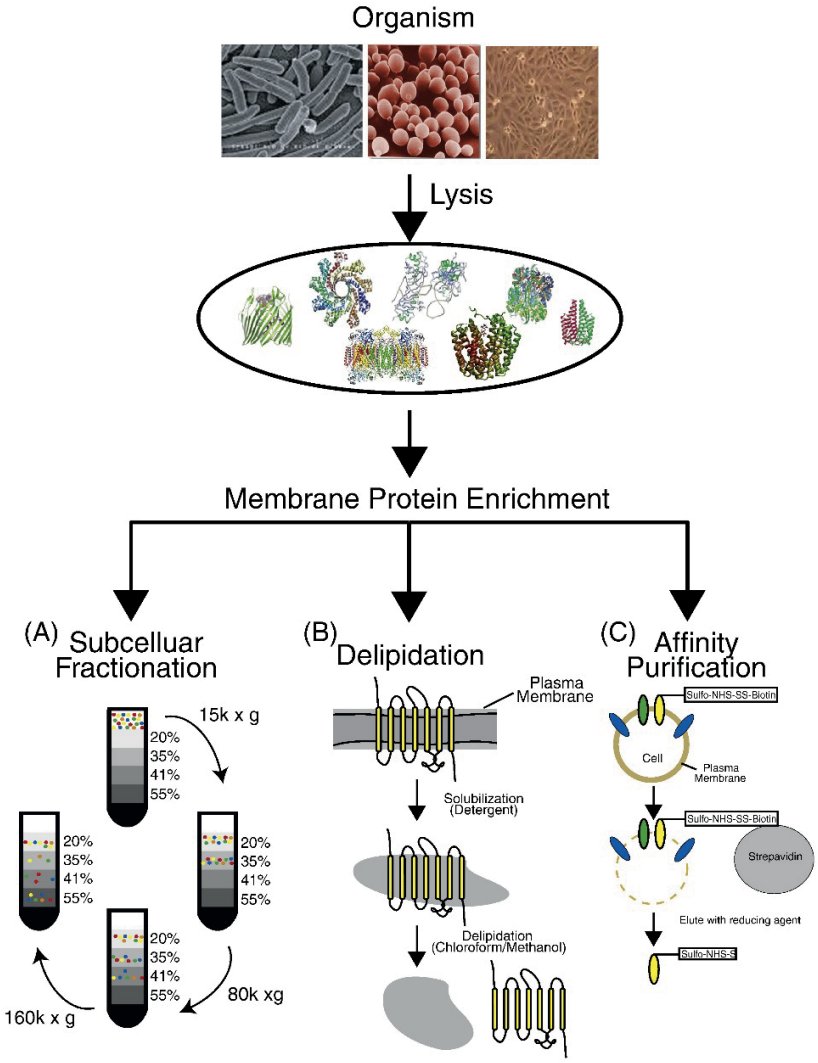

Figure 6: Membrane protein enrichment strategies

Reprinted from (Gilmore & Washburn, 2010), with permission from Elsevier depicting different approaches for membrane protein enrichment.

1.3.2 Enrichment of plasma membrane proteins

The fact that PM proteins represent a subset of membrane proteins can be exploited for their isolation by refining existing membrane purification strategies such as subcellular fractionation (Zhang et al, 2005) or two-phase partitioning systems (Schindler et al, 2006). Yet, these approaches require high sample loads and often suffer from weak enrichment

and contaminations from other membranous compartments (Elschenbroich et al, 2010; Schindler et al, 2006; Tan et al, 2008; Weekes et al, 2010).

At the same time, cell surface proteins have a number of distinct characteristics distinguishing them from other membrane proteins that enable more selective purification approaches. The first target in that regard are their extracellular domains, which provide means for affinity purification enrichment. In particular, the targeted moieties can either be primary amines or certain glycosylation patterns (Gilmore & Washburn, 2010; Lu et al, 2008).

Sulfo-biotinylation reagents are specifically designed to not penetrate the cell membrane allowing the selective modification of lysine residues and protein N-termini displayed on the extracellular side. Their biotin moiety is connected to an amine-reactive *N*-hydroxysulfosuccinimide (NHS) via a linker containing an internal disulfide bridge. These disulfide bonds can later be cleaved to elute proteins or peptides from the avidin/streptavidin affinity support (Nunomura et al, 2005; Zhao et al, 2004). Topological information such as the identification of domains exposed at the cell surface can be deduced from enrichments on the peptide level. Enrichment on the protein level has, however, resulted in broader sequence coverage and is therefore applied more frequently (Lu et al, 2008) (Figure 7).

One of the most common PTMs seen in extracellular domains of PM proteins are glycosylations. Corresponding affinity purifications are either lectin-mediated (Ghosh et al, 2004) or involve chemical derivatization of the carbohydrate side chain by hydrazone (Zhang et al, 2003) or oxime ligations (Zeng et al, 2009) (Figure 7).

Although exogenous labels confer specificity, respective moieties persisting throughout protocols are not reliably detected by MS (Schiapparelli et al, 2014; Weekes et al, 2010). This may in part reflect varying labeling efficiencies influenced by extracellular regions of PM proteins lacking the respective moieties or having them masked by PTMs. Moreover, labeling extends to cells that have lost their structural integrity, potentially contaminating PM purifications with cytoplasmic components (Lu et al, 2008).

Surface coating by colloidal silica beads relies on electrostatic interactions between the negatively charged phospholipid head groups of the PM and the cationic beads. After an initial coating step, beads are attached to the cell surface with a crosslinking agent. The

resulting increased PM density allows its subsequent isolation by centrifugation. While the technique has been applied for proteomic analyses of the PM in different cell types and tissues (Durr et al, 2004; Oh et al, 2004; Rahbar & Fenselau, 2004; Rahbar & Fenselau, 2005), the yields in terms of absolute numbers of PM proteins have rather been low (Arjunan et al, 2009; Choksawangkarn et al, 2012; Choksawangkarn et al, 2013) (Figure 7).

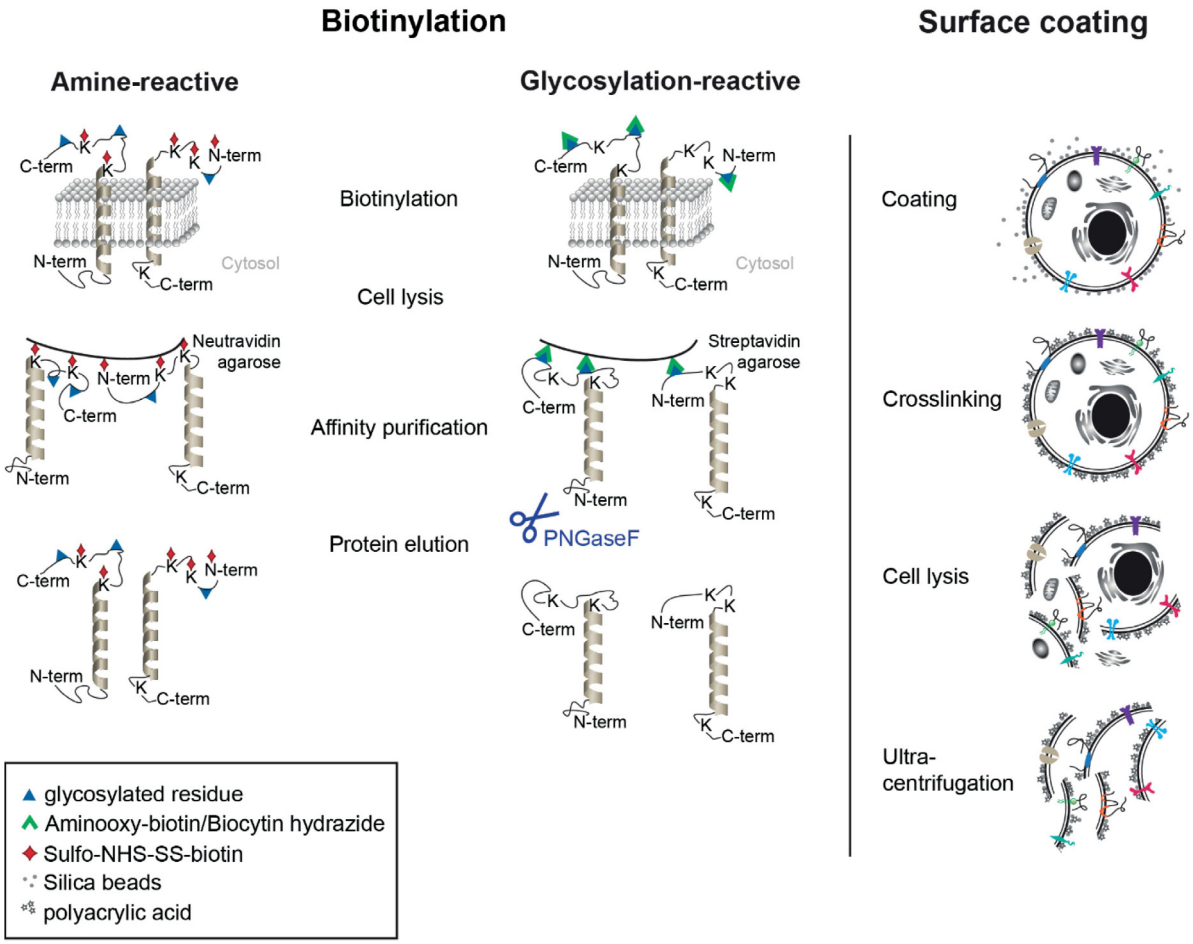

Figure 7: Cell surface protein enrichment strategies

Adapted with permission from (Hörmann et al, 2016). Copyright 2016 American Chemical Society. The figure illustrates enrichment strategies based on distinct plasma membrane protein features.

1.3.3 Digestion techniques for membrane proteomics

Tryptic digestion is considered the gold standard in shotgun proteomics (Zhang et al, 2013) displaying high proteolytic activity and cleavage specificity. Moreover, tryptic peptides typically have size and charge parameters favoring their detection in MS runs (Hanne Kolsrud Hustoft, 2012). The characteristic hydrophobic patches of membrane proteins are, however, poor in the basic tryptic cleavage sites arginine and lysine, resulting in larger peptide fragments that have a lower detectability by MS (Baldwin, 2004). Therefore, a couple of less specific proteases, as well as combinations of several digestive enzymes have been tested. While proteinase K has been successfully applied to a mammalian membrane proteome using tightly controlled reaction parameters (Wu et al, 2003), analyses using other proteases with decreased specificity such as elastase or pepsin have so far only been demonstrated to work in bacterial samples (Rietschel et al, 2009a; Rietschel et al, 2009b). A major complication therein are the large number of peptides generated from non-specific proteases that are hard to predict with random locations of positive charges directing fragmentation during MS analysis (Gilmore & Washburn, 2010). Multiple ways of manipulating charge-directed fragmentation have been published, including nicotinylation and N-terminal addition of basic groups to peptides (Jansson et al, 2008; Munchbach et al, 2000). Yet, none of them found further application in more recent membrane proteomics protocols.

As far as the combination of several digestive enzymes is concerned, isolated bacterial membrane proteins have been subjected to a trypsin/cyanogen bromide or trypsin/chymotrypsin mixture, respectively, with the trypsin/chymotrypsin digestion achieving distinctly higher sequence coverage of bacterial transmembrane regions (Fischer et al, 2006). Still, parallel enzymatic digestions with several enzymes displaying distinct cleavage sites lead to an exponential increase in the potentially resulting peptides, complicating confident peptide assignments and ultimately protein identifications.

Conversely, pushing tryptic cleavage specificity at lysine sites by adding Lys-C to the digestive solution, decreases both, the number of missed cleavage sites as well as the required search space, boosting confidence in the peptide assignments and proteins identified (Wisniewski et al, 2009a).

1.3.4 Label-free quantification in membrane proteomics

Quantitative proteomic workflows applied to the large-scale identification of membrane proteins can be categorized into labeled and label-free approaches (Figure 8). The first group introduces isobaric or isotopic mass tags to proteins or peptides. The resulting mass differences allow to decipher intensity signals and to deduce quantitative information from MS spectra. By design, this category provides relative quantitation, even though adding a synthetic, labeled peptide at known concentration can make the method readily accessible for absolute quantification (Vaudel et al, 2010).

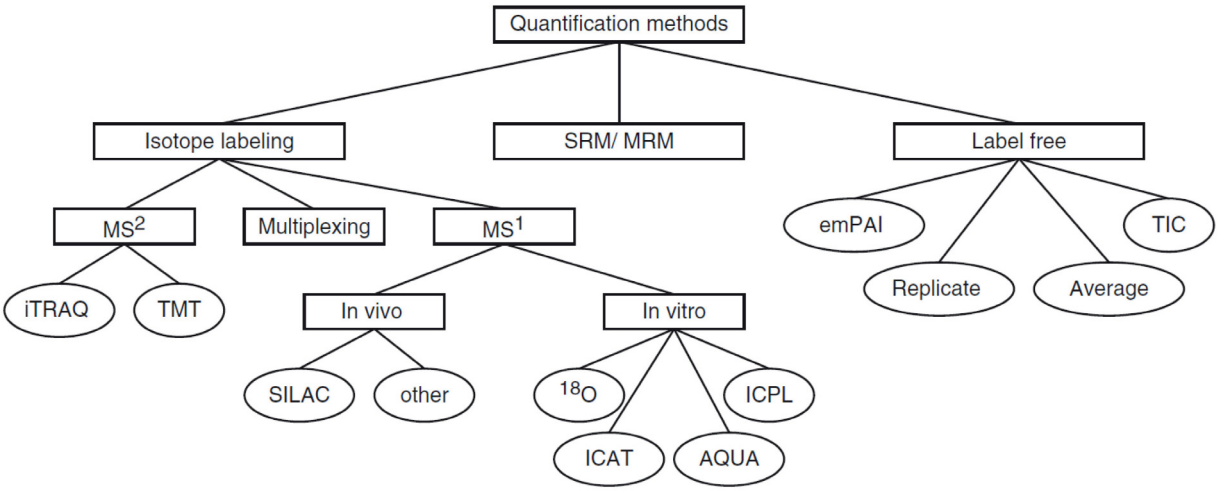

Figure 8: Quantification strategies in proteomic workflows

Reused with permission from (Vaudel et al, 2010). Copyright 2010 WILEY‐VCH Verlag GmbH & Co. KGaA, Weinheim depicting commonly used quantification methods sorted by the presence or absence of a label, respectively.

Label-free quantification, on the other hand, does not use any labels circumventing additional labor-intensive and expensive sample processing steps (Bluemlein & Ralser, 2011). Instead, label-free approaches aim to find indicators of protein abundance directly in the MS output. Henceforth, they are technically simple, cover a higher dynamic range and allow quantitative comparisons between an unlimited number of samples including previously acquired data. Yet, it also means that the quality of the quantifications heavily

depends on the accuracy and precision of the data with complex protein mixtures requiring high-resolution mass spectrometers and corresponding data processing tools. Label-free approaches seem to result in slightly higher coefficients of variation than their labeled counterparts. At the same time, however, they have shown higher reproducibility between replicate samples and enabled up to 60 % more protein quantification (Liu et al, 2013).

There are different metrics used for label-free quantification based on either spectral counting or peak intensities. The former approach assumes a linear correlation between a protein´s sampling in MS and its relative abundance, which indeed has been demonstrated over two orders of magnitude (Liu et al, 2004). Spectral counting depends on peptide identifications limiting its accuracy, while reproducibility and quantitative proteome coverage have been graded high (Bantscheff et al, 2007; Mosley et al, 2011). The spectral counting metric has been adapted in order to account for protein length by the so-called protein abundance index (PAI) (Rappsilber et al, 2002), later revised to the exponentially modified protein abundance index (emPAI) (Ishihama et al, 2005) or peptides shared between different proteins by calculation of distributed normalized spectral abundance factors (dNSAFs) (Zhang et al, 2010).

Label-free quantification based on peak intensities uses a larger amount of the information available from high precision mass spectra. The extracted ion chromatograms metric, for example, defines a peptide concentration as the total intensity of the corresponding precursor on the MS^1 level (Wang et al, 2003). Accordingly, quantitative comparability strongly depends on reproducible peptide separation and the performance of complex, so-called feature finder algorithms that detect precursors in MS^1 spectra. Altogether, correct data processing of the acquired MS data is still a major limiting factor for label-free quantification approaches based on peak intensities (Vaudel et al, 2010).

1.4 Aims of this thesis

The purpose of this thesis was to identify and establish two robust protocols empowering the routine evaluation of both, whole membrane proteomes and PM proteomes. To this end, several isolation approaches were assessed and systematically compared for their enrichment and reproducibility parameters.

Moreover, the research performed within the framework of this thesis was directed at improving existing protocols with particular attention to FASP-free sample preparation in light of the conflicting results concerning peptide recovery and sequence coverage in quantitative, filter-based experiments (Bereman et al, 2011; Erde et al, 2014; Glatter et al, 2015; Leon et al, 2013; Nel et al, 2015).

Further aims of this thesis were to improve the characterization of non-PM annotated proteins commonly co-purified with PM extraction protocols and to benchmark the two membrane proteomic workflows using different cell lines and perturbations.

2. Results

2.1 A Surface Biotinylation Strategy for Reproducible Plasma Membrane Protein Purification and Tracking of Genetic and Drug-Induced Alterations

Katrin Hörmann, Alexey Stukalov, André C. Müller, Leonhard X. Heinz, Giulio Superti-Furga, Jacques Colinge, and Keiryn L. Bennett

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In this article, the three most popular strategies, namely sulfo-NHS-SS-biotinylation, aminooxy-biotinylation and surface coating with silica beads to isolate PM proteins for subsequent analysis by one-dimensional gel-free LCMS were compared. Assessing absolute and relative numbers of PM proteins and reproducibility parameters on a qualitative and quantitative level indicated sulfo-NHS-SS-biotinylation as superior for most of the monitored criteria.

The procedure was further simplified by introducing a competitive biotin elution strategy that circumvents FASP, yet yields an average PM-annotated protein fraction of 54 % (347 proteins). Moreover, the non-PM annotated data were found to be extremely enriched for interactors of purified PM proteins and computational analysis using additional databases and prediction tools jointly suggested over 90 % of the purified proteins to be associated with the PM.

As a validation, changes in the PM proteome composition induced by genetic alteration and drug treatment were tracked: GPI-anchored proteins were depleted in PM purifications from cells deficient in the GPI transamidase component *PIGS;* and treatment

of cells with the *N*-glycosylation inhibitor tunicamycin significantly reduced the abundance of *N*-glycoproteins in surface purifications. These results demonstrate that the improved, filter-free sulfo-NHS-SS-biotinylation protocol is a specific, effective and reproducible method to isolate proteins associated with the PM.
Altogether, this study combined comparative analyses and technological advancement with proteomic-driven data analysis paving the way for high-performance differential PM proteomics to become a more widely and routinely used tool in the characterization and comparison of different cell types, tissues or disease states.

The author of this thesis contributed to the design and performed all experiments, analyzed the data and wrote the manuscript. Alexey Stukalov helped with statistical analyses. André C. Müller operated the mass spectrometers and was responsible for running the samples. Leonhard X. Heinz gave experimental advice and feedback to the manuscript. Giulio Superti-Furga, Jacques Colinge and Keiryn L. Bennett jointly determined the experimental design and analysis strategy and provided feedback to the manuscript.

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A Surface Biotinylation Strategy for Reproducible Plasma Membrane Protein Purification and Tracking of Genetic and Drug-Induced Alterations

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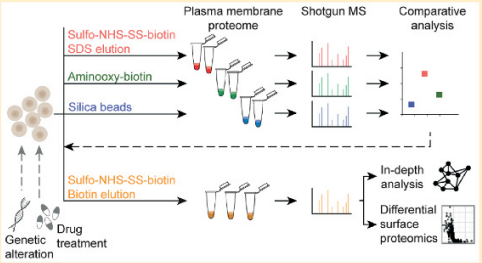
Supporting Information

ABSTRACT: Plasma membrane (PM) proteins contribute to the identity of a cell, mediate contact and communication, and account for more than two-thirds of known drug targets.[1−8] In the past years, several protocols for the proteomic profiling of PM proteins have been described. Nevertheless, comparative analyses have mainly focused on different variations of one approach.[9−11] We compared sulfo-NHS-SS-biotinylation, aminooxy-biotinylation, and surface coating with silica beads to isolate PM proteins for subsequent analysis by one-dimensional gel-free liquid chromatography mass spectrometry. Absolute and relative numbers of PM proteins and reproducibility parameters on a qualitative and quantitative level were assessed. Sulfo-NHS-SS-biotinylation outperformed aminooxy-biotinylation and surface coating using silica beads for most of the monitored criteria. We further simplified this procedure by a competitive biotin elution strategy achieving an average PM annotated protein fraction of 54% (347 proteins). Computational analysis using additional databases and prediction tools revealed that in total over 90% of the purified proteins were associated with the PM, mostly as interactors. The modified sulfo-NHS-SS-biotinylation protocol was validated by tracking changes in the plasma membrane proteome composition induced by genetic alteration and drug treatment. Glycosyl-phosphatidylinositol (GPI)-anchored proteins were depleted in PM purifications from cells deficient in the GPI transamidase component *PIGS*, and treatment of cells with tunicamycin significantly reduced the abundance of *N*-glycoproteins in surface purifications.



KEYWORDS: *plasma membrane, biotin, silica beads, cell surface, aminooxy-biotin, comparative analysis, shotgun proteomics, tunicamycin, PIGS*

INTRODUCTION

The plasma membrane (PM) of human cells not only safeguards the cellular content by delimiting the boundaries to the environment, but also is richly populated with proteins that coordinate such functions as nutrient uptake, cellular growth, motility, and physical interactions with other cells and the extracellular matrix. Still, these pharmacologically important proteins are underrepresented in standard liquid chromatography−mass spectrometry (LC−MS) analyses.[3] Reasons for such poor representation include low membrane-to-cytosol protein ratios, overall low cellular abundance, and highly hydrophobic character.[12−14] These challenges recently have been addressed by techniques that both enrich the PM fraction and improve the level of peptide detection during analysis by LC−MS.[3,9,15−17] In principle, PM proteins are either selectively isolated via chemical labels or extracted from cells as part of the entire PM fraction based on physicochemical properties. There have been conflicting reports, however, with respect to which of these techniques results in the highest yield and purity of PM proteins and what type of non-plasma membrane annotated proteins are predominantly co-purified.[9,18] Although targeting of either primary amines or preoxidized sialic acid residues on both *N*- and *O*-linked glycoproteins confers specificity, respective moieties that persist throughout the protocol are poorly detected by MS.[9,19] Beyond that, purifications based on exogenous labels rely heavily on labeling efficiency. Biases may be introduced by excluding proteins that do not carry the targeted residue or do not expose the residue to a degree that enables derivatization. Surface coating by silica beads, which solely relies on electrostatic interactions between the catioinic beads and the negatively charged cell surface, has proven to be a very specific approach.[20] Nevertheless, the return in terms of absolute numbers of PM proteins has been rather

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small.[10,11,21,22] Apart from isolation, the detection of PM proteins by MS is complicated by their highly hydrophobic character requiring the use of detergents for proper solubilization. The development of filter-aided sample preparation (FASP)[17] has greatly facilitated the removal of detergents from protein lysates to meet LC−MS requirements. Results concerning peptide recovery and sequence coverage in quantitative, filter-based experiments remain controversial.[23−27] PM proteins often display hydrophobic patches and are thus particularly compounded by such potential losses. To this end, we substituted the detergent-containing elution step in the sulfo-NHS-SS-biotinylation strategy with a detergent-free, competitive elution using D-biotin. This alteration resulted in high relative and absolute amounts of purified surface proteins, refined reproducibility parameters, and proved to account for genetically- or drug-induced changes. Surface purifications from cells deficient in the glycosylphosphatidylinositol (GPI) transamidase component *PIGS* were depleted of all detectable GPI-anchored proteins, and the abundance of *N*-glycosylated proteins in PM fractions from cells treated with the *N*-glycosylation inhibitor tunicamycin was significantly decreased.

■ EXPERIMENTAL PROCEDURES

Cell Culture

KBM7 and HAP1 cells were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin−streptomycin.

Purification and Enrichment of Plasma Membrane Proteins

Method 1A: Cell Surface Protein Isolation via Sulfo-NHS-SS-biotin and Elution with Sodium Dodecyl Sulfate. Cell surface proteins were isolated according to the instructions provided by the manufacturer using the reagents supplied with the "Pierce® Cell Surface Protein Isolation Kit" (Thermo Scientific, Rockford, IL). Cells (40×10^6) were washed twice with ice-cold phosphate-buffered saline (PBS) and reconstituted in 4 mL of biotin solution (10×10^6 per mL). The mixture was agitated for 30 min at 4 °C, the labeling reaction halted with 200 μL of quenching solution, and the cell pellets washed twice with tris-buffered saline (TBS). The cells were resuspended in 500 μL of lysis buffer and lysed by sonication on ice with 5 × 1 s pulses. Sonication was repeated twice over a period of 30 min with vortexing of the lysate for 5 s every 5 min. The resultant cell lysate was centrifuged at 10 000 × *g* for 2 min at 4 °C and the clarified supernatant used for the subsequent affinity purification. NeutrAvidin agarose slurry (500 μL) was added to a SnapCap spin column (Thermo Scientific, Rockford, IL), washed three times with wash buffer, and incubated with the clarified cell lysate for 60 min at room temperature (RT) with end-overend mixing. After centrifugation at 1000 × *g* for 1 min, the flow-through was discarded, and the beads washed three times with wash buffer. Proteins were eluted with 400 μL of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 50 mM dithiothreitol (DTT) to cleave the disulfide bridge in the biotin label. After incubation for 60 min, the proteins were collected via centrifugation at 1000 × *g* for 2 min and then further prepared for LC−MS analysis via FASP as originally described[17] using filtration units with nominal molecular weight cutoffs of 30 000 Da.[28] For the predigestion with PNGaseF (New England Biolabs, Frankfurt am Main, Germany), cells were suspended in 1 × G7 buffer containing 5000 U PNGaseF and incubated at 37 °C overnight.

Method 1B: Cell Surface Protein Isolation via Sulfo-NHS-SS-biotin and Elution with Biotin. Samples were prepared as described in the preceding section; however, the SDS-PAGE sample buffer used to elute the proteins was replaced with a solution of 2.5 mM D-biotin dissolved in 50 mM HEPES pH 8.0, 150 mM NaCl, 50 mM DTT, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitors (1:1000). After incubation and collection via centrifugation, the protein concentration was determined. An aliquot of ∼20−30 μg was alkylated by adding iodacetamide to a final concentration of ∼55 mM and incubation for 30 min in the dark. Samples were adjusted to a pH of 7.5−8.5 by adding 1 M triethylammonium bicarbonate (TEAB) and digested overnight at 37 °C using a trypsin/protein ratio of ∼1:30 (w/w). The samples were then acidified with 30% TFA and subsequently concentrated and purified by solid phase extraction (SPE) using MicroSpin columns (5−60 μg, The Nest Group, Southborough, MA). All centrifugation steps for the SPE were performed at 800−1000 rpm for 1 min. If these parameters were not sufficient to force the liquid through the column material, centrifugation time or speed was slightly increased. SPE columns were activated with 200 μL of methanol and equilibrated twice with 100 μL of stage tip buffer (STB: 0.5% formic acid (FA), 2% TFA). After the digested samples were loaded, the columns were washed with 100 μL of STB buffer. Peptides were eluted with 50 μL of elution buffer (0.4% FA + 90% ACN) and then concentrated in a vacuum centrifuge at 45 °C. For the LC−MS analyses, peptides were reconstituted in 5% FA.

Method 2: Aminooxy-biotin Labeling of Plasma Membrane Proteins. Cell surface sialylated glycoproteins were isolated essentially as described[16,29] with minor modifications. Cells (120×10^6) were washed twice with ice-cold PBS and resuspended in 1 mM sodium meta-periodate, 10 mM aniline, and 250 μM aminooxy-biotin (Thermo Scientific, Rockford, IL). After incubation at 4 °C for 30 min in the dark, the reaction was quenched by glycerine to a final concentration of 1 mM. The cell pellets were washed once each with PBS containing 5% FCS and PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The cells were lysed in 10 mM Tris-HCl pH 7.6, 1% Triton X-100, 150 mM NaCl, protease inhibitors (1 μL/mL), 5 mM IAA, and 0.1 mg/mL PMSF at 4 °C for 30 min. Cell debris and nuclei were removed by centrifugation at 4 °C once at 2800 × *g* and twice at 16 000 × *g*. To isolate labeled glycoproteins, 144 μL of streptavidin agarose beads (life technologies, Eugene, OR) were added to Snap Cap spin columns and incubated with the cell lysate for 2 h at 4 °C. To eliminate nonspecifically bound proteins, multiple washing steps were performed (20 × 600 μL followed by centrifugation at 1000 × *g* for 1 min). The washing was initiated with lysis buffer, followed by PBS supplemented with 0.5% SDS and 6 M urea in 100 mM Tris-HCl pH 8.5. Further washes included higher salt solutions (5 M NaCl and 100 mM Na_2CO_3) before completion with PBS and water. The proteins were digested on the beads overnight using 2.5 μg of trypsin in 50 mM NH_4HCO_3. The tryptic peptides were collected via centrifugation at 1000 × *g* for 1 min. The beads were rinsed with 200 μL of 50 mM NH_4HCO_3 and tryptic fractions pooled. Peptides were washed three times each with 300 μL of PBS, water, and G7 buffer (New England Biolabs, Frankfurt am Main, Germany). After incubation at RT for 5 h, glycopeptides were eluted with 15 000 units glycerol-free PNGaseF in 200 μL of G7 buffer. After the first collection of glycopeptides via

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J. Proteome Res. XXXX, XXX, XXX−XXX

centrifugation at 1000 × *g* for 1 min, the beads were rinsed with 200 μL of G7 buffer and the glycopeptide fractions combined. For the LC−MS analyses, aliquots of tryptic and glycopeptide fractions were pooled.

Method 3A: Colloidal Silica Beads. Samples were prepared as previously described.[15] Cells (30×10^6 or 50×10^6) were washed three times with ice-cold MES-buffered saline (MBS: 25 mM MES pH 6.5, 150 mM NaCl) and then added to a 10% silica-bead solution. The mixture was incubated on ice and rocked gently for 10 min to allow the silica beads to attach to the cells. The cells were pelleted at 1000 × *g* for 5 min at 4 °C and washed three times with ice-cold MBS. To cross-link the cells to the silica beads, 0.1% polyacrylic acid (PAA) in MBS was prepared and cells added dropwise. The samples were incubated on ice for 10 min and then centrifuged at 1000 × *g* for 5 min at 4 °C. The resultant cell pellets were washed three times with MBS and lysed in 1 mL of 250 mM sucrose, 25 mM HEPES, 20 mM KCl, 1 μL/mL protease inhibitor cocktail. Cells were resuspended and sonicated on ice until the solution became cloudy. For the density gradient centrifugation, a discontinuous Nycodenz (Axis-Shield PoC, Oslo, Norway) gradient consisting of 1.5 mL layers of 40, 35, 30 and 27.5% Nycodenz solutions was prepared. The cell lysate was diluted to a final concentration of 25% Nycodenz in the sample layer and then placed on top of the prepared gradient. Lysis buffer (1 mL) was added with care to the gradient, and ultracentrifugation was performed at 100 000 × *g* for 1 h at 4 °C. The supernatant was discarded, and the plasma membrane pellet resuspended in 500 μL of 25 mM Na_2CO_3. After incubation with agitation for 30 min at 4 °C, the pellet was collected at 5000 × *g* for 20 min at 4 °C and the supernatant removed. Proteins were eluted from the silica beads and solubilized with 200 μL of 8 M urea buffer containing 2 mM DTT by incubation with agitation for 30 min at 37 °C. After centrifugation at 5000 × *g* for 20 min at 4 °C, the supernatant was collected and the protein concentration determined. Proteins were alkylated with 8 mM IAA for 30 min at 37 °C and the urea concentration diluted to ~1.5 M with 100 mM NH_4HCO_3. $CaCl_2$ was added to a final concentration of 2 mM before initiating the digestion by adding trypsin at an enzyme-to-protein ratio of 1:50 (w/w). The digest was incubated overnight at 37 °C and quenched the following day with 2.5% TFA. For all subsequent desalting steps of the peptides, centrifugation was performed at 200 × *g*. MacroSpin columns (The Nest Group, Southborough, MA) were conditioned with 500 μL of ACN and washed twice with ultrapure water. Samples were loaded onto the column, washed twice with 0.1% TFA, and eluted with 70% ACN, 0.1% TFA in two steps to yield an eluate of 400 μL. The peptides were concentrated in a vacuum concentrator at 45 °C and then resuspended in 5% FA.

Method 3B: Alternative Colloidal Silica Beads Protocol. Since the original publication,[15] Kislinger et al. had further optimized sections of the protocol (personal communication). First, the discontinuous gradient for the ultracentrifugation step ranged from 55−70% Nycodenz and centrifugation parameters were increased to 130 000 × *g* for 90 min. Second, to improve the removal of nonspecific proteins, two 150−200 μL of Na_2CO_3 wash steps were performed. Additionally, elution of the proteins from the beads was extended to a two-step process. In the first step, the bead-cell pellet was resuspended in 150 mM NaCl and rotated at 4 °C overnight. After centrifugation at 5000 × *g* for 20 min at 4 °C, the remaining pellet was subjected to the second step of elution with 200 μL of 25 mM HEPES, 1% Triton X-100, and a higher salt concentration of 400 mM NaCl. This second step was incubated with agitation at 4 °C for a minimum of 1 h and centrifuged as for the first elution. The resultant supernatants were combined and the proteins precipitated with acetone overnight at −20 °C. The protein pellet was collected by centrifugation and the acetone precipitation repeated twice. The final pellet was dried at 37 °C for 30 min, resuspended in 100 μL of 8 M urea, 100 mM Tris pH 8.5, 2 mM DTT, and agitated for 30 min at 37 °C. The protein concentration was determined and the sample prepared for LC−MS as described in the preceding section.

Reversed-Phase Liquid Chromatography−Mass Spectrometry

Peptide mixtures were separated by LC and analyzed by collision-induced dissociation (CID) on a hybrid linear trap quadrupole (LTQ) Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to an Agilent 1200 HPLC nanoflow system (Agilent Biotechnologies, Palo Alto, CA) via a nanoelectrospray ion source using liquid junction (Proxeon, Odense, Denmark). Two solvents were used for the separation of digested samples: solvent A consisted of 0.4% FA in water and solvent B of 0.4% FA in 70% methanol and 20% isopropanol. Samples were stored in a thermostatic microautosampler before being automatically loaded onto a trap column (Zorbax 300SB-C18 5 μm, 5 × 0.3 mm, Agilent Biotechnologies) by a binary pump at a flow rate of 45 μL/min. For loading and washing, 0.1% TFA was used. After washing, peptides were back-flushed onto a 16 cm fused silica analytical column with an inner diameter of 50 μm packed with C18 reversed phase material (ReproSil-Pur 120 C18-AQ, 3 μm, Dr. Maisch, Ammerbuch-Entringen, Germany). Peptides were eluted at a constant flow rate of 100 nL/min by three subsequent gradients: (1) 3 to 30% solvent B in 27 min, (2) 30 to 70% solvent B in 25 min, and (3) 70 to 100% solvent B in 7 min.

Peptides were identified in a data-dependent acquisition mode using a top 15 CID method. A single lock mass at m/z 445.120024 was chosen,[30] and selected ions were dynamically excluded for 60 s. Maximal ion accumulation times were 500 ms for MS^1 and 50 ms for MS^2 mode, respectively. To prevent overfilling of the ion traps, automatic gain control (AGC) was set to 10^6 ions for MS^1 mode and 5000 for MS^2 mode. The threshold for switching from MS^1 to MS^2 was 2000 counts. Peptides were detected with a resolution of 60 000 (m/z 400). All samples were analyzed as technical, back-to-back replicates.

Data Processing and Database Searching

The acquired raw MS data files were processed with msconvert (ProteoWizard Library v2.1.2708) and searched against the human Swiss-Prot database (v2013.01, 37 398 sequences) with the search engines Mascot (v2.3.02, MatrixScience, London, U.K.) and Phenyx (v2.5.14, GeneBio, Geneva, Switzerland).[31] A maximum of one missed tryptic cleavage site was allowed for the aminooxy-biotin and the silica beads approach, while a maximum of two missed tryptic cleavage sites was enabled for the sulfo-NHS-SS-biotin method. Initial searches were performed with relatively broad mass tolerances via a Perl script on both precursor and fragment ions (±10 ppm and ±0.6 Da, Mascot only). All precursor and fragment ion masses were recalibrated based on high-confidence peptide identifications and subsequently subjected to a second search with narrower mass tolerances (±4 ppm, ± 0.3 Da). Carbamidomethylated cysteine was defined as a fixed modification, while oxidation of

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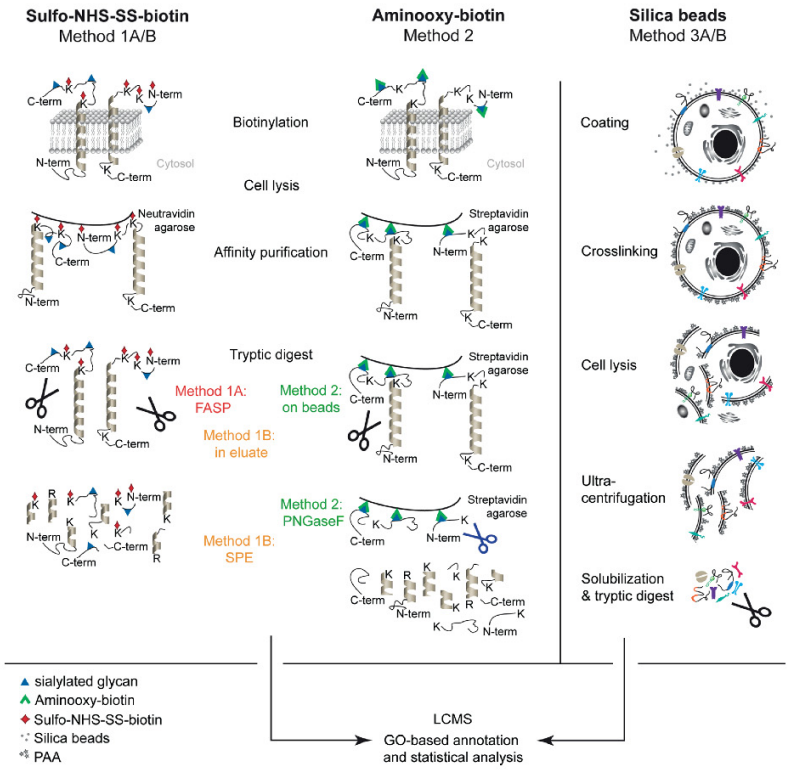

Figure 1. Experimental outline. Biotinylation strategies started by labeling either primary amines (Method 1A/1B) or oxidized sialylated glycoproteins (Method 2) on intact cells. After cell lysis, biotin modifications were used to separate PM proteins from other cellular content. Bound proteins were eluted and digested by FASP (Method 1A), digested in solution (Method 1B), or directly digested on the beads (Method 2). For the biotin elution approach, resultant peptides were concentrated via SPE before LC−MS analysis. For the aminooxy-biotin approach, glycopeptides were eluted from the beads by PNGaseF and pooled with the tryptic fraction. For the silica beads protocol (Method 3A/3B), intact cells were coated with the beads and attached to the cell surface by cross-linking prior to cell lysis and isolation of the PM fraction via ultracentrifugation. PM proteins were solubilized and tryptically digested prior to analysis by LC−MS.

methionine residues was selected as a variable modification. For the sulfo-NHS-SS-biotin experiments, the biotin moiety conjugated to lysine residues and protein N-termini was added as a variable modification. Release of *N*-linked glycopeptides by PNGaseF treatment deamidates asparagine residues to aspartic acid. This was set as additional variable modification as required. False positive detection rates (FDRs) of <1% and <0.1% were determined for proteins and peptides, respectively, by applying the same procedure against a reversed database. UniProtKB/Swiss-Prot Gene Ontology cellular component (GO.CC) annotations of the identified proteins were retrieved via BioMart.[32] Data are means of two biological replicates from different experimental days unless otherwise stated. Abundance data of multiple isoforms were averaged. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium[33] via the PRIDE partner repository with the data set identifier PXD002141.

RESULTS

Three alternative methods, namely sulfo-NHS-SS-biotinylation with SDS elution, aminooxy-biotinylation, and surface coating using silica beads, were assessed with respect to their yield of PM proteins (Figure 1). Along the lines of previous studies,[9,16,18,34] the PM fraction was defined as the subpopulation of proteins annotated as "plasma membrane", "cell surface", "cell membrane", or "extracellular". These represent surface-exposed proteins targeted by the extraction methods. All three approaches led to the identification of comparable or improved numbers of PM proteins than previously reported.[9,16,18,20,29] In our hands, the sulfo-NHS-SS-biotinylation approach coupled to SDS elution (Method 1A) yielded the highest absolute number of PM proteins; however, the "glycocapture" with aminooxy-biotin (Method 2) resulted in the highest relative amount of PM proteins compared to non-PM proteins. Despite increasing the purity of the silica beads method by changing different protocol parameters, absolute numbers of PM proteins were compromised (Methods 3A and 3B; Table 1).

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J. Proteome Res. XXXX, XXX, XXX−XXX

Table 1. Relative and Absolute Numbers of PM Proteins Purified with the Three Methods Tested[a]

		sulfo-NHS-SS-biotin SDS elution	aminooxy-biotin	silica beads
PM	relative	49%	74%	55%
	absolute	650	340	187
	total protein IDs	1306	468	364

[a]Absolute and relative numbers of annotated PM proteins identified with three previously published PM extraction protocols. Data represent means of two biological replicates.

Proteins labeled with sulfo-NHS-SS-biotin should carry a carbamidomethyl (CAM)-thiopropanoyl modification after the isolation procedure. Determining the number of detected proteins with such a biotin moiety not only provides a measure of quality control for the non-cell-membrane-permeability of the sulfo-NHS-SS-biotin reagent, but also could aid as an additional annotation-independent layer of evidence for the localization of proteins at the cell surface. We found at least one CAM-thiopropanoyl modification for around a quarter of all proteins identified. Within the biotinylated protein fraction, PM proteins clearly represented the main subpopulation (Table 2). For the non-PM annotated proteins carrying a biotin moiety, no recurring subcellular localization pattern was apparent. These numbers are consistent with earlier, similar analyses,[9,18] and reports that biotinylated peptides originating from labeling at the protein level are difficult to identify.[19]

Table 2. Identification of Biotinylated Proteins[a]

			proteins with detected biotinylated peptide(s)		
	total protein IDs	annotated PM proteins	relative	absolute	biotinylated PM fraction
sulfo-NHS-SS-biotin SDS elution	937	452	17%	162	33%
sulfo-NHS-SS-biotin SDS elution + PNGaseF	780	494	39%	307	20%

[a]Identification of the CAM-thiopropanoyl modification in two samples either directly processed with sulfo-NHS-SS-biotin coupled to SDS elution or pretreated with PNGaseF. Total numbers of all proteins identified and absolute numbers of PM annotated proteins are indicated in the first two columns. Proteins with at least one peptide identified with a biotin moiety are indicated with relative and absolute numbers. The annotated PM subset thereof is indicated in the last column.

Reproducibility and Sensitivity of the Plasma Membrane Enrichment Procedures

To assess the reproducibility of the methods, annotated PM proteins from two biological replicates per method were characterized on a qualitative and quantitative level. First, PM proteins were identified and their distributed normalized spectral abundance factors (dNSAFs) calculated.[35] Then overlaps in annotated PM proteins between biological replicates were determined, their dNSAFs plotted, and Spearman's rank correlation coefficient calculated for each method (Figure 2a). All approaches yielded extremely significant correlations, with the sulfo-NHS-SS-biotinylated replicates (Method 1A) returning the strongest p-value ($<2 \times 10^{-16}$) and the correlation coefficient closest to 1 ($\rho = 0.83$). Moreover, neither the aminooxy-biotin nor the silica beads method tapped into significantly different space within the PM proteome (Figure 2b). To estimate the sensitivity of the protocols, the average number of spectra per annotated PM protein was calculated and plotted against the absolute number of annotated PM proteins identified with each method (Figure 3a). Since sulfo-NHS-SS-biotinylation, followed by SDS elution scored second in terms of sensitivity, yielded the highest absolute number of PM proteins and covered "plasma membrane" as well as "extracellular" protein annotations to the same extent (Figure 3b), optimization of this strategy was pursued further.

Cell surface proteins are usually highly glycosylated, often to a degree where the accessibility of primary amines to derivatization by reagents such as sulfo-NHS-SS-biotin is hindered. Although pre-digestion of the cells with PNGaseF to remove *N*-linked sugars prior to derivatization led to an increase in the number of proteins identified with a biotin moiety, the subset of PM proteins among the labeled fraction surprisingly decreased to a comparable degree (Table 2). Consequently, further development of this particular aspect of the protocol was ceased. Instead, the SDS in the elution step of the protocol was substituted with D-biotin (Method 1B). Switching to a detergent-free, competitive elution yielded high and reproducible numbers of surface proteins among the purified fractions (Figure 4a). Compared to the results obtained with SDS elution, purity and reproducibility improved. The relative number of plasma membrane proteins purified rose from 49% to 54%, the reproducible fraction of plasma membrane proteins between independent biological replicates increased from 55% to 77%, and on the quantitative level, the Spearman correlation coefficient ρ strengthened from 0.83 for Method 1A to 0.93 for Method 1B (Table 1; Figures 2a and 4a). Intriguingly, these numbers were confirmed in the morphologically distant, fibroblast-like cell line HAP1 (Figure S1).

The Majority of Non-PM Annotated Proteins Are Co-purified Due to Interactions with the PM Annotated Protein Set

Sulfo-NHS-SS-biotinylation followed by biotin elution (Method 1B) also purified the majority of non-PM-annotated proteins robustly and reproducibly (Figure 4b). From this observation, we reasoned that at least some of these proteins might be "false negatives", that is, proteins that are either (i) located at the surface, but not yet annotated as such, or (ii) not physically located on the surface, but interacting with a PM protein. To test this hypothesis and deduce potential subgroups, publically available protein−protein interaction (PPI) data were retrieved. Out of 201 consistently identified non-surface-annotated proteins, 140 were found to share interactions with at least one PM protein. Interestingly, half of all interactors were annotated as intracellular membrane proteins (Figure S2). To assess the statistical significance of these numbers, sets of PM and non-PM proteins corresponding in their size to the numbers deemed as reproducible, subtracted by those with no PPI data available, were randomly drawn from the KBM7 proteome.[36] Assessment of direct interactions between these sets in 10 000 simulations revealed a slightly right-skewed, bell-shaped distribution with a mean of 43 non-PM proteins having at least one interaction with a PM protein (Figure 4c). Consequently, the non-PM proteins identified as

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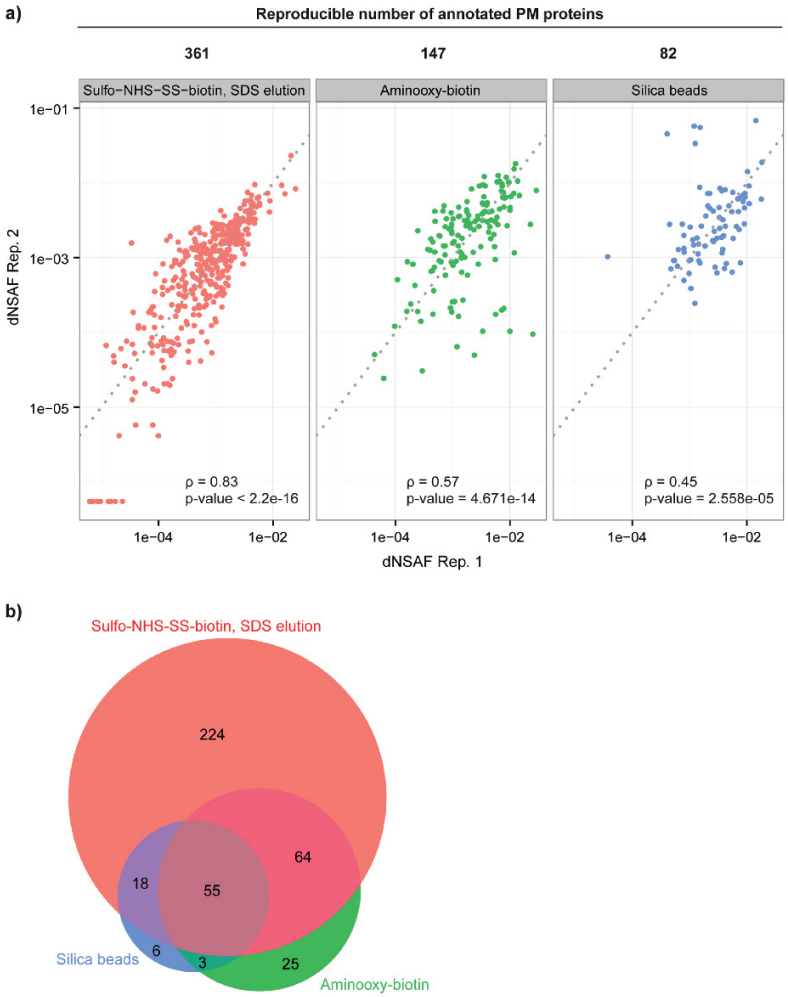

Figure 2. Reproducibility of PM extraction protocols. (a) Quantitative reproducibility of three previously published PM extraction protocols (Methods 1A, 2, and 3A/B). For each protocol, the reproducible subset of annotated PM proteins was determined and the Spearman correlation coefficient ρ calculated between two biological replicates prepared and analyzed on different days and quantified by dNSAF. (b) Venn diagram depicting the overlap in reproducibly detected PM proteins between the different protocol types.

interacting with at least one PM protein were attributed to the set of "PM-associated proteins" with extreme significance (p-value $<1 \times 10^{-4}$). The remaining 61 non-PM proteins were controlled for PM localization by consulting the manually curated Human Protein Reference Database,[37−39] the antibody-based Human Protein Atlas,[40,41] and two independent protein subcellular localization prediction tools (WoLF PSORT[42] and SherLoc2[43]). Seven proteins were annotated as PM by at least one of the databases. Ten more proteins were unanimously reported as PM by both subcellular localization prediction tools. Three of these 17 were proteins annotated by UniProtKB/Swiss-Prot[44] as "integral component of membrane" without subcellular assignment, a subgroup that has previously been suspected to be dominated by PM proteins.[9] The remaining 44 proteins were queried against the CRAPome database of common contaminants in AP-MS experiments.[45] Only two proteins were not documented within the CRAPome: the zinc transporter SLC39A10 and the mitochondrial proteolipid MP68. Interestingly, both are annotated as "integral component of membrane". The SLC is an example of a membrane protein without precise subcellular assignment and was thus attributed to the set of highly probable PM proteins. Conversely, MP68 is inferred to be a single-pass mitochondrial membrane protein and was therefore allocated to the

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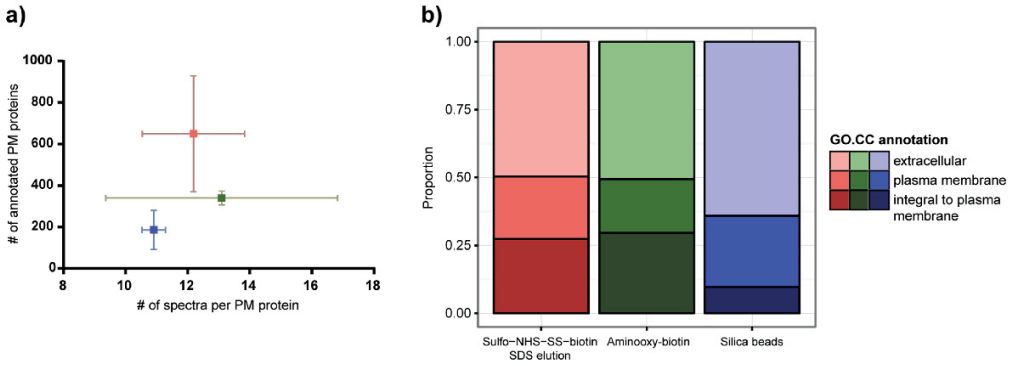

Figure 3. Sensitivity and subcomposition of established PM extraction protocols. (a) The average number of spectra per annotated PM protein was calculated and plotted against the absolute number of annotated PM proteins identified with each method. Data represent mean and standard deviation from two independent biological replicates. (b) Subcompositions of the annotated PM fractions shown as stacked bar plots for the three assessed PM extraction methods.

improbable PM fraction. Altogether, the non-PM-annotated proteins were divided into 158 highly probable (79%) and 43 improbable PM proteins (21%) (Supplementary Table 1). These inferences were additionally supported by abundance data for the three subsets: (i) annotated PM proteins, (ii) highly probable PM proteins, and (iii) improbable PM proteins. While dNSAF data for the annotated and highly probable PM proteins showed similar abundances (Welch two sample *t* test, *p*-value $\geq$ 0.05), indicating that these indeed form the joint subgroup of PM and PM-associated proteins, improbable PM proteins displayed significantly reduced abundances compared to both (Welch two sample *t* test, *p*-value: 8.968×10^{-8} compared to the annotated PM fraction and 8.115×10^{-4} compared to the highly probable PM proteins, respectively) (Figure 4d).

Sulfo-NHS-SS-biotinylation with Biotin Elution Can Track Changes in the PM Proteome Composition

Next, we tested our protocol by monitoring changes in the surface proteome composition introduced by genetic or chemical perturbations. The GPI transamidase component *PIGS* is essential for the transfer of GPI to proteins and hence for the attachment of certain extracellular membrane proteins to the lipid bilayer.[46] As a consequence, PM purifications from *PIGS* deficient cells should be depleted of any GPI-anchored proteins. In fact, our data proved all three GPI-anchored proteins robustly detected in the KBM7 wt samples, namely CD59, BST2, and NEGR1, to be absent from the surface of *PIGS* KO cells (Figure 5; Supplementary Table 2).

For the chemical perturbation, tunicamycin was chosen, an antibiotic known to block the synthesis of *N*-acetylglucosamin-phosphotransferase during protein glycosylation.[47−50] Cells were exposed to 50 nM tunicamycin for 24 h before isolation and analyzation of their cell surface proteome. Intriguingly, *N*-glycoproteins were overrepresented within the subset of proteins with significantly decreased surface expression levels (hypergeometric test, *p*-value: 2.442×10^{-3}). Specifically, 21 *N*-glycoproteins were not identified and another 18 significantly reduced in abundance following drug treatment. An additional 13 *N*-glycoproteins were diminished by tunicamycin; however, their changes were not significant (Welch two sample *t* test, *p*-value $\geq$ 0.05). Inversely, 12 *N*-glycoproteins increased in PM abundance compared to the wt (Figure 6a; Supplementary Table 3).

On the basis of the observation that non-PM annotated proteins in the purifications were significantly enriched for interactors of annotated PM proteins, abundance patterns between all *N*-glycoproteins and their interactors were controlled for congruence. Indeed, nearly 90% of interactors displayed abundance changes that paralleled the alterations observed with their *N*-glycoprotein counterparts (Figure 6a; Supplementary Tables 3 and 4).

Proteins might be affected to different extents by the tunicamycin treatment depending on their individual turnover rates. We retrieved turnover rates from a quantitative spatial proteomic analysis of proteome turnover in human cells[51] that covered nearly 80% of the proteins purified with our protocol. Data integration revealed that proteins depleted or significantly reduced in abundance by the tunicamycin treatment were underrepresented in a subset filtered for turnover rates of $\geq$ 24 h (hypergeometric test, *p*-value: 2.053×10^{-2}), while the proportion of proteins with any other treatment effect was significantly elevated (hypergeometric test, *p*-value: 1.196×10^{-2}) (Figure 6b).

■ DISCUSSION

The purpose of this study was to identify a robust protocol for the routine evaluation of plasma membrane proteomes in future studies of surface marker expression, membrane transport and signaling. We systematically evaluated three PM isolation strategies for enrichment efficiencies and characteristics. On average, between 187 and 650 PM proteins were detected, depending on the applied protocol. Importantly, all protocols presented in this study isolated PM proteins with high purification efficiencies as documented by the universally high percentage rates of PM proteins among all identified proteins. This is particularly noteworthy considering that all our experiments were analyzed as one-dimensional, gel-free LC−MS experiments, that is, without protein or peptide fractionation. Quereshi et al.[18] recently compared human cancer cell surface proteomes using a nearly identical biotinylation strategy supplemented with peptide fractionation. Interestingly, fractionation did not appear to boost the number

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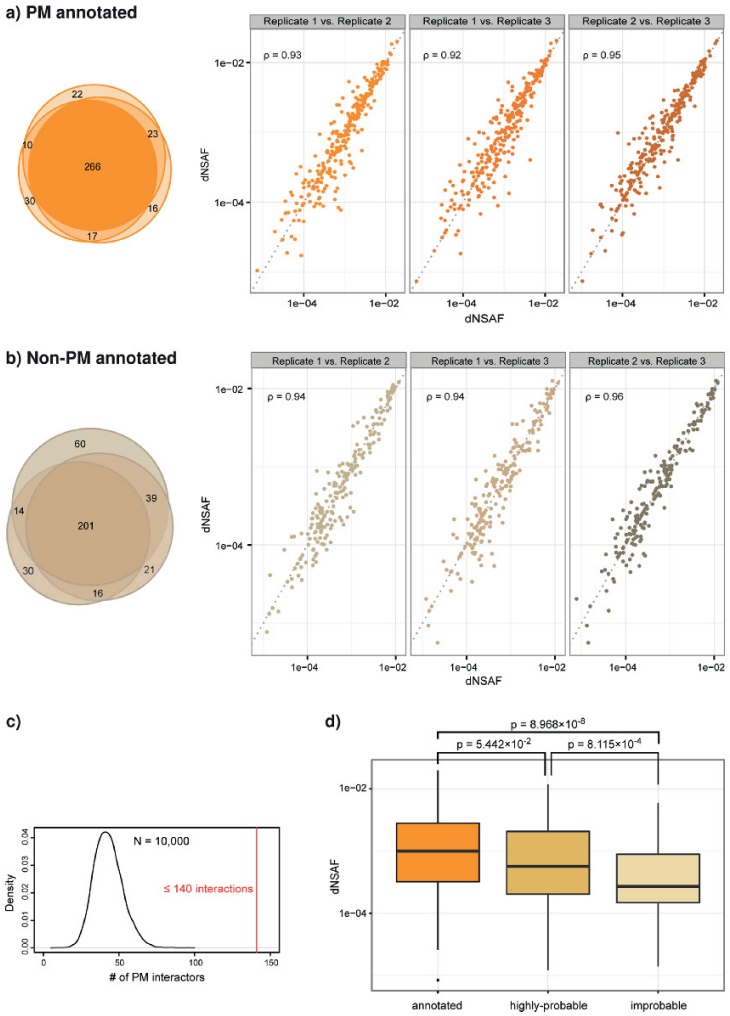

Figure 4. Reproducibility and subcomposition of sulfo-NHS-SS-biotinylation using competitive biotin elution. Qualitative and quantitative reproducibility of (a) PM annotated proteins and (b) non-PM annotated proteins. The Venn diagrams each represent overlaps in identified proteins between three biological replicates. The scatterplots assess quantitative reproducibility of the optimized protocol by correlating dNSAF from three biological replicates. All correlations indicated in the respective panels were extremely significant (p-value $<2 \times 10^{-16}$, Spearman method). (c) Density distribution for the number of direct interactions present in 10 000 sets of annotated PM and non-PM annotated proteins randomly drawn from the KBM7 proteome. (d) Boxplots of dNSAF data from the three subsets (i) annotated PM proteins, (ii) highly probable PM proteins, and (iii) improbable PM proteins. Interactors of annotated PM proteins, proteins annotated as PM by additional databases, and proteins unanimously predicted as PM by two subcellular prediction tools were summarized into the set of highly probable PM proteins. P-values were calculated using the Welch two-sample t test.

of identified PM proteins in their study. Conversely, non-PM proteins were enhanced impairing the protocol's purification power and sensitivity.

Reproducibility represents a key feature of any scientific method and is an essential criterion in the evaluation of a protocol's overall performance. To our knowledge, none of the PM isolation techniques used within this work has been previously assessed for robustness. We found the overlaps of identified proteins between biological replicates to be quite variable depending on the protocol type. While the shared fraction was relatively small for samples obtained with the aminooxy-biotin (Method 2) or silica beads approaches (Methods 3A and B), the sulfo-NHS-SS-biotinylation strategies (Methods 1A and B) resulted in increased numbers of mutual

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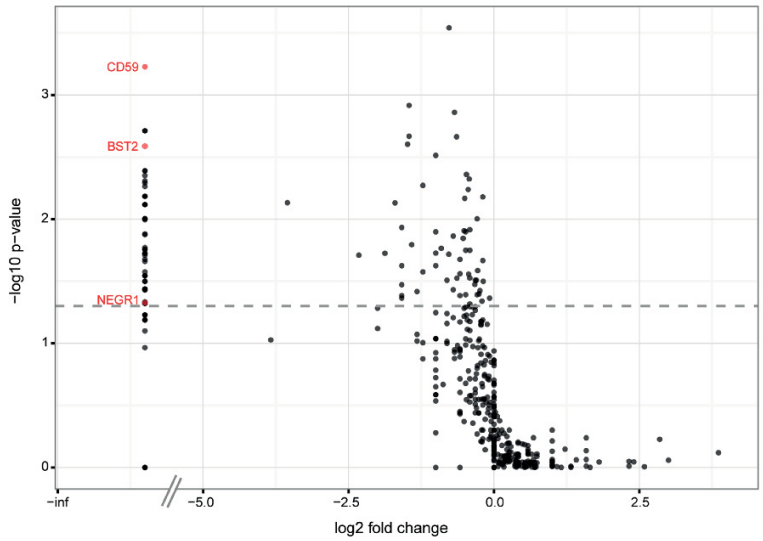

Figure 5. GPI-anchored proteins are depleted from PM purifications of GPI attachment factor *PIGS* KOs. Scatterplot representing abundance changes in surface proteins between KBM7 wt and *PIGS* KO cells. The three GPI-anchored proteins recurrently identified in surface purifications of wt cells, but depleted from the surface of *PIGS* deficient cells, are marked in red. The *x*-axis is broken to display proteins abrogated upon *PIGS* KO. Data are based on three independent biological replicates.

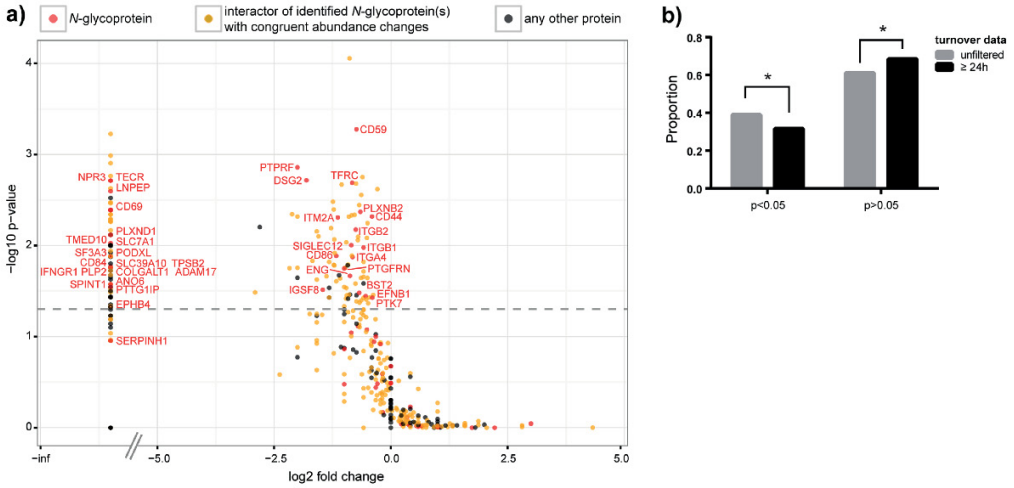

Figure 6. Surface protein abundance changes upon tunicamycin treatment. (a) Scatterplot showing surface protein abundance changes after treatment with 50 nM tunicamycin for 24 h. *N*-glycoprotein, red; interactor of identified *N*-glycoproteins with congruent abundance changes, orange; any other protein, black. The *x*-axis is broken to display proteins abrogated upon tunicamycin treatment. Data are based on three independent biological replicates. (b) Bar graphs indicating proportions for two different tunicamycin treatment effect groups and two data sets. The unfiltered data set represents all identified proteins for which turnover data were available from Boisvert et al.[51] The second data set is a subset thereof with protein turnover ≥ 24 h. *, $p < 0.05$.

protein identifications and higher degrees of positive correlation in abundance measured by dNSAF. Moreover, establishing a detergent-free elution strategy (Method 1B) enabled us to omit FASP and thereby precluded any potential problems that may arise from poor peptide recovery and sequence coverage reported in quantitative FASP experiments before.[25−27] Proteins purified with this method were in high agreement with *RaftProt*, a mammalian lipid raft proteome

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J. Proteome Res. XXXX, XXX, XXX−XXX

database[52] strengthening our protocol's sensitivity and reliability. Computational analysis demonstrated the majority of non-PM annotated proteins to be co-purified due to direct interactions with the PM annotated protein set and suggested additional non-PM annotated proteins to be likely located on the surface further improving confidence in the method.

As a proof-of-concept for our workflow, changes in the PM composition were monitored for two exemplary perturbations: gene deletion and drug treatment. Results from previous single FACS analyses[36] of GPI attachment factor *PIGS* deficient cells were confirmed and extended to prove that all GPI-anchored proteins identified in the wt cells were indeed abrogated in the *PIGS* KO clones. The picture resulting from the comparison of wt and tunicamycin treated cells was not as straightforward. Tunicamycin is known to prevent protein glycosylation by blocking the formation of *N*-acetylglucosamine-lipid intermediates;[47−50] however, several studies have shown that the fate of *N*-glycoproteins in the presence of tunicamycin varies.[53−56] While protein synthesis is not significantly affected per se, the secretion of immunoglobulins is inhibited to variable extents, independent of their glycosylation degree.[56] By integrating publically available data, we determined protein turnover rates to be one source of variation for tunicamycin treatment effects after 24 h. Additional ones remain to be elucidated.

Altogether, our surface biotinylation strategy yielded high numbers of reproducible PM annotated proteins. Furthermore, our study shed light on the previously poorly characterized subset of non-PM annotated proteins identifying the majority of these proteins as associated with the PM via PPIs. Ultimately, the work presented here provides for a procedure that empowers routine cell surface proteomics as a widely used tool in the characterization of the complement of proteins operating at the plasma membrane. Quantitative evaluation of surface proteins will allow functional correlations and assessment of transport, signaling, and adhesion properties. A more detailed comparison of cellular differentiation and disease states may aid in uncovering new functional cell and disease subtypes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b01066.

Table indicating the subsets within the reproducible set of proteins obtained with sulfo-NHS-SS-biotinylation coupled to biotin elution (XLS)

Table showing surface protein abundance changes upon *PIGS* KO (XLS)

Table giving abundance changes of purified proteins upon tunicamycin treatment (XLS)

Table indicating PPIs for surface proteins affected by tunicamycin treatment (XLS)

Figures showing reproducibility of the developed protocol on HAP1 cells and the localization profile for co-purified interactors of annotated plasma membrane proteins (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CAM, carbamidomethylation; Da, Dalton; dNSAF, distributed normalized spectral abundance factors; FA, formic acid; FASP, filter-aided sample preparation; FDR, false discovery rate; GO.CC, Gene Ontology cellular component; GPI, glycosylphosphatidylinositol; IAA, iodoacetamide; IMDM, Iscove's modified Dulbecco's medium; LTQ, linear trap quadrupole; MBS, MES-buffered saline; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PAA, polyacrylic acid; PM, plasma membrane; PNGase, peptide-*N*4-(*N*-acetyl-beta-glucosaminyl)-asparagine amidase; PPI, protein−protein interaction; rpm, revolutions per minute; SPE, solid phase extraction; STB, stage tip buffer; TEAB, triethylammonium bicarbonate; Tris, tris-(hydroxymethyl)aminomethane; wt, wild type

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2.2 Triton X-114 phase separation coupled to FASP for membrane proteomics of human cells

After having successfully established a reproducible purification strategy for the large-scale analysis of PM proteins, a similar protocol covering the whole membrane proteome still needed to be found. Following the pioneering work of Bordier *et al.* (Bordier, 1981) and its first applications in the proteomic analysis of human tissues (Donoghue et al, 2008; English et al, 2012), TX114 phase separation was selected for the enrichment of membrane proteins. Isolated membrane proteins were then prepared for LCMS via FASP, given the much higher number of proteins that had been detected upon its inclusion (Wisniewski et al, 2009a). The resulting workflow was validated as a method for the global analysis of membrane proteins in the human leukemia cell line KBM7 used in the comparative study of surface enrichment strategies before. In particular, absolute and relative numbers of identified membrane proteins, their reproducibility and selected features, such as their number of transmembrane helices were assessed (Figure 9).

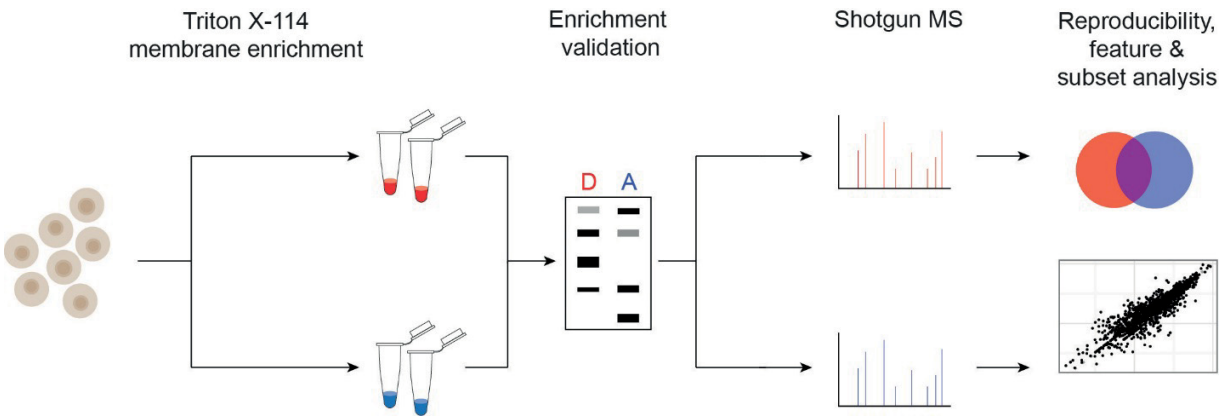

Figure 9: Experimental workflow of Triton X-114 phase separation for membrane proteomics of human cells

Hydrophilic and hydrophobic proteins were separated using TX114 phase separation. The enrichment was validated via Western blot using selected membrane proteins. Aqueous and detergent phases were then analyzed using shotgun MS and the identified proteins assessed for enrichment, reproducibility and feature parameters. A, aqueous phase; D, detergent phase.

In this approach, the separation of hydrophilic and hydrophobic proteins is achieved by simple stepwise centrifugation steps. In detail, heating the samples to 37 °C lets TX114 reach its cloud point, *i.e.* the temperature where densely packed micelle groups are formed increasing the molecular weight and ultimately resulting in phase separation with the aqueous phase hosting the hydrophilic and the detergent phase hosting the more hydrophobic proteins (Donoghue et al, 2008).

However, Western blot analysis probing for a panel of diverse types of membrane proteins revealed that they are hardly ever selectively recovered in only one of the phases (Table 1) (Figure 10). Yet, the method might still be of great value despite this limitation, as long as the composition of the individual fractions and especially the detergent fraction can be judged reproducible.

Table 1: Panel of membrane proteins probed for enrichment validation

Protein	Type
LAMP2	Single-pass membrane protein
LAMTOR1	Lipid anchored (*N*-myristoylated) protein
SMPDL3B	GPI-anchored protein

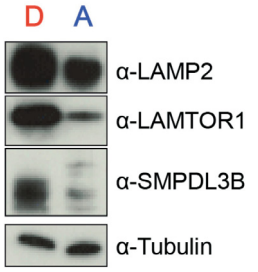

Figure 10: Triton X-114 enrichment validation via Western blot
Aliquots of detergent (D) and aqueous phases (A) were probed for different types of membrane proteins.

Hence, overlaps in protein identifications between two biological replicates were determined for both, aqueous and detergent phase. Then, dNSAF were calculated for every reproducible protein and plotted. Intriguingly, reproducible protein identifications made up at least 86 % of all samples and Spearman's rank correlation coefficient ρ

indicated high and extremely significant correlations for both phases, whereas the number of proteins recurrently detected in both phases remained below 35 % (Figure 11).

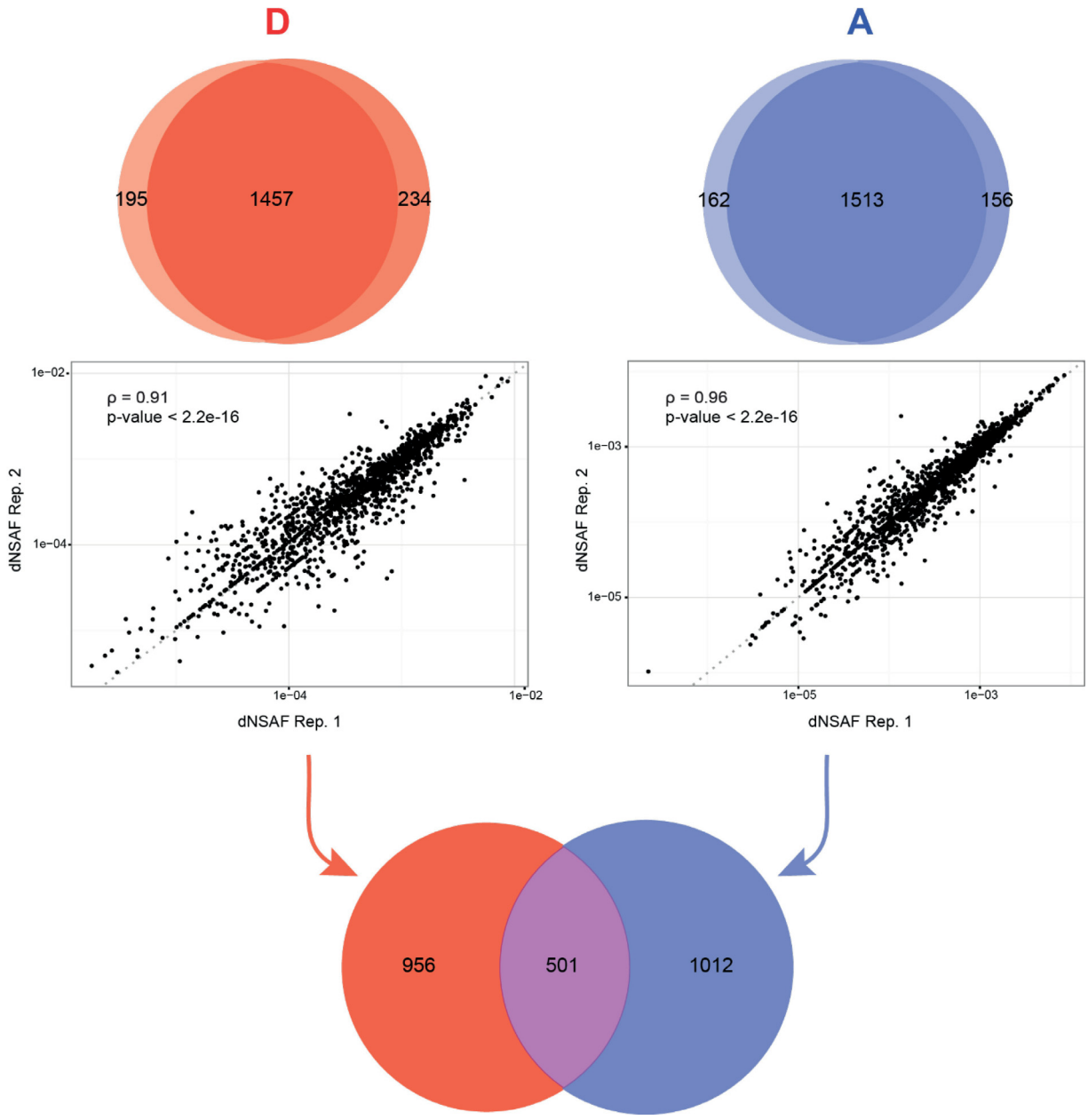

Figure 11: Reproducibility of Triton X-114 phase separation

Quantitative reproducibility of proteins detected in the detergent (D; red) and aqueous (A; blue) phase. Proteins were quantified by dNSAF and the Spearman correlation coefficient ρ calculated between two biological replicates.

Moreover, equal numbers of proteins were identified in both phases. Still, membrane proteins and specifically, integral membrane proteins were selectively enriched in the detergent phase. Numerically, membrane proteins amounted to 79 % in the detergent, as opposed to 50 % in the aqueous phases and integral membrane proteins represented no less than 40 % of the detergent versus only 6 % of the aqueous phases, respectively (Figure 12).

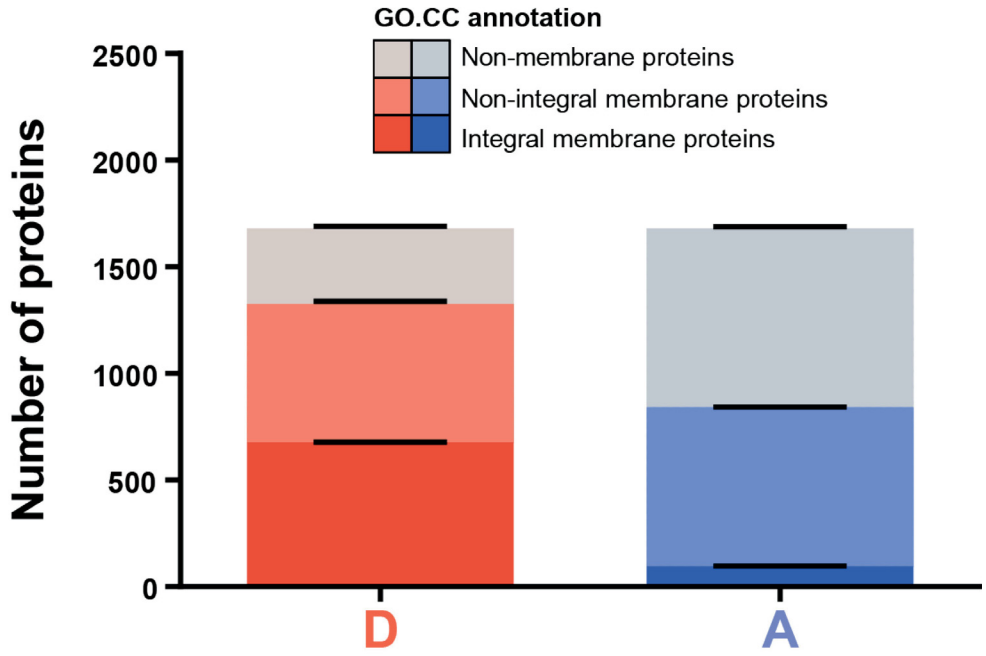

Figure 12: Sub composition of aqueous and detergent phases

GO.CC annotations shown as stacked bar plots. Error bars represent the standard deviation between two biological replicates. D, detergent phases; A, aqueous phases.

This enrichment was also ascertainable in the phase distribution of major functional classes of membrane proteins, namely channels, transporters and receptors (Figure 13).

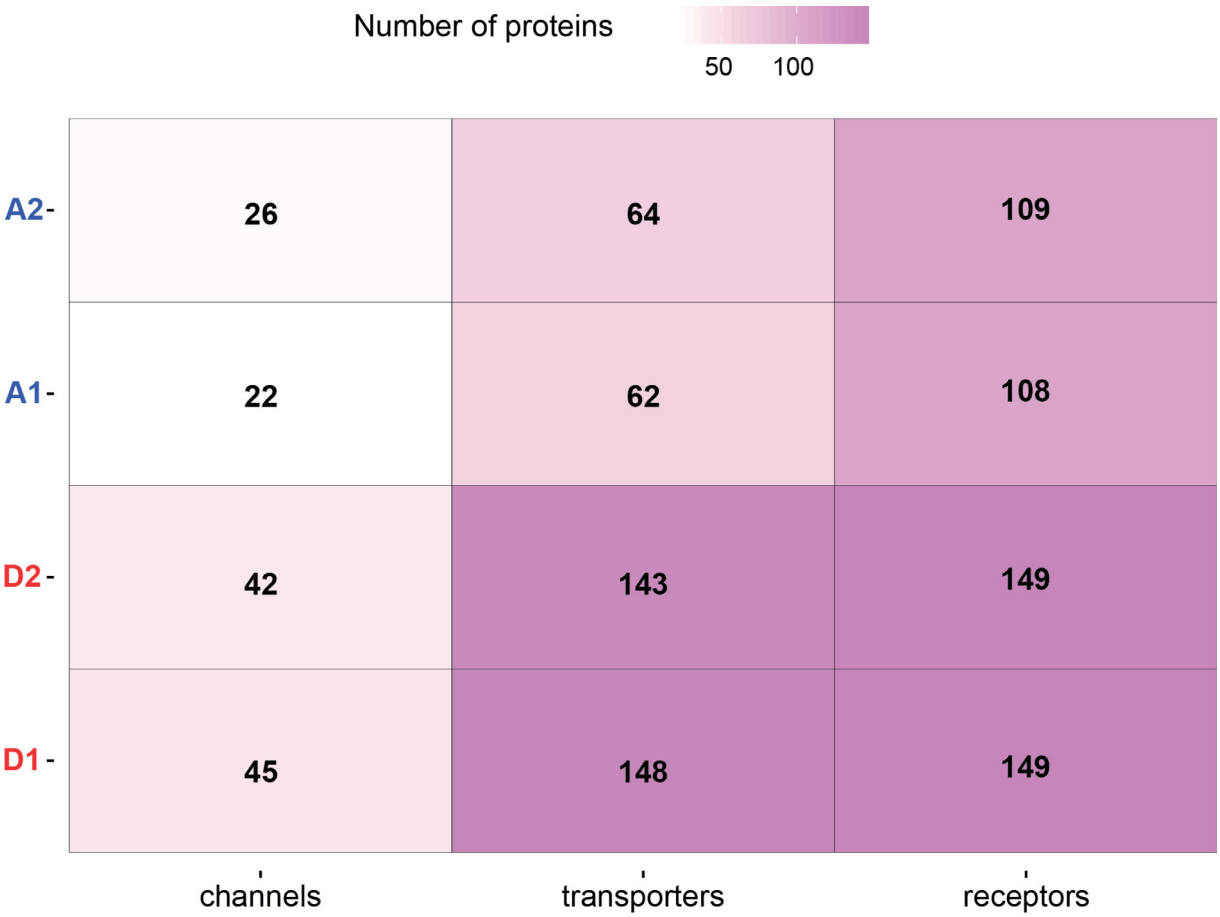

Figure 13: Phase distribution of major functional classes of membrane proteins
Levelplot indicating the number of proteins per functional class detected in the individual phases with each row representing a biological replicate and each column displaying the data of one functional class. A, aqueous phase; D, detergent phase.

Additionally, predictions about the number of transmembrane helices per protein via the combined transmembrane topology and signal peptide predictor Phobius (Kall et al, 2004; Kall et al, 2007) indicated the average number of transmembrane helices per protein to be twice as high for proteins recovered in the detergent than for those stemming from aqueous phase samples (Table 2) (Figure 14).

Table 2: Summary of transmembrane topology prediction analysis using Phobius
Numbers of proteins with at least one predicted transmembrane helix detected in the different biological replicates and phases, respectively.

Number of predicted transmembrane helices	D1	D2	A1	A2
1	285	295	65	63
2	70	74	18	17
3	47	47	6	9
4	57	56	2	3
5	25	24	1	1
6	31	26	2	2
7	37	34	5	1
8	29	27	3	3
9	25	25	0	2
10	17	17	0	0
11	17	14	0	0
12	25	24	0	0
13	6	6	1	1
14	5	5	1	1
15	1	1	0	0
16	2	2	0	0
17	0	1	0	0
39	1	1	0	0

The most extreme observation in this regard was certainly the Piezo-type mechanosensitive ion channel component 1 (PIEZO1), for which 36 out of the 39 transmembrane helices predicted by Phobius have been manually asserted by Swiss-Prot as well.

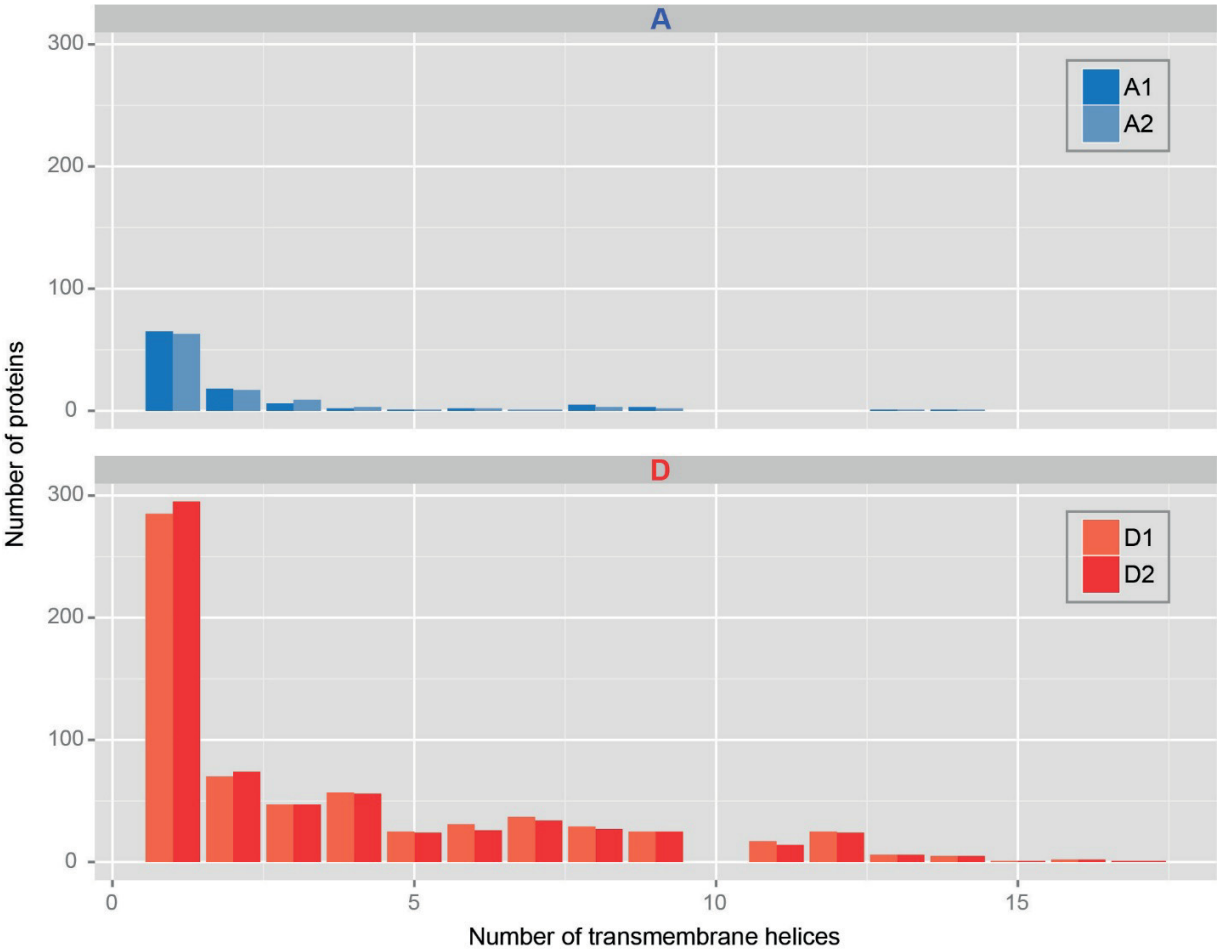

Figure 14: Distribution of transmembrane helices predicted by Phobius
The two panels represent results for aqueous (A; blue) and detergent phases (D; red) and proteins ranging between 1 and 17 predicted transmembrane helices by Phobius.

The sequence coverage of membrane proteins in MS experiments has been proclaimed improvable (Fischer et al, 2006; Wu & Yates, 2003). Yet, peptides detected with the experimental workflow depicted in Figure 9 and described in detail under section 4.3 covered individual proteins to an almost even extent (Figure 15). Likewise, sequence coverages did not differ between integral and non-integral membrane proteins (Wilcoxon-Mann-Whitney test, p-value ≥ 0.05) underlining the protocol's robustness and reliability for membrane proteomics.

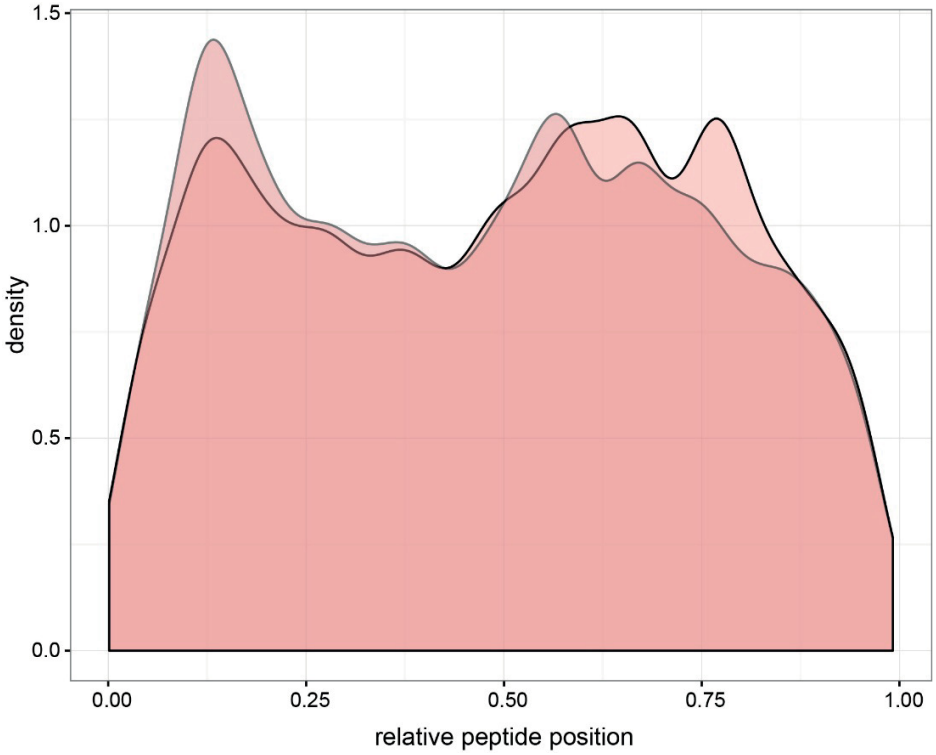

Figure 15: Protein coverage of detected peptides Density plot representing data from two biological replicates of detergent phases. The relative peptide position was defined as the start position of the respective peptide divided by the protein length.

In order to assess to what extent the incorporation of a peptide fractionation step and the modification of gradient time and composition could further increase the number of identified proteins, aliquots of the detergent phases were separated off-line into 10 fractions and then analyzed using a prolonged gradient of either the regular composition (methanol (MeOH), isopropanol (IPA), formic acid (FA)) or alternatively, consisting of 100 % acetonitrile (ACN) (Table 3). Although fractionation consistently resulted in higher absolute numbers of identified membrane proteins, relative numbers decreased. This general trend was even more pronounced for the subgroup of integral membrane proteins suggesting off-line separation to predominantly enhance non-membrane proteins. Conversely, fractionation did result in a significant increase in sequence coverages obtained for integral membrane proteins as opposed to all other proteins purified within the detergent phases (Wilcoxon-Mann-Whitney test, p-value = 3.13×10^{-11} for the samples run with the MeOH, IPA, FA gradient and p-

value = 2.954×10^{-9} for the sample analyzed using the alternative ACN gradient, respectively).

Table 3: Comparison of unfractionated and fractionated samples

Numbers are means of two biological replicates for the unfractionated data and based on one biological replicate for the fractionated samples, respectively.

	Total protein IDs	Membrane proteins		Integral membrane proteins	
		relative	absolute	relative	absolute
unfractionated	1672	79 %	1326	40 %	676
fractionated MeOH, IPA, FA	6424	57 %	3679	25 %	1633
fractionated ACN	6043	58 %	3516	26 %	1563

Finally, the membrane proteomics workflow was applied to two additional leukemic cell lines, namely K562 and HL60, and the results compared with previously published global proteomics data (Moghaddas Gholami et al, 2013). Intriguingly, the datasets overlapped to an almost even degree (83 % for K562 and 82 % for HL60) proving that targeted membrane protein isolation coupled to one-dimensional gel-free LCMS analysis is able to robustly extend the proteome recovered with two-dimensional global proteomic approaches. More specifically, the proteins exclusively detected with the protocol presented herein consisted of significantly higher proportions of integral membrane proteins than those unanimously identified with both approaches (hypergeometric test, p-value < 0.05) (Figure 16).

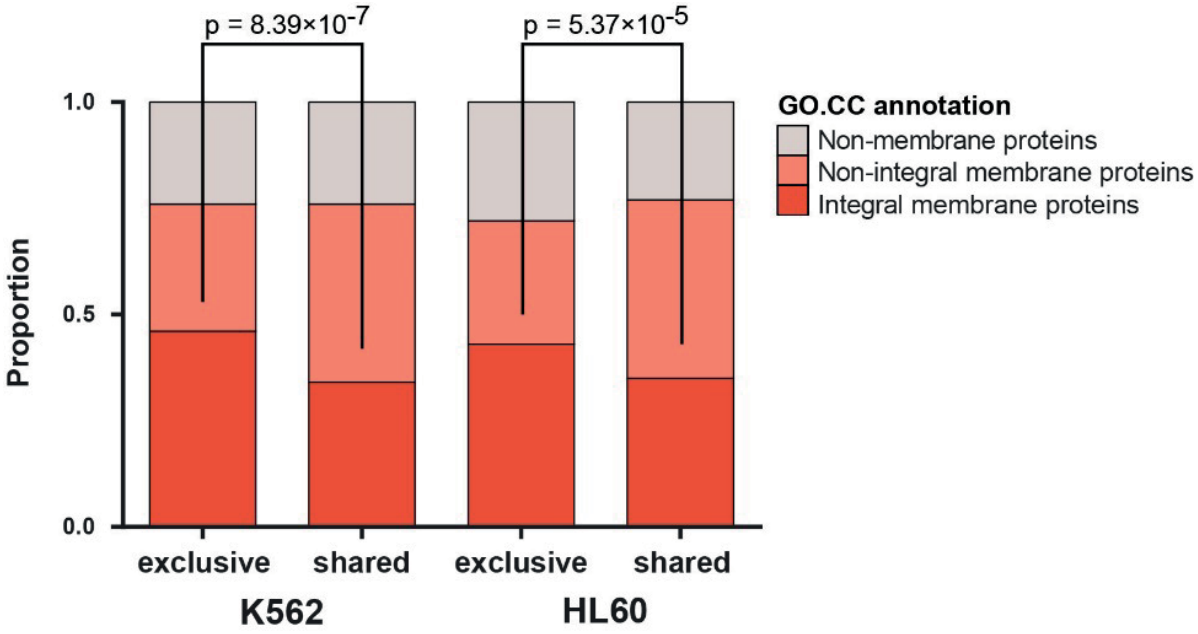

Figure 16: Comparative sub composition analysis with global proteomics data

GO.CC annotations shown as stacked bar plots. Bars labeled as “exclusive” represent proteins solely identified with the membrane proteomics workflow, while bars labeled as “shared” represent proteins identified in the global proteomics dataset as well.

3. Discussion

3.1 General discussion

Membrane proteins are located at the interface of living cells and their external environments making them key players in signaling and cellular transport processes. Most importantly, they represent the vast majority of known protein drug targets. Still, their large-scale proteomic analysis is challenging, while deeper insights into this specific sub-proteome might enable a better understanding of pathologic situations and new therapeutic approaches identifying surface markers or transporter specificities.

This thesis aimed to identify two protocols allowing a more routine evaluation of whole membrane and cell surface proteins. To this end, shotgun proteomics and specifically one-dimensional, gel-free LCMS experiments were applied throughout. As far as whole membrane proteomics are concerned, coupling a TX114 phase separation approach to FASP identified equal numbers of proteins in the detergent and aqueous phases, enabling an unbiased and systematic assessment of reproducibility parameters at an unprecedented scale.

Within the detergent phase extracts, an average of 1672 proteins was identified in a single 60 min MS run. Thereof, 79 % were annotated membrane proteins, with more than half being even annotated as integral membrane proteins. This represents a more than two fold enrichment in terms of membrane proteins and a more than six fold enrichment in terms of integral membrane proteins compared to control samples subjected to the same experimental workflow excluding the phase separation procedure. The aqueous phase extracts, on the other hand, showed no evidence of integral membrane protein enrichment, even though the relative numbers of membrane proteins were still found to be slightly elevated compared to the controls. A fact that is most probably explicable by the amphiphilic nature of some membrane proteins, accompanied by their detection in both of the phases as documented in the TX114 phase separation enrichment validation via Western blot (Figure 10).

The detergent phases not only consistently hosted more integral membrane proteins, but the proteins recovered therein also contained significantly higher numbers of

transmembrane helices confirming similar observations in human heart and brain tissue samples (Donoghue et al, 2008; English et al, 2012). Namely, proteins identified within the detergent phases included pharmacological key targets, such as GPCRs (S1PR4, F2RL1, GPR155, GPRC5C) and integrins (ITGB1, ITGB2), a panel of ABC transporters covering six of their seven human subfamilies (ABCC4, ABCB7, ABCD3, ABCC1, ABCD1, ABCE1, ABCA3, ABCB10, ABCB8, ABCF2) and over 60 distinct SLCs.

In contrast to English *et al.*, who reported poor overlaps in protein identifications between detergent extracts of different biological replicates (English et al, 2012), all indicated numbers and enrichments were highly reproducible on both, the qualitative as well as the quantitative level. Therefore, the experimental workflow developed within this thesis cannot only be deemed reliable and robust, minimizing the number of biological replicates required, but also provides further proof of the applicability of label-free quantification in membrane proteomics.

Moreover, the relative and absolute number of membrane proteins recovered in the detergent phases exceeded previous results (Donoghue et al, 2008; English et al, 2012), even though this may partly be explained by a newer and hence more sensitive generation of MS machines running the samples for this thesis. Interestingly, fractionation of the detergent phases selectively improved the sequence coverage of integral membrane proteins as well as the absolute numbers of membrane proteins identified. Relative numbers were however diminished, confirming similar observations for PM protein targeted protocols (Ozlu et al, 2014; Weekes et al, 2010). In addition, the tested alternative gradient composition did not affect monitored yield parameters, even though the effect of *e.g.* heavily prolonged gradient times remains to be determined.

The rather low sequence coverage of membrane proteins and specifically the underrepresentation of peptides from transmembrane domains has been ascribed to the inherent lack of tryptic cleavage sites in these hydrophobic patches together with their poor solubility and decreased accessibility to proteases (Tan et al, 2008). Yet, the peptide identifications resulting from the workflow introduced within this thesis suggest that the combination of TX114 phase separation and FASP effectively overcomes this complication, delivering an almost even coverage of all proteins isolated within the

detergent phase and extends the data retrieved with global proteomic approaches, while keeping the required sample input to a minimum.

With regard to PM proteins, the publication arising from this thesis provides a systematic comparison of three PM isolation strategies allowing the scientific community to choose a protocol based on their individual needs, *i.e.* tailored to achieve either a higher absolute or relative number of PM protein identifications, as no specific enrichments were detected within the sub fractions exclusive to each protocol. Intriguingly, purification efficiencies of all tested protocols analyzed as one-dimensional, gel-free LCMS experiments were rather high, surpassing similar approaches supplemented with peptide fractionation (Ozlu et al, 2014; Weekes et al, 2010).

Reproducibility represents another key feature determining the power of any scientific technique. Sulfo-NHS-SS-biotinylation strategies displayed higher numbers of mutual protein identifications than samples obtained with the aminooxy-biotin or silica beads approaches. Furthermore, they scored equally better in the degree of positive correlation on the quantitative level.

The introduction of FASP and thus, the possibility to use detergents for the solubilization of proteins, while allowing their removal via filter-based devices prior to LCMS analysis, clearly represented a major breakthrough for the in-depth analysis of membrane proteomes (Wisniewski et al, 2009a). Still, there have been opposing results concerning peptide recovery and sequence coverage in quantitative, filter-based experiments (Bereman et al, 2011; Erde et al, 2014; Glatter et al, 2015; Leon et al, 2013; Nel et al, 2015). Similarly, switching from sodium dodecyl sulfate (SDS) elution followed by FASP to a detergent-free, competitive elution increased the relative number of PM proteins purified with sulfo-NHS-SS-biotinylation from 49 % to 54 %, expanded the reproducible fraction between independent biological replicates from 55 % to 77 % and strengthened the quantitative correlation from 0.83 to 0.93. Contrary to this, the absolute number of surface proteins decreased from 650 to 347 and data from the whole membrane proteomic approach presented herein that employs FASP has not given any line about poor peptide recovery or reproducibility.

Another major concern in the application of exogenous labels for PM protein isolation are PTMs, especially glycosylations that may hinder the accessibility of residues, mask them from derivatization by the reagent and ultimately decrease the labeling efficiency. Indeed, efforts to pre-digest cells with PNGaseF to remove *N*-linked sugars prior to derivatization, led to an increase in purification efficiency as well as in the number of proteins identified with a biotin moiety. Yet, the two increases were not related. In fact, the subset of PM proteins amongst the labeled fraction surprisingly decreased to a comparable degree suggesting pre-digestion to compromise cellular integrity.

In other respects, computational analysis of proteins co-purified with the detergent-free PM strategy developed within this thesis, identified the majority of non-PM annotated proteins as interactors of the surface annotated set. Despite this finding being solely based on PPI data, it was further supported by defining the number of false positive interactors, *i.e.* proteins purified from unlabeled cells subjected to an otherwise unchanged protocol, which amounted to less than 5 %. Additionally, a subset of non-PM annotated proteins was found to likely be located on the surface further improving purity and confidence of the method. This first dissection of the previously poorly characterized subset of non-PM annotated proteins might enable future biologically or medically relevant deductions. Particularly, given that upon drug treatment with tunicamycin, nearly 90 % of interactors showed differential patterns that paralleled those observed for their *N*-glycoprotein counterparts.

The question to which extent the presented PM isolation protocols still offer room for optimization might be put forward. As far as sulfo-NHS-SS-biotinylation with biotin elution is concerned, results in the morphologically distant, fibroblast-like cell line HAP1 demonstrated the general utility of the developed protocol. Conversely, adapted parameters for the silica bead protocol that had achieved better results in certain cell types, failed to do so in my hands. Hence, cell type centered alterations might well not be generally applicable optimizations, but rather become overly cell specific. Still, that does not preclude the possibility of valuable optimization potential in studies where differential surface proteomics are limited to one cell or tissue type. Optimizations on the LCMS side, as brought up before, might also apply here. In particular, labeling approaches, specifically those where labeling occurs at tryptic cleavage sites, like sulfo-NHS-SS-

biotinylation might require an even more elevated number of missed tryptic cleavage sites allowed during peptide assignments. Yet, this comes at the cost of having to redefine subsequent thresholds in order to maintain a defined false discovery rate (FDR).

3.2 Conclusion & future prospects

Altogether, the two experimental workflows developed within this thesis yielded high numbers of reproducible membrane and surface proteins providing two independent, yet complimentary procedures suited to the routine evaluation of both, whole membrane and PM proteomes.

While it is already important to accomplish a more reliable and in-depth membrane centered mapping of cell lines and tissues, combining the two approaches might be particularly interesting in the study of trafficking perturbations or selective internalizations of surface proteins.

In general, enabling a more detailed understanding of disease subtypes may pave the way for new therapeutic approaches, especially given that it has been shown before that membrane proteomic technologies are not limited to human samples, but can well be extended to the complementary analysis of human pathogens (Wolff et al, 2008).

Moreover, empowering routine whole membrane and surface proteomics may further progress knowledge on basic biological processes, such as cell cycle progression (Ozlu et al, 2014) and stem cell differentiation and reprogramming (Rugg-Gunn et al, 2012).

Similarly, activation of human T-cells is known to be accompanied by surface remodeling, namely, amongst others, the increased surface expression of specific interleukin receptors and SLCs (Fazekas de St Groth et al, 2004; Hayashi et al, 2013; Macintyre et al, 2014). A more global approach to compose a surface atlas and determine the changes induced by T-cell activation in more detail was, however, only published last year and still limited to glycoproteins in the proteomic strategy applied (Graessel et al, 2015). Thus, it is probable that the two experimental workflows presented herein would greatly refine the existing T-cell atlas, aiding in a better understanding of T-cell biology and corresponding therapeutic immune targets.

Naturally, the characterization of cancer cells and specifically the identification of membrane molecules contributing to tumor intravasation, metastasis and drug resistance represent another area of medically urging application (Conn et al, 2008; Rahbar & Fenselau, 2005; Sun et al, 2014). In the past, different membrane proteomic strategies already led to the identification of candidate drug targets (Bock et al, 2012; Hoover et al, 2015). Therefore, increased sensitivity and robustness of the membrane proteomic protocols applied are likely to directly be reflected in the number and quality of identified biomarkers and protein targets.

In addition, recent data indicates that metabolic competition can drive cancer progression and that cancer cells selectively reprogram their cellular metabolism to gain evolutionary and thermodynamic advantage (Alfarouk et al, 2014; Chang et al, 2015). Differential expression of membrane proteins is a key feature allowing tumor cells to cope with highly heterogeneous microenvironments. Consequently, assessing such membrane centered alterations on an omics scale might unravel further causes of therapy resistance, yet at the same time reveal currently hidden Achilles' heels.

4. Materials & Methods

4.1 Cell culture

KBM7 and HAP1 cells were grown in Iscove's modified Dulbecco's medium (IMDM), while K562 and HL60 cells were cultured in RPMI-1640 medium. Both media were supplemented with 10 % fetal calf serum (FCS) and 1 % Penicillin-Streptomycin.

4.2 Purification and enrichment of plasma membrane proteins

4.2.1 Sulfo-NHS-SS-biotinylation and elution with sodium dodecyl sulfate

Cell surface proteins were isolated according to the instructions provided by the manufacturer using the reagents supplied with the "Pierce® Cell Surface Protein Isolation Kit" (Thermo Scientific, Rockford, IL). Cells (40×10^6) were washed twice with ice-cold phosphate-buffered saline (PBS) and reconstituted in 4 mL biotin solution (10×10^6 per mL). The mixture was agitated for 30 min at 4ºC, the labeling reaction halted with 200 µL quenching solution and the cell pellets washed twice with tris-buffered saline (TBS). The cells were re-suspended in 500 µL lysis buffer and lysed by sonication on ice with 5 × 1 s pulses. Sonication was repeated twice over a period of 30 min with vortexing of the lysate for 5 s every 5 min. The resultant cell lysate was centrifuged at 10,000 × *g*, for 2 min at 4ºC and the clarified supernatant used for the subsequent affinity purification. NeutrAvidin agarose slurry (500 µL) was added to a SnapCap spin column (Thermo Scientific, Rockford, IL), washed three times with wash buffer, incubated with the clarified cell lysate for 60 min at room temperature (RT) with end-over-end mixing. After centrifugation at 1,000 × *g* for 1 min, the flow-through was discarded and the beads washed three times with wash buffer. Proteins were eluted with 400 µL sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 50 mM dithiothreitol (DTT) to cleave the disulfide bridge in the biotin label. After incubation for 60 min, the proteins were collected via centrifugation at 1,000 × *g* for 2 min and then further prepared for LCMS analysis via FASP as originally described (Wisniewski et al, 2009b) using filtration units with nominal molecular weight cutoffs of 30,000 Da (Wisniewski et al, 2011). For the pre-

digestion with PNGaseF (New England Biolabs, Frankfurt am Main, Germany), cells were suspended in 1×G7 buffer containing 5,000 U PNGaseF and incubated at 37ºC overnight.

4.2.2 Sulfo-NHS-SS-biotinylation and elution with biotin

Samples were prepared as described in the preceding section, however, the SDS-PAGE sample buffer used to elute the proteins was replaced with a solution of 2.5 mM D-biotin dissolved in 50 mM HEPES pH 8.0, 150 mM NaCl, 50 mM DTT, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitors (1:1,000). After incubation and collection via centrifugation, the protein concentration was determined. An aliquot of ~20-30 µg was alkylated by adding iodacetamide to a final concentration of ~55 mM and incubation for 30 min in the dark. Samples were adjusted to a pH of 7.5 - 8.5 by adding 1 M triethylammonium bicarbonate (TEAB) and digested overnight at 37ºC using a trypsin:protein ratio of ~1:30 (w/w). The samples were then acidified with 30 % trifluoroacetic acid (TFA) and subsequently concentrated and purified by solid phase extraction (SPE) using MicroSpin columns (5-60 µg, The Nest Group, Southborough, MA). All centrifugation steps for the SPE were performed at 800-1,000 rpm for 1 min. If these parameters were not sufficient to force the liquid through the column material, centrifugation time and/or speed were slightly increased. SPE columns were activated with 200 µL methanol and equilibrated twice with 100 µL stage tip buffer (STB: 0.5 % FA, 2 % TFA). After loading the digested samples, the columns were washed with 100 µL STB buffer. Peptides were eluted with 50 µL elution buffer (0.4 % FA + 90 % ACN) and then concentrated in a vacuum centrifuge at 45ºC. For the LCMS analyses, peptides were reconstituted in 5 % FA.

4.2.3 Aminooxy-biotinylation

Cell surface sialylated glycoproteins were isolated essentially as described (Weekes et al, 2012; Zeng et al, 2009) with minor modifications. Cells (120×10^6) were washed twice with ice-cold PBS and re-suspended in 1 mM sodium meta-periodate, 10 mM aniline and 250 µM aminooxy-biotin (Thermo Scientific, Rockford, IL). After incubation at 4ºC for 30 min in the dark, the reaction was quenched by glycerine to a final concentration of 1 mM. The cell pellets were washed once each with PBS containing 5 % FCS and PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The cells were lysed in 10 mM Tris-HCl pH 7.6, 1 % Triton X-100, 150 mM NaCl, protease inhibitors (1 µL/mL), 5 mM iodoacetamide (IAA), 0.1 mg/mL PMSF at 4ºC for 30 min. Cell debris and nuclei were removed by centrifugation at 4ºC; once at 2,800 × *g* and twice at 16,000 × *g*. To isolate labeled glycoproteins, 144 µL streptavidin agarose beads (life technologies, Eugene, OR) were added to Snap Cap spin columns and incubated with the cell lysate for 2 h at 4ºC. To eliminate non-specifically bound proteins, multiple washing steps were performed (20×, 600 µL followed by centrifugation at 1,000 × *g* for 1 min). The washing was initiated with lysis buffer, followed by PBS supplemented with 0.5 % SDS and 6 M urea in 100 mM Tris-HCl pH 8.5. Further washes included higher salt solutions (5 M NaCl and 100 mM Na_2CO_3) before completion with PBS and water. The proteins were digested on the beads overnight using 2.5 µg trypsin in 50 mM NH_4HCO_3. The tryptic peptides were collected via centrifugation at 1,000 × *g* for 1 min. The beads were rinsed with 200 µL 50 mM NH_4HCO_3 and tryptic fractions pooled. Peptides were washed three times each with 300 µL PBS, water and G7 buffer (New England Biolabs, Frankfurt am Main, Germany). After incubation at RT for 5 h, glycopeptides were eluted with 15,000 units glycerol-free PNGaseF in 200 µL G7 buffer. After the first collection of glycopeptides via centrifugation at 1,000 × *g* for 1 min, the beads were rinsed with 200 µL G7 buffer and the glycopeptide fractions combined. For the LCMS analyses, aliquots of tryptic and glycopeptide fractions were pooled.

4.2.4 Colloidal silica beads

Samples were prepared as previously described (Kim et al, 2011). Cells (30×10^6 or 50×10^6) were washed three times with ice-cold MES-buffered saline (MBS: 25 mM MES pH 6.5, 150 mM NaCl), and then added to a 10 % silica bead solution. The mixture was incubated on ice and rocked gently for 10 min to allow the silica beads to attach to the cells. The cells were pelleted at 1,000 × *g* for 5 min at 4ºC and washed three times with ice-cold MBS. To crosslink the cells to the silica beads, 0.1 % polyacrylic acid (PAA) in MBS was prepared and cells added drop-wise. The samples were incubated on ice for 10 min and then centrifuged at 1,000 × *g* for 5 min at 4ºC. The resultant cell pellets were washed three times with MBS and lysed in 1 mL 250 mM sucrose, 25 mM HEPES, 20 mM KCl, 1 µL/mL protease inhibitor cocktail. Cells were re-suspended and sonicated on ice until the solution became cloudy. For the density gradient centrifugation, a discontinuous Nycodenz (Axis-Shield PoC, Oslo, Norway) gradient consisting of 1.5 mL layers of 40, 35, 30 and 27.5 % Nycodenz solutions was prepared. The cell lysate was diluted to a final concentration of 25 % Nycodenz in the sample layer and then placed on top of the prepared gradient. Lysis buffer (1 mL) was added with care to the gradient and ultracentrifugation was performed at 100,000 × *g* for 1 h at 4ºC. The supernatant was discarded and the plasma membrane pellet re-suspended in 500 µL 25 mM Na_2CO_3. After incubation with agitation for 30 min at 4ºC, the pellet was collected at 5,000 × *g* for 20 min at 4ºC and the supernatant removed. Proteins were eluted from the silica beads and solubilized with 200 µL 8 M urea buffer containing 2 mM DTT by incubation with agitation for 30 min at 37ºC. After centrifugation at 5,000 × *g* for 20 min at 4ºC, the supernatant was collected and the protein concentration determined. Proteins were alkylated with 8 mM IAA for 30 min at 37ºC and the urea concentration diluted to ~1.5 M with 100 mM NH_4HCO_3. $CaCl_2$ was added to a final concentration of 2 mM, before initiating the digestion by adding trypsin at an enzyme-to-protein ratio of 1:50 (w/w). The digest was incubated overnight at 37ºC and quenched the following day with 2.5 % TFA. For all subsequent desalting steps of the peptides, centrifugation was performed at 200 × *g*. MacroSpin columns (The Nest Group, Southborough, MA) were conditioned with 500 µL ACN and washed twice with ultrapure water. Samples were loaded onto the column, washed twice with 0.1 % TFA and eluted with 70 % ACN, 0.1 % TFA in two steps

to yield an eluate of 400 µL. The peptides were concentrated in a vacuum concentrator at 45ºC and then re-suspended in 5 % FA.

4.2.5 Alternative colloidal silica beads protocol

Since the original publication (Kim et al, 2011), Kislinger *et al.* had further optimized sections of the protocol (personal communication). Firstly, the discontinuous gradient for the ultracentrifugation step ranged from 55 to 70 % Nycodenz and centrifugation parameters were increased to 130,000 × *g* for 90 min. Secondly, to improve the removal of non-specific proteins, two 150 - 200 µL Na_2CO_3 wash steps were performed. Additionally, elution of the proteins from the beads was extended to a two-step process. In the first step, the bead-cell pellet was resuspended in 150 mM NaCl and rotated at 4ºC overnight. After centrifugation at 5,000 × *g* for 20 min at 4ºC, the remaining pellet was subjected to the second step of elution with 200 µL 25 mM HEPES, 1 % Triton X-100 and a higher salt concentration of 400 mM NaCl. This second step was incubated with agitation at 4ºC for a minimum of 1 h, and centrifuged as for the first elution. The resultant supernatants were combined and the proteins precipitated with acetone overnight at -20ºC. The protein pellet was collected by centrifugation and the acetone precipitation repeated twice. The final pellet was dried at 37ºC for 30 min, re-suspended in 100 µL 8 M urea, 100 mM Tris pH 8.5, 2 mM DTT and agitated for 30 min at 37ºC. The protein concentration was determined and the sample prepared for LCMS as described in the preceding section.

4.3 Purification and enrichment of whole membrane proteins

TX114 phase separation experiments were performed as described in (Bordier, 1981). Briefly, 30×10^6 cells were harvested, washed once with PBS, resuspended in 500 µL PBS and 100 µL 6 % pre-condensed TX114, mixed by pipetting/inversion and incubated for 15 min on ice. The samples were centrifuged for 1 min at 13,000 rpm, the supernatants were transferred to new tubes, the pellets, which correspond to the insoluble fractions, were resuspended in 200 µL SDS-PAGE sample buffer by sonication. The supernatants were incubated for 5 min at 37 °C to induce phase separation and centrifuged for 1 min at 13,000 rpm at room temperature. The upper aqueous phases were transferred to new tubes. To wash, the lower, detergent phase was mixed with 500 µL PBS, the upper phase with 100 µL 6 % TX114 and incubated for 5 min on ice and for 5 min at 37 °C. Samples were centrifuged again and the initial phases were kept for further processing.

Proteins were precipitated by adding 500 µL MeOH and 125 µL chloroform to the aqueous phases and 450 µL PBS, 500 µL MeOH and 125 µL chloroform to the detergent phases followed by vortexing. Samples were centrifuged for 4 min at 13,000 rpm, 750 µL of the upper phases was removed and 400 µL MeOH was added and mixed by pipetting. Samples were centrifuged again for 1 min at 13,000 rpm, supernatants were removed and the pellets were dried under the chemical hood. Precipitated proteins were solubilized in SDS-PAGE sample buffer by sonication and then either subjected to Western blot analysis or further prepared for LCMS via FASP as originally described (Wisniewski et al, 2009b) using filtration units with nominal molecular weight cutoffs of 30,000 Da (Wisniewski et al, 2011).

4.4 Reversed-phase liquid chromatography mass spectrometry

Peptide mixtures were separated by liquid chromatography and analyzed by collision-induced dissociation (CID) on a hybrid linear trap quadrupole (LTQ) Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to an Agilent 1200 high-performance liquid chromatography (HPLC) nanoflow system (Agilent Biotechnologies, Palo Alto, CA) via a nanoelectrospray ion source using liquid junction (Proxeon, Odense, Denmark).

Two solvents were used for the separation of digested samples: solvent A consisted of 0.4 % FA in water and solvent B of 0.4 % FA in 70 % MeOH and 20 % IPA. Samples were stored in a thermostatic microautosampler before being automatically loaded onto a trap column (Zorbax 300SB-C18 5 µm, 5 × 0.3 mm, Agilent Biotechnologies) by a binary pump at a flow rate of 45 µL/min. For loading and washing, 0.1 % TFA was used. After washing, peptides were back-flushed onto a 16 cm fused silica analytical column with an inner diameter of 50 µm packed with C18 reversed-phase material (ReproSil-Pur 120 C18-AQ, 3 µm, Dr. Maisch, Ammerbuch-Entringen, Germany). Peptides were eluted at a constant flow rate of 100 nL/min by three subsequent gradients: (1) 3 to 30 % solvent B in 27 min, (2) 30 to 70 % solvent B in 25 min and (3) 70 to 100 % solvent B in 7 min (Bennett et al, 2011).

For the fractionated samples, peptide mixtures were first separated into 10 off-line fractions using a Phenomenex column (150 × 2.0 mm Gemini-NX 3 µm C18, 110 Å, Phenomenex, Torrance, CA, USA) on an Agilent 1200 series HPLC system (Agilent Biotechnologies, Palo Alto, CA) with UV detection at 214 nm. HPLC solvent A consisted of 20 mM NH_4OH pH 10.5 in 5 % acetonitrile and solvent B consisted of 20 mM NH_4OH pH 10.5 in 90 % ACN. Peptides were separated at 35 °C with a flow rate of 100 µL/min and eluted from the column with a 41 min gradient ranging from 0 to 35% solvent B, followed by a 4 min gradient from 35 to 70% solvent B and, finally, a 2 min gradient from 70 to 100 % solvent B (Bennett et al, 2011).

The 10 fractions were then separated in a second dimension on-line using a Dionex UltiMate 3000 LC system (Dionex Corporation, Sunnyvale, CA) before being analyzed on

a hybrid quadrupole Orbitrap Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Microautosampler, trap column and analytical column parameters were the same as described before. However, the total gradient time was extended to 100 min and, in addition to the regular composition of solvent B indicated before, an alternative composition consisting exclusively of 100 % ACN was employed. Peptides were eluted at a constant flow rate of 100 nL/min using the following respective gradients:

ACN	
0 - 3 %	5 min
3 - 13 %	3 min
13 - 32 %	47 min
32 - 45 %	10 min
45 - 100 %	5 min
100 %	7 min
100 - 3 %	3 min
3 %	20 min

MeOH, IPA, FA	
0 - 3 %	5 min
3 - 13 %	2 min
13 - 35 %	43 min
35 - 70 %	12 min
70 - 100 %	5 min
100 %	10 min
100 - 3 %	3 min
3 %	20 min

Peptides were identified in a data-dependent acquisition mode using a top 15 collision-induced dissociation (CID) method. A single lock mass at *m/z* 445.120024 was chosen (Olsen et al, 2005) and selected ions were dynamically excluded for 60 s. Maximal ion accumulation times were 500 ms for MS^1 and 50 ms for MS^2 mode, respectively. To prevent overfilling of the ion traps, automatic gain control (AGC) was set to 10^6 ions for MS^1 mode and 5,000 for MS^2 mode. The threshold for switching from MS^1 to MS^2 was 2,000 counts. Peptides were detected with a resolution of 60,000 (*m/z* 400). All samples were analyzed as technical, back-to-back replicates.

4.5 Data processing and database searching

The acquired raw MS data files were processed with msconvert (ProteoWizard Library v2.1.2708) and searched against the human Swiss-Prot database (v2013.01, 37,398 sequences) with the search engines Mascot (v2.3.02, MatrixScience, London, U.K.) and Phenyx (v2.5.14, GeneBio, Geneva, Switzerland) (Colinge et al, 2003). A maximum of one missed tryptic cleavage site was allowed for the aminooxy-biotin and the silica beads approach, whilst a maximum of two missed tryptic cleavage sites was enabled for the sulfo-NHS-SS-biotin method. Initial searches were performed with relatively broad mass tolerances via a Perl script on both precursor and fragment ions (±10 ppm and ±0.6 Da, Mascot only). All precursor and fragment ion masses were recalibrated based on high-confidence peptide identifications and subsequently subjected to a second search with narrower mass tolerances (±4 ppm, ±0.3 Da). Carbamidomethylated cysteine was defined as a fixed modification, whilst oxidation of methionine residues was selected as a variable modification. For the sulfo-NHS-SS-biotin experiments, the biotin moiety conjugated to lysine residues and protein N-termini was added as a variable modification. Release of *N*-linked glycopeptides by PNGaseF treatment deamidates asparagine residues to aspartic acid. This was set as additional variable modification as required. FDRs of <1 % and <0.1 % were determined for proteins and peptides, respectively, by applying the same procedure against a reversed database.

UniProtKB/Swiss-Prot Gene Ontology cellular component (GO.CC) annotations of the identified proteins were retrieved via BioMart (Guberman et al, 2011). For the prediction of transmembrane topology and signal peptides, FASTA file formats of proteins were downloaded from UniProtKB/Swiss-Prot and then subjected to Phobius at http://phobius.sbc.su.se/index.html (Kall et al, 2004; Kall et al, 2007).

Data are means of two biological replicates from different experimental days unless otherwise stated. Abundance data of multiple isoforms were averaged.

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6. CV

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Date of birth: 21-Jan-1988 in Munich, Germany

Education:

since October 2012
PhD studies: Medical Informatics, Biostatistics & Complex Systems, Medical University of Vienna & CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences

October 2010 until September 2012
Master: Genetics, PLU Salzburg (Final grade: pass with distinction)

October 2007 until August 2010
Bachelor: Pharmaceutical Sciences, LMU Munich (Final grade: 2.11)

September 1998 until Juni 2007
Rupprecht-Gymnasium, Munich
Graduation: High-school diploma of European secondary school (Final grade: 1.0)

Scholarships:

December 2012
merit based scholarship by the University of Salzburg

since June 2010
scholarship holder of *e-fellows.net*

January 2008 until September 2012
scholarship holder of the German National Academic Foundation

Languages:

English: CEFR Level C2
French: CEFR Level B2
German: mother tongue
Italian: CEFR Level C1
Spanish: CEFR Level B2
Swedish: CEFR Level A2

IT-skills:	MS Office (expert) R, Adobe Illustrator, GraphPad Prism (advanced) Linux, Python, Java (basic)
Selected courses and workshops:	July until August 2013 Case-based introduction to Biostatistics, Johns Hopkins University
	02-Mar until 06-Mar-2013 13th Spring School of Bioinformatics for Molecular Biologists, Helmholtz Zentrum, Institute for Bioinformatics and Systems Biology
	17-Nov until 18-Nov-2011 Technik^{Business} workshop, Collège des Ingénieurs, Paris
	07-Sep until 09-Sep-2011 Entrepreneurial training using the business game TOPSIM GENERAL MANAGEMENT II, Roche Diagnostics GmbH, Penzberg
	07-Sep 2008 until 20-Sep-2008 Gene therapy of multifactor diseases, Head: Prof. Dr. med. Thomas Pap, Department of Medicine, Institute of myoskeletal medicine, WWU Münster, German National Academic Foundation, Olang
Academic conferences:	09-Aug until 13-Aug-2015 BioMedical Transporters 2015, Membrane Transporters – from basic science to drug discovery, Lugano
	24-Apr until 29-Apr-2015 Keystone Symposium on the Human Proteome, Stockholm
	20-Jul until 25-Jul-2014 Gordon Research Conference on Protein Processing, Trafficking & Secretion, New London (NH)
Publications:	Hörmann et al., A surface biotinylation strategy for reproducible plasma membrane protein purification and tracking of genetic and drug-induced alterations, Journal of Proteome Research, 2016
	Schultz et al., Evaluation of drug-induced neurotoxicity based on metabolomics, proteomics and electrical activity measurements in complementary CNS in vitro models, Toxicology in Vitro, 2015
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