Unraveling Sterol-dependent Membrane Phenotypes by Analysis of Protein Abundance-ratio Distributions in Different Membrane Fractions Under Biochemical and Endogenous Sterol Depletion*

Henrik Zauber‡, Witold Szymanski‡, and Waltraud X. Schulze‡§¶

During the last decade, research on plasma membrane focused increasingly on the analysis of so-called microdomains. It has been shown that function of many membrane-associated proteins involved in signaling and transport depends on their conditional segregation within sterol-enriched membrane domains. High throughput proteomic analysis of sterol-protein interactions are often based on analyzing detergent resistant membrane fraction enriched in sterols and associated proteins, which also contain proteins from these microdomain structures. Most studies so far focused exclusively on the characterization of detergent resistant membrane protein composition and abundances. This approach has received some criticism because of its unspecificity and many co-purifying proteins. In this study, by a label-free quantitation approach, we extended the characterization of membrane microdomains by particularly studying distributions of each protein between detergent resistant membrane and detergent-soluble fractions (DSF). This approach allows a more stringent definition of dynamic processes between different membrane phases and provides a means of identification of co-purifying proteins. We developed a random sampling algorithm, called Unicorn, allowing for robust statistical testing of alterations in the protein distribution ratios of the two different fractions. Unicorn was validated on proteomic data from methyl-β-cyclodextrin-treated plasma membranes and the sterol biosynthesis mutant smt1. Both, chemical treatment and sterol-biosynthesis mutation affected similar protein classes in their membrane phase distribution and particularly proteins with signaling and transport functions. Molecular & Cellular Proteomics 12: 10.1074/mcp.M113.029447, 3732–3743, 2013.

From the ‡Max Planck Institute of molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany; §Department of Plant Systems Biology, University of Hohenheim, Garbenstraße 30, 70599 Stuttgart, Germany

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1 The abbreviations used are: DRM, detergent resistant membrane fraction; DSF, detergent soluble fraction; SP, soluble protein fraction; IM, intracellular membrane fraction; PPI, protein-protein interaction mβcd methyl-β-cyclodextrin; FDR, false discovery rate; Llo, phase liquid ordered phase; Lld, phase liquid disordered phase.
mains (10–12), research on protein-sterol interactions is possible by usage of sterol depleting agents like methyl-β-cyclo-dextrin mjcd (13). Therefore mjcd is suitable for detecting false positive cholesterol protein interactions in DRM studies (14–19). Proteins depleted on mjcd treatment are finally considered to be sterol dependent (15–17). To compare the mjcd treatment for disturbing the sterol distribution in the Lₐ fraction, we studied the sterol biosynthesis deficient mutant smt1. (20) smt1 carries a point mutation in the smt1 locus, encoding the sterol methyltransferase 1 and it exhibits a dwarf-like phenotype on whole plant level (20). In total, three sterol methyltransferases are encoded in Arabidopsis where SMT1 catalyzes the first step in the sterol biosynthesis by adding a methyl group at C24 of the sterol precursor cycloartenol. SMT2 and SMT3 act at later steps and were shown to be functionally redundant as C-24 sterol methyltransferases at SMT2 and SMT3 act at later steps and were shown to be functionally redundant as C-24 sterol methyltransferases at the branching in sterol synthesis that either leads to sitosterol or campesterol (21). The total sterol composition in the branching in sterol synthesis that either leads to sitosterol or campesterol (21). The total sterol composition in

The treated PM pellet was recovered and resuspended after sample dilution and ultracentrifugation. (15) Protein content in PM fraction was determined by Bradford assay (25). All samples were adjusted to equal protein amounts of 100 μg. All PM fractions were mixed for 30 min with TritonX-100 at a protein/detergent ratio of 1:15 with final detergent concentration being diluted below 1%. (15) Detergent resistant membranes (DRM) and a detergent soluble fraction (DSF) could be separated in a sucrose gradient (1.8 mM; 1.6 mM; 1.4 mM, and 0.15 mM) using ultracentrifugation at 150,000 g for 18 h. From the four collected fractions, DRM, DSF, SP, and IM proteins were extracted using methanol/chloroform extraction. An overview of the sample preparation is provided in Fig. 1.

Proteomeic Analysis—Extracted proteins were dissolved in 6 M urea, 2 M thiourea, pH 8 and protein concentration of SP and IM fractions determined. Total protein content from DRM and DSF fraction and 75 μg of protein from SP and IM fraction was reduced, carbamidomethylated (26) and subsequently digested with LysC for 3 h at room temperature. Afterward, samples were diluted four times with 2 mM Tris-HCl pH 8 and trypsin was added for overnight digestion at room temperature (27, 28). The reaction was stopped by adding trifluoroacetic acid to reach a pH of around 2. Tryptic peptides were desalted over C18 Stop and Go Extraction tips (Empire Disk; Varian Inc, Palo Alto, CA) (29).

LC-MS/MS Analysis and Protein Identification—Injections containing 25 μg of protein were analyzed by LC-MS/MS using nano-flow HPLC (Easy nLC, Thermo Scientific, Waltham, MA) and an Orbitrap hybrid mass spectrometer (LTQ-Orbitrap XL, Thermo Scientific, Waltham, MA) as mass analyzer. Peptides were eluted from 75 μm analytical column (Reposil C18, Dr. Maisch GmbH) on a linear gradient, running from 5 to 80% acetonitrile in 90 min at a flow rate of 250 nl/min. Up to five data-dependent MS/MS spectra were acquired in the linear ion trap for each FTMS full-scan spectrum, acquired at 60,000 full-width half-maximum resolution settings with an overall cycle time of ~1 s and precursor mass tolerance of 10 ppm. Raw file peak extraction, protein identification, and quantitation of peptides was performed by MaxQuant (30) (version 1.3.0.5) using a protein sequence database of Arabidopsis thaliana (TAIR10, 35386 entries, www.arabidopsis.org). For protein identification, carbamidomethylation and N-terminal protein acetylation were used as fixed modifications and methionine oxidation as a variable modification. Multiplicity was set to 1 for label-free quantitation. Standard settings in MaxQuant involving peptide false-discovery rate of 0.01, minimum peptide length of 6 amino acids, MAMS mass tolerance of 0.5 Da, two maximal allowed missed cleavages, and enabled retention time correlation, using a time window of 2 min were used. The full list of all identified peptides in all replicates and treatments can be downloaded as supplemental Table S2.

Calculation of Label-free Protein Intensities—Peptide lists derived from MaxQuant (evidence.txt) were directed to cRacker (31) analysis for normalization between samples and for merging peptide intensities to protein intensities using razor protein identifications from MaxQuant. Peptides, which were quantified in less than 70% among all fraction specific samples, were filtered out. The principal steps of the reference protein normalization were: (1) All peptide intensities within each sample were normalized to fraction of total ion-intensity sums. (2) To compensate effects of normalization because of number of detected peptides per samples, intensity was proportionally corrected to reciprocal number of identified peptides in each sample using the integrated normalization option in cRacker (31). (3) Peptide intensities with missing values in more than the permitted numbers of samples within replicates of each fractions were filtered out. (4) Remaining normalized peptide intensities were median scaled and me-
Analysis of Protein Distribution by Random Sampling Algorithm

**Fig. 1. Scheme of fractionation workflow starting from suspension culture cell material on the left and the Unicorn algorithm for statistical testing of ratios on the right.** The four fractions soluble proteins (SP), intracellular membranes (IM), detergent resistant fraction (DRM) and detergent soluble fraction (DSF) were collected and subjected to proteomic analysis. The IM and plasma membrane (PM) fraction were separated using two-phase system (24). PM was optionally treated with mβcd and after TritonX 100 treatment the fractions DRM and DSF could be separated in a sucrose density gradient. Calculated protein intensities were directed to the bootstrap based algorithm Unicorn for analyzing DRM/DSF protein intensity ratios. Protein specific scores were calculated by multiplying counts of p values ≤ 0.05 with log2 transformed ratios (log2 (ratio)). A significance threshold was determined by randomizing p value and ratio tables using a false discovery rate (FDR) ≤ 1%. The sterol-dependent protein candidates could be determined after filtering out IM and SP abundant fractions using results from t test based pairwise comparisons from cRacker (31).

**RESULTS**

**Reproducibility of Unicorn Results**—The algorithm Unicorn was developed to enable statistical testing of protein abundance distributions between two fractions, here DRM and DSF membrane subcompartments. For proving reproducibility of the results obtained from the Unicorn algorithm, effects of mβcd treatment and smt1 analysis was done in R by calculating pairwise Pearson correlation coefficients using the function “cor” supplied by the R base distribution. For a detailed comparison, responding candidate proteins were manually assigned to functional protein groups based on protein descriptions supplied in TAIR (35).

**Supplemental File S1.**

Bootstrap Approach for Analysis of DRM/DSF Abundance Ratios—We developed a bootstrap based algorithm, named Unicorn, to statistically test differences between abundance ratios between DRM/DSF fractions in wild type compared with DRM/DSF abundance ratios on mβcd treatment or in the sterol-biosynthesis mutant smt1. Unicorn uses preprocessed protein intensities, relative standard deviation and protein specific counts of analyzed peptides from cRacker files “proteinlist.csv,” “proteinlist-sd.csv,” and “n-protein.csv”). In Unicorn, the first step involves a random generation of an ion-intensity distribution matching the median, standard deviations (DRM sd + DSF sd) and numbers of analyzed peptides (n1 + n2) per protein of all measured DRM/DSF protein intensity ratios. Second, the two resulting pools of ion-intensity ratios from wildtype and smt1 mutant or mβcd treatment were subjected to pairwise t-testing (Fig. 1). To control variability in using such a stochastic approach, the analysis was iterated 10 times and p values were collected. A score for each protein was calculated by multiplying number of positive tests (α = 0.05) with the log2 transformed abundance ratio of a protein. A threshold score indicating significantly different ratios at a false discovery rate lower than 1% was calculated using randomized p value and ratio matrices for each experiment. Protein candidates were filtered for DRM/DSF specific proteins using cross comparisons with IM or SP fraction (t test; α = 0.01). The full Unicorn algorithm was written in R and is designed to work semi-automatically with cRacker output. The R scripts of Unicorn can be provided on demand.

Statistical Analysis of Sterol-dependent Protein Candidates—Statistical analysis was performed using free available scripting language R (32). All experimental data are based on three biological replicates and up to three technical replicates. For testing the efficiency of filtering co-purifying proteins as well as for analyzing protein subcellular localization distribution, information on subcellular location was derived from SUBA3 (33) and functional information was annotated based on MapMan functional categories (34). A correlation analysis of protein abundances in DRM and DSF in replicates from mβcd and smt1 analysis was done in R by calculating pairwise Pearson correlation coefficients using the function “cor” supplied by the R base distribution. For a detailed comparison, responding candidate proteins were manually assigned to functional protein groups based on protein descriptions supplied in TAIR (35).
proteins were overlapping in mβcd treatment and smt1 respectively (Figs. 2C, 2D). Proteins were considered as responding to mβcd or smt1 (sterol-dependent proteins) if their distribution between DRM and DSF was significantly different from untreated control or wild type (FDR ≤ 1%). Across all iterations, the overlap of unique proteins between the iterations is smaller but still, the largest fraction of commonly identified responding proteins is significant in all iterations (Figs. 2E, 2F). The number of nonoverlapping proteins between iterations is related to the stochastic nature of random sampling. Therefore, some ratio comparisons expressing lower scores, can by chance fall below the stringent score threshold. If a less stringent analysis needs to be applied we propose running several iterations to identify the proteins in this lower scoring range. However, with increasing number of iterations, false positives are also likely to accumulate. Therefore, only one random sampling iteration was applied for analyzing mβcd and smt1. Comparing the score distributions from mβcd treatment and smt1, more proteins in the lower scoring range were positively tested in smt1, explaining the slightly higher number of differentially abundant proteins in total for smt1. Without including prior information, the ratio-based testing algorithm does not allow to distinguish whether effects mainly in DRM or DSF caused the distribution differences. Because we can assume that on the mβcd treatment as well as in the smt1 mutant the observed alterations in protein distributions were especially induced effects on sterol composition of membranes, in the following sections we will interpret changes in DRM/DSF ratios as especially induced by changes in abundance in the sterol enriched DRM fraction.

**Significant Alterations in DRM/DSF Distribution Ratios On mβcd Treatment and in smt1 Mutant**—In total, 3028 proteins were quantified, out of which distribution ratios between DRM and DSF were compared for 1184 proteins in mβcd treatment versus control, and 1788 in the smt1 versus wild type. Several proteins showed a significantly depleted DRM/DSF ratio both on mβcd treatment and in the smt1 mutant (Fig. 3). On mβcd treatment 99 proteins and in the smt1 mutant 108 proteins were significantly depleted from DRM compared with untreated control or wildtype (Fig. 3, red triangles). Two hundred and seventy-four proteins in mβcd treatment and in the smt1 mutant (Fig. 3, blue triangles) were significantly depleted from DRM compared with untreated control or wildtype (Fig. 3, blue triangles), whereas only 37 (mβcd) and 32 (smt1) proteins were significantly enriched in DRM fractions, as concluded from their significantly higher DRM/DSF ratio relative to the controls (Fig. 3, red triangles). Two hundred and seventy-four proteins in mβcd comparisons and 353 proteins in smt1 comparisons were excluded from the further analysis, because of significantly higher abundances in soluble protein fraction (SP) or intracellular membrane (IM) fractions based on pairwise comparisons of all fractions (t test, α = 0.01). The number of such contaminants being enriched in DRM fraction of smt1 was with 238 proteins up to two times higher than in the mβcd treatment. The coefficient of correlation between treated and untreated samples was slightly higher in mβcd treatment in comparison to smt1. Considering that in the mβcd treatment both untreated and mβcd-treated plasma membranes are extracted from the same sample aliquot, a higher correlation between
non-responding proteins in treated and untreated sample is expected. Therefore, the slightly weaker correlation in smt1 distributions is most likely related to the increased biological variation by doubling the number of analyzed individuals from smt1 and the wild-type control. In contrast, a correlation analysis between combined biological and technical replicates showed a higher correlation of Unicorn ratio scores between DRM preparations of smt1, suggesting detection of highly reproducible protein distribution changes in samples of the smt1 mutant (supplemental Fig. S1).

In total, 31 DRM-depleted proteins were overlapping between mβcd and smt1 comparisons, whereas among the DRM-enriched proteins, only two proteins were overlapping (Fig. 4A). However, when comparing functional classification of DRM depleted and enriched proteins in the two treatments affecting sterol composition, the overlap between the biochemical mβcd treatment and genetic mutation in smt1 is considerably higher (Fig. 4B). In total, 27 protein functional groups (blue circles) exhibited a depletion on altered membrane sterol composition in both experiments (Fig. 4B). Largest overlap of identified protein functional groups was observed for the two controls, untreated plasma membrane and untreated wild type with a high fraction of individual proteins in each functional group. In contrast, for other overlapping proteins, the number of proteins was close to the number of functional groups defined. Thirty-eight proteins assigned to two protein functional groups (14 protein kinases; 28 proteins of unknown functions) displayed DRM depletion as well as enrichments on individual protein basis in mβcd or smt1 experiments (Fig. 4B). The largest fraction of overlapping

Fig. 4. Venn diagrams of proteins (A) and protein functional groups (B) found to be DRM enriched (red) or depleted (blue) on mβcd treatment or in smt1 mutant. From 231 proteins with altered DRM/DSF distribution ratio, in total 76% where depleted from DRM fractions in both experiments whereas the total overlap between mβcd treatment and smt1 mutant was low with 31 proteins (13%). The count numbers of individual proteins and the number of functional protein groups (B). Most protein functional groups being depleted from DRM on mβcd treatment and in smt1 fall into few groups with many proteins per group.
proteins was depleted in response to both sterol modifying strategies and includes 41 proteins from 15 protein functional groups such as ATPases, fasciclin-like arabinogalactan proteins, cupredoxins, or remorin. Interestingly among overlapping protein functional groups, many proteins were enriched on \( m\beta cd \) treatment but depleted in \( smt1 \) (Fig. 4B).

Efficient Removal of Copurifying Proteins by Pairwise Analysis of Fractions—Several responding proteins appeared to be mainly copurifying proteins from IM or SP fractions and could be successfully filtered out (Fig. 5). Thereby, responding proteins were excluded from all samples if they showed a significant higher abundance in IM or SP fractions based on pairwise \( t \) test \( (p < 0.01) \). To check specificity and efficiency of the removal of copurifying proteins in DRM and DSF fractions based on their abundance profile to IM and SP, proteins were mapped against the \( A. thaliana \) specific subcellular localization database SUBA3 (33). General protein representations of subcellular compartments were similar in \( m\beta cd \) and \( smt1 \) experiments. Indeed, most of the proteins with established cytosolic location and subcellular localizations to plastid, nucleus, mitochondrial, and endoplasmic reticulum were filtered out, as indicated by a high fraction of SP and IM proteins for these compartments. However, a large number of copurifying proteins with high abundance in SP and IM was not yet assigned to any subcellular location. The highest number of copurifying proteins was found in \( smt1 \) DRM and was of cytosolic origin or unassigned localization.

Sterol-dependent Proteins Have Major Functions in Signaling and Transport—Functional categories of all proteins with altered DRM/DSF distribution were analyzed based on MapMan functional categories (36). In total, 29 functional categories out of 59 were affected commonly in \( m\beta cd \) treatment and \( smt1 \) mutant. Furthermore, vesicle transport and lipid metabolism associated proteins were responding in both \( m\beta cd \) treatment and \( smt1 \) mutant, but referred to equal numbers of enriched and depleted proteins on treatment. Major differences between \( m\beta cd \) treatment and \( smt1 \) were observed with respect to the representations of transport related bins. Particularly in \( smt1 \) many categories of transporters, such as four major intrinsic proteins, two calcium and one potassium transporters (supplemental Fig. S2) were strongly depleted from DRM in addition to proteins from the ATPase protein class. In contrast, \( m\beta cd \) treatment resulted in a stronger retainment of transport proteins in DRM fractions compared with \( smt1 \) mutant.

The removal of all protein synthesis related proteins through the subcellular location filter (Fig. 5) efficiently cleaned the functional categories of sterol-dependent proteins from typical copurifying contaminants in DRM preparations. However, still a small number of potential contaminants remained to be in the list of responding proteins. For instance, three proteins assigned to protein synthesis functions were still considered as depleted from DRM in \( smt1 \) (supplemental Fig. S2). Nevertheless, given the high number of copurifying contaminations in \( smt1 \) the filtering process was efficient and remaining “contaminants” may also be attributed to errors in functional categorization. For instance remorin, one of the most dominant protein microdomain markers in the plasma membrane of plants, is functionally still assigned to the “RNA.regulation of transcription” in MapMan and mistakenly predicted to be localized in the nucleus in SUBA3 (status November 2012). Because functions of many proteins in Arabidopsis are experimentally uncharacterized, databases such as MapMan or
SUBA3 remain to contain some degree of inaccuracies. In addition we found, that proteins unassigned to functions and location represented the largest category among proteins responding to altered sterol environments. Therefore, a more detailed analysis based on hand-curated protein descriptions was carried out. Proteins with significant changes in DRM/DSF ratios on mβcd treatment or in smt1 were manually assigned to functional groups using description information provided by The Arabidopsis Information Resource (TAIR) (35) and Uniprot (37). Altogether, 119 groups were defined (supplemental Table S1) out of which 44 groups were selected for further analysis containing a minimum of two representing proteins. These groups included 73% of all responding proteins (Fig. 6). For most protein functional groups, consistent strong depletion from DRM could be observed for mβcd treatment as well as in the sterol-biosynthesis mutant smt1. Particularly, ATPases, protein kinases, leucine-rich repeat kinases, and glycosyl hydrolases (Fig. 6) were depleted from DRM on alterations of the sterol environment. Also, many protein functional groups previously described of being sterol-dependent were depleted in both mβcd treatment and smt1. Most prominent examples are remorins, SPFH protein family, cupredoxins (SKU), fasciclin (FLA), ATPases, and ABC transporters. (3, 15, 38–46) Some proteins showed an increased DRM/DSF ratio on alterations of the sterol-environment, with 20 protein functional groups being affected in mβcd treatment but only 11 in smt1. Additionally, three DRM relevant groups including major intrinsic protein family and receptor-like kinases were depleted in smt1 but not responding or even DRM-enriched on mβcd treatment. In contrast three protein functional groups, namely clathrin, calcium-binding EF hand family proteins, and ENTH domain-containing proteins, were depleted on mβcd treatment but enriched or not responding in smt1.

**DISCUSSION**

By analyzing DRM/DSF protein intensity ratio distributions on mβcd treatment and in the sterol-biosynthesis mutant smt1, we could stringently define DRM specific proteins using label-free proteomics. Plasma membrane microdomains are likely to be of diverse nature, exhibiting altering substable Lc states (9, 47–52). Therefore, the DRM/DSF protein abundance ratios can better reflect global changes in protein-sterol interactions and account for total protein abundance changes compared with studying abundance changes in DRM only. Although the applied DRM preparation protocol is based on enrichment of membranes and proteins by taking advantage overall depletion from DRM on mβcd treated and in smt1 mutant. In both experiments, most of published microdomain markers like remorin or members of the SPFH family proteins were depleted from the DRM fraction. Largest fractions of depleted protein functional groups are of unknown function, ATPases, protein kinases, ABC transporters or receptor-like kinases. Specific protein functions were found to be enriched in mβcd treated DRM.
of their different physicochemical properties, most plasma membrane proteins are still present in both DRM as well as DSF fractions. Examples for stimulus-driven protein-sterol interactions and associated changes in DRM/DSF abundance ratios were described for the flagellin receptor FLS2 and the ion channel SLAH3. FLS2 was shown to be recruited to DRM fractions on stimulation of cells with flagellin (1). Also for SLAH3 and its kinase CPK21 an ABA-stimulus-dependent recruitment to sterol-rich environments was demonstrated which even involved post-translational modification (53).

**Random Sampling as a Suitable Scoring Method**—The segregation of plasma membrane proteins between DRM and DSF is not absolute. Moreover, most of proteins can be detected in both fractions. Even though microdomains can be short lived dynamic structures, a measurable steady state equilibrium of proteins interacting with the $L_o$ or $L_d$ phase (9, 47, 51, 54) is likely to exist, reflecting the strength of specific protein-sterol interactions. Applying label-free quantitation on proteomic data, one way to express this steady state distribution of plasma membrane protein between $L_o$ and $L_d$ phases is to use abundance ratios of two fractions. With the Unicorn algorithm, we developed a systems biology based approach for robust testing of ratio distributions between two fractions. Unicorn score values are useful for expressing alterations in sterol-protein interactions, but in general could be applied to other systems in which distributions of proteins between compartments needs to be quantified. Advantage of Unicorn algorithm over log$_2$ fold comparisons of ratios lays in the integration of variance information into the statistical analysis, which raises robustness of statistical analysis and allows identification of significant alterations lower a strictly set log$_2$ fold threshold. Further, score distributions from Unicorn, were highly reproducible showing that bias related to the conversion of ratios to simulated intensities is minimal. Proteins not repeatedly identified as significant may therefore result from randomized data used for defining FDR-controlled Unicorn-score thresholds. Even though the number of such single protein occurrences was shown to be low, repetitive rounds of the running algorithm allow for specific selection of proteins repeatedly identified as significant. Future applications for score-based analysis of DRM/DSF protein abundance ratios can be envisaged in time series experiments analyzing DRM protein dynamics for example on specific stress treatments. Although plasma membrane dynamics are likely to be closely related to cell wide changes in protein abundances, future experimental designs and algorithms need to integrate multiple biological information levels such as inputs from different protein fractions as shown in this study.

**Efficient Characterization of Sterol-dependent Proteins by Abundance Distributions**—Label-free proteomic approaches gained in importance besides isotopically labeled experiments because of their cheap and simple experimental setup. Nevertheless, the major drawback is that analysis has to be performed on relative abundances and dependent on sample complexity, relative abundance values must not always correlate with the absolute ones. Therefore, label-free analyses of DRM protein abundances only might be biased by normalization and will always be influenced by total abundance changes of respective proteins. The analysis of abundance ratios of protein comparing DRM and DSF fractions reflects the transient and heterogenous nature of sterol-protein interactions. Nevertheless, even conclusions of DRM compositions based on DRM/DSF ratios require control experiments for determining false positives and defining copurifying contaminants. These contaminants can originate from soluble proteins that are trapped in microsome vesicles or from impurities during plasma membrane purifications by aqueous two-phase system preparations (24). Up to 5% (55–57) of proteins purified with plasma membrane preparations were shown to be from other co-purifying membranes or soluble proteins. For example, ribosomes have a very similar density to DRM and are highly abundant cellular components. Therefore, they are the most frequent contaminants within DRM fractions. One way to reduce co-purifying proteins could involve more purification steps (58), but this is usually accompanied by an overall loss of total protein yield. On the other hand, as demonstrated in this study, by co-analysis of intracellular membrane (IM) fractions and soluble protein (SP) fractions, a large number of responding proteins could be unambiguously identified as copurifying proteins from IM or SP and thus were excluded from further considerations in the biological context applied. Our final set of sterol-dependent proteins on m$jcd$ treatment was defined by analyzing protein functional groups, in large parts matches with other reported lists of sterol-dependent proteins (15, 38, 59, 60). Apart from several proteins involved in transport and signaling, known microdomain residents such as remorin, SPFH proteins, and fasciclins could consistently and reproducibly be defined as sterol-dependent. We particularly compared the list of sterol-dependent proteins identified with the Unicorn algorithm to two other strategies for determining sterol-dependent proteins: a combination of one-dimensional and two-dimensional gel electrophoresis combined with mass spectrometry for protein identification (61, 62) and metabolic labeling using $^{15}$N isotopes of DRM and m$jcd$ treatment as a control (15). Borner et al. (2005) identified 45 DRM-dependent proteins using total membrane protein as a reference to define DRM specificity of proteins. Kierszniewska et al. (2009) defined 38 DRM-dependent proteins by testing for sterol dependent coreduction in protein abundance on m$jcd$ treatment. Minami et al. (2009) identified 96 proteins in DRM fractions from which 60 proteins showed differential alteration in protein abundance on cold treatment. According to the stress specific focus of the letter study, no classic control was used to reduce contaminants in the DRM fractions. Therefore, for comparing DRM dependent protein classes we will refer to the total number of DRM dependent observations only. Using the ratio dependent analysis of DRM/DSF abundances, we could identify at least two times...
more DRM-dependent proteins being depleted in mbdcd treatment or in the smt1 mutant (Fig. 7A) when comparing our dataset with the studies from Borner et al. and Kierszniowska et al., whereas the number of identified proteins was comparable to the study of Minami et al. Nevertheless it needs to be pointed out, that the comparison with the Minami data set is biased, because no comparable filtering of contaminants was applied. The overlap across all four treatments shows that up to 40% of sterol-dependent proteins identified using Unicorn algorithm overlapped with both published datasets from Borner et al. and Kierszniowska et al. In contrast, the data sets from Borner and Kierszniowska overlapped only with five proteins. Again, the Minami data set shared a higher number of observed proteins in the DRM fraction with the Unicorn analysis, whereas the relative fraction to the total number of proteins is considerably lower (17%). The observed differences between all independent DRM studies might be partly attributed to different experiment specific analysis strategies including applied software tools and algorithms (search engine, application of retention time correlation, handling of protein unique peptides). In addition the applied methods might have an impact on the availability of observable proteins, but because the overlap among comparable methods was not significantly different, the variance among data sets related to specific technical differences in methodology seems to be small. In addition, functional redundancy between protein orthologs, as well as high specificity of protein lipid interactions to particular cell specific environments can result in low reproducibility between different DRM data sets.

In order do increase comparability, a less stringent comparison between different experiments should be based on protein functional groups (Fig. 7B). In this study, more than 80% of the sterol-dependent protein functions defined by Borner et al. and Kierszniowska et al. could be confirmed with our ratio distribution approach. In contrast, only 36% of unfiltered DRM identifications from the Minami dataset could be confirmed. Because the Minami data set is unfiltered against contaminants the lower overlap could be explained by remaining DRM unspecific proteins that were not significantly identified as DRM dependent with the Unicorn algorithm. Overall, the overlap of proteins among the analyzed datasets confirm the specificity and applicability of the Unicorn algorithm to identify sterol-dependent proteins. Thereby, the better performance of this study can be further attributed to the use of protein presence in DRM and DSF fractions for defining DRM dependent proteins, rather than analyzing the protein presence from the DRM fraction alone.

DRMs are likely to relate to a mixture of different microdomains with various specific protein compositions (50). This can make the analysis using detergent resistance of sterol lipid clusters just like scratching at the tip of an iceberg, seeing only the very abundant interacting proteins. A recent study introduced a new technique for targeted analysis of sterol interacting proteins using photoreactive cholesterol probes in human cell lines (63). This method could well complement the strategy introduced here and might increase the specificity of sterol dependent protein identifications. However, the chemoproteomic strategy has some drawbacks as

![Venn diagrams of sterol-dependent unique proteins](A) and protein functional groups (B) from different approaches for definition of sterol-dependent proteins. DRM specific responding proteins (blue) from ratio based DRM/DSF analysis were compared with DRM analysis using combinatorial 1D and 2D gel electrophoresis combined with mass spectrometry based proteomics (Borner et al. 2005 in black; Minami et al. 2009 in green) and a proteomic approach including isotopic metabolic labeling with 15N using false discovery controlled thresholds for defining significant protein intensity ratios (red; Kierszniowska et al. 2012). Numbers denote unique protein counts in compared samples and the associated unique protein functional group counts.
well, especially regarding the research of sterol dependent proteins in plant systems. Considering the sterol diversity in plants, such a method requires new synthesis of several different sterol probes and tedious evaluation for plant systems, because cholesterol is only a minor sterol species in plants. Nevertheless, using photoreactive sterol probes is a promising strategy for robust confirmation of sterol-protein interactions and an alternative to cross-linking strategies (48).

Altered Sterol–Protein Interactions in Sterol-biosynthesis Mutant smt1—With the finding that the ratio based analysis based on Unicorn is capable for analysis of sterol-dependent proteins, we expanded the study of protein–sterol interactions to the sterol-biosynthesis mutant smt1. smt1 had a similar DRM/DSF protein distribution as resulted from mβcd treatments with most proteins being depleted from DRM. In conclusion the smt1 mutant seems to exhibit a phenotype of a constitutive-sterol depleted plasma membrane proteome. This finding is supported by reported reduced levels of several major plasma membrane sterols like sitosterol, stigmasterol and brassicasterol in smt1 mutant (21, 64). However, other minor sterol species like cycloartenol, isofucosterol, and cholesterol accumulate in smt1.

Additional to its structural function in membranes, sterols were shown to be involved in signaling (13, 65–67), with the phytohormone brassinosteroid as the main signaling active sterol in plants (21, 66, 68). Brassinosteroids influence plant growth and development and regulate cell division and elongation (68). Since smt1 is impaired in early sterol synthesis steps, brassinosteroid synthesis could be affected as well and therefore lead to imbalances in brassinosteroid signaling (21). Dwarf phenotypes of sterol synthesis mutants could not be rescued if mutants were impaired in synthesis steps upstream of the substrate 24-methylenelophenol including smt1 (21, 66, 68). Therefore the observed phenotype in smt1 is likely not to be a major result of alterations in brassinosteroid signaling. Additionally the impact of brassinosteroid signaling on undifferentiated callus systems as used in this study is presumably lower in comparison to fully differentiated plants. Consistent with our observations, smt1 callus was reported to be actively dividing (64) without showing strong impairments in growth as was described for fully differentiated smt1 plants (20). Therefore, a callus system was used in this study for minimizing effects caused by unbalances in brassinosteroid signaling processes and to provide on the other hand sufficient amounts of material for plasma membrane preparations. Even though additional alterations in cell signaling and proposed sterol signaling probabilities (21, 66, 69) might still contribute to the phenotype of smt1 even in callus system, for studying cell mechanistic relationships between sterols and proteins in the plasma membrane, smt1 remains to be an useful target for studying protein sterol interactions by taking advantage of the endogenous altered sterol levels.

It remains unclear how sterol sorting processes (70–72) and endo-/exocytosis are also affected and it is unknown to which degree the remaining sterol species in smt1 are efficiently incorporated into the plasma membrane. Our findings suggest that either depletion of sterols in general or altered sterol compositions in the plasma membrane inhibit efficient protein-sterol interactions in smt1. The twofold higher number of copurifying proteins in the mβcd DRM fraction in comparison to mβcd treatment supports this finding of true DRM relevant proteins being much lower abundant than the copurifying contaminants. Furthermore, this also indicates that the membrane trafficking machinery may be severely affected in smt1. Thus, plasma membrane proteins tended to accumulate in other fractions such as IM and SP. Even though the loss of smt1 function shows a pleiotropic whole plant phenotype (20, 21, 64, 73), a ratio based analysis, using the Unicorn algorithm, allows to draw conclusions specifically on protein-sterol interactions. General protein abundance levels, membrane trafficking and internalization processes of proteins might be affected, but analyzing DRM and DSF fractions in parallel, focused the analysis on to the relative distribution of plasma membrane incorporated proteins. Although a single fraction based analysis might be significantly affected by general changes in plasma membrane protein abundances, the ratio based analysis can compensate this effect and makes proteomic characterization of DRM composition changes in sterol mutants possible.

We propose the sterol biosynthesis mutant smt1 as an additional system for membrane microdomain research to mβcd treatment. The mutant smt1 showed a better correlation of protein ratios between individual DRM preparations, whereas mβcd treatment seems to result in lower reproducibility between the DRM fractions but this did not affect DRM/DSF protein abundance distributions (Fig. 3). Furthermore, only a small number of proteins related to signaling or transport was found to be in smt1 DRM fractions, whereas in mβcd this number was much higher. Although it is possible that these proteins, following the usual mβcd analysis strategy, are false positives, we cannot exclude that this enrichment of specific protein classes is an artificial effect of the sterol depleting mechanism of mβcd itself. It has been shown that mβcd dissolves sterol clusters from the side (16), and additionally mβcd removes only a fraction of plasma membrane sterols (16), with microdomain centers likely to be unaffected. The remaining proteins in these central clusters would finally be considered as enriched. Therefore, instead of defining an enriched protein in the microdomain fraction as a false positive, an especially tight interaction with microdomain structures needs to be considered as well.

CONCLUSION

Protein-sterol interactions are complex and the proteomic analysis based on detergent resistance requires appropriate strategies for controlling false positives. We demonstrated that a systems approach involving several cellular fractions is suited for defining sterol-dependent proteins using label-free
normalization in shotgun proteomics. Thereby, by integrating high level statistics, our ratio based analysis of DRM/DSF protein abundances outperformed previous testing strategies in terms of reproducibility among replicates and with regards to total numbers of identified responding protein functional groups. Using standardized result-outputs from cRacker, the unicorn algorithm is semi-automated to carry out ratio based statistical analyses and can be applied to a wide range of questions regarding protein distributions. We could show that the sterol biosynthesis mutant smt1 mimicked a mβcd-treated like phenotype in DRM/DSF protein distributions. Therefore, we propose smt1 as a useful alternative to mβcd treatment in biochemical studies of sterol–protein interactions and their dynamics in signaling and transport pathways.

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†† To whom correspondence should be addressed: Department of Plant Systems Biology, Universität Hohenheim, Garbenstraße 30, 70599 Stuttgart, Germany.

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