Fractionation profiling: a fast and versatile approach for mapping vesicle proteomes and protein–protein interactions

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ABSTRACT We developed “fractionation profiling,” a method for rapid proteomic analysis of membrane vesicles and protein particles. The approach combines quantitative proteomics with subcellular fractionation to generate signature protein abundance distribution profiles. Functionally associated groups of proteins are revealed through cluster analysis. To validate the method, we first profiled >3500 proteins from HeLa cells and identified known clathrin-coated vesicle proteins with >90% accuracy. We then profiled >2400 proteins from Drosophila S2 cells, and we report the first comprehensive insect clathrin-coated vesicle proteome. Of importance, the cluster analysis extends to all profiled proteins and thus identifies a diverse range of known and novel cytosolic and membrane-associated protein complexes. We show that it also allows the detailed compositional characterization of complexes, including the delineation of subcomplexes and subunit stoichiometry. Our predictions are presented in an interactive database. Fractionation profiling is a universal method for defining the clathrin-coated vesicle proteome and may be adapted for the analysis of other types of vesicles and particles. In addition, it provides a versatile tool for the rapid generation of large-scale protein interaction maps.

INTRODUCTION

Several thousand proteins are associated with the eukaryotic endomembrane system, yet the characterization of their functional interactions remains a mostly unresolved question (Havugimana et al., 2012). Numerous large-scale studies have been carried out to determine the localization and interactome of individual proteins using fluorescence or affinity tags (Huh et al., 2003; Ewing et al., 2007), but these approaches are extremely labor intensive and also suffer from potential interference introduced by tagging. An alternative strategy is to separate organelles by density gradient centrifugation and identify proteins associated with individual fractions through mass spectrometry (Dunkley et al., 2004; Foster et al., 2006). Such comparative proteomic approaches have yielded valuable insights into the composition of major organelles. However, transport vesicles, which are responsible for the selective exchange of contents between organelles, are not resolved by these methods.

Clathrin-coated vesicles (CCVs) mediate transport between the trans-Golgi network and endosomes and also facilitate plasma membrane endocytosis (Robinson, 2004; Faini et al., 2013). Their function is critically important in all eukaryotes: in multicellular organisms, defects in CCV trafficking are embryonic lethal (Robinson, 2004; Borner et al., 2007; Umasankar et al., 2012) or associated with severe developmental phenotypes (Tarpey et al., 2006; Montpetit et al., 2008). Similarly, unicellular trypanosomes, the causative agents of sleeping sickness, are not viable without clathrin-mediated endocytosis (Allen et al., 2003). To date, the CCV composition from only three sources has been characterized by proteomics: rat brain (Blondeau et al., 2004), rat liver (Girard et al., 2005), and HeLa cells (Borner et al., 2006, 2012; Hirst et al., 2012). Because standard biochemical fractionation procedures never yield “pure” fractions,
assignment of identified proteins as genuine CCV components or contaminants is highly ambiguous (Blondeau et al., 2004; Girard et al., 2005). Recently we showed how selective interference with CCV formation through gene silencing (Borner et al., 2012) or drug-induced knocksideways (Hirst et al., 2012) in conjunction with quantitative mass spectrometry can be used to define the contents of CCVs objectively and with high accuracy. A limitation of these perturbation-based approaches, however, is that they require gene knockdown or transgene expression and are thus restricted to cell types amenable to these techniques. There is no universal method for the proteomic analysis of CCVs and, more generally, transport vesicles. To fill this niche, we developed “fractionation profiling,” which is based exclusively on quantifying subcellular fractionation behavior. The method requires no chemical or genetic manipulation and reveals the composition of CCVs with high specificity and sensitivity. Furthermore, our data suggest that fractionation profiling will work in a wide variety of cell types. Although we initially designed the method for the analysis of transport vesicles, we also demonstrate its potential for discovering and characterizing protein complexes.

RESULTS
Development of the fractionation profiling approach
HeLa cells are still the only human cell type with a well-characterized CCV proteome (Borner et al., 2012), and we hence developed and validated fractionation profiling in these cells. We previously showed that a “mixed vesicle fraction” can be prepared from HeLa cells by standard biochemical fractionation techniques (Borner et al., 2006, 2012). This fraction is highly enriched in CCVs but also contains other membrane vesicles, as well as large cytosolic protein complexes. The aim of fractionation profiling is not to obtain a “pure” CCV fraction but to determine the subcellular fractionation behavior of CCVs and identify all proteins that share it. First, we established differential centrifugation conditions that allow the further division of the vesicle fraction into three subfractions of roughly equal total protein content (Figure 1A). In parallel, we prepared a reference fraction from metabolically “heavy” labeled cells (stable isotope labeling by amino acids in cell culture [SILAC] method; Ong et al., 2002). This reference combines the contents of all three subfractions. Electron microscopy shows that CCVs and other structures have characteristic enrichment and depletion profiles across the subfractions (Figure 1B). Gel electrophoretic analysis (Figure 1, C and D) confirms that CCV-associated proteins share the same sharply defined profile, with a pronounced peak in fraction 1 and almost complete depletion from fraction 3. This signature profile is clearly resolved from coat components of major non-CCV transport vesicles and other particles, such as AP-3, AP-4, and ribosomes.

For proteomic analysis, an aliquot of the SILAC heavy reference fraction was pooled with each (SILAC light) subfraction. The experiment was then repeated with reversed metabolic labels. Thus six sample pairs were obtained and analyzed by quantitative mass spectrometry (Figure 1E). In total, we identified >4500 proteins (Supplemental Table S1), with 2827 proteins quantified across all six samples. For every protein, we determined the abundance in each subfraction relative to the reference. The resulting six ratios constitute a protein’s profile. As expected, coat components of CCVs show very closely matched profiles and are clearly distinguishable from non-CCV proteins (Figure 1F).

To sort proteins into groups with related profiles, we first performed principal components analysis (PCA). This allowed the representation of the variation in our six-dimensional data set as a two-dimensional scatter plot and clustered proteins based on profile similarity (Figure 2). Known CCV proteins clustered tightly in the periphery of the plot. Hence fractionation profiling successfully segregated CCV from non-CCV proteins. Of importance, the cluster analysis extends to all other proteins in the data set; numerous known protein complexes with diverse functions also formed tight clusters throughout the plot, as discussed later.

The Predictor database
To harness the profiling data, we designed an interactive analysis tool (see Materials and Methods). The “Predictor” (Supplemental Table S1) searches the profiling data against query proteins and provides a classified output of proteins with similar profiles. Profile similarity calculations are based on distances in multidimensional log space. Of importance, the Predictor is not based on PCA. The purpose of the PCA plot in Figure 2 is to provide a graphical illustration of profile clustering. The reduction of a six-dimensional data set to a two-dimensional scatter plot necessarily causes a loss of discriminating power. As a result, some clusters of known protein complex subunits (colored dots) appear to overlap with unrelated proteins (gray dots). The Predictor, however, achieves much higher resolution than shown in Figure 2 by calculating distances in six-dimensional space. See Materials and Methods for further details.

Clathrin heavy chain (CHC) is the major CCV protein, and proteins with similar profiles are candidate CCV constituents. To test whether the Predictor can recover the known HeLa CCV proteome, we searched the database of 2827 complete profiles against CHC. The Predictor classified 93 hits as “very high confidence” CCV proteins; 84 of these are previously reported CCV proteins. Based on their functional annotation, eight of the remaining nine proteins are strong candidate novel CCV proteins, and only one is a definite false positive, suggesting a predictive accuracy of 90–98% (at the 60% sensitivity threshold; see Supplemental Table S2 for a detailed analysis of the predictions and new CCV proteins). A further 75 proteins were classified as “high-confidence” predictions, bringing the total number of predicted CCV proteins to 168. These include 112 known CCV proteins, as well as 45 strong candidate novel coat and cargo CCV proteins, with <7% definite false positives (at the 80% sensitivity threshold; Supplemental Table S2). Collectively these data suggest that fractionation profiling can identify known and novel CCV proteins with exceptional accuracy and coverage.

Application of fractionation profiling to S2 Drosophila cells reveals the first insect CCV proteome
Although Drosophila is a widely used model organism, the composition of its CCVs remains poorly characterized. To test whether fractionation profiling is transferable to other cell systems, we chose to investigate Drosophila S2 cells. We applied fractionation profiling without further optimization (Figure 3A). Clathrin and associated proteins had profiles similar to those in HeLa cells (Figure 3B). For proteomic analysis, we prepared a reference and three subfractions from SILAC-labeled S2 cells, repeated the preparation with reversed labeling, and analyzed the six sample pairs by mass spectrometry. In total, we identified >3000 proteins, of which 1799 were quantified across all six samples. PCA shows that subunits of known protein complexes form clusters, as expected (Figure 3C). Known fly CCV proteins, including clathrin, AP-1, and AP-2, formed a distinct cluster in the periphery of the plot. We then constructed a Predictor for the S2 profiling data (Supplemental Table S3). A search against Drosophila clathrin heavy chain revealed a list of 29 candidate CCV proteins predicted with the highest level of confidence (Table 1). Remarkably, the human homologues of 27 of these are known CCV proteins; most of these proteins have not been characterized in
FIGURE 1: Development of the fractionation profiling approach. (A) Workflow. HeLa cells were metabolically labeled with heavy (Lys-8/Arg-10) or light (Lys-0/Arg-0) amino acid isotopes. Equivalent vesicle-enriched membrane fractions (Ves Frac) were prepared by biochemical fractionation. The light Ves Frac was subfractionated by a series of three centrifugation steps with increasing speed (pellets 1–3). In parallel, the heavy Ves Frac was centrifuged once at high speed to obtain the reference pellet. (B) Characterization of subfractions by electron microscopy. Pellets were fixed and cross-sectioned. CCVs are the predominant profiles in pellet 1 and very abundant in pellet 2. Pellet 3 contains mostly smooth membrane vesicles and nonvesicular protein particles (e.g., proteasomes and ribosomes). The reference fraction has an intermediate composition. (C) Analysis by gel electrophoresis (Coomassie stain). All fractions have distinct compositions. Clathrin heavy chain (small arrow) is enriched in fractions 1 and 2 and depleted from fraction 3. Several other prominent bands show the opposite trend. (D) Analysis by Western blot. Clathrin heavy chain (CHC) and
Fractionation profiling reveals the composition of protein complexes

Stably associated proteins are expected to show near-identical fractionation behavior and thus similar profiles; in turn, this may be exploited to predict the composition of protein complexes. To test this, we focused on the HeLa data set, as the annotation of the human protein database is much more comprehensive than that of Drosophila. We first investigated the well-characterized and abundant CCT chaperonin T-complex (Figure 4A). All eight associated proteins (AP-1, AP-2, and GGA2) have equivalent profiles. Nonclathrin vesicle coats (AP-3, AP-4) and large protein particles (EF-2, ribosomal elongation factor 2) have distinct patterns. Data in B-D are in excellent agreement. (E) Fractionation profiling as a proteomic method. Fractions 1–3 and Ref1 were prepared as in A. Fractions 4–6 and Ref2 were prepared similarly but with reversed metabolic labels (heavy 4–6, light Ref2). Fractions 1–3 were pooled with Ref1 and fractions 4–6 with Ref2 and analyzed by mass spectrometry. For each identified protein, six ratios of enrichment/depletion relative to the reference fraction were obtained. (F) Proteomic profiles of clathrin (AP-1, AP-2) and nonclathrin (AP-3, AP-4) adaptor protein subunits, showing the relative abundance distribution across the six subfractions (log2 scale). Subunits of the same AP-complex have near-identical profiles, demonstrating the accuracy of the method. Clathrin, AP-1, and AP-2 have extremely similar profiles, suggesting that they are all part of the same subcellular structure (i.e., CCVs). In contrast, nonclathrin adaptors AP-3 and AP-4 have profiles different from clathrin and different from each other. Note that each profile consists of two independent data triplets obtained with slightly different spin parameters to improve profile discrimination (deliberately “imperfect” repeats; see Materials and Methods). Hence the two parts of the profiles (1–3 and 4–6) are not identical; they show similar patterns, which appear shifted on the y-axis.
FIGURE 3: Application of fractionation profiling to S2 cells reveals the composition of Drosophila CCVs. (A) Drosophila S2 lysates were subfractionated as in Figure 1A. Gel electrophoresis (Coomassie stain) reveals that all subfractions have different compositions. The probable clathrin heavy chain band is indicated (arrow). (B) Western blotting of fractions.
subunits have extremely similar profiles, and querying the Predictor with any subunit retrieves the other seven subunits as top hits. Furthermore, the mass spectrometric data allow an estimation of relative protein abundance. For the T-complex, an equimolar stoichiometry of all subunits is suggested (Figure 4B), as previously reported (Yebenes et al., 2011). Hence profiling correctly predicts the composition of the T-complex de novo. Next we tested whether fractionation profiling can also predict the organization of more elaborate complexes. The 26S proteasome consists of two subcomplexes—the 20S core and the 19S regulatory particle. The latter is further divided into lid and base (Tomko and Hochstrasser, 2013). A search of the predictor with consensus profiles for the 19S or 20S particles identified 32 (of 33) subunits of the 26S proteasome, as well as several accessory factors. To gain further insights into complex arrangement, we applied PCA to the subunit profiles (Supplemental Figure S1A). Core and regulatory particles were clearly discriminated. Furthermore, the separation of lid and base, as well as the arrangement of core inner and outer ring, were also discernible. Finally, profiling predicts that most core and regulatory subunits are present in roughly equimolar quantities, unlike most accessory factors, which are present in substoichiometric amounts (Supplemental Figure S1B).

Encouraged by this proof of principle, we investigated the structure of less-well-studied mammalian protein complexes. TRAPP is a multisubunit vesicle-tethering complex (Yu and Liang, 2012). In yeast, the core complex TRAPPI contains subunits 1–6 that can associate with subunits 9 and 10 to form TRAPPII or with subunit 8 to form TRAPPIII; all three TRAPPs have different functions. The mammalian TRAPP has only recently been characterized through genetic interactions and pull-down experiments (Bassik et al., 2013). PCA of our profiling data clearly support the postulated existence of a mammalian TRAPPIII complex consisting of subunits 1, 2, 2L, 3–5, 6A/6B, 8, and 11–13 (Figure 4C). In addition, we provide the first experimentally determined estimated TRAPPIII subunit stoichiometry (Figure 4D). In agreement with the yeast model, our data suggest an equimolar stoichiometry for all core subunits 1–6, except for subunit 3, which is present in two copies. In addition, it appears that the recently identified subunits 11 and 12 are also present in single copies, with subunit 13 probably less tightly associated. Finally, the fact that subunit 8 (the defining feature of TRAPPIII) is almost as abundant as the core subunits 1–6 implies that the majority of TRAPPI is part of TRAPPIII and that very light TRAPPI is “free” or part of TRAPPII in HeLa cells.

The yeast Gid complex is a multisubunit E3 ligase with key regulatory functions in glucose metabolism (Menssen et al., 2012). The mammalian homologue has been termed the CTLH complex (Kobayashi et al., 2007), and its components have been implicated in regulation of endosomal trafficking (Heisler et al., 2011) and cell spreading (Valiyaveettil et al., 2008). Pull-down experiments have identified six members of the CTLH complex: ARMC8, C20orf11, MACEA, MLK1, RANBP9, and RMD5D (Kobayashi et al., 2007). Our profiling data support this composition (Figure 4, E and F) but clearly indicate the presence of three further subunits: C17orf39 and WDR26, the human homologues of yeast GID4 and GID7, respectively; and VRBP (the HIV-VPR binding protein), which has no homologue in yeast. VRBP is a particularly intriguing new discovery. This protein associates as a substrate adaptor with the CRL4A E3 ligase CUL4A/DBB1/RBX1 (Romani and Cohen, 2012). The CRL4A complex is also present in our profiling data, but its profile is distinct from that of the CTLH complex (Figure 4, G and H). Furthermore, VRBP has a LisH protein interaction domain, which is also present in several CTLH subunits (Menssen et al., 2012). Our data therefore suggest that VRBP may have an additional function as a substrate adaptor in CTLH. Of importance, the HIV protein VPR is known to “highjack” VRBP and its associated E3 ligase to trigger degradation of as-yet-identified targets (Romani and Cohen, 2012). We propose that the CTLH complex may also become a target of HIV-VPR and that it may play a role in HIV pathogenesis.

Fractionation profiling predicts the existence of novel protein complexes and trafficking pathways

The foregoing data demonstrate the predictive power of our approach for known protein complexes. However, profiling also predicts the existence of novel complexes (Figure 5, Supplemental Figure S2, and Supplemental Table S5). For example, we provide the first evidence that the BAR-domain proteins SNX4 and SNX30 (van Weering et al., 2010) form a stable dimer (Figure 5, A and B). A recent study identified a novel protein dimer C17orf75/WDR11, implicated in protection against ricin toxicity (Bassik et al., 2013). Our profiling data suggest that these proteins are in fact part of a trimeric complex, which also includes the protein FAM91A1 (Figure 5, C and D). The protein C10orf32 has been implicated in susceptibility to arsenic poisoning (Pierce et al., 2012); here we propose that C10orf32 is in complex with the uncharacterized protein LOIH12CR1 (Figure 5, E and F). These examples illustrate that our approach covers a broad spectrum of important novel protein associations.

shown in A confirms that CHC and the associated adaptor AP-1 have similar profiles, which are distinct from the nonclathrin adaptor AP-3. (C) Proteomic analysis of S2 fractions from two independent profiling experiments identified 1799 proteins with complete profiles. PCA of the profiles reveals clustering of subunits of known protein complexes, including AP-1, AP-2, AP-3, the anaphase-promoting complex (APC), the T-complex (CCT), clathrin heavy and light chains (Cla), the Exocyst (Exo), the mitochondrial ribosome (mRP), the proteasome core (PS20) and regulatory particle (PS19), signalosome (Sig), and the V-ATPase (vATP0, integral membrane subcomplex; vATP1, peripheral subcomplex). Of importance, proteins whose mammalian homologues are known CCV proteins cluster in the vicinity of clathrin (marked in red, CCV). Fractionation profiling successfully reveals the composition of the Drosophila CCV proteome. (x-, y-axes = first and second principal components; cumulative R² = 0.917). (D) To illustrate that even a single fractionation profiling experiment is sufficient to produce meaningful clustering, this plot shows PCA of the first triplet only (i.e., three data points). Joined PCA of both triplets (all six data points) results in a very similar plot. (D) Four candidate Drosophila CCV coat components predicted by fractionation profiling were C-terminally tagged and transiently expressed in S2 cells. Proteins were visualized by immunofluorescence microscopy. All four proteins colocalize with established markers of clathrin-coated vesicles, confirming their association with CCVs in S2 cells. LeF RasGAP, SCYL2, and SMAP2 colocalize tightly with AP-1, a marker of TGN/endosomal CCVs. SES1/2 localizes to plasma membrane clathrin-coated pits. Because clathrin light chain (CLC) marks both endocytic and intracellular clathrin-coated structure, it shows only partial colocalization with SES1/2. Some areas of colocalization are indicated (white arrowheads). Scale bar, 10 μm.
Finally, we investigated whether this pathway is related to flotillin- or caveolin-mediated endocytosis (Sandvig et al., 2011). PCA shows that profiles of these markers of clathrin-independent endocytic pathways are clearly different from those for the ARF6 cargo cluster (Figure 5H).

DISCUSSION

Here we developed proteomic fractionation profiling, a simple yet powerful tool for mapping functional protein associations. We have mainly used fractionation profiling for the analysis of CCVs and protein complexes, but the method is likely to work equally well for the characterization of other types of vesicular transport intermediates. Our results are presented in the interactive Predictor database (Tables S1 and S3).

### TABLE 1: The Drosophila S2 CCV proteome: top predictions.

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The Drosophila S2 cell Predictor (Supplemental Table S3) was searched against clathrin heavy chain (Chc). Predictions are ranked in order of similarity to Chc. Only the predictions with the highest level of confidence and a minimum of five data points are shown here (see Supplemental Table S4 for the complete list of predicted Drosophila S2 CCV proteins). Relative abundance is indicated in arbitrary units. The last column indicates whether the human homologue of an identified protein is a known CCV constituent.

*Protein subcellular localization investigated by immunofluorescence microscopy (Figure 3D).
Fractionation profiling for the analysis of vesicle composition

Fractionation profiling is based on identifying proteins with similar subcellular distributions. This concept has been exploited in several published methods, including localization of organelle proteins by isotope tagging (LOPIT; Dunkley et al., 2004) and protein correlation profiling PCP; Foster et al., 2006). Both approaches rely on density gradient centrifugation to separate organelles; quantification of protein distribution across the gradient is achieved by isotope tagging (LOPIT) or label-free quantification (PCP). Although these methods are conceptually powerful, they necessitate large numbers of fractions, long measuring times, and complex data analysis. Crucially, neither method was able to resolve transport vesicles, such as CCVs. Fractionation profiling pairs the superior accuracy of SILAC quantification with the robustness and simplicity of differential centrifugation. It requires minimal amounts of starting material and instrument time and is computationally straightforward. We show that fractionation profiling can determine the composition of CCVs from HeLa cells with excellent sensitivity and specificity, comparable to our perturbation-based multivariate profiling approach (Berner et al., 2012). Unlike our previous method, however, fractionation profiling does not involve the use of small interfering RNA knockdown, which is a distinct advantage, since not all cell types are amenable to knockdown. To demonstrate that the approach is not limited to HeLa cells, we applied it to evolutionarily distant Drosophila S2 cells. Without requiring any cell-specific optimization, fractionation profiling provided the first comprehensive insect CCV proteome. Furthermore, our data also allow the first specific interspecies comparison of CCV content and thus identify evolutionarily conserved core machinery and cargo (Table 1).

Our findings suggest that the approach will be applicable to most cell types amenable to SILAC labeling and thus provide a long-awaited universal tool for characterizing the composition of CCVs. Additional strengths of the method are its relative simplicity and rapid yield of results. In our experience, even a single fraction triplet already generates a very accurate draft CCV proteome (illustrated in Figure 3C). An exciting prospect is the application of the method to unicellular eukaryotes. For example, trypanosome pathogenicity depends on clathrin-mediated endocytosis (Allen et al., 2003), yet the trypanosome CCV composition remains largely unknown. Fractionation profiling could quickly provide answers and thus identify new drug targets for treatment of sleeping sickness. We expect that fractionation profiling has the potential to become a standard tool for the proteomic analysis of CCVs.

Fractionation profiling as a tool for mapping protein complexes

A second major result of this work is that fractionation profiling allows the characterization of protein complexes. Several previous studies used comparative approaches to investigate protein interaction networks. For example, Havugimana et al. (2012) analyzed cell lysates by multiple chromatographic techniques. The distribution of 3000 proteins was quantified by mass spectrometry across >1000 subfractions, and 622 putative complexes were identified (from ~14,000 binary interactions). Similarly, Kristensen et al. (2012) used size-exclusion chromatography to separate cell lysates into 50–100 fractions and applied protein correlation profiling (Foster et al., 2006) to identify 291 complexes from 3400 quantified proteins (and ~7000 binary interactions). Although both studies yielded impressive catalogues, they are largely restricted to cytosolic protein complexes, as the used separation techniques cannot easily cope with membrane fractions. Furthermore, both methods require substantial mass spectrometric analysis time, owing to the large number of fractions. Here we introduced a comparatively simple method—fractionation profiling—which requires analysis of only six fractions, yet provides very accurate predictions. Furthermore, the method is particularly suitable for the analysis of membrane-associated protein complexes. The HeLa data cover >3500 protein profiles, approximately one-third of the HeLa proteome (Nagaraj et al., 2011). The Predictor does not rigidly assign complex boundaries. However, we identified 6980 pairs of profiles with “very high similarity,” which are comparable to predicted binary interactions. We therefore estimate that at least several hundred protein complexes are covered by our data set. These include large cytosolic particles such as ribosomes and proteasomes (megadalton range), but also smaller assemblies, such as the CTLH complex (~600 kDa). Very small cytosolic complexes are probably not pelletted under the conditions used here; however, complexes associated with membranes are very well resolved, regardless of size. Even small dimers (such as SNX4/SNX30; ~100 kDa) are readily identified. Indeed, our analysis includes numerous membrane-interacting complexes that not represented in either Havugimana’s (2012) or Kristensen’s (2012) study, such as AP4, BLOC-1, CTLH, LAMTOR, and TRAPP. In addition, our method predicts groups of integral membrane proteins with similar trafficking pathways, such as ARF6-dependent endocytic cargo proteins, and thus goes beyond the analysis of complexes.

The information gleaned from a profiling analysis is threefold. First, searching the Predictor against a protein of interest identifies candidate functionally associated proteins. The Predictor provides a classified output, which allows users to evaluate the relevance of the retrieved proteins. The Predictor can hence identify novel complexes or candidate novel subunits of known complexes. Second, the Predictor provides estimated relative abundances of the retrieved proteins. We demonstrated the validity of this feature by correctly predicting the stoichiometry of numerous known complexes (Figure 4 and Supplemental Figure S1). Third, the retrieved profiles can be further analyzed by PCA to give insights into the structural organization of protein complexes. To test this, we first predicted the subcomplex arrangement of the 265 proteasome (Supplemental Figure S1A); we then used the method to define the composition of the HeLa TRAPPIII complex (Figure 4C) and also to map the association of VPRBP with CTLH versus the CRL4A E3 ligase (Figure 4H). To our knowledge, there is no published method as simple as fractionation profiling that grants similarly detailed insights into protein complex composition and organization.

Conclusion

Here we exploited fractionation profiling as a tool for characterizing CCV composition. We also used it to analyze a number of known and uncharacterized protein complexes to illustrate the power of the approach. These examples represent only a small proportion of the information in the profiling data sets. We therefore present the complete data as a community resource (the Predictor, Supplemental Tables S1 and S3). Readers are strongly encouraged to search the database against proteins of interest. The Predictor is very easy to use; a quick-start guide is included in the file, and a detailed manual is provided separately (Supplemental Predictor Manual).

Finally, we propose that fractionation profiling can be adapted to other cell biological questions. In principle, any homogeneous subcellular structure should be amenable to a profiling analysis, provided it is possible to prepare an enriched fraction as the starting point. Good candidate targets include, for example, neuronal secretory granules (Bonnemaison et al., 2013) and the elusive “Glut4 storage vesicles” in adipocytes (Leto and Saitel, 2012).
FIGURE 4: Fractionation profiling predicts the composition and stoichiometry of protein complexes. (A, B) The chaperonin T-complex (eight subunits). (A) Fractionation profiles, showing the protein abundance in each fraction relative to the reference (log₂ scale). Profiles of individual T-complex subunits are nearly superimposable. (B) Estimated stoichiometry, normalized to the median of all subunits. Fractionation profiling correctly predicts the equimolar stoichiometry of the complex. (C, D) The TRAPP complex (14 subunits). Yeast TRAPP exists in three configurations: the TRAPPI core (1, 2, 2L, 3–5, 6A, and 6B) can associate either with subunits 9 and 10 to form TRAPPII or with subunit 8 to form TRAPPIII. (C) PCA of subunit profiles reveals that in HeLa cells, the majority of TRAPP is assembled into
Conversely, the approach may be focused primarily on protein complexes, for example, by subfractionating nuclear or mitochondrial extracts. The transfer to other biological targets will probably require some optimization of the fractionation conditions. Nevertheless, the experimental design and subsequent data analysis can be modeled on the approach presented here, as discussed further in a dedicated section at the end of Materials and Methods. Fractionation profiling is an extremely versatile yet conceptually straightforward tool for mapping functional protein interactions, with many potential future applications beyond those demonstrated here.

MATERIALS AND METHODS

Fractionation profiling

The following protocol describes the procedure as used in this study for the analysis of CCVs. The final section discusses conceptual aspects of the approach, limitations, and caveats, as well as considerations for future applications.

Overview

A fractionation profiling experiment consists of two parallel sample preparations: a “reference” sample (e.g., from SILAC heavy-labeled cells) and a set of “subfractions” (e.g., prepared from SILAC light-labeled cells). Labels are usually swapped for a repeat experiment. The first part of the protocol follows the method for preparing a CCV-enriched fraction described in Borner et al. (2012).

Preparation of cell lysates

All operations were performed at 4°C. Adherent HeLaM cells (recommended 1000 cm² confluent/lysate) were washed once in ice-cold phosphate-buffered saline (PBS) and once in ice-cold MES buffer (0.1 M 2-(N-morpholino)ethanesulfonic acid [MES], pH 6.5 [adjusted with NaOH], 0.2 mM ethylene glycol tetraacetic acid, and 0.5 mM MgCl₂). Cells were drained carefully and scraped into 10 ml 0.5–1 ml of ice-cold MES buffer. Cells were lysed in a 30-ml Potter-Elvehjem homogenizer (clearance 100–150 μm) with 20 strokes of a motorized pestle.

Suspension Drosophila S2 cells (recommended ~0.5–1 ml of pelleted cells/lysate) were washed once in ice-cold PBS and once in ice-cold MES buffer. Cells were resuspended in 6 ml of ice-cold MES buffer and first lysed in a Potter-Elvehjem homogenizer as described. To improve the lysis of the relatively small S2 cells, primary lysates were additionally passed through a 21-gauge (0.8-mm) needle eight times.

Subcellular fractionation

All operations were performed at 4°C. Lysates were centrifuged at 4000 × g for 32 min to pellet unbroken cells and large debris. Supernatants were treated with 50 μg/ml ribonuclease A (≥2.5 Kunitz units/ml) for 1 h, and partially digested ribosomes were pelleted by centrifugation at 4000 × g for 3 min. Supernatants were then centrifuged at 209,000 × g (55,000 rpm, MLA-80 rotor; Beckman Coulter, High Wycombe, UK) for 40 min to pellet membranes. Pellets were resuspended in 400 μl of MES buffer with 60 strokes in a 1-ml Wheaton homogenizer (tight pestle, clearance ~25–75 μm). An equal volume of F/S buffer (12.5% [wt/vol] Ficoll [PM400; GE Life Sciences, Little Chalfont, UK] and 12.5% [wt/vol] sucrose in MES buffer) was added and carefully mixed, and samples were centrifuged at 21,700 × g (20,000 rpm, TLA-110; Beckman Coulter) for 34 min. Supernatants were diluted 1:5 in MES buffer. Up to this stage, the preparation procedure is identical for the (SILAC heavy) reference and (SILAC light) subfraction samples (and essentially as described in Borner et al., 2012).

1. For the preparation of the “reference” fraction, the diluted supernatant was centrifuged at 195,500 × g (60,000 rpm, TLA-110) for 40 min to yield the “reference pellet.”

2. For the generation of “subfractions,” the diluted supernatant was first centrifuged at 66,500 × g (35,000 rpm, TLA-110) for 20 min to yield the 35K pellet. The supernatant was transferred to a fresh centrifuge tube, mixed by pipetting, and centrifuged at 110,000 × g (45,000 rpm, TLA-110) for 20 min to yield the 45K pellet. The supernatant was again transferred to a fresh tube and centrifuged at 195,500 × g (60,000 rpm, TLA-110) for 40 min to obtain the final 60K pellet.

The spin parameters of the reference pellet correspond to those of the 60K subfraction pellet. Hence the contents of the reference pellet correspond to those of all three subfraction pellets combined (35K + 45K + 60K).

The recommended procedure for sample resuspension is to resuspend all pellets in 1× sample buffer (made from NuPAGE 4× LDS sample buffer [Life Technologies, Paisley, UK] diluted with water). Each subfraction pellet should be redissolved in 20 μl and the reference pellet in 60 μl. This method is particularly suitable for small sample yields (e.g., if limited starting material is available). Alternatively, samples may be dissolved in a suitable volume of SDS buffer (2.5% [wt/vol] SDS, 50 mM Tris-HCl, pH 8) for estimation of protein yields before gel electrophoresis.

Note that for HeLa cells, the two repeats of the fractionation profiling were deliberately performed with slightly different spin parameters (35K, 45K, and 60K, first triplet; 30K, 40K, and 60K, second triplet). The profiles obtained with the different sets of spins are correlated but appear somewhat shifted on the y-axis in Figure 1F. The intention behind this “imperfect” repeat was to enhance the resolution of subcellular structures by teasing out more differences in fractionation behavior. Inspection of the PCA loadings plot corresponding to Figure 2, however, indicates that both sets of spins make very similar contributions to the analysis. The gain in resolution is thus likely to be relatively small. For the S2 cells, we used identical spin conditions for both repeats. In our experience, either method performs well.

TRAPPIII. Subunits 11–13 are also digested part of this complex. Core subunits are shown in blue, the TRAPPIII subunit 8 is shown in red, the TRAPPII subunits 9 and 10 are shown in green, and subunits 11–13 are shown in gray. (D) Estimated stoichiometry of mammalian TRAPPII (E–H) The CTLH complex (six known plus three novel subunits). Yeast homologues of mammalian proteins are indicated (GID; Mensten et al., 2012). (E) Subunit profiles. Three candidate novel subunits—C17orf39, WDR26, and VPRBP—were identified. (F) Estimated stoichiometry of the mammalian CTLH complex predicts a core of ARMc8, C20orf11, MAEA, RanBP9, and WDR26, with Muskelin, C17orf39, RMND5a, and VPRBP possibly less stably associated. (G) VPRBP has been proposed to function as a substrate adaptor in the E3 ligase CRL4A (Romani and Cohen, 2012). The CRL4A ligase consists of three proteins (CUL4A, DDB1, RBX1), which are also present in the profiling set. Consensus (median) profiles of CRL4A and CTLH were plotted. The profile of VPRBP matches that of CTLH. (H) PCA of subunit profiles confirms that VPRBP (red) profiles closely with CTLH subunits (blue) and not with CRL4A subunits (black). Error bars of abundance scores correspond to MADs; n = 6 replicate measurements.
FIGURE 5: Fractionation profiling predicts novel protein complexes and trafficking pathways in HeLa cells. (A–F) As a primer for a systematic analysis of novel protein–protein associations, the Predictor database was searched for pairs of near-identical profiles. We identified 989 such pairs, derived from 266 proteins in 58 groups (Supplemental Table S5). Clustering by PCA (Supplemental Figure S2) revealed many known stable protein complexes but also novel associations among uncharacterized proteins. A few examples are shown here. (A, B) The SNX4/SNX30 dimer. (A) Fractionation profiles, showing the protein abundance in each fraction relative to the reference (log$_2$ scale). (B) Estimated stoichiometry. (C, D) The C17orf75/WDR11/FAM91A1 trimer. A previous report suggested the association of C17orf75...
Sample preparation for mass spectrometric analysis

Two alternative protocols were used for sample mixing:

1. Equal-proportions mixing (recommended): Each subfraction sample (35K, 45K, and 60K, all SILAC light labeled if the reference is SILAC heavy) was combined with one-third of the reference sample. Using the recommended resuspension volumes, each of the three samples should be ∼40 µL. Two microliters of 1 M dithiothreitol (DTT) was added to each sample (50 mM final), and samples were incubated at 90°C for 3 min.

2. Equal-protein mixing: An alternative protocol was to estimate the protein concentration in each fraction by bicinchoninic acid assay (Thermo Fisher Scientific, Cramlington, UK) and mix equal protein quantities of reference and subfractions. This method optimizes protein load, but a proportion of the sample is lost for the protein assay. It is hence recommended only for preparations with large yields. DTT was added from a 1 M stock to a final concentration of 50 mM, and samples were incubated at 90°C for 3 min.

The two methods result in slightly different ratios; it is hence recommended to use the same method between repeat fractionation profiling experiments to ensure reproducibility of profiles.

Mass spectrometry analysis and raw data processing

Samples were separated by one-dimensional gel electrophoresis, using precast gradient gels (NuPAGE; Invitrogen). Gels were stained with colloidal Coomassie, and each lane was cut into 10 pieces. A typical fractionation profiling experiment thus comprised 30 gel slices, which were subjected to tryptic digest (Shevchenko et al., 2006). Peptide extracts were cleaned up and stored at 4°C on StageTips (Rappsilber et al., 2007). Samples were eluted from StageTips, separated on a 20-cm reverse-phase column, and analyzed on a Q Exactive mass spectrometer (Thermo Fisher Scientific), essentially as described (Nagaraj et al., 2012).

Each fractionation profiling experiment included two triplets of samples; each sample consisted of 10 gel slices/mass spectrometry runs. All (60) raw files were jointly processed with MaxQuant, version 1.3.0.5 (Cox and Mann, 2008), and its built-in Andromeda search engine (Cox et al., 2011), using UniProt (www.uniprot.org) reference databases for human or Drosophila proteins. Cam (C) was set as a fixed modification and oxidized (M) and acetyl (Protein N-terminus) as variable modifications. Both peptide and protein false discovery rates were set to 0.01; the minimum peptide length was seven amino acids. Calculations of ratios were performed on razor and unique peptides with the requantify and match-between runs features enabled. The minimum ratio count was two.

Data analysis

The protein identification lists from MaxQuant (Cox and Mann, 2008) were filtered by removing matches to the reverse database, proteins only identified with modified peptides, and common contaminants. Abundance ratios were then log2 transformed. For HeLa cells, we identified 4523 proteins (minimum, one ratio in the six samples); 3549 had at least one complete fractionation triplet (i.e., a usable profile), and 2827 had complete profiles (six ratios). For S2 cells, the corresponding numbers were 3163 (minimum, one ratio), 2483 (minimum, one data triplet), and 1799 (complete profile). All identifications are presented in Supplemental Tables S1 (HeLa) and S3 (Drosophila).

PCA was performed as described in Borner et al. (2012), using SIMCA-P+ 11.5 (Umetrics, Crewe, UK). Scatter plots in Figures 2–5 and Supplemental Figures S1 and S2 were also generated in SIMCA-P+ and further annotated in PowerPoint (Microsoft, Reading, UK). Line and column plots shown in Figures 1, 4, and 5 and Supplemental Figure S1 were prepared within Prism 6 (GraphPad Software, La Jolla, CA) and further annotated in PowerPoint (Microsoft).

Construction of the Predictor database

The Predictor provides a very simple interface that allows users to query the profiling data (Supplemental Tables S1 and S3). Its purpose is to predict groups of functionally associated proteins on the basis of common subcellular fractionation behavior. Using the Predictor requires only the most basic familiarity with Excel (Microsoft). The Predictor contains all proteomic data presented in this study in a compact yet highly accessible format. A quick-start guide is included in the same file. In addition, a detailed manual, including guidance to the interpretation of results, is provided as a separate document (Supplemental Predictor Manual).

Overview

Abundance profiles were tabulated in the raw data tables (Supplemental Tables S1 and S3; see Complete Data tab). Using standard Excel spreadsheet functions, an interactive interface (the Predictor) was created, which allows users to input one or several query genes and specify various search parameters. The Predictor then retrieves the corresponding profiles from the raw data table and calculates a consensus query profile. This query profile is automatically compared with all other profiles in the raw data table. Profiles are then sorted by similarity to the query profile, as shown in the results table. In addition, the Predictor provides various graphical summaries for both query and output profiles, including estimates of relative protein abundance. The details of the calculations are provided in what follows.

Distance calculations and ranking

A complete profile consists of six (log-transformed) abundance ratios, R1–R6, which indicate the protein’s distribution across different subcellular fractions. Proteins with similar profiles have similar subcellular distributions. To evaluate profile similarities, users can choose between two alternative measures of profile distance. For a given query profile, the distance to any other Protein X in the database is calculated as follows:

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and WDR11; profiling clearly indicates a stable complex that includes FAM91A. (C) Profiles. (D) Estimated stoichiometry. (E, F) The LOH12CR1/C10orf32 dimer. (E) Profiles. (F) Estimated stoichiometry. (G, H) Cargo of the ARF6-dependent endocytic pathway. CD44, CD98 (SLC3A2 heavy chain, SLC7A5 light chain), ICAM1, and BSG (CD147) are known cargo proteins of a clathrin-independent endocytic pathway. (G) Profiles of these proteins are almost identical, confirming their similar trafficking itineraries. A further protein, SLC16A1 (monocarboxylate transporter 1), is identified as part of the ARF6 cluster. Error bars of abundance scores correspond to MADs (n = 6 replicate measurements).
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1. Average absolute profile distance ("Manhattan distance"): 
   \[ \text{Av abs distance} = \left| \text{ABS}(R_1^{\text{Query}} - R_1^{\text{Protein}}) \right| + \left| \text{ABS}(R_2^{\text{Query}} - R_2^{\text{Protein}}) \right| + \ldots + \left| \text{ABS}(R_6^{\text{Query}} - R_6^{\text{Protein}}) \right|/6 \]

2. Average squared profile distance (the average "squared Euclidean distance"): 
   \[ \text{Av squared dist} = (R_1^{\text{Query}} - R_1^{\text{Protein}})^2 + (R_2^{\text{Query}} - R_2^{\text{Protein}})^2 + \ldots + (R_6^{\text{Query}} - R_6^{\text{Protein}})^2/6 \]

Both methods produce distance values \( \geq 0 \). However, they produce slightly different hierarchies within the output. The squared-distance method is more stringent; because all differences are squared, large deviations from the query profile are accentuated much more than with the absolute-distance calculation. Conversely, the squared distance is more likely to produce false negatives, since a single inaccurate ratio can "distort" the whole profile similarity.

The predictor also allows to search the database with and for "incomplete" profiles, that is, profiles with fewer than six abundance ratios. In such cases, only the differences of available ratios are summed and divided by the number of available data points. Distances are thus normalized to the number of available data points, and these "average" distances are comparable between complete and incomplete profiles. To maintain high predictive power, however, it is recommended that query and output profiles should contain at least one complete fraction triplet (see earlier discussion).

Once the predictor has calculated the distance of the query profile to every protein in the database, proteins are ranked according to similarity with the query (hierarchical output).

Note that the Predictor's distance calculations do not involve prior PCA. The PCA plots in Figures 2-5 and Supplemental Figures S1 and S2 represent graphical illustrations of profile clustering but are not required for the Predictor.

**Similarity evaluation**

In addition to the hierarchical output, the absolute distances to the query are also provided, together with a simple "star" system to guide the interpretation of the results (e.g., three stars denote proteins whose profile is almost identical to the query; two stars denote a useful cut-off for proteins commonly found in a complex, etc.). To derive these qualifiers, we scored distances of subunits within well-established mammalian protein complexes. Guided by the CORUM database (Ruepp et al., 2010), we assembled a calibration set of known protein complexes represented in our HeLa fractionation profiling data set. We included only complexes with at least four subunits with complete profiles. Our reference set included 21 complexes, with 232 proteins in total (Supplemental Table S6). PCA showed that the subunits of most calibration complexes cluster tightly. Therefore subunits within a calibration cluster have mostly very similar profiles. For each of the 21 complexes, we calculated a consensus "center" (i.e., median) profile and determined the distance of each subunit to the center of its complex. The resulting 232 distances are representative of typical profile distances encountered in known stable protein complexes. Distances were sorted from highest to lowest, and distance values were identified to include the top 40% (almost identical; three stars), 80% (very similar; two stars), 90% (similar; one star), or 95% (borderline similar, annotated as "B") of all calibration profiles. Cut-offs were determined for both distance scoring methods (see earlier description). Similarity filters are automatically applied to the Predictor's output and included in the annotation.

Because the CORUM database (Ruepp et al., 2010) covers only mammalian proteins, a proxy was devised for the Drosophila data set, using the information derived from the HeLa data set. First, a distance matrix of all complete HeLa profiles was generated (2827 × 2827 profiles). The corresponding 3,994,551 distances were sorted from highest to lowest. These calculations were performed in the Perseus module of the MaxQuant software suite (www.perseus-framework.org) using a custom plug-in, which is available on request. In this ranked list of distances, we located the distances corresponding to the star rankings determined as described and converted them into proportions (e.g., only the top 989 distances were smaller than the three-star cut-off, corresponding to the top 0.0247%).

**CCV scoring**

A particular strength of the Predictor is to identify proteins associated with CCVs. The indispensable core component of all CCVs is clathrin heavy chain (commonly known as CHC, but CLTC in UniProt nomenclature). Hence all proteins with profiles similar to CLTC are candidate CCV proteins. Although all CCV proteins occupy the same subcellular compartment, the profile distances between known CCV proteins are still somewhat larger than those encountered in tight CORUM protein complexes, and a different scoring method was devised to account for this. The HeLa CCV proteome is well characterized, with 151 predicted constituents (Borner et al., 2012; Hirst et al., 2012). Of these, 140 were represented with complete profiles in our analysis. For each CCV protein, we calculated the profile distance to CHC and sorted distances from lowest to highest. We then determined the distance cut-offs that include the top 40% of known CCV proteins (three-star similarity), top 60% (two stars), top 80% (one star), and top 90% (B, borderline similarity). Classifiers are automatically applied to the predictor's output and included in the annotation. For example, the predictor classifies 93 proteins as two- or three-star CCV proteins, of which 84 (60% of 140) are in the reference data set of 140.

Because no suitable reference database of fly CCV proteins was available, a simple proxy was used to determine similarity guides. We assumed that the S2 data set contains roughly the same proportion of CCV proteins as the HeLa set, scaled down by the lower complexity of the Drosophila genome. Thus the number of HeLa proteins in each star category was multiplied by (1799/2827) to account for the smaller number of proteins identified in the S2 set and further multiplied by the ratio of estimated genes in Drosophila relative to humans (∼14,000/21,000). For example, there are 93 × 1799/2827 × 14,000/21,000 = 39 proteins classified as two- or three-star CCV components in the S2 set of proteins with complete profiles.

In Figure 2, all known CCV proteins predicted with two- or three-star confidence are annotated as CCV (in red), except clathrin, AP-1, and AP-2 subunits, which are shown in black, blue, and green, respectively. In Figure 3C, proteins predicted as CCV proteins with two- or three-star confidence are annotated as CCV (red) if their human homologues are known CCV proteins, except clathrin, AP-1, and AP-2 subunits, which are shown in black, blue, and green, respectively.

**Abundance and stoichiometry estimation**

The relative abundance of identified proteins was estimated from the mass spectrometric data using the iBAQ method (Schwanhausser et al., 2011), as implemented in MaxQuant (Cox and Mann, 2008).
Each fractionation profiling data set consists of six sample pairs (SILAC heavy/light). In all cases, half the sample is the invariant “reference.” Thus for each protein there are up to six reference iBAQs. These were normalized to total reference iBAQ for each sample to account for differences in mass spectrometer performance between runs. The median iBAQ value was then calculated for each protein. All median iBAQs were normalized to the iBAQ of clathrin heavy chain (one of the most abundant proteins in the preparation). Clathrin heavy chain was then arbitrarily assigned an “abundance score” of 1,000,000. Hence a protein with an abundance score of 10,000 is present with 1% of the copy number of clathrin.

For the stoichiometry estimates in Figures 4 and 5 and Supplemental Figure S1, abundance scores were additionally normalized to the median abundance score of the core subunits of the complex of interest. Figures show the fold differences relative to the median score, on a log2 scale, to create equal distances for substoichiometric and superstoichiometric deviations. As a robust measure of the spread of individual abundance estimations, the median absolute deviation from the median (MAD) abundance score was calculated for each protein (indicated in Supplemental Tables S1 and S3). Error bars in Figures 4 and 5 and Supplemental Figure S1 also show MADs.

Cell culture and SILAC labeling
HeLa cells were maintained and SILAC (Ong et al., 2002) labeled as described (Borner et al., 2012). Drosophila S2 cells were grown in Schneider’s medium supplemented with 10% (vol/vol) fetal calf serum at 26–28°C in 175-cm² tissue culture flasks. SILAC labeling was performed based on the method described in Bonaldi et al. (2008). Schneider’s medium for SILAC labeling (without arginine and lysine) was purchased from Dundee Cell Products (Dundee, UK).

Gel electrophoresis, Western blotting, and immunofluorescence microscopy
Gels were Coomassie stained and scanned as described (Antrobus and Borner, 2011). Western blotting was performed as in Borner et al. (2012). Immunofluorescence microscopy was performed as described for Dmel2 cells (Hirst et al., 2009). The following antibodies were used: dAP1G (Hirst et al., 2009), dCLC (Heerssen et al., 2008), and anti-V5 (46-0705; Life Technologies). Images were adjusted for brightness and contrast in Photoshop (Adobe Systems Europe, Maidenhead, UK) or PowerPoint (Microsoft).

Drosophila constructs and transient gene expression
LqfR-V5, SCYL2-V5, SMAP2-V5, and SES1/2/V5 constructs for expression in S2 cells were assembled in the constitutive vector pAc5.1V5 (Life Technologies). Genes were amplified using RedTaq from Drosophila Dmel2 cDNA made in-house from Dmel2 lysates, and various restriction sites were added at the 5’ and 3’ ends: SCYL2 (EcoR1 and Apa1), LqfR (Kpn1 and Apa1), SES1/2 (EcoR1 and Xba1), and SMAP2 (EcoR1 and Xba1). All constructs were fully sequenced; SMAP2 and SES1/2 were free of all changes, and LqfR and SCYL2 had one conservative substitution each. Constructs were transfected using TransIT-2020 (Mirus Bio, Madison, WI) following the manufacturer’s instructions, and cells were fixed 5–7 d posttransfection to moderate expression levels. In brief, for each transfection, we combined 1 μl of TransIT and 500 μg of DNA in 50 μl of serum-free medium, added this to 0.6 × 10⁶ cells in serum-free medium, incubated for 4 h, and then recovered to full growth medium.

Electron microscopy
Pelleted fractions were fixed with 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at room temperature for 30 min, washed with 0.1 M cacodylate buffer, and postfixed using 1% osmium tetroxide for 1 h. Pellets were then washed before being incubated with 1% tannic acid in 0.05 M cacodylate buffer, pH 7.2, for 40 min to enhance contrast. Pellets were dehydrated in ethanol before being embedded in Araldyte CY212 epoxy resin (Agar Scientific, Stansted, UK). Ultrathin sections (70 nm) were cut using a diamond knife mounted to a Reichert Ultracut S ultramicrotome (Leica Microsystems, Milton Keynes, UK) and picked up onto coated electron microscopy grids. The sections were stained with lead citrate and observed in a FEI Tecnai Spirit (Eindhoven, Netherlands) transmission electron microscope at an operating voltage of 80 kV.

Fractionation profiling: conceptual aspects, limitations of the method, and suggestions for future applications
We expect that fractionation profiling in its present form can be used for the characterization of clathrin-coated vesicles from most cell types, provided they are amenable to SILAC labeling and mechanical lysis. In addition, the approach can be adapted to other biological questions. Here we discuss key aspects of the method that we consider critical to the success of future applications.

Suitable targets
Fractionation profiling is best suited for the analysis of vesicles of fairly uniform size and density, as these produce sharply defined abundance distribution profiles. We expect the method to perform well with different types of coated vesicles, synaptic vesicles, and dense-core vesicles. In addition, our data show that large protein particles, such as proteasomes, can also generate highly characteristic profiles. Conversely, if a vesicle population is very heterogeneous (e.g., intrinsically heterogeneous or rendered variable through fragmentation during cell lysis), it is likely to be more evenly distributed across the gradient, and such a profile may be less easily discerned from those of other heterogeneous vesicles. However, even in those cases, the method will generate useful information, albeit in a more limited way. Subunits of stable, obligatory protein complexes will always produce similar profiles, and these are usually very tightly clustered.

In essence, fractionation profiling is capable of providing insights at two cellular levels: whole vesicles and particles, as well as smaller assemblies, such as protein complexes and membrane domains. The difference is the “range” of the predicted associations. For homogeneous vesicles such as CCVs, it is possible to predict the complement of the entire structure, as well as to predict protein complexes within the assembly (e.g., the AP-1 and AP-2 complexes are readily predicted as individual complexes and as components of CCVs). For other proteins with less characteristic profiles, it is possible to predict immediately close proteins with confidence but not the composition of the associated compartment. An example is the AP-3 complex, which has a broad distribution in our analysis (Figure 1D). The different subunits are readily predicted as a tight complex, as is the known interaction with the BLOC-1 complex, but there are few high-confidence predictions beyond these immediate associations. This suggests that our vesicle preparation contains endosomal membrane patches coated with AP-3 domains but perhaps not many free AP-3 vesicles (which would most likely produce a sharper profile). Thus, if the aim of a fractionation analysis is to characterize a whole organelle, a fairly homogeneous population will be required. The analysis of tight protein complexes, however, will work even with very heterogeneous preparations.

Profile optimization
The most discriminating profiles show strong differences among the different subfractions, ideally with a clear peak or depletion in one
fraction. The CCV profile is a case in point, with a moderate peak in fraction 1 and strong depletion from fraction 3. Of importance, the depletion from fraction 3 is by far the most characteristic trait of the CCV profile and contributes most to setting it apart from other profiles.

Spin conditions (speed and duration) will need to be optimized for each individual target vesicle (initially by Western blotting for marker proteins). The speeds we used here for CCVs may serve as a starting point, as most membrane vesicles will pellet in this range. Ideally, the different subfractions should have fairly similar total protein content to simplify downstream processing.

Note that vesicle profiles may differ between cell types. For example, the profiles of CCVs from HeLa and S2 are similar but not identical. Analysis of several marker proteins associated with the organelle of interest (e.g., by Western blotting) may help to identify such profile shifts and, if required, fine-tune the spin conditions. In the case of S2 cells, however, cell-type-specific optimization was not required; small shifts do not appear to pose a problem.

Sample purity

Every fractionation profiling experiment begins with a preparation enriched in the organelle of interest. How enriched/pure this preparation needs to be is difficult to predict in general. As a reference, we quantified the proportion of CCV proteins in our preparation, based on the relative abundance estimates that are part of the Predictor output. Briefly, we summed the relative abundance scores of all CCV proteins and divided this by the summed abundance scores of all proteins in the preparation. This gives the proportion of protein molecules (copy number) associated with CCVs in the preparation. Next we weighted these numbers by the molecular weights of individual proteins, to estimate the proportion of protein mass associated with CCVs. According to these calculations, the HeLa CCV preparation contains 4.6% CCV proteins (proportion of copy number) and ~9.6% CCV protein (proportion of total protein mass). The main reason for the discrepancy between these numbers is that clathrin heavy chain—the predominant CCV protein—has a very high molecular weight. The S2 preparation is about half as pure, with 2.4% CCV proteins (copy number) and 5.1% CCV protein (total mass).

Although we have not investigated the requirements for CCV enrichment systematically, it seems likely that higher purity will improve the analysis. First, it will increase the number of identified relevant proteins. It will also increase the average number of quantification events per protein and thus the quantification accuracy. Of importance, if a protein is associated with more than one organelle, it will only be classified as a CCV protein if its predominant association within the preparation is with CCVs, and so a high proportion of CCVs in the preparation will enhance the discriminating power (see also the final section, Caveats and limitations of the approach). Nevertheless, as our analysis shows, even a preparation that contains >95% “contaminating” non-CCV proteins is readily amenable to fractionation profiling.

Number of subfractions

The number of analyzed subfractions is another important aspect of the experimental design. Here we tried to minimize this number to keep the mass spectrometric analysis time as short as possible while maintaining high accuracy of prediction for CCVs. Theoretically, a larger number of fractions or experimental repeats will improve accuracy, but the trade-off is that more gaps will appear in the data matrix, as proteins are less likely to be identified in all subfractions. In addition, the yield of the CCV preparation is rather low, and if this low amount of protein is distributed over more subfractions, it may decrease the number of protein identifications and indirectly (via reduced sequencing coverage) even the quantification accuracy. Finding the optimum number of subfractions hence depends on a number of considerations. The more complex the mixture, the more fractions are advisable, if the quantity of starting material is not limiting. For CCVs, two biological replicates of three fractions (i.e., six data points) yield very solid data; for other organelles or subcellular preparations, the number will need to be optimized case by case. Based on our experience, we expect that 6–10 data points will be sufficient for most applications.

Mass spectrometric analysis

Our experimental setup maximizes the accuracy of the quantitative mass spectrometry. Tight profile clustering is hence achieved with a relatively small number of subfractions/data points. Key to this strategy is the use of the SILAC method (Ong et al., 2002), which is extremely accurate for measuring small or medium differences in protein abundance by mass spectrometry. The approach is based on metabolic labeling of cells in culture by supplementing the growth medium with different isotopes of the amino acids arginine and lysine before subcellular fractionation. All proteins from the “reference” sample are distinguishable from proteins in the subfractions by a mass shift. This allows pooling of reference and subfraction samples before enzymatic digestion for mass spectrometric analysis, thus avoiding variability introduced by sample preparation. Furthermore, mass spectrometric analysis of SILAC samples compares ion intensities of isotopic variants of the same peptide within the same mass spectrometry run. This avoids normalization errors required for “label-free” quantification, which compares ion intensities of the same peptide between different mass spectrometry runs. Instrument performance differences between runs are therefore less problematic with SILAC. A drawback of the method is that the dynamic range of quantification is limited by the isotope labeling efficiency. In practice, SILAC is extremely accurate for the determination of relative differences less than ~20-fold. In fractionation profiling, the differences in protein abundance between subfractions and the reference fraction are deliberately kept small to be within that range. Furthermore, the subfractions and the reference have almost the same overall protein composition; they differ mostly in the relative abundance of proteins (i.e., the same species of proteins are present but in different proportions). This facilitates the identification of proteins across all subfractions, avoiding gaps in the data matrix. Finally, the high-performance mass spectrometer (Q Exactive) we used here allows the quantification of multiple peptides/SILAC pairs per fraction for each protein, resulting in very robust quantification.

Caveats and limitations of the approach

Fractionation profiling identifies groups of proteins with similar fractionation behavior and, by inference, similar subcellular distribution at steady state. Both false-positive and -negative classifications can occur. Similar profiles may arise by chance; structures with similar size and density will have similar fractionation properties and thus may not be resolved by the method. The resolution is also lower for similar profiles near the baseline (i.e., broad uniform distribution across the subfractions), and the Predictor alerts users when queried with such a profile.

Conversely, there are several reasons why the predictor may not show a previously established interaction. First, the interaction may be transient and thus not substantial at steady state. Second, a binding partner may be mostly engaged in another interaction and thus
profile with this predominating partner. Third, a protein may have multiple stable binding partners, resulting in a “mixed” profile.

A related situation arises when a protein is present at multiple subcellular localizations. As with multiple binding partners, the outcome depends highly on the composition of the preparation. For example, assume that protein X is equally found in compartments A and B. If A and B are both present in similar proportions in the sample preparation, protein X will have a hybrid profile, which may be difficult to interpret or even without predictive value. If compartment A is strongly enriched in the preparation but compartment B is not, then the A profile will dominate, and vice versa. A good example of this situation is the cation-independent mannose 6-phosphate receptor (IGF2R), a cargo protein of CCVs. It is abundant in CCVs but also present at the trans-Golgi network and on endosomes, fragments of which are present in the vesicle fraction. Because the CCVs are strongly enriched in the preparation compared with the other membrane compartments, IGF2R has a clear CCV profile. This example further highlights how fractionation profiling benefits from a sample preparation highly enriched in the organelle of interest.

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