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Using stable isotope tagging and mass spectrometry to characterize protein complexes and to detect changes in their composition

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Running head: Protein complex analysis using stable isotope tagging and MS

Key Words: mass spectrometry, stable isotope tagging, ICAT reagents, quantification, protein complex, dynamics, affinity purification, SEQUEST, Peptide prophet, Protein prophet, ASAPratio.

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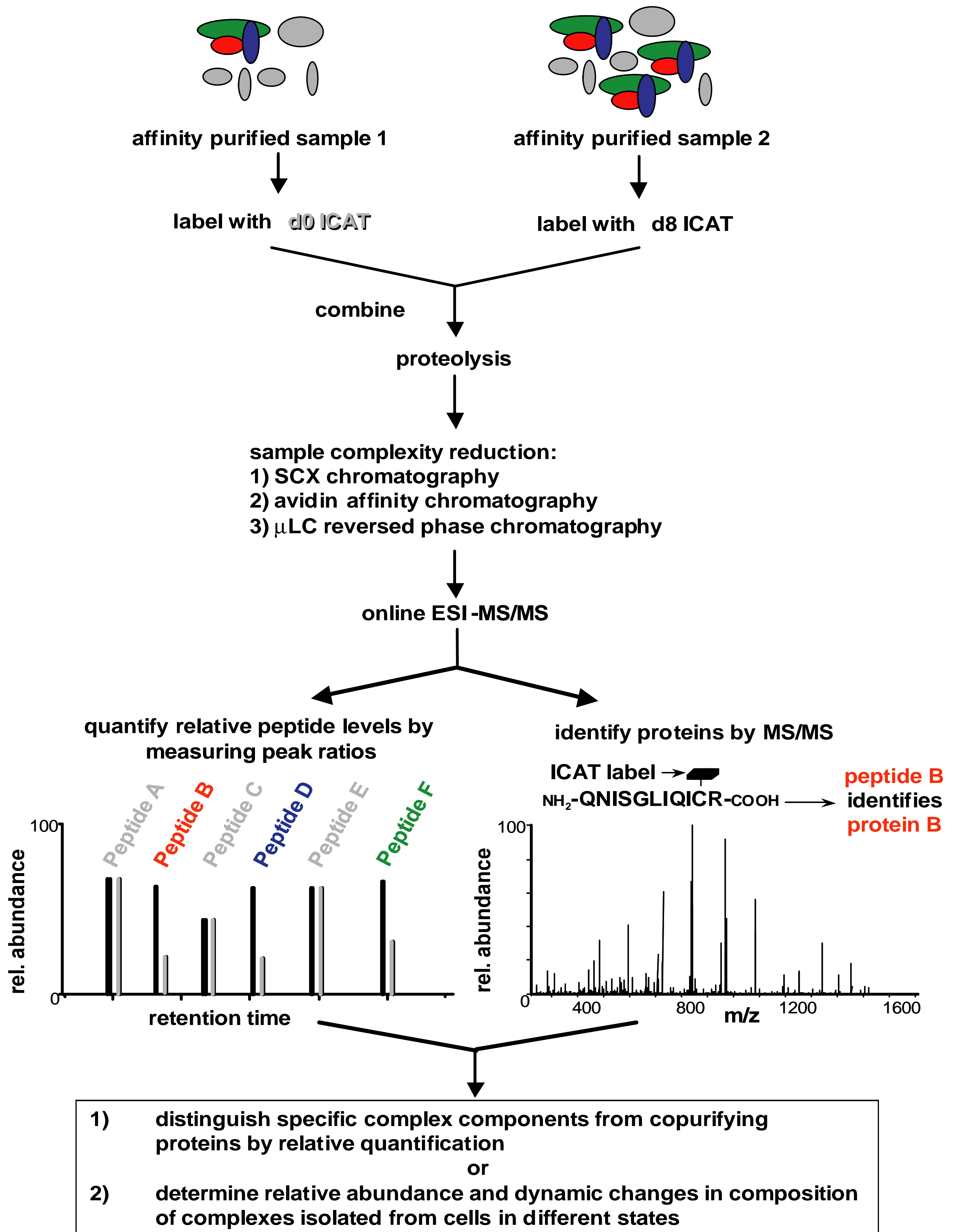
Figure 1



Abstract

One of the primary goals of proteomics is the description of the composition, dynamics and connections of the multiprotein modules that catalyze a wide range of biological functions in cells (1). Mass spectrometry has proven to be an extremely powerful tool for characterizing the composition of purified complexes (2-4). However, because mass spectrometry is not a quantitative technique, the usefulness of the data is limited. For example, without quantitative measurements, it is difficult to detect dynamic changes in complex composition, and it can be difficult to distinguish bona fide complex components from nonspecifically co-purifying proteins. Here we describe a strategy for characterizing the composition of protein complexes and their dynamic changes in composition by combining affinity purification approaches with stable isotope tagging and mass spectrometry. The use of software tools for statistical analysis of the data is also described.

1. Introduction

Mass spectrometric analysis of purified protein complexes is an extremely powerful tool for identification of protein components and their post translational modifications (5), and the development of rapid methods for protein complex purification, such as tandem affinity tagging (6), has enabled the isolation of numerous complexes for mass spectrometric analysis (2, 3, 7). However, even with the best purification protocols, it is often difficult to purify a complex to homogeneity. Without proper controls, this can lead to the identification of nonspecifically co-purifying proteins. Furthermore, protein complexes are dynamic. Individual subunits have a wide range of affinities for the complex, and composition can change depending on the status of the cell. Because mass spectrometry is not an inherently quantitative technique, it is difficult to detect these changes.

The development of stable isotope tagging approaches permit quantification of the relative levels of proteins in two or more samples (8). Peptides derived from proteins, which are differentially labeled with stable isotopes, can be distinguished by a characteristic mass shift in the mass spectrometer. Importantly, the isotopically labeled peptides are virtually chemically identical and thus generate similar specific signal intensities in a mass spectrometer. Therefore, the relative levels of the isotopically labeled peptides can be determined by comparing the signal intensities of sibling peptides. Application of isotope tagging and mass spectrometry to the analysis of protein complexes can guide the identification of bona fide complex components by comparing the relative abundances of peptides derived from a specific purification and a control purification in which the complex of interest is not enriched (9-12) (Figure 1). It can also

be used to detect changes in the composition of complexes by comparing the relative abundances of peptides derived from complexes isolated from cells exposed to different growth conditions (10, 13). Here we describe methods for using stable isotope tagging and mass spectrometry to characterize the composition of protein complexes and to detect changes in their composition. There are now several approaches available for isotope tagging, including ICAT and related approaches, oxygen 18 (18O) labeling, and SILAC, see (8) for review. Here we concentrate on the ICAT labeling technique because, like 18O labeling, it is a post isolation approach, and these approaches are the most general. In addition, they are compatible with labeling tissues.

2. Materials

2.1 Characterization of protein complexes

2.1.1 Preparation of yeast nuclear extracts (http://www.fhcrc.org/labs/hahn/methods/biochem_meth/)

1.YPD (for cell growth): 10 g yeast extract, 20 g peptone, water to 950 ml, autoclave, add 50 ml 40% glucose before use.

2. YPD/S: 20 g yeast extract, 40 g peptone, 40 g glucose, 364 g sorbitol, water to 2 liters. Prepare fresh on the day extracts are being prepared.

3. YPD/S(4°C): 20 g yeast extract, 40 g peptone, 40 g glucose, 364 g sorbitol, water to 2 liter. Prepare fresh on the day extracts are being prepared. Store at 4°C.

4. 1M Sorbitol (4°C): 182 g sorbitol, water to 1 liter. Prepare fresh. Store at 4°C.

5. Zymolyase (ICN Biomedicals, Irvine, CA): Dissolve at 6 mg/ml in 50 mM Tris-HCl pH 8 with 2X concentrated protease inhibitors. Incubate 10 min on ice

before using. This material does not dissolve well, so keep in suspension as well as possible. Zymolyase is reportedly contaminated with proteases, so extra care is needed to wash spheroplasts.

6. Buffer A (400 ml): 18% polysucrose 400 (or Ficoll 400), 10 mM Tris acetate pH 7.5, 20 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA. The polysucrose takes many hours to dissolve and is frequently stirred overnight. Add 0.5 mM spermidine, 0.15 mM spermine, 3 mM DTT, and protease inhibitors before use.

7. Buffer B: 100 mM Tris acetate, 50 mM potassium acetate, 10 mM magnesium sulfate, 20% glycerol, 2 mM EDTA. Adjust pH to 7.9 with KOH. Store buffer at 4°C. 3 mM DTT and protease inhibitors are added before use.

8. Buffer C: 20 mM HEPES, 10 mM magnesium sulfate, 1 mM EGTA, 20% glycerol. Adjust pH to 7.6 with KOH. Store buffer at 4°C. 3 mM DTT and protease inhibitors are added before use.

9. 1.5 liters Buffer C + 75 mM ammonium sulfate: Store buffer at 4°C. 3 mM DTT and protease inhibitors are added before use.

10. Protease inhibitors: 0.1 mM PMSF (100X), 16 mg/ml in ethanol. Store at -20°C. Benzamidine (100X), 32 mg/ml in water. Store at -20°C. Leupeptin (500X), 0.15 mg/ml in ethanol. Store at -70°C for less than 6 months. Pepstatin (200X), 0.28 mg/ml in methanol. Store at -20°C. Chymostatin (2,500X), 5 mg/ml in DMSO. Store at -20°C.

2.1.2 Immunopurification of protein complexes

1. Buffer IP: 20 mM HEPES, pH 7.6, 100 mM potassium acetate, 1 mM EDTA. 5 mM magnesium acetate, 0.05% NP-40. Add protease inhibitors before use.

2. Buffer EL: 20 mM HEPES, pH 7.6, 100 mM potassium acetate, 1 mM EDTA. 5 mM magnesium acetate, 0.0025% NP-40.

3. FLAG-M2 agarose (Sigma-Aldrich, St. Louis, MO)

4. 3XFLAG peptide (Sigma-Aldrich)

2.1.3 Stable isotope labeling with Cleavable ICAT reagents

1. Microcon 10 concentrators, (Millipore, Billerica, MA)

2. TE 8.3: 20 mM Tris-HCl, pH 8.3, 1 mM EDTA

3. Tributylphosphine (TBP) (Sigma-Aldrich), TBP is toxic, work in the hood. Prepare a 5% solution (0.2 M) in 1-propanol (HPLC grade) (see Note 1)

4. Cleavable ICAT reagents (Applied Biosystems, Foster City, CA). The reagents are light sensitive. Perform labeling reactions in tubes supplied by vendor.

2.1.4 Proteolysis, fractionation and purification of ICAT labeled peptides

1. Endoproteinase LysC, sequencing grade (Roche Diagnostics Corporation, Indianapolis, IN) Resuspend in 0.4% acetic acid at 0.25 mg/ml.

2. Trypsin, sequencing grade modified (Promega Corporation, Madison, WI). Resuspend in buffer supplied by vendor at 1 mg/ml.

2.1.5 Strong cation exchange (SCX) fractionation of peptides

1. SCX Buffer: 5 mM KH_2PO_4, pH to 3 with 10% phosphoric acid, then add acetonitrile to 25%.

2. SCX Buffer + 1M KCl: 5 mM KH_2PO_4, 1M KCl, pH to 3 with 10% phosphoric acid, then add acetonitrile to 25%.

3. 200 microliter SCX cartridges (Applied Biosystems) (see Note 2)

2.1.6 Purifying ICAT labeled peptides

1. 10X PBS (Fisher Scientific International Inc., Hampton, NH): 1.37M Sodium Chloride, 0.027M Potassium Chloride, and 0.119M Phosphate Buffer. Dilute 10X PBS 1/5 and 1/10 for 2X and 1X solutions, respectively.

2. Monomeric avidin cartridge (Applied Biosystems) (see Note 3)

3. Avidin elution buffer: 30% acetonitrile, 0.4% trifluoroacetic acid (TFA) Prepare fresh.

4. Avidin wash buffer: 50 mM ammonium bicarbonate, 20% methanol. Prepare fresh.

5. Glass collection vials (Waters, Milford, MA cat. # WAT025054)

2.1.7 Removing biotin from ICAT labeled peptides

1. Cleaving Reagent A (Applied Biosystems) contains concentrated TFA. TFA is toxic, work in the hood.

2. Cleaving Reagent B (Applied Biosystems) contains a scavenger that reduces side reactions during the cleaving reaction.

2.1.8 Mass spectrometry analysis

1. 75 micron ID X 360 OD micron fused silica capillary tubing (Polymicro Technologies, Cedar Hill, TX)

2. Pressure bomb (Mass Evolution, Houston, TX)

3. Magic C18 resin (Michrom Bioresources, Inc., Auburn, CA), 5 micron, 200A.

4. HPLC Buffer A: 0.4% acetonitrile, 0.005% heptafluorobutyric acid (HFBA)

5. HPLC Buffer B: 100% acetonitrile, 0.4% acetonitrile, 0.005% HFBA

6. LCQ ion trap mass spectrometer with nanoelectrospray ionization source (Thermofinnigan, Waltham, MA)

2.1.9 Data analysis

1. SEQUEST software (Thermofinnigan)

2. Protein Prophet, Peptide Prophet, Xpress, ASAPratio (http://sashimi.sourceforge.net/)

2.2 Characterizing changes in complex composition

2.2.1 Preparation of MEL cell nuclear extracts

1. Cell culture medium: RPMI 1640, 10% Fetal Bovine Serum, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin, 2mM Glutamine.

2. Buffer A: 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl. Store at 4^0C. Add 0.5 mM DTT and Protease Inhibitors Cocktail EDTA-free (Roche) before use.

3. Buffer B: 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 600 mM KCl, 25% Glycerol, 0.2 mM EDTA. Store at 4^0C. Add 0.5 mM DTT and Protease Inhibitors Cocktail EDTA-free (Roche) before use.

4. Buffer C: 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 100 mM KCl, 20% Glycerol. Store at 4^0C. Add 0.3 mM DTT and Protease Inhibitors Cocktail EDTA-free (Roche) before use.

2.2.2 Immunopurification of protein complexes

1. p18-specific rabbit polyclonal antibodies (Santa Cruz, Santa Cruz, CA, sc-477)

2. Normal rabbit IgG (Santa Cruz, sc-2027)

3. Protein A-Sepharose resin (Pharmacia, New York, NY)

3. DMP: Dimethylpimelimidate (Sigma-Aldrich)

4. Crosslink buffer A: 3M NaCl, 50 mM Na Borate pH 9.0

5. Crosslink buffer B: 3M NaCl, 200 mM Na Borate pH 9.0

6. Ethanolamine 0.2 M, pH 8.0

7. IP buffer: 25 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 10 % Glycerol, 0.1 % NP40. Store at 4^0C. Add 0.3 mM DTT and Protease Inhibitors Cocktail EDTA-free (Roche) before use.

8. Elution buffer: 6M urea. Prepare fresh.

2.2.3 Stable isotope labeling with ICAT reagents-see 2.1.3

1. Nanosep 3K centrifugal devices (Pall Corporation, New York, NY)

2.2.4 -2.2.6 see 2.1.4-2.1.6

2.2.7 Preparation of control peptides

1. 1cc MCX cartridges (Waters)

3. Methods

We describe methods for characterizing the composition of protein complexes and for detecting changes in the composition of complexes using stable isotope tagging and mass spectrometry. In both approaches, single-step affinity purifications can be used for

complex isolation. Due to the high resolving power of liquid chromatography-mass spectrometry (LC-MS) techniques, the co-purifying contaminants are not detrimental. In fact, they are actually useful for calibrating a common ratio. In addition, the potential for sample losses and for the dissociation of weakly interacting factors is minimized by using single-step affinity purifications. In the method for characterizing the composition of protein complexes, specific complex components are distinguished from nonspecific, co-purifying proteins, by comparing the relative abundance of isotopically labeled peptide pairs derived from affinity purification of the complex of interest and a control purification that is performed in parallel (9, 10). In the example presented here, a previously uncharacterized yeast protein is tagged with a FLAG epitope at its' chromosomal locus, and extracts are prepared from this strain and from an untagged strain (11). Immobilized anti-FLAG antibodies are used to purify the FLAG tagged protein along with associated proteins. In an attempt to preserve complex interactions, the antibody resin is washed with a buffer containing low concentrations of salt and detergent (100 mM potassium acetate, 0.05% NP40). Proteins are eluted from the resin by competition with a triple FLAG containing peptide and prepared for labeling with ICAT reagents by concentration and buffer exchange with filtration devices. After labeling with ICAT reagents, the proteins mixtures are combined and digested with endoproteinase Lys-C and trypsin. Peptides are fractionated on strong cation exchange columns and ICAT labeled peptides are then isolated by avidin affinity chromatography. Labeled peptides are analyzed by microcapillary reversed phase liquid chromatography (μLC) electrospray ionization (ESI) tandem mass spectrometry (MS/MS), and peptides are identified by sequence database searching using the search algorithm SEQUEST (14).

The relative abundances of identified ICAT labeled peptide pairs are determined from the ratio of the peptides' signal intensities using Xpress (15) or ASAPratio software (16). Bona-fide components of the complex (or complexes) are identified by their increased abundance in the specific purification compared to the nonspecific purification. The significance of each peptide and protein identification is estimated using the software tools Peptide Prophet (17), Protein Prophet (18). ASAPratio provides a statistical assessment to help distinguish potential complex components with significant abundance changes from the population of nonspecific proteins.

In the method for detecting changes in complex composition, the transcription factor p18NF-E2/MafK is immunopurified from murine erythro-leukemia (MEL) cell extracts, derived from either proliferating or differentiating cells, using an immobilized p18-specific antibody (13). After washing the antibody resin, the bound proteins are eluted by incubation with 6 M urea for 2h at 37ºC. Control immunopurifications are performed in parallel using normal rabbit IgG. Eluted proteins from the p18 immunopurifications are prepared for isotopic tagging and mass spectrometry analysis as described above. Proteins from the control purification are directly analyzed by mass spectrometry without isotopic tagging (see Note 4). Protein identification and relative abundance ratios are determined using SEQUEST (14) and Xpress (15) or ASAPratio (16), respectively. Only proteins which are enriched in the p18 specific purifications and are not detected in the control fractions (as tested by mass spectrometry and/or Western blot) are considered p18-interacting proteins. The significance of each peptide and protein identification was estimated using the Peptide Prophet (17) and Protein Prophet (18).

3.1 Characterizing the composition of protein complexes

3.1.1 Preparation of yeast nuclear extracts

(**http://www.fhcrc.org/labs/hahn/methods/biochem_meth/**)

Day1

1. The *Saccharomyces cerevisiae* strains JRY14 (TFB5-FLAG) and BWG1-7a (untagged) are grown in YPD media to an OD600 of 3 at 30°C. For wild type cells, ~2.5 ml of a saturated overnight culture inoculated per liter at 5:30 pm gives A600 of ~3 at 9:00 am. Grow 3 liters of cells.

2. Harvest cells in 1 liter bottles (4.5 K x G for 10 min, *i.e.,* 4 K rpm in a Beckman J6-HC centrifuge). Drain excess media as well as possible and weigh cells. Expected yield is 20-35 g cells. If cells are overgrown, zymolyase will work poorly in spheroplasting cells.

3. Resuspend cell pellets in 35 ml 50 mM Tris-HCl 7.5, 30 mM DTT. Usually this can be done by gently shaking the centrifuge bottles. Leave cells in 1 liter bottles. Incubate at 30°C for 15 min.

4. Pellet cells (4.5 K x G for 8 min) and resuspend in 20 ml YPD/S. Add 2 to 3 ml 2M sorbitol and an equal volume 6 mg/ml zymolyase solution. The amount required can vary from ~12-18 mg depending on the yeast strain. Incubate at 30°C with occasional gentle mixing. (see Note 5)

5. Check progress of spheroplasting every 15 min. To check, mix 4 microliters of cells with 4 microliters 1% SDS on a glass slide. Observe the number of cell ghosts under microscope. Incubate cells until about 80% spheroplasts are obtained. This can take anywhere from 30 min. to 2 1/2 hours. If cells are spheroplasting slowly after 1 hr, an

extra 1 to 2 ml of zymolyase can be added. However, if cells were overgrown (A600 >5), the cells may never spheroplast. Spheroplasting is also somewhat strain dependent.

6. After spheroplasting has reached about 80%, add 100 ml YPD/S (room temp) and pellet cells (4.5 K x G for 12 min).

7. Resuspend cells in 250 ml YPD/S (room temp) and incubate at 30°C for 30 min to allow cells to recover. The resuspension of spheroplasts works best if a small volume (~50 ml) of YPD/S is first added and cells are resuspended using a baking spatula. Then add the remaining YPD/S.

8. Pellet cells (4.5 K x G for 12 min) and resuspend in 200 ml cold YPD/S (4°C). Resuspend as in the previous step. Keep everything cold from this point on. Cells can be kept on ice for an hour or so if other cells are still spheroplasting.

9. Repeat step 8.

10. Pellet cells (4.5 K x G for 12 min) and resuspend in 250 ml cold 1M sorbitol.

11. Pellet cells (4.5 K x G for 12 min) and drain sorbitol media as well as possible. (careful- sometimes the spheroplast pellet is not very tight). Resuspend in 100 ml Buffer A at 4°C.

12. Dounce the spheroplasts three times using a B type pestle. Transfer dounced cells to GSA bottles.

13. Spin 4.1 K x G for 8 min (5 K rpm in a GSA rotor). Transfer supernatant to new GSA bottles. Do not worry about the slimy loose pellet that also transfers. Repeat.

14. Spin supernatant 4.1 K x G for 5 min. Transfer supernatant to new GSA bottle. Repeat. By the last (fourth) spin, the slimy non pelleted material should be nearly gone and the pellets firm.

15. Transfer supernatant to 50 ml centrifuge tubes and pellet crude nuclei. Spin 20.2 K x G for 30 min (13 K rpm in an SS34 rotor). Remove supernatant by dumping and discard. Drain pellets.

16. Resuspend crude nuclear pellets with a small spatula in 10 ml Buffer B and transfer to 50 ml screwcap tubes. The prep can be stopped at this point. Quick freeze and store resuspended nuclear pellets at -70°C.

Day 2

1. Thaw nuclei on ice and measure volume. Add 3M ammonium sulfate (pH 7.5) to 0.5 M final concentration (1/5 original volume of nuclei) and immediately mix and incubate on roller in cold room for 30 min. After 10 min, break up any lumps with a glass rod. This step lyses nuclei.

2. Transfer to SW28 thick walled ultracentrifuge tubes and spin at 28 K rpm (141 K x G) for 90 min at 4°C in an ultracentrifuge.

3. Carefully remove supernatant with a 5 ml pipette (and pasteur pipette if necessary) being careful to avoid the pellet. Do not worry about the white floating material. Transfer to 50 ml screwcap tube.

4. Add 0.35g solid ammonium sulfate/ml supernatant and immediately incubate on cold room roller for 30 min. The ammonium sulfate can be added all at once if a number of preps are being done. However, it is best if ammonium sulfate is added slowly while stirring supernatant in a beaker. The pH should remain above 7 (it almost always does) but should be checked. Adjust pH with 1M NaOH if necessary.

5. Transfer to thick walled ultracentrifuge tubes and spin in SW28 at 10 K rpm (18 K x G) for 20 min at 4°C. Remove supernatant by dumping and re spin pellets at 10 K rpm for 4 min. Carefully remove all remaining supernatant with a pasteur pipette.

6. Resuspend pellets in Buffer C containing DTT and protease inhibitors. Depending on protein pellet size, resuspend in 0.4-1.5 ml buffer. This can be done with a small dounce homogenizer or a blue pipette tip depending on the amount of protein. Extracts can be frozen on dry ice and stored at -70°C at this point.

7. Dialyze nuclear extracts against 500 ml Buffer C + 75 mM ammonium sulfate at 4°C. Exchange buffer after two and four hours.

8. Aliquot extract and store at -70°C.

9. Measure the protein concentration using a Bradford assay. Extracts are sometimes difficult to get a reproducible measurement of protein concentration using the BioRad assay. This modified method works well. Dilute extract 1/4 in 0.1% SDS. Add 1-2 microliters of diluted extract to 0.8 ml water in a 13x100 mm disposable test tube. Add 1 microliter 0.1% SDS to protein standards. Add 0.2 ml dye reagent. After 10 min, read absorbance at A595. Extracts should be 25-50 mg/ml in protein.

3.1.2 Immunopurification of protein complexes

1. 12.5 mg nuclear extract from JRY14 (TFB5-FLAG) and BWG1-7a is diluted to 5 ml in Buffer IP, incubated at 22°C for 10 min, and then centrifuged at 3000 x G for 2 minutes. The supernatant is retained.

2. 2 ml of a 50 % slurry of FLAG-M2 agarose beads is prepared by washing with 20 ml Buffer IP in a 10 ml Bio-Rad column, followed by washing with ~4 ml 0.1 M glycine pH

3.5 over 15 minutes. Next, the beads are equilibrated with 10 ml Buffer IP. Be careful not to overexpose the beads to acidic glycine.

3. Transfer 1 ml of the 50% slurry of FLAG-M2 beads to a 15 ml tubes. Gently pellet the beads by centrifuging at 1000 x G for 5 min. Remove the supernatant, and add the clarified extracts. Extracts are incubated with the beads for 2 hours at 4°C with gentle agitation, after which the beads are pelleted at 1000 x G for 5 min and the supernatants are removed. *Save the supernatants for monitoring binding efficiency*.

4. The beads are washed twice with 12 ml Buffer IP, and then twice with 12 ml Buffer EL, by incubating them for 5 min at 4°C with gentle agitation.

5. Beads are transferred to mini spin columns (Bio-Rad, Hercules, CA), the supernatant is discarded and proteins are eluted by incubating beads in 0.5 ml Buffer EL containing 0.1 mg/ml 3XFLAG peptide for 30 minutes at 22°C. The supernatant is collected and the elution step is repeated. The beads are washed with 0.5 ml Buffer EL and the wash is combined with the previous elutions. *Save ~1/40 of the eluates for analysis by western blotting and silver stained SDS-PAGE*.

3.1.3 Stable isotope labeling with ICAT reagents

1. Eluted protein samples (each containing approximately ~30 µg of total protein) are concentrated to ~50 microliters in Microcon 10 devices by centrifuging at 3000 x G in the cold room. The buffer is exchanged by addition of 500 microliters of TE 8.3 containing 50 mM NaCl, and the volume is reduced to ~25 microliters. *Save an aliquot of the concentrated samples for analysis by SDS-PAGE* (see Note 6).

2. SDS is added to 0.3%, and the samples are boiled for 5 min.

3. Proteins are reduced with 5 mM TBP at 37°C for 30 min, and then diluted with 125 µl TE 8.3 containing 7.2 M urea.

4. Isotopically heavy or light ICAT reagents are added to 1.5 mM. (see Note 7). There are 175 nmoles of reagent per tube. Briefly spin 2 tubes of isotopically heavy and 2 tubes of isotopically light ICAT reagent in a microcentrifuge to bring the reagent to the bottom of the tubes. Resuspend one tube of heavy and one tube of the light reagent in 17.5 microliters methanol (10 nmol/microliter). Add each protein solution to one tube of heavy or light ICAT reagent. To reach 1.5 mM ICAT concentration, add an additional 5 microliters of the appropriate reagent to each tube. Vortex the samples thoroughly to resuspend the ICAT reagent, and incubate for 90 min at 22 °C.

5. Reactions are quenched by addition of 10 mM β–mercaptoethanol or DTT for 20 min at 37°C. *Optional: Save an aliquot (~1/40) of each sample to monitor labeling efficiency by SDS-PAGE analysis.*

3.1.4 Proteolysis and SDS-PAGE analysis

1. Samples are combined, and proteins are digested by addition of endoproteinase Lys-C (1:100 w/w) at 37°C for 3 h. SDS and urea concentrations are reduced to 0.01% and 1.2 M respectively, by addition of TE 8.3, and samples are digested with trypsin (1:20 w/w) overnight at 37 °C. *Optional: Save an aliquot (~1/80) to monitor digestion efficiency by SDS-PAGE analysis.*

2. Before performing SCX fractionation, it is advisable to analyze the samples by SDS-PAGE and silver staining to monitor digestion efficiency. Analyze an aliquot of the starting samples, the ICAT labeled samples, and the combined digested sample. The lane

containing the digested sample should contain very little full length proteins. Trypsin migrates at 24kDa and it may be visible. If the sample is not completely digested, add more trypsin (1:20, w/w), and incubate at 37°C for 3 hours.

3.1.5 SCX fractionation of peptides

This step removes SDS and trypsin, and permits reduction of sample complexity.

1. Peptides are diluted with an equal volume of SCX Buffer and the pH is adjusted to 3 with 10% trifluoroacetic acid (TFA). 10% TFA is added in 1-5 microliter increments and the pH is monitored by spotting 1 microliter of sample onto pH paper.
2. SCX cartridges are prepared by washing with 3 ml SCX Buffer containing 1 M KCl, and equilibrated with 3 ml SCX Buffer (see Note 2). To wash SCX cartridges, fill a 1-2.5 ml Hamilton syringe with the appropriate solution, remove air bubbles, insert the syringe needle into the needle port adapter and slowly depress the plunger. For washing and equilibrating, inject the solution so that 2 to 3 drops/second flow from the outlet.
3. Peptides are slowly loaded onto equilibrated SCX cartridges (~1 drop/second). Save the flow through. Peptides are successively eluted with 0.75 ml SCX Buffer containing 40 mM, 200 mM, 350 mM, and 600 mM KCl into eppendorf tubes (see Note 8).
4. To clean the SCX cartridge, wash with 2 ml SCX Buffer containing 1 M KCl. To store the column, wash with 2 ml SCX Buffer. For long term storage include 0.1% sodium azide in the wash. Cartridges can be reused ~20 times depending on the complexity of the samples.

3.1.6 Purifying ICAT labeled peptides

1. Reduce the acetonitrile concentration by drying the samples under reduced pressure to ~350 microliters, and dilute the samples 2-fold with 2X PBS (pH 7.2). Adjust the pH to ~7 with ~10 microliters 1M ammonium bicarbonate.

2. Prepare a monomeric avidin cartridge by washing with 2 ml avidin elution buffer, followed by 2 ml 2X PBS (pH 7.2) (~2 drops/second) (see Note 3).

3. Slowly load peptides onto avidin cartridges (~1 drop/second). Save the flow through.

4. Wash the cartridge with 2 ml 2X PBS (pH 7.2), followed by 1 ml 1X PBS (pH 7.2) to reduce the salt concentration.

5. Wash the cartridge with 1 ml avidin wash buffer. This step removes nonspecifically bound peptides.

6. Wash with 1 ml MilliQ water.

7. Peptides are eluted by slowly injecting 800 microliters avidin elution buffer (1 drop/second). Allow the first 50 microliters to go to waste. Collect the remaining 750 microliters in a glass vial.

8. To purify additional samples, repeat the procedure beginning at step 2.

9. To store the cartridge, wash with 2 ml avidin elution buffer, followed by 2 ml 2X PBS (pH 7.2). Include 0.1% sodium azide for long-term storage.

3.1.7 Removing biotin from ICAT labeled peptides

1. Purified peptides are dried under reduced pressure.

2. Prepare the final cleaving reagent by combining Cleaving Reagent A and Cleaving Reagent B in a 95:5 ratio. ~ 90 µL of final cleaving reagent are needed for each fraction.

3. Vortex to mix, then centrifuge for a few seconds to bring the solution to the bottom of the tube.

4. Add ~90 µL of freshly prepared cleaving reagent to each sample tube.

5. Vortex to mix, then centrifuge for a few seconds to bring the solution to the bottom of the tube.

6. Incubate for 2 hours at 37 °C.

7. Centrifuge the tube for a few seconds to bring the solution to the bottom of the tube.

8. Evaporate the sample to dryness in a centrifugal vacuum concentrator (~30 to 60 min).

3.1.8 Mass spectrometry analysis

1. Peptides are resuspended in 10% acetonitrile, 0.1% TFA.

2. Peptides are pressure loaded onto in-house prepared 10 cm x 75 µm fused silica microcapillary columns packed with 5 micron Magic C18 beads and equilibrated with HPLC Buffer A containing 10% acetonitrile using a pressure cell at 1000 psi helium. (see Note 10).

3. Columns are placed in-line with a mass spectrometer that is capable of acquiring tandem mass spectra. Peptides are resolved by running 80 min gradients from 10-40% HPLC Buffer B at 0.3 µl/min, and analyzed by automated data-dependent tandem mass spectrometry. The mass spectrometer is set to scan from 400-1800 m/z followed by one data dependent MS/MS scan on the most abundant ion. Dynamic exclusion is set to exclude ions that have been selected for MS/MS analysis for 2 minutes with a mass window of 2 daltons.

3.1.9 Data analysis

1. Peptides are identified by searching MS/MS spectra against an appropriate database using the SEQUEST algorithm. For ion trap data, peptide mass tolerance is set at 3 daltons, and average masses are used for the precursor masses and for fragment masses. The mass of cysteine is statically modified by 227.13 daltons which accounts for the mass of the isotopically normal form of ICAT after the cleavage reaction that is added to the peptides. In addition, the mass of cysteine is differentially modified by 9.03 daltons to account for peptides modified with the isotopically heavy form of the ICAT reagent. Methionine is also differentially modified with 16 daltons to account for oxidized methionine residues.

2. Next, the search results are analyzed with the programs Peptide Prophet and Protein Prophet (17, 18). These programs estimate the probability of each peptide and protein identification using a statistical model that is based on a number of criteria including Sequest search scores and the number of tryptic termini of peptides. We typically select proteins with probability values $\geq$ 0.9 for further analysis.

3. The relative abundance ratios of correctly identified ICAT labeled peptide pairs are determined using XPRESS (15) or ASAPratio (16) programs. Ratios are calculated by comparing the summed ion intensity of each peptide over its elution peak. To adjust for any systematic error due to sample handling, ASAPratio normalizes the abundance ratios. This is done by generating a ratio distribution from the logarithm (base 10) of all peptide ratios, fitting it with a normal distribution, and setting the most common ratio to 1.

4. Specific complex components are distinguished from nonspecifically co-purifying proteins by inspection of the distribution of all abundance ratios. ASAPratio calculates a

P-value for each protein from the distribution that can be used to distinguish true interactor's from the background of co-purifying proteins (see Note 12).

3.2 Characterizing changes in complex composition

3.2.1 Preparation of Mouse Erythro-Leukemia (MEL) cell nuclear extracts

1. MEL cells are grown in spinner flasks in RPMI cell culture medium up to a concentration of $1.5x10^6$ cell/ml, in the absence (proliferating) or presence (differentiated state) of 2% DMSO. Grow 8 L of each differentiation state.

2. Nuclear extracts from proliferating and differentiating cells are prepared separately.

3. Cells are harvested in 500 ml conical tubes (500 x G for 10 min at 4^oC, *i.e.,* 1.5 K rpm in a Beckman Allegra X-15R, rotor SX 4750), and washed with 5 Packed Cell Volumes (PCVs) of ice-cold PBS buffer.

4. Cells are resuspended in 5 PCVs of ice-cold buffer A, and incubated on ice for 10 min to allow swelling.

5. Cells are pelleted (500 x G for 10 min), resuspended in 2 PCVs of ice-cold buffer A and lyzed on ice by 10 dounces using a B-type pestle (Kimble / Kontes, Vineland, NJ). Cell lysis is checked under a microscope using Trypan blue.

6. Nuclei are pelleted at 25,000 X G for 30 min at 4^oC (Beckman Coulter, Inc., Fullerton, CA, rotor JA25.50) and the supernatants are discarded.

7. Nuclei are resuspended in one Nuclear Pellet Volume (NPV) of ice-cold Buffer B, and nuclear proteins are extracted on ice by 10 dounces using a B-type pestle (Kimble / Kontes), followed by a 30 min incubation on a roller in cold room.

8. Nuclear extracts are recovered after centrifugation at 25,000 x G for 30 min at 4^oC (Beckman Coulter, Inc., rotor JA25.50), and dialyzed twice against 50 volumes of Buffer C for 2 and 12h, respectively.

9. After dialysis, nuclear extracts are further centrifuged (17 K x G for 15 min at 4^oC) to eliminate proteins that precipitated during dialysis. The supernatants are then recovered and NP40 is added up to 0.1% final.

10. Nuclear extracts are aliquoted, snap frozen in liquid nitrogen and stored at -80^oC.

11. The total protein concentration is measured using a Bradford assay, and should be 1-5 mg/ml for the differentiated extract and 5-10 mg/ml for the proliferating extract.

3.2.2 Immunopurification of protein complexes

3.2.2.1 Crosslink of p18-specific antibodies on protein A Sepharose

1. The pH of the antibody solution (containing 1.6 mg of p18-specific antibodies) is adjusted to 9.0 with NaOH. NaCl is added to this solution up to a final concentration of 3M. Check the pH again and eventually re-adjust to 9.0 with NaOH.

2. 1.6 ml of protein A-Sepharose resin is washed with 10 volumes H_2O and equilibrated with 10 volumes of IP buffer containing 100 mM KCl.

3. Mix the antibody solution with the protein A Sepharose resin for 1h at room temperature with rotation.

4. The beads are washed twice with 10 volumes of crosslink buffer A and resuspended in 10 volumes of crosslink buffer B. Save an aliquot for Coomassie stained SDS-PAGE analysis of the crosslink.

5. Add DMP to bring the final concentration to 20 mM and mix for 30 min at room temperature with rotation. Save an aliquot for Coomassie stained SDS-PAGE analysis of the crosslink.

6. The reaction is stopped by washing the beads once in 0.2M ethanolamine and incubating in 0.2M ethanolamine for 2h at room temperature with rotation.

7. The antibody-bound beads are equilibrated with IP buffer containing 100 mM KCl by washing 3 times with 10 bed volumes.

8. The efficiency of the crosslink is verified by the absence of the antibody heavy chain in the samples after crosslink as analyzed on the Coomassie gel.

3.2.2.2 Crosslink of normal rabbit IgG on protein A Sepharose

1. Normal rabbit IgG (200 µg) is cross-linked to protein A-Sepharose (200 µl) resin with 20 mM final of dimethylpimelimidate as described in 3.2.2.1.

3.2.2.3 Immunopurification using p18-specific antibodies

1. 8 ml of nuclear extract from proliferating and differentiating cells (containing equal amounts of p18) are separately incubated with the p18-specific antibody-bound resin (0.8 ml each) at 4°C for 12h with rotation. Beads are recovered by centrifugation at 500 x G for 5 min and the supernatants (unbound proteins) are kept for analysis by Western blot.

2. Antibody-bound proteins are washed twice with 10 bed volumes of ice-cold IP buffer containing 300 mM KCl, and equilibrated similarly with IP buffer containing 100 mM KCl. All washes are kept for analysis by Western blot.

3. Bound proteins are then eluted by incubation with 1 bed volume of pre-heated 6 M urea for 2h at 37°C with rotation. ~ 1/40 of the eluates are saved for analysis by Western blot and silver stained SDS-PAGE.

3.2.2.4 Control immunopurification using normal rabbit IgG

1. 1 ml of nuclear extract from proliferating and differentiating cells (containing equal amounts of p18) are separately incubated with the normal rabbit IgG resin (0.1 ml each) at 4°C for 12h with rotation.

2. Samples are then treated as described in 3.2.2.3 and proteins are eluted in 100 µl 6 M urea.

3.2.3 Stable isotope labeling with ICAT reagents

1. Eluted proteins from the p18 purifications (each containing approximately 3 µg of total protein) are concentrated to 25 µl each in Nanosep 3 K centrifugal devices (see Note 6).

2. The solutions are adjusted to 20 mM Tris-HCl 8.3, and 1 mM EDTA. Keep an aliquot for analysis by silver stained SDS-PAGE.

3. The proteins are denatured by adding SDS to 0.3% and heating to 37°C (see Note 13).

4. The samples are reduced by addition of TBP to 5 mM and incubation at 37°C for 30 min. Samples are then diluted with 125 µl TE 8.3 buffer containing 45 mg of urea, in order to obtain final concentrations of 0.05% SDS and 6M urea.

5. One tube of heavy and one tube of light ICAT reagent are briefly spun in a microfuge to pellet the reagent. Protein solutions are added separately to each tube of ICAT reagent and after vortexing thoroughly, the tubes are incubated with shaking for 90 min at 22°C.

6. Reactions are quenched by addition of 10 mM β–mercatopethanol or DTT for 20 min at 37 °C. Optional: Save an aliquot (~1/40) of each sample to monitor labeling efficiency by SDS-PAGE analysis.

3.2.4 Proteolysis and SDS-PAGE analysis-see 3.1.4

3.2.5 SCX fractionation of peptides-see 3.1.5

3.2.6 Purifying ICAT labeled peptides-see 3.1.6

3.2.7 Preparation of control peptides

A mixed bed cation exchange column is used to remove small molecules such as salts, SDS and urea, prior to mass spectrometry.

1. The solutions containing eluted proteins from the control purifications are adjusted to 20 mM Tris-HCl 8.3, and 1 mM EDTA. Keep an aliquot for analysis by silver stained SDS-PAGE.

2. Proteins are reduced by addition of DTT to 2 mM and incubation at 37°C for 30 min.

2. Reduced cysteines are alkylated by addition of iodoacetamide to 10 mM and samples are incubated at 22°C for 20 min in the dark.

3. Proteins are digested by addition of endoproteinase Lys-C (1:100 w/w) at 37 ^{o}C for 3 h. The urea concentration is reduced to 1.2 M, by addition of TE 8.3, and samples are digested with trypsin (1:20 w/w) overnight at 37 ^{o}C. (Optional: Save an aliquot (~1/40) to monitor digestion efficiency by SDS-PAGE analysis).

4. The pH of the samples is adjusted to ~3 by addition of 10% TFA in small aliquots (~1-5 microliters) Check by spotting ~1 microliter to pH paper.

6. Prepare a 1cc MCX mixed bed cation exchange cartridge by wetting with 2 ml methanol, followed by washing with 5 ml 80% acetonitrile, 0.1%TFA, 2 ml water, and 2 ml 10% ammonium hydroxide, 90% methanol, and 2 ml water.

7. Equilibrate with 2 ml 0.1% TFA.

8. Load sample slowly to the cartridge.

9. Wash with 5 ml 80% acetonitrile, 0.1% TFA

10. Wash with 2 ml water.

11. Elute with 1ml 10% ammonium hydroxide, 90% methanol, prepared fresh.

12. Dry samples in speedvac (avoid drying acidic samples at the same time)

and resuspend samples in 0.5 % acetonitrile, 0.1 % TFA.

3.2.8 Mass spectrometry analysis-see 3.1.7

3.2.9 Data analysis-see 3.1.8

Data was analyzed as described in 3.1.8, and a histogram of the measured abundance ratios was plotted. A ratio of 1.2 was assessed as indicative of a significant enrichment either before or after differentiation. This value was chosen after examining the abundance ratios obtained for p18 and for the non specific proteins (the ones present in both the p18 fractions and the control fractions), which served as internal controls, and displayed an average ratio < 1.2 (see Note 4). It should be noted that due to the variation of the signal-to-noise ratios between different peptides, in some cases the abundance ratios should only be regarded as tendency of enrichment and not as absolute enrichment values between different proteins.

4. Notes

1. Tris (2-Carboxyethyl) Phosphine Hydrochloride (TCEP·HCl, Pierce) can be use instead of TBP. It is water soluble. A 0.2M solution is prepared in 100 mM Tris-HCl, 8.3.

2. SCX microcolumns can be prepared in cases where there is a small amount of sample (i.e., ~10 micrograms). (Western Analytical Products, Murrieta, CA) sells 1 mm X 10 mm SCX cartridges.
3. When working with small amounts of sample (i.e., ~10 micrograms starting material) immobilized monomeric avidin (Pierce, Rockford, IL) can be used by packing them into a microcolumn from Western Analytical Products or into a pasteur pipette blocked with glass wool.
4. With the use of iTRAQ reagents (Applied Biosystems), it is now possible to compare the relative abundance ratios of up to four samples in one mass spectrometry experiment. This is very useful in experiments directed at detecting changes in complex composition because the proteins from control experiments can be isotopically tagged and analyzed along with the proteins from the specific purifications. Thus, in one experiment it is possible to accurately distinguish specific complex components from nonspecific proteins, and to detect dynamic changes in complex composition. Mass spectrometers that can detect fragment ions between 114 and 117 m/z are required for quantification of iTRAQ labeled peptides.
5. Recombinant lyticase (Sigma-Aldrich) can be used instead of zymolyase. It is supposed to contain lower levels of proteases than zymolyase. When using lyticase, step 9 of the extract prep can be omitted.
6. A fraction of the sample can be analyzed on a silver stained SDS-polyacrylamide gel along with a titration of a standard protein. This is useful for assessing the quantity, quality, and complexity of the sample.

7. In cases where larger amounts of protein are being labeled, it is important to use enough ICAT reagent to label all of the cysteine residues in the sample. To estimate the total amount of cysteine in the sample, measure the protein concentration of the sample, calculate the number of moles of protein assuming an average molecular weight of 50 kDa, and multiply this number by 6 (~6 cysteine residues/protein). ICAT can be added in a five-fold molar excess over the total moles of cysteine.
8. The number of fractions collected depends on the complexity and quantity of the sample.
9. Many laboratories use autosamplers (i.e. Famos autosampler) in place of pressure bombs for loading samples onto C18 columns.
10. When analyzing the data, it is important to consider the following factors. 1) In a typical experiment, the accuracy of quantification is around 20-25%. 2) For low abundance peptides, or peptides with low ionization efficiency, the measured enrichment values may be affected by signal to noise issues.
11. If urea is used for eluting proteins, the sample should not be boiled before reducing and labeling. Urea can carbamylate proteins at high temperatures. Incubation at 37°C for 30 min is sufficient for denaturing the proteins.

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References

1. Hartwell, L. H., Hopfield, J. J., Leibler, S., and Murray, A. W., From molecular to modular cell biology, *Nature, 402*, C47 (1999).

2. Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G., Functional organization of the yeast proteome by systematic analysis of protein complexes, *Nature, 415*, 141 (2002).

3. Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P., Bennett, K., Boutilier, K., Yang, L., Wolting, C., Donaldson, I., Schandorff, S., Shewnarane, J., Vo, M., Taggart, J., Goudreault, M., Muskat, B., Alfarano, C., Dewar, D., Lin, Z., Michalickova, K., Willems, A. R., Sassi, H., Nielsen, P. A., Rasmussen, K. J., Andersen, J. R., Johansen, L. E., Hansen, L. H., Jespersen, H., Podtelejnikov, A., Nielsen, E., Crawford, J., Poulsen, V., Sorensen, B. D., Matthiesen, J., Hendrickson, R. C., Gleeson, F., Pawson, T., Moran, M. F., Durocher, D., Mann, M., Hogue, C. W., Figeys, D., and Tyers, M., Systematic

identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry, *Nature, 415*, 180 (2002).

4. Krogan, N. J., Peng, W. T., Cagney, G., Robinson, M. D., Haw, R., Zhong, G., Guo, X., Zhang, X., Canadien, V., Richards, D. P., Beattie, B. K., Lalev, A., Zhang, W., Davierwala, A. P., Mnaimneh, S., Starostine, A., Tikuisis, A. P., Grigull, J., Datta, N., Bray, J. E., Hughes, T. R., Emili, A., and Greenblatt, J. F., High-definition macromolecular composition of yeast RNA-processing complexes, *Mol Cell, 13*, 225 (2004).

5. Aebersold, R., and Mann, M., Mass spectrometry-based proteomics, *Nature, 422*, 198 (2003).

6. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B., A generic protein purification method for protein complex characterization and proteome exploration, *Nat Biotechnol, 17*, 1030 (1999).

7. Butland, G., Peregrin-Alvarez, J. M., Li, J., Yang, W., Yang, X., Canadien, V., Starostine, A., Richards, D., Beattie, B., Krogan, N., Davey, M., Parkinson, J., Greenblatt, J., and Emili, A., Interaction network containing conserved and essential protein complexes in Escherichia coli, *Nature, 433*, 531 (2005).

8. Julka, S., and Regnier, F., Quantification in proteomics through stable isotope coding: a review, *J Proteome Res, 3*, 350 (2004).

9. Ranish, J. A., Yi, E. C., Leslie, D. M., Purvine, S. O., Goodlett, D. R., Eng, J., and Aebersold, R., The study of macromolecular complexes by quantitative proteomics, *Nat Genet, 33*, 349 (2003).

10. Blagoev, B., Kratchmarova, I., Ong, S. E., Nielsen, M., Foster, L. J., and Mann, M., A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling, *Nat Biotechnol, 21*, 315 (2003).

11. Ranish, J. A., Hahn, S., Lu, Y., Yi, E. C., Li, X. J., Eng, J., and Aebersold, R., Identification of TFB5, a new component of general transcription and DNA repair factor IIH, *Nat Genet, 36*, 707 (2004).

12. Flory, M. R., Carson, A. R., Muller, E. G., and Aebersold, R., An SMC-domain protein in fission yeast links telomeres to the meiotic centrosome, *Mol Cell, 16*, 619 (2004).

13. Brand, M., Ranish, J. A., Kummer, N. T., Hamilton, J., Igarashi, K., Francastel, C., Chi, T. H., Crabtree, G. R., Aebersold, R., and Groudine, M., Dynamic changes in transcription factor complexes during erythroid differentiation revealed by quantitative proteomics, *Nat Struct Mol Biol, 11*, 73 (2004).

14. Eng, J. K., McCormack, A. L., and Yates, J. R., An approach to correlate tandem mass spectral data of peptides with amino acid sequences in protein databases, *J. Am. Soc. Mass Spectrom., 5*, 976 (1994).

15. Han, D. K., Eng, J., Zhou, H., and Aebersold, R., Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry, *Nat Biotechnol, 19*, 946 (2001).

16. Li, X. J., Zhang, H., Ranish, J. A., and Aebersold, R., Automated statistical analysis of protein abundance ratios from data generated by stable-isotope dilution and tandem mass spectrometry, *Anal Chem, 75*, 6648 (2003).

17. Keller, A., Nesvizhskii, A. I., Kolker, E., and Aebersold, R., Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search, *Anal Chem, 74*, 5383 (2002).

18. Nesvizhskii, A. I., Keller, A., Kolker, E., and Aebersold, R., A statistical model for identifying proteins by tandem mass spectrometry, *Anal Chem, 75*, 4646 (2003).

Figure legend

Figure. 1 Schematic representation of the quantitative proteomics approach for the analysis of affinity purified macromolecular complexes. To distinguish specific complex components from copurifying proteins, a control purification (sample 1) is performed in which the complex of interest is not enriched. To detect quantitative changes in the abundance and composition of a complex isolated from cells in different states, the samples are prepared identically. Affinity purified proteins from specific and control purifications are reduced, labeled with either the isotopically heavy or normal version of the ICAT reagent, and combined. After proteolysis, sample complexity is reduced in three sequential chromatographic steps, followed by ESI-MS/MS analysis. During this process, peptides pairs are quantified by measuring their peak ratios as they coelute from the C18 column into the mass spectrometer. In every other scan, peptides are selected for fragmentation. The resulting MS/MS spectra are used to search sequence databases using SEQUEST to identify the peptides and thus the proteins from which they originated. Depending on the experiment, the relative quantification can be used to distinguish specific complex components from copurifying proteins, or to detect changes in the abundance and composition of complexes isolated from cells in different states. (Reproduced from ref. 9)