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Review Article

Analyzing protein–protein interactions by quantitative mass spectrometry

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ABSTRACT

Since most cellular processes depend on interactions between proteins, information about protein–protein interactions (PPIs) provide valuable insights into protein function. Over the last years, quantitative affinity purification followed by mass spectrometry (q-AP-MS) has become a powerful approach to investigate PPIs in an unbiased manner. In q-AP-MS the protein of interest is biochemically enriched together with its interaction partners. In parallel, a control experiment is performed to control for non-specific binding. Quantitative mass spectrometry is then employed to compare protein levels in both samples and to exclude non-specific contaminants. Here, we provide two detailed q-AP-MS protocols for pull-downs with immobilized bait proteins or transient transfection of tagged expression constructs. We discuss benefits and limitations of q-AP-MS and highlight critical parameters that need to be considered. The protocols and background information presented here allow the reader to adapt the generic q-AP-MS strategy for a wide range of biological questions.

1. Introduction

Most biological processes require direct physical interactions between proteins. While some interactions are binary, other cellular events involve large multi-protein complexes. Identifying and characterizing protein–protein interactions (PPIs) is crucial to gain molecular insights into cell function and physiology. Importantly, PPIs are frequently regulated in response to a specific stimulus or cell state [1,2]. It is therefore important to identify binding partners of a protein in its specific cellular context. An ideal system for detecting PPIs would take the cellular background into account, work with endogenous levels of the protein of interest and have both a high sensitivity (i.e. low false-negative rate) and specificity (i.e. low false-positive rate).

One of the most popular methods of studying PPIs is the yeast-two-hybrid (Y2H) approach [3]. A great advantage of this system is that it is scalable and can be used to identify many PPIs in relatively short time. However, the Y2H method provides only a static picture and cannot yield immediate clues about the cellular processes that convert genetic information into complex phenotypes [4]. Other disadvantages are caused by using yeast as a heterologous system. For example, mammalian proteins expressed in yeast may not carry all post-translational modifications relevant for their function. This is especially problematic since PPIs are often regulated by reversible modifications such as phosphorylation [1,5]. These and other factors contribute to the notorious high false-positive and false-negative rate of the assay.

An attractive alternative to Y2H is affinity purification followed by mass spectrometry or AP-MS (see [6] for an excellent review). The general principle is that a protein of interest is purified biochemically from an appropriate biological sample. In most cases tissue culture cells are employed but *in vivo* samples such as whole organs can also be used. After purification, mass spectrometry-based proteomics [7,8] identifies the proteins in the sample. This list of identified proteins is expected to contain the protein of interest and its cellular interaction partners. AP-MS detects PPIs among proteins derived from their native cellular environment that carry all relevant post-translational modifications. The method reveals the composition of entire protein complexes and thus potentially the function of large molecular machines. When combined with quantification, the method can also uncover dynamic changes in PPIs and thereby directly provide information about cell signaling.

The biggest challenge in AP-MS experiments is to distinguish between true interaction partners and co-purifying contaminants. One way to alleviate this problem is tandem affinity purification (TAP). In this method, tagged bait proteins are purified by two successive purification steps in order to reduce the non-specific background binders [9]. Although the TAP method is still widely used it has considerable disadvantages. Most importantly, due to the increased sensitivity of mass spectrometers it is impossible to remove contaminants completely. Furthermore, two successive stringent purification steps can also remove biologically important but weak or substoichiometric interactors. Thus, TAP suffers from a trade-off between specificity and sensitivity.

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The best way of distinguishing true interaction partners from non-specific contaminants available to date is to use quantitative proteomics. This strategy is based on comparing the abundance of proteins identified in the sample with a suitable control. The general idea is to perform two experiments in parallel: In addition to the affinity purification of the protein of interest a control experiment is performed that is not expected to yield any interaction partners. Quantitative mass spectrometry can then be used to compare the abundance of proteins in both pull-downs. True interaction partners are more abundant in the actual AP-MS sample compared to the control. In contrast, non-specific contaminants have a 1:1 ratio since they are equally abundant in both pull-downs. In this way, quantification circumvents the trade-off between sensitivity and specificity and can confidently identify PPIs even under low stringency conditions.

Quantitative AP-MS (q-AP-MS) experiments have been employed successfully in different ways to address a wide range of biological questions [10–20]. A detailed discussion of this work is beyond the scope of this manuscript, see [21] for an excellent review. Here, we describe two strategies of how q-AP-MS can be used to detect PPIs (Fig. 1). Both methods are generic and can be employed to a broad spectrum of biological questions. We provide both step-by-step protocols and background information to allow researchers adapting the workflow to their specific experimental system. We begin with a discussion of general considerations important for any q-AP-MS experiment.

2. General considerations

2.1. Exogenous or endogenous bait?

The gold-standard assay for PPIs is still the co-immunoprecipitation (coIP) of untagged proteins at their endogenous level. The only available screening method based on this assay is *quantitative immunoprecipitation combined with knock-down* (QUICK) [14]. In QUICK, the protein of interest is knocked-down by RNA interference in control cells but not in cells used for the real coIP. The protein of interest is then precipitated from both samples. The target protein itself and its interaction partners are more abundant in the real coIP compared to the control and can thus be identified. QUICK assesses interactions between untagged endogenous proteins at their normal cellular levels within the appropriate cell type. It can therefore identify PPIs with very high confidence.

The disadvantage of QUICK is that antibodies are often not available. Therefore, we describe two alternative strategies based on tagged bait proteins that can be used even when bait-specific antibodies are not available. In the first approach an exogenous bait is coupled to a matrix to fish-out interaction partners from cell lysates (Fig. 1A). This approach is very flexible and allows for the application of different types of baits. For example, recombinant proteins, synthetic peptides or other small molecules can be used [10–12,15,17,19,20,22]. Another interesting feature is that the bait can be used in modified forms to screen for modification-specific interactions. For instance, differences in interaction partners of tyrosine-phosphorylated peptides and their non-modified versions can reveal phosphorylation-dependent interactions. In the example presented here we use GDP- versus GTPγS-loaded states of the small GTPase Cdc42. The experiment is therefore designed to identify specific interaction partners of both the inactivated and the activated GTPase.

In the second approach presented here the bait protein is expressed in cell lines by transient transfection with an expression vector (Fig. 1B). Bait proteins are fused to a biochemical tag to facilitate purification. A wide range of different tags is available with different advantages and disadvantages (see Table 1 for an overview and [23] for a detailed review). These tags differ in size, ranging from short peptide motifs to proteins of several kDa. Tags bind to different types of binding partners such as antibodies (e.g. α-FLAG, α-HA), proteins (e.g. streptavidin, calmodulin), or small molecules (e.g. biotin, glutathione). Smaller tags may be advantageous since they are expected to interfere less with bait protein function. On the other hand, larger tags can help to increase the solubility of the bait protein which can facilitate subsequent purification. Another important factor to consider is the affinity of the interaction between the tag and its binding partner: The interaction must be strong enough to efficiently purify the bait from the lysate.

Pull-downs with exogenous or transfected baits have unique strengths and weaknesses. Using exogenous baits uncouples bait production from the interaction experiment. Therefore, large amounts of cell lysates and bait proteins can be prepared in advance and used for many experiments. Even cells which cannot be transfected efficiently can be used without restriction. However, it should be kept in mind that this assay may erroneously detect interactions among proteins that never co-localize *in vivo*. Transfected bait proteins have the advantage that they are produced in their native environment and in the correct subcellular location. An important caveat here is that overexpression of the bait protein might result in non-specific interactions. Expression systems based on bacterial artificial chromosomes (BACs) circumvent this problem but are not available for all proteins of interest or specific variants [13]. Irrespective of the method used it should always be kept in mind that tagging may alter protein function. Therefore, interaction partners should be verified by coIP with the endogenous proteins and/or functional follow-ups.

2.2. Which method should be used for quantification?

q-AP-MS depends on reliable quantification of relative differences in protein abundance between in the pull-down of interest and the control. Different methods allow for protein quantification by mass spectrometry [24,25]. Some of these methods rely on stable isotope labeling while others use computational approaches to obtain quantitative information from MS data (i.e. label-free quantification). In principle, any method can be used for q-AP-MS as long as it is accurate enough for unambiguous comparison of both samples. Stable isotope-based approaches are usually more precise than label-free approaches. For cell culture experiments, *stable isotope labeling by amino acids in cell culture* (SILAC) is both the easiest and most accurate stable isotope-based approach [26,27]. SILAC can now even be used to label model organisms like flies and mice [28,29]. Therefore, SILAC is generally the method of choice for q-AP-MS experiments and also used in this protocol.

Label-free quantification has also been used recently for q-AP-MS and is the method of choice when SILAC is not possible [13,16,19,20]. However, care should be taken when selecting the algorithm for label-free quantification. Although still popular, spectral counting provides only a very rough estimate of protein abundance and is thus error-prone [30]. Label-free algorithms based on pair-wise comparison of peptide peak intensities are more accurate and therefore preferable [13].

2.3. What is a suitable control?

q-AP-MS relies on comparing the abundance of proteins co-purified with the bait with a suitable control. This is a critical point since any change in protein abundance between the actual AP-MS sample and the control will be considered as a potential interaction partner. The choice of a suitable control depends a lot on the context of the experiment. In general, the closer the control resembles the actual experiment the better. For example, if the goal is to iden-

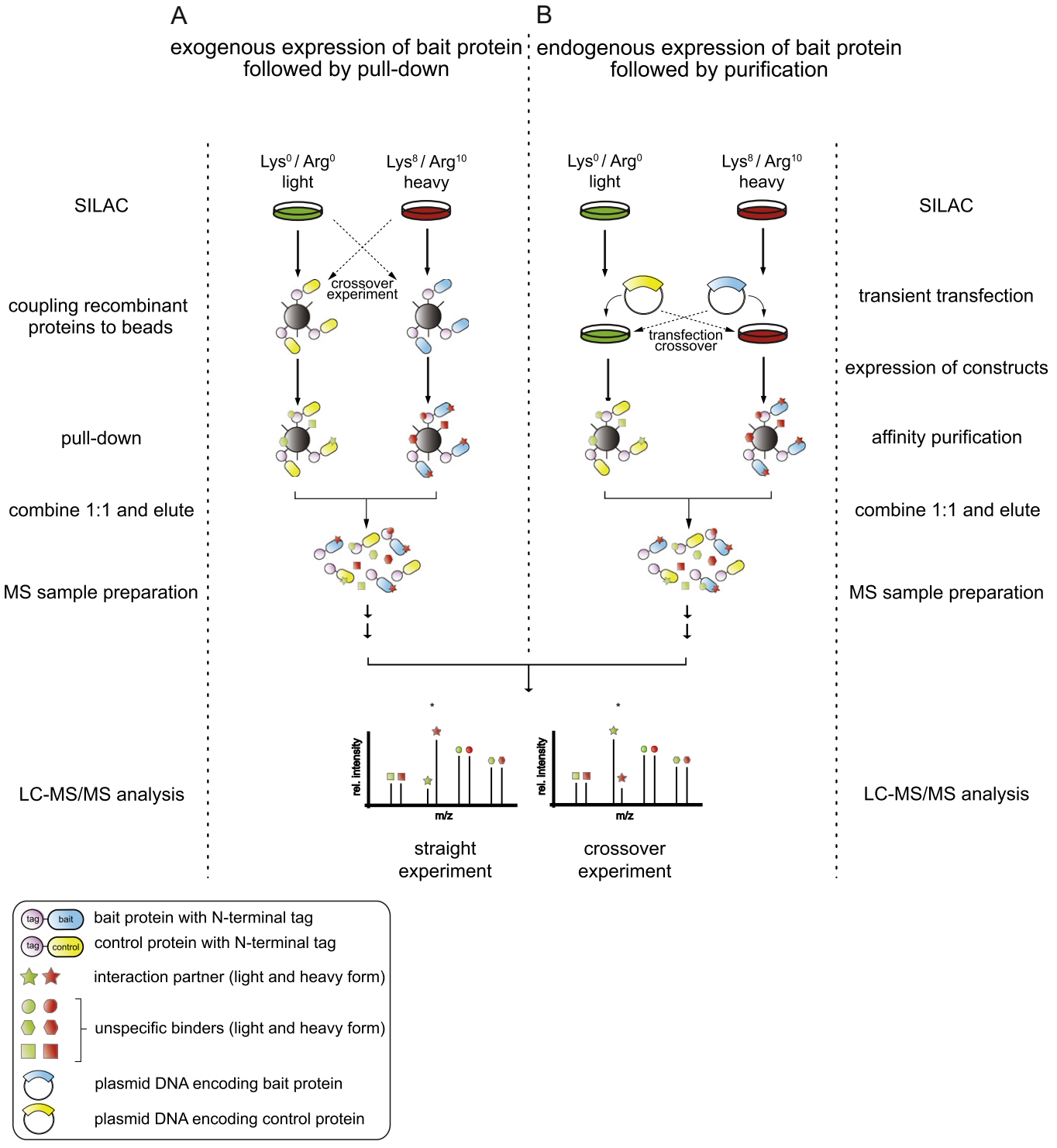

Fig. 1. Experimental workflow for quantitative interaction proteomics. (A) Scheme for pull-down procedure with exogenous expression of the bait protein. Either light or heavy stable-isotope labeled cell lysate is incubated with tagged bait or control proteins, which has been crosslinked to the affinity matrix. After the pull-down beads are mixed and captured bait/prey complexes are eluted and analyzed by LC-MS/MS. Crossover experiments are performed by swapping the lysate. (B) Scheme for affinity purification with endogenous expression of the bait protein. Stable-isotope labeled cells are transiently transfected with an expression plasmid encoding a bait or control protein with an appropriate tag. After bait expression, the tagged constructs are immunoprecipitated and the eluates are combined before subsequent MS sample preparation and LC-MS/MS analysis. The crossover experiment is performed by swapping the transfected plasmids. Specific interaction partners are observed by high heavy-to-light ratios in the straight and low heavy-to-light-ratios in the crossover experiments, whereas non-specific binders have 1:1 heavy-to-light ratio in both experimental conditions (A and B).

tify proteins interacting with a modified peptide, the control should be the same peptide in the non-modified form. In case of endogenously expressed baits control cells should be transfected with the empty expression vector. In the latter case, the same affinity matrix must be used for both the experiment and the control. This is important since different affinity matrixes have different cross-reactivities. For example, different antibodies can cross-react with different cellular proteins. Therefore, a "control" antibody,

Table 1
Peptide and protein affinity tags commonly used in purification of fusion proteins.

Tag name	Sequence	Origin	Size	Binding partner	Comment	Reference
Peptide tags						
Calmodulin-binding peptide	KRRWKKNFIAVSAANRFKKISSSGAL	Rabbit skeletal muscle	26 aa – 2960 Da	Calmodulin	Binding in presence of Calcium chloride	[41]
c-myc	EQKLISEEDL	Human oncogene c-myc	10 aa – 1202 Da	Anti-c-myc antibody (9E10)		[42]
FLAG	DYKDDDDK	Bacteriophage T7	8 aa – 1012 Da	Anti-FLAG antibody (M1, M2, M5)	Improved 3XFLAG system	[43]
HA	YPYDVPDYA	Hemaglutinin from human influenza virus	9 aa – 1102 Da	Anti-HA antibody (12CA5)		[44]
Hexa-Histidine (His-Tag)	HHHHHH	Artificial	6 aa – 840 Da	Ni^{2+}-NTA, Co^{2+}-CMA		[45]
Isopeptag	TDKDMTITFTNKKDAE	*Streptococcus pyogenes*	16 aa	Pilin-C	Forms covalent bond	[46]
Strep-tag II	WSHPQFEK	*Streptomyces avidinii* (Streptavidin)	8 aa – 1060 Da	Streptavidin		[47]
Protein tags						
BioEase™/ AviTag™	http://www.tools.invitrogen.com/content/sfs/manuals/pmtbioeasedest_man.pdfGLNDIFEAQKIEWHE	*K. pneumonia*/*E. coli*	72/15 aa	Biotinylation by birA (*E. coli*) binding by Streptavidin/Avidin	Variation of the BCCP approach	[48]
Biotin Carboxyl Carrier Protein (BCCP)		*Sulfolobus tokodaii*	169 aa	Biotin Protein-ligase OR Streptavidin/Avidin	kD = 1.2 nm	[49]
Green fluorescent protein (GFP)	http://www.colorado.edu/mcdb/MCDB1151/indproj/gfpseq.html	*Aequorea victoria*	238 aa – 26.9 kDa	Anti-GFP Antibody	Numerous derivates	[50,51]
Glutathione S-transferase (GST)	http://www.ncbi.nlm.nih.gov/nuccore/U58012.1	*Schistosoma japonicum*	26 kDa	Glutathione	Forms dimers	[52,53]
Maltose-binding protein (MPB)	http://www.ncbi.nlm.nih.gov/nuccore/J01648.1	*Escherichia coli*	40 kDa	Amylose		[54]
Tandem-Affinity-Purification (TAP)	http://www.embl.de/ExternalInfo/seraphin/TAPdescription.html	Rabbit + *Staphylococcus aureus*	~20 kDa	1st step: IgG matrix2nd step: Calmodulin	ProteinA + TEV cleavage size + CBP	[9]

even of the same isotype, is not a suitable control for an immunoprecipitation experiment.

2.4. Should crosslinking be used?

The results of q-AP-MS experiments should reflect the composition of endogenous protein complexes. This may, however, not be true in all cases: Although q-AP-MS captures transient interactions, very labile complexes may fall apart during purification. Conversely, PPIs may artificially form in a cell lysate between proteins that do not interact *in vivo*. The latter point is also the reason why even SILAC samples and their controls should generally be mixed after affinity purification [17]. Crosslinking offers the opportunity to "freeze" PPIs inside cells before lysis. The most popular crosslinker is formaldehyde: Due to its small size, formaldehyde can permeate cell walls and membranes and induce efficient, reversible cross-links between proteins [31]. Crosslinking can also provide structural information about proteins and PPIs, although this approach is challenging both technically and bioinformatically [32]. Due to these complications most q-AP-MS experiments are performed without crosslinking of the proteins in the lysate.

A related question is whether or not the bait protein (for exogenous bait) or the antibody (for coIP) should be crosslinked covalently to the affinity matrix. Without crosslinking, the bait protein or antibody is often by far the most abundant protein in the purified sample. Since mass spectrometry is mainly limited by dynamic range, this may cause less abundant interaction partners to escape detection. Thus, it is generally recommended to crosslink the bait protein or the antibody to the affinity matrix.

For exogenous bait experiments we tested two alternatives: the homobifunctional crosslinker dimethyl pimelimidate (DMP, [33]) and N-Hydroxysuccinimide (NHS) activated Sepharose. Both crosslinkers clearly increased the number of identified interaction partners, showing that coupling baits to the matrix is indeed beneficial (see Fig. 2 and Section 4.). Many anti-tag-antibodies can be pur-

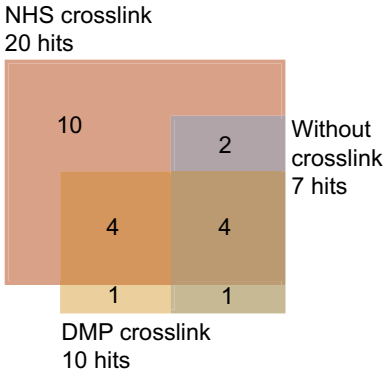

Fig. 2. Pulldown of Cdc42 effector proteins is most efficient with NHS-Sepharose. The pull-downs of recombinantly expressed and nucleotide-loaded Cdc42-GTPγS and Cdc42-GDP were carried out in a q-AP-MS experiment. Three experiments were performed, first with glutathione beads without crosslinker, second with glutathione beads with DMP crosslinking and third with NHS-activated Sepharose. Effector proteins were pulled-down from SILAC labeled HeLa cell lysate. Depicted is the number of proteins known to interact with Cdc42 in the list of thirty identified proteins with the highest ratio. The highest amount of known effectors is recovered using the NHS-Sepharose approach.

chased directly in a matrix-bound form. If crosslinked antibodies are not available, it is usually advisable to crosslink them to protein A or protein G Sepharose using DMP or another bifunctional crosslinker (see [34] for a detailed overview of different crosslinkers).

2.5. Which kind of biological replicates are advisory?

As for all biological experiments, replicates also increase confidence in q-AP-MS experiments. In case of SILAC-based quantification such a replicate is most conveniently performed as a so-called crossover experiment with swapped isotope labels (Fig. 1). Swapping the labels should result in reciprocal SILAC ratios for true interaction partners (see Fig. 3 and Conclusions). In contrast, exogenous contaminants such as proteins derived from fetal bovine serum are always unlabeled (i.e. light) and can thus easily be identified. For pull-down experiments with exogenous baits a crossover experiment can also control for differences in protein abundance in the cell lysates used for the experiment. Such differences in cellular protein abundance could otherwise be misinterpreted as specific interactions. Since label-free quantification is generally less accurate than SILAC replicates are even more important in this case. Label-free experiments should usually be performed at least in triplicates since this permits statistical tests like the Student's *t*-test for reliable identification of interaction partners [13].

3. Experimental protocols

3.1. General remarks

In this chapter, we describe a step-by-step procedure for the identification of PPIs using either a pull-down with recombinant bait protein or a coIP with a transfected bait protein. We use either stable isotope-labeled HeLa or HEK293T cells but in principle other cell lines that are amenable to SILAC can be utilized as well. We also propose different cell lysis buffers (Section 3.2.2) for each purification protocol, but again, other lysis buffers with protease and phosphatase inhibitors can be used as well, if the conditions are not too denaturing and work efficiently for the protein of interest. A considerable practical advantage of q-AP-MS over tandem affinity purification is that less material is needed. We routinely use the protocols described here to identify interaction partners from about 2×10^7 cells using in-solution digests. However, if expression levels of bait protein in transient transfections are very low or if known interaction partners are missed the experiments can be scaled up by using more cells or bait protein. Another limiting factor is the dynamic range of protein concentrations in the precipitates. Therefore, a prefractionation step by SDS–PAGE can enhance identification rates. Since only a fraction of the loaded protein amount is recovered during in-gel digestion [35] it is often advisable to prefractionate and scale up at the same time. In our experience, both protocols can be scaled up easily to five to ten 15-cm plates per condition.

3.2. Materials

3.2.1. Cell Culture and stable isotope labeling by amino acids in cell culture (SILAC)

Medium: Dulbecco's Modified Eagle's Medium (DMEM) lacking arginine and lysine (custom preparation from Gibco)

- *Amino acids:* $^{13}C_6{}^{15}N_4$ L-arginine and $^{13}C_6{}^{15}N_2$ L-lysine (Cambridge Isotope Laboratories). $^{12}C_6{}^{14}N_4$ L-arginine and $^{12}C_6{}^{14}N_2$ L-lysine (Sigma),
- *Supplements:* dialyzed fetal bovine serum (dFBS, Gibco), L-glutamine (Gibco), penicillin/streptomycin (Invitrogen),
- *Cells:* adherent HeLa epithelial adenocarcinoma (LGC Promochem) or adherent HEK293TN human embryonic kidney (System Biosciences) cells.

3.2.2. Cell lysis, pull-down, transfection, affinity purification and mass spectrometry

- Cell lysis:
  - Pull-down lysis buffer: 50 mM HEPES pH 7.5, 150 mM NaCl, 0.2% NP-40, Complete Protease Inhibitor cocktail (Roche) 1:25, Phosphatase Inhibitor Mix 1 (Sigma) 1:100
  - Radioimmunoprecipitation (RIPA) buffer: 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 1% Na-Deoxycholate, 0.1% SDS, Benzonase (Sigma), Complete Protease Inhibitor cocktail (Roche) 1:25, Phosphatase Inhibitor Mix 1 (Sigma) 1:100
  - Dulbecco's phosphate buffered saline (D-PBS, Invitrogen) without calcium chloride and magnesium chloride
- Pull-down experiment
  - Beads: NHS-activated Sepharose 4 Fast Flow (GE Healthcare)
  - Chemicals: hydrochloric acid (Roth)
  - Coupling buffer: the composition of the coupling buffer depends on the ligand to be coupled. Phosphate or carbonate based buffers with pH 6–9 are standard. Avoid primary amines like Tris.
  - Quenching buffer: 0.2 M ethanolamine pH 8.0.
  - Low pH buffer (e.g. 0.1 M acetate, 0.5 M NaCl pH 5)
  - High pH buffer (e.g. 0.1 M Tris HCl pH 8)
  - Stringency wash buffer: e.g. 50 mM HEPES pH 7.5, 300 mM NaCl, 0.2% NP-40 (low stringency)
  - Pre-elution buffer: e.g. 3 mM HEPES pH 7.5, 300 mM NaCl (reduces the buffer capacity to facilitate subsequent acidic elution)
  - Elution buffer: 0.1 M glycine pH 3.0
- Transient transfection of mammalian cells: linear polyethylenimine (PEI 'Max', nominally Mw 40,000, Polysciences), 1 μg/μl in dH_2O stock solution
- Affinity purification: μMACS Epitope Isolation Kit and respective M columns (Miltenyi Biotec), elution buffer: 0.1 M glycine pH 3.0
- Protein precipitation: 2.5 M sodium acetate pH 5.0 (Merck), Glycoblue (Ambion), 1 M Tris pH 8.2 (Roth), 100% LC-MS grade ethanol (Merck)
- In-solution digest and LC–MS/MS analysis
  - In-solution digest: Lysyl endopeptidase (Lys-C) (Wako) and sequencing grade modified trypsin (Promega). Stop and go extraction tips containing C_{18} Empore disks (3 M)
  - LC–MS/MS analysis: ReproSil-Pur C_{18}-AQ 3 μm resin (Dr. Maisch), LC–MS grade acetonitrile (Sigma), LC-MS grade water (Sigma), LC-MS grade formic acid (Fluka)

3.3. Cell culture

HeLa or HEK293T cells are cultivated at 37 °C with 5% CO_2 and split every second or third day. SILAC media is essentially prepared as described [26]. Briefly, DMEM lacking arginine and lysine is supplemented with 10% dialyzed fetal bovine serum, 4 mM glutamine and 1% penicillin/streptomycin. To prepare heavy (H) SILAC media we add 28 mg/l $^{13}C_6{}^{15}N_4$ L-arginine plus 49 mg/l $^{13}C_6{}^{15}N_2$ L-lysine. Light (L) SILAC medium is prepared by adding the corresponding non-labeled amino acids.

HeLa or HEK293T cells are labeled in the corresponding SILAC medium for at least seven cell divisions. For one quantitative

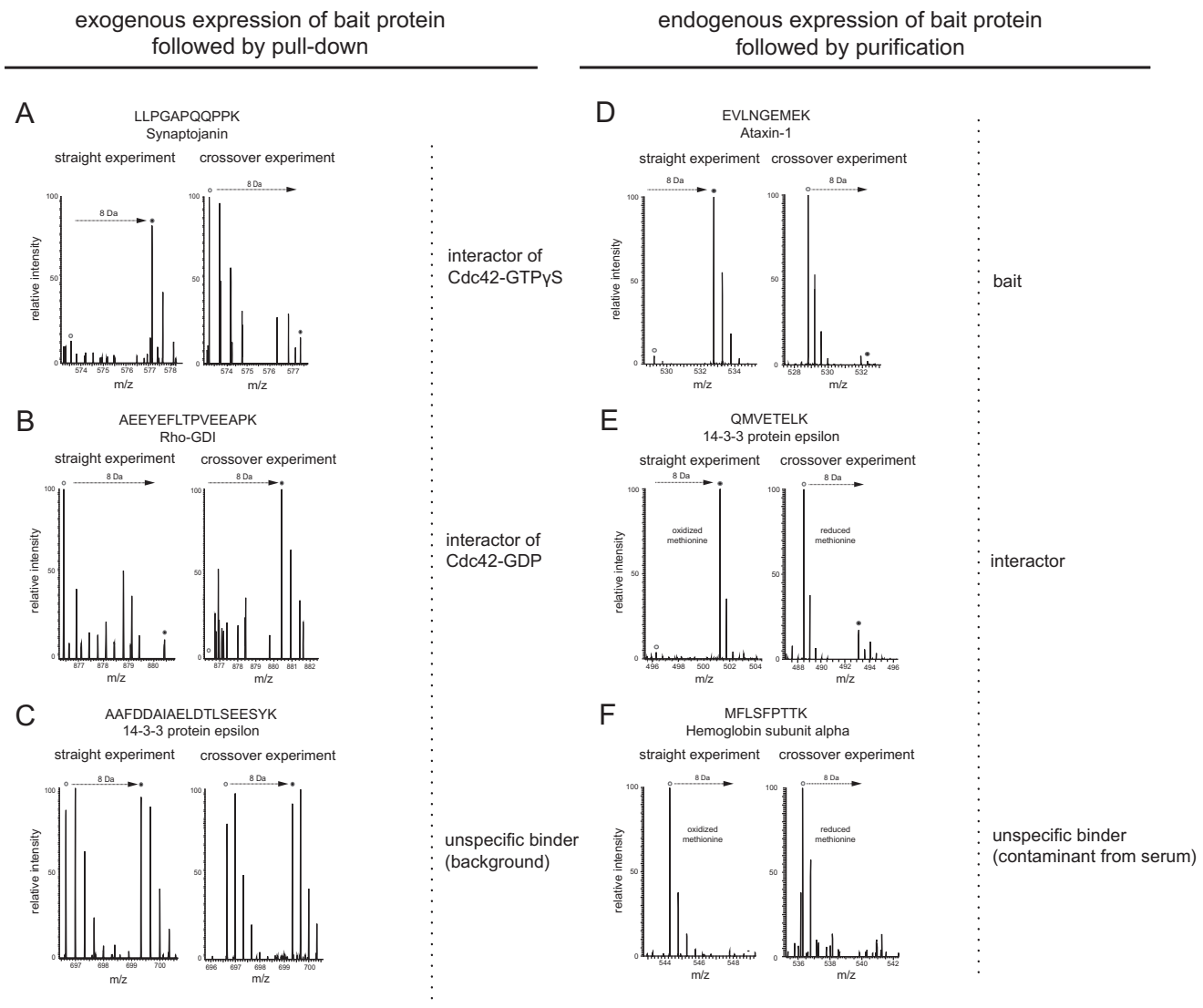

Fig. 3. Exemplary mass spectra of identified peptides. SILAC pairs for the straight and the corresponding crossover experiment are depicted next to each other. All peptides contain one lysine, resulting in a mass difference of 8 Da between the light (white circle) and the corresponding heavy (black circle) peak. (A) Cdc42-GTPγS used as bait in a pull-down specifically interacts with Synaptojanin, SILAC ratios swap according to the label state swap. (B) Cdc42-GDP used as bait in a pull-down specifically interacts with Rho-GDI, SILAC ratios swap according to the label state swap. (C) The 14-3-3 protein shows a 1:1 ratio in the Cdc42 experiment, indicating non-specific binding. (D) Ataxin-1 used as the bait in an immunoprecipitation with swapped ratio in the crossover experiment. (E) The known Ataxin-1 binding protein 14-3-3 exhibits a ratio similar to the bait protein itself, indicating a specific interaction with Ataxin-1. (F) The unlabeled serum protein hemoglobin shows very low heavy-to-light ratios in both experimental conditions, indicating an non-specific contamination derived from serum.

immunoprecipitation, usually one 15 cm dish of light and heavy cells, respectively, is sufficient (2×10^7 cells of each SILAC state).

3.4. Exogenous expression of bait protein followed by pull-down

N-Hydroxysuccinimides (NHS) are activated esters which react with amine groups to amides. The following protocol is based on the standard protocol for NHS-activated Sepharose 4 Fast Flow (GE Healthcare). The recombinant protein should not be stored in solutions containing primary amines (e.g. Tris- or ammonium-based buffers) because they will react with NHS and decrease available binding sites. The protocol describes a single pull-down, with a respective amount of 100–150 µg of recombinant protein and 100 µl NHS-resin. The NHS protocol can also be performed for immunoprecipitations using 30 µl NHS-Sepharose and 5–10 µg of antibody. A complete quantitative q-AP-MS experiment requires 4 pull-downs (2 for the actual experiment and 2 for the crossover experiment). Optimal ratios of slurry to antibody to lysate are dependent on the protein used and should be experimentally titrated.

Notes of volumes refer to the initial amount of slurry (e.g. '4 volumes' for an initial volume of 30 µl slurry means 120 µl). To avoid hydrolysis of the activated ester all steps preceding coupling should be performed without delays.

3.4.1. Preparation of NHS-beads

Pipette 100 µl of NHS-beads slurry into a 1.5 ml Eppendorf tube. Centrifuge (1000*g*, 2 min) and remove the supernatant. To remove isopropanol used for storing the beads wash once with 10–15 vol cold 1 mM hydrochloric acid. Centrifuge again (1000*g*, 2 min, 4 °C).

3.4.2. Wash

Wash twice with at least 4 volumes coupling buffer, centrifuge (1000*g*, 2 min, 4 °C). Check the pH and adjust if necessary with additional washing steps. The pH should not be acidic to avoid

denaturation of the recombinant protein. The pH can range between 6–9, higher pH will result in hydrolysis of the ester bound.

3.4.3. Crosslinking

Mix the recombinant protein (or antibody) with coupling buffer to a final volume of 0.5 vol and add the solution to the slurry. Beads should always stay wet and be able to freely move within the dispersion. If beads tend to dry add coupling buffer (optimal ratio coupling solution:medium is 0.5:1). Incubate by head over tail rotation for 120 min at room temperature.

3.4.4. Quenching

Centrifuge and remove the supernatant. Take a sample of the supernatant and check for binding efficiency via western blot. Add 2.5 vol quenching buffer. Incubate for 30 min at room temperature by head over tail rotation. Quenching is needed to saturate free binding sites.

Q1 *3.4.5. Wash cycles*

Centrifuge and remove the supernatant. Wash with alternating wash buffers to remove not covalently coupled bait protein. To use a low and a high pH buffer has proven to be practical. Repeat this step three times.

Finally wash twice with at least 4 vol lysis buffer. The affinity matrix is ready for use after this step. For storage add ethanol (20%) or azide and store at 4 °C. However, some coupled proteins are not very stable, so immediate usage is normally advisable.

3.4.6. Pull-down from cell lysate

Save a sample of cell lysate (sample "*Input*"). Add the cell lysate to the Protein-Sepharose. Again, beads should stay wet and be able to freely move within the dispersion (use a matrix:liquid phase ratio of ~1:1, if beads seem too dry add a bit of lysis buffer). Incubate the pull-down on a spinning wheel at 4 °C (incubation time from 30 min to overnight, depending on the bait to prey affinity and stability). Centrifuge and save a sample of the supernatant (sample "*Out*").

3.4.7. Stringency wash

Pool the respective samples (two pull-downs from the "straight" experiment and the two pull-downs from the crossover). Wash one to three times with 4 vol washing buffer (e.g. 50 mM HEPES pH 7.5, 300 mM NaCl, 0.2% NP-40). If pull-downs result in a high number of non-specific background binders (ribosomal proteins, histones) increase the stringency by using a higher salt or detergent concentration (0.5 M NaCl, 0.1% SDS).

3.4.8. Pre-elution wash

Wash once with at least 4 volumes of a low buffer non-detergent solution. This step facilitates acidic elution of the bound interaction partners for in-solution digestion. In case in-gel digestion is used beads can be directly boiled in SDS–PAGE sample buffer after the stringency wash.

3.4.9. Elution

Incubate the beads with 100 µl elution buffer, then incubate for 2 min on a spinning wheel or thermo shaker, check the pH (use a pH indicator stick, the pH should be acidic, if not add more elution buffer, incubate again). Centrifuge and save the supernatant, it should contain the protein of interest and interaction partners. Elute three times in total. Pool the three eluates and continue immediately with protein precipitation.

3.4.10. Protein precipitation

Transfer the sample (volume not more than 300 µl) to a 2 ml dust-free tube. Add 70 µl sodium acetate and 2 µl Glycoblue. The addition of Glycoblue is recommended if the protein concentration is low. Fill up to 2 ml with 100% ethanol and mix briefly by inverting the Eppendorf tube. Incubate the precipitation overnight at room temperature or at 4 °C and centrifuge the next day with 20,000*g* at 4 °C for at least 30 min. Remove the supernatant carefully and air dry the protein pellet. Continue with MS sample preparation (Section 3.6).

3.4.11. Control pull-down efficiency

To check if the pull-down was efficient, compare the "input", "output" and bead sample by SDS–PAGE and western blotting. Known interaction partners of the bait protein should be detected in the input sample but significantly depleted in the output. A high amount of interaction partners in the bead sample suggests inefficient elution.

3.5. Transient transfection of bait protein followed by immunoprecipitation

Here, we provide a protocol optimized for immunoprecipitations of epitope-tagged proteins overexpressed in the HEK293T system by using the µMACS Epitope Isolation Kit (Miltenyi Biotec). These magnetic microbeads gave best results for our immunoprecipitations and offer other important advantages [13]. However, other affinity matrices may work as well. We use linear PEI as the transfection reagent for HEK293T, as it is a highly efficient (usually $\geqslant$ 95%) and much cheaper than many other commercially available alternatives. Different cell lines may require different transfection reagents.

3.5.1. Transient transfection

Mix the expression plasmid with polyethylenimine in a 1:2 DNA to PEI ratio (transfection ratio may need optimization depending on the cell line). For a 15 cm cell culture dish mix 15 µg plasmid DNA with 30 µg PEI and add 2 ml of serum-free DMEM. Incubate for 30–45 min at room temperature to assure efficient binding of the DNA to PEI. Add additional 2 ml of serum-free DMEM and mix briefly before transferring your transfection setup to the cells. Cell confluency should be 60–70% on the day of transfection. For control transfections use an empty vector carrying only the tag sequence or a more appropriate control (see Section 2.3).

3.5.2. Cell harvesting

Harvest the cells 24 h post-transfection. Aspire the medium, wash once with ice-cold DPBS, then add 500 µl of ice-cold RIPA lysis buffer. Use cell scrapers to harvest the cells and incubate them for 30 min on ice. Cell debris should be cleared by centrifugation at 20,000*g* for 10 min at 4 °C.

3.5.3. Immunoprecipitation from cell lysate

Add 50 µl of µMACS beads (directed against the tag of choice) to each lysate from different SILAC conditions separately and incubate on ice for 45 min.

3.5.4. Column preparation

Equilibrate the MACS column with 200 µl ice-cold RIPA buffer. Transfer the IP slurries to the MACS column, the different IP setups are now combined on the column.

3.5.5. Wash

Wash the µMACS column three times with 200 µl lysis buffer and once with 100 µl Miltenyi wash buffer 2 (included in the Epitope Isolation kit), to reduce the salt concentration before elution of the proteins.

3.5.6. Elution

The proteins are eluted from the μMACS column in a dust-free 2 ml tube by adding 300 μl elution buffer to the column. Continue with protein precipitation as described in Section 3.4.11. Precipitated proteins are ready for MS sample preparation (Section 3.6).

3.6. In-solution digest

MS sample preparation including reduction and alkylation of cysteine residues is done as described [35]. Lysyl endopeptidase (Lys-C) (Wako) and sequencing grade modified trypsin (Promega) are used for in-solution digestion. Stop and go extraction tips containing C_{18} Empore disks (3 M) are used to purify and store peptide extracts [36].

3.7. LC-MS/MS

On-line LC-MS/MS analysis is performed as described previously [28]. In brief, peptide mixtures are separated by reversed phase chromatography using the Eksigent NanoLC-1D Plus system (Eksigent) on in-house manufactured 10-cm fritless silica microcolumns with an inner diameter of 75 μm. Columns are packed with ReproSil-Pur C_{18}-AQ 3 μm resin (Dr. Maisch GmbH) [37]. Separation is performed using a 10–60% ACN gradient (240 min) with 0.5% formic acid at a flow rate of 200 nl/min. Eluting peptides are directly ionized by electrospray ionization and transferred into the orifice of a linear trap quadrupole Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific). Mass spectrometry is performed in the data-dependent mode with one full scan in the Orbitrap (m/z 300–1700; resolution of 60,000; AGC target value of 1×10^6). The five most intense ions with a charge state greater than 1 are selected (target value 3000; monoisotopic precursor selection enabled) and fragmented in the linear trap quadrupole using CID (35% normalized collision energy and wideband activation enabled). Dynamic exclusion for selected precursor ions is 60 s.

3.8. Processing of MS data

The MaxQuant software package (version 1.0.13.13) is used to identify and quantify proteins [38,39]. SILAC duplets are extracted from isotope patterns, recalibrated, and quantified by the Quant module (heavy label Arg10 and Lys8, maximum of three labeled amino acids per peptide, polymer detection enabled, and top six MS/MS peaks per 100 Da). Peak lists are searched on a Mascot search engine (version 2.2, Matrix Science) against an in-house curated database of *H. sapiens* (IPI.Human, version 3.64) plus common contaminants. When performing interaction studies with recombinant proteins expressed in a different organism (e.g. *E. coli*) the database should also contain all protein entries from this organism. This is important since the purified recombinant protein often still contains trace amount of contaminants derived from this species, and these may be falsely assigned to for example human proteins (when searching a human database). All protein sequences are also reversed to generate a target-decoy database [40]. Carbamidomethylation of cysteine is selected as a fixed modification, and oxidation of methionine and acetylation of the protein N-terminus are used as variable modifications. Lys-C and trypsin are selected as proteases (full specificity) with a maximum of two missed cleavages. A mass tolerance of 0.5 Da is selected for fragment ions. A minimum of six amino acids per identified peptide and at least one peptide per protein group are required. The false discovery rate is set to 1% at both the peptide and protein levels. Protein ratios are calculated from the median of all normalized peptide ratios using only unique peptides or peptides assigned to the protein group with the highest number of peptides. Only protein groups with at least three SILAC counts are considered for further analysis.

4. Conclusions

Combining classical pull-down or coIP techniques with SILAC-based quantitative proteomics is a powerful approach to detect PPIs with high sensitivity and, perhaps more importantly, high specificity. As an example for exogenous bait expression, we show that recombinantly expressed GTPγS-loaded Cdc42 versus the GDP-loaded form can be used to identify specific interaction partners of both forms of this Rho-GTPase (Fig. 3A–C). As an example for cellular expression of tagged bait we used Ataxin-1 and show specific interaction with 14-3-3 protein epsilon (Fig. 3D–F). In all cases, heavy-to-light SILAC ratios of specific interaction partners are inverted in the crossover experiment, validating their specific binding. In contrast, non-specific background binders show a 1:1 ratio in both the straight and the crossover experiment (Fig. 3C). A contaminating serum protein is always detected in the light (i.e. unlabeled) form and can thus easily be excluded (Fig. 3F). We also show that covalently crosslinking the bait to the matrix yields a higher number of literature-reported interaction partners (Fig. 2). We assume that crosslinking reduces the amount of bait-derived peptides in the sample which facilitates MS analysis. The highest number of known interaction partners was detected using the NHS-approach, where 10 interaction partners were exclusively found. Only two interaction partners were not covered by the NHS-based pull-down. In summary, the two protocols described and the background information provided should facilitate adapting the generic q-AP-MS strategy for a wide range of biological questions.

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