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Reports

Improved methodology for the affinity isolation of human protein complexes expressed at near endogenous levels

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An efficient and reliable procedure for the capture of affinity-tagged proteins and associated complexes from human cell lines is reported. Through multiple optimizations, high yield and low background affinity-purifications are achieved from modest quantities of human cells expressing endogenous-level tagged proteins. Isolations of triple-FLAG and GFP-tagged fusion proteins involved in RNA metabolism are presented.

Keywords: endogenous protein complex purification; affinity isolation; cryogenic grinding; 3×FLAG; GFP-tag; exosome complex; NEXT complex; CBC

Affinity isolation of proteins and associated complexes has facilitated the rapid growth of proteomic analyses (1). Model organisms amenable to targeted genetic engineering, such as the yeast *Saccharomyces cerevisiae*, have been at the center of this development (2–7)—providing a simple, direct path for the capture of endogenous protein complexes, ideal for both proteomic and biochemical analyses (8). While gene targeting systems for mammalian cell-types have been around for decades (9–12), simple, efficient methods for expressing affinity-tagged fusion proteins at or near endogenous levels are a more recent development (13–18). Moreover, this development was necessary; it is widely acknowledged that protein over-expression can lead to experimental artifacts, including mislocalization, and the formation of spurious interactions or altered activities (1,19–21). Thus, for biomedical studies it is critical that tagged proteins be expressed at physiological levels.

Simultaneously, the technologies and methods for cell breakage and affinity isolation have themselves evolved. Our laboratory has shown that near complete cell breakage can be achieved by mechanical grinding at liquid N_2 temperatures, resulting in a fine, granular material (cell grindate) with excellent properties for subsequent affinity isolation of tagged proteins and complexes (22–24) (e.g., http://lab.rockefeller.edu/rout/media/grinding.html). With respect to affinity isolations, the triple-FLAG (3×FLAG)-tag exhibits superiority to the single FLAG-tag through increased avidity of interaction with M2 anti-FLAG antibodies (25–27). We have observed that in many cases 3×FLAG-tagged protein complexes can be isolated at stringencies where traditional FLAG-tagged proteins fail to be immunoprecipitated. In addition, GFP makes an excellent tag not only for protein localization studies but also for the reliable capture of protein complexes (13,22) since high-quality anti-GFP antibodies are available. Here we present our own polyclonal anti-GFP immunoglobulin as well as a bacterially expressed anti-GFP nanobody (28,29). Further, antibody coupled, micron-scale magnetic beads have been indispensable in the development of high-yield, high-fidelity isolations of protein complexes from a variety of cell-types and using various affinity-tags (22–24).

We have applied these tools in a streamlined approach for the optimization of expression and capture of proteins involved in human RNA metabolism including the exosome complex, the NEXT complex, and the nuclear cap binding complex (CBC). The eukaryotic exosome complex has been shown to play a central and evolutionarily conserved role in the processing and degradation of a broad array of RNA species. Commensurate with its diverse activities, the exosome varies in composition along with cellular localization and requires activating cofactors (30). The NEXT complex, composed of RBM7, ZCCHC8, and SKIV2L2, also known as hMTR4, has recently been shown to target the exosome for the specific degra-

Method summary:

Here we present a streamlined affinity isolation approach for the analysis of human protein complexes. A synthesis of methods is used to achieve quality results without the protein over-expression typically required by such experiments. We have chosen several protein complexes related to RNA biology as examples to present the method, and we focus on the use of the triple-FLAG (3×FLAG) and GFP-tags and cryogenic grinding with cell lines that express the protein of interest at near endogenous levels. This method provides for high yield, low background affinity isolations using only modest quantities of cell material.

dation of promoter upstream transcripts (PROMPTs) (17). However, the exosome and its cofactors have proven difficult to isolate from human cells at high yield and purity using modest quantities of starting material. Published methods have typically required long incubations and handling times during affinity isolation, and provided only silver stainable quantities of complex, requiring liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses or significant scale-up (16,17,31). Using a robust and rapid procedure of less than 2 h from extraction to elution that typically requires only 100 mg or less of starting material (wet cell weight; WCW), we affinity isolate the multicomponent human RNA exosome complex via both 3×FLAG-tagged RRP6 and RRP41 subunits, respectively, as well as the exosome cofactor NEXT complex, via LAP-tagged RBM7. These purifications provide Coomassie-stainable yield of coprecipitating proteins at high purity and apparent stoichiometry and are amenable to standard peptide mass fingerprinting analyses by MALDI-TOF MS. Finally, via a 3×FLAG-tagged NCBP2 (CBP20), we extend this strategy to the human CBC, important for pre-mRNA splicing and nuclear export of 5′-7-methyl guanosine (m7G) capped RNAs (32–35). In addition to the expected coprecipitation of NCBP1 (CBP80), we identify the SRRT, KPNA2, and PHAX proteins. This general strategy may provide for more facile analyses of human protein-protein interaction networks.

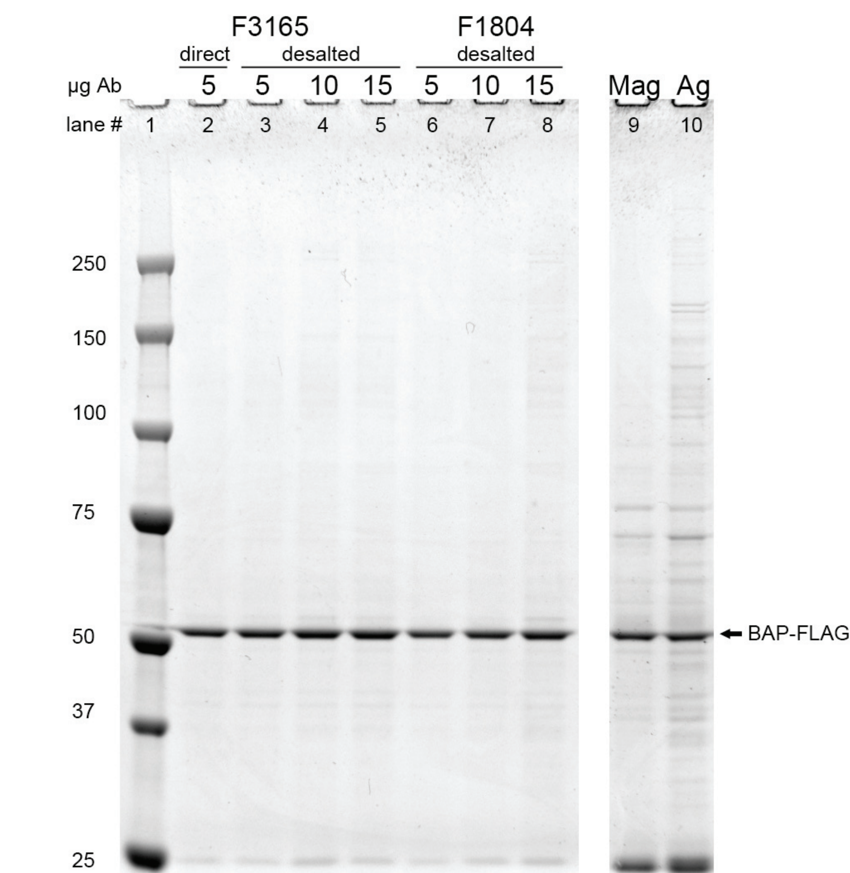

Figure 1. Magnetic beads coupling optimization. Magnetic beads coupled to anti-FLAG M2 antibodies (nos. F3165 and F1804, respectively; Sigma-Aldrich) in three different concentrations—5, 10, and 15 µg/mg of MBs—were used for immunoprecipitation of the reporter protein, BAP-FLAG, in the presence of HEK cell lysate. Anti-FLAG M2 antibodies were either directly coupled to MBs (F3165) or subjected to desalting before coupling (F3165 and F1804). Mag (iron impregnated agarose)-mediated and Ag (agarose)-mediated affinity isolations were performed in parallel (nos. M8823 and A2220, respectively; Sigma-Aldrich). Protein marker (Bio-Rad Precision Plus Protein All Blue Standards 161-0373).

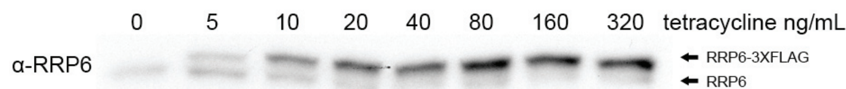

Figure 2. Titration of RRP6-3×FLAG expression. DMEM containing 320 ng/mL tetracycline was serially diluted with DMEM to 5 ng/mL tetracycline, and each dilution added to cells to induce RRP6-3×FLAG expression. Ten micrograms WCE were run on a 6% Tris-glycine gel and transferred to a PVDF membrane. The membrane was probed with antibodies against the endogenous RRP6 protein.

Materials and methods

Cell line construction

For 3×FLAG-tag affinity isolation experiments we used HEK293 Flp-In TREx cell lines expressing the tagged protein of interest, established according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Fusion proteins were introduced using a modified pcDNA5/FRT/TO vector that contains the protein of interest followed by a C′-terminal 3×FLAG-tag (Supplementary Figure S1). For GFP-tag affinity isolation, HeLa Kyoto cell lines expressing LAP-tagged proteins of interested were provided by Poser et al. (14); the LAP-tag includes enhanced GFP (EGFP) (13).

Protein expression

For 3×FLAG-tagged proteins, expression was induced following the manufacturer's instructions (Invitrogen) by replacing cell growth media (DMEM, high glucose, GlutaMAX, no. 61965; Invitrogen), supplemented with 10% FBS (dialyzed, U.S., no. 26400; Invitrogen) and 1:100 v/v penicillin-streptomycin liquid (no.15140; Invitrogen), with fresh media containing tetracycline (no. 87128; Sigma-Aldrich, St. Louis, MO, USA). Expression levels of the endogenous and 3×FLAG-tagged RRP6 protein were monitored by Western blotting analysis using antibodies to the endogenous protein (no. ab95028; Abcam, Cambridge, MA, USA) and an anti-rabbit, HRP-conjugated secondary antibody (no. P0160; Dako, Glostrup, Denmark). Tetracycline quantities used during protein induction were titrated accordingly, to yield near wild-type (WT) expression levels for the tagged-protein. For LAP-tagged proteins, HeLa cells were grown in the same media as HEK cells, but additionally supplemented with 400 µg/mL G418 (no. 11811; Invitrogen). LAP-tagged proteins are expressed under control from the WT promoter and gene context (14,36,37).

Cell lysis methods

Cells were lysed by either sonication or

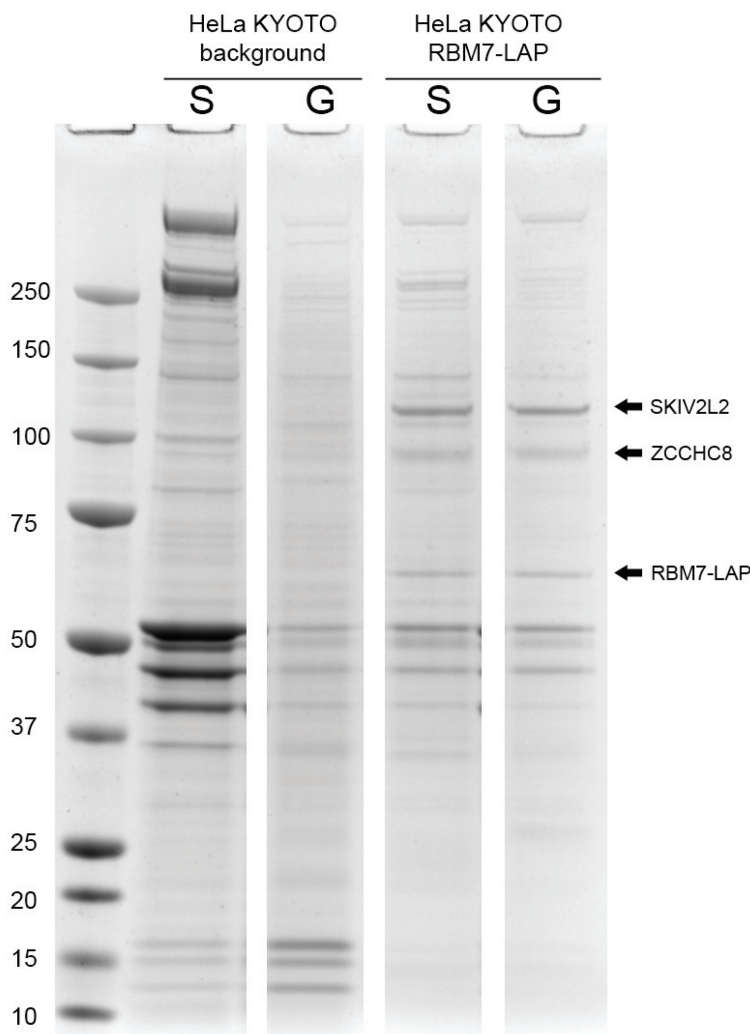

Figure 3. Cryogenic disruption provides lower background purifications. Control HeLa cells or HeLa cells expressing LAP-tagged RBM7 protein were lysed using either sonication or cryogenic grinding and extracted in 20 mM HEPES, pH 7.4, 300 mM NaCl, 0.5% v/v Triton X-100. Clarified cell lysates were subjected to affinity isolation using MBs coupled to anti-GFP antibodies (S, sonication, G, grinding).

cryogenic grinding. Briefly, freshly grown cells were scraped from one 150-cm^2 culture dish (Dishes Nunclon Δ, no. 168381; Nalge Nunc, Rochester, NY, USA) in extraction buffer and were sonicated: 20 W, 3 cycles of 10 s each, using a microtip probe and cooling on ice 10 s in between each cycle. For cryogenic disruption, cells were ground using a Retsch (Haan, Germany) PM 100 planetary ball mill by the following program: ~1 g WCW of frozen cells were placed in a 50-mL grinding jar with 3 × 20 mm stainless steel balls, all pre-cooled with liquid N_2, and run for three cycles of 3 min, 400 rpm, cooling with N_2 in between each 3 min cycle. The 20-mm balls were then replaced with 18 × 10 mm pre-cooled stainless steel balls. The cell powder was subjected to an additional six cycles of 1 min each, 400 rpm, with N_2 cooling in between. The resulting cell powder is stored at -80°C. A detailed protocol is provided in supplement. In both cases >90% cell disruption was confirmed by light microscopy.

Antibody coupling

All antibodies were coupled to Dynabeads M-270 epoxy (no. 143-02D ; Invitrogen). The optimal concentration for coupling to anti-FLAG antibodies (nos. F3165 and F1804; Sigma-Aldrich) was determined as described in the text. In all subsequent cases, anti-FLAG and anti-GFP antibodies were coupled at a concentration of 10 µg antibody/mg of Dynabeads (22,38). After antibody coupling and final washing, 200 µL storage buffer were added to 30 mg equivalent Dynabeads (dry weight). Dynabeads can be stored at 4°C in PBS supplemented with 0.02% w/v sodium azide or PBS in 50% v/v glycerol for storage at -20°C. Polyclonal anti-GFP antibodies were generated in llama, otherwise prepared as described (22). See Rothbauer et al. (28) for details of the anti-GFP nanobody.

Affinity isolation and SDS-PAGE

Briefly, affinity media were added to clarified cell extracts, centrifuged at 14,000 rpm, 10 min in a microfuge, and incubated with rotation at 4°C for 1 h. Affinity media were prewashed with 3 × 1 mL extraction buffer and again with 3 × 1 mL extraction buffer after the 1 h incubation. For elution of the exosome and NEXT complexes, 1 × LDS sample buffer (no. NP0007, no reducing agent; Invitrogen) was added directly to the affinity media, with incubation at 75°C for 10 min, and the supernatant collected. A detailed protocol is provided in the supplementary material. For elution of the CBC complex, 1 mg/mL 3×FLAG peptide (no. F4779; Sigma-Aldrich) was added to the affinity media and incubated 15 min at room temperature. Electrophoresis was performed using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) as per manufacturer's instructions or as specified in text. Bands were visualized by Coomassie staining (39). Bands of interest were excised and analyzed using a MALDI-prOTOF mass spectrometer (PerkinElmer, Waltham, MA, USA) and mass-to-charge ratio (M over Z) (40), PeakErazor (41), and ProFound (42) (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) software. See the supplementary materials for sample work-up details, raw data, and a full results summary; additional details contained in the text.

Results and discussion

Optimization of Dynabeads M270 antibody conjugate

Given the advantages of magnetic particles for affinity isolation, we generated our own anti-FLAG M2 (27,43) antibody-coupled magnetic beads (MBs, Dynabeads M-270 epoxy; Invitrogen) and tested them via affinity isolation of the reporter protein BAP-FLAG (no. P7457; Sigma-Aldrich). The comparison to affinity isolations performed with commercially available agarose resins from Sigma-Aldrich (products A2220 and M8823) demonstrates several points of note (Figure 1 and Supplementary Figure S2). First, the MBs exhibit significantly reduced nonspecific protein binding when compared with agarose beads, even at low stringency of purification. Secondly, although Sigma-Aldrich offers two grades of anti-FLAG antibody, purified immunoglobulin (F3165) or affinity isolated antibody (F1804), we see no significant

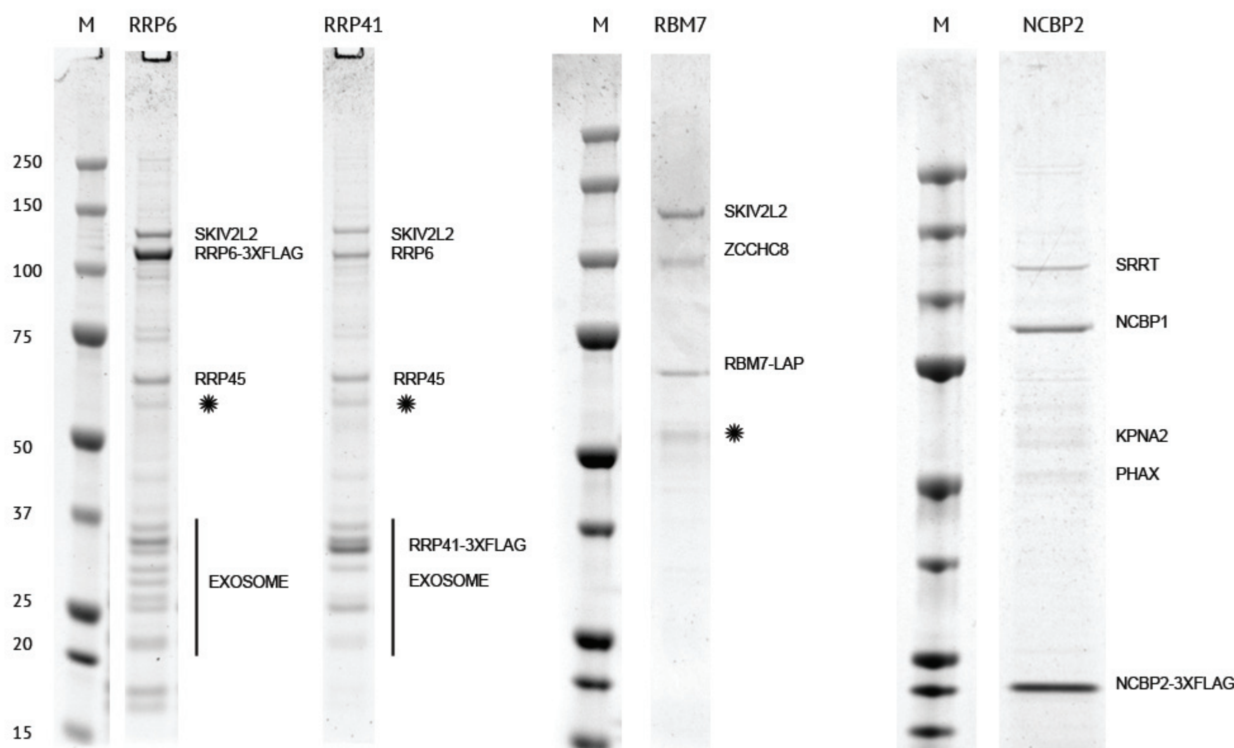

Figure 4. Copurification of complexes with 3×FLAG- and LAP-tagged proteins using magnetic beads. Cryogenically disrupted HEK cells (100 mg) expressing RRP6-3×FLAG or RRP41-3×FLAG proteins and HeLa cells expressing RBM7-LAP were used in affinity isolation experiments with MBs coupled to anti-FLAG or anti-GFP antibodies, and eluted with LDS; 200 mg disrupted HEK cells expressing CBC20-3×FLAG were affinity isolated with MBs coupled to anti-FLAG antibodies and eluted with 3×FLAG peptide. All isolations were done in 20 mM HEPES, pH 7.4. Exosome isolations were done at 300 mM NaCl with 0.5% v/v Triton X-100; NEXT complex at 500 mM NaCl, 0.5% Triton X-100; and CBC at 100 mM NaCl, 1.0% v/v Triton X-100. Relevant proteins identified by MALDI-MS are indicated. *Ig heavy chain.

difference in performance between the two in our assay. Moreover, the F3165 product requires no preparative handling, whereas F1804 requires desalting to remove glycerol prior to coupling. While the F3165 product does contain NaN_3 (0.02%), which can react with the epoxy functionalized M-270 Dynabeads used here, interference is minimal at the concentration used for antibody preservation (typically <10% reduction in performance; Figure 1, compare lanes 2 and 3). On the other hand, the F1804 product contains glycerol (50%), which significantly reacts with the epoxy-functionalized beads, necessitating desalting (data not shown). We observed a modest increase in the binding of our reporter protein, BAP-FLAG, with increasing concentration of the antibody used during conjugation over the range presented. We settled on conjugating at 10 µg/mg MBs directly, without desalting, using purified immunoglobulin (F3165) given the relative performance and antibody cost. The same strategy has been successfully applied to coupling polyclonal llama anti-GFP antibodies and anti-GFP nanobodies (see below), as well as anti-Myc E910 monoclonal antibodies (data not shown) to MBs.

Expression of tagged proteins at near endogenous levels

Due to the artifact prone nature of protein over-expression (1,19–21) (Supplementary Figure S3), we set out to create cell lines expressing our proteins of interest at near endogenous levels via the HEK293 Flp-In T-REx system. We previously generated cell line derivatives stably expressing tetracycline-inducible versions of RRP6, RRP41, and SKIV2L2, each with a C-terminal FLAG-tag, respectively (17). For point of comparison in affinity isolation, this work was repeated using C′-terminal 3×FLAG-tag constructs. In all cases tested, the 3×FLAG-tagged proteins have exhibited superior affinity isolation in terms of total yield and the stringency at which the purification can be successfully carried out (Supplementary Figure S4). We have observed that 3×FLAG-tagged proteins can be readily affinity isolated in the presence of 1 M NaCl, 2 M urea, or 1% (w/v) N-laurylsarcosine, respectively, without apparent loss of yield for the handle protein (although many coprecipitating proteins are lost under these conditions; data not shown); hence we have adopted 3×FLAG-tags moving forward. Comparable results to those acquired with 3×FLAG have also been obtained using a 3×Myc-tag, in concert with the E910 monoclonal antibody (data not shown), but this lacks a well-established method for native elution by competitive displacement achievable with the 3×FLAG-tag (26). As can be seen in Figure 2, RRP6-3×FLAG was readily titrated to near the endogenous level by using tetracycline at a concentration of 5 ng/mL in the media, demonstrating the ability of this system to achieve physiological expression of proteins of interest. An alternative approach is to express your protein of interest as a stable, affinity-tagged, BAC-cloned transgene from the endogenous promoter and context (18).

Cryogenic disruption of human cells shows favorable properties for protein affinity isolation using magnetic beads

In order to characterize the relative merits of cell disruption by cryogenic grinding versus standard sonication in human cells, we utilized anti-GFP conjugated MBs and HeLa Kyoto cells, expressing the EGFP-containing RBM7-LAP protein (13,14) (Figure 3). Each experiment utilized equivalent material from only one ~90% confluent 150-cm^2 culture dish, which is

~100 mg WCW in the case of cryogenic cell grindate. The control cell line clearly shows that sonication generates a vastly increased level of nonspecific protein binding in the absence of the expressed tagged protein. In stark contrast, although some nonspecific binding remains apparent in the sonicated sample when RBM7-LAP is expressed, the differences between sonicated and cryogenically disrupted cells are much less pronounced. Nonspecific binding of protein to affinity resins can vary significantly with extraction and washing solution compositions, but these data suggest that mechanical grinding provides increased uniformity between the tagged protein expressing and control cell lines. Given these and the above observations, we conclude that cryogenic disruption of cells followed by affinity isolation using magnetic beads is highly efficient, reliable, and robust. Figure 4 shows a panel of purifications using tagged proteins purified via the methods optimized as described here. The exosome and NEXT complexes were eluted with LDS sample loading buffer at 75°C, while the CBC was eluted with 3×FLAG peptide to avoid contamination of the sample with IgG, which can occur when eluting with sample buffer at elevated temperature.

While we believe the purifications presented to be among the highest quality preparations of the human exosome and NEXT complexes produced by a single step affinity isolation at the given scale, the expected coprecipitation patterns of specific interactors have previously been established using affinity isolation and protein staining (17,31). However, to our knowledge, Figure 4 also presents the first example of a Coomassie-stained coprecipitation pattern for the human CBC, and this result is well supported by published results obtained using other analytical means: the interaction between NCBP1 and NCBP2 is well-established and leads to formation of a heterodimer that binds to m7G-capped RNA (32); KPNA2 (human importin-α) is required for the nuclear export of CBC bound to capped RNAs and the subsequent re-import of CBC along with importin-β (44,45); PHAX was shown to be a bridging factor between CBC and CRM1, an export factor for U small nuclear RNAs (snRNA) (46); and SRRT (ARS2) was reported to interact with the CBC and shown to be crucial for microRNA (miRNA) biogenesis (47). While it is not certain how many different complexes this purification comprises—or in what proportions these other proteins interact with the CBC in concert or separately—it will be interesting to determine unambiguously if the PHAX containing CBC population overlaps with the SRRT-containing population, as these two components have not previously been reported in mutual coprecipitation, and they are considered to have differing physiological roles in RNA metabolism.

Polyclonal Ig versus nanobody

Conducting identical purifications of RBM7-LAP using magnetic beads conjugated with either affinity isolated anti-GFP polyclonal Ig or a bacterially expressed and purified anti-GFP nanobody (28,29), we observe the loss of the nonspecific Fc binding protein TRIM21 (data not shown) from our isolations and, as expected, reduced pollution from Ig chains when the nanobody is used. However, at equivalent proportions by mass used in coupling to Dynabeads, the anti-GFP nanobody reagent exhibited lower yields than our polyclonal Ig (Supplementary Figure S5).

Here we have applied an optimized workflow for the isolation of endogenous protein complexes from human cells at quantities detectable by Coomassie staining of SDS-PAGE gels, with low nonspecific background, and without protein over-expression. Expression at or near the endogenous level mitigates the possibility of induced artifacts that can lead to erroneous conclusions. Using magnetic beads coupled to high-affinity antibodies, 100 mg WCW of material typically affords superior quality isolations for most protein complexes we have examined, even at low stringency of extraction—providing yields in the range of tens to hundreds of nanograms per protein band, amenable to identification by MALDI-TOF MS. We often use 50 mg WCW, corresponding to 1/2 of one 150-cm^2 culture dish, to great success in pilot experiments.

It is often desirable to make several grams of cell grindate at one time and to store it at -80°C for a subsequent period of continued experimentation. (We have done so 6 months or more with no apparent loss of performance in affinity isolations.) Most of the time we have achieved excellent initial results with the usual "standard buffers" (e.g., HEPES, pH 7.4, 100 mM NaCl, 0.5% v/v Triton X-100). However, as individual experiments do not consume exorbitant quantities of material, it is reasonable to explore several affinity isolation conditions in parallel, rapidly converging on an optimized result with efficient use of time and material. A main advantage of 3×FLAG-based and GFP-based affinity isolations is the ability to use high stringency conditions. Therefore, we commonly screen increasing NaCl concentrations up to 1 M, as well as numerous different detergents at different concentrations (e.g., Triton X-100, Tween-20, CHAPS).

Once a favored result is identified, it is simple to scale-up several-fold if required for downstream analysis. Due to the yield and purity that can be achieved, native release of protein complexes via competitive elution, or protease cleavage of the tagged protein, provide the possibility of carrying out enzymatic or biochemical assays, as well as biophysical analyses such as negative stain EM.

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Competing interests

The authors declare no competing interests.

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