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Abstract

The Microprocessor complex catalyzes the first step of miRNA biogenesis in the nucleus of mammalian cells. The minimal catalytically active complex is formed by two essential factors, the dsRNA binding protein DGCR8 and the RNAse III endonuclease Drosha. Importantly, several co-factors can associate to this complex and modulate either positively or negatively the cleavage and binding efficiency of this complex. Here, we describe a simple method for purification of DGCR8 and Drosha coupled to mass spectrometry or western blot which allows robust identification of unknown associated factors. This approach has recently revealed the presence of a new DGCR8-dependent, Drosha-independent complex involved in RNA turnover.

Keywords: Immunoprecipitation, mass spectrometry, pri-miRNA processing, miRNAs, DGCR8, Drosha, RNA processing, western blot.

Running Head: DGCR8 and Drosha purification

1. Introduction

Co-immunoprecipitation or immunoprecipitation (IP) coupled to mass spectrometry techniques aim to identify novel factors associated with the proteins of interest. An important challenge of these approaches is the discrimination amongst true interacting partners versus contaminants. The direct comparison of results obtained with different tagged versions of the same protein, or alternatively, different primary antibodies, plus the inclusion of appropriate negative controls allows the identification of robust interactions and excludes common contaminants obtained by these techniques (1). Recent efforts towards describing common contaminants in these approaches have recently been described, such as the 'bead proteome' (2) and the 'Crapome' (3).

After the discovery of the minimal catalytically active Microprocessor complex (4, 5), many other factors have been shown to modulate the activity as well as the RNA binding capacity of this complex, either by direct protein–protein interactions (6) or by binding to the same substrate and precluding or exposing signals necessary for efficient processing (7).

The Microprocessor complex consists of two different essential proteins, the dsRNA binding protein DGCR8 and the RNAse III endonuclease Drosha. Here, we present a method to identify new interacting partners for this complex, either using overexpressed and endogenous core Microprocessor components. This approach can be applied to the detailed study of the relative composition of the Microprocessor associated factors in different cellular contexts, as well as, during different cellular conditions, such as stress responses.

For co-IP of associated factors to overexpressed Microprocessor components, total cell lysates of cells expressing FLAG- or T7-tagged version of DGCR8 and Drosha are prepared and purified using FLAG- or T7-antibdy coated beads. After extensive washes of the beads, immunoprecipitates are loaded in a SDS-PAGE gel to be sequenced by mass spectrometry, or analysed by western blot for specific factors. This protocol is run in parallel with untransfected

extracts to discriminate the non-specific binding of proteins to beads and other reagents used. For the purpose of endogenous proteins purification, total cell extracts are prepared and incubated with beads coupled to primary antibodies against DGCR8 or Drosha, and as a negative control, beads coupled to an IgG antibody from the same serotype are used.

In addition, we have included an RNAse treatment step that allows discrimination between those interactions that are RNA-dependent (binding to common RNA substrates) vs independent (direct protein-protein interactions). This approach has been successfully employed to identify many common factors bound to the Microprocessor core, as well as a new DGCR8-dependent, Drosha-independent complex involved in RNA decay (1).

2. Materials

2.1. Cell culture and transfections

1. Dulbecco's Modified Eagle Medium (DMEM)
2. FCS
3. Penicillin/Streptomycin
4. Phosphate buffered saline (PBS) pH 7.4
5. 0.25% Trypsin-EDTA, Phenol Red
6. Lipofectamine 2000
7. Table top centrifuge
8. Refrigerated Microfuge
9. 15mL & 50mL centrifuge tubes
10. Flag-DGCR8, T7-DGCR8 and Flag-Drosha plasmids
11. 1.5mL tubes

2.2 Cell Lysis and lysate clearance

1. Buffer D: 20mM Hepes-KOH pH 7.9, 100mM KCl, 0.2mM EDTA, 5% glycerol

2. IP buffer: 50mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA, 1% Triton X100
3. Bioruptor (or probe sonicator)
4. Refrigerated microfuge
5. 1.5mL tubes
6. DTT 1M (1.54g DTT dissolved in 10mL of H20)
7. PMSF
8. EDTA-free protease inhibitor cocktail
9. RQ1 DNase
10. RNase A
11. RNase Inhibitors

2.3 Immunoprecipitation

1. FLAG M2 beads (Sigma)
2. T7 beads (Novagen)
3. Protein A magnetic beads (Dynabeads)
4. Antibody against DGCR8 (ab90579)
5. Antibody against Drosha (NBP1-03349)
6. Serotype matched IgG control
7. Rotating wheel
8. High-salt Buffer D: 20mM Hepes-KOH pH 7.9, 200mM KCl, 0.2mM EDTA, 5% glycerol
9. IP High-salt buffer: 50mM Tris pH 7.5, 200 mM NaCl, 1mM EDTA, 1% Triton X100

2.4 SDS-PAGE and mass spectrometry/western blot

1. Thermomixer or thermoblock
2. 4x Loading buffer and 10x Reducing Agent (Invitrogen)

3. 4-12% Bis Tris Novex gels
4. 20x MOPS running buffer
5. Prestained Benchmark protein ladder
6. Gel tank
7. Western blot transfer unit (iBLOT2)
8. ECL and secondary antibodies coupled to HRP
9. Colloidal blue staining kit
10. Sterile razor blades
11. 15cm sterile plates for gel staining

2.5 Confirmation of RNase treatment

1. Thermocycler
2. Reverse Transcriptase
3. DNA polymerase
4. 1 x TBE
5. Agarose
6. 6 x DNA Loading Dye
7. UV transluminator
8. SYBR Safe

23. Methods

3.1 Cell Culture and transfections

Detailed below is a simple method to expand and transiently transfect HeLa cells with plasmid vectors expressing Drosha and DGCR8 (FLAG- and/or T7-) and their respective controls using a lipid based transfection reagent. This has previously generated a complex protein mixture after immunoprecipitation to perform Mass Spectrometry analyses on (see **Figure 1**).

3.1.A Preparation of transiently transfected HeLa cells overexpressing DGCR8 and Drosha

1. Seed HeLa cells (or the cell line of choice) into a 6 well plate and expand to 70% confluency. In total, 3 wells of a 6 well plate should be seeded per tagged protein to be transfected [**note 1**].
2. Briefly rinse cells with PBS, and aspirate the liquid. Add 1.5mL of DMEM (10%FCS) without antibiotics [**note 2**], and place cells back in the incubator.
3. Each well of the 6 well plate is to be transfected with 4μg of plasmid DNA and 10 μL of Lipofectaime 2000 following basic manufacturer's instructions. Cells are transfected with the following plasmids: pcDNA3-Flag (empty control plasmid), Flag-Drosha, Flag-DGCR8, pCG-T7 (empty control plasmid), and T7-DGCR8 [**note 3**].
4. Remove the cells from incubator and add the transfection complex mixture dropwise to each of the 3 wells. The final volume should be 2 mL. Place cells back into the incubator and leave overnight [**note 4**].
5. After 24 hours, trypsinize the cells and pool the transfected cells of the same identity. Reseed the pooled cells in a 15cm plates and allowed to expand for a further 24 hours.

6. Collect the cells by trypsinization or scraping 48 hours after the initial transfection to allow for optimal protein overexpression. Cells are briefly washed with ice-cold PBS, centrifuged (200 x g for 5 mins), and cell pellets can be snap-frozen on dry ice and store at -80°C until required, or immediately processed.

3.1.B Preparation of HeLa whole cell extract

1. In total 3 IPs will be performed (IgG control, Drosha and DGCR8) and 1 x 10 cm dish is at least required per IP. Therefore, expand HeLa cells in 3 x 10 cm tissue culture dishes to 90% confluency.
2. Trypsinize the cells and pool into one centrifuge tube. Cells are then pelleted by centrifugation (5min at 200 x g) in a tabletop centrifuge.
3. Aspirate the supernatant from the pelleted cells and wash the cell pellets in ice-cold 10mL PBS and spin again at 200 x g for 5 mins.
4. Aspirate the supernatant and resuspend the cell pellet in 3mL of ice-cold PBS and transfer 1mL to 3 x 1.5mL tube. Pellet cells by spinning at 200 x g for 5 min using a microfuge at 4°C [**note 5**].
5. Carefully remove supernant and snap-freeze cell pellet on dry ice and store at -80°C until required, or process immediately.

3.2 Bead preparation

Please note that different buffers (IP and Buffer D) and beads (Magnetic and Agarose) are used to purify Microprocessor-associated complexes from transiently transfected cells or HeLa whole cell lysate (see **Figure 1**).

3.2.A For immunoprecipitation of overexpressed epitope tagged proteins

1. Pre-wash 10-20µL of magnetic FLAG or agarose T7 antibody beads per IP by resuspending the beads in 1mL of buffer D.
2. Microfuge at 1000 x g for 30 seconds, and if using magnetic beads precipitate the beads on a magnetic rack for 30 seconds.
3. Aspirate as much of the supernatant without disturbing the beads and repeat for a total of three washes [**note 6**]. On last wash resuspend beads in 100µL of buffer D and keep on ice until required.

3.2.B For immunoprecipitation of endogenous proteins: coupling antibodies to beads

1. Pre-wash 10-20µL of Protein A Dynabeads per IP by resuspending the beads in 1mL of IP buffer.
2. Microfuge at 1000 x g for 10 sec and precipitate the beads on a magnetic rack.
3. After aspirating the supernatant [**note 6**], repeat for a total of three washes. On last wash resuspend beads in 500µL of IP buffer and add 1µg of the primary antibody of your choice and include the negative species matched IgG as a control. Incubate for 3-4 hours at 4°C with end-over-end rotation.
4. Microfuge at 1000 x g for 30 sec and magnetically precipitate the beads. Perform 3 washes with 1mL of IP buffer, and resuspend the beads in 100µL of IP buffer and keep on ice until needed.

3.3 Cell lysis and lysate clearance

We have employed a sonication based cell disruption method to lyse cells and shear their DNA content in order to release chromatin associated proteins and reduce sample viscosity. Please note that different buffers (IP and Buffer D) and beads (Magnetic and Agarose) are used to purify Microprocessor-associated complexes from transiently transfected cells or HeLa whole cell lysate (see **Figure 1**).

Prepare all buffers fresh and keep chilled on ice when adding protease inhibitors, DTT and PMSF to correct final concentration, cool a microfuge to 4°C and begin chilling the Bioruptor's water tank.

1. If using HeLa cells for IP of Microprocessor-associated complexes using e**pitope-tagged overexpressed** components resuspend the cell pellets in 1mL of **Buffer D** with freshly added 0.5mM DTT, 0.2mM PMSF and 1x Protease Inhibitors final concentration. Alternatively, if using HeLa cells for IP of Microprocessor-associated complexes using **endogenous** Microprocessor components, resuspend the pellet in 1mL of **IP buffer** with freshly added 0.5mM DTT, 0.2mM PMSF and 1x Protease Inhibitors final concentration.
2. Add 10µL of RQ1 DNase to the cell lysates. If testing for RNA-independent interactions, add 10µL of RNase A. If testing for RNA-dependent interactions add 10µL of RNase Inhibitor.
3. Sonicate in the Bioruptor (Diagenode) using the low setting for a total of 5 cycles of 30 sec on/30 sec off [**note 7**].
4. Spin at 16,000 x g for 10 min using a microfuge at 4°C, and transfer the supernatant to a new 1.5mL tube.
5. Microfuge samples again at 16,000 x g for 10 min at 4°C, and transfer 95% of the supernatant to a new 1.5mL tube, taking care not to disturb any pelleted debris and store on ice [**note 8**].
6. Transfer 50µl (5%) of the cleared cell lysate to 1.5mL tube labelled INPUT and freeze on dry ice. This can be processed later for input analyses.

3.4 Immunoprecipitation and washes

1. Microfuge the beads at 1000 x g for 30 sec to initially pellet the beads (from step **3.2A** and **3.2B**), and then magnetically precipitate the magnetic beads. Fully aspirate the last wash, being careful not to disturb beads.
2. Transfer the cleared cell lysate to the 1.5mL tube containing the beads.
3. Rotate the immunoprecipitations end-over-end at 4°C from 1hr to overnight [**note 9**]. Overexpressed proteins IPs can be carried out at shorter times, whereas endogenous IPs are usually incubated overnight.
4. Microfuge the IPs at 1000 x g for 30 sec to collect beads at the base of the tube [**note 10**], and then magnetically precipitate the paramagnetic beads.
5. Remove 50µl and transfer to a 1.5mL tube labelled UNBOUND and immediately snap freeze on dry ice [**note 11**].
6. Aspirate remaining lysate from IPs being careful not to disturb beads.

At this step, the standard salt buffers are replaced with high-salt IP buffer (200mM NaCl) or high-salt buffer D (200mM KCl) to perform high-stringency washes with rotation for 5 min. Please note that different buffers are used for different IPs (see **Figure 1**).

7. To wash beads, resuspend in 1mL of high-salt buffer, and rotate at room temperature for 5 min at 16 rpm. Microfuge for 10 sec at 1000 x g to pellet beads, and if using magnetic beads precipitate them on a magnetic rack. Fully aspirate the wash buffer.
8. Repeat step 7 for a total of five washes. After rotating for the last wash, remove 100µL of bead suspension and place in a clean 1.5mL tube labelled 10% IP. This can be used to assess IP efficiency.
9. Microfuge all tubes for 10 sec at 1000 x g and then magnetically precipitate the magnetic beads. Completely aspirate the last wash buffer and resuspend the beads in 15µl of 2x LD buffer with 2x Reducing agent (7.5µL 4 x LD buffer, 3µL 10x Reducing agent, and 4.5µL of PBS) and incubate at 70°C for 10 min in a thermomixer with 1100 rpm shaking.

10. If IPs are to be stored and not processed immediately, microfuge for 10 sec at 1000 x g to pellet beads, and then magnetically precipitate the beads from the sample and transfer the eluate to a labelled 1.5mL tube and snap freeze on dry ice. This can then be stored at -80°C until required [**note 12**].

3.5 SDS PAGE and Sample Isolation

To obtain the complete interactome of Microprocessor-associated components we chose to sequence whole lanes from the IPs. For this, it is necessary to perform the staining and the gel excision steps within a laminar hood to prevent contamination from aerosols.

1. Spin the 1.5mL tubes at 1000 x g for 10 sec to collect liquid at the base of the tube and heat at 70°C for 10 min. Load a prestained protein ladder, and all of the 15µl mass spectrometry IPs into a 10 well 1.0mm 4-12% Bis-Tris Novex gel [**note 13**]. Leave at least one empty lane between samples to prevent cross-contamination when cutting lanes.
2. Run the gel at 170V for 5 min or until the samples have entered the gel and begin to separate. This can be judged by separation of the protein marker and when the dye front reaches the first graduation of the gel case (see **Figure 2A**). For confirmation of optimal immunoprecipitation conditions, 10% of the IP is run in a separate gel and stained with Colloidal coomassie blue prior to send samples to mass spec (see **Figure 2B**).
3. Transfer the unopened Novex gel cassette to a flow laminar hood. Break open the cassette and transfer gel to a clean 15cm dish and stain with Colloidal coomassie blue staining kit as per manufacturer's instructions.
4. After visualizing the lanes with protein stain, return the gel to the laminar hood and use a clean blade to excise the whole stained lane from the gel (as seen in **Figure 2A**), and transfer to a labeled sterile tube and store at 4°C. For each individual sample, use a new pair of gloves and razor blade to prevent cross contamination.
5. Send the individual mixture of proteins for mass spectrometry analyses.

3.6 Input Processing and Analysis Controls

3.6A Analysis of bona-fide interactions by western blot

To define whether the IPs have good signal to noise ratios and are robust enough, we performed protein staining and western blot analysis on samples of the cell lysate before and after IP to check for antigen depletion. Co-IP of bonafide protein interactors by western blot, such as reciprocal DGCR8 and Drosha co-immunoprecipitation is also used as a control for good experimental methodology. In addition, confirmation of RNAse treatment can be assessed by analyzing RNA content from the UNBOUND fraction (see **Figure 3**).

1. Defrost INPUT, UNBOUND, and 10% IP samples on ice.
2. Transfer 20µL of the lysates (INPUT and UNBOUND) to a new 1.5mL tube and add 7.25µL of 4x LD dye and 3µL of 10X Reducing agent, mix, centrifuge briefly to collect liquid at the base of the tube. Add the same amount of these to the 10% IP sample and incubate all samples for 10 min at 70°C with 1100 rpm agitation.
3. Briefly centrifuge tubes after heating and place on a magnetic stand to precipitate the beads. Load 15µL into a 12 well 4-12% Bis Tris Novex gel, along with 10µL of protein ladder. The gel should be run in 1xMOPS for 1hour at 150V, or until the dye front has reached the bottom of the gel.
4. If assessing IP samples for noise, stain the gel using Colloidal blue staining kit as per the manufacturer's instructions (as shown in **Figure 2B**). For assessment of co-IP of bonafide Microprocessor associated proteins by western blot, transfer the gel to a nitrocellulose membrane using a blotting systems (**Figure 3A**, for overexpressed proteins, and **Figure 3B and 3C** for endogenous proteins).

5. Block the membrane by agitating it in 5% non-fat powder PBS-T (PBS supplemented with Tween-20 to a final concentration of 0.05%) for 30 mins to 1 hour at room temperature.
6. Dilute the appropriate primary antibody (usually 1:500- 1:1,000 for anti-Drosha and DGCR8, and 1:10,000 for overexpressed proteins for anti-FLAG or T7) in 5% non-fat powder PBS-T and leave to incubate overnight, rotating at 4°C.
7. Perform 3 PBS-T washes with agitation for 10 min each, at room temperature.
8. Dilute the appropriate secondary antibody in 5% non-fat powder PBS-T and leave to incubate at room temperature with agitation for over 1 hour.
9. Perform 3 PBS-T washes with agitation for 20 min each, at room temperature.
10. Image western blot with ECL and chemiluminescent film (see **Figure 3**).

3.6B Confirmation of RNase treatment

1. Take the UNBOUND fraction (50ul) and perform a Trizol LS extraction, or alternatively Proteinase K treatment followed by an acidic phenol-chloroform extraction. After precipitation with ethanol or isopropanol, resuspend pellets in 10 µl of RNase-free water.
2. Perform retrotranscription against an abundant house-keeping gene, such as GAPDH, 7SK or actin. Amplify the transcript of interest by PCR. Next, run the PCR products in a 1% TBE-agarose gel and stain with SYBR safe to check for efficient RNase treatment (as seen in **Figure 3A**, **3B**, and **3C** bottom panels).

4. Notes

Note 1: To achieve 70% confluency 20-24 hours after seeding, seed 0.18-0.2 x 10^6 cells per well of a 6 well plate. This equates to roughly $1/48^{th}$ of a confluent T75 flask of cells (250µL of 12mL suspension)

Note 2: Using antibiotics in combination with lipid based transfection reagents can be highly cytotoxic and negatively affect transfection efficiency of your experiment.

Note 3: Ensure plasmid DNA concentration is at least 0.5µg/µL. Adding large volumes low concentration plasmid DNA can carry over contaminants from the plasmid DNA prep, which can negatively affect transfection efficiency.

Note 4: Transfection complex can be remove from cells after 6 hours. This may increase cell viability and has no effect on transfection efficiency.

Note 5: Doing this ensures that equal volumes of protein are used for the individual IP and avoids the need to normalize the input material.

Note 6: Accidently aspirating the magnetic beads is not usually a problem as they are isolated against the tube wall away from the base of the tube. However, agarose beads will remain pelleted at the bottom. To aid aspiration we recommend placing a p1000 tip into a p10 tip to improve aspiration accuracy and reduce turbulent flow of supernatant into the pipette, which could result in bead loss.

Note 7: If snotty DNA pellet remains in the lysate after sonication, this can be due to the cell pellet being too concentrated. Resuspend in larger volume, or alternatively use a Branson probe sonicator (5 cycles of 10sec on followed by 30 sec off using; 10 AMP) to lyse cells and shear DNA. It is important to shear the cellular DNA content as this will aid the release of chromatin-associated proteins.

Note 8: Proteous columns can be used for further cell lysate clearance if required.

Note 9: The incubation time depends on quality of the antibody used and/or the abundance of the antigen in the cell lysate. Adjust incubation times depending on these factors. To asses IP quality see Note 11.

Note 10: Pelleting beads before applying to a magnet aids magnetic precipitation, and clears liquid/beads from the tube cap.

Note 11: Western blot analyses can be used to assess IP efficiency by comparing protein depletion of equivalent fractions of the UNBOUND with the INPUT samples. The UNBOUND also serves to monitor optimal RNase treatment.

Note 12: The use of buffer D, which is high in glycerol, allows freezing of 'active' immunoprecipitates with overexpressed proteins, which can be used in 'in vitro' processing assays.

Note 13: Do not use homemade SDS-PAGE running buffer here, rather segregate a commercially available premade buffer specifically for your Mass Spectrometry analyses to prevent contamination.

5. References

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Figure 1

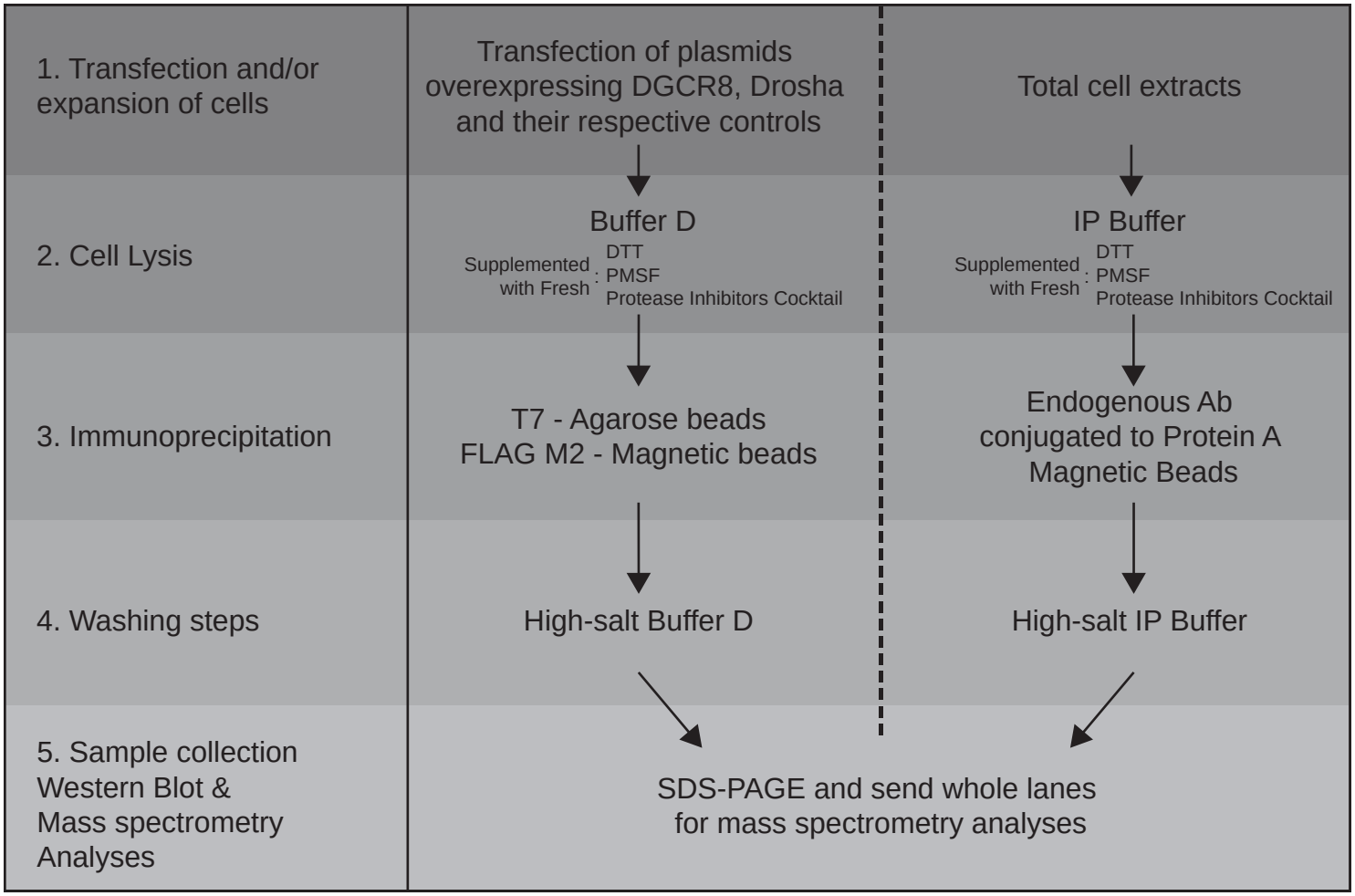

Figure 1. Experimental overview schematic highlighting different buffer and beads used during the protocol.

Figure 2

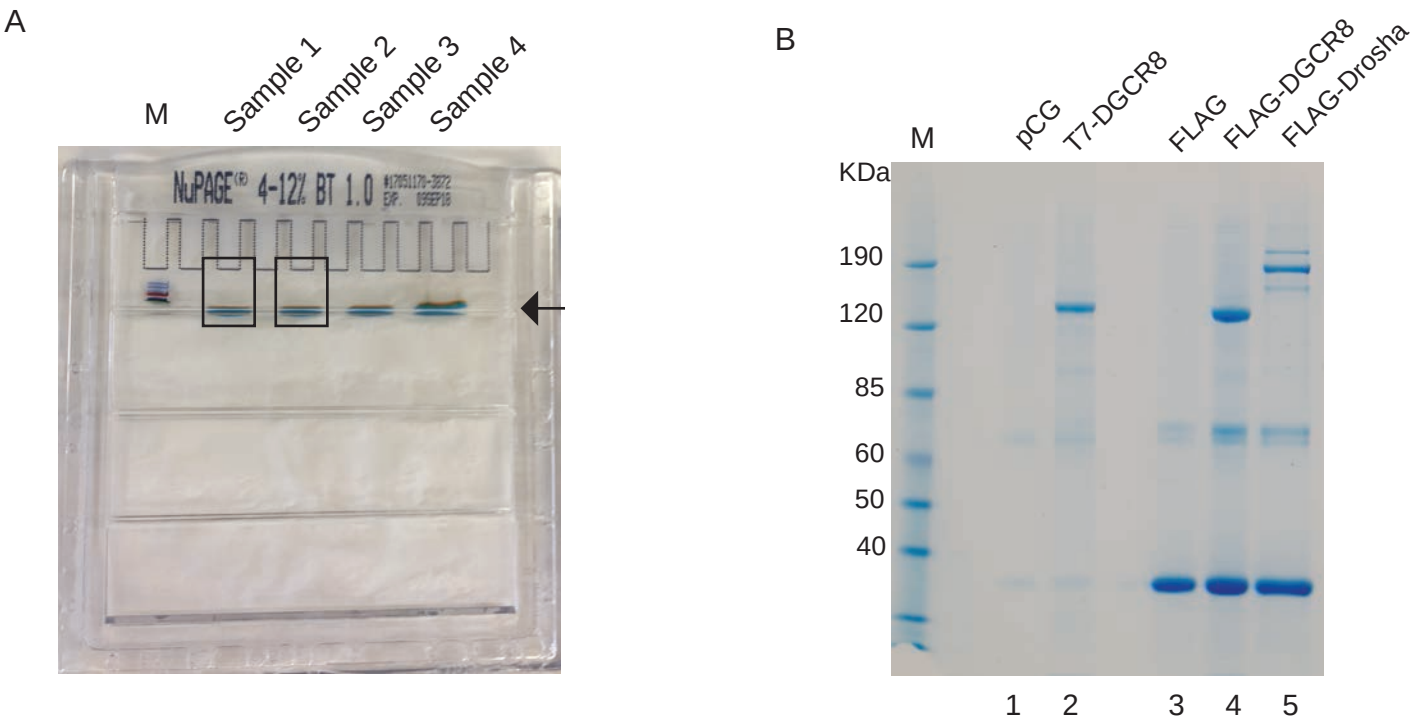

Figure 2. Purification of associated factors with overexpressed Microprocessor components DGCR8 and Drohsa. (a) SDS-PAGE separation of immunoprecipitates after 5mins running at 170V or until the protein dye front reaches the first graduation on the Novex gel casette. Samples are sliced as depicted by the black rectangle **(b)** Coomassie blue staining of 10% of the immunopurified complexes with overexpressed T7-DGCR8, FLAG-DGCR8 and FLAG-Drosha (lanes 2, 4 and 5). As negative controls, cells transfected with control plasmids were immunoprecipitated using T7 (pCG-T7, lane 1) and FLAG antibody coated beads (pcDNA3-FLAG, lane 3). M, denotes marker (reproduced from (1) under a CC BY 4.0, DOI 10.1016/j.molcel.2015.11.011)

Figure 3

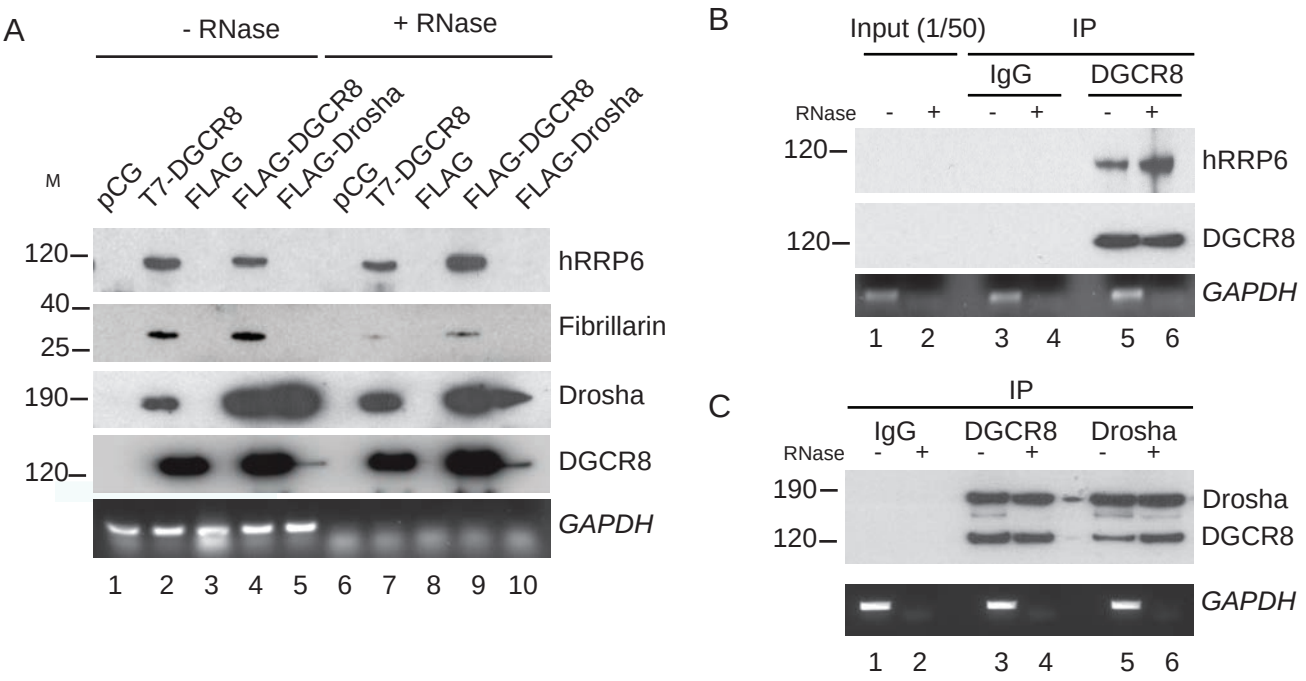

Figure 3. Immunoprecipitation followed by western blot analyses with overexpressed and endogenous Microprocessor factors (**a**) Validation of proteins interacting with T7-DGCR8, FLAG-DGCR8, and FLAG-Drosha by immunoprecipitation followed by western blot analysis with specific antibodies, in the presence (lanes 6–10) or absence of RNase A (lanes 1–5). The exosome component hRRP6 and Drosha are coimmunoprecipitated with DGCR8 independently of the presence of RNA, whereas Fibrillarin co-immunoprecipitation depends on RNA presence. The RT-PCR amplification of Gapdh serves as a control for RNase treatment (bottom panel). (**b, c**) The co-immunoprecipitation of hRRP6 (**b**, compare lane 5 and 6) and Drosha (**c**, compare lanes 3 and 4) with DGCR8 is independent of the presence of RNA. Drosha co-immunoprecipitates DGCR8 independently of the presence of RNA (**c**, lanes 5 and 6). Gapdh mRNA PCR amplification serves as a control for RNase treatment.