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TITLE:

Identification of Nucleolar Factors During HIV-1 Replication Through Rev Immunoprecipitation and Mass Spectrometry

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HIV-1 replication, rev, immunoprecipitation, mass spectrometry, nucleolus, B23

SUMMARY:

Here we describe Rev immunoprecipitation in the presence of HIV-1 replication for mass spectrometry. The methods described can be used for the identification of nucleolar factors involved in the HIV-1 infectious cycle and are applicable to other disease models for the characterization of understudied pathways.

ABSTRACT:

The HIV-1 infectious cycle requires viral protein interactions with host factors to facilitate viral replication, packaging, and release. The infectious cycle further requires the formation of viral/host protein complexes with HIV-1 RNA to regulate the splicing and enable nucleocytoplasmic transport. The HIV-1 Rev protein accomplishes the nuclear export of HIV-1 mRNAs through multimerization with intronic *cis*-acting targets—the Rev response element (RRE). A nucleolar localization signal (NoLS) exists within the COOH-terminus of the Rev arginine-rich motif (ARM), allowing the accumulation of Rev/RRE complexes in the nucleolus. Nucleolar factors are speculated to support the HIV-1 infectious cycle through various other functions in addition to mediating mRNA-independent nuclear export and splicing. We describe an

immunoprecipitation method of wild-type (WT) Rev in comparison to Rev nucleolar mutations (deletion and single-point Rev-NoLS mutations) in the presence of HIV-1 replication for mass spectrometry. Nucleolar factors implicated in the nucleocytoplasmic transport (nucleophosmin B23 and nucleolin C23), as well as cellular splicing factors, lose interaction with Rev in the presence of Rev-NoLS mutations. Various other nucleolar factors, such as snoRNA C/D box 58, are identified to lose interaction with Rev mutations, yet their function in the HIV-1 replication cycle remain unknown. The results presented here demonstrate the use of this approach for the identification of viral/host nucleolar factors that maintain the HIV-1 infectious cycle. The concepts used in this approach are applicable to other viral and disease models requiring the characterization of understudied pathways.

INTRODUCTION:

The nucleolus is postulated as the interaction ground of various cellular host and viral factors required for viral replication. The nucleolus is a complex structure subdivided into three different compartments: the fibrillar compartment, the dense fibrillar compartment, and the granular compartment. The HIV-1 Rev protein localizes specifically within granular compartments; however, the reason for this localization pattern is unknown. In the presence of single-point mutations within the NoLS sequence (Rev mutations 4, 5, and 6), Rev maintains a nucleolar pattern and has previously been shown to rescue HIV-1_{HXB2} replication, however, with reduced efficiency compared to WT Rev[1]. All single-point mutations are unable to sustain the HIV-1_{NL4-3} infectious cycle. In the presence of multiple single-point mutations within the NoLS sequence (Rev-NoLS mutations 2 and 9), Rev has been observed to disperse throughout the nucleus and cytoplasm and has not been able to rescue HIV-1_{HXB2} replication[1]. The goal of this proteomics study is to decipher nucleolar as well as nonnucleolar cellular factors involved in the Rev-mediated HIV-1 infectious pathway. Rev immunoprecipitation conditions are optimized through interaction with the nucleolar B23 phosphoprotein, which has previously been shown to lose interaction with Rev in the presence of nucleolar mutations.

Rev cellular factors have been extensively studied in the past; however, this has been done in the absence of viral pathogenesis. One protein, in particular, that is characterized in this study through Rev interaction during HIV-1 replication is the nucleolar phosphoprotein B23—also called nucleophosmin (NPM), numatrin, or NO38 in amphibians[2,3,4]. B23 is expressed as three isoforms (NPM1, NPM2, and NPM3)—all members of the nucleophosmin/nucleoplasmin nuclear chaperone family[5,6]. The NPM1 molecular chaperone functions in the proper assembly of nucleosomes, in the formation of protein/nucleic acid complexes involved in chromatin higher-order structures[7,8], and in the prevention of aggregation and misfolding of target proteins through an N-terminal core domain (residues 1–120)[9]. NPM1 functionality extends to ribosome genesis through the transport of preribosomal particles between the nucleus and cytoplasm[10,11], the processing of preribosomal RNA in the internal transcribed spacer sequence[12,13], and arresting the nucleolar aggregation of proteins during ribosomal assembly[14,15]. NPM1 is implicated in the inhibition of apoptosis[16] and in the stabilization of tumor suppressors ARF[17,18] and p53[19], revealing its dual role as an oncogenic factor and tumor suppressor. NPM1 participates in the cellular activities of genome stability, centrosome replication, and transcription. NPM1 is found in nucleoli during cell cycle interphase, along the chromosomal periphery during mitosis,

and in prenucleolar bodies (PNB) at the conclusion of mitosis. NPM2 and NPM3 are not as well-studied as NPM1, which undergoes altered expression levels during malignancy[20].

NPM1 is documented in the nucleocytoplasmic shuttling of various nuclear/nucleolar proteins through an internal NES and NLS[9,21] and was previously reported to drive the nuclear import of HIV-1 Tat and Rev proteins. In the presence of B23-binding-domain-β-galactosidase fusion proteins, Tat mislocalizes within the cytoplasm and loses transactivation activity; this demonstrates a strong affinity of Tat for B23[2]. Another study established a Rev/B23 stable complex in the absence of RRE-containing mRNAs. In the presence of RRE mRNA, Rev dissociates from B23 and binds preferably to the HIV RRE, leading to the displacement of B23[22]. It is unknown where, at the subnuclear level, Tat transactivation and the Rev exchange process of B23 for HIV mRNA take place. Both proteins are postulated to enter the nucleolus simultaneously through B23 interaction. The involvement of other host cellular proteins in the HIV nucleolar pathway is expected. The methods described in this proteomics investigation will help elucidate the interplay of the nucleolus with host cellular factors involved during HIV-1 pathogenesis.

The proteomics investigation was initiated through the expression of Rev NoLS single-point mutations (M4, M5, and M6) and multiple arginine substitutions (M2 and M9) for HIV-1_{HXB2} production. In this model, a HeLa cell line stably expressing Rev-deficient HIV-1_{HXB2} (HLfB) is transfected with WT Rev and Rev nucleolar mutations containing a flag tag at the 3' end. The presence of WT Rev will allow viral replication to occur in HLfB culture, in comparison to Rev-NoLS mutations that do not rescue Rev deficiency (M2 and M9), or allow viral replication to occur but not as efficiently as WT Rev (M4, M5, and M6)[1]. The cell lysate is collected 48 h later after viral proliferation in the presence of Rev expression and subjected to immunoprecipitation with a lysis buffer optimized for Rev/B23 interaction. Lysis buffer optimization using varying salt concentrations is described, and protein elution methods for HIV-1 Rev are compared and analyzed in silver-stained or Coomassie-stained SDS-PAGE gels. The first proteomics approach involves the direct analysis of an eluted sample from expressed WT Rev, M2, M6, and M9 by tandem mass spectrometry. A second approach by which the eluates of WT Rev, M4, M5, and M6 underwent a gel extraction process is compared to the first approach. Peptide affinity to Rev-NoLS mutations in comparison to WT Rev is analyzed and the protein identification probability displayed. These approaches reveal potential factors (nucleolar and nonnucleolar) that participate in HIV-1 mRNA transport and splicing with Rev during HIV-1 replication. Overall, the cell lysis, IP, and elution conditions described are applicable to viral proteins of interest for the understanding of host cellular factors that activate and regulate infectious pathways. This is also applicable to the study of cellular host factors required for the persistence of various disease models. In this proteomics model, HIV-1 Rev IP is optimized for B23 interaction to elucidate nucleolar factors involved in nucleocytoplasmic shuttling activity and HIV-1 mRNA binding. Additionally, cell lines stably expressing infectious disease models that are deficient for key proteins of interest can be developed, similar to the HLfB cell line, to study infectious pathways of interest.

PROTOCOL:

1. Cell culture

1.1. Maintain HLfB in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1 mM sodium pyruvate within tissue-culture-treated 100 mm plates. Keep the cell cultures at 37 °C in a humidified incubator supplied with 5% CO_2. Passage confluent cells to a cell density of 1 x 10^6 cells/mL.

1.2. Discard the cell culture media. Gently rinse the cells with 10 mL of 1x phosphate-buffered saline (PBS). Remove and discard the 1x PBS without disrupting the cell layer.

1.3. Add 2 mL of 1x trypsin-EDTA solution to the cells. Rock the dish to coat the monolayer and incubate at 37 °C within a humidified chamber for 5 min.

1.4. Firmly tap the side of the dish with the palm of the hand to detach the cells. Resuspend the detached cells in 8 mL of fresh culture media. Spin the cells at 400 x *g* for 5 min.

1.5. Discard the culture media without disrupting the cell pellet. Resuspend the cell pellet in 10 mL of fresh culture media. Subculture 1 mL of concentrated cells with 9 mL of fresh culture media within tissue-culture-treated 100 mm plates.

NOTE: For each Rev-NoLS mutation, 3x 100 mm HLfB or HeLa culture plates will yield enough protein lysate for western blot analysis and mass spectrometry. Add extra plates for a WT Rev positive control and negative control. The subculture cell volume will require optimization with the use of different cell types.

2. Expression of Rev-NoLS-3'flag mutations during HIV-1 replication

2.1. Grow the HLfB cell culture to a cell density of 2 x 10^6 cells/mL. Prepare 4 mL of calcium phosphate-DNA suspension for each 100 mm plate as follows.

2.1.1. Label two 15 mL tubes as 1 and 2. Add 2 mL of 2x HBS (0.05 M HEPES, 0.28 M NaCl, and 1.5 mM Na_2HPO_4 [pH 7.12]) to tube 1. Add TE 79/10 (1 mM Tris-HCl and 0.1 mM EDTA [pH 7.9]) to tube 2. The volume of TE 79/10 is 1.760 mL—the volume of DNA.

2.1.2. Add 20 µg of plasmid containing the Rev-NoLs-3'flag mutation of interest to tube 2 and mix its contents through resuspension. Add 240 µL of 2 M $CaCl_2$ to tube 2 and mix again through resuspension.

2.1.3. Transfer the mixture of tube 2 to tube 1 dropwise, gently mixing. Allow the suspension to sit at room temperature for 30 min. Vortex the precipitation.

2.2. Add 1 mL of the suspension dropwise to each of the 3-cell culture, 100 mm plates while gently swirling the media. Return the plates to the incubator and leave the transfection mixture

for 6 h. Replace the transfection mixture with 10 mL of fresh culture media and incubate the cells for 42 h.

3. Collection of viral protein lysate

3.1. Discard the cell media 48 h posttransfection. Place each 100 mm plate on a bed of ice. Label 15 mL tubes for each Rev-NoLS mutation sample and place the tubes on ice.

3.2. Gently rinse the cells with 10 mL of prechilled 1x PBS without disrupting the cell layer. Discard the 1x PBS. Add 3 mL of lysis buffer (50 mM Tris-HCl [pH 8.0], 137 mM NaCl, and 1% X-100 detergent [see the **Table of Materials**]), treated with protease inhibitor cocktail, to each of the 100 mm plates.

3.3. Use a cell scraper to disrupt the cell layer. Tilt the plate and gently scrape and gather the cells into a pool. Collect the cell lysate using a 1,000 µL micropipette and mix the cell lysates from each of the three 100 mm plates in the prelabeled 15 mL tube.

3.4. Incubate the cell lysate on ice for 15 min, vortexing every 5 min. Centrifuge the cell lysate at 15,000 x *g* for 5 min.

3.5. Collect the protein supernatant without disrupting the cell debris pellet and transfer it to another sterile 15 mL tube. Obtain the viral protein lysate concentration using the Bradford method (see section 4).

3.6. Save an aliquot of the input sample (20 µg) for western immunoblot analysis. Add 2x sample buffer (20% glycerol, 0.02% bromophenol blue, 125 mM Tris-Cl [pH 6.8], 5% SDS, and 10% 2-mercaptoethanol) to the final volume. Boil it at 95 °C for 10 min, and store the input sample at -20 °C.

4. Bradford assay

NOTE: Prepare 10x bovine serum albumin (BSA) from 100x BSA stock before generating protein standard curves.

4.1. Aliquot water into microcentrifuge tubes for the following blank and standards: Blank = 800 µL; Standard 1 = 2 mg/mL, 798 µL; Standard 2 = 4 mg/mL, 796 µL; Standard 3 = 6 mg/mL, 794 µL; Standard 4 = 8 mg/mL, 792 µL; Standard 5 = 10 mg/mL, 790 µL. Aliquot 10x BSA into the following designated standards: Standard 1 = 2 µL; Standard 2 = 4 µL; Standard 3 = 6 µL; Standard 4 = 8 µL; Standard 5 = 10 µL.

4.2. Prepare a mixture of protein samples by mixing 795 µL of water with 5 µL of protein samples. Add 200 µL of protein assay dye reagent (see the **Table of Materials**) to each blank, standard, and protein sample. Vortex the samples briefly for an even mixture and incubate at room temperature (18–20 °C) for 5 min.

4.3. Transfer the blank, standards, and protein samples to cuvettes. Measure the protein concentrations at an OD of 595 nm.

5. Coimmunoprecipitation of Rev-NoLS-3'flag

5.1. Rinse 25 µL of M2 affinity gel beads (see the **Table of Materials**) with 500 µL of lysis buffer, treated with protease inhibitor cocktail. Rinse more affinity gel beads for every mutation sample and controls.

NOTE: Prepare enough M2 affinity gel beads for two gels (50 µL)—one for western immunoblot analysis and the other for protein staining and mass spectrometry.

5.2. Spin at 820 x *g* for 2 min at 4 °C. Remove the supernatant. Rinse 2x more.

5.3. Add viral protein lysate (1 mg/mL in 5 mL of total volume) to the prerinsed M2 affinity beads. Adjust the total volume using lysis buffer.

5.4. Incubate the reaction for 3 h, rotating at 4 °C. Centrifuge the M2 affinity beads/viral protein lysate at 820 x *g* for 1 min.

5.5. Collect the supernatant and save an aliquot of post-IP sample (20 µg) for western immunoblot analysis. Measure the protein concentration of the post-IP lysate. Collect 20 µg for western immunoblotting.

5.6. Add 2x sample buffer to the final volume. Boil it at 95 °C for 10 min, and store the post-IP sample at -20 °C.

5.7. Rinse the M2 beads with 750 µL of lysis buffer and wash the beads on a rotator at 4 °C for 5 min. Centrifuge the M2 beads at 820 x *g* and discard the supernatant.

5.8. Repeat steps 4.7 to 4.8 for two more washes on a rotator at 4 °C for 5 min. After the third wash, remove any traces of lysis buffer from the M2 beads/co-IP complex using a long gel-loading tip.

NOTE: Pinch the end of the gel-loading tip with flat tweezers before removing any trace amounts of the lysis buffer. This will prevent the disruption and uptake of the M2 beads.

5.9. Resuspend the M2 beads in 55 µL of 2x loading buffer. Boil the sample at 95 °C for 10 min.

5.10. Load 25 µL of eluate onto two separate SDS-PAGE gels (one gel for western immunoblotting and the other for Coomassie staining).

6. Preparation of SDS-PAGE gels

6.1. Cast two 15% SDS-acrylamide resolving gels by mixing the following reagents in a 50 mL tube (at a final volume of 40 mL, enough for four gels): 4.16 mL of ultrapure water, 15 mL of 40% acrylamide:bisacrylamide (29:1), 10 mL of 1.5 M Tris-HCl (pH 8.8), 400 µL of 10% SDS, 400 µL of 10% ammonium persulfate, and 40 µL of TEMED.

6.2. Mix the resolving gel by inverting the 50 mL tube several times. Pipette the resolving gel mixture into a precleaned western gel apparatus (four gels—three for western immunoblotting and one for Coomassie/silver staining).

6.3. Gently pipette enough water to cover the top layer of the gel mixture. Allow the resolving gel to polymerize.

6.4. Pour the water layer from the resolving gel, using a delicate task wiper (see the **Table of Materials**) to absorb any excess water.

6.5. Cast two 5% SDS-acrylamide stacking gels by mixing the following reagents in a 50 mL tube (at a final volume of 20 mL, enough for four gels): 11.88 mL of ultrapure water, 2.5 mL of 40% acrylamide:bisacrylamide (29:1), 5.2 mL of 1.5 M Tris-HCl (pH 8.8), 200 µL of 10% SDS, 200 µL of 10% ammonium persulfate, and 20 µL of TEMED.

6.6. Mix the stacking gel by inverting the 50 mL tube several times. Pipette the stacking gel mixture above the resolving gel to the top of the apparatus.

6.7. Place a gel cassette comb containing the appropriate number of lanes into the stacking gel. Absorb any overflow of the gel mixture using a delicate task wiper (see the **Table of Materials**). Allow the stacking gel to polymerize completely.

6.8. Flood the Western gel apparatus with 1x western running buffer (5x concentration: 250 mM Tris-Cl [pH 8.3], 1.92 M glycine, 0.5% SDS, and 10 mM EDTA).

6.9. Gently pull the gel cassette comb from the stacking gel. Allow the 1x western running buffer to fill the loading wells. Flush each well with 1x western running buffer using a syringe prior to loading the samples.

6.10. Load the western immunoblot samples into each corresponding gel (input samples, coimmunoprecipitated samples, and post-IP samples). Load the western gel protein markers.

6.11. Load the coimmunoprecipitated samples for the Coomassie/silver staining into another gel. Load the western gel protein markers.

6.12. Connect the running gel apparatus to a power source and run the gels at 100 V until the loading dye reaches the resolving gel. Increase the voltage to 140 V until the loading dye reaches the bottom of the resolving gel.

7. Western blot transfer

7.1. Disassemble the western gel apparatus. Slice and discard the stacking gel, leaving the resolving gel intact.

7.2. Gently transfer the resolving gels to a clean tray filled with western transfer buffer (25 mM Tris, 194 mM glycine, 0.005% SDS, 20% methanol) and soak them for 15 min.

7.3. Assemble the gel transfer apparatus as follows.

7.3.1. Cut three PVDF transfer membranes and six pieces of filter paper (see the **Table of Materials**) to the size of the resolving gel.

7.3.2. Soak the PVDF membrane in methanol for 5 min. Hydrate it in water for 5 min. Place the PVDF membrane in the western transfer buffer until ready to use.

7.3.3. Place the gel holder cassette in a glass baking tray filled partially with western transfer buffer, with the black side at the bottom.

7.3.4. Place a foam pad soaked with western transfer buffer against the black side of the gel holder cassette.

7.3.5. Wet a piece of filter paper in western transfer buffer and place it on top of the foam pad. Place the resolving gel on top of the filter paper.

NOTE: Place the resolving gel in the correct loading orientation to be transferred to the PVDF membrane.

7.3.6. Place one PVDF transfer membrane on top of the resolving gel. Wet a piece of filter paper with western transfer buffer and place it on top of the PVDF transfer membrane.

7.3.7. Place another foam pad soaked with western transfer buffer on top of the filter paper. Carefully fold the white side of the gel holder cassette on top of the soaked foam pad. Lock the cassette tightly.

7.3.8. Place the gel holder cassette into the transfer apparatus electrode assembly. Repeat steps 7.3.4–7.3.8 for each remaining resolving gel.

7.3.9. Fill the transfer apparatus tank with western transfer buffer. Place a stirring rod into the apparatus tank.

7.3.10. Place the apparatus tank on top of a stir plate. Adjust the stir setting to 5–6, making sure that the stir bar is not stuck or hitting the gel holder cassettes.

7.3.11. Connect the gel transfer apparatus to a power source and transfer the gel at 100 V for 1 h at 4 °C.

8. Immunoblotting

8.1. Remove the gel holder cassette and place the black side down against a clean glass baking tray. Open the cassette and carefully discard the foam pad and filter paper. Mark a corner of the PVDF membrane to identify the correct loading orientation. Keep the membrane wet.

NOTE: The PVDF membrane can be air-dried and stored in a clean, sealed container. Rehydrate the membrane by repeating steps 7.3.2.

8.2. Place the membrane in 100 mL of blocking solution (5% milk, 1x TBS, and 0.1% Tween 20). Block the membrane by gentle rocking at room temperature (18–20 °C) for 1 h.

8.3. Cut across the membrane above the 25 kDa protein marker. Place the top portion of the membrane, containing protein bands larger than 25 kDa, in blocking solution containing B23 mouse monoclonal IgG_1 (1:500 dilution). Block overnight, rocking at 4 °C.

8.4. Place the bottom portion of the membrane, containing protein bands smaller than 25 kDa, in blocking solution containing M2 mouse monoclonal IgG_1 (1:1,000 dilution, see the **Table of Materials**). Block overnight, rocking at 4 °C.

8.5. Wash the membrane 3x for 10 min in 25 mL of western wash solution (1x TBS, 0.1% Tween 20) on a rocking platform.

8.6. Incubate the membranes in goat-anti-mouse IgG_1-HRP (1:5,000 dilution) diluted in blocking solution for 1 h at room temperature. Wash the membrane 3x for 10 min in 25 mL of western wash solution on a rocking platform.

8.7. Prepare chemiluminescence western blotting substrate. Use a p1000 micropipette to add the substrate to the membrane.

8.8. Develop each membrane in chemiluminescence western blotting substrate for 5 min. Remove the membrane from the substrate. Absorb excess substrate using a delicate task wiper (see the **Table of Materials**).

8.9. Place the membrane into a clean sheet protector taped to the inside of a cassette. Take the cassette into a dark room and place one sheet of film into the cassette. Lock the cassette in place and incubate for 5–15 min. Remove the film from the cassette and develop it.

9. Coomassie staining

9.1. Disassemble the western gel apparatus. Slice and discard the stacking gel, leaving the resolving gel intact. Gently transfer the resolving gel to a clean tray filled with 25 mL of ultrapure water.

9.2. Incubate the gel on a rocking platform for 15 min. Use gentle rocking to prevent the resolving gel from breaking. Discard the ultrapure water and repeat the washing step 2x more.

NOTE: If SDS bubbles remain after the washing steps, the gel can be washed in ultrapure water overnight. Residual SDS can cause high background staining of the gel.

9.3. Mix the Coomassie stain reagent by inverting the bottle (see the **Table of Materials**). Place 100 mL of Coomassie stain reagent to cover the resolving gel and incubate the gel on a rocking platform for 1 h. Discard the Coomassie stain reagent and wash the gel in deionized water on a rocking platform for 15 min.

9.4. Discard the deionized water. Repeat the washing step 2x more. Continue to wash the gel until the desired resolution of protein bands is observed.

10. Silver staining

10.1. Disassemble the western gel apparatus. Slice and discard the stacking gel, leaving the resolving gel intact. Gently transfer the resolving gel to a clean tray filled with 25 mL of ultrapure water.

10.2. Incubate the gel on a rocking platform for 15 min. Use gentle rocking to prevent the resolving gel from breaking. Discard the ultrapure water and repeat the washing step 2x more.

NOTE: If SDS bubbles remain after the washing steps, the gel can be washed in ultrapure water overnight. Residual SDS can cause high background staining of the gel.

10.3. Fix the gel in 30% ethanol:10% acetic acid solution (6:3:1 water:ethanol:acetic acid) overnight at room temperature. Wash the gel in a 10% ethanol solution for 5 min at room temperature. Replace the ethanol solution and wash for another 5 min.

10.4. Prepare sensitizer working solution from the Pierce Silver Stain Kit by mixing one-part silver stain sensitizer with 500 parts ultrapure water (50 µL of sensitizer with 25 mL of ultrapure water). Incubate the resolving gel in the sensitizer working solution for 1 min. Wash the gel in ultrapure water for 1 min, replace the water, and wash the gel again for 1 min.

10.5. Prepare stain working solution by mixing one-part silver stain enhancer with 50 parts silver stain (500 µL of enhancer with 25 mL of silver stain). Incubate the gel in stain working solution for 30 min.

10.6. Prepare developer working solution by mixing 1 part silver stain enhancer with 50 parts silver stain developer (500 µL of enhancer with 25 mL of developer). Prepare 5% acetic acid solution as stop solution. Wash the gel with ultrapure water for 1 min, replace the water, and wash the gel for an additional 1 min.

10.7. Replace the water with developer working solution and incubate until the desired protein band intensity is resolved (5 min). Replace the developer working solution with stop solution and incubate for 10 min.

11. In-gel reduction, alkylation, and digestion of Coomassie-stained gel bands

11.1. Cut the gel bands from the gel using a clean razor blade. Cut each gel band into approximately 5 mm cubes and place them in a clean 0.5 mL microcentrifuge tube.

11.2. Destain the gel pieces by covering them with 100 mM ammonium bicarbonate in 1:1 acetonitrile:water at room temperature for 15 min. Discard the supernatant. Repeat this step.

11.3. Dry the gel pieces for 5 min in a vacuum centrifuge. Reduce the proteins by covering the dried gel pieces with 10 mM dithiothreitol in 100 mM ammonium bicarbonate and incubating them for 1 h at 56 °C.

11.4. Pipette off any supernatant. Alkylate the proteins by covering the gel pieces with 100 mM iodoacetamide in water and incubating them for 1 h at room temperature in the dark.

11.5. Pipette off the supernatant and shrink the gel pieces by covering them with acetonitrile and shaking them gently at room temperature for 15 min. Pipette off the supernatant and reswell the gel pieces by covering them with 100 mM ammonium bicarbonate and shaking them gently at room temperature for 15 min.

11.6. Repeat step 11.5. Dry the gel pieces for 5 min in a vacuum centrifuge.

11.7. Cover the gel pieces with 50 ng/µL sequencing grade modified trypsin (see the **Table of Materials**) in 100 mM ammonium bicarbonate. Allow the gel to swell for 5 min; then, pipette off any remaining solution. Cover the gel pieces with 100 mM ammonium bicarbonate and allow them to reswell completely, adding additional 100 mM ammonium bicarbonate so the gel pieces are completely covered.

11.8. Incubate the gel pieces overnight at 37 °C. Stop the reaction by adding 1/10 of the volume of 10% formic acid in water. Collect the supernatant from each tube.

11.9. Extract the gel pieces by covering them with 1% formic acid in 60% acetonitrile and incubating them for 15 min with gentle shaking.

11.10. Reduce the volume of the combined supernatants to less than 20 µL in a vacuum centrifuge, while taking care to avoid drying the supernatants completely. Add 1% formic acid to bring the total volume back to 20 µL.

12. Liquid chromatography/mass spectrometry

NOTE: The samples were analyzed using a mass spectrometer equipped with ultra HPLC, a nanospray source, and a column (see the **Table of Materials**). Solvents A and B are 0.1% formic acid in water and acetonitrile, respectively.

12.1. Load the digested proteins into high recovery polypropylene autosampler vials. Load the vials into the sample manager of a UPLC system.

12.2. Inject 6 µL of each sample. Load each sample onto the trapping column of the nanotile for 1.5 min at 8 µL/min, using 99% solvent A/1% solvent B.

12.3. Elute the peptides into the mass spectrometer with a linear gradient from 3% to 35% of solvent B over 30 min, followed by a gradient from 35% to 50% of solvent B over 4 min and 50% to 90% of solvent B over 1 min. Maintain 90% acetonitrile for 3 min; then reduce the %B back to 3% over 5 min.

12.4. Acquire positive ion profile mass spec data in resolution (20,000 resolution) mode. Acquire data from 100 to 2,000 Da at a rate of one scan every 0.6 s. Acquire data in MS^E mode by alternating scans with no collision energy and scans with elevated collision energy.

12.5. For the elevated collision energy, ramp the collision energy in the Trap cell from 15 V to 40 V. Acquire a lock mass scan every 30 s, using the +2 ion of [Glu_1]-Fibrinopeptide B as the lock mass. Acquire a data file using a blank injection of solvent A, using the same acquisition method between each pair of samples to control the carryover.

13. Data analysis for mass spectrometry

13.1. Copy the mass spectrometry results files to the computer running a quantitative and qualitative proteomics research platform (e.g., ProteinLynx Global Server). Data analysis is highly CPU-intensive and should be performed on a separate, high-performance data analysis computer.

13.2. Create a new project for the data. Create a new microtiter plate representing the autosampler plate. Assign the samples to the same position in the microtiter plate as their position in the autosampler.

13.3. Assign each sample processing parameters. Parameters to use are automatic chromatographic peak width and MSTOF resolution; low-energy threshold, 100 counts; elevated-energy threshold, 5 counts; intensity threshold, 500 counts.

13.4. Assign each sample workflow parameters. Parameters to use are database, concatenated human SwissProt and HIV, with reversed sequences; automatic peptide and fragment tolerance; min fragment ion matches per peptide, 3; min fragment ion matches per protein, 7; min peptide matches per protein, 1; primary digest reagent, trypsin; missed cleavages, 1; fixed modifier reagents, carbamidomethyl C; variable modifier reagents, oxidation M; false discovery rate, 100.

13.5. Select the samples and choose **Process Latest Raw Data**. When the search completes, select the samples and choose **Export Data to Scaffold** (version 3). Open **Scaffold**, create a new file, and import each file exported from the proteomics platform as a new biosample using precursor ion quantitation.

13.6. When all files have been imported, proceed to the **Load and Analyze Data** screen. Select the same database used for the search and import data using LFDR scoring and standard experiment-wide protein grouping. Set display options to **Protein Identification Probability**, the protein threshold to 20%, the minimum number of peptides to 1, and the peptide threshold to 0% during the analysis.

REPRESENTATIVE RESULTS:

Rev-NoLS single- and multiple-point arginine mutations, corresponding to a variety of subcellular localization patterns, were examined in their ability to interact with cellular host factors in comparison to WT Rev. WT Rev-3'flag and p*cDNA*-flag vector were expressed in HLfB culture. Protein complexes were processed from total cell lysate and stained with silver stain reagent. Rev-NoLS-3'flag is detectable (approximately 18 kDa) in three different lysis buffer conditions containing various concentrations of NaCl (137 mM, 200 mM, and 300 mM) in **Figure 1**. B23 was detectable (37 kDa) in lysis buffer containing lower salt concentration (137 mM, lanes 2–4), barely detectable in lysis buffer containing 200 mM NaCl, and undetectable in lysis buffer containing a high salt concentration (300 mM NaCl). In **Figure 2**, WT Rev-3'flag, Rev-NoLS M1-3'flag, and p*cDNA*-flag vector were expressed in HLfB culture. Protein complexes were processed from total cell lysates prepared from two lysis buffer conditions (137 mM and 200 mM NaCl). M2 mouse monoclonal IgG_1 was used in the detection of Rev-3'flag expression from cell lysates. B23 detection was optimal in lysis buffer containing 137 mM NaCl with WT Rev-3'flag (input and α-flag-Rev IP) but lost affinity with Rev-NoLS M1-3'flag. B23 affinity with WT Rev-3'flag decreased with a higher salt concentration in lysis buffer containing 200 mM NaCl. p*cDNA* negative control did not yield nonspecific immunodetection in input and α-flag-Rev IP of all lysis buffer conditions.

Elution conditions were optimized for Rev-NoLS-3'flag IP in **Figure 3**. WT Rev-3'flag and p*cDNA*-flag vector were expressed in HLfB culture. Protein complexes were processed from total cell lysates and eluted using three different conditions to eradicate light and heavy chain background (25 and 50 kDa)—2x sample loading buffer at 37 °C for 15 min, 2x sample loading buffer at 95 °C for 3 min, and 3x flag peptide at 4 °C for 30 min. Rev NoLS-3'flag (~18 kDa) was most detectable after elution through boiling in 2x sample loading buffer. B23 (~37 kDa) was detectable under two conditions—37 °C incubation in 2x sample loading buffer for 15 min and 95 °C incubation in 2x sample loading buffer for 3 min.

M2 (nuclear/nucleolar in localization, nonfunctional in HIV-1_{HXB2} production), M6 (nucleolar in pattern, functional in HIV-1_{HXB2} production), and M9 (dispersed in the cytoplasm/nucleus, nonfunctional in viral production) were expressed in HLfB culture. Protein complexes were processed from total cell lysate, eluted through 95 °C incubation in 2x sample loading buffer for 3 min, and resolved in silver stain reagent (**Figure 4**). WT Rev was detectable after IP flag reaction. Rev-NoLS M2, M6, and M9 were also detectable at 18 kDa. Bands corresponding to B23 protein were observed at the 37 kDa marker. Protein complexes were further observed in each lane corresponding to IP reactions of WT Rev, M2, M6, M9, and p*cDNA* negative background control. Protein lysates were analyzed by immunodetection for α-flag-Rev and B23. Abundant WT Rev and moderate levels of M6 were expressed (α-flag input) and detectable after flag IP (α-flag-Rev IP, **Figure 5**). M2 and M9 were not highly expressed from 20 μg of protein lysate input but detectable at low intensity after flag IP from 5 mg of protein lysate. p*cDNA* negative control did not yield nonspecific immunodetection in input and α-flag-Rev IP. B23 affinity with WT Rev was observed after IP flag reaction (B23 co-IP). B23 affinity was slightly observed with M2 (two single-point mutations R48,50G) and M6 (single-point mutation R50G). B23 affinity was lost in the presence of three single-point mutations of M9 (R46,48,50G) within Rev-NoLS.

Total lysates from immunoprecipitated WT Rev and Rev-NoLS-3'flag mutations (4, 5, 6, and 8) were processed, stained with Coomassie reagent, and visualized in comparison to BSA serial dilutions (right panel). Rev-NoLS-3'flag is detectable (12.5–25 μg) in the presence and absence of mutations at 18 kDa (**Figure 6**). Protein complexes processed from IP reactions of WT Rev-3'flag, nucleolar-localizing Rev-NoLS-3'flag mutations (M4, M5, and M6), and negative control p*cDNA*-flag were visualized in SDS-PAGE gels stained with Coomassie reagent (**Figure 7**). WT Rev (WT1 and WT2) was detectable after IP flag reaction at 18 kDa. Rev-NoLS mutations were slightly detectable after expression in HLfB and IP flag reaction. p*cDNA* negative control did not yield nonspecific background at 18 kDa.

Immunoprecipitated lysates prepared from WT Rev-3'flag, M2, M6, M9, and negative control p*cDNA*-flag (shown in **Figure 4**) were analyzed by tandem mass spectrometry. Protein identification probability (in percentages) is displayed for comparison of protein interactions occurring with each nucleolar-localizing Rev-NoLS mutation versus WT Rev (**Table 1**). Cellular proteins, some of which are nucleolar in localization pattern (ribosomal isoforms, eukaryotic translation initiation factor 48, snoRNA C/D box 58B, and nucleophosmin B23), were identified as direct/indirect binding partners of WT Rev. These nucleolar factors lost binding affinity to M2 (two single-point mutations R48,50G), M6 (single-point mutation R50G), and M9 (three single-point mutation R46,48,50G), similar to p*cDNA* negative control. Each lane of the Coomassie-stained gel in **Figure 6** was processed for tandem mass spectrometry (**Table 2**). Peptide affinity to Rev-NoLS mutations was analyzed and displayed using protein identification probability (in percentages). A variety of cellular proteins, some of which are nucleolar in localization pattern (nucleolin C23, nucleophosmin B23, and nucleosome assembly protein), were identified as direct/indirect protein binding factors of WT Rev. These nucleolar factors were not identified to bind with M4, M5, and M6. Transport factors ARHGEF1 (rho guanine nucleotide exchange factor 1) and TBC1D24 (TCB1 domain family, member 24) were lost in affinity in the presence of Rev-

NoLS mutations. Splicing factors hnRNPC (heterogeneous ribonuclear protein C) and PNN (pinin, desmosome-associated protein) were additionally observed to bind to WT Rev, which lost interaction with Rev single-point nucleolar mutations.

FIGURE AND TABLE LEGENDS:

Figure 1: Optimization of cell lysis conditions for Rev-3'flag co-IP during HIV-1 production. WT Rev-NoLS-3'flag and negative control p*cDNA*-flag IP conditions were optimized in three different NaCl concentrations (137 mM, 200 mM, and 300 mM) in lysis buffer. As observed through silver staining, 137 mM NaCl was the optimal salt concentration for Rev immunoprecipitation and B23 binding.

Figure 2: Optimization of lysis conditions for Rev-3'flag co-IP and B23 immunodetection during HIV-1 production. WT Rev-NoLS-3'flag, M1-3'flag, and negative control p*cDNA*-flag IP conditions were optimized in two different NaCl concentrations (137 mM and 200 mM) in lysis buffer. As observed through immunodetection, 137 mM NaCl was the optimal salt concentration for Rev immunoprecipitation and B23 binding.

Figure 3: Optimization of Rev-3'flag co-IP elution during HIV-1 production, visualized by silver staining. Protein complexes were processed from total cell lysates prepared during WT Rev-3'flag and p*cDNA*-flag IP. Three different elution conditions were tested—2x sample loading buffer at 37 °C for 15 min, 2x sample loading buffer at 95 °C for 3 min, and 3x flag peptide at 4 °C for 30 min. The optimal elution conditions of WT Rev occurred after the 15 min incubation period in 2x sample loading buffer at 37 °C and after the 3 min incubation period in 2x sample loading buffer at 95 °C.

Figure 4: Immunoprecipitation of Rev-3'flag mutations 2, 6, and 9 during HIV-1 production. Protein complexes that bound directly/indirectly with WT Rev, M2 (R48,50G), M6 (R50G), M9 (R46,48,50G), and negative control p*cDNA*-flag in the presence of HIV-1 replication are shown in a silver-stained SDS-PAGE gel.

Figure 5: Rev-3'flag co-IP of mutations 2, 6, and 9 for B23 immunodetection during HIV-1 production. The protein lysates and IP reactions prepared from WT Rev, M2, M6, M9, and negative control p*cDNA*-flag in **Figure 4** were further analyzed by immunodetection for α-flag-Rev and B23. WT Rev and mutant expression, as well as interaction with B23 nucleocytoplasmic protein, are observed.

Figure 6: Quantification of Rev-NoLS mutations 4, 5, 6, and 8 after flag IP reaction. IP reactions from HLfB expressing WT Rev and nucleolar-localizing M4 (R46G), M5 (R48G), M6 (R50G), M8 (ΔRQ), and negative control p*cDNA*-flag were measured for protein concentration using BSA serial dilutions. A protein concentration of 12.5–25 μg was observed for each sample.

Figure 7: Immunoprecipitation of nucleolar-localizing Rev-NoLS mutations 4, 5, and 6 during HIV-1 production. Coomassie-stained SDS-PAGE gel containing immunoprecipitated protein

complexes of WT Rev (1 and 2), M4, M5, and M6 are shown.

Table 1: Identification of cellular host factors that interact with WT Rev during HIV-1 production. Protein eluates prepared from WT Rev, M2, M6, and M9 were directly analyzed by tandem mass spectrometry. Protein interactions that are directly/indirectly bound to WT Rev versus M2, M6, and M9 are summarized by protein identification probability (in percentages).

Table 2: Identification of cellular host factors complexed with nucleolar-localizing Rev mutations 4, 5, and 6 during HIV-1 production. The Coomassie-stained SDS-PAGE gel in **Figure 6** was processed for tandem mass spectrometry. Protein interaction results of WT Rev versus nucleolar mutations M4, M5, and M6 are summarized by protein identification probability (in percentages).

DISCUSSION:

Mass spectrometric analyses comparing Rev-NoLS mutations and WT Rev in the presence of HIV-1 were assessed to understand nucleolar factors involved in the viral replication cycle. This would identify nucleolar components required for viral infectivity. Nucleolar B23 has a high affinity to Rev-NoLS and functions in the nucleolar localization of Rev[3] and nucleocytoplasmic transport of Rev-bound HIV mRNAs[22]. The affinity of B23 with Rev-NoLS mutations, which contained single or multiple arginine substitutions, was assessed through immunoprecipitation of Rev-3'flag during viral production (**Figure 2**; WT and mutations M2, M6, and M9). B23 affinity to single-point Rev-NoLS mutations M4, M5, and M6 were previously examined in the presence of HIV-1 replication. In the previous study, IP eluates of M4, M5, and M6 were subjected to western immunoblotting with an antibody specific to α-flag for Rev and B23 affinity[1]. In the background of Rev single-point mutations, B23 binding affinity was significantly reduced. B23 maintained affinity with WT Rev during HIV replication. Single-point mutations induced within Rev-NoLS were expected to decrease binding affinity to other cellular host factors facilitating HIV mRNA binding and transport. Rev-NoLS single- and multiple-point mutations in this model abolished nucleophosmin B23 affinity to Rev, indicating a disruption in nucleocytoplasmic shuttling and HIV mRNA transport. It is likely that nucleolar factors (nucleolin C23 and nucleosome assembly protein 1), transport factors (ARHGEF1 and TCB1D24), and splicing factors (hnRNPC and PNN) are involved in the HIV-1 infectious cycle through interaction with Rev protein complexes. **Table 1** and **Table 2** reveal various other cellular factors, both nucleolar and nonnucleolar in pattern, that are potentially involved in the HIV-1 replication cycle through Rev. The nucleolar factor—snoRNA C/D box 58, implicated in snoRNA processing, snoRNA transport to the nucleolus, and 2'-O-methylation of ribosomal RNA—was identified yet the function of this protein in the HIV-1 replication cycle remains unknown. The specific roles of these cellular factors in the HIV-1 infectious cycle are currently being investigated.

The results presented here demonstrate the use of this approach for the identification of viral/host nucleolar factors that maintain the HIV-1 infectious cycle. Viral/host nucleolar factors that participate in other disease models could be identified using this approach. It is further likely that one nucleolar factor could involve multifunctional roles in various disease models. For example, B23 has been implicated in the transport of nucleolar viral proteins, viral assembly,

encapsidation, replication, and latency in other viral infectious models. B23 is characterized in interactions with NoLS of cellular factors for nucleocytoplasmic transport—p120 growth factor (amino acids 40–57)[23] and C23 pre-rRNA processor (amino acids 540–628)[24]. B23 is also documented to interact with the human T-cell lymphotropic virus (HTLV-1) protein Rex (amino acids 1–22)[25], HIV-1 Tat (amino acids 49–57)[2], and HIV-1 Rev (amino acids 37–47)[26]. The Japanese encephalitis virus (JEV) genome encodes a nucleolar-localizing core protein, through which amino acids Gly42 and Pro43 interact with the N-terminal region of B23 during JEV infection, resulting in the transportation of viral core protein/B23 into the nucleus[27]. The single-stranded RNA genome of the Hepatitis B virus (HBV) is composed partially of double-stranded DNA, which encodes a nucleolar core protein. The HBV core protein associates with nucleolin and B23 in the nucleolus[28]; B23 was demonstrated in the HBV assembly through interaction with the core protein N-terminal domain. Specifically, B23 amino acids 259–294 bind to the N-terminal domain of the HBV core protein to allow viral encapsidation[29]. The negative-sense, single-stranded RNA hepatitis D virus (HDV) expresses HDVAg antigen in two isoforms; the small isoform aids in RNA replication, and the large isoform facilitates viral assembly. RNA replication takes place within the nucleolus and requires B23 interaction with HDVAg[30,31]. HDV infection causes an upregulation of B23, which interacts mostly with the small HDVAg isoform and less with the large HDVAg isoform. Interactions take place through the small HDVAg NLS domain, through which B23 binds and achieves nuclear accumulation. Upon deletion of the HDV binding site to B23, RNA replication was impaired. HDVAg was shown to colocalize with B23 and nucleolin in the nucleolus. Nucleolin was discovered to possess transcriptional properties as a repressor[32], revealing the nucleolus as a compartment for regulation of HDV replication. B23 is also involved in the latency of the Kaposi's sarcoma-associated herpesvirus (KSHV) genome. KSHV latent protein—v-cyclin—with host CDK6 kinase, phosphorylates B23 at Thr199, facilitating B23 interaction with latency-associated nuclear antigen[33]. The latency-associated nuclear antigen acts to prevent viral lytic replication. Depletion of B23 leads to KSHV reactivation, revealing B23 as a regulator of KSHV latency. B23 function in the HIV-1 replication cycle is characterized in the nucleocytoplasmic transport activity of Tat and Rev, and it is unknown if B23 can induce latency during HIV infection. B23's involvement in the replication, encapsidation, and assembly of HIV-1 is currently unknown.

Adaptation of this method to other disease models would require much effort and time for the generation of protein-deficient infectious backbones expressed in the appropriate cell lines. The advantage of this Rev-deficient HIV-1_{HXB2} backbone is the ability to examine Rev-NoLS mutations in the presence of the full viral backbone, viral infectious factors, and host factors involved in the HIV-1 replication cycle. Other studies have examined Rev nucleolar function in the absence of the full HIV-1 infectious system. Thorough characterization of infectious and disease pathways must include representative environments that support the natural course of disease progression. Two different types of analyses were conducted and compared in the ability to identify nucleolar factors. **Table 1** lists factors that remained bound to WT Rev as a result of direct mass spectrometry analysis of protein eluate. This analysis yielded several known factors of HIV-1 Rev. This direct method was compared to another process involving the extraction of protein separated within SDS-PAGE gels and mass spectrometry analysis of such proteins. This second method yielded a variety of known and potential factors that are bound to the Rev protein complex but lacked the following proteins previously identified in **Table 1**: signal recognition

particle 14 kDa, eukaryotic translation initiation factor 4B, ribosomal protein L22, small nucleolar RNA C/D box 58B, and zinc finger CCHC domain containing 11. Ultimately, the superior method chosen for mass spectrometry analyses in this protocol involved the extraction of peptides from SDS-PAGE gels. The first direct method included 2x sample buffer in the eluate without bromophenol blue; the remaining components of the 2x sample buffer could interfere with complete trypsin treatment and could yield incompletely processed peptides for mass spectrometry analysis. The second indirect method was able to purify trypsin-treated peptides from potential contaminants of SDS-PAGE.

The mass spectrometry preparation methods described here could be utilized for the identification of therapeutic interventions to eradicate HIV-1 infection through targeting Rev. All deletion and single-point Rev-NoLS mutations could be examined for dominant-negative activity and utilized in the arrest of Rev function. Dominant negative characteristics of interest for Rev functional arrest are the following: Rev/RRE binding affinity; relocalization of nucleolar WT Rev through multimerization with Rev mutants; loss in affinity to key cellular factors involved in HIV-1 mRNA transport and splicing. Rev multimerization involving the coexpression of Rev-NoLS mutations with WT Rev could be examined. Dominant negative mutations in this model are expected to multimerize with WT Rev and shift nucleolar patterns toward the nucleus and cytoplasm, leading to a Rev functional arrest. Mass spectrometry could be used to identify the loss of key cellular factors involved in HIV-1 mRNA splicing and transport. The identification of missing interactions with WT Rev as a result of coexpression with dominant-negative Rev-NoLS mutations would reveal the involvement of nucleolar-specific pathways in HIV-1 pathogenesis. Alternatively, viral HIV-1_{NL4-3} particles generated in the background of Rev-NoLS mutations could be investigated for all packaged cellular factors. Cellular and viral factors packaged within viral particles may be further identified through mass spectrometry. This would reveal the presence of nucleolar factors within viral particles and the role of identified nucleolar factors in viral infection. The methods described are applicable to other viral and disease models for the identification and characterization of understudied pathways. This would allow the development of therapeutic interventions against diseases by which limited treatments are available.

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DISCLOSURES:

The authors have nothing to disclose.

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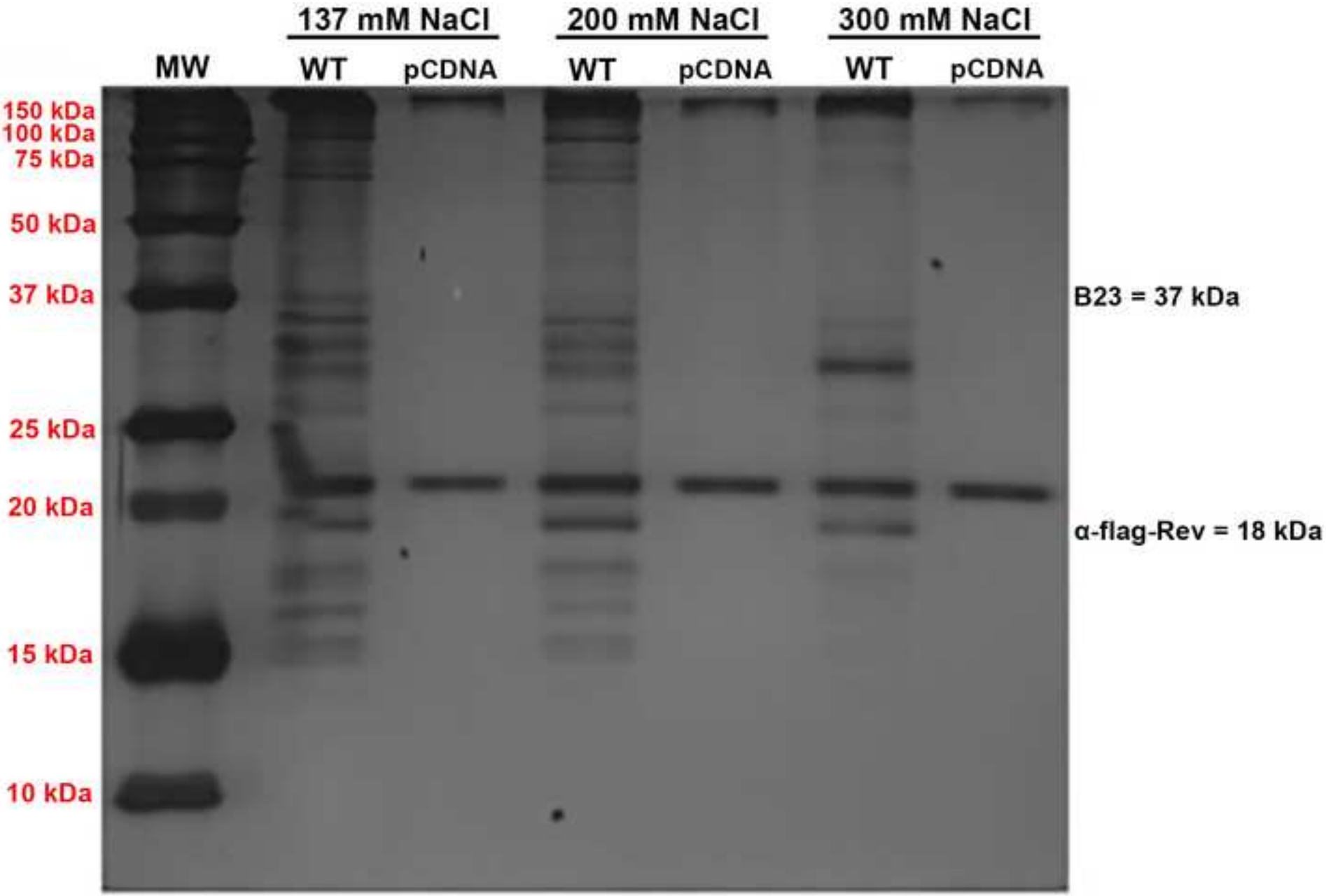
Figure 1



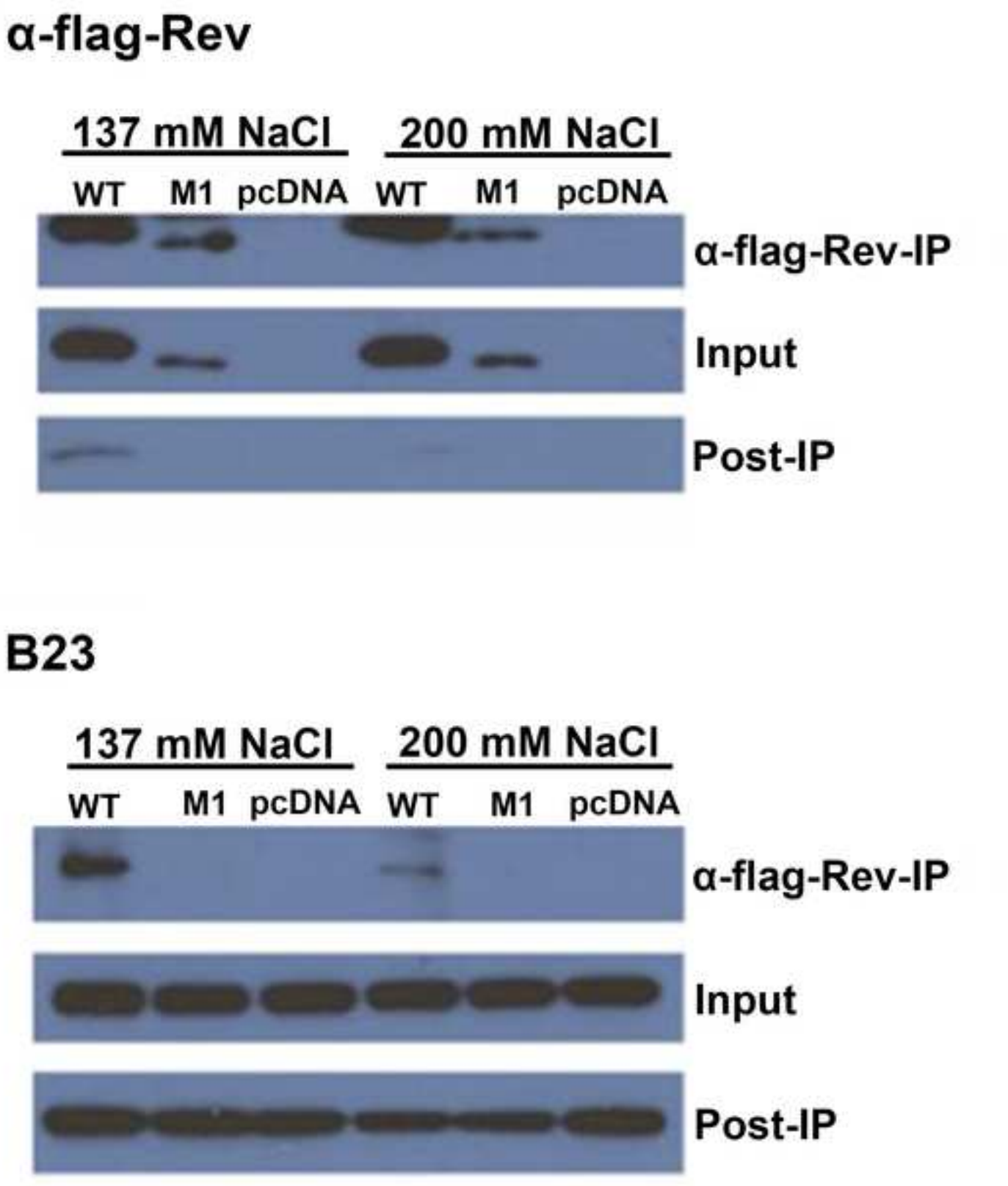


Figure 3

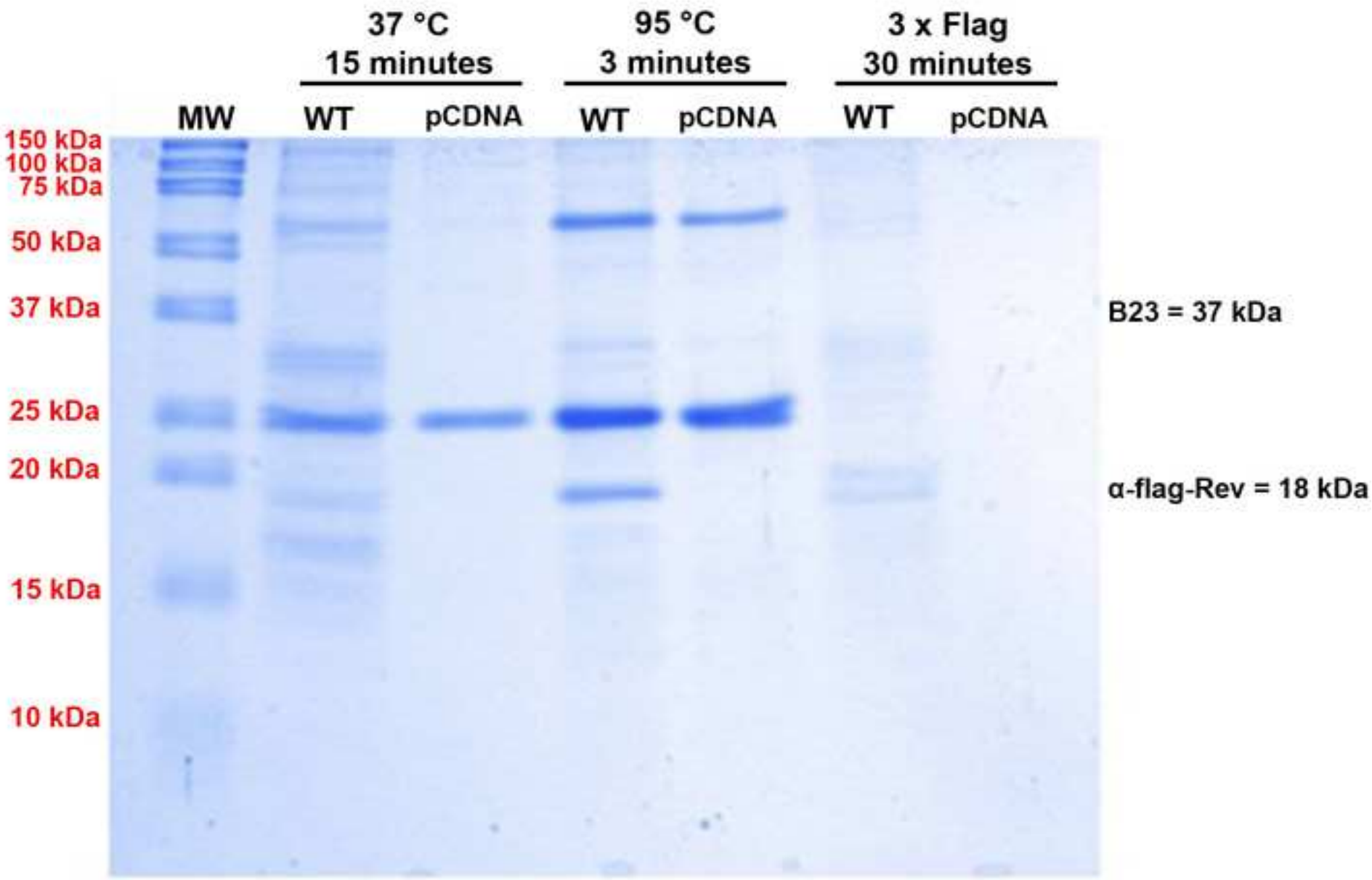


Figure 4

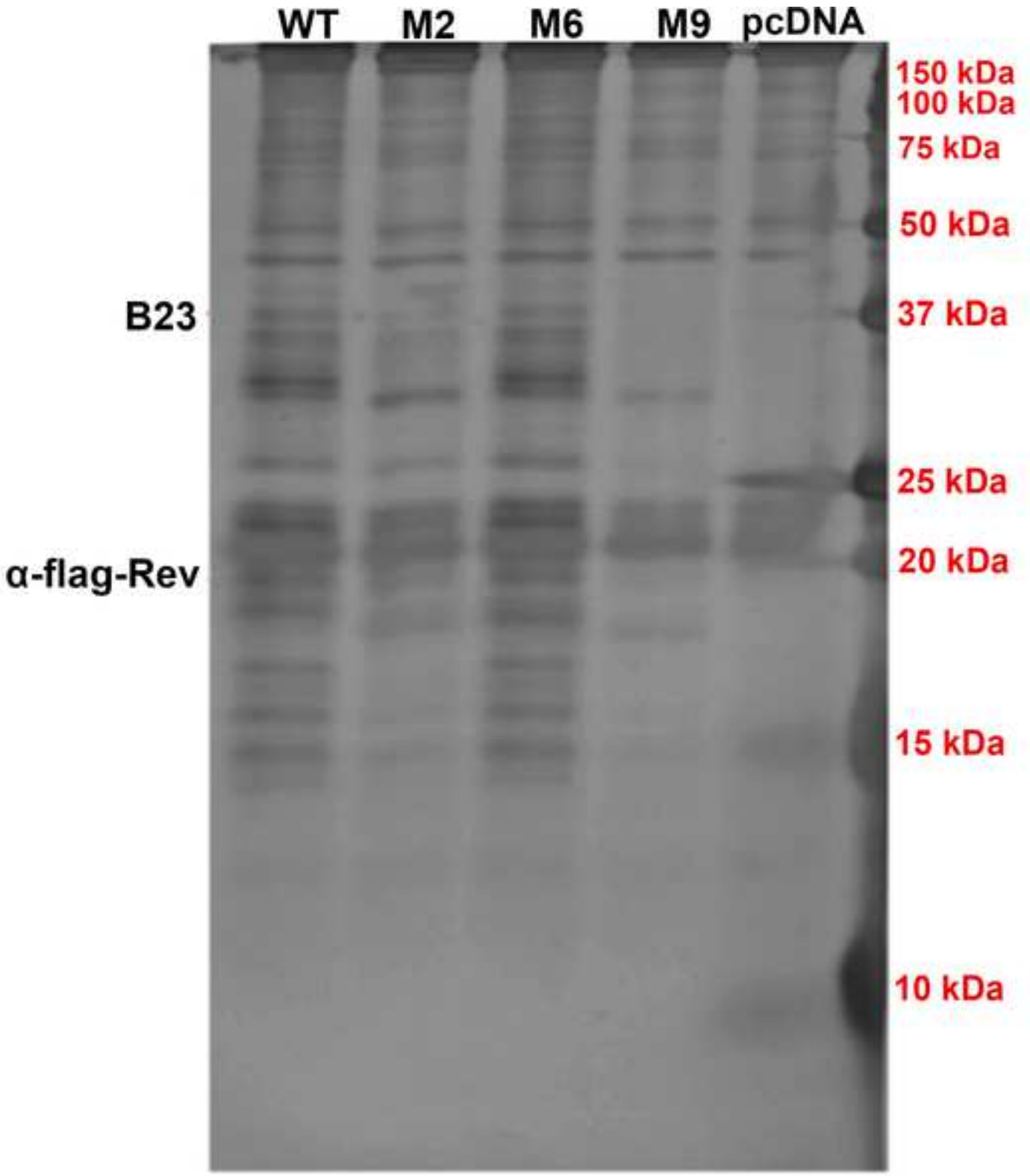


Figure 5

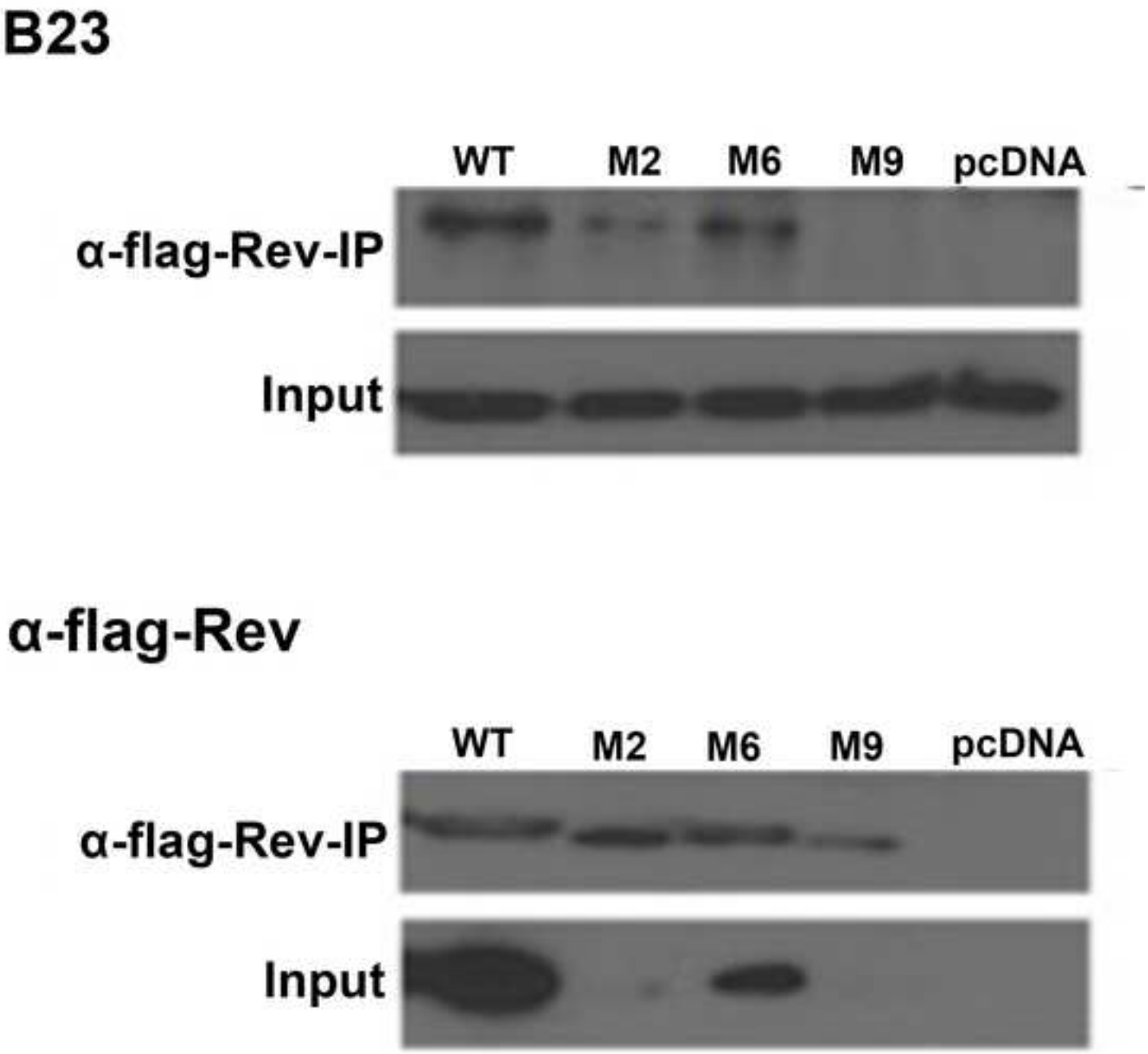


Figure 6

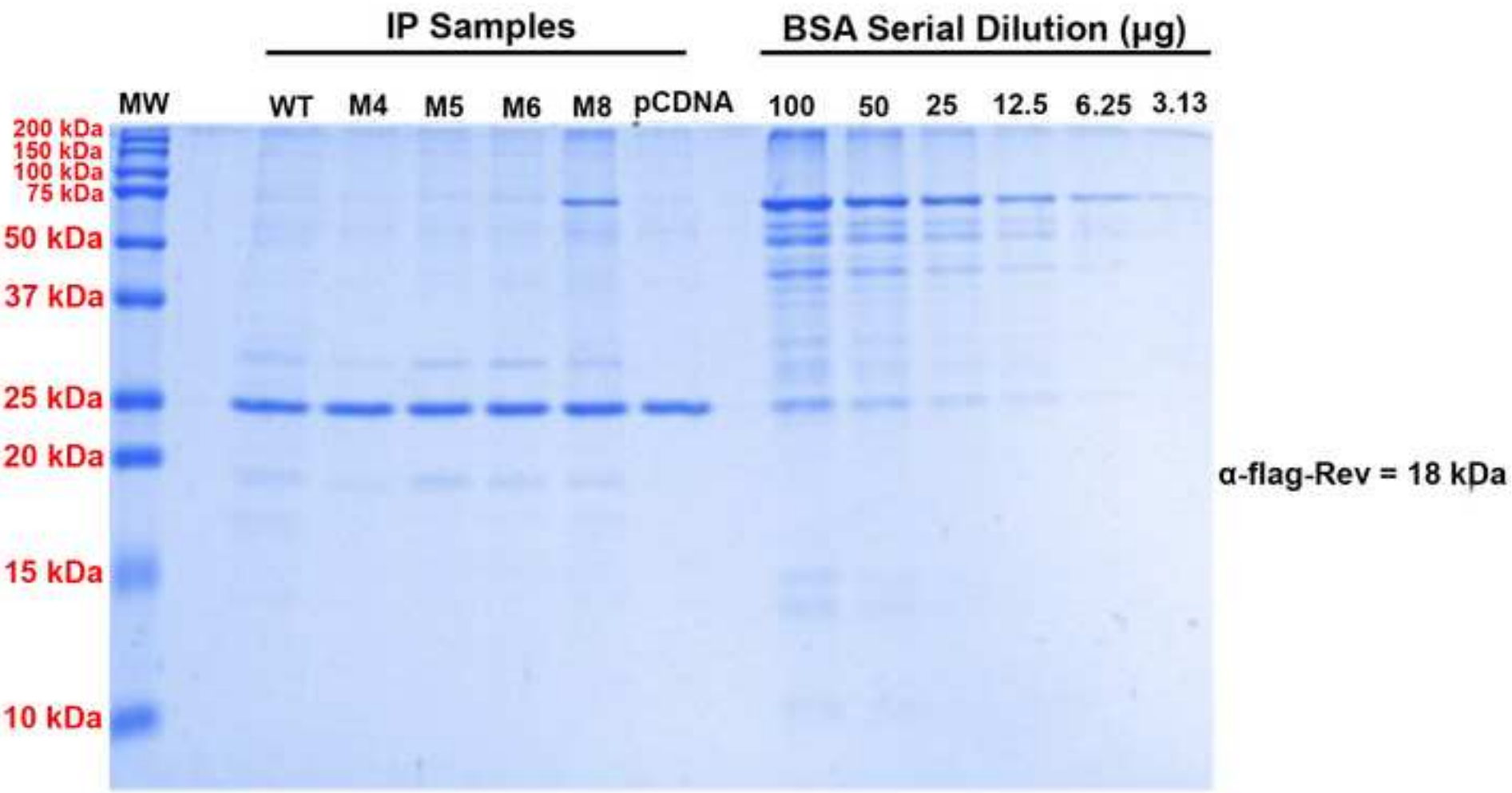
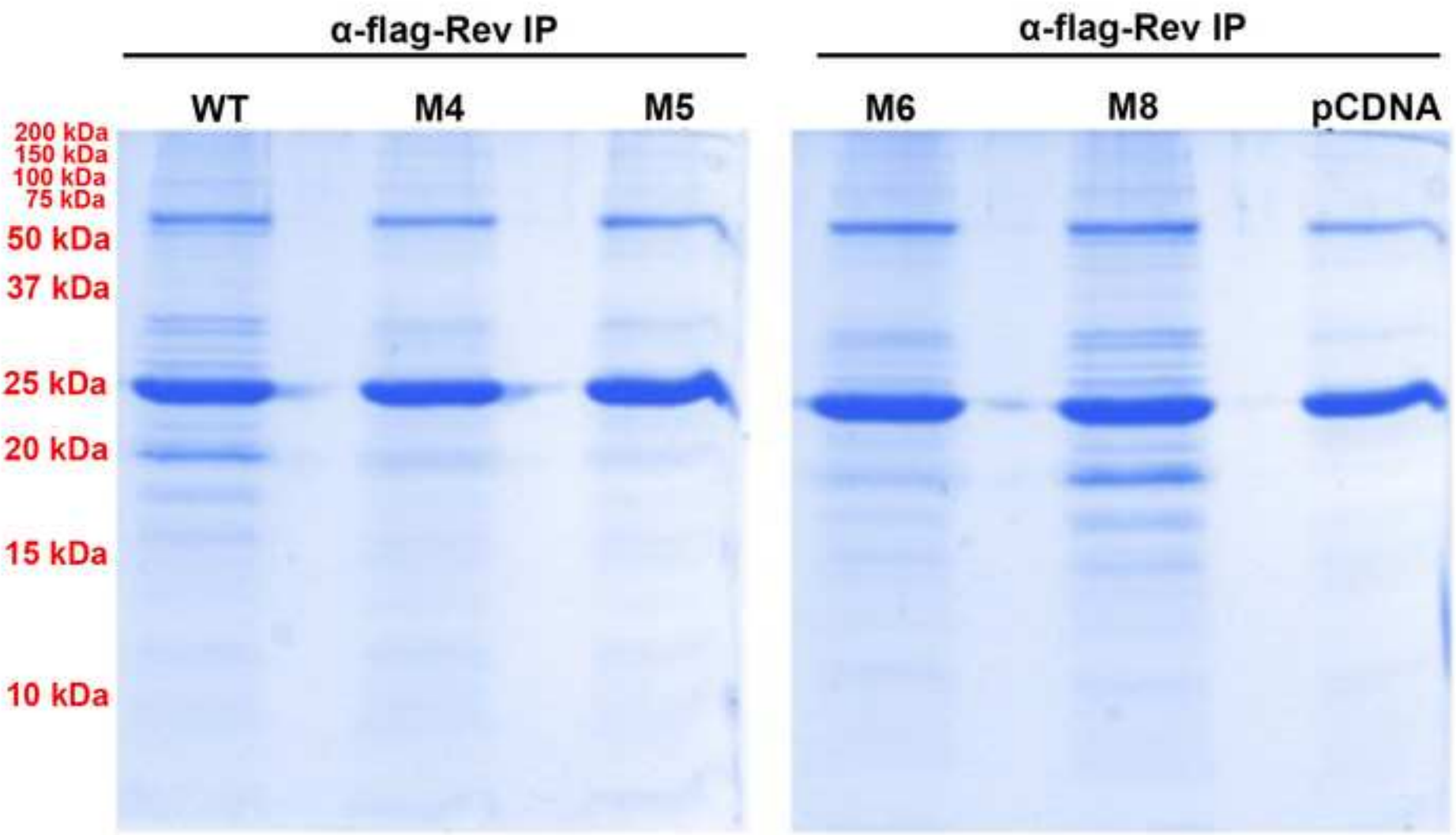


Figure 7



Identified Proteins
signal recognition particle 14kDa (homologous Alu RNA binding protein)
ribosomal protein S3A
eukaryotic translation initiation factor 4B
ribosomal protein L31
ribosomal protein L12
ribosomal protein L22
small nucleolar RNA, C/D box 58B
zinc finger, CCHC domain containing 11
actin binding LIM protein 1
ribosomal protein S13
nucleophosmin (nucleolar phosphoprotein B23, numatrin)

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Molecular Weight	WT Rev	M2	M6	M9
15 kDa	95%	0	0	0
30 kDa	95%	0	0	0
69 kDa	95%	0	0	0
14 kDa	91%	0	0	0
18 kDa	93%	0	0	0
15 kDa	91%	0	0	0
15 kDa	87%	0	0	0
185 kDa	87%	0	0	0
79 kDa	79%	0	0	0
17 kDa	78%	0	0	0
33 kDa	73%	0	0	0

Table 2

mitochondrial ribosomal protein S31	45 kDa	0	0	0	74%	0
galactose-3-O-sulfotransferase 1	49 kDa	74%	0	0	0	0
suppressor of variegation 4-20 homolog 1 (Drosophila)	99 kDa	0	72%	0	0	0
mitochondrial ribosomal protein S25	20 kDa	0	0	0	70%	0
ribosomal L1 domain containing 1	55 kDa	0	0	0	70%	0
family with sequence similarity 110, member D	29 kDa	69%	0	0	0	0
ribosomal protein L36a-like	12 kDa	0	0	0	66%	0
cerebellin 4 precursor	22 kDa	0	0	0	64%	0
N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	144 kDa	64%	0	0	0	0
RAD51 homolog B (S. cerevisiae)	38 kDa	0	0	0	64%	0
transcription elongation regulator 1	124 kDa	63%	0	0	0	0
homeobox A1	15 kDa	0	0	0	62%	0
phospholipid transfer protein	49 kDa	0	0	0	62%	0
Rho GTPase activating protein 33	137 kDa	0	0	54%	0	0
mitochondrial ribosomal protein S18B	29 kDa	0	0	0	52%	0
endoplasmic reticulum aminopeptidase 2	106 kDa	51%	0	0	0	0
tripartite motif containing 28	89 kDa	0	50%	0	0	0
immature colon carcinoma transcript 1	24 kDa	0	0	0	50%	0
AT rich interactive domain 1A (SWI-like)	242 kDa	0	0	49%	0	0
mitochondrial ribosomal protein S17	15 kDa	0	0	0	48%	0
pinin, desmosome associated protein	82 kDa	0	0	0	48%	0
protein phosphatase, Mg2+/Mn2+ dependent, 1G	59 kDa	0	0	0	45%	0
G patch domain and ankyrin repeats 1	39 kDa	45%	0	0	0	0
mitochondrial ribosomal protein L3	39 kDa	0	0	0	44%	0
budding uninhibited by benzimidazoles 3 homolog (yeast)	37 kDa	0	44%	0	0	0
WD and tetratricopeptide repeats 1	76 kDa	0	0	0	44%	0
protein disulfide isomerase family A, member 2	58 kDa	0	0	0	42%	0
kazrin, periplakin interacting protein	86 kDa	0	41%	0	0	0
coiled-coil-helix-coiled-coil-helix domain containing 2	16 kDa	0	0	40%	0	0
heat shock protein 90kDa alpha (cytosolic), class A member 1	85 kDa	0	0	40%	0	0
retinitis pigmentosa GTPase regulator	83 kDa	0	0	40%	0	0
CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1	104 kDa	0	39%	0	0	0
mitochondrial ribosomal protein L37	48 kDa	0	0	0	39%	0
C2CD2-like	76 kDa	0	38%	0	0	0
DnaJ (Hsp40) homolog, subfamily C, member 6	106 kDa	0	0	38%	0	0
mitochondrial ribosomal protein L51	15 kDa	0	0	0	38%	0
bystin-like	50 kDa	0	0	0	38%	0
huntingtin-associated protein 1	76 kDa	0	0	0	37%	0
zinc finger protein 263	77 kDa	0	36%	0	0	0
cell division cycle and apoptosis regulator 1	133 kDa	0	0	34%	0	0
protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)	256 kDa	0	0	34%	0	0
KRAB-A domain containing 2	56 kDa	0	0	0	31%	0
activating signal cointegrator 1 complex subunit 2	28 kDa	0	0	0	31%	0
centrosomal protein 76kDa	74 kDa	0	30%	0	0	0
polymerase (RNA) III (DNA directed) polypeptide C (62kD)	61 kDa	0	0	0	30%	0
5-hydroxytryptamine (serotonin) receptor 6	47 kDa	0	0	0	29%	0
T cell receptor alpha joining 56	2 kDa	0	26%	0	0	0
biorientation of chromosomes in cell division 1-like	330 kDa	0	0	0	25%	0
Ras association and DIL domains	114 kDa	0	0	25%	0	0
WD repeat domain 66	130 kDa	0	0	0	24%	0
chromosome 6 open reading frame 25	13 kDa	0	0	22%	0	0
transmembrane protein 177	34 kDa	0	0	0	21%	0
neural precursor cell expressed, developmentally down-regulated 4-like	101 kDa	0	20%	0	0	0

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number
Acetic acid	Fisher Chemical	A38S-212
Acetonitrile	Fisher Chemical	A955-500
Acrylamide:Bisacrylamide	BioRad	1610158
Ammonium bicarbonate	Fisher Chemical	A643-500
Ammonium persulfate	Sigma-Aldrich	7727-54-0
ANTI-Flag M2 affinity gel	Sigma-Aldrich	A2220
anti-Flag M2 mouse monoclonal IgG	Sigma-Aldrich	F3165
BioMax MS film	Carestream	8294985
Bio-Rad Protein Assay Dye Reagent Concentrate, 450 mL	Bio-Rad	5000006
B23 mouse monoclonal IgG	Santa Cruz Biotechnologies	sc-47725
Bromophenol blue	Sigma-Aldrich	B0126
Carnation non-fat powdered milk	Nestle	N/A
Cell scraper	ThermoFisher Scientific	179693PK
C18IonKey nanoTile column	Waters	186003763
Corning 100-mm TC-treated culture dishes	Fisher Scientific	08-772-22
Dithiothreitol	Thermo Scientific	J1539714
1 x DPBS	Corning	21-030-CVRS
ECL Estern blotting substrate	Pierce	32106
Ethanol, 200 proof	Fisher Chemical	A409-4
FBS	Gibco	16000044
Formic Acid	Fisher Chemical	A117-50
GelCode blue stain reagent	ThermoFisher	24590
Glycerol	Fisher Chemical	56-81-5
goat-anti-mouse IgG-HRP	Santa Cruz Biotechnologies	sc-2005
Iodoacetamide	ACROS Organics	122270050
KimWipe delicate task wiper	Kimberly Clark Professional	34120
L-glutamine	Gibco	25030081
Methanol	Fisher Chemical	67-56-1
NanoAcuity UPLC	Waters	N/A
Pierce Silver Stain Kit	Thermo Scientific	24600df
15-mL Polypropylene conical tube	Falcon	352097

Prestained Protein Ladder, 10 to 180 kDa	Thermo Scientific	26616
Protease inhibitor cocktail	Roche	4693132001
Purified BSA	New England Biolabs	B9001
PVDF Western blotting membrane	Roche	3010040001
Sodium Pyruvate	Gibco	11360070
10 x TBS	Fisher Bioreagents	BP2471500
TEMED	BioRad	1610880edu
Triton X-100 detergent solution	BioRad	1610407
Trizaic source	Waters	N/A
trypsin-EDTA	Corning	25-051-CIS
Tween 20	BioRad	1706531
Synapt G2 mass spectrometer	Waters	N/A
Whatman filter paper	Tisch Scientific	10427813

Comments/Description

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Title of Article:	Identification of Nucleolar Factors during HIV-1 Replication through Rev Immunoprecipitation and Mass Spectrometry
Author(s):	Jerlisa Ann C. Arizala, Pritsana Chomchan, Haitang Li, Dominique L. Ouellet, Roger Moore, Helen Ge, and John J. Rossi

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CORRESPONDING AUTHOR

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Signature: Jerlisa Ann C. Arizala Date: 01/07/2019

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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise lines 89-91 to avoid previously published text.
3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Agilent, Gelcode, Falcon, Flag, Triton-X 100, kimwipe, Whatman, etc.
4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.
5. 1.7, 4.2, 4.5, 4.8, etc.: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).
6. 2.1: Please specify culturing conditions.
7. 4.6.1: Please describe how to measure the protein concentration of the post-IP lysate.
8. 8.3: Please specify the incubation temperature.
9. Please include mass spectrometry methods in the protocol.
10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
11. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
14. Figures: Please include a space between the numerical values and their corresponding units (e.g., 137 mM, 150 kDa, 15 min, 37 °C, etc.).
15. Please shorten the figure legends. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
16. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.
17. References: Please do not abbreviate journal titles.
18. Table of Materials: Please sort the items in alphabetical order according to the name

of material/equipment.

Author's rebuttal to editorial comments:
1-2. The manuscript was proofread.
3. ANTI-Flag was changed to M2 affinity gel or M2 mouse monoclonal IgG.
Other trademarked names were changed to general reagent terms and were referenced to the table of materials.
4. The introduction was rephrased to include a clear statement of the overall goal of this method.
5. Centrifuge speeds were changed to g-force.
6. Culturing conditions were specified.
7. The Bradford assay was incorporated into the protocol section (Section 4)
8. Room temperature was specified (18 to 20 degrees Celcius).
9. Mass spectrometry methods were added to the protocol section.
10.Protocol steps were shortened.
11-13. Essential steps of the protocol were highlighted after shortening protocol steps.
14. Spaces were added between all numerical valudes and corresponding units for all figures.
15. Figure legends were shortened and the results thoroughly described in the Results section.
16. Critical steps of the protocol, modifications and troubleshooting of the technique, and limitations were included in a separate paragraph.
17. Journal titles in references were revised to not contain abbreviation.
18. Items in table of materials were sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript from Arizala and colleagues describes an IP-Mass Spec method to investigate cellular factors that associate with the wild type HIV-1 Rev protein during the viral replication cycle. Using this method, the Authors compare the wild type Rev protein with a variety of point mutations in the Rev nucleolar localization signal. A number of cellular proteins are identified in IP-Mass Spec experiments that are enriched for the association with the wt Rev protein relative to the various mutant proteins.

This clearly presented manuscript describes a general method for detecting cellular co-factors that associate with viral proteins during infection. Although IP-Mass Spec is now an established method that has been used by numerous labs in many publications, this manuscript does describe a step-by-step procedure that should prove useful for labs not experienced in this type of methodology. Some minor concerns with the manuscript are described below.

Minor Concerns:
1) Figure 4 is of rather poor quality relative to the other figures.

2) Tables 1 and 2 present a "%" measurement in the columns of wt Rev and mutants. This measurements need clarification.
3) The majority of proteins that co-IP with wt Rev (and some mutants) are unlikely to make direct protein-protein interactions with Rev. Rather, they are most likely in protein complexes that co-IP via a direct-protein-protein interaction with Rev and another protein in the complex. Thus, most of the proteins in the Tables are likely to associate indirectly with Rev. It would be useful to state this explicitly so that readers/viewers appreciate this key point.

Author's rebuttal to Reviewer #1 comments:
1. Figure 4 resolution was improved for publication quality.
2. The proteomics results were displayed and compared using protein identification probability (%). This was changed and indicated throughout the manuscript.
3. The cellular factors identified in tables 1 and 2 were explicitly described to interact directly/indirectly with the Rev protein complex during HIV-1 replication.

Reviewer #2:
Manuscript Summary:
This manuscript is to accompany a JOVE video describing a comparative IP-mass spec approach for identifying host cell factors interacting with the HIV-1 Rev protein. The included methods and sample results focus on a refined IP and gel separation strategy for recovery of 3xFLAG-tagged Rev and associated proteins. The methods are easy to follow, some of the hurdles faced to IP purification and refinements are described, and the screen yields what looks to be a pretty interesting dataset of proteins differentially-bound to wild-type Rev vs. a panel of Rev mutants. However, the methods included are relatively generic (e.g., how to perform SDS-PAGE, etc.) and don't provide a technically-detailed description of sample prep for mass spec, mass spec details, analysis workflow, statistics, etc.- these things would be much more useful to readers. Thus the paper could be significantly improved. The text would also benefit from some additional clarifications and a few modifications to static figures and additional details added to tables, as outlined below.

Major Concerns:
1. Lines 108-120. The paper indicates that two techniques were compared (direct analysis of eluates vs. extraction after SDS-PAGE) but the pro and cons of the two approaches are not discussed and, unless I'm mistaken (Table 1 vs. Table 2?), dealt with in the data. How did the results differ for the two methods? Which method was superior and what were the pros and cons? As noted below, discussion should spend less time on B23 and more time discussing technical nuances.
2. How was sample prep for mass spec carried out? Even if done by core would be useful to / needed by readers attempting to replicate process.
3. Both Table 1 and 2; What are the percentages and what do they mean? How were the computational and statistical analyses performed? How many replicates? Should be addressed in more detail than just listing names of software.

4. Figure 5. Why are input levels of Rev so low for M2 and M9? Doesn't really make sense if the IP is robust...enrichment should be equivalent to input unless beads are saturated?

Minor Concerns:
1. Line 46. Please clarify why snoRNA C/D box58 is highlighted, e.g., was this also a differential interactor?
2. Lines 58, 62. Not meaningful here to list the mutation #, I would just re-write to say a subset of mutant Rev proteins were ID'd that localize to the NOLC but with reduced activity, while an additional subset were ID'd that do not localize to the NOLC and don't function. So then the 3 comparators are set-up in a simple fashion: WT, nucleolus/defective, non-nucleolus/defective. A figure to explain Rev organization and clearly define the mutations and predicted effects would also be very valuable.
3. IP-MS screens make no judgment regarding if or how identified proteins are relevant to infection. As the authors appreciate, data are hypothesis-generating followed by validation and/or functional assays. Lines 48 and 667, 683-684, 687-88 should be edited and discussed accordingly.
4. Lines 690-720; why devote a paragraph to discussing B23 function? This is a technical report and there are no validation or structure-function type analyses. Would be more valuable to use this space to discuss pros and cons of approach, how it could be improved, adapted, etc.
5. Intro paragraphs 2 and 3 are very long/expansive and should be broken up for readability.
6. Paragraph 2 seems to be trying to set up a straightforward hypothesis, that B23 will be bound by wild-type Rev but less-so by the mutants, but never quite gets there….could be more clearly articulated to better set up control IP experiments in Figures 1 and 2.7.
7. Lines 158-161. Cell number would be more useful than plate #.
8. Figure 2. Need to adjust top panel, cutting off the bands.
9. Table 1- Legend title says that these are factors that interact with Rev NoLS "mutations", text says and table shows they were detected with wild-type Rev but not with M2, M6, or M9. Something here needs to be corrected.

Author's rebuttal to Reviewer #2 comments:
Major Concerns:
1. Pros and cons, and differences of both approaches were discussed.
2. The mass spectrometry procedures were added to the protocol.
3. Percentages in tables 1 and 2 represent the protein identification probability during analysis using Scaffold software, version 3. The protein threshold was set to 20%, minimum number of peptides was set to 1, and the peptide threshold was set to 0%. This description was included in section 13.8 in the Results section. Although data is available for three replicate experiments for WT Rev versus mutations, this manuscript is showing representative data for one experiment.
4. The input levels of M2 and M9 in Figure 5 are not highly expressed as WT Rev. 20 ug was used for input during immunoblot analysis, versus the IP reaction, which used 5 mg of protein lysate. This was indicated in the results section.

Minor Concerns:
1. snoRNA C/D box58 was highlighted because Rev mutants lost interaction with this factor in comparison to WT Rev (Table 1). This was briefly described in the abstract.
2. The specific mutation numbers were mentioned and described for the purpose of referencing a published figure showing localization pattern of the Rev mutations under examination in this manuscript for mass spectrometry analyses.
3. The proteins identified from the mass spectrometry results and briefly discussed eluded to the involvement in HIV-1 infection through Rev functionality, which is mRNA-independent nucleocytoplasmic transport and HIV-1 mRNA splicing regulation. Factors (nucleolar and non-nucleolar) that bind to the Rev protein complex were expected to participate in these Rev functions to facilitate HIV-1 infection.
4. Pros and cons of the approach, improvement, and adaptation to other disease models were previously discussed throughout the Discussion section. An emphasis on the cons of this method was included in a separate paragraph. B23 function was also emphasized to explain that one nucleolar factor in one disease model could serve multifunctional roles in other disease models, which is why this approach could benefit the characterization of pathways – nucleolar o non-nucleolar – in other infectious and disease models.
5. Paragraphs 2 and 3 were revised into multiple paragraphs for readability.
6. B23 was previously shown to lose interaction with Rev in the presence of Rev NoLS mutations.
7. Reference to plate number was replaced with cell number.
8. Figure 2 top panel could not be adjusted.
9. Table 1 legend was corrected to indicate that the host cellular factors bind to WT Rev.

Editorial Comments Part II:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.
2. Please address the following specific comments marked in the manuscript:
    a. Please ensure that the title is in line with the highlighted portion of the protocol. Presently mass spectrometry portion is not highlighted.
    b. Please ensure that the protocol is no more than 10 pages and highlighted section is no more than 2.75 pages including headings and spacings. This is a hard-cut limit. Presently the protocol is around 11 pages and highlights are around 4 pages. Please combine some shorter steps so that there are around 203 action per steps.
    c. Notes cannot be filmed hence highlights removed.
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3. Please ensure that the protocol is no more than 10 pages and the highlight is no more than 2.75 pages including headings and spacings. Presently both exceeds the limit. Please combine some shorter steps into one so that each step has no more than 2-3 actions per step.

Author Response Part II:

1. The manuscript’s formatted style was retained.
2. The following comments in the manuscript were addressed:
    a. The mass spectrometry portion was not highlighted because it is a common method used to analyze protein interactions.
    b. The protocol was shortened to 10 pages.
    c. Notes were not included in the highlighted sections.
    d. The figures in this manuscript were not previously published.
3. The protocol was shortened to 10 pages, and the highlight shortened to 2.75 pages. Shorter steps were combined into one step.