Chapter 12

Affinity-Based Interactome Analysis of Endogenous LINE-1 Macromolecules

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Abstract

During their proliferation and the host's concomitant attempts to suppress it, LINE-1 (L1) retrotransposons give rise to a collection of heterogeneous ribonucleoproteins (RNPs); their protein and RNA compositions remain poorly defined. The constituents of L1-associated macromolecules can differ depending on numerous factors, including, for example, position within the L1 life cycle, whether the macromolecule is productive or under suppression, and the cell type within which the proliferation is occurring. This chapter describes techniques that aid the capture and characterization of protein and RNA components of L1 macromolecules from tissues that natively express them. The protocols described have been applied to embryonal carcinoma cell lines that are popular model systems for L1 molecular biology (e.g., N2102Ep, NTERA-2, and PA-1 cells), as well as colorectal cancer tissues. N2102Ep cells are given as the use case for this chapter; the protocols should be applicable to essentially any tissue exhibiting endogenous L1 expression with minor modifications.

Key words Retrotransposon, Ribonucleoprotein, Affinity capture, Protein complexes, Interactomics

1 Introduction

LINE-1 (L1) is the only known active, autonomous, protein-coding human retrotransposon. Retrotransposons are mobile DNA sequences that multiply via a "copy and paste" mechanism termed retrotransposition: a parent genomic DNA locus is transcribed, producing an RNA copy of the L1 genome; the L1 RNA is reverse transcribed, giving rise to a cDNA copy of the L1 genome; the cDNA is inserted into the host cell genome at a new location, giving rise to a new genomic L1 locus [1, 2]. Although the interplay between L1 proliferation and human evolution, development, and health is an area of ongoing scholarly study, L1s may be con-

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ceptualized as genetic parasites [3–5]. Over evolutionary time, L1 retrotransposition has deposited approximately 500,000 sequences into the human genome, constituting ~20% of the genome in modern-day humans. The overwhelming majority of these L1-derived genome sequences are truncated and mutated degenerate "fossils" although ~100 loci are predicted to be capable of replication [6, 7]. The active L1 sequences are sources of genetic structural variation between humans and cause genome instability in cancer [1, 8–10].

Being streamlined genetic parasites, L1 DNA sequences only possess two open reading frames, *ORF1* and *ORF2*, encoding two polypeptides coined ORF1p and ORF2p, both of which are essential for L1 proliferation [1, 2]. A putative third open reading frame, *ORF0*, may produce a polypeptide of uncertain significance, which has been proposed to enhance L1 proliferation [11, 12]. ORF1p is ~40 kDa in mass and forms a homotrimeric nucleic acid chaperone protein [13, 14] that is highly expressed in human cancers and cell culture models [15, 16]; ORF1p can also adopt higher-order oligomeric states [17] and can undergo liquid-liquid phase separation, which may produce compartments within the cell that are required for retrotransposition and may also have implications for the biochemical isolation of ORF1p-containing complexes [18, 19]. Although ORF1p is required for retrotransposition, its roles at the molecular level are not well understood [14, 17, 20–23]. ORF2p is a ~150 kDa multidomain polypeptide, which possesses endonuclease and reverse transcriptase activities that are required for LINE-1 mobilization [24, 25]. In contrast to ORF1p, ORF2p is expressed at such low levels that direct detection of the endogenous protein has yet to be robustly and reproducibly demonstrated [26–28]. Both ORF1p and ORF2p assemble on L1 mRNAs and form ribonucleoprotein (RNP); in ectopic expression systems, this process has been shown to preferentially occur between the ORF proteins and the L1 mRNA that encoded them [29–31].

On account of encoding few protein activities, L1s must co-opt host cellular machinery in order to replicate; conversely, host cells also possess multiple mechanisms to suppress L1 activity and defend against its cytotoxicity [32–44]. L1-associated macromolecules may therefore exhibit variable compositions depending on the context of expression and gamut of host responses. Notably, L1-mediated cytotoxicity is not limited to its DNA-damaging capabilities: L1s are also implicated as a source of pathological cytoplasmic nucleic acids [45, 46]; they may also sequester and accumulate normally homeostatic host proteins, such as RNA-binding proteins, away from their typical functions and into (phase separated) L1 macromolecular assemblies or granules, among other possibilities.

Although the teleological roles of retrotransposition in evolution are somewhat controversial [47–49], critical interactions with the host functions are highly conserved; for example, the PIP box, a motif required for L1 ORF2p-PCNA binding and retrotransposition, is conserved from corn to humans [32]. The necessary motifs and modes of interactions of other host factors are not as well-defined, making the degree of conservation challenging to assert. L1 RNP interactions with PABPC1/4, UPF1, MOV10, and ZCCHC3, among others, are likely conserved and rely, at least in part, on interactions with the L1 RNA [50]. Although not an L1 RNP constituent, another important example of a conserved L1 regulator is P53, which suppresses L1 expression and arrests cell growth in response to L1 activation in humans; P53 has been shown to suppress many classes of mobile elements in other organisms and may have evolved to combat transposons before developing its better-understood functions in apoptosis [51, 52]. Several studies have explored the landscape of L1 interactors, by different means, in model cells (e.g., [32, 36, 50, 52–56]); these have typically relied on ectopic L1 overexpression from transfected plasmids. However, studies of L1 interactors in endogenous expression contexts are increasingly common (e.g., [26, 57–59]).

To aid the study of L1 molecular biology—including physical interactions with proliferative and defensive host proteins—we outline our methods for immunoprecipitation of L1 RNPs with subsequent characterization by mass spectrometry and RNA sequencing [26, 32, 50, 60]. For this, we use cryomilled cells and antibody-coupled magnetic beads, previously described in detail [61]. While our prior work centered on ectopic L1 expression systems, here we focus on L1 RNP capture and analysis using endogenously expressing cell lines and patient tissues. The protocols described have been applied to embryonal carcinoma cell lines that are popular model systems for L1 molecular biology (e.g., N2102Ep, NTERA-2, and PA-1 cells), as well as colorectal cancer tissues. N2102Ep cells are given as the use case for this chapter; the protocols should be applicable to essentially any tissue exhibiting endogenous L1 expression with minor modifications.

2 Materials

Use cell culture grade (e.g., Subheading 2.1), *molecular biology grade* (e.g., Subheadings 2.3, 2.4, and 2.5), *and mass spectrometry grade* (e.g., Subheading 2.6) *reagents where appropriate.*

2.1 Culture and Harvest of N2102Ep Cells

1. N2102Ep cl. 2/A6 (European Collection of Authenticated Cell Cultures).
2. Humidified, CO_2-controlled tissue culture incubator.

3. Water bath (set to 37 °C).
4. DMEM, high glucose, GlutaMAX™ medium.
5. Fetal Bovine Serum.
6. 100× Penicillin-streptomycin (P/S).
7. 200 mM L-Glutamine.
8. Dulbecco's phosphate-buffered saline (DPBS, no calcium, no magnesium).
9. TrypLE™ Express Enzyme (1×), no phenol red (Gibco).
10. 70% Ethanol (for cleaning).
11. Tissue culture flasks in 75 cm^2, and 175 cm^2 sizes, and 500 cm^2 tissue culture plates.
12. 230 mm Glass Pasteur pipettes (for biosafety cabinet vacuum system).
13. Serological pipettes in 1, 5, 10, 25, and 50 mL volumes.
14. Pipette controller.
15. Micropipettes and corresponding filter tips.
16. 1.5 mL microcentrifuge tubes and a tube rack.
17. Hemocytometer for counting.
18. Refrigerated benchtop centrifuge with 50 mL adaptors.
19. 40 cm Cell scrapers.
20. 50 mL Conical tubes.
21. BD Microlance™ 3, 18 gauge needles or similar.
22. BD Luer lock™ syringes 30 mL.
23. Luer lock™ syringe end caps.
24. Liquid nitrogen (LN_2) tolerant plastic funnel and small pitcher/beaker (~500 mL).
25. LN_2 and Dewar flask.
26. Gloves and goggles for handling LN_2.
27. Small Styrofoam box.
28. Parafilm.

2.2 Cryomilling Cells

1. ~9 L ice pan 25% filled with LN_2.
2. Nalgene® Resmer 3 × 3 half rack or comparable LN_2 tolerant rack.
3. Small clean styrofoam cooler half filled with LN_2.
4. Retsch Planetary ball mill PM 100.
5. Retsch Stainless steel milling balls 20 mm diameter.
6. Retsch Stainless steel "comfort" milling jars, 50 mL.

7. Small (~50–100 mL) and large (1–10 L) decanters for LN_2 to be used for pouring.
8. Stainless steel spatulas.
9. Extra-large forceps.
10. 50 mL Conical tubes.
11. Epredia™ UltraFIT™ Nylon Laboratory/Cleanroom Glove Liners (Fisher Scientific).

2.3 Coupling Antibodies to Magnetic Medium

1. Dynabeads M270 Epoxy (Invitrogen).
2. α-ORF1 Antibody 4H1 (MilliporeSigma, cat. #MABC1152).
3. IgG from mouse serum (MilliporeSigma, cat. #I5381).
4. Magnetic separator for microcentrifuge tubes.
5. Zeba™ Spin Desalting Columns 7 K MWCO, 0.5 mL (Pierce).
6. Antistatic microspatula.
7. Nutating mixer or similar.
8. Tube revolver rotator in a 37 °C environment.
9. 100 mM Sodium phosphate, pH 7.4.
10. 3 M Ammonium sulfate, buffered with 100 mM sodium phosphate, pH 7.4.
11. 100 mM Glycine-Cl pH 2.5.
12. 10 mM Tris-Cl pH 8.8.
13. 100 mM Triethylamine, freshly prepared.
14. 1× PBS pH 7.4.
15. 1× PBS with 0.5% v/v Triton X-100.
16. 1× PBS with 50% v/v glycerol and 0.5 mg/mL BSA.

2.4 Immunoprecipitation for Protein Analyses (Including IP-MS)

1. Styrofoam cooler ~$^1/_3$ full with LN_2, Nalgene® Resmer 3 × 3 half rack or comparable LN_2 tolerant rack, inside.
2. Weighing spatula and large forceps.
3. Ice pan with ice.
4. Extraction solution: 20 mM HEPES-Na pH 7.4, 500 mM NaCl, 1% v/v Triton X-100 (or your own extraction solution of choice).
5. Protease inhibitor cocktail: cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche).
6. Ultrasonic liquid processor with micro tip (QSonica S-4000 generator with low-intensity 1/16 in. microprobe or similar).
7. Refrigerated benchtop microcentrifuge.
8. Magnetic affinity media (from 2.4 Coupling of Magnetic Medium).

9. Safe-lock Eppendorf tubes in 1.5, 2.0, and 5.0 mL sizes.
10. Rotating wheel or Tube Revolver Rotator.
11. Magnetic separator for microcentrifuge tubes.
12. Vortex.
13. Benchtop minifuge.
14. Thermomixer.
15. SDS-polyacrylamide gel electrophoresis system (we use Invitrogen™ Mini Gel Tank for mini gels and Invitrogen™ XCell4 SureLock™ Midi-Cell for midi gels) and power supply.
16. NuPAGE™ 4–12% Bis-Tris gel, 1.0 mm, 15-well (ThermoScientific) or appropriate precast acrylamide gels for your electrophoresis system.
17. 20× MOPS (3-morpholinopropane-1-sulfonic acid) gel running buffer.
18. 4× Lithium dodecyl sulfate (LDS) sample loading buffer.
19. NuPAGE™ 10× Sample Reducing Agent (ThermoScientific); or 500 mM dithiothreitol (DTT).
20. Precision Plus Protein™ All Blue Prestained Protein Standards, 10–250 kDa (Bio-Rad).
21. Blue silver Coomassie Stain (modified as described in [62]).
22. Criterion™ Blotter with Wire Electrodes (BioRad) or comparable Western blot transfer system.
23. Immun-Blot® PVDF Membrane (BioRad) or similar.
24. Extra Thick Blot Filter Paper (Precut, 15 × 20 cm), Thick Blot Filter Paper (Precut, 15 × 20 cm), or similar.
25. Foam Pads for Mini Trans-Blot® Cell and Criterion™ Blotter (BioRad) or similar.
26. Amersham ECL Mouse IgG, HRP-linked whole Ab (from sheep) (MilliporeSigma).
27. Immobilon® Forte Western HRP substrate, 100 mL (MilliporeSigma).
28. ImageQuant LAS-4000 Luminescent Image Analyzer (FujiFilm) or similar.

2.5 Immunoprecipitation for RNA Analyses (RIP-Seq)

The procedure for capturing RNA in L1 RNP is essentially identical to protein capture, except for the elution method. Extra steps to protect RNA quality are also proposed (*see* Subheading 3.7). The materials required for the RNA-specific steps are as follows.

1. Nuclease-free water or similar.
2. RNaseZap™ RNase Decontamination Solution (Fisher Scientific) or similar RNase decontamination solution.
3. SoftFit-L™ Filtered Pipette Tips (ThermoScientific) or similar.

4. Recombinant RNasin® Ribonuclease Inhibitor (Promega).
5. Direct-zol™ RNA microprep kit (Zymo).
6. Phasemaker™ Tubes (Invitrogen).
7. TRIzol™ Reagent.
8. Chloroform.
9. Ethanol (absolute).
10. Agilent Bioanalyzer 2100 instrument (Agilent).
11. Agilent RNA 6000 Pico Kit (Agilent).

2.6 Protein Mass Spectrometry (MS)

Different MS-based instruments and analytical approaches may require different sample work-up procedures; we suggest conferring with your expert collaborator or proteomics core facility prior to embarking on IP-MS experiments. Because we analyze our own samples on our own MS instrument, we provide a detailed description of that process, which other MS-expert laboratories can adopt directly; these procedures can otherwise be shared with MS collaborators and/or core facilities to enable successful analyses (*see* **Note 1**).

1. ESI mass spectrometer with Orbitrap detector (e.g., Exploris™ 480, from ThermoScientific™).
2. CalMix (Calibration mixture for the Mass Spectrometer, contact your provider).
3. UHPLC system (*u*ltra-*h*igh-*p*erformance *l*iquid *c*hromatography—for example, UltiMate™ 3000, ThermoScientific™) (*see* **Note 2**).
4. Analytical column (e.g., 50 cm nanoViper™, ThermoScientific™).
5. Pre-column (optional).
6. MS-grade solvents for LC procedures, for example, water, acetonitrile, methanol, isopropanol, formic acid.
7. 1 M Triethylammonium Bicarbonate (TEAB), pH 8.5.
8. S-trap™ columns (Protifi) [63], and associated reagents: Tris (2-carboxyethyl)phosphine (TCEP), methylmethanethiosulfonate (MMTS), trypsin, Lys-C (protease mixture), sodium dodecyl sulfate (SDS), urea, glycine, phosphoric acid.
9. Vortex mixer.
10. Thermomixer w/2 mL thermoblock.
11. Benchtop centrifuge suitable for 2 mL microfuge tubes.
12. Centrifugal vacuum concentrator or "SpeedVac."
13. Microcentrifuge.
14. Micropipettes and pipette tips (compatible with organic solvents/no polymer leakage).

15. Lo-bind 2 mL microfuge tubes.
16. Regular 2 mL microfuge tubes (to discard the washes).
17. 500 μL microfuge tubes.
18. MS vials (Chromatography Direct), consisting of 2 mL thread vial with a label, 250 μL conical glass inserts and Blue Screw Caps.

2.7 RNA Sequencing (RIP-Seq)

We conduct our RNA immunoprecipitation (RIP-seq) [64] sample analyses with dedicated core facilities. If not running the sequencing instrument yourself, considerations for RNA sample and cDNA library preparation should be discussed with the expert collaborator or core facility: specific expectations and requirements will vary depending on the number of samples to be run and the depth of sequence coverage desired (contributing to the amount of sample multiplexing that can be achieved). We recommend a read length of ≥100 bp with paired-end sequencing reads: given the high copy number of L1 sequences in the human genome, a single read can align to thousands of locations in the genome (a.k.a. multimapping); shorter reads align ambiguously to more candidate loci than longer reads. We recommend 150 bp reads because they are the longest currently available using Illumina's high output sequencing by synthesis (SBS) reagent kits, in conjunction with the HiSeq and NovaSeq platforms (as of March 14, 2022) [65]. Paired-end sequencing data is important for L1 RNA expression because, for example, it facilitates the detection of read pairs that map both to L1s and to the host genome sequences located immediately upstream of an L1 locus. Such reads help to discriminate when a locus is "passively co-transcribed" from a nearby promoter rather than actively expressed from the L1 5′ UTR/promoter [66, 67]. We recommend a read depth of ≳40 M reads per sample. Analysis with the L1EM program gives the best results at loci that are covered by at least 100 read pairs [66]; 40 M+ reads will typically yield read counts exceeding this threshold and the most abundant loci, especially in samples that have been enriched for LINE-1 RNA (e.g., by α-ORF1p RIP-seq). We have carried out matched total RNA- and RIP-seq analyses successfully using the above-stated parameters on the NovaSeq 6000 platform in conjunction with the Trio RNA-Seq™ library preparation kit. This library preparation kit facilitates DNase treatment and rRNA depletion during library construction and it accepts as little as 500 pg of RNA as input; RIP samples are typically lower yield than typical total and poly(A) + RNA samples. However, other RNA sequencer and cDNA library preparation configurations could provide excellent results and should be determined based on the infrastructure available to you; we therefore do not provide a stepwise description of this procedure.

2.8 Bioinformatics Analyses

We use the RStudio/R programming environment [68] for protein and RNA bioinformatics analyses. A repository of relevant code for protein and RNA analyses described in this chapter can be accessed here: https://github.com/moghbaie/endogenous_l1. L1EM source code and installation instructions are available here: https://github.com/FenyoLab/L1EM.

2.8.1 Label-Free Quantitative (LFQ) Protein Analysis

1. Workstation or high-performance computing (HPC) cluster [69] with MaxQuant (MQ) installed [70, 71].
2. Proteomics Quality Control R-package for MQ: PTXQC [72].
3. NormalyzerDE [73].
4. clusterProfiler [74].

2.8.2 Differential Enrichment RNA-seq Analysis

1. Workstation or high-performance computing (HPC) cluster with STAR aligner installed [75].
2. featureCounts from Rsubread [76].
3. edgeR [77–79].
4. clusterProfiler [74].

2.8.3 Locus-Specific L1 Quantification

1. L1EM software package [66]. The default L1EM parameters are intended for a workstation or cluster node with the following specifications (*see* **Note 3**): Unix-like operating system (e.g., Linux or Mac OS X), 16 CPU cores, and 64 GB RAM.

3 Methods

3.1 Cell Culture

2102Ep cells are an established model cell line demonstrating endogenous L1 expression and activity [80–83]. N2102Ep cells [84] are a clonal isolate of 2102Ep; in culture, they typically present as relatively small, uniform cells with a tendency to grow in clusters (Fig. 1a). We commonly observe larger individual cells in addition to the clusters. In addition, we have noticed more floating cell debris in this cell line than in, for example, HEK-293(T) or HeLa cells. N2102Ep cells are advantageous for L1 interactome analysis due to their relatively high endogenous ORF1p expression level (e.g., higher than NTERA2 cl.D1 and PA-1 cells; Fig. 1b).

1. Thaw one N2102Ep stock in a 37 °C water bath until only a small ice crystal remains (1–2 min). Do not completely submerge the tube or you risk contamination. Clean the tube with ethanol, open it inside the biosafety cabinet and transfer thawed cells by micropipette to a 15 mL conical tube containing 10 mL DMEM Glutamax. Pellet in benchtop centrifuge at room temperature (RT) for 5 min at 1000 × RCF.

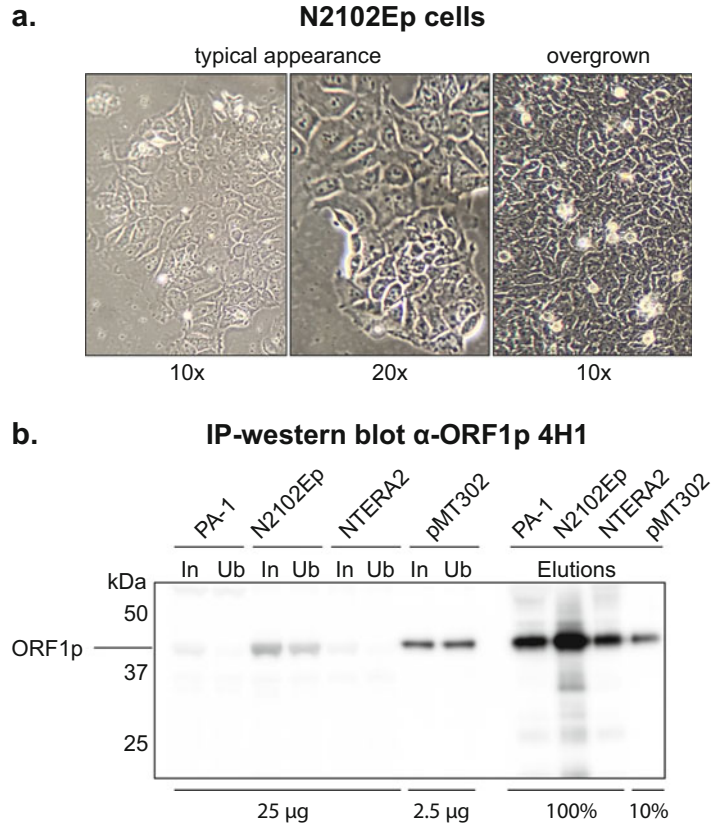


Fig. 1 N2102Ep cell images and example IP-western results. (**a**) Light microscopy of N2102Ep cells, the phase contrast magnification is indicated. On the left, typical log-phase cells are displayed; on the right, overgrown (fully confluent) cells are displayed. (**b**) Western blot (on PVDF membrane after separation on 4–12% Bis-Tris SDS-polyacrylamide gel). Clarified protein extracts (*In*put and *Un*bound; micrograms loaded, given) and IP elutions (percent of elution volume loaded, given) analyzed by Western blot. Embryonal carcinoma cell lines are compared with HEK-293T$_{LD}$ cells transfected with pMT302—containing an L1 sequence derived from L1RP [32]—provided for comparison between endogenous and ectopic expression contexts

2. During the spin, prepare your flask. Seed the cells into a 1× 150 cm^2 flask with a 35 mL medium. Adherent cells are maintained in DMEM Glutamax supplemented with 10% (v:v) FBS and 2 mM L-Glutamine (*see* **Note 4**). In the flasks and volumes suggested here, N2102Ep cells will require new media every 2–3 days. This is evident by the change in media color from reddish pink to orange. Cells need attention when the media is orange, do not let the color progress to yellow. Using the maximum recommended media volume for the flasks you are using can maximize the time between media changes.

3. Once cells reach ~90% confluency, split 1:5 into 150 cm^2 flasks. Once these reach ~90% confluency, split 1:4 into 500 cm^2 plates. This cell line is a lower yield than, for example, HEK-293(T), so 20 plates will yield ~5 g of cell powder (vs ~10 g of HEK-293(T), *see* **Note 5**).
4. To split the cells, aspirate media and wash the flask with 10 mL PBS. Aspirate PBS. Add 1.5 mL TrypLE™ Express Enzyme and tilt the flask to ensure even coverage. Tap the side of the flask firmly with an open palm two to three times, then return the flask to the incubator for 5–7 min.
5. Check that all cells are loosened. If not, you can tap the flask again with an open palm, return it to the incubator for another minute or use a cell scraper.
6. Resuspend cells in medium and return to the incubator.

3.2 Harvest and Freeze Cells in Liquid Nitrogen

To harvest, the cells are released from the plates by physical scraping, pelleting in a syringe, and then injecting them into a tube containing liquid nitrogen [85]. A video protocol is available at [60].

1. Gather an ice bucket and 16× 50 mL conical tubes (or 3–4 clean centrifuge bottles if using floor centrifuge).
2. Count the cells; cell counts may aid downstream normalizations and keep track of the yield of cells, for example, per plate and per mg wet cell weight obtained.
3. Transfer a 1 mL aliquot(s) of culture for Western blotting to a microcentrifuge tube. Spin at 1000 × RCF for 30 s, aspirate the media, and freeze.
4. Harvest the cells plate by plate on ice in a 9 L ice pan. With the ice pan oriented horizontally, place the plate of cells to one side, and arrange a row of 50 mL conical tubes for cell collection on the other side (or in another, nearby ice bucket; a video of this procedure has been recorded [60] and many figures related to this process, as further elaborated below, have been published in the previous version of this protocol [61]).
5. Add 20 mL ice-cold PBS to the plate, then scrape the plate twice with a large cell scraper, for example, from left to right in a series of rows (once), then top to bottom in a series of columns (twice).
6. Rotate the plate 45°, add another 10 mL ice-cold PBS and scrape again.
7. Inspect the plates after scraping and, if needed, add an additional 5 mL of PBS to recover the last remaining cells.
8. Repeat **steps 4–7** for each dish; cell suspensions from different dishes may be combined to reduce sample number and plastic waste.

9. Spin the cells at 1000 × RCF for 5 min at 4 °C to pellet them. Carefully pour off the supernatant. Hold all the tubes on ice.
10. Resuspend pellets in a minimal volume (<3 mL) of ice-cold PBS + 1× protease inhibitor. Pool pellets into two conical tubes. Spin again, 1000 × RCF for 5 min at 4 °C. Carefully pour off the supernatant.
11. Pellet cells inside a syringe. Use a 30 mL syringe(s) for cell pelleting; 1× 30 mL syringe is sufficient for the harvest of 20 plates. This syringe size fits securely inside a 50 mL conical tube.
12. Remove the plunger and set it aside.
13. Securely cap the syringe with a Luer lock end cap and place it inside of a 50 mL conical tube. Parafilm on the top of the syringe. Select an appropriate balance for the centrifuge.
14. Spin at 1000 × RCF for 5 min at 4 °C. Transfer the syringe to an ice bucket.
15. Aspirate the PBS, leaving wet cells in the syringe (*see* **Note 6**).
16. Form the frozen cell pellets (BBs) following the steps below. Use care and best practices in handling LN_2. Appropriate protective gear and goggles should be used to prevent injury. In lieu of cryo gloves, which are not ideal for fine manipulations, we use nylon glove liners underneath thick nitrile gloves.
17. Insert a 3 × 3, 50 mL conical tube rack in a 5–7 L styrofoam box; fill the box with LN_2 to the top of the rack. The setup that follows is displayed in Fig. 2.
18. Place a 500 mL beaker or pitcher on top of the rack and fill it with 50–100 mL of LN_2. Pre-cool a funnel whose spout will fit inside the opening of a 50 mL conical tube. Beaker and funnel should be composed of Nalgene® or other LN_2-compatible material. Pre-label a 50 mL conical tube, transfer it to the rack, and partly fill with LN_2 (to prevent it from floating). You may punch several holes in the cap of the 50 mL tube using an 18 ga needle; this permits careful drainage of the LN_2 from the tube (described below).
19. Pre-chill a small stainless steel spatula, standing it vertically in the LN_2 by placing it in one of the rack positions or leaning in the corner.
20. Hold the syringe over the beaker containing 50 mL LN_2, remove the Luer lock cap (it may be necessary to use pliers to grip and turn the nozzle cap), gently work the plunger into the syringe at an angle, and inject the cells gradually into the beaker containing LN_2. If injected too fast, they will form large clumps. Use the pre-chilled spatula as needed to break apart any clumps.

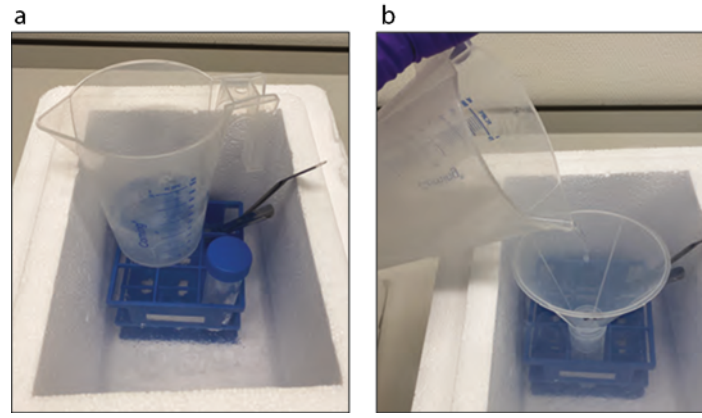


Fig. 2 Setup for freezing cell BBs in LN_2. (**a**) Pitcher containing LN_2, seated on top of a 50 mL tube rack in LN_2 bath, including a 50 mL tube (also containing LN_2) and metal utensils. Frozen cells are squirted from the syringe into the pitcher containing LN_2, producing BBs. (**b**) The BBs are transferred to the 50 mL tube by pouring them through, using the funnel. If holes are poked through the tube cap ("punched cap," not shown), it can be replaced on the tube when needed so that excess LN_2 can be removed by inverting the tube into a sink without losing the cell BBs. More BBs in LN_2 may then be transferred by pouring. The same can be done once all BBs are successfully transferred. When finished, close the tube using an intact cap and store the cell BBs at −80 °C or colder

21. Place the funnel inside the 50 mL conical tube in the rack in the styrofoam cooler. Slowly and carefully pour the contents of the beaker into the funnel in the conical tube. You can periodically cap the tube, using a cap with pre-punched holes (e.g., made by piercing the cap with a needle [we use 18 gauge], multiple times), and pour out the LN_2 to make more space (*see* **Note 7**). Replace the punched cap with an intact cap and store tubes at −80 °C until cryomilling (*see* **Note 8**). Cell BBs may be stored at −80 °C (or colder) indefinitely.

3.3 Cryomilling

We cryomill cells under liquid nitrogen in a Retsch PM 100 planetary ball mill [60]. For ~1–8 g cells, we use a 50 mL jar with two 20 mm diameter balls (*see* **Note 9**). Custom-made PTFE insulators [60, 61, 86] minimize warming of the sample during milling and improve safety and performance (*see* **Note 10**); the below assumes a "sleeve and puck" insulator is used. We use a homemade LN_2 decanter made using a spatula and a 50 mL conical tube to pour LN_2 into and over the milling jars [85].

1. Pre-clean milling jar, lid, balls, two small steel spatulas, and large forceps using Windex® glass cleaner or similar. Inspect the PTFE gasket for signs of damage. Weigh the jar + insulators + balls and adjust the PM 100 counterbalance accordingly (*see* **Note 11**).

2. Pre-cool the jar, balls, spatulas, forceps, LN_2 decanter, and PTFE insulators in a 9 L ice pan containing LN_2 until the LN_2 stops boiling (*see* **Note 12**).
3. Transfer the cold PTFE base to the PM100.
4. Transfer the frozen cell BBs into the milling jar.
5. Fill with LN_2 to within ~0.5–1 cm of the top. Cover with the lid and Teflon top insulator. Move the jar plus lids, en bloc, into the PM100, and clamp in place (*see* **Note 13**).
6. Pour LN_2 over the jar using the large decanter until it overflows.
7. Mill that material with three cycles of the following program: 400 rpm, 3 min, reverse rotation every 30 s, no interval breaks (*see* **Note 14**). Between milling cycles, the jar will need to be cooled. This can be achieved by pouring LN_2 over the jar, in situ, using the homemade decanter.
8. After the completion of the three cycles, remove the jar. Slowly release clamping pressure (*see* **Note 15**). Transfer jar assembly to the pan of LN_2.
9. Put a pre-labeled 50 mL tube in a rack in the LN_2 pan. Remove the steel balls from the milling jar with forceps, dislodging large chunks of cell powder with a spatula.
10. Transfer milled cells to conical tubes with chilled spatulas or spoons. Once the sample is fully transferred, cap the tube loosely and move it to a rack inside the Styrofoam box.
11. Store vertically at −80 °C overnight with the caps loose to allow LN_2 to evaporate, and then seal and store (*see* **Note 16**).

3.4 Conjugation of Dynabeads with α-ORF1p Antibody and mIgG

Nucleophilic side chains and N-termini on the antibody react with epoxide functional groups on the bead surface (*see* **Note 17**). It is critical that all other nucleophiles are absent from the solution, or these will react with the beads and prevent antibody coupling. This includes tris, glycerol, azide, and other common antibody storage solution components. It is safest to buffer exchange antibodies from commercial sources unless the absence of nucleophiles can be assured (*see* **Note 18**). The below protocol is given with the α-ORF1p clone 4H1 antibody in mind (*see* [87] for a general protocol). The MilliporeSigma product contains 0.1 M Tris-Glycine (pH 7.4), 150 mM NaCl, and 0.05% (w/v) sodium azide. The Tris and azide are interfering species and should be removed by desalting them into 0.1 M sodium phosphate, pH 7.4. Mock IPs used as nonspecific binding controls can be done using beads coupled to naïve mouse polyclonal IgG, or isotype-matched mouse IgG1κ, (mIgG) following the identical procedures.

3.4.1 Antibody Buffer Exchange with Microcentrifuge Desalting Columns

For the "Antibody Coupling" procedure described below, use Zeba™ Spin Desalting Columns 7 K MWCO, 0.5 mL, if your antibody contains any species that will interfere with the Dynabeads epoxy coupling chemistry (*see* **Note 19**).

1. Pre-equilibrate Zeba™ Spin Desalting columns in 0.1 M sodium phosphate pH 7.4; three times according to the manufacturer's instructions.
2. Load, centrifuge, and recover exchanged antibody solution according to the manufacturer's instructions.

3.4.2 Antibody Coupling

This protocol is for the coupling of 60 mg Dynabeads. In the last step, the beads are slurried by the addition of 400 μL storage solution (*see* **Note 20**). We also routinely couple with larger amounts of Dynabeads, slurried accordingly. For a larger scale coupling, scale all volumes in the protocol linearly. A commercial coupling kit for Dynabeads is available from Life Technologies; this uses proprietary components but is effective. We want the final antibody concentration in the antibody mix to be 0.5 mg/mL. This requires the original antibody concentration to be at least 0.75 mg/mL. If the antibody concentration is less than 0.75 mg/mL, 4 M ammonium sulfate stock can be used instead of 3 M stock to reduce the volume. Alternatively, antibodies can be concentrated before being added to the antibody mix. Low antibody concentration (<0.5 mg/mL) in the antibody mix may cause lowing coupling efficiency. The procedure can be performed in 2 days, with coupling on day 1 (**steps 1–8**), and bead washing on day 2 (**steps 9–17**).

1. Calculate how much antibody to use. One milligram of Dynabeads M-270 Epoxy has been estimated to immobilize 7–8 μg of antibody; coupling is not 100% efficient, and excess antibody appears to help drive the reaction [88]. For α-ORF1p, we use 10 μg antibody/mg Dynabeads (*see* **Note 21**).
2. Before beginning, allow the bottle of Dynabeads to equilibrate to RT (~10–15 min). Do not uncap the bottle while cold, or you will risk getting condensation inside the bottle, which can compromise bead quality.
3. Weigh out 60 mg of Dynabeads M270 Epoxy in a 1.5 mL microfuge tube using antistatic scoops. These scoops are especially useful for amounts <100 mg. Add 1 mL of 100 mM phosphate buffer, pH 7.4 to the tube. Vortex briefly and transfer beads to a 5 mL Eppendorf tube. Rinse the microfuge tube twice with 1 mL of 100 mM phosphate buffer and transfer the wash to the 5 mL Eppendorf tube (3 mL total). Vortex for 30 s, then incubate for 10 min on a nutator or end-over-end mixer.
4. While the beads mix, prepare the antibody mixture (20 μL/mg Dynabeads, 1.2 mL total). Calculate the volume of 0.6 mg of

antibody solution based on the antibody concentration. Transfer the appropriate amount of antibody to a 5 mL Eppendorf tube. Add 100 mM phosphate buffer, pH 7.4, to 800 µL to the antibody solution, and mix well. Add 400 µL 3 M ammonium sulfate (final concentration will be 1 M) to the tube, drop-wise, with constant mixing to avoid creating a high local ammonium sulfate concentration (which could cause the antibody to precipitate). The final volume of the antibody mix will be 1.2 mL.

5. Transfer the beads to a magnetic separator. Aspirate the buffer.
6. Wash again with 3 mL 100 mM phosphate buffer. Add buffer, vortex for 15 s, apply magnet, and aspirate; no incubation is necessary.
7. Add the antibody mixture to the beads in a 2 mL locking microcentrifuge tube. Mix well.
8. Incubate overnight (18–24 h) on a rotating wheel (or thermomixer set to 1000 rpm) at 37 °C (30 °C is also effective). If a climate room is unavailable, we have successfully placed the rotating wheel or thermomixer inside a small 37 °C incubator.
9. Bead washing is generally performed using a vacuum aspiration system. We have observed that in the absence of detergent, a small fraction of beads can be lost when using the vacuum, so these bead washing steps can also be performed by pipette. When using a solution that contains detergent, the vacuum system is ideal.
10. Separate beads from the antibody mixture with a magnet. Carefully remove the antibody mixture and set it aside in a clean tube: it still contains 30–50% of the antibody, unreacted, which can be recovered for reuse (*see* **Note 22**; Fig. 3a). We routinely save antibody mixtures for direct use in other assays such as immunoblotting; recovery also allows concentration of antibodies and transfer into a more permanent storage solution.
11. Wash beads once with 3 mL 100 mM glycine pH 2.5. Add the solution, vortex briefly, and take it off as fast as possible.
12. Wash once with 3 mL 10 mM Tris–HCl, pH 8.8.
13. Prepare fresh 100 mM triethylamine: Add 42 µL stock to 2.958 mL water. Apply, mix, remove, and proceed to the next step as fast as possible.
14. Wash the beads with 3 mL 1× PBS, incubating for 5 min on the nutator. Repeat four times for a total of five washes.
15. Wash twice with 3 mL PBS + 0.5% (v/v) Triton X-100, incubating each wash for 10 min on the nutator.
16. Resuspend beads in 400 µL storage solution: 1x PBS, with 50% (v/v) glycerol and 0.5 mg/mL BSA (*see* **Note 23**).

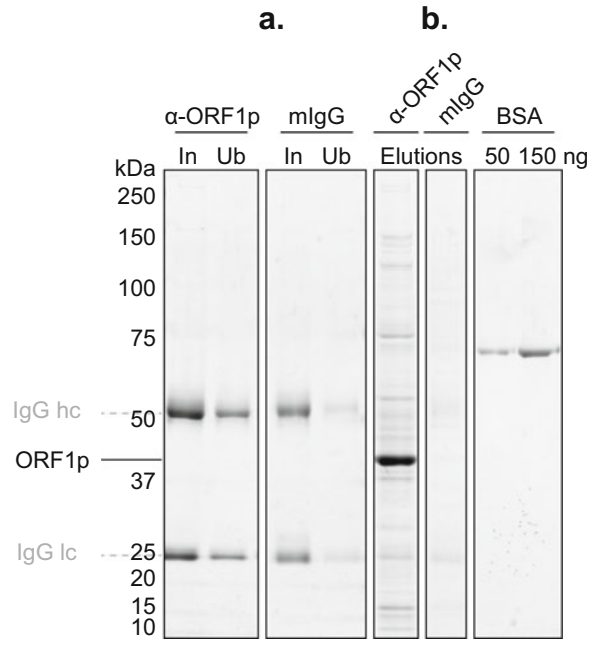


Fig. 3 Antibody coupling and example IP-Coomassie stain results. This Coomassie blue G-250 stained, 4–12% Bis-Tris SDS-polyacrylamide gel illustrates various protein bands and relative intensities expected when making affinity media and conducting IPs. (**a**) Antibody solutions used in bead coupling (*In*put and *Un*bound from coupling reactions using *α-ORF1p* IgG and naïve *m*ouse *IgG*). (**b**) Elutions from IPs conducted using those beads. ORF1p and IgG *h*eavy *c*hain and *l*ight *c*hain are indicated. 50 and 150 ng of BSA have been loaded as a comparative quantity standard

17. Mix well and aliquot 100 μL each into Eppendorf tubes. Store at −20 °C.

3.5 Affinity Capture Using Conjugated Magnetic Medium

For a comprehensive review of considerations affecting expression systems, epitope tagging, and affinity medium choice *see* [89]. In brief, we find that Dynabeads, when conjugated to high-quality antibodies, provide for high-fidelity recovery of endogenous protein complexes from human cells [85, 88]. We have successfully applied this approach to L1 RNPs [32]. When combined with neodymium magnet racks, antibody-conjugated magnetic medium (beads) is rapidly separated from the solution and immobilized on the side of the tube. This allows near complete aspiration of the buffer without the risk of aspirating the beads. Before beginning, ensure all solutions to be used have the appropriate additives and are at the correct temperature (RT for extraction solution, ice-cold for wash solution).

3.5.1 Prepare Clarified Cell Extracts

1. Fill a ~4 L ice pan with ice.
2. Place a Nalgene® rack inside a small styrofoam cooler and fill it to the middle of the rack with LN_2. Pre-cool all tools in a styrofoam cooler. Pre-label locking, 2 mL microcentrifuge tubes by dropping them with open caps into the LN_2. Retrieve them with pre-cooled, large forceps.
3. Remove cell powder from −80 °C and place it in Nalgene® rack inside cooler prepared in **step 2**. Cell powders must always be kept on LN_2 when not at −80 °C.
4. Using a microbalance and pre-cooled tools, weigh out 100 mg of cell powder into a pre-labeled, pre-cooled tube—hold on LN_2 (*see* **Note 24**).
5. Repeat **step 4** for as many purifications as needed; multiple purifications can be pooled after elution if a larger scale is required.
6. Move the tubes to RT for 1–2 min (*see* **Note 25**).
7. Add 400 μL of extraction solution (*see* **Note 26**) (20 mM Na-HEPES pH 7.4, 500 mM NaCl, 1% v/v Triton X-100; plus protease inhibitors) to each tube, vortex for ~30 s until powders are resuspended, and then place the crude extracts on ice. If large chunks of visible powder remain, another round of vortexing can be completed after the sample rests briefly on ice. Some membrane aggregates may be observed.
8. Sonicate each tube with a micro-tip probe at 4 °C. We calibrate sonication energy in the following way: for 100 mg of cell powder, combined with 400 μL extraction solution, we apply ~15 J of energy spread across 5 × 2 s bursts with 2 s pauses between each pulse [60] (*see* **Notes 27** and **28**). Membrane aggregates should typically no longer be visible.
9. Centrifuge for 10 min at full speed (20,000 × RCF) in a refrigerated microcentrifuge at 4 °C. During this step, the affinity medium can be pre-washed (Subheading 3.5.2, **step 2**).
10. Remove supernatant—this is your clarified extract—and add to the tube containing α-ORF1 Dynabeads or mIgG control beads (Subheading 3.5.2, **step 5**).
11. Set a fraction aside before combining with beads to compare pre- and post-bead binding to assess the efficacy of the affinity capture.

3.5.2 Affinity Capture

1. To prepare beads, place one 1.5 mL safe-lock tube per reaction onto a magnet. Pipette 1 mL extraction solution into each empty 1.5 mL microcentrifuge tube.
2. Pipette 20 μL of α-ORF1 or mIgG control Dynabeads slurry into the solution, with the microcentrifuge tube on the magnet

(*see* **Note 29**). Pipette up and down until the pipette tip is clear (no beads left behind). When the beads are adhered to the side of the microfuge tube, remove the solution.

3. Wash the beads two additional times with 1 mL of extraction solution and remove it.
4. Hold the beads on ice until needed.
5. Combine the clarified extract with the beads. Incubate at 4 °C for 30 min.
6. Separate beads on a magnetic separator. Set a fraction aside to compare with the input and aspirate the remainder.
7. Wash the beads with 1 mL of the extraction solution and then remove the supernatant. The wash protocol for beads described below (**steps 8–10**) is used throughout.
8. Add the solution and vortex at full power for 2–3 s.
9. Pulse-spin in a benchtop microcentrifuge to remove any magnetic beads from the cap.
10. Separate beads on a magnetic separator and remove the solution using a vacuum aspirator.
11. Resuspend the beads in 1 mL of extraction solution, transfer to a fresh microcentrifuge tube, place them on the magnet and then aspirate the solution (*see* **Note 30**).
12. Wash again with 1 mL of extraction solution, place them on the magnet, and then aspirate the solution.
13. Pulse-spin in a benchtop microcentrifuge to get any residual solution to the bottom of the tube; put the tube back on a magnetic separator and then aspirate the last bit of solution before elution.
14. Depending on the downstream analysis, beads can be eluted in different reagents:
    (a) For basic protein analysis (SDS-PAGE/Western): Elute in 25 µL of 1.1× LDS sample loading buffer; *see* Subheading 3.6.
    (b) For RNA analysis: Elute in 250 µL of Trizol (*see* Subheading 3.7).
    (c) For MS analysis: Elute in 50 µL of 2.5% w/v SDS (buffered in, e.g., 10–40 mM Tris or HEPES pH 7.4–8); *see* Subheading 3.8.

3.6 SDS-PAGE and Western Analysis

3.6.1 SDS-PAGE

1. To elute samples in LDS, add 25 µL of LDS to the beads.
2. Incubate at RT for 10 min or 70 °C for 5 min with mixing.
3. Collect the eluate.
4. Add 500 mM DTT to a final concentration of 50 mM (*see* **Note 31**).
5. Heat samples at 70 °C for 10 min.

6. Load 90% of each sample on a 15-well or 26-well, 4–12% NuPAGE™ Bis-Tris gel, following the manufacturer's instructions.
7. Run at 200 V until the tracking dye reaches the bottom of the gel cassette.
8. Remove the gel from the plastic cassette and proceed to stain with, for example, colloidal Coomassie brilliant blue G-250 (*see* **Note 32**); we use modified "Blue silver" staining [62, 90] (Fig. 3b).

3.6.2 Western Analysis

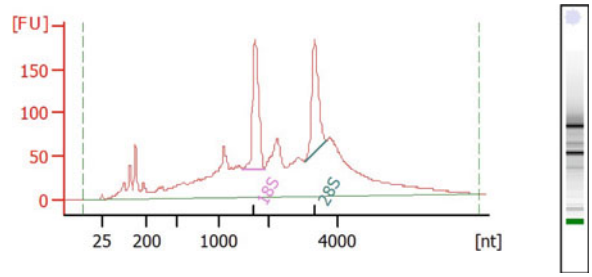
1. Run SDS-PAGE as described above; this time load 10% of each sample.
2. Conduct a wet transfer at 70 V for 1.5 h at 4 °C using PVDF membrane [91].
3. Block the membrane in Tris-buffered saline with 0.1% (v/v) Tween® 20 (TBST) with 5% (w/v) nonfat dry milk, at RT for 2 h.
4. Apply primary antibody to membrane: mouse α-ORF1p (MilliporeSigma, clone 4H1 at 0.4 μg/mL or ~1:5000 dilution), 4 °C overnight; secondary antibody: ECL anti-mouse HRP 1:10,000, RT 1 h.
5. Wash the membrane with TBST after primary and secondary antibody incubation (3 quick rinses followed by 3 × 10 min wash).
6. After the final wash, remove any excess TBST.
7. Add Immobilon Forte Western HRP substrate to the membrane. Make sure the substrate is enough to cover the entire surface of the membrane.
8. Remove any excess reagent and cover the membrane in transparent plastic wrap.
9. For signal development, we use the GE FujiFilm ImageQuant LAS-4000 Luminescent Image Analyzer.

3.7 RNA Extraction

Before beginning, clean the bench surface and micropipettes with Invitrogen™ RNaseZap™ or a similar RNase decontamination solution according to the manufacturer's recommendation. Recombinant RNasin® should be added to the extraction and wash solutions. All steps should be performed using certified nuclease-free, filter pipette tips. Lab coats and gloves should be clean. Change gloves as often as needed. Ideally solutions should be made with nuclease-free water. Once the affinity media is in Trizol, all subsequent steps can be performed at RT.

1. Perform the Affinity Capture as described above in Subheading 3.5. Stop after the last bead wash.

2. Elute beads in 250 μL Trizol at RT. Add 250 μL Trizol to each tube, and vortex for 1 min. Pulse spin, then return tubes to the magnet.
3. Spin the Phasemaker tube at 16,000 × RCF 30 s.
4. Add 50 μL chloroform and 25 μL nuclease-free water to the Phasemaker tube. Do this immediately before adding Trizol elutions.
5. Collect the elution and transfer it to the Phasemaker tube.
6. Mix by hand, vigorously for 15 s.
7. Incubate for 2 min at RT with end-over-end mixing. This step is almost certainly dispensable for IP—it is supposed to be time for RNPs to dissociate.
8. Spin 16,000 × RCF, 5 min.
9. Transfer the aqueous phase to a new tube by pressing the pipette tip against the wall of the tube; avoid puncturing the Phasemaker gel.
10. Pool replicates at this step, if necessary (must be from the same cell line, same beads). Check the total volume of the combined aqueous phase.
11. Check the volume.
12. Add an equal volume of 100% EtOH to each sample and mix thoroughly, vortex, and pulse spin.
13. Check the volume again.
14. The remaining steps are completed using the Zymo kit. Transfer the mixture to a Zymo-Spin IC column in a collection tube and centrifuge at 16,000 × RCF for 30 s. The maximum capacity of each Zymo column is 700 μL. To process a sample with a greater volume, reload the sample and spin again.
15. DNase I treatment: Add 400 μL RNA Wash Buffer to the column and centrifuge at 16,000 × RCF for 30 s (in an RNase-free tube, add 5 μL DNase I 95 U/μL), 35 μL DNA Digestion Buffer and mix. Add the mix directly to the column. Incubate at RT (20–30 °C) for 15 min.
16. Add 400 μL Direct-zol RNA PreWash to the column and centrifuge at 16,000 × RCF for 30 s. Discard the FT and repeat this step. Use the vacuum-trap system to clear the liquid from the tubes both times.
17. Add 700 μL RNA Wash Buffer to the column and centrifuge for 2 min at 16,000 × RCF to ensure complete removal of the wash buffer. Transfer the column carefully into an RNase-free tube.



RNA Area: 3,239.1
RNA Concentration: 6,447 pg/µl
rRNA Ratio [28s / 18s]: 0.8
RNA Integrity Number (RIN): 6.5

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,461	1,904	320.3	9.9
28S	3,045	3,667	252.2	7.8

Fig. 4 RNA quality control: example Agilent 2100 Bioanalyzer output. Representative electropherogram, pseudo-gel image, and sample quality metrics. With N2102Ep cells, RNAs obtained following the described protocols provided RINs OF between 6 and 7. While these samples are depleted of DNA by TRIzol extraction, they have not been treated with DNase (this is an optional step using the Zymo kit). DNase treatment was done during the Trio library preparation. If DNase treatment is required before your library preparation, this can be done on the column during the Zymo RNA microprep

18. Collect RNA samples: To elute RNA, add 6 µL of RNase-Free Water directly to the column matrix and centrifuge at 16,000 × RCF for 30 s.
19. Store the two sets (1 and 5 µL for each) of tubes at −80 °C.
20. Use the 1 µL aliquot for Bioanalyzer analysis using the pico kit—to obtain RNA quality and concentration information (Fig. 4).
21. Use the 5 µL aliquot for cDNA library production prior to RNA-seq.

3.8 MS Sample Preparation

Proper preparation of samples is critical for mass spectrometry, as a number of interfering species can reduce sensitivity and compromise protein identification (for discussion and advice, *see* [89]). For identification of the species that can be readily observed by standard protein staining techniques (reviewed in [92, 93]), we excise the region of interest in the gel and use GeLC-MS [94, 95]. For sensitive detection and identification of proteins in the entire fraction, we use liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) approaches (reviewed in [96, 97]. For IP elutions, we prefer to use the Ultra High Recovery S-trap™ protocol (https://protifi.com/pages/s-trap) given the small amount of protein coming from this type of samples. A version of the S-trap protocol that we use in our lab follows:

1. Dry down the sample completely in a SpeedVac at RT and resuspend protein from IPs in 25 μL of 8 M urea, 100 mM glycine buffer, pH 7.55—the suspension must contain a final concentration of 5% (w/v) SDS, which is achieved by following the instructions in Subheading 3.5.2, **step 14.c**. (*see* **Note 33**).
2. Reduce disulfide bonds by adding 1 μL of 120 mM TCEP (*see* **Note 34**).
3. Incubate at 37 °C for 15 min (Higher temperatures are not recommended due to the presence of urea).
4. Put the tube briefly on ice to bring the temperature down.
5. Alkylate-free cysteines by adding 1 μL of 0.5 M MMTS to the sample (*see* **Note 34**).
6. Incubate at RT for 10 min (*see* **Note 34**).
7. Add 2.5 μL 64.2% (w/w) phosphoric acid to the sample (*see* **Note 35**).
8. Add 165 μL of S-Trap™ binding/wash buffer (9:1 methanol: TEAB 1 M pH = 7.55) into the S-Trap™ column. The following two steps must be done as quickly as possible.
9. Add 10 μL Trypsin/Lys-C stock solution (200 ng/μL in 50 mM TEAB pH 8.5) to the sample and immediately mix by pipetting up and down.
10. Immediately transfer the sample into the S-Trap™ column and mix by pipetting up and down.
11. Spin down in a centrifuge in a 2.0 mL microfuge tube at 4000 × RCF until all the solution has passed through (30 s is enough). Discard flowthrough.
12. Wash by adding 150 μL S-Trap™ binding/buffer to the spin column and centrifuge 4000 × RCF, 30 s (*see* **Note 36**).
13. Repeat the washing step twice for a total of three washes. For the final wash, centrifuge for 1 min to get rid of all the solvents.
14. Place the S-Trap™ column into a new 2 mL Lo-bind tube. Dry the tip of the column with tissue when necessary.
15. Add 25 μL of Trypsin/Lys-C working solution (20 ng/μL in 50 mM TEAB pH 8.5) to the top of the S-trap™. Make sure bubbles are not formed on the surface of the packing material.
16. Cap the S-Trap™ column loosely and incubate for 1 h at 47 °C (*see* **Note 37**).
17. Elute the peptide mixture in three steps by adding the solvent on top of the column, then centrifuging at 4000 × RCF for 1 min before adding the next elution buffer:
    (a) 40 μL of 50 mM TEAB pH 8.5.
    (b) 40 μL of water:formic acid 99.8:0.2 (v:v).
    (c) 35 μL of water-acetonitrile 1:1 (v:v).

18. Dry down in a SpeedVac at a max temperature of 30 °C (if the samples are going to be left on the SpeedVac overnight, use RT instead).

3.9 Protein Analysis by Mass Spectrometry

Always wear gloves when handling any sample, reagent, flask, or tube that is going to be analyzed via MS in order to avoid keratin contaminations.

1. Resuspend the sample in 20–25 μL of an adequate solvent (*see* **Note 38**).
2. Vortex and spin down the tubes.
3. Pipette up and down their content to maximize the peptide's resuspension and transfer to an MS vial.
4. Get rid of any bubble that is formed at the bottom of the MS vial by shaking it (*see* **Note 39**).
5. MS vials are placed in the HPLC autosampler.
6. LC solvents must be degassed and can be stored in glass bottles that are typically placed above the LC system (*see* **Note 40**).
7. Before running any sample, make sure that instrument maintenance has been performed satisfactorily (*see* **Note 41**).
8. Samples can be run using many different MS methods, depending on their nature. Some of our standard parameters are listed in **Note 42**, and an example chromatogram can be found in Fig. 5.

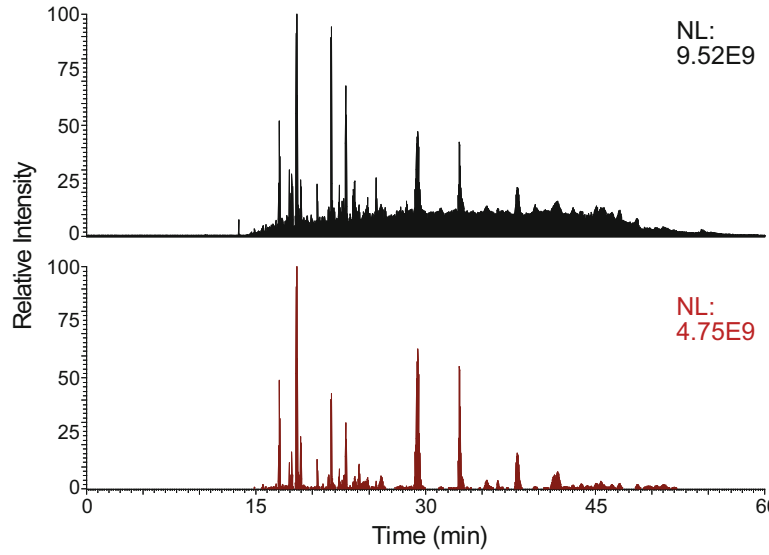


Fig. 5 Mass spectrometry: example mass chromatograms. Total Ion Current (TIC, top panel) and Base Peak Intensity (BPI, bottom panel) LC-MS chromatograms for an α-ORF1p IP sample from N2102Ep, generated in the manner described by these protocols. 473 proteins were identified and quantified after data analysis under these conditions

9. Replicates of the same type can be run one after the other. When changing the type of sample to be run, a blank should be run in between two different types of samples to help get rid of any remaining carryover from the previous ones.

3.10 Protein Bioinformatics Analysis

After MS data acquisition, you will have a collection of Thermo RAW files that accord to each IP-MS run. These files will typically need to be transferred from the MS-instrument-attached PC to a workstation or HPC cluster for processing using MQ. The computational requirements and related considerations can be found on the MQ website (https://www.maxquant.org) and in [69]. The features of the MQ software [71] are updated/changed periodically as new versions are released, and this affects the settings for optimal use—a guide has been published recently [70]. We provide general guidance for MQ parameters that apply as of the time of writing, for LFQ analysis. For example analyses, you can refer to these publications [26, 98]. The primary output is one or more volcano plots exhibiting the associated adjusted *p*-values and effect sizes of protein enrichments in the α-ORF1p IPs (cases) compared to mock IPs (controls) (Fig. 6). The proteins exhibiting statistically significant enrichment are candidate-specific interactors of ORF1p; these proteins can be initially explored for their potential functions in L1 biology via, for example, their common memberships in macromolecular complexes, their participation in common metabolic pathways, and their ontological associations. This information can be used to prioritize targets for orthogonal biological validation.

3.10.1 RAW Data Processing Using MQ

1. Load the IP and mock IP (or other control) RAW files into MQ—depending on the nature of your analysis and the context of different IP/control experiments, you will want to group them accordingly; MQ has settings that apply globally and settings that can be differentially applied to limited groups (*see* **Note 43**). Many settings can be left as default—but attention is drawn to the following. *Number of threads*: every assigned thread will process one RAW file at a time (the maximum number of files that will be processed in parallel is equal to the number of threads); leave two threads unused to carry out system tasks and background processes (to avoid system hanging or crashes during MQ processing); each thread will draw upon 2 GB of system memory. *Fixed Modifications*: if using S-traps as described in this protocol, the MMTS-derived dithomethane modification of cysteine residues must be specified. *Modifications included in protein quantification*: variable modifications such as oxidation (M) and acetyl (Protein N-term) are typically included, as is the fixed modification used to block free cysteine residues. Phospho (STY) can be included in the search, but because these regulatory modifications are highly variable, they should be excluded from

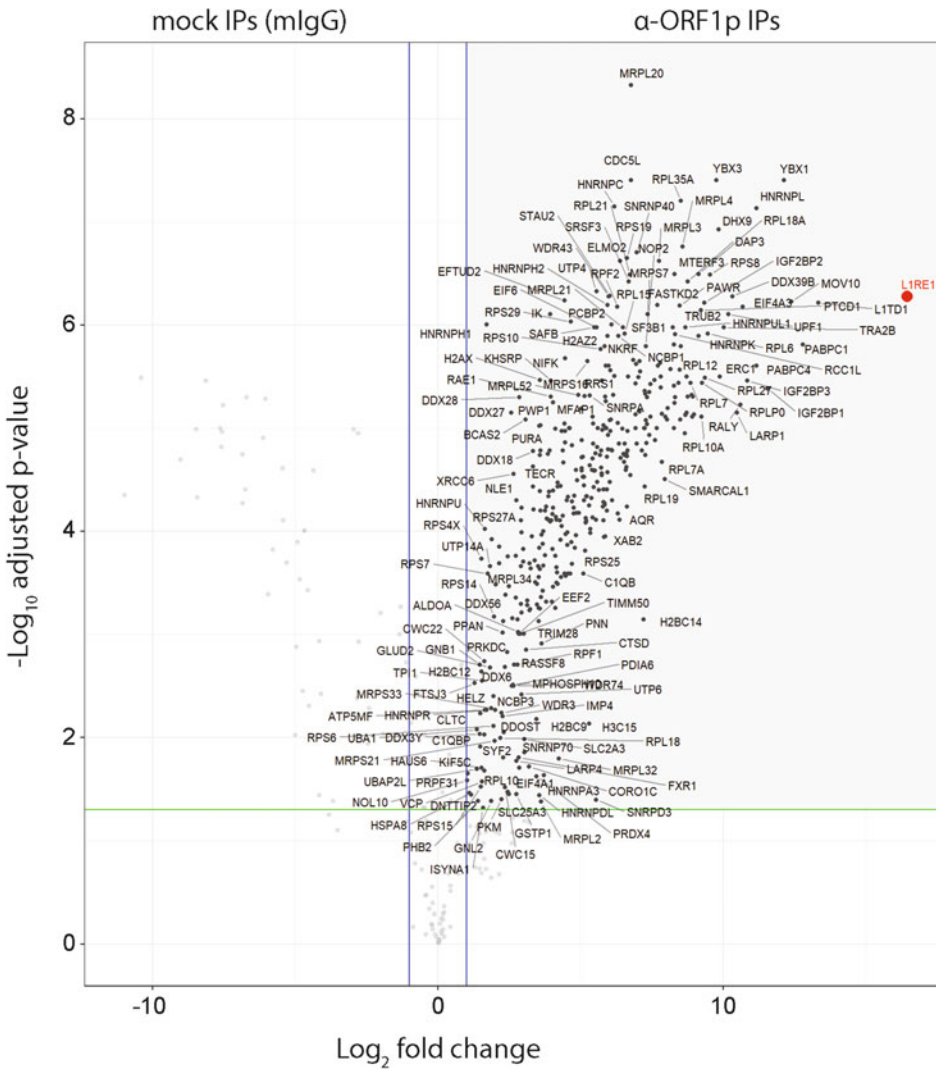


Fig. 6 Label-free quantitative MS: example volcano plot; IP-MS of α-ORF1p vs. naïve mouse IgG IPs (IP-MS) from N2102Ep cells. Using at least three LC-MS/MS sample replicates of the case (α-ORF1p IP) and control (mIgG IP), the effect sizes of protein enrichment differences between the case and control IPs (*x*-axis; $\log_2$ of LFQ intensity case/control) were graphed against the probability of observing a difference at least that large when the null hypothesis is true, corrected for multiple hypothesis testing (*y*-axis; $-\log_{10}$ of *p*-values obtained from an ANOVA-like test with Benjamin-Hochberg correction). Statistical significance is assigned at an adjusted *p*-value ≤ 0.05 (green line) and $\log_2$ fold change ≥ 1 (blue lines, 2× effect size). Black dots represent proteins that were significantly co-enriched with ORF1p upon α-ORF1p IP (right side, between green and blue lines); gray dots are proteins that were not co-enriched with α-ORF1p IP or were de-enriched compared to the control. ORF1p (Uniprot gene symbol L1RE1) is indicated in red

quantification along with their unmodified counterpart peptides. *Match between runs*: True (*see* **Note 44**). *Second peptides*: True. *Stabilize large LFQ ratios*: True. *Separate LFQ in*

parameter groups: False (*see* **Note 43**). *Require MS/MS for LFQ comparisons*: True.

2. We suggest using a protein database composed of the Uniprot human proteome (reviewed) with isoforms, *see*: https://www.uniprot.org/help/human_proteome. The appropriate FASTA file can be obtained by querying: https://www.uniprot.org/uniprot/?query=proteome:up000005640+AND+reviewed:yes&format=fasta&include=yes. This database includes a single entry of representative protein sequences for L1 ORF1p (L1RE1/Q9UN81) and ORF2p (O00370).
3. Once satisfied with your MQ settings, save them: the mqpar.xml file produced contains all the settings and can be loaded to expedite future analyses.

3.10.2 Post-processing in R

Below, we briefly describe a basic analysis. Aside from this, you should construct a variety of plots at each step to validate the accuracy of the analysis, including histograms of intensities pre-imputation and post-imputation (**step 5**), density plots of *p*-values and adjusted *p*-values, volcano plots (**step 6**), scatter plots of the average LFQ intensity values of the case replicates against the average LFQ intensity values of the control replicates, and finally heat maps to compare the LFQ intensities between the most significant records or selected proteins of interest.

1. Once the MQ run has completed, you should run quality control (QC) on the output to make sure all the samples pass certain criteria, such as sufficient peptide intensity, low contaminant abundance, low frequency of missed trypsin cleavages, among others [99, 100]. We use the PTXQC package to prepare quality control reports [72]. Samples that do not pass QC metrics should be omitted from downstream analyses.
2. Create volcano plots and conduct GO enrichment analysis. First, from the MQ proteingroups.text file, filter out reverse sequences and potential contaminants.
3. Prepare LFQ intensity tables to perform, for example, an F-test: $\log_2$ transform LFQ intensities for all proteins in all case (IP) and control (mock IP) replicates, assorting them into one "comparison table."
4. Normalize the case and control replicates in the comparison table using linear regression from the NormalyzerDE package [73].
5. Impute missing values: because of the properties of LC-MS/MS and data-dependent acquisition, some protein intensity measurements will be missing from some samples (usually proteins that are not present or are relatively low abundance in the sample), and this will hinder the representation of those

proteins in terms of "fold change" because dividing a number by zero is undefined; imputing small values to replace missing values, instead of using zero, will allow the estimation of a fold change for all proteins detected and quantified in all samples (example methods and rationale described in [98]).

6. Fit a linear model between case and control replicates' $\log_2$ LFQ intensities to calculate the $\log_2$ fold change and perform an ANOVA-like test (e.g., F-test) between case and control replicate $\log_2$ LFQ intensities, producing associated *p*-values for each protein; calculate the adjusted *p*-values using the Benjamini-Hochberg procedure, to reduce type I error; the results can be visualized together using a volcano plot.
7. Filter protein records using the criteria adjusted *p*-value ≤ 0.05 and $\log_2$ fold change ≥ 1, to separate significant proteins from noise.
8. Perform GO enrichment analysis on the list of significant proteins [74].

3.11 RNA Bioinformatics Analysis

After RNA-seq data acquisition, you will have a collection of FASTQ files that accord to each sequencing run; these files will typically need to be transferred from a server at the sequencing facility to a workstation or HPC cluster for processing. Here, we provide general guidance for methods and parameters that apply at the time of writing. The final output will be, for example, (1) a table of calculated sample RNA abundances, differential abundances obtained from cross-sample comparisons, and the adjusted *p*-values for the observed differences. as well as (2) one or more plots to summarize and visualize the results (e.g., volcano plots, heat maps), exhibiting the associated adjusted *p*-values and effect sizes of RNA enrichments in the α-ORF1p IPs (cases) compared to mock IPs and total RNA, respectively (controls); *see* Fig. 7a, b. The RNAs exhibiting statistically significant enrichment are candidate-specific interactors of ORF1p; these RNAs are presumed to be assembled into large heterogeneous RNPs containing L1. This information can be used to compare the RNA content of different L1 RNP enriched fractions and to explore the context of L1 RNP assembly along with other RNAs [50, 58].

3.11.1 RIP-Seq Analysis Steps Are Similar to Typical RNA-Seq Analysis

1. Align FASTQ files with STAR, using the following parameters, to produce BAM files [75].
2. Make counts from aligned genes using featureCounts [102].
3. Construct a differential gene expression list (DGEList) using edgeR, including the counts matrix (output of *featureCounts*), the groups vector (e.g., data originates from RIP-seq or total RNA-seq, etc.), and a list of genes as inputs [77].

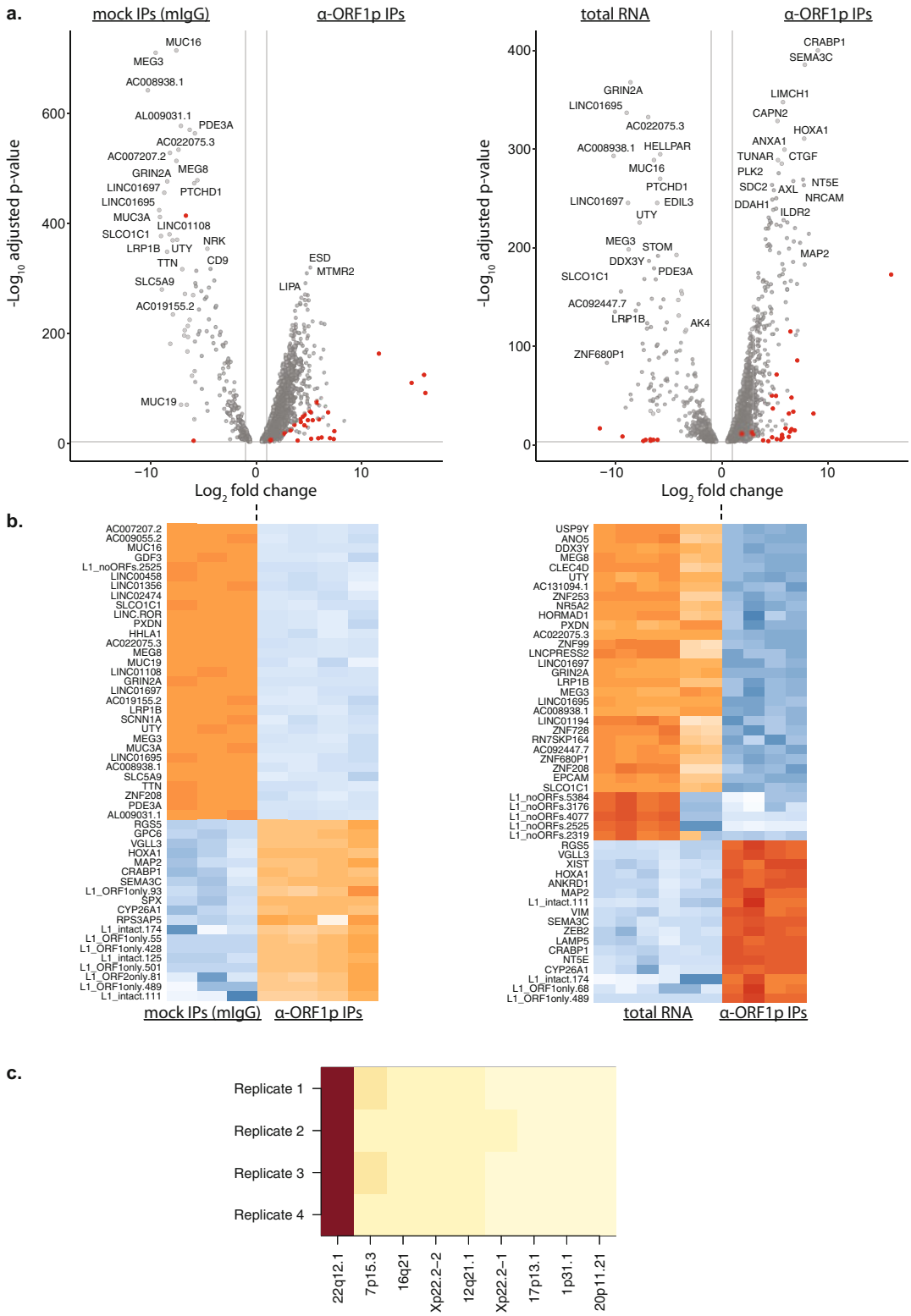


Fig. 7 L1 and other RNAs: example volcano plots and heat maps; RIP-seq of α-ORF1p vs. naïve mouse IgG IPs and total RNA from N2102Ep cells; L1EM ranking of abundant loci. Using at least three sample replicates of

4. Filter records, retaining genes with more than 100 cpm (counts per million reads mapped) in at least two replicates.
5. Normalize the DGEList using the *calcNormFactors* function to find scale factors that minimize $\log_2$ fold changes between samples.
6. Calculate log-counts-per million (logCPM) using the *cpm* function with parameter log = TRUE
7. Perform exploratory analysis on logCPM, producing a PCA plot.
8. Construct a model matrix and estimate dispersions using the DGEList and the model matrix.
9. Use, for example, the *glmFit* function to fit a generalized linear model to the read count matrix.
10. Perform an ANOVA-like test between case and control values defined in the model matrix using, for example, the *glmLrt* function.
11. Extract the differential expression table, including adjusted *p*-values, using the "BH" method.
12. Make volcano plots to visualize upregulated (adjusted *p*-value ≤ 0.05 and $\log_2$ fold change ≥ 1) and downregulated (adjusted *p*-value ≤ 0.05 and $\log_2$ fold change ≤ -1) genes.

3.11.2 L1 Locus Detection Using L1EM

Because the youngest, most active L1 loci are highly repetitive, most reads will not map uniquely to a single locus. While it is possible to identify expressed L1 loci from the fraction of reads

Fig. 7 (continued) the case (α-ORF1p IP) and control (mIgG IP or total RNA, as indicated), (**a**) the effect sizes of RNA enrichment differences between the case and control replicates (*x*-axis; $\log_2$ of relative RNA abundances) were graphed against the probability of observing a difference at least that large when the null hypothesis is true, corrected for multiple hypothesis testing (*y*-axis; $-\log_{10}$ of Benjamin-Hochberg corrected *p*-values from edgeR). Statistical significance is assigned at adjusted *p*-value ≤ 0.05 (horizontal gray line) and $\log_2$ fold change ≥ 1 (vertical gray lines, 2× effect size). Gray dots represent RNAs that were significantly co-enriched with ORF1p upon α-ORF1p IP (right side) or de-enriched (left side); red dots represent L1 RNAs originating from different candidate loci. (**b**) A subset of RNAs from the same data (top 50 most different) are instead represented in heat maps. Each heat map corresponds to the volcano plot above it and has been truncated. *On the left*, four α-ORF1p IPs were compared to three mock IPs. Three mock IPs (the minimum) were performed to limit the cost and because they do not teach us anything about biology, instead providing a useful filter to remove false positive "sticky" RNAs from our hits. In comparison, *on the right*, six total RNA analyses were performed [101]. These were prepared in two batches (four and two), and a batch effect is visible in the heat map. The total RNA control provides biological insight: what transcripts are normally present in these cells and are extractable under our IP conditions; comparing these with the α-ORF1p IPs provides a basis to assess the affinity of association of cellular RNAs with L1 RNPs. (**c**) Using the output of L1EM, each locus (*x*-axis) that accounts for at least 2.5% of the *ORF1* intact L1 RNA in the ORF1p RIP-seq data was plotted against the experimental replicates (*y*-axis); they are ordered from most (dark color) to least (light color) abundant

that do align uniquely [67], accurate locus-specific quantification requires an expectation maximization approach [66, 103, 104]. We use L1EM because it is specifically tailored to capture the types of transcripts known to be generated from L1 loci [66]; these include, for example, L1-promoter-driven expression of the ~6 knt L1 mRNA (with cleavage and polyadenylation at its canonical site) as well as those L1 transcripts that are polyadenylated at a downstream site (i.e., 20–30% of L1s include read-through transcription of downstream human genome sequences), and RNAs containing L1 sequences which originate from a non-L1 promoter (a.k.a., passive co-transcription). To perform locus-specific analysis using L1EM (Fig. 7c):

1. Follow the instructions at https://github.com/FenyoLab/L1EM. This will perform all the necessary installation and setup needed to run L1EM:
   (a) Cloning of the L1EM git repository to your local machine
   (b) Downloading and indexing of the human "hg38" reference genome
   (c) Building of the L1EM custom reference using *generate_L1EM_fasta_and_index.sh*
2. Make the following adjustments to the *parameters.sh* file to account for the increased volume of LINE-1 reads in a RIP-seq experiment:
   (a) Optionally, adjust threads to the number of available cores on your machine. Make sure to have at least 4 GB of memory available to each thread.
   (b) Set realign_NM = 2
   (c) Set L1EM_NM = 2
   (d) Set NMdiff = 1
3. Determine whether your data are strand-specific. Strand specificity can easily be determined by looking at reads aligning to a housekeeping gene such as *GAPDH* or *ACTB*. In strand-specific data, the read 1 s will be antisense to the gene, while the read 2 s are sense. For data that is not strand-specific, a roughly equal number of read 1 and 2 s will align on each strand.
4. Run L1EM. This will likely take several hours. If the data is strand-specific, follow the instructions on GitHub: https://github.com/FenyoLab/L1EM. If the data is not strand-specific, use *run_L1EM_unstranded.sh* rather than *run_L1EM.sh*, but otherwise follow the instructions on GitHub.
5. Find the L1EM output. For strand-specific data, the *full_counts.txt* output provides a table of read count estimates

for 5 possible L1-containing transcripts at each locus (*see* **Note 45**):

(a) "only" = L1 RNAs that start at the 5′ UTR and end at the poly A site of the 3′ UTR

(b) "3prunon" = L1 RNAs that start at the 5′ UTR and end at a downstream polyadenylation site.

(c) "sense_runthrough" = L1 RNAs that start upstream of the locus and are sense to the L1 element.

(d) "antisense_runthrough" = L1 RNAs that start upstream of the locus, and are antisense to the L1 element.

(e) "antisense" = L1 RNAs that start ~500 bases into the 5′ UTR and are antisense to the locus.

6. For data that is not strand-specific, "antisense" expression is not calculated, and "sense_runthrough" and "antisense_runthrough" are combined into a single estimate of "runthrough" L1 RNA.

7. Count "active" LINE-1 RNA expression and normalize. In most cases, one is interested in L1 RNA driven by L1 promoter activity, which would be the sum of "only" and "3prunon." For each sample, add up the "only" and "3prunon" values for each L1Hs locus and then divide by the total, to get the percent of LINE-1 RNA that is derived from each L1Hs locus. Alternatively the estimated counts can be normalized to the total number of mapped reads or rounded down to the nearest integer and passed to a differential expression algorithm depending on the desired analysis.

4 Notes

1. All reagents used for mass spectrometry handling and preparation require a higher level of purity (MS quality) due to the high sensitivity of the technique. The materials used need to be carefully chosen in order to minimize protein loss and plastic leakage into the solution.

2. Other separation methods can also be coupled to it (e.g., electrophoresis [105, 106]). In recent years, ion mobility has gained a lot of attention as the second dimension of analytical separation [107–109].

3. For use with lighter compute resources, adjust line 2 of parameters.sh. You will need 1 core per thread and 4 GB of memory per thread.

4. Most of our cultures are antibiotic-free; the routine use of penicillin-streptomycin can suppress contamination, but

ultimately will result in delayed detection of that contamination and in our view encourages poor sterile technique.

5. The morphology of the cell line affects the total yield. HEK-293(T) and HeLa cell lines tend to grow in large clumps and on top of one another in confluent flasks and plates. N2102Ep also grows in clumps, but when the monolayer is confluent, the cells do not continue to grow on top of each other. PA-1 cells grow in a stretched-out, star-like pattern. A confluent plate of PA-1 cells will therefore have a significant amount of empty space, due to the particular morphology of the cells. For N2102Ep, you can expect a yield of 5–7 g of cell powder from 20 plates, and for PA-1 (in the pluripotent state), you can expect ≲4 g from 20 plates.
6. In order to thoroughly remove the liquid, we commonly suck off the very top layer of wet cells at the meniscus (~100 μL) in addition to the PBS. We use a vacuum aspirator and a glass Pasteur pipet.
7. Be careful! Pressure will build up and LN_2 can shoot out; decant into a sink.
8. Storage with a punched cap is acceptable for a few days; however frost will form on cells over longer periods. This is undesirable as it will contribute water weight to the cell material, which will affect batch-to-batch reproducibility.
9. 1 g wet cell weight (WCW) is approximately the minimum amount of cells for milling. ~300 mg of cell material may be lost on the surfaces of the jar, lids, and balls, so we aim to produce at least 1.5 g WCW, ensuring ≳1 g of recovered material (i.e., at least ~10 experiments worth at the 100 mg WCW scale). The manufacturer's guidelines [110] indicate that the maximum volume of sample loaded should be ~1/3 of jar volume; the total volume of the balls should be ~1/3 and the remaining ~1/3 free space is for free movement of the balls. The manufacturer recommends the use of three balls in the 50 mL container, yet we routinely use just two—which, in combination with wet milling in the presence of LN_2, has been effective at achieving complete cell lysis.
10. Custom PTFE insulators are not required for this protocol [85]. However, we find that milling is greatly aided by the inclusion of LN_2 within the jar ("wet milling"). The insulators prevent warming of the jar, evaporation of the LN_2, and pressure buildup, resulting in faster, more reliable milling. To have insulators made at The Rockefeller University, contact Vadim Sherman of the High Energy Physics Instrument Shop.
11. This will be equal to the mass of the jar, jar lid, the milling balls being used, and the quantity of cells to be added to the jar. If any PTFE insulators are used, their mass should also be

included. We suggest recording the masses of the jar, lid, and balls (and their combined mass, including any insulator used) in advance and recording them on an informational sheet stored near the mill.

12. We use two containers: a Styrofoam box filled with enough LN2 to completely submerge the jar, and a pan for sample prep and intermediate cooling steps in which the jar is not completely submerged. When grinding multiple samples, we put a tube rack in the Styrofoam box for storage of BBs and grindate and cover the box with its lid.
13. Clamping force should be firm, but not excessive such that removal of the jar becomes a problem.
14. During milling, a distinct clunking noise should be heard as the balls collide. This noise may stop at some point during a rotation but should resume when the rotation is reversed. If these sounds are not heard, inspect the jar between cycles: if the cell material is frozen to the sides of the jar and not a powder, it will reduce or negate the milling efficiency. In this case, chip the frozen material from the side with a nitrogen-cold spatula. Add LN_2 to the jar and resume milling. Milling is happening when clunking sounds are heard.
15. Releasing too quickly can cause rapid depressurization and loss of cell powder. A controlled release allows gentle depressurization, which can be heard as a gentle hiss.
16. Cell powder can be stored at −80 °C essentially indefinitely, without affecting performance.
17. At pH 7.4, reactivity with the epoxy resin is expected to be dominated by ($R\text{-}S^-$) thiolate anions of deprotonated cysteine sulfhydryl side chains; deprotonated ($\text{-}NH_2$) protein amino termini may also participate. As the pH gets more alkaline (e.g., as with borate buffer), deprotonated ($R\text{-}NH_2$) lysine amine side chains will also become reactive targets.
18. Conjugation reaction reagent-compatibility note: avoid the presence of BSA, gelatin, proteins or peptides aside from your antibody, and primary-amine-containing chemicals; avoid sodium azide (although this is tolerable up to ~0.02% (w/v) with minimal effects on coupling efficiency); avoid glycerol and when it is present at high concentrations (e.g., ~50% v/v), double desalt the sample to thoroughly remove it [85].
19. Different volumes of Zeba™ columns are available when needed. Other desalting products are also available and have worked well enough in our hands, for example, Micro Bio-Spin™ 6 (BioRad, cat. #73262211) or Slide-A-Lyzer™ Dialysis Cassettes (ThermoFisher Scientific).
20. This results in a slurry of approximately 15% (w:v).

21. Excess antibodies remaining in the binding solution can be saved, recaptured, and reused. The optimal quantity in μg antibody/mg Dynabeads can vary between antibody clones and between batches of the same antibody. Because the surplus antibody can be recaptured and reused, increasing the quantity of antibodies is an option to help drive the reaction; 10 μg antibody/mg beads is our default [60, 85]. It is always wise to store several aliquots of working beads aside, for the long term, to compare with future batches (quality control) and for direct troubleshooting when performance differences are observed.
22. Approximately 30–50% of the antibody is unbound after bead coupling. The ammonium sulfate solution can be stored at 4 °C and used for, for example, Western blotting directly. Additionally, recovery from the ammonium sulfate allows the same antibody to be reused: it can be reconcentrated and transfered into a bead-coupling-compatible storage solution (we prefer sodium phosphate buffer, but PBS is also viable). Briefly, put the post-coupling solution in a SpeedVac and reduce the volume to 1/2 to 1/3 of the initial volume. The ammonium sulfate concentration will become sufficient to precipitate most antibodies, and you will then see a pellet at the bottom of the tube. In rare cases, antibodies do not precipitate upon concentration. The antibody is then in the concentrated supernatant and can be passed across a desalting column to remove the concentrated ammonium sulfate and exchange it for, for example, sodium phosphate. After concentration, move the tube to a microcentrifuge, centrifuge at top speed for 2 min, save the supernatant, and resuspend the pellet in one-third of the initial post-coupling mix volume in 100 mM sodium phosphate buffer, pH 7.4. Protein recovery can be verified by BCA or 660 nm protein assays, or SDS PAGE by comparing input and output aliquots. For BCA or 660 nm assays, use bovine gamma globulin as the standard to get an accurate concentration. Please note: a saturated solution of ammonium sulfate is 4.1 M at 25 °C or 3.8 M at 0 °C. The volume reduction of the antibody mix should be done at RT. Thus, even if the final concentration of ammonium sulfate increases from 1 to 3 M, it still does not reach the saturation point. Ammonium sulfate should remain in solution, not be pelletted with the antibody. If the pellet is resuspended in phosphate buffer before doing the protein assay, the residual ammonium sulfate (traces trapped in the pellet) should not cause a problem. All fractions can be checked by SDS-PAGE for any doubts.
23. With proper storage, coupled Dynabeads may be used without loss of performance for >1 year. Alternatively, if the beads will be completely consumed within ~8 weeks, storage at 4 °C is

suitable—resuspend with PBS, 0.5 mg/mL BSA, and 0.02% sodium azide and store at 4 °C.

24. Use a small Styrofoam rack on a microbalance and pre-chill tubes and weighing instruments in LN_2. We have found that this is easiest when using inexpensive small stainless steel measuring spoons designed for culinary use.
25. Allowing the tube to briefly warm prevents the extraction solution from flash freezing on the side of the tube.
26. Determination of the optimal extraction solution for each complex is critical but beyond the scope of this chapter. For more information, *see* LaCava et al. [89].
27. We use QSonica S4000 and Q700 systems, each equipped with a low-intensity 1/16 in. microprobe (QSonica, cat. #4717). On the S4000, 5 × 2 s pulses at 2 Amp generate 15–20 J of energy; on the Q700, 5 × 2 s pulses at 4 Amp generate comparable energy output. The J of energy output will vary depending on the system being used and should be determined empirically. The solution temperature should not rise above ~4 °C during ultrasonication.
28. Hold tubes on ice between each subsequent manipulation — working at RT is otherwise acceptable.
29. The ratio of beads to lysate can be optimized for your particular affinity medium and cell type. This can be accomplished by titrating the affinity medium against recovery of the target protein, for example, by Western blotting.
30. We find that transfer to a fresh tube at this step reduces background because some protein nonspecifically sticks to the tube.
31. Reducing agent is omitted to reduce the release of IgG from the beads. It should be added after separation from the magnetic medium, before denaturation for SDS-PAGE.
32. When staining with increased sensitivity is required, we use SYPRO™ Ruby Protein Gel Stain (ThermoScientific).
33. The values correspond to final concentrations. 100 mM TEAB can be used instead of glycine. However, the use of glycine is preferred to TEAB to limit carbamylation from urea.
34. DTT and IAA (iodoacetamide) can be used in combination as reducing and alkylating reagents respectively as an alternative to TCEP and MMTS. It is important to keep in mind, however, that IAA may present solubility problems and IAA stock solutions as well as any sample containing this reagent must be kept away from light as much as possible (e.g., by covering the tubes with aluminum foil).
35. The 55% solution (no units are specified by the manufacturer) is an error in the official S-trap protocol, which assumes the

original 85% phosphoric acid stock is prepared in units of % w/v, instead of w/w (it is w/w as specified by most manufacturers). Therefore, the 64.2% (w/w) that we specify here is the correct final concentration that corresponds to the "55%" specified by the manufacturer. To prepare approx. 50 mL of this solution, add 17.7 mL of HPLC-MS grade water to 32.3 mL of phosphoric acid 85% (w/w).

36. Rotate the S-Trap™ column 180 degrees in between washing/elution steps to get the best results.
37. DO NOT SHAKE/MIX. The cap MUST NOT form an airtight seal.
38. 99.9:0.1 water:formic acid (v:v) is a sensitive choice, although a small v:v quantity of methanol up to 5% might be added in order to aid the resuspension of less hydrophilic peptides. We empirically observed a small increase in peptide IDs when using 5% v:v of methanol.
39. It is of utmost importance that no air bubble is formed at the bottom of the MS vial, otherwise the needle will inject air into the LC system.
40. Only LC-MS quality solvents should be used and extra attention must be paid so that they do not run out while the HPLC system is running.
41. It is recommended that the Ion Transfer Tube from the mass spectrometer be cleaned at least once a week and the system be calibrated as indicated by the software using CalMix. After calibration, a standard sample should be run (typically, a HeLa peptide digest, which is commercially available). After the run is done and before getting to the actual samples, it must be checked that the BPI and the width of the peaks of the standard meet the required values.
42. For MS2 analysis, HCD fragmentation at the instrument's default intensity is recommended. A mass range of 375–1500 m/z is appropriate. As a rule of thumb, the largest mass in the range should not be larger than 4× the smallest one. For IPs, a 45-min linear gradient going from 3% to 45% acetonitrile (MeCN, ACN) provides enough separation. After that, adding a step that goes to 80% MeCN for 5–10 min and then going back to 3% MeCN for column stabilization is recommended. Thus, the total running time for such methods is usually around 60 min. It is good practice to keep an eye on the chromatograms as the samples run to check for contaminants, peak intensity and width, etc. Ideally, the protein amount loaded should be between 0.5 and 2 μg, depending on the column's loading capacity.

43. When all the samples are related, separating them into groups is not needed. When the samples are of very different nature and/or use very different experimental parameters and/or instrument settings, they should be separated into groups. When simply comparing ORF1p IPs and cognate controls as outlined in this chapter, no separate grouping is needed.
44. This setting enables the transfer of MS/MS-based peptide identifications across samples. This improves protein identification in individual runs by accessing the MS/MS data from all of the runs.
45. The EM algorithm that underlies L1EM assigns reads fractionally to transcripts, thus the read count estimates will not be integer values.

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Bibliography

1. Ostertag EM, Kazazian HH (2001) Biology of mammalian L1 retrotransposons. Annu Rev Genet 35:501–538
2. Moran JV, Gilbert N (2002) Mammalian LINE-1 retrotransposons and related elements. In: Craig NLC, Cragie R, Gellert M, Lambowitz AM (eds) Mobile DNA II. American Society of Microbiology, Washington, DC, pp 836–869
3. Beauregard A, Curcio MJ, Belfort M (2008) The take and give between retrotransposable elements and their hosts. Annu Rev Genet 42: 587–617
4. Goodier JL, Kazazian HH (2008) Retrotransposons revisited: the restraint and rehabilitation of parasites. Cell 135(1):23–35
5. Paço A, Adega F, Chaves R (2015) LINE-1 retrotransposons: from "parasite" sequences to functional elements. J Appl Genet 56(1): 133–145
6. Brouha B, Schustak J, Badge RM, Lutz-Prigge S, Farley AH, Moran JV et al (2003) Hot L1s account for the bulk of retrotransposition in the human population. Proc Natl Acad Sci U S A 100(9):5280–5285
7. Yang L, Metzger GA, McLaughlin RN. Characterization of LINE-1 transposons in a human genome at allelic resolution. BioRxiv. 1 Apr 2019
8. Beck CR, Garcia-Perez JL, Badge RM, Moran JV (2011) LINE-1 elements in structural variation and disease. Annu Rev Genomics Hum Genet 12:187–215
9. Burns KH, Boeke JD (2012) Human transposon tectonics. Cell 149(4):740–752
10. Hancks DC, Kazazian HH (2012) Active human retrotransposons: variation and disease. Curr Opin Genet Dev 22(3):191–203
11. Denli AM, Narvaiza I, Kerman BE, Pena M, Benner C, Marchetto MCN et al (2015) Primate-specific ORF0 contributes to retrotransposon-mediated diversity. Cell 163(3):583–593
12. Boeke JD, Fenyo D (2015) Much ado about zero. Cell 163(3):534–535
13. Khazina E, Weichenrieder O (2018) Human LINE-1 retrotransposition requires a metastable coiled coil and a positively charged N-terminus in L1ORF1p. elife 7:e34960
14. Khazina E, Truffault V, Büttner R, Schmidt S, Coles M, Weichenrieder O (2011) Trimeric structure and flexibility of the L1ORF1 protein in human L1 retrotransposition. Nat Struct Mol Biol 18(9):1006–1014
15. Rodić N, Sharma R, Sharma R, Zampella J, Dai L, Taylor MS et al (2014) Long interspersed element-1 protein expression is a hallmark of many human cancers. Am J Pathol 184(5):1280–1286

16. Ardeljan D, Taylor MS, Ting DT, Burns KH (2017) The human long interspersed element-1 retrotransposon: an emerging biomarker of neoplasia. Clin Chem 63(4): 816–822
17. Callahan KE, Hickman AB, Jones CE, Ghirlando R, Furano AV (2012) Polymerization and nucleic acid-binding properties of human L1 ORF1 protein. Nucleic Acids Res 40(2):813–827
18. Newton JC, Naik MT, Li GY, Murphy EL, Fawzi NL, Sedivy JM et al (2021) Phase separation of the LINE-1 ORF1 protein is mediated by the N-terminus and coiled-coil domain. Biophys J 120(11):2181–2191
19. Sil S, Boeke JD, Holt LJ. Condensation of LINE-1 is required for retrotransposition. BioRxiv. 11 Apr 2022
20. Kolosha VO, Martin SL (2003) High-affinity, non-sequence-specific RNA binding by the open reading frame 1 (ORF1) protein from long interspersed nuclear element 1 (LINE-1). J Biol Chem 278(10):8112–8117
21. Martin SL, Bushman FD (2001) Nucleic acid chaperone activity of the ORF1 protein from the mouse LINE-1 retrotransposon. Mol Cell Biol 21(2):467–475
22. Martin SL, Cruceanu M, Branciforte D, Wai-Lun Li P, Kwok SC, Hodges RS et al (2005) LINE-1 retrotransposition requires the nucleic acid chaperone activity of the ORF1 protein. J Mol Biol 348(3):549–561
23. Martin SL, Branciforte D, Keller D, Bain DL (2003) Trimeric structure for an essential protein in L1 retrotransposition. Proc Natl Acad Sci U S A 100(24):13815–13820
24. Feng Q, Moran JV, Kazazian HH, Boeke JD (1996) Human L1 retrotransposon encodes a conserved endonuclease required for retrotransposition. Cell 87(5):905–916
25. Moran JV, Holmes SE, Naas TP, DeBerardinis RJ, Boeke JD, Kazazian HH (1996) High frequency retrotransposition in cultured mammalian cells. Cell 87(5):917–927
26. Ardeljan D, Wang X, Oghbaie M, Taylor MS, Husband D, Deshpande V et al (2020) LINE-1 ORF2p expression is nearly imperceptible in human cancers. Mob DNA 11:1
27. Briggs EM, Spadafora C, Logan SK (2019) A re-evaluation of LINE-1 ORF2 expression in LNCaP prostate cancer cells. Mob DNA 10: 51
28. Dai L, LaCava J, Taylor MS, Boeke JD (2014) Expression and detection of LINE-1 ORF-encoded proteins. Mob Genet Elements 4:e29319
29. Kulpa DA, Moran JV (2005) Ribonucleoprotein particle formation is necessary but not sufficient for LINE-1 retrotransposition. Hum Mol Genet 14(21):3237–3248
30. Kulpa DA, Moran JV (2006) Cis-preferential LINE-1 reverse transcriptase activity in ribonucleoprotein particles. Nat Struct Mol Biol 13(7):655–660
31. Wei W, Gilbert N, Ooi SL, Lawler JF, Ostertag EM, Kazazian HH et al (2001) Human L1 retrotransposition: cis preference versus trans complementation. Mol Cell Biol 21(4): 1429–1439
32. Taylor MS, LaCava J, Mita P, Molloy KR, Huang CRL, Li D et al (2013) Affinity proteomics reveals human host factors implicated in discrete stages of LINE-1 retrotransposition. Cell 155(5):1034–1048
33. Arjan-Odedra S, Swanson CM, Sherer NM, Wolinsky SM, Malim MH (2012) Endogenous MOV10 inhibits the retrotransposition of endogenous retroelements but not the replication of exogenous retroviruses. Retrovirology 9:53
34. Dai L, Taylor MS, O'Donnell KA, Boeke JD (2012) Poly(A) binding protein C1 is essential for efficient L1 retrotransposition and affects L1 RNP formation. Mol Cell Biol 32(21):4323–4336
35. Goodier JL, Cheung LE, Kazazian HH (2012) MOV10 RNA helicase is a potent inhibitor of retrotransposition in cells. PLoS Genet 8(10):e1002941
36. Goodier JL, Cheung LE, Kazazian HH (2013) Mapping the LINE1 ORF1 protein interactome reveals associated inhibitors of human retrotransposition. Nucleic Acids Res 41(15):7401–7419
37. Niewiadomska AM, Tian C, Tan L, Wang T, Sarkis PTN, Yu X-F (2007) Differential inhibition of long interspersed element 1 by APOBEC3 does not correlate with high-molecular-mass-complex formation or P-body association. J Virol 81(17): 9577–9583
38. Suzuki J, Yamaguchi K, Kajikawa M, Ichiyanagi K, Adachi N, Koyama H et al (2009) Genetic evidence that the non-homologous end-joining repair pathway is involved in LINE retrotransposition. PLoS Genet 5(4):e1000461
39. Peddigari S, Li PW-L, Rabe JL, Martin SL (2013) hnRNPL and nucleolin bind LINE-1 RNA and function as host factors to modulate retrotransposition. Nucleic Acids Res 41(1): 575–585

40. Hata K, Sakaki Y (1997) Identification of critical CpG sites for repression of L1 transcription by DNA methylation. Gene 189(2): 227–234
41. Soifer HS, Zaragoza A, Peyvan M, Behlke MA, Rossi JJ (2005) A potential role for RNA interference in controlling the activity of the human LINE-1 retrotransposon. Nucleic Acids Res 33(3):846–856
42. Yang N, Kazazian HH (2006) L1 retrotransposition is suppressed by endogenously encoded small interfering RNAs in human cultured cells. Nat Struct Mol Biol 13(9): 763–771
43. Mandal PK, Ewing AD, Hancks DC, Kazazian HH (2013) Enrichment of processed pseudogene transcripts in L1-ribonucleoprotein particles. Hum Mol Genet 22(18):3730–3748
44. Belancio VP, Whelton M, Deininger P (2007) Requirements for polyadenylation at the 3′ end of LINE-1 elements. Gene 390(1–2): 98–107
45. Šulc P, Solovyov A, Marhon SA, Sun S, LaCava J, Abdel-Wahab O, et al. Repeats mimic immunostimulatory viral features across a vast evolutionary landscape. BioRxiv. 4 Nov 2021
46. De Cecco M, Ito T, Petrashen AP, Elias AE, Skvir NJ, Criscione SW et al (2019) L1 drives IFN in senescent cells and promotes age-associated inflammation. Nature 566(7742):73–78
47. Malik HS, Burke WD, Eickbush TH (1999) The age and evolution of non-LTR retrotransposable elements. Mol Biol Evol 16(6): 793–805
48. Khan H, Smit A, Boissinot S (2006) Molecular evolution and tempo of amplification of human LINE-1 retrotransposons since the origin of primates. Genome Res 16(1):78–87
49. Muotri AR, Chu VT, Marchetto MCN, Deng W, Moran JV, Gage FH (2005) Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. Nature 435(7044):903–910
50. Taylor MS, Altukhov I, Molloy KR, Mita P, Jiang H, Adney EM et al (2018) Dissection of affinity captured LINE-1 macromolecular complexes. eLife 7:e30094
51. Wylie A, Jones AE, Abrams JM (2016) p53 in the game of transposons. BioEssays 38(11): 1111–1116
52. Ardeljan D, Steranka JP, Liu C, Li Z, Taylor MS, Payer LM et al (2020) Cell fitness screens reveal a conflict between LINE-1 retrotransposition and DNA replication. Nat Struct Mol Biol 27(2):168–178
53. Moldovan JB, Moran JV (2015) The zinc-finger antiviral protein ZAP inhibits LINE and Alu retrotransposition. PLoS Genet 11(5):e1005121
54. Pizarro JG, Cristofari G (2016) Post-transcriptional control of LINE-1 retrotransposition by cellular host factors in somatic cells. Front Cell Dev Biol 4:14
55. Mita P, Wudzinska A, Sun X, Andrade J, Nayak S, Kahler DJ et al (2018) LINE-1 protein localization and functional dynamics during the cell cycle. eLife 7:e30058
56. Liu N, Lee CH, Swigut T, Grow E, Gu B, Bassik MC et al (2018) Selective silencing of euchromatic L1s revealed by genome-wide screens for L1 regulators. Nature 553(7687):228–232
57. Vuong LM, Pan S, Donovan PJ (2019) Proteome profile of endogenous retrotransposon-associated complexes in human embryonic stem cells. Proteomics 19(15):e1900169
58. Briggs EM, McKerrow W, Mita P, Boeke JD, Logan SK, Fenyö D (2021) RIP-seq reveals LINE-1 ORF1p association with p-body enriched mRNAs. Mob DNA 12(1):5
59. Garland W, Müller I, Wu M, Schmid M, Imamura K, Rib L et al (2022) Chromatin modifier HUSH co-operates with RNA decay factor NEXT to restrict transposable element expression. Mol Cell 82(9): 1691–1707.e8
60. LaCava J, Jiang H, Rout MP (2016) Protein complex affinity capture from cryomilled mammalian cells. J Vis Exp 118:54518
61. Taylor MS, LaCava J, Dai L, Mita P, Burns KH, Rout MP et al (2016) Characterization of L1-ribonucleoprotein particles. Methods Mol Biol 1400:311–338
62. Jiang H, Taylor MS, Molloy KR, Altukhov I, LaCava J (2019) Identification of RNase-sensitive LINE-1 ribonucleoprotein interactions by differential affinity immobilization. Bio Protoc 9(7):e3200
63. Zougman A, Selby PJ, Banks RE (2014) Suspension trapping (STrap) sample preparation method for bottom-up proteomics analysis. Proteomics 14(9):1006–1000
64. Gagliardi M, Matarazzo MR (2016) RIP: RNA immunoprecipitation. Methods Mol Biol 1480:73–86
65. Maximum read length for illumina sequencing platforms [Internet]. [cited 2022 Apr 8]. Available from: https://support.illumina.com/bulletins/2020/04/maximum-read-

length-for-illumina-sequencing-platforms.html
66. McKerrow W, Fenyö D (2020) L1EM: a tool for accurate locus specific LINE-1 RNA quantification. Bioinformatics 36(4):1167–1173
67. Deininger P, Morales ME, White TB, Baddoo M, Hedges DJ, Servant G et al (2017) A comprehensive approach to expression of L1 loci. Nucleic Acids Res 45(5):e31
68. http://www.rstudio.com/ [Internet]. [cited 2022 Apr 8]. Available from: http://www.rstudio.com/
69. Neuhauser N, Nagaraj N, McHardy P, Zanivan S, Scheltema R, Cox J et al (2013) High performance computational analysis of large-scale proteome data sets to assess incremental contribution to coverage of the human genome. J Proteome Res 12(6):2858–2868
70. Tyanova S, Temu T, Cox J (2016) The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc 11(12):2301–2319
71. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26(12):1367–1372
72. Bielow C, Mastrobuoni G, Kempa S (2016) Proteomics quality control: quality control software for maxquant results. J Proteome Res 15(3):777–787
73. Willforss J, Chawade A, Levander F (2019) NormalyzerDE: online tool for improved normalization of omics expression data and high-sensitivity differential expression analysis. J Proteome Res 18(2):732–740
74. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z et al (2021) clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. Innovation (N Y) 2(3):100141
75. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S et al (2013) STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29(1):15–21
76. Liao Y, Smyth GK, Shi W (2019) The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. Nucleic Acids Res 47(8):e47
77. Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26(1):139–140
78. McCarthy DJ, Chen Y, Smyth GK (2012) Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Res 40(10):4288–4297
79. Chen Y, Lun ATL, Smyth GK (2016) From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline. [version 2; peer review: 5 approved]. F1000Res 5:1438
80. Leibold DM, Swergold GD, Singer MF, Thayer RE, Dombroski BA, Fanning TG (1990) Translation of LINE-1 DNA elements in vitro and in human cells. Proc Natl Acad Sci U S A 87(18):6990–6994
81. Goodier JL, Zhang L, Vetter MR, Kazazian HH (2007) LINE-1 ORF1 protein localizes in stress granules with other RNA-binding proteins, including components of RNA interference RNA-induced silencing complex. Mol Cell Biol 27(18):6469–6483
82. Garcia-Perez JL, Morell M, Scheys JO, Kulpa DA, Morell S, Carter CC et al (2010) Epigenetic silencing of engineered L1 retrotransposition events in human embryonic carcinoma cells. Nature 466(7307):769–773
83. Philippe C, Vargas-Landin DB, Doucet AJ, van Essen D, Vera-Otarola J, Kuciak M et al (2016) Activation of individual L1 retrotransposon instances is restricted to cell-type dependent permissive loci. eLife 5:e13926
84. Andrews PW, Goodfellow PN, Shevinsky LH, Bronson DL, Knowles BB (1982) Cell-surface antigens of a clonal human embryonal carcinoma cell line: morphological and antigenic differentiation in culture. Int J Cancer 29(5):523–531
85. Domanski M, Molloy K, Jiang H, Chait BT, Rout MP, Jensen TH et al (2012) Improved methodology for the affinity isolation of human protein complexes expressed at near endogenous levels. BioTechniques 0(0):1–6
86. Obado SO, Field MC, Chait BT, Rout MP (2016) High-efficiency isolation of nuclear envelope protein complexes from trypanosomes. Methods Mol Biol 1411:67–80
87. Cristea IM, Chait BT (2011) Conjugation of magnetic beads for immunopurification of protein complexes. Cold Spring Harb Protoc 2011(5):pdb.prot5610
88. Cristea IM, Williams R, Chait BT, Rout MP (2005) Fluorescent proteins as proteomic probes. Mol Cell Proteomics 4(12):1933–1941
89. LaCava J, Molloy KR, Taylor MS, Domanski M, Chait BT, Rout MP (2015) Affinity proteomics to study endogenous protein complexes: pointers, pitfalls, preferences

and perspectives. BioTechniques 58(3): 103–119

90. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B et al (2004) Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25(9):1327–1333
91. Rosenberg IM (1996) Protein analysis and purification. Birkhäuser Boston, Boston
92. Miller I, Crawford J, Gianazza E (2006) Protein stains for proteomic applications: which, when, why? Proteomics 6(20):5385–5408
93. Gauci VJ, Wright EP, Coorssen JR (2011) Quantitative proteomics: assessing the spectrum of in-gel protein detection methods. J Chem Biol 4(1):3–29
94. Piersma SR, Warmoes MO, de Wit M, de Reus I, Knol JC, Jiménez CR (2013) Whole gel processing procedure for GeLC-MS/MS based proteomics. Proteome Sci 11(1):17
95. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1(6):2856–2860
96. Dunham WH, Mullin M, Gingras A-C (2012) Affinity-purification coupled to mass spectrometry: basic principles and strategies. Proteomics 12(10):1576–1590
97. Oeffinger M (2012) Two steps forward--one step back: advances in affinity purification mass spectrometry of macromolecular complexes. Proteomics 12(10):1591–1608
98. Dou Y, Kalmykova S, Pashkova M, Oghbaie M, Jiang H, Molloy KR et al (2020) Affinity proteomic dissection of the human nuclear cap-binding complex interactome. Nucleic Acids Res 48(18): 10456–10469
99. Wang X, Chambers MC, Vega-Montoto LJ, Bunk DM, Stein SE, Tabb DL (2014) QC metrics from CPTAC raw LC-MS/MS data interpreted through multivariate statistics. Anal Chem 86(5):2497–2509
100. Bittremieux W, Tabb DL, Impens F, Staes A, Timmerman E, Martens L et al (2018) Quality control in mass spectrometry-based proteomics. Mass Spectrom Rev 37(5):697–711
101. Schurch NJ, Schofield P, Gierliński M, Cole C, Sherstnev A, Singh V et al (2016) How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use? RNA 22(6):839–851
102. Liao Y, Smyth GK, Shi W (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30(7):923–930
103. Yang WR, Ardeljan D, Pacyna CN, Payer LM, Burns KH (2019) SQuIRE reveals locus-specific regulation of interspersed repeat expression. Nucleic Acids Res 47(5):e27
104. Jin Y, Tam OH, Paniagua E, Hammell M (2015) TEtranscripts: a package for including transposable elements in differential expression analysis of RNA-seq datasets. Bioinformatics 31(22):3593–3599
105. Chen D, Yang Z, Shen X, Sun L (2021) Capillary zone electrophoresis-tandem mass spectrometry as an alternative to liquid chromatography-tandem mass spectrometry for top-down proteomics of histones. Anal Chem 93(10):4417–4424
106. Lubeckyj RA, Sun L (2022) Laser capture microdissection-capillary zone electrophoresis-tandem mass spectrometry (LCM-CZE-MS/MS) for spatially resolved top-down proteomics: a pilot study of zebrafish brain. Mol Omics 18(2):112–122
107. Charkow J, Röst HL (2021) Trapped ion mobility spectrometry reduces spectral complexity in mass spectrometry-based proteomics. Anal Chem 93(50):16751–16758
108. Gerbasi VR, Melani RD, Abbatiello SE, Belford MW, Huguet R, McGee JP et al (2021) Deeper protein identification using field asymmetric ion mobility spectrometry in top-down proteomics. Anal Chem 93(16): 6323–6328
109. Ihling CH, Piersimoni L, Kipping M, Sinz A (2021) Cross-linking/mass spectrometry combined with ion mobility on a timsTOF Pro instrument for structural proteomics. Anal Chem 93(33):11442–11450
110. Retsch GmbH. Ball Mills – guidelines for sample amount and ball charge. 19 July 2021. https://www.retsch.com/dltmp/www/5aba547e-5c30-40f3-9813-350c8ac9dd60-c416d7e1c7dd/info_ball_mills_ball_charge_en.pdf