

Chapter 1

Cell Type-Specific Affinity Purification of Nuclei for Chromatin Profiling in Whole Animals

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Abstract

Analyzing cell differentiation during development in a complex organism requires the analysis of expression and chromatin profiles in individual cell types. Our laboratory has developed a simple and generally applicable strategy to purify specific cell types from whole organisms for simultaneous analysis of chromatin and expression. The method, termed INTACT for Isolation of Nuclei Tagged in specific Cell Types, depends on the expression of an affinity-tagged nuclear envelope protein in the cell type of interest. These nuclei can be affinity-purified from the total pool of nuclei and used as a source for RNA and chromatin. The method serves as a simple and scalable alternative to FACS sorting or laser capture microscopy to circumvent the need for expensive equipment and specialized skills. This chapter provides detailed protocols for the cell-type specific purification of nuclei from *Caenorhabditis elegans*.

Key words Nuclei, Cell type, *Caenorhabditis elegans*, Expression profiling, Chromatin profiling, INTACT

1 Introduction

Most multicellular organisms are comprised of different tissues and cell types. The differentiation of a specific cell type from undifferentiated progenitors requires the expression of specific genes at specific time points. This is achieved by a combination of chromatin-based mechanisms involving transcription factor binding, nucleosome remodeling, deposition of histone variants, and post-translational histone modifications [1, 2]. As a consequence, each cell type is characterized by a specific chromatin landscape that gives rise to a specific gene expression signature. To understand cell type-specific function and differentiation, it is important to understand what chromatin changes underlie these processes and what expression profiles arise from them. However, these questions are intractable when studying whole organisms, as differences in expression and chromatin profiles between cell types are

marginalized in mixed populations. In recent years, several approaches have been developed to obtain pure populations of specific cell types or to extract RNA or DNA from specific cell types. These methods include the use of cell lines derived from specific tissues [3, 4], modified RNAs [5, 6], FACS-based approaches either for dissociated cells or nuclei [7–12], Dam-methylase expression in specific tissues [13], laser capture microscopy [14–18], and affinity-purification of nuclei [19–21].

Affinity-purification of nuclei is fast, simple and can be carried out without specialized equipment. The method, termed *isolation of nuclei tagged in specific cell types* (INTACT), relies on the expression of an affinity tag on the nuclear envelope specifically in the cell type of interest (Fig. 1).

The method was initially developed in our lab for *Arabidopsis thaliana* and we have since adapted it to *Caenorhabditis elegans* and *Drosophila melanogaster* [19–21]. Similar strategies have also been developed by other labs to purify nuclei from *D. melanogaster* and *Xenopus* [22, 23]. The method allows for the simultaneous isolation of RNA and chromatin, thus allowing comparison of gene expression profiles directly to the underlying chromatin landscapes. We use a two-component system for nuclear tagging where a nuclear pore fusion protein serves as a substrate for

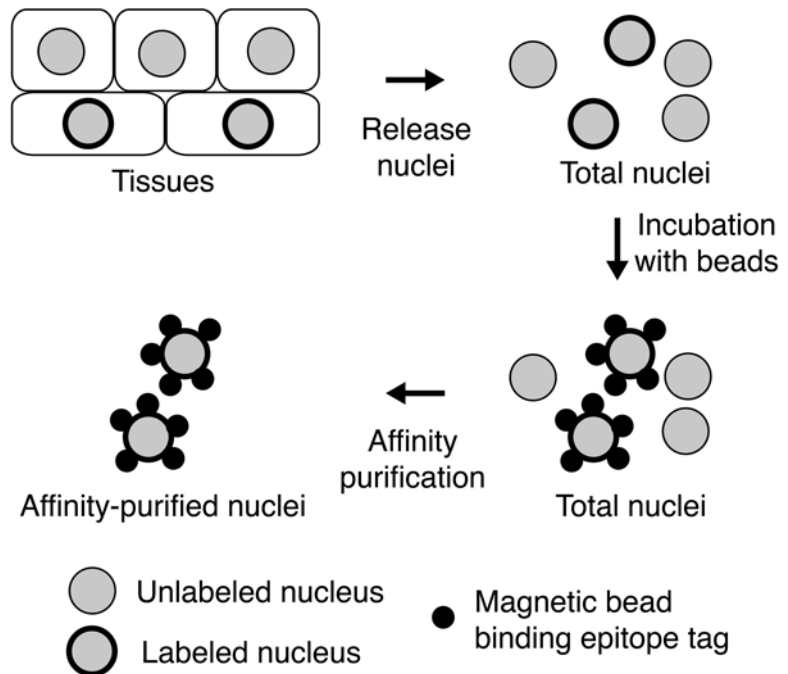


Fig. 1 Scheme of tissue-specific nuclei purification. Nuclei are epitope-labeled by the expression of a nuclear-tagging fusion protein specifically in the tissue of interest. Total nuclei are released and mixed with magnetic beads which recognize the epitope tag. The bead-bound nuclei are subsequently affinity-purified

biotinylation by *E. coli* biotin ligase (BirA), which is co-expressed in the same cells to mediate specific biotinylation. For *C. elegans*, we selected the outer nuclear pore protein NPP-9 and fused it to a tagging cassette that includes mCherry for visualization, biotin ligase recognition peptide (BLRP), a preferred substrate for BirA, and 3xFLAG for immunodetection. We call the NPP-9 fusion protein nuclear tagging fusion (NTF). We express a BirA::GFP fusion ubiquitously using the *his-72* promoter to enable biotinylation of the NTF in vivo [21].

Application of the method to purify muscle nuclei from adult *C. elegans*, using the *myo-3* promoter to express tagged NPP-9 in muscle, resulted in yields of 1–2 million nuclei with >90 % purity. Analysis of these nuclei revealed hundreds of genes that were specifically upregulated in muscle tissue, but also showed that the nucleosome occupancy was reduced over the promoters and within the bodies of these genes. The method also greatly increased the sensitivity of detecting changes in gene expression upon knock-down of the muscle-specific transcription factor HLH-1, underlining the importance of analyzing pure populations of nuclei when analyzing changes that only affect a subset of cells within an organism [21].

Here we provide a detailed protocol for the purification of tissue-specific nuclei from *C. elegans*. We divide the protocol into three stages: (1) *C. elegans* culture and fixation; (2) Isolation and affinity-purification of nuclei; and (3) Assessment of quality. The purified nuclei can be used as a source of RNA for gene expression profiling, of chromatin for micrococcal nuclease- or sonication-mediated fragmentation and chromatin immunoprecipitation, or of proteins for proteomic profiling.

2 Materials

2.1 Culture and Fixation of *C. elegans* Adults

1. Peptone-rich agar plates, 150 mm diameter: 12.5 g agar, 10 g peptone, 0.6 g NaCl, 1.5 g KH₂PO₄, 0.25 g K₂HPO₄, H₂O to 1 L.
2. *E. coli* strain NA22. Available from the Caenorhabditis Genetics Center (CGC), <http://www.cbs.umn.edu/research/resources/cgc>.
3. *C. elegans* strain expressing both NTF and BirA, e.g., strain JJ2300 expressing NTF in body wall muscle cells and BirA ubiquitously, available from the CGC.
4. *C. elegans* strain expressing only NTF (negative control), e.g., strain JJ2286 expressing NTF in body wall muscle cells, available from the CGC.
5. M9 solution: 22 mM KH₂PO₄, 42 mM Na₂HPO₄, 86 mM NaCl, 1 mM MgSO₄.

6. Phosphate-buffered saline (PBS): 8 mM Na_2HPO_4 , 1.46 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4.
7. 50-mL conical tubes.
8. Sodium hypochlorite solution, 10–15 % available chlorine (Sigma).
9. 5 M NaOH.
10. *N,N*-dimethylformamide (Sigma).

2.2 Isolation and Affinity-Purification of Nuclei

1. Nuclei purification buffer (NPB): 10 mM Tris pH 7.5, 40 mM NaCl, 90 mM KCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol-bis(2-aminoethylethanes)- $\text{N}_1\text{N}_1\text{N}_1\text{N}_1$ - tetraacetic acid (EGTA), 0.5 mM spermidine, 0.2 mM spermine, 0.2 mM DTT, 0.1 % Triton X-100.
2. NPB-0.5: NPB supplemented with Triton X-100 (0.5 % v/v).
3. Ceramic mortar and pestle.
4. Liquid nitrogen.
5. Glass dounce homogenizer, 7 mL.
6. Refrigerated tabletop centrifuge.
7. Bioruptor sonicator (Diagenode).
8. Refrigerated centrifuge for 50-mL conical tubes with swinging-bucket rotor (e.g., Sorvall Instruments RC5C).
9. Streptavidin M-280 Dynabeads (Invitrogen).
10. End-over-end rotator for Eppendorf tubes (e.g., Labquake Shaker, Thermo Scientific).
11. MiniMacs magnet (Miltenyi Biotec).
12. 10-mL serological pipettes.
13. NPB supplemented with 1 % (w/v) bovine serum albumin (BSA).
14. 1-mL micropipette tips.
15. Tygon tubing.
16. Hoffman tubing clamp.

2.3 Quality Control and Estimation of Yield

1. 4',6-diamidino-2-phenylindole (DAPI).
2. Glass coverslips, 22 × 40 mm, No 1.5.
3. Microscope slides 3 well, 25 × 75 mm.
4. Upright fluorescence microscope (e.g., Zeiss Axioplan).
5. SDS-PAGE gel loading buffer (2×): 10 mM Tris-HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 2 % (v/v) β -mercaptoethanol, 0.04 % (w/v) bromophenol blue.
6. 100 °C heat block.
7. 6 and 18 % Tris-Glycine polyacrylamide gels.

8. Western blot transfer system.
9. Nitrocellulose membranes.
10. 2 % (w/v) bovine serum albumin (BSA) in PBS.
11. Anti-histone H3 C-terminus antibody (Abcam).
12. Anti-FLAG M2 antibody (Sigma).
13. Horseradish peroxidase (HRP)-conjugated streptavidin.
14. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies.
15. Autoradiography film (Kodak).

3 Methods

3.1 Culture and Fixation of Adult *C. elegans*

1. Seed peptone-rich plates with 2 mL of an overnight culture of *E. coli* NA22. Incubate the plates overnight at 37 °C, then overnight at room temperature.
2. Grow the worm strain expressing both NTF and BirA (sample) and the strain expressing only NTF (negative control) on these plates until they are almost starved (*see Note 1*).
3. Wash the worms off the plates with M9 solution.
4. Wash the worms three times in M9 and resuspend them in 10 mL of M9 in a 50-mL conical tube.
5. Add 1 mL of sodium hypochlorite solution and 1 mL of 5 M NaOH.
6. Incubate with occasional shaking until the worm bodies have mostly disappeared, leaving behind embryos.
7. Pellet the embryos by centrifugation at $2,000 \times g$ for 1 min.
8. Wash the embryos three times with M9.
9. Plate the embryos on fresh NA22 plates (*see Note 2*).
10. Grow synchronized cultures to the young adult stage.
11. For one preparation, use worms from 2 to 4 plates or about 1–2 mL worm pellet, 1.5–2 million worms (*see Note 3*).
12. Wash the worms off the plates with M9 and collect in 50-mL conical tubes.
13. Pellet the worms to the bottom of the tubes by incubation on ice for 10–15 min.
14. Wash the worms 3–4 times with M9 and twice with PBS.
15. Centrifuge at $1,000 \times g$ for 2 min, remove residual PBS.
16. Add *N,N*-dimethylformamide cooled to –20 °C to a volume of 50 mL (*see Notes 4 and 5*).
17. Incubate at room temperature for 1 min (*see Note 6*).

18. Pellet the worms by centrifugation at $1,000\times g$ for 1 min.
19. Remove the *N,N*-dimethylformamide and wash the worms three times with PBS cooled at 4 °C.
20. Freeze the worms dropwise in liquid nitrogen (*see* **Note 7**).
21. Grind the worms to a fine powder under liquid nitrogen.

3.2 Isolation and Affinity-Purification of Nuclei

1. All subsequent steps are carried out on ice or in the cold room. Buffers and equipment are precooled.
2. Add NPB to the worm powder to a total volume of 6 mL.
3. Transfer the suspension to a glass dounce homogenizer.
4. Break up the tissues with 30 strokes of the tight-fitting piston.
5. Distribute into six 1.5-mL centrifuge tubes.
6. Pellet debris by centrifugation at $100\times g$ for 2 min in a refrigerated tabletop centrifuge.
7. Collect the supernatants containing nuclei and pool in a 50-mL conical tube.
8. Resuspend each pellet in 1 mL of NPB.
9. Sonicate with a Bioruptor sonicator at the lowest power output setting (130 W) twice for 30 s to release more nuclei (*see* **Notes 8 and 9**).
10. Pellet debris by centrifugation at $100\times g$ for 2 min in a refrigerated tabletop centrifuge.
11. Collect the supernatants containing nuclei, and pool in the same 50-mL conical tube as above.
12. Repeat the sonication, low speed centrifugation and supernatant collection two more times.
13. Discard the pellets, which contain worm fragments and debris (*see* **Note 10**).
14. Bring the volume of nuclei collected in the 50-mL conical tube to 50 mL with NPB.
15. Pellet residual debris by centrifugation at $100\times g$ for 5 min in a refrigerated centrifuge, then transfer the supernatant to a new 50-mL conical tube, discarding the pellet.
16. Add a 3-mL cushion of OptiPrep at the bottom of the tube (*see* **Note 11**).
17. Collect the nuclei as a layer on the cushion by centrifugation at $1,000\times g$ for 10 min in a refrigerated centrifuge (*see* **Note 12**).
18. Transfer the nuclei into new 50-mL tube (*see* **Note 13**).
19. Bring the volume to 50 mL with NPB, add an OptiPrep cushion, and collect the nuclei on the cushion by centrifugation at $1,000\times g$ for 10 min in a refrigerated centrifuge. Repeat for a total of three centrifugation steps (*see* **Note 14**).

20. Collect the nuclei in a 15-mL conical tube.
21. Retain 5 % of the sample for Western blot analysis and 1 % for microscopy (*see* Subheading 3.3, **steps 1** and **6**).
22. Bring the volume to 5 mL with NPB.
23. Add 30 μ L of washed magnetic streptavidin-coated Dynabeads (*see* **Notes 15** and **16**).
24. Incubate for 45 min at 4 °C using an end-over-end rotator.
25. Bring the volume to 10 mL with NPB-0.5 (final concentration of Triton X-100 is 0.3 %).
26. Incubate 1-mL micropipette tips in NPB supplemented with 1 % (w/v) BSA (*see* **Note 17**).
27. Draw the nuclei into a 10-mL serological pipette.
28. Insert the pipette into a 1-mL micropipette tip that is wedged into a MiniMacs magnet. A schematic view of the assembled column is shown in Fig. 2.
29. Run the nuclei through the column at a flow rate of 1 mL/min. Control the flow rate with a Hoffman tubing clamp.

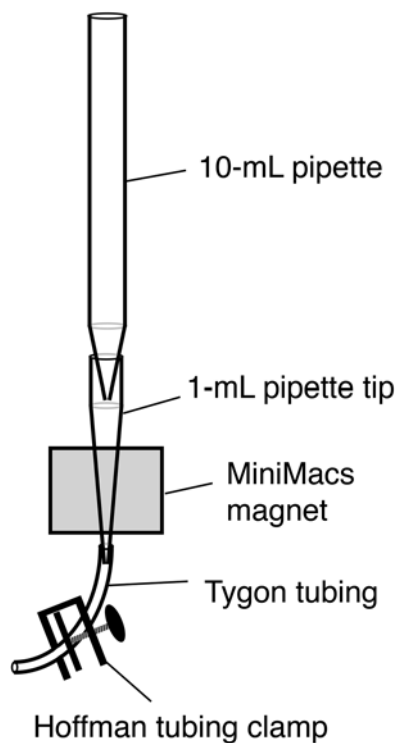


Fig. 2 Column setup for affinity purification. A 10-mL serological pipette is inserted into a 1-mL pipette tip which has been wedged into a MiniMacs magnet. A piece of Tygon tubing is attached to the pipette tip, and flow is controlled by a Hoffmann tubing clamp

30. Elute the beads into 10 mL of NPB-0.5.
31. Run the eluate through a second column (*see* Subheading 3.2, **steps 26–30** and **Note 18**).
32. Elute the beads into 500 μ L of NPB.
33. Retain 5 % of the eluate for Western blot analysis and 1 % for microscopy (*see* Subheading 3.3, **steps 1** and **6**).
34. The remaining nuclei can be used for RNA, chromatin or protein isolation.

3.3 Quality Control and Estimation of Yield

1. To 1 % aliquots collected at **steps 21** and **33** of Subheading 3.2, add DAPI to a final concentration of 1 μ g/mL.
2. Place a 1- μ L aliquot of this sample onto a 3-well microscope slide.
3. Cover with a coverslip.
4. Count bead-bound vs. non-bead-bound nuclei under a compound microscope (*see* **Note 19**).
5. Compare to the number of nuclei in the negative control.
6. Mix 5 % aliquots collected at **steps 21** and **33** of Subheading 3.2 with equal amounts of SDS-PAGE sample buffer.
7. Incubate at 100 °C for 5–10 min.
8. Resolve the samples on two 6 % SDS-PAGE gels (one for detection of the FLAG epitope and one for detection of the biotinylated BLRP) and on one 18 % gel (for detection of histone H3).
9. Transfer the proteins to nitrocellulose membranes using a Western blot transfer system.
10. Block the membranes with 2 % (w/v) BSA in PBS (*see* **Note 20**).
11. Detect biotinylated BLRP with streptavidin-HRP, FLAG with anti-FLAG antibody followed by anti-mouse-HRP antibody, and histone H3 with anti-H3 antibody followed by anti-rabbit-HRP antibody (*see* **Note 21**).
12. Expose the membranes to autoradiography film.

4 Notes

1. We use a negative control for every purification to assess the quality of the pull-down. Nuclear lysis leads to clumping of the nuclei that can subsequently stick to the magnetic beads and lead to a large number of false positive nuclei. This problem is most easily recognized when a negative control is used. The problem of false positives caused by nuclear lysis and clumping is best addressed by gentler handling of the nuclei.

2. Treating adult worms with sodium hypochlorite and letting embryos hatch on fresh NA22 plates generates age-synchronized populations. This is important, as cells from the same tissue will have different expression and chromatin profiles depending on the stage of development.
3. The amount of worms needed to prepare nuclei of a given cell type will vary greatly depending on the required yield, the abundance of the cell type, and the efficiency of release of these nuclei from surrounding tissue. The first and third factors need to be determined empirically.
4. The purification can be done without fixation, as we have demonstrated for *D. melanogaster* mesoderm nuclei [21]. For native purifications, we omit detergents from the NPB to prevent lysis of the nuclei and use HB125 buffer (0.125 M sucrose, 15 mM Tris, pH 7.5, 15 mM NaCl, 40 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, Roche Complete protease inhibitor cocktail) instead of NPB. However, when using the *C. elegans* NPP-9 tag we found that avoidance of any fixative leads to lower yields, possibly due to dissociation of NPP-9 from the nuclear pore.
5. 1 % formaldehyde can be used in the place of DMF for fixation. In this case, the 10 mM Tris is replaced by 50 mM HEPES in the NPB buffer because Tris quenches formaldehyde. We recommend the use of formaldehyde cross-linking if chromatin immunoprecipitation is the downstream application.
6. It is important to fix the worms lightly, as over-fixing will hinder the liberation of nuclei from the surrounding tissue.
7. Worms can be kept at -80°C for a few days. However, we have experienced a reduction in pull-down efficiency upon longer storage.
8. The sonication steps may be unnecessary for some tissues, e.g., germ cells.
9. It is important to sonicate lightly. Too much sonication will lead to the formation of small debris that can stick to nuclei and beads and will decrease the purity of the final preparation.
10. The pellet can be examined under a microscope for the degree of fragmentation and presence of labeled nuclei.
11. We use an OptiPrep cushion to prevent nuclei from being forced against the wall of the conical tube, which would cause lysis and clumping.
12. It is important to use a swinging bucket rotor so that the nuclei are pelleted on top of the OptiPrep cushion and do not collect on the side of the tube.

13. We collect the nuclei by first removing the supernatant, then removing the OptiPrep through the layer of nuclei, to leave the nuclei in approximately 500–1,000 μ L in the conical tube.
14. Washing the nuclei is necessary because of the abundance of endogenous biotinylated proteins in *C. elegans*, which compete with the biotinylated BLRP tag for binding of the streptavidin beads. As most of these proteins are cytoplasmic, background levels can be reduced by washing the nuclei. We found that a minimum of two washes are necessary.
15. It is possible to use beads conjugated to an anti-FLAG antibody to affinity-purify the nuclei via FLAG-tag without the need for in vivo biotinylation by BirA. We found that this reduced the purity of the affinity-purified nuclei from >90 to 80–90 %.
16. We tested several different sizes of beads and found that 2- μ m beads gave the best results because of their size relative to the nuclei. Larger beads crushed the nuclei, whereas smaller beads were less efficient in capturing the nuclei.
17. Coating the 1-mL tip with BSA reduces sticking of the beads and nuclei to the tip, which increases the yield. BSA from some sources can contain RNases and should be avoided when the purified nuclei are used for expression profiling.
18. We found that two passes over the column give the best combination of yield and purity.
19. Bead-bound nuclei are considered positives, and non-bead-bound nuclei are considered false positives. The ratio of the number of non-bead-bound to bead-bound nuclei represents the purity of the pull-down, which should be >0.9. The purity can also be assessed by counting mCherry-positive and mCherry-negative nuclei. However, this approach is less practical, as there is strong auto-fluorescence of the magnetic beads. We have found that the two approaches result in very similar numbers. From the number of bead-bound nuclei and the size of the aliquot, the total number of nuclei in the pull-down sample (yield) can be extrapolated.
20. Milk should be avoided as a blocking agent, as it contains biotin that will cause background when probing with streptavidin-HRP.
21. Streptavidin detection tests for the successful in vivo biotinylation of the BLRP tag within the NTF. However, the signal is often relatively weak, possibly due to the presence of relatively large amounts of endogenous biotinylated proteins. We therefore also routinely detect the NTF with an anti-FLAG antibody, which also confirms the size of the NTF. We detect histone

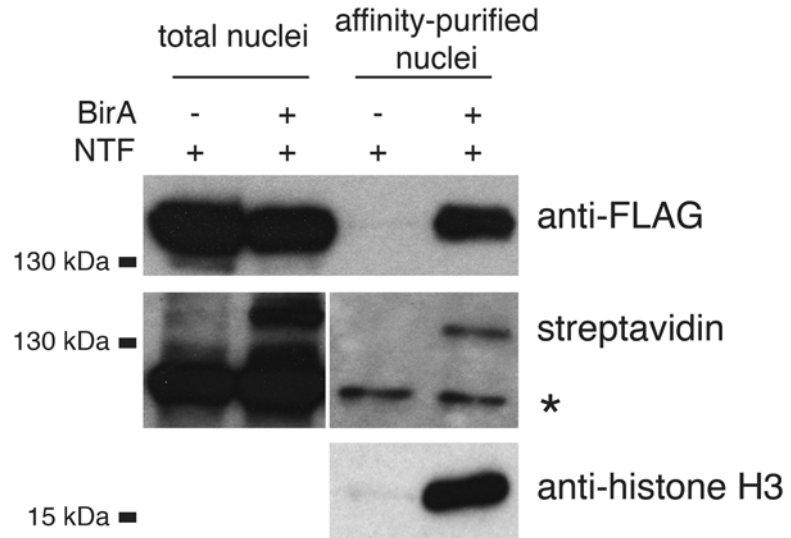


Fig. 3 Representative Western blots. Blots were probed by anti-FLAG, streptavidin, and anti-histone H3. The FLAG tag (*top*) is present in both input samples, but only in the successful pull-down, indicating the successful pull-down of the nuclear-tagging fusion (NTF) protein. Streptavidin (*middle*) confirms the *in vivo* biotinylation of the NTF. Endogenous biotinylated proteins are also visible and marked with an asterisk. Histone H3 (*bottom*) is only present in the successful pull-down, confirming that nuclei and not just the NTF were pulled down (image reproduced from [21])

H3 in the pull-down samples to confirm successful purification containing chromatin. Detection of histone H3 is also very sensitive to false positives in the negative control. Representative Western blots are shown in Fig. 3.

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