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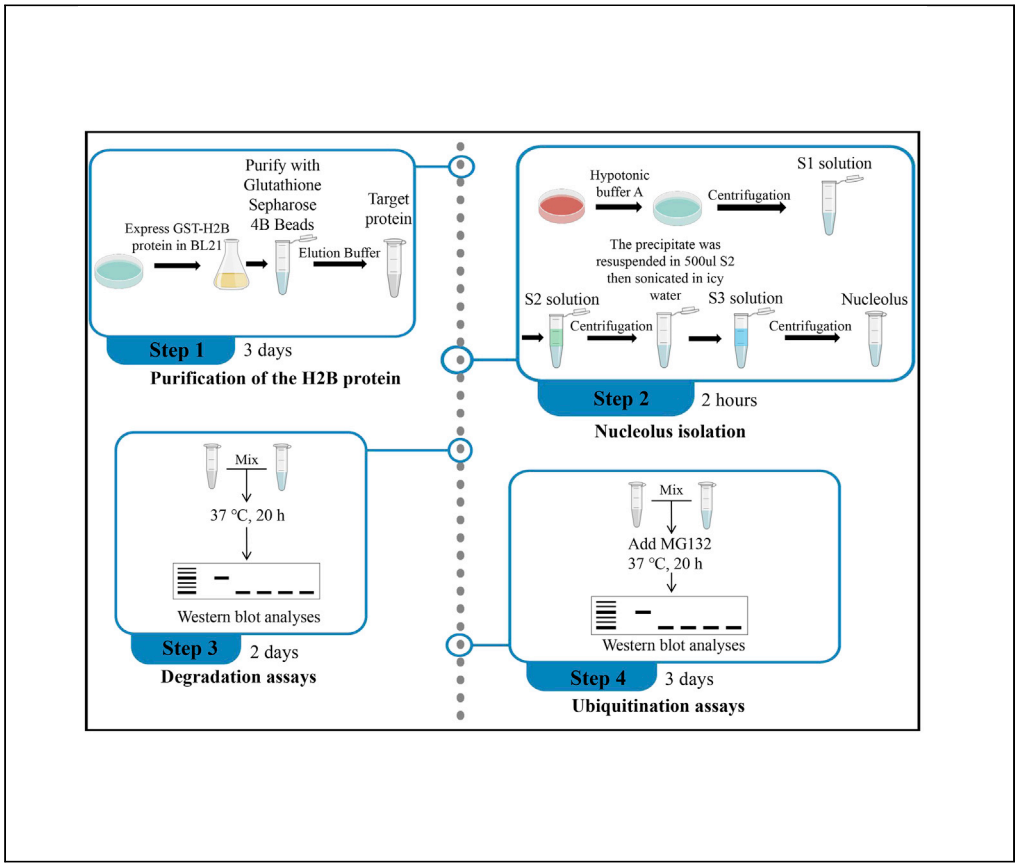
In vitro ubiquitination and degradation of bacteria-purified human histone H2B by the nucleoli fractions



Histones are the main components of chromatin, and the protein levels of histones significantly affect chromatin assembly. Here, we describe detailed protocols for histone H2B purification from bacteria and for the separation of nucleolar fractions and cytoplasmic and nucleoplasmic fractions. Finally, the in vitro ubiquitination and degradation of H2B by distinct cellular fractions are described.

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Highlights

Protocols to purify nucleoli, cytoplasm, and nucleoplasm

In vitro H2B degradation and ubiquitination assays by these cellular components

In vitro ubiquitination and degradation of bacterial histone H2B by the nucleoli fractions

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In vitro ubiquitination and degradation of bacteria-purified human histone H2B by the nucleoli fractions

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SUMMARY

Histones are the main components of chromatin, and the protein levels of histones significantly affect chromatin assembly. Here, we describe detailed protocols for histone H2B purification from bacteria and for the separation of nucleolar fractions and cytoplasmic and nucleoplasmic fractions. Finally, the in vitro ubiquitination and degradation of H2B by distinct cellular fractions are described. For complete details on the use and execution of this protocol, please refer to Liu et al. (2021).

BEFORE YOU BEGIN

1. The protocol below describes the specific steps for *in vitro* degradation of histone H2B by the nucleolar fraction. This protocol can also be applied to study the degradation of other proteins by the nucleolar fraction.
2. The protease inhibitor PMSF (1 mM, final concentration) was added fresh to the following buffers immediately before use: hypotonic buffer, buffers S1, S2, S3 and RIPA buffer.
3. GST epitope tags are used for purification in this protocol. Other tags can be used but will require additional optimization.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Nucleolin (NCL)	GeneTex	GTX16940
Glutathione S-transferase (GST) Tag	ABclonal Technology	AE006
Actin	Abmart	M20011
Ubiquitin	Abcam	ab7780
Bacterial and virus strains		
BL21(DE3)	TIANGEN	CB105
Chemicals, peptides, and recombinant proteins		
MG-132	Sigma-Aldrich	M8699

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sucrose	Sinopharm	10021418
HEPES	Solarbio Life Sciences	H8090
Glutathione (reduced)	Solarbio Life Sciences	G8180
Glutathione Sepharose 4B Beads	GE Healthcare	71024800-EG
Isopropyl-β-D-thiogalactoside, IPTG	Sigma-Aldrich	I6758
Phenylmethanesulfonyl fluoride, PMSF (100 mM)	Beyotime	ST506
Trypsin-EDTA Solution	Beyotime	C0203
Dulbecco's modified Eagle's medium, DMEM	HyClone	SH30243.01
Fetal Bovine Serum, FBS	Upsilon	UB68506
5 × SDS-PAGE loading buffer	Beyotime	P0286
Prestained Protein Ladder	Thermo Fisher Scientific	26617
RIPA buffer	Beyotime	P0013D
DTT solution, 0.5M	Beyotime	ST041
Tris-HCl, 1 M, pH8.0	Beyotime	ST780
Coomassie Brilliant Blue Staining Kit	Beyotime	P0017F
Kanamycin (10 mg/mL)	TIANGEN	RT503
Experimental models: cell lines		
HEK-293T cells	Chinese National Collection of Authenticated Cell Lines	GNHu17
Recombinant DNA		
GST-H2B plasmid	This paper	N/A
Others		
Glass Dounce homogenizer, 15 mL	Nantong Synlab	N/A

MATERIALS AND EQUIPMENT

Buffers and other solutions

LB media		
Reagent	Final concentration	Amount
Yeast extract	0.5% (w/v)	1 g
Tryptone	1% (w/v)	2 g
NaCl	1% (w/v)	2 g
ddH2O	N/A	For 200 mL final
Total	N/A	200 mL

Add the above reagents to an autoclavable flasks, and autoclave it.
Can be stored at +4°C for 1 week.

LB agar plates		
Reagent	Final concentration	Amount
Yeast extract	0.5% (w/v)	0.25 g
Tryptone	1% (w/v)	0.5 g
NaCl	1% (w/v)	0.5 g
Agar	1.5% (w/v)	0.75 g
ddH2O	N/A	Up to 50 mL final
Total	N/A	50 mL

Add the above reagents to an autoclavable flasks, and autoclave it. Add 100 μg/mL Kanamycin (50 μL here) when the media become cool enough to be touched by hands but do not solidify (~60°C). Swirl the agar bottle to ensure even distribution of the antibiotic throughout the agar. Pouring approximately 15 mL molten agar to 10 cm plates in a biosafety cabinet. Leave the plates in a biosafety cabinet to solidify. The plates are then stored at 4°C for 1 week.

IPTG solution		
Reagent	Final concentration	Amount
IPTG	200 mM	0.476 g
ddH2O	N/A	Up to 10 mL
Total	N/A	10 mL
Use filtration for sterilization. Aliquot and store at −20°C for 6 months.		

Elution buffer		
Reagent	Final concentration	Amount
Glutathione	5 mM	0.154 g
Tris-HCl (1 M, pH8.0)	50 mM	5 mL
ddH2O	N/A	Up to 100 mL
Total	N/A	100 mL
Prepare the solution just before use.		

Hypotonic buffer A solution		
Reagent	Final concentration	Amount
1 M HEPES-KOH (pH 7.5)	10 mM	10 mL
4 M KCl	10 mM	2.5 mL
1 M $MgCl_2$	1.5 mM	1.5 mL
ddH2O	N/A	Up to 1000 mL
Total	N/A	1000 mL
Stored at +4°C for 1 year. Add DTT just before use (final concentration 0.5 mM).		

S1 solution		
Reagent	Final concentration	Amount
Sucrose	0.25 M	8.558 g
1 M $MgCl_2$	10 mM	1 mL
ddH2O	N/A	Up to 100 mL
Total	N/A	100 mL
Stored at +4°C for 1 week.		

S2 solution		
Reagent	Final concentration	Amount
Sucrose	0.35 M	11.981 g
1 M $MgCl_2$	0.5 mM	50 µL
ddH2O	N/A	Up to 100 mL
Total	N/A	100 mL
Stored at +4°C for 1 week.		

S3 solution		
Reagent	Final concentration	Amount
Sucrose	0.88 M	30.122 g
1 M $MgCl_2$	0.5 mM	50 µL
ddH2O	N/A	Up to 100 mL
Total	N/A	100 mL
Stored at +4°C for 1 week.		

STEP-BY-STEP METHOD DETAILS

Purification of the GST-H2B protein

Timing: 3 days

This major step describes the transformation of BL21 cells with the pET42b (+) plasmid encoding the H2B protein (GST-H2B) and purification of this protein. Please also refer to (Jing et al., 2020) for an example of this step.

1. Take the competent BL21 cells from the −80°C freezer and thaw the cells on ice.
2. Mix 2 μL of the GST-H2B plasmids (approximately 1 μg) with 20 μL of BL21 competent cells in a microcentrifuge tube. Gently mix the cells and the plasmids.
3. Place the tube with cells on ice for 30 min.
4. Heat shock the tube by placing it into a 42°C water bath for 90 s.
5. Put the tube back on ice for 2 min.
6. Add 800 μL LB medium (without antibiotic) to the tube and incubate in a bacterial shaker at 250 rpm for 60 min at 37°C.
7. Centrifuge the tube at 4000 rpm for 5 min and discard supernatant.
8. Resuspend it by adding 100 μL LB liquid medium (without antibiotic) and transfer the entire amount on one 10 cm LB plate containing 100 μg/mL kanamycin antibiotic. And then, a spreader is used to spread the sample evenly over the surface of a section of the plate. Thereafter, using a fresh, sterile spreader, drag through the first section and spread the bacteria over a second section of the plate, and the step was repeated to create the third section.

Note: This step could also be done by inoculating loops.

9. Incubate the plate at 37°C for 1 h. After that, invert the plate and continue the incubation at 37°C for approximately 12 h.
10. Using a sterile pipette tip, select a single colony from the LB agar plate.
11. Drop the tip into 3 mL LB medium with 100 μg/mL kanamycin. Grow the cells in a bacterial shaker at 250 rpm at 37°C for approximately 12 h.
12. Incubate 2 mL of the cell culture into 200 mL LB medium with kanamycin (the dilution ratio was 1:100) and grow the cell culture in a bacterial shaker at 250 rpm at 37°C until the $OD_{600} \approx 0.6$.
13. Add IPTG at a final concentration of 0.2 mM to the cell culture and incubate it in a bacterial shaker at 250 rpm at 20°C for approximately 12 h.

⚠ CRITICAL: it is important the final concentration of IPTG is 0.2 mM. Do not exceed this concentration. Otherwise, insoluble proteins will be produced, leading to a lower production of the target proteins.

14. Pellet the cells down by centrifuging them at 1000 *g* for 5 min (if using a 50 mL centrifuge tube, add about 45 mL of cell culture to the tube, centrifuge at 1000 *g* for 5 min. Discard the supernatant and keep the pellet. Then add another 45 mL of cell culture to this tube and repeat steps 4–5 times until all the 200 mL of cell culture was pelleted.).
15. Resuspend the pellet with 3 mL PBS supplemented with 1 mM PMSF (insoluble precipitates may exist) and transfer them to 5 mL centrifuge tube.
16. Sonicate the pellet solution (with 1 min on/3 min off intervals, with a sonicator at 50% power) in an ice/water slurry bath for approximately 30 min until most of the insoluble precipitates disappear. In our lab, this requires approximately 10 min.
17. Transfer the cells to a 50 mL centrifuge tube and add 17 mL PBS supplemented with 1 mM PMSF. Sonicate again (with 1 min on/3 min off intervals) in an ice/water slurry bath with a sonicator at 50% power until the mixture becomes clear. In our lab, this requires approximately 30 min.

Note: We use a QSonica Q125 (with a 1/8 inch tip), whose maximum power is 125 W. Many other suitable sonicators can be used. The above parameters can be used as initial parameters for optimization.

18. Centrifuge the lysate at 4°C at 12000 rpm for 10 min and collect the supernatant, which contains the target protein. Save the supernatant and the pellet for analysis (Figure 1).
19. Prepare the glutathione sepharose 4B beads (steps 20–23).
20. Gently shake the bottle of glutathione sepharose 4B to resuspend the slurry.
21. Transfer 1 mL beads to a 5 mL microcentrifuge tube. Centrifuge at 500 *g* for 5 min. Carefully discard the supernatant.
22. Wash the beads by adding 4 mL PBS and invert to mix. Sediment the medium by centrifugation at 500 *g* for 5 min. Carefully discard the supernatant.
23. Repeat step 22 two more times.
24. Mix the supernatant from step 18 with the prepared beads from step 23 together in a 50 mL tube and rotate it gently for approximately 12 h at 4°C.
25. Centrifuge it at 4°C at 500 *g* for 5 min to collect beads, carefully discard the supernatant until there are approximately 3 mL solution left, resuspend the beads gently.
26. Transfer the solution to a 5 mL centrifuge tube, sediment the beads by centrifugation at 500 *g* for 5 min. Carefully discard the supernatant.
27. Wash the beads by adding 1 mL PBS supplemented with 1 mM PMSF and resuspend gently.
28. Rotate at 4°C for 10 min. Sediment the beads by centrifugation at 500 *g* for 5 min. Carefully discard the supernatant.
29. Repeat steps 27 and 28 two more times for a total of three washes.
30. Elute the bound protein by adding 2 mL elution buffer and rotate it at 4°C for 6 h.

⚠ CRITICAL: Prepare elution buffer just before use. Long time storage of elusion buffer will make glutathione oxidized and lose its function.

31. Centrifuge at 4°C at 2000 rpm for 5 min, after which the target protein is contained in the supernatant. Save the supernatant at −80°C for analysis (Figure 1).

Pause point: The supernatant can be stored at −80°C for analysis later.

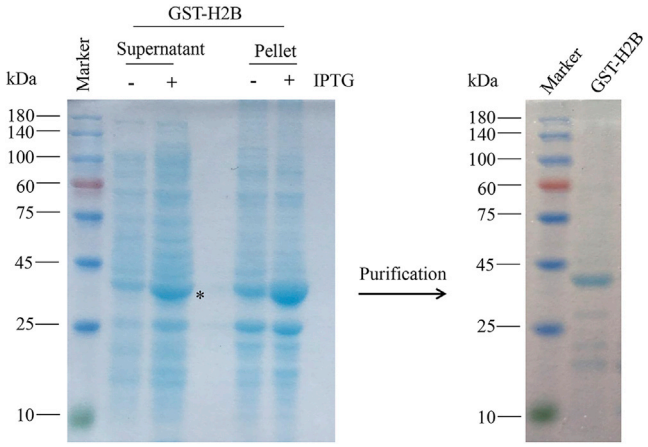

Figure 1. Purification of GST-H2B protein

Left panel: BL21 cells with GST-H2B plasmids treated with or without IPTG were incubated for 20 h and lysed by sonication. The supernatant and pellet were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (step 18). * indicates the GST-H2B protein. Right panel: The supernatant in step 31 was analyzed by SDS-PAGE and Coomassie Brilliant Blue staining, showing that target protein (H2B) was purified by glutathione sepharose 4B. This figure is from previously published data [Figure 5A in (Liu et al., 2021), with modification] and is reprinted with permission.

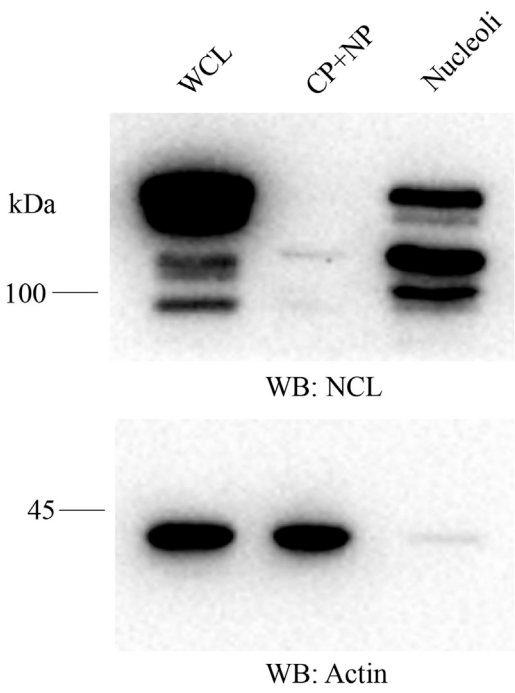

Figure 2. Western blot analysis of the isolated nucleolar fractions and non-nucleolar fractions (cytoplasmic + nucleoplasmic fractions, CP+NP)

The isolated nucleolar fractions and non-nucleolar fractions (cytoplasmic + nucleoplasmic fractions, CP+NP) were subjected to western blot and indicated proteins were immunoblotted (antibody dilution: NCL, 1:1000; Actin, 1: 1000). Actin: marker of non-nucleoli fraction. NCL: marker of nucleoli fraction (Ginisty et al., 1999). WCL: whole cell lysate. This figure is identical to previously published data [Figure 5B in (Liu et al., 2021)]and is reprinted with permission (Liu et al., 2021).

Nucleolus isolation

Timing: 2 h

This major step describes the isolation of nucleoli from HEK293T cells.

32. HEK293T cells grown to 80%–90% confluence (approximately 2.5 × 10^6) in one 6 cm dish are sufficient to obtain a sufficient nucleolar fraction for GST-H2B degradation by cellular components (other cell types may need optimization). Please note that the solutions used in the following steps are for a 6 cm dish.
33. When the cells have grown to 80%–90% confluence, wash the cells gently with 3 mL prewarmed PBS carefully and slowly, and discard the PBS completely. Add 1 mL prewarmed trypsin-EDTA solution and incubate at 37°C for approximately 1 min to detach cells. After adding 2 mL prewarmed complete growth medium, transfer the cells to a 5 mL conical tube and centrifuge at 200 × *g* for 5 min.
34. Discard the supernatant and add 1 mL hypotonic buffer A supplemented with 1 mM PMSF to the cell pellet. Resuspend cells and incubate them on ice for 30 min.

Note: The cells will be swollen at the end of this step. It is important to observe the changes in cell size under a microscope to determine the time of incubation. Other cell types may need optimization.

35. Transfer the solution into a prechilled glass Dounce homogenizer on ice.
36. Stroke up and down promptly and steadily approximately 10 times on ice, making sure the membranes of most cells (>90%) get fragmented while the nuclei remain intact.

Note: the number of strokes needs optimization according to the cell type and Dounce homogenizer used. Insufficient rupture will lead to the cytoplasmic fraction from unbroken cells contaminating the nuclear fraction. In contrast, excessive homogenization will result in

breakdown of the nuclear envelope, leading to the loss of soluble nuclear proteins from nuclear fractions (Liang and Cohn, 2021).

37. Centrifuge at 220 *g* for 5 min at 4°C. The nuclei will be collected in the precipitate after centrifugation. The cytoplasm (cytoplasmic fraction, CP fraction) will be contained in the supernatant. Save the CP fraction in another 1.5 mL microcentrifuge tube.
38. Resuspend the nuclei with 500 μL S1 solution supplemented with 1 mM PMSF at 4°C.
39. Add 500 μL S2 solution supplemented with 1 mM PMSF to a new 1.5 mL microcentrifuge tube and then add the S1 solution of step 38 to S2 solution carefully and slowly along the tube wall. Two separated layers can be observed, which represent S2 at the bottom and S1 at the top.
40. Centrifuge at 220 *g* for 5 min and discard the supernatant. Clean nuclei will be in the pellets.
41. Resuspend the pellets in 500 μL S2 solution supplemented with 1 mM PMSF and then sonicate in an ice/water slurry bath with a sonicator at 40% power six times, each time for 10 s on and 30 s off.

Note: We used a QSonica Q125 (with a 1/8 inch tip), whose maximum power is 125 W. Many other suitable sonicators can be used. The above parameters can be used as initial parameters for optimization.

42. Take a new 1.5 mL tube and add 500 μL S3 solution supplemented with 1 mM PMSF to it. Carefully add the solution obtained from step 41 to the top of the S3 solution along the tube wall. Two separated layers can be observed, which represent S3 at the bottom and S2 at the top.
43. Centrifuge at 3000 *g* for 10 min at 4°C. The nucleoli will be in the pellets, and the nucleoplasm fraction (nucleoplasm fraction, NP fraction) will be in the supernatant. Save the supernatant in another microcentrifuge tube.
44. Resuspend the nucleoli pellet with 500 μL S2 supplemented with 1 mM PMSF and centrifuge at 4°C at 1430 g for 5 min. Discard the supernatant.
45. Repeat step 44 two more times for a total of 3 washes.
46. Resuspend the nucleoli pellet in 500 μL RIPA solution supplemented with 1 mM PMSF. Western blotting analyses can be performed to determine the purification efficiency of the nucleolar fractions (step 46) and non-nucleolar fractions (cytoplasmic + nucleopasmic fractions, CP+NP) (step 37 and step 43) (Figure 2). Up to this step, it is ready for the GST-H2B degradation assay and ubiquitination assay.
47. If long-term preservation of nucleoli is needed, keep the nucleoli in RIPA solution at −80°C.

Pause point: store the nucleoli (step 47), the nucleoplasm fraction (step 43) and cytoplasmic fraction (step 37) at −80°C for analysis later.

In vitro analysis of GST-H2B degradation by the nucleoli fraction.

Timing: 2 days

48. *In vitro* analyses of GST-H2B degradation by different cellular fractions are performed by establishing the reaction systems indicated in Figure 3.
49. Incubate the established reaction systems at 37°C for 20 h.

⚠ CRITICAL: keep the temperature 37°C.

50. Add 5 × SDS-PAGE loading buffer to the samples and analyze the protein expression in the samples by Western blot.

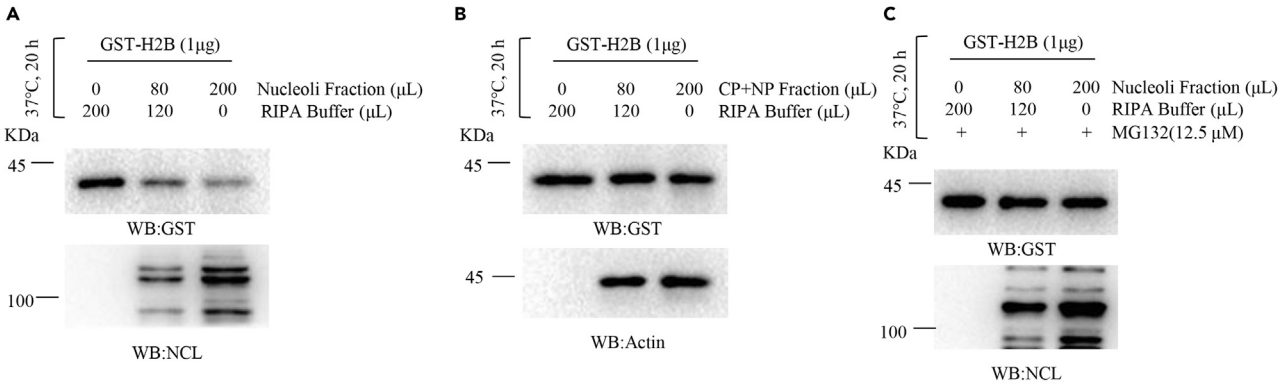

Figure 3. *In vitro* degradation assays of GST-H2B by the nucleoli and non-nucleoli fractions

Reaction systems were established as indicated and incubated at 37°C for 20 h. Samples were then subjected to Western blot analysis. A: GST-H2B protein (1 μg) was added to a solution of the nucleoli fraction and RIPA buffer, incubated at 37°C for 20 h, and then analyzed by Western blotting. This figure is identical to previously published data [Figure 5C in (Liu et al., 2021)] and is reprinted with permission. B: GST-H2B protein (1 μg) was added to a solution of the CP+NP fraction and RIPA buffer, incubated at 37°C for 20 h, and then analyzed by Western blotting. This figure is identical to previously published data [Figure 5F in (Liu et al., 2021)] and is reprinted with permission. C: GST-H2B protein (1 μg) was added to a solution of the nucleoli fraction, RIPA buffer and MG132, subjected to incubation at 37°C for 20 h, and then analyzed by Western blotting (antibody dilution: NCL, 1:1000; Actin, 1: 1000; GST, 1: 1000). This figure is identical to previously published data [Figure 5D in (Liu et al., 2021)] and is reprinted with permission. NCL: Nucleolin. GST: Glutathione S-transferase tag.

In vitro analysis of GST-H2B ubiquitination by the nucleoli fraction.

Timing: 3 days

51. *In vitro* GST-H2B ubiquitination analysis of different cellular fractions is performed by establishing the reaction systems (500 μL in total) as indicated in Figure 4.
52. After adding MG132 to a final concentration of 25 μM, the established reaction systems are then incubated at 37°C for 20 h.

⚠ CRITICAL: keep the temperature 37°C.

53. Prepare 50 μL glutathione sepharose 4B beads according to steps 20–23.
54. Add the solution from step 52 to the bead pellet from step 53 and resuspend the pellet by pipetting several times gently and slowly.
55. Rotate at 4°C slowly for approximately 12 h.
56. Collect the beads by sedimentation at 4°C at 500 g for 5 min.
57. Wash the beads by adding 1 mL PBS supplemented with 1 mM PMSF and resuspended gently. Rotate at 4°C for 10 min. Sediment the beads by centrifugation at 500 *g* for 5 min. Carefully discard the supernatant.
58. Repeat step 57 two more times for a total of three washes.
59. Add 300 μL elution buffer to the pellet. Resuspend the pellet by pipetting several times gently and slowly.

⚠ CRITICAL: prepare elution buffer just before use. Long time storage of elusion buffer will make glutathione oxidized and lose its function.

60. Rotate at 4°C for 6 h.
61. Sediment the pellet by centrifugation at 4°C at 500 *g* for 5 min.
62. Transfer the supernatant to a new 1.5 mL microcentrifuge tube. The target protein is contained in the supernatant.
63. Add 5 × SDS-PAGE loading buffer to the supernatant and analyze the protein expression by Western blot.

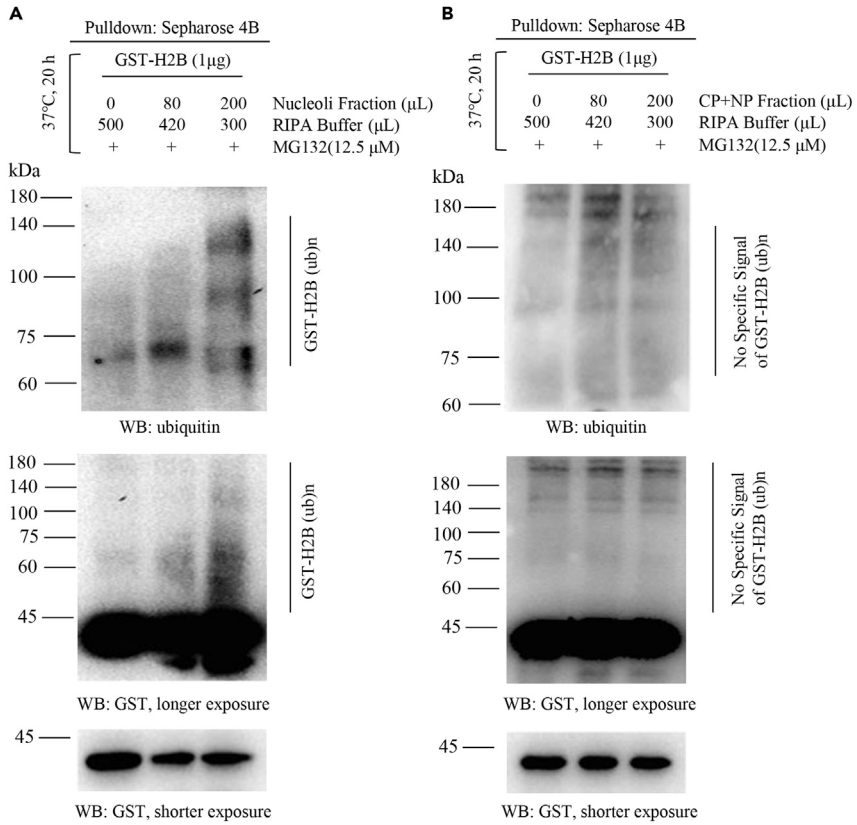

Figure 4. *In vitro* ubiquitination analysis of GST-H2B by the nucleoli or non-nucleoli fractions

(A) GST-H2B (1 μg) was mixed with the nucleoli, RIPA buffer and MG132 and incubated at 37°C for 20 h. Samples were pulled down by Sepharose 4B beads and then subjected to Western blot analyses. This figure is identical to previously published data [Figure 5E in (Liu et al., 2021)] and is reprinted with permission.

(B) GST-H2B (1 μg) was mixed with the cytoplasmic+nucleoplasmic (CP+NP) fraction, RIPA buffer and MG132 and incubated at 37°C for 20 h. Samples were pulled down by Sepharose 4B beads and then subjected to Western blot analyses (antibody dilution: ubiquitin, 1:1000; GST, 1: 1000). This figure is identical to previously published data [Figure 5G in (Liu et al., 2021)] and is reprinted with permission.

EXPECTED OUTCOMES

Large amounts of highly purified protein of interest can be collected after expression in BL21 cells. This protocol allows us to test the degradation and ubiquitination of proteins of interest by the cytoplasmic fraction, nucleoplasmic fraction, and nucleoli fraction. An example of this is shown in Figures 3 and 4, which show the degradation and uniquitination of GST-H2B, respectively, by the nucleoli fraction and the cytoplasmic fraction plus nucleoplasmic fraction (CP+NP) fraction.

LIMITATIONS

This is an *in vitro* assay that can only detect the degradation of H2B by nucleoli or other cellular components.

TROUBLESHOOTING

Problem 1

Low yield of target protein in BL21 cells (step 13).

Potential solution

Do not incubate it for too long time in step 13, and do not use a temperature over 20°C. Otherwise, insoluble proteins will be produced.

In addition, IPTG should be used at a final concentration of 0.2 mM. Do not exceed this concentration. Otherwise, insoluble proteins will be produced, leading to a lower production of the target proteins.

Problem 2

No separated layers are observed between the S1, S2 or S3 solutions when mixing them (steps 39 and 42).

Potential solution

When adding S1, S2, or S3 solutions to other solutions, do it slowly along the microcentrifuge tube wall.

Check the contents of the S1, S3, and S3 to make sure they were made correctly.

Problem 3

The quantity of nucleoli extracted is not sufficient (step 32).

Potential solution

Culture the cells in two or three 6-cm dishes. Scale up the solutions used accordingly. In our study, HEK293T cells grown to 80%–90% confluence (approximately 2.5×10^6) in one 6 cm dish are sufficient to obtain a sufficient nucleolar fraction for GST-H2B degradation by cellular components. However, different cell type might have very different cell size which might make the cell number in a confluent plate varies a lot. So, the cells used may require optimization.

Problem 4

Low yield of GST-tagged protein by glutathione Sepharose 4B beads (step 30).

Potential solution

Reduce the time for sonication, which may cause GST-tagged protein denaturation.

Increase the time used for elution.

Problem 5

No degradation or ubiquitination by nucleoli is observed (steps 48 and 51).

Potential solution

The ratio of the protein of interest to the nucleoli components affects the efficiency. Increase the number of groups to find the best ratio.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Su Chen (chensubio@163.com or chensu@xjtu.edu.cn).

Materials availability

Plasmids used in this study are available by contacting the lead contact, Su Chen (chensubio@163.com or chensu@xjtu.edu.cn).

DATA AND CODE AVAILABILITY

This study did not generate new data or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

S.L., G.Z., Y.L., Q.S. L.Y., J.D., and B.Z. developed and optimized the method. S.L., G.Z., and S.C. wrote the method. S.C. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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