

Submission ID #: 70934

Scriptwriter Name: Kathyayani Sridharan

Project Page Link: <https://review.jove.com/account/file-uploader?src=21634918>

Title: Identification of Modified Histones as Binding Substrates of Human Spindlin Family Member 4 (SPIN4) by Peptide Arrays and Native Nucleosome Pulldown

Authors and Affiliations:

Chen-I Hsu¹, Aastha Dave¹, Chih-Han Tu¹, Sujin Park², Bin Yu¹, Youn Hee Jee^{2,3,4}, Jia-Ray Yu^{1,5}

¹Division of Cellular and Molecular Pathogenesis, Department of Pathology, Virginia Commonwealth University School of Medicine

²Center for Precision Medicine and Genomics Research, Children's National Research Institute

³Department of Endocrinology, Children's National Hospital

⁴Department of Pediatrics, George Washington University School of Medicine and Health Sciences

⁵Massey Comprehensive Cancer Center, Virginia Commonwealth University School of Medicine

Corresponding Authors:

Jia-Ray Yu

yuj12@vcu.edu

Youn Hee Jee

yjee@childrensnational.org

Email Addresses for All Authors:

chenihsu003@gmail.com

daveaastha23@gmail.com

chihhan.tu@vcuhealth.org

spark1@childrensnational.org

bin.yu@vcuhealth.org

yuj12@vcu.edu

yjee@childrensnational.org

Author Questionnaire

1. **Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**
Note: Screen captures are obtained from authors as image files for 2 steps and this has been annotated in the file
3. **Filming location:** Will the filming need to take place in multiple locations? **No**
4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 18

Number of Shots: 52

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

REQUIRED: ~~Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Chen-I Hsu:** SPIN4 is a bone development regulating histone reader. SPIN4 mutations cause overgrowth but its pathogenic role remains unclear.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Chen-I Hsu:** This protocol can be adapted and applied to study binding substrates of histone binding proteins across all species.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Chen-I Hsu:** Fast and targeted validation of the binding substrates of histone binding proteins.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Chen-I Hsu:** The conditions required for expression and purification of each candidate histone binding proteins may vary.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Chen-I Hsu:** Histone mass spectrometry following native mono-nucleosome pulldown would give the full scope of histone PTMs.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

NOTE: LAB MEDIA for protocol were added at the postshoot stage. Please contact the postshoot note integrator (Kathyayani Sridharan) for queries regarding lab media.

2. Histone Peptide Array

Demonstrator: Chen-I Hsu

- 2.1. To begin, weigh five grams of bovine serum albumin, or BSA (*B-S-A*) to prepare the blocking buffer [1]. Dissolve into hundred milliliters of BC150 (*B-C-one-fifty*) Protein Storage Buffer [2]. Use a hydrophobic pen to outline the reaction area on the peptide array to conserve the reagents [3]. Place filter papers moistened with water in a fifteen-centimeter petri dish [4].
 - 2.1.1. Talent weighing 5 g of BSA.
 - 2.1.2. Talent dissolving BSA in 100 ml buffer.
 - 2.1.3. Talent outlining the reaction area on array with marker.
 - 2.1.4. Talent placing moist filter papers in petri dish.
- 2.2. Position the arrays on moistened filter papers [1] and add 300 microliters of blocking buffer to each array area carefully [2]. Cover and incubate the petri dish at room temperature for one to three hours [3].
 - 2.2.1. Talent placing the arrays on filter paper.
 - 2.2.2. Talent adding blocking buffer to the array.
 - 2.2.3. Talent incubating the covered plate at room temperature.
- 2.3. Prepare one micromolar of recombinant FLAG (*Flag*)-tagged Spindlin 4 or FLAG-SPIN4 (*Flag-Spin-four*) protein and 1X FLAG (*one-X-flag*) peptide control, each in blocking buffer [1]. Transfer the reagents and array dish to a cold room to reduce background and improve the signal-to-noise ratio [2].
 - 2.3.1. Talent preparing 1 μ M FLAG-SPIN4 and 1X FLAG peptide solutions.
 - 2.3.2. Talent moving the array plate and reagents to cold room.
- 2.4. Remove the excess blocking buffer from the arrays [1]. Add the FLAG-SPIN4 protein or FLAG peptide control onto each block [2]. Cover the petri dish for overnight incubation in the cold room [3].
 - 2.4.1. Talent removing excess buffer from the arrays.
 - 2.4.2. Talent adding desired proteins and control to the arrays.

- 2.4.3. Talent covering the plate for overnight incubation.
- 2.5. Add 50 milliliters of peptide array-wash buffer to a wash box [1]. Discard the protein solution from the arrays [2] and transfer them into wash buffer with reaction side up [3].
- 2.5.1. Talent adding 50 ml wash buffer to a wash box.
- 2.5.2. Talent discarding the protein solution.
- 2.5.3. Talent transferring the arrays to wash buffer with reaction side up.
Videographer: Obtain multiple reusable takes of 2.5.2 and 2.5.3 as these will be used later in the protocol
- 2.6. Wash the arrays five times- perform the first two washes for one minute each, followed by three subsequent washes of five minutes each [1]. Then, rinse with blocking buffer to remove residual detergents [2].
- 2.6.1. Talent washing the arrays in wash buffer.
- 2.6.2. Talent rinsing the arrays with blocking buffer. *Videographer: Obtain multiple reusable takes of 2.6.1 and 2.6.2 as these will be used later in the protocol*
- 2.7. Place the arrays back into the petri dish [1]. Add 300 microliters of anti-FLAG antibody to each block and incubate for one hour at room temperature [2-TXT]. Discard the protein solution, transfer the arrays to the wash buffer and repeat the washes as demonstrated previously [3].
- 2.7.1. Talent placing the arrays in petri dish.
- 2.7.2. Talent adding primary antibody and placing the plate aside. **TXT: Primary Ab: 1:2000 dilution** *Videographer: Obtain multiple reusable takes of 2.7.2 as these will be used later in the protocol*
- 2.7.3. *Reuse 2.5.2, 2.5.3, 2.6.1 and 2.6.2*
- 2.8. Add 300 microliters of HRP (*H-R-P*)-conjugated goat anti-mouse IgG (*I-G-G*) secondary antibody solution to each block and incubate for one hour at room temperature [1-TXT]. Discard the protein solutions and wash the array five times as demonstrated previously [2].
- 2.8.1. *Reuse 2.7.2. TXT: Secondary Ab: 1:6000 dilution*
- 2.8.2. *Reuse 2.5.2, 2.5.3, 2.6.1 and 2.6.2*
- 2.9. Place the arrays inside a transparent sheet protector [1] and add one milliliter of Enhanced Chemiluminescence or ECL (*E-C-L*) substrate ensuring even coverage [2].
- 2.9.1. Talent placing the arrays inside a transparent sheet protector.
- 2.9.2. Talent adding 1ml ECL covering the arrays' surface.
- 2.10. Remove trapped air bubbles [1]. Capture chemiluminescent and bright-field images using digital imager [2]. ~~Quantify the signal intensity using peptide array software and~~

online analysis tools [3], then interpret the results [4]. After uploading the raw data file to the **MODified Histone Peptide Array Analysis** software, in the **Spot Alignment** tab, confirm each grid is centered on its peptide spot [3]. In **Spot Statistics**, check duplicate spot correlation [4] and ensure that the error is less than 5% percent [5]. Use the **Reactivity** tab to assess binding across various histone modifications [6], then use the **Specificity** tab to determine the target peptide's relative binding preference among different histone modifications [7]. **Note:** The numbers, narration and shots are updated as per the LAB MEDIA provided by the authors.

2.10.1. Talent smoothing out air bubbles.

2.10.2. LAB MEDIA: raw data.

2.10.3. ~~SCREEN: Signal analysis using software tools and result interpretation.~~ **Note:** This shot is removed, and new shots are added to accomodate the LAB MEDIA files provided by the authors.

Added shot: LAB MEDIA: software1. Video Editor: Please highlight the Spot Alignment tab on the top left under the File option and emphasize the blue grid lines encircling the black dots in the 2 panels on the left.

Added shot: LAB MEDIA: software4. Video Editor: Please highlight the Spot Statistics tab, and in the scatter plot on the left side of the image, please highlight the diagonal clustering of the data points.

Added shot: LAB MEDIA: software4. Video Editor: In the bar graph on the right side of the image, please highlight the tallest bar and the corresponding X-axis value 0-5%.

Added shot: LAB MEDIA: software2. Video Editor: Please highlight the Reactivity tab and simultaneously emphasize the heights of the bars corresponding to their respective X-axis labels.

Added shot: LAB MEDIA: software3. Video Editor: Please highlight the Specificity tab and the toggled 'Multiple Peptide Average', then emphasize the heights of orange bars corresponding to their respective X-axis labels simultaneously.

3. Modified nucleosome pulldown

3.1. To prepare 2X (*two-X*) bead blocking buffer, dissolve 1 gram of BSA into 10 milliliters of BC150 lysis buffer via vortexing [1]. Transfer FLAG-SPIN4-WT (*Wildtype*) and the FLAG-SPIN4-D82H (*Dee-eighty-two-etch*) mutant beads containing equimolar protein concentrations into separate 1.5 milliliter tubes [2]. To the WT sample, add equal volume of hundred millimolar 1X FLAG peptide negative control beads [3]. Adjust all samples to uniform volumes using appropriate amount of BC150 lysis buffer [4].

3.1.1. Talent dissolving 1 g BSA in 10 ml buffer.

3.1.2. Talent adding protein samples to two separate 1.5 ml tubes.

3.1.3. Talent adding negative control to wild type sample.

3.1.4. Talent making up the volume in all tubes to be identical.

- 3.2. Add an equal volume of 2X bead blocking buffer to each tube to make up the final volume to 1 milliliter [1]. Incubate overnight on a tube rotator set at four degrees Celsius and 20 rpm (*R-P-M*) [2].
 - 3.2.1. Talent adding bead blocking buffer to each tube.
 - 3.2.2. Talent incubating the tubes on rotator.
- 3.3. To initiate nucleosome binding, thaw 1X (*one-X*) mononucleosomes extracted from HEK293FT (*H-E-K-two-ninety-three-F-T*) cells on ice [1]. For initial optimization, calculate one-in-one molar ratio of 1X nucleosome to SPIN4 protein [2].
 - 3.3.1. Talent thawing the nucleosomes on ice.
 - 3.3.2. Talent calculating the required volume of nucleosome.
- 3.4. In a fifteen-milliliter tube, mix 1X nucleosome with an equal volume of 2X bead blocking buffer [1]. Combine the mixture with pre-blocked beads [2] and incubate for 4 hours on a tube rotator at 4 degrees Celsius and 20 rpm for complexation [3].
 - 3.4.1. Talent mixing the nucleosome solution with bead blocking buffer.
 - 3.4.2. Talent combining nucleosome and bead solutions in 15 ml tubes.
 - 3.4.3. Talent placing the mixture on a rotator.
- 3.5. Centrifuge the mixture at 3000 *g* for three minutes, at four degrees Celsius [1]. After discarding the supernatant, resuspend the beads in one milliliter of BC150 lysis buffer [2] and transfer to one-point-five milliliter tubes [3].
 - 3.5.1. Talent placing the tube in the centrifuge and closing the lid.
 - 3.5.2. Talent resuspending the beads in lysis buffer.
 - 3.5.3. Talent transferring the mixture to 1.5 ml tubes.
- 3.6. To wash the beads thrice, centrifuge at 3000 *g* for one minute [1]. After discarding the supernatant, resuspend the pellet in one milliliter of BC150 lysis buffer [2]. Agitate the tube occasionally for uniform distribution of beads between repeat washes [3].
 - 3.6.1. Talent placing the tubes in a centrifuge.
 - 3.6.2. Talent resuspending the pellet in lysis buffer. *Videographer: Obtain multiple reusable takes of 3.6.1 and 3.6.2, as these will be used later in the protocol*
 - 3.6.3. Talent agitating the tubes gently to suspend the beads evenly.
- 3.7. Perform two additional washes as demonstrated previously [1] and incubate the tubes on the rotator set at twenty rpm, four degree Celsius for ten minutes [2]. Resuspend the beads in nine column volumes of BC150 lysis buffer [3].
 - 3.7.1. *Reuse shots 3.6.1 and 3.6.2.*
 - 3.7.2. Talent placing the sample on a rotator.

3.7.3. Talent resuspending the beads in 9 CV lysis buffer.

3.8. Mix 160 microliters' beads from each sample with 40 microliters of 5X Laemmli (*five-X-LEM-lee*) buffer [1]. Heat at 95 degrees Celsius for five minutes for denaturation [2]. Perform a FLAG peptide elution of the protein-nucleosome complex for cleaner results with reduced background [3]. Centrifuge at 3000 *g* for five minutes at room temperature [4]. Then, perform silver staining and Western blotting to validate and detect the modified histone-SPIN4 binding [5].

3.8.1. Talent mixing beads with dye.

3.8.2. Talent heating the tubes in a heating block.

3.8.3. Talent performing FLAG peptide elution

Author's Note: Most of the filming was completed as per the script. For the final step, 3.8.3 (Talent performing FLAG peptide elution), the videographer suggested filming the entire process and sending back for review. This part is divided into three steps, and the videographer should have annotated them accordingly.

Note for Video Editor: If you have any doubt for shot 3.8.3, reach out to Kathyayani Sridharan.

3.8.4. Talent centrifuging the tubes containing mixture.

3.8.5. WIDE: Talent loading samples on SDS-PAGE gels with silver staining and Western blot transfer equipment on the workbench.

Results

4. Results

- 4.1. SPIN4 significantly binds to histone H3 (*H-three*) lysine four, or H3K4 (*H-three-K-four*) monomethyl, dimethyl and trimethyl peptides [1]. Conversely, the SPIN4D82H (*Spin-four-dee-eighty-two-H*) loss-of-function mutant showed reduced binding to all targets, confirming that Tudor domain one is essential for substrate binding. [2]. The binding affinity of SPIN4 is negatively impacted by other post-translational modifications in-cis conformations, such as H3R2me2s (*H-three-R-two-M-E-two-S*) [3] and H3R2me2a (*H-three-R-two-M-E-two-A*) [4].
 - 4.1.1. LAB MEDIA: Figure 2A, B, C: *Video editor: Please emphasize the blue lines immediately next to the Y-axis of all three images.*
 - 4.1.2. LAB MEDIA: Figure 2A, B, C: *Video editor: Please emphasize the red lines near the blue line immediately next to the Y-axis of all three images.*
 - 4.1.3. LAB MEDIA: Figure 2A, B, C: *Video editor: Please highlight the blue lines corresponding to the text R2me2s in all three images.*
 - 4.1.4. LAB MEDIA: Figure 2A, B, C: *Video editor: Please highlight the blue lines corresponding to the text R2me2a in all three images.*
- 4.2. Consistent with peptide array observations, SPIN4D82H exhibited an overall reduction of nucleosome binding relative to wildtype SPIN4 [1]. Western blot analysis comparing the pull-down samples of SPIN4 and SPIN4D82H further showed no quantifiable binding of SPIN4D82H to monomethyl, dimethyl or trimethyl H3K4 peptides, validating these dual approaches for rapidly identifying relevant histone post translation modification, or PTM (*P-T-M*) substrates [2].
 - 4.2.1. LAB MEDIA: Figure 4A: *Video editor: Please emphasize the band between values 25 and 37 in Flag-SPIN4 and Flag-SPIN4^{D82H} lanes.*
 - 4.2.2. LAB MEDIA: Figure 4B: *Video editor: Please emphasize the Flag-SPIN4 and Flag-SPIN4^{D82H} lanes corresponding to H3K4me1, H3K4me2 and H3K4me3 sequentially.*

Pronunciation guide:

1. Histone
 Pronunciation link: <https://www.merriam-webster.com/dictionary/histone>
 IPA: /'hɪstəʊn/
 Phonetic Spelling: his·tohn
2. Peptide
 Pronunciation link: <https://www.merriam-webster.com/dictionary/peptide>

IPA: /'pɛp.taɪd/

Phonetic Spelling: pep·tyde

3. Bovine

Pronunciation link: <https://www.merriam-webster.com/dictionary/bovine>

IPA: /'boʊ.vaɪn/

Phonetic Spelling: boh·vyne

4. Albumin

Pronunciation link: <https://www.merriam-webster.com/dictionary/albumin>

IPA: /æl'bjʊ.mɪn/

Phonetic Spelling: al·byoo·min

5. Hydrophobic

Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrophobic>

IPA: /,haɪdrə'foʊbɪk/

Phonetic Spelling: hy·druh·foh·bik

6. Microliters

Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>

IPA: /'maɪkroʊ.li:tər/

Phonetic Spelling: my·kroh·lee·terz

7. Incubate

Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>

IPA: /'ɪŋkjəˌbeɪt/

Phonetic Spelling: in·kyuh·bayt

8. Recombinant

Pronunciation link: <https://www.merriam-webster.com/dictionary/recombinant>

IPA: /rɪ'kɑːmbɪnənt/

Phonetic Spelling: ree·kahm·buh·nuhnt

9. Antibody

Pronunciation link: <https://www.merriam-webster.com/dictionary/antibody>

IPA: /'æn.tɪˌbɑː.di/

Phonetic Spelling: an·tee·bah·dee

10. Chemiluminescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/chemiluminescence>

IPA: /ˌkɛmɪluːməˈnɛsəns/

Phonetic Spelling: keh·mee·loo·muh·ness·uhns

11. Nucleosome

Pronunciation link: <https://www.merriam-webster.com/dictionary/nucleosome>

IPA: /'nuːkliəˌsɒm/

Phonetic Spelling: noo·klee·uh·sohm

12. Pulldown

Pronunciation link: <https://www.merriam-webster.com/dictionary/pulldown>

IPA: /'pʊlˌdaʊn/

Phonetic Spelling: puhl·down

13. Vortexing
Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>
IPA: /'vɔːr.tɛks/
Phonetic Spelling: vor·teks·ing
14. Equimolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/equimolar>
IPA: /,iːkwɪ'moʊlər/
Phonetic Spelling: ee·kwih·moh·ler
15. Lysis
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysis>
IPA: /'laɪsɪs/
Phonetic Spelling: ly·sis
16. Mononucleosomes
Pronunciation link: No confirmed link found
IPA: /,mɑːnoʊ'nuːkliə,soʊmz/
Phonetic Spelling: mah·noh·noo·klee·uh·sohmz
17. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sentrɪ,fjuːdʒ/
Phonetic Spelling: sen·trih·fyooj
18. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /,suːpər'netənt/
Phonetic Spelling: soo·per·nay·tuhnt
19. Aspirate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aspirate>
IPA: /'æspə'reɪt/
Phonetic Spelling: as·puh·rayt
20. Laemmli
Pronunciation link: No confirmed link found
IPA: /'ləmli/
Phonetic Spelling: lem·lee
21. Denaturation
Pronunciation link: <https://www.merriam-webster.com/dictionary/denaturation>
IPA: /diː,neɪtʃə'reɪʃən/
Phonetic Spelling: dee·nay·chuh·ray·shuhn
22. Elution
Pronunciation link: <https://www.merriam-webster.com/dictionary/elution>
IPA: /iː'luːʃən/
Phonetic Spelling: ee·loo·shuhn
23. SDS-PAGE
Pronunciation link: No confirmed link found

IPA: /ˌɛsdiːˈɛs peɪdʒ/

Phonetic Spelling: es-dee-es-payj

24. Western blotting

Pronunciation link: <https://www.merriam-webster.com/dictionary/Western%20blot>

IPA: /ˈwɛstərn blɑːtɪŋ/

Phonetic Spelling: wes-tern blot-ing