Protocol
Tandem immuno-purification of affinity-tagged proteins from mouse testis extracts for MS analysis

Here, we describe a protocol for extraction and tandem immunoprecipitation of the cytoplasmic and nuclear proteins from mouse testis for mass spectrometry. This protocol has been applied to knockin mice that express a meiotic protein of interest tagged with 3xFLAG-HA in the testis. The protocol is optimized for salt extraction of cytoplasmic and nuclear proteins from mouse frozen testes and thus can be used for a variety of proteins.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Tandem immuno-purification of affinity-tagged proteins from mouse testis extracts for MS analysis

Naoki Tani,1 Nobuhiro Tanno,2 and Kei-ichiro Ishiguro2,3,4,*

1Liaison Laboratory Research Promotion Center, IMEG, Kumamoto University, Kumamoto 860-0811, Japan
2Department of Chromosome Biology, Institute of Molecular Embryology and Genetics (IMEG), Kumamoto University, Kumamoto 860-0811, Japan
3Lead contact
4Technical contact
*Correspondence: ishiguro@kumamoto-u.ac.jp
https://doi.org/10.1016/j.xpro.2022.101452

SUMMARY

Here, we describe a protocol for extraction and tandem immunoprecipitation of the cytoplasmic and nuclear proteins from mouse testis for mass spectrometry. This protocol has been applied to knockin mice that express a meiotic protein of interest tagged with 3xFLAG-HA in the testis. The protocol is optimized for salt extraction of cytoplasmic and nuclear proteins from mouse frozen testes and thus can be used for a variety of proteins.

For complete details on the use and execution of this protocol, please refer to Ishiguro et al. (2020) and Tanno et al. (2022).

BEFORE YOU BEGIN

This protocol is designed to identify the proteins potentially interacting with the protein of your interest in the cytoplasmic and nuclear fractions of mouse testis. Fusing epitope tags such as 3xFLAG and HA to the N- or C-terminus of a protein allows enrichment of that particular protein and its interacting proteins (Nakatani and Ogryzko, 2003). Tandem immunoprecipitation is generally beneficial since relatively lower background of non-specific proteins is detected in the subsequent mass spectrometry (MS) analysis compared to conventional immunoprecipitation methods. Thus, the method has been widely used in culture cell lines and transgenic animals that express tagged proteins (Ikura et al., 2000; Nakatani and Ogryzko, 2003) (Odajima et al., 2016). In our case, since any cell lines that undergo meiosis were not available, we tagged 3xFLAG-HA to the protein of interest in the testis by ssODN-mediated Crispr-CAS9 genome editing and generated knock-in mice (Figure 1A) (Yoshimi et al., 2016). This enabled us to identify the interacting proteins with the protein of interest during meiosis. We previously generated Stra8-3xFLAG-HA-p2A-GFP knock-in (Stra8-3FH-GFP KI) mice, which had 3xFLAG-HA tagged STRA8 protein expressed in the testis (Ishiguro et al., 2020) From the nuclear fraction of Stra8-3FH-GFP KI testes, we identified MEIOSIN as a STRA8 interacting protein that directs the switching from mitosis to meiosis (Ishiguro et al., 2020). The same strategy is essentially applicable to any proteins that are expressed in the testis (Tanno et al., 2022).

Due to the nature of spermatocytes that are adherent to each other, and seminiferous tubules that are rigid consisting of epithelial and connective tissues, it is rather difficult to apply ordinal nuclear extraction methods used for culture cells, such as Dignam method (Dignam et al., 1983). Since the number of spermatocytes at a specific stage is limited in the testis, we often collect numerous frozen testes to obtain sufficient amount for immunoprecipitation experiment. Thus, we optimized the processes to extract cytoplasmic and nuclear proteins from mouse frozen testes. Our protocol for the preparation of testis extracts is also applicable to conventional immunoprecipitation (Fujiwara et al., 2020; Horisawa-Takada et al., 2021; Ishiguro et al., 2011, 2014; Kim et al., 2015; Takemoto et al., 2020). Essentially, extraction of cytoplasmic and nuclear proteins in this protocol depends on salt solubility, which can be theoretically applicable to the proteins that are expressed in any tissues other than the testis.
Institutional permissions
Fbxo47-3xFLAG-HA knock-in mice was C57BL/6 background (Tanno et al., 2022). Skp1-3xFLAG-HA knock-in mice (Tanno et al., 2022) and Stra8-3xFLAG-HA-p2A-GFP knock-in (Stra8-3FH-GFP KI) mice (Ishiguro et al., 2020) were congenic strains of C57BL/6 background. Male mice were used for immunoprecipitation of testis extracts. Animal experiments were approved by the Institutional Animal Care and Use Committee (approval F28-078, A30-001, A28-026, A2020-006). Permissions should be acquired from related institutions.

Figure 1. Expression of 3xFLAG-HA fusion protein in knockin mouse testis
(A) Schematic illustrations of the Fbxo47-3xFLAG-HA knock-in alleles, which were used in our experiments (Tanno et al., 2022). Blue boxes represent exons. The stop codon in the last coding exon is replaced with in-frame 3xFLAG-HA and endogenous 3’UTR in the indicated alleles. Red arrows indicate the FBXO47-3xFLAG-HA protein. Note that small amount of the FBXO47-3xFLAG-HA protein in the IP from the nuclear extract was detected by LC-MS/MS analysis (Tanno et al., 2022), and a faint band was visible in the western blot.
(B) Shown is an example of tandem immunoaffinity purification of 3xFLAG-HA fusion protein from cytosolic and nuclear fractions of the Fbxo47-3xFLAG-HA knock-in mouse testes. For the control, mock immunoaffinity purification was performed using non-tagged control testes. * indicates a band that was detected by anti-FLAG antibody. This may represent a degraded product or an isoform of FBXO47 protein. The image was adapted from Supplementary Figure 4 in our previous paper (Tanno et al., 2022).

**Institutional permissions**
Fbxo47-3xFLAG-HA knock-in mice was C57BL/6 background (Tanno et al., 2022). Skp1-3xFLAG-HA knock-in mice (Tanno et al., 2022) and Stra8-3xFLAG-HA-p2A-GFP knock-in (Stra8-3FH-GFP KI) mice (Ishiguro et al., 2020) were congenic strains of C57BL/6 background. Male mice were used for immunoprecipitation of testis extracts. Animal experiments were approved by the Institutional Animal Care and Use Committee (approval F28-078, A30-001, A28-026, A2020-006). Permissions should be acquired from related institutions.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse anti-HA 5D8 monoclonal antibody-coupled Magnet agarose | MBL | Cat# M132-10 RRID: AB_11142502 |
| anti-FLAG M2 monoclonal antibody agarose affinity gel | Sigma-Aldrich | Cat# M8823 RRID: AB_2637089 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse anti-FLAG M2 (IB, 1:1000) | Sigma-Aldrich | Cat# F1804 RRID: AB_262044 |
| Rabbit anti-HA (WB, IF, 1:1000) | Abcam | Cat# ab9110 RRID: AB_307019 |

### Chemicals, peptides, and recombinant proteins

- 3xFLAG peptide | Sigma-Aldrich | Cat# F4799 |
- SimplyBlue | Thermo Fisher Scientific | Cat# LC6065 |
- cComplete, EDTA-free | Roche | Cat# 4 693 132 |
- NuPAGE 4%–12% Bis-Tris Protein Gel | Thermo Fisher Scientific | Cat# NP0322 |
- NuPAGE MOPS-SDS Running Buffer | Thermo Fisher Scientific | Cat# NP0001 |
- NuPAGE LDS Sample Buffer (4x) | Thermo Fisher Scientific | Cat# NP0007 |
- NuPAGE Antioxidant | Thermo Fisher Scientific | Cat# NP0005 |
- NuPAGE Sample Reducing Agent (10x) | Thermo Fisher Scientific | Cat# NP0004 |
- Bio-Rad Protein Assay Dye Reagent Concentrate (20x) | Bio-Rad | Cat# 5000006JA |
- Pierce DTT, No-Weight Format (48 x 7.7 mg) | Thermo Fisher Scientific | Cat# 20291 |
- Pierce Iodoacetamide, Single-Use (24 x 9.3 mg) | Thermo Fisher Scientific | Cat# 90034 |
- Tryptsin/Lys-C Mix, Mass Spec Grad (5 x 20 μg) | Promega | Cat# V5073 |
- Ammonium Hydrogencarbonate (for Proteomics) | FUJIFILM Wako Pure Chemical | Cat# 018-21742 |
- Acetonitrile for LCMS | FUJIFILM | Cat#8012-19851 |
- Trifluoroacetic acid (for High Performance Liquid Chromatography) | FUJIFILM Wako | Cat#206-10731 |
- Ultrapure Water (for LC/MS) | FUJIFILM Wako | Cat#214-01301 |
- Formic acid (abt. 99%) | FUJIFILM Wako | Cat#067-04531 |
- Silver Quest | Thermo Fisher Scientific | Cat# 6070 |
- 1 M Tris-HCl (pH 8.0) | Sigma-Aldrich | Cat# T2694 |
- 1 M Tris-HCl (pH 7.5) | Sigma-Aldrich | Cat# T2319 |
- HEPES | Sigma-Aldrich | Cat# H4034 |
- NaCl | Sigma-Aldrich | Cat# S3014 |
- KCl | Sigma-Aldrich | Cat# D4541 |
- D-PBS | Nacalai Tesque | Cat# 14249-24 |
- EDTA | Nacalai Tesque | Cat# 15105-35 |
- Triton X | Nacalai Tesque | Cat# 35501-15 |
- Tween20 | Nacalai Tesque | Cat# 35624-15 |
- HCI | Nacalai Tesque | Cat# 18321-05 |
- KOH | Nacalai Tesque | Cat# 28616-45 |
- MgCl₂ | Wako | Cat# 135-00165 |
- Glycine | Wako | Cat# 073-00737 |
- Glycerol | Wako | Cat# 075-00616 |
- β-mercaptoproethanol | Wako | Cat# 139-07525 |

### Experimental models: Organisms/strains

- Mouse: Stra8-3xFLAG-HA-p2A-GFP knock-in, Male homozygous (Postnatal 10–12 days old) | Ishiguro et al. (2020) | CARD ID 2555 |
- Mouse: Fbxo47-3xFLAG-HA knock-in, Male, homozygous (Postnatal 14–21 days old) | Tanno et al. (2022) | CARD ID 2972 |
- Mouse: Skp1-3xFLAG-HA knock-in, Male heterozygous (Postnatal 14–21 days old) | Tanno et al. (2022) | CARD ID 2638 |
- Mouse: C57BL/6N, Male (Postnatal 10, 14–21 days old) | SLC | N/A |

### Software and algorithms

- Proteome Discoverer version 1.4 | Thermo Fisher Scientific | N/A |
- Mascot search engine version 2.7 | Matrix Science | N/A |
- Xcalibur 4.027.19/Tune 2.8 SP1 | Thermo Fisher Scientific | N/A |
- FBXO47 IP Raw Data | Mendeley Data | https://data.mendeley.com/datasets/ct84bbswv5/2 |

### Other

- Cell strainer 100 μm | FALCON #352360 | N/A |
- Homogenizer HK-1 | AS ONE, 1-2050-11 | N/A |
- Potter-type tissue grinder pestle/tube (15 mL) | AS ONE, #5-5721-04 | N/A |
- Potter-type tissue grinder pestle/tube (30 mL) | AS ONE, #5-5721-05 | N/A |

(Continued on next page)
### MATERIALS AND EQUIPMENT

#### Buffer A

| Reagent                          | Final concentration | Amount       |
|----------------------------------|---------------------|--------------|
| Tris-HCl (pH 7.5) (1 M)          | 20 mM               | 2 mL         |
| KCl (2 M)                        | 200 mM              | 10 mL        |
| EDTA (0.5 M)                     | 0.4 mM              | 0.8 mL       |
| TritonX100 (10%)                 | 0.1%                | 1 mL         |
| β-mercaptoethanol (0.1 M)        | 1 mM                | 1 mL         |
| Glycerol                         | 10%                 | 10 mL        |
| ddH2O                            | n/a                 | 75.2 mL      |
| **Total**                        | n/a                 | 100 mL       |

Stock can be stored at 4°C for a week. Freshly add 2 tablets of Complete Protease Inhibitor to 100 mL of stock before use.

#### Buffer B

| Reagent                          | Final concentration | Amount       |
|----------------------------------|---------------------|--------------|
| HEPES-KOH pH 7.0 (0.5 M)         | 20 mM               | 4 mL         |
| KCl (2 M)                        | 400 mM              | 20 mL        |
| MgCl2 (1 M)                      | 5 mM                | 0.5 mL       |
| Tween20 (10%)                    | 0.1%                | 1 mL         |
| β-mercaptoethanol (0.1 M)        | 1 mM                | 1 mL         |
| Glycerol                         | 10%                 | 10 mL        |
| ddH2O                            | n/a                 | 63.5 mL      |
| **Total**                        | n/a                 | 100 mL       |

Stock can be stored at 4°C for a month. Freshly add 2 tablets of Complete Protease Inhibitor to 100 mL of stock before use.

#### Buffer C

| Reagent                          | Final concentration | Amount       |
|----------------------------------|---------------------|--------------|
| HEPES-KOH pH 7.0 (0.5 M)         | 20 mM               | 4 mL         |
| EDTA (0.5 M)                     | 0.4 mM              | 0.8 mL       |
| β-mercaptoethanol (0.1 M)        | 1 mM                | 1 mL         |
| Glycerol                         | 10%                 | 10 mL        |

(Continued on next page)
### Reagent Final concentration Amount

| Reagent          | Final concentration | Amount   |
|------------------|---------------------|----------|
| ddH2O            | n/a                 | 84.2 mL  |
| Total            | n/a                 | 100 mL   |

Stock can be stored at 4°C for a month. Freshly add 2 tablets of Complete Protease Inhibitor to 100 mL of stock before use.

### Buffer D

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| HEPES-KOH pH 7.0 (0.5 M) | 20 mM               | 4 mL     |
| KCl (2 M)                | 200 mM              | 10 mL    |
| MgCl2 (1 M)              | 5 mM                | 0.5 mL   |
| Tween20 (10%)            | 0.1%                | 1 mL     |
| β-mercaptoethanol (0.1 M)| 1 mM                | 1 mL     |
| Glycerol                 | 10%                 | 10 mL    |
| ddH2O                    | n/a                 | 73.5 mL  |
| Total                    | n/a                 | 100 mL   |

Stock can be stored at 4°C for a month. Freshly add 2 tablets of Complete Protease Inhibitor to 100 mL of stock before use.

### TBS

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Tris-HCl pH7.4 (1 M)     | 50 mM               | 5 mL     |
| NaCl (5 M)               | 150 mM              | 3 mL     |
| ddH2O                    | n/a                 | 72 mL    |
| Total                    | n/a                 | 100 mL   |

Stock can be stored at 4°C for a month.

### 50× stock solution of 3xFLAG peptide (5 mg/mL)

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| 3xFLAG peptide powder (Sigma-Aldrich, F4799) | 5 mg/mL | 4 mg     |
| TBS                      | 150 mM              | 0.8 mL   |
| Total                    | n/a                 | 0.8 mL   |

Stock can be stored at –30°C.

### Acidic elution buffer

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Glycine-HCl pH 2.5 (1 M) | 100 mM              | 5 mL     |
| NaCl (5 M)               | 150 mM              | 1.5 mL   |
| ddH2O                    | n/a                 | 43.5 mL  |
| Total                    | n/a                 | 50 mL    |

Stock can be stored at 4°C for a month.

### Mobile phase A

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Formic acid              | 0.1%                | 1 mL     |
| LCMS grade ultrapure water | n/a             | 999 mL   |
| Total                    | n/a                 | 1000 mL  |

Stock can be stored at 4°C for a month.
### Mobile phase B

| Reagent                  | Final concentration | Amount |
|--------------------------|---------------------|--------|
| Formic acid              | 0.1%                | 1 mL   |
| LCMS grade acetonitrile  | n/a                 | 999 mL |
| Total                    | n/a                 | 1000 mL|

Stock can be stored at 4°C for a month.

### Digestion buffer

| Reagent                                  | Final concentration | Amount |
|------------------------------------------|---------------------|--------|
| Ammonium bicarbonate pH 8.0 (2 M)        | 40 mM               | 0.2 mL |
| ddH₂O                                    | n/a                 | 99.8 mL|
| Total                                    | n/a                 | 100 mL |

Stock can be stored at 4°C for a month.

### 10% Triton X-100

| Reagent   | Final concentration | Amount |
|-----------|---------------------|--------|
| Triton X-100 | 10%               | 5 mL   |
| ddH₂O     | n/a                 | 45 mL  |
| Total     | n/a                 | 50 mL  |

Stock can be stored at 20°C–25°C (room temperature) up to 3 months.

### 10% Tween20

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Tween20 | 10%                 | 5 mL   |
| ddH₂O   | n/a                 | 45 mL  |
| Total   | n/a                 | 50 mL  |

Stock can be stored at 20°C–25°C (room temperature) up to 3 months.

### 1 M Glycine-HCl (pH 2.5)

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Glycine | 1 M                 | 7.5 g  |
| HCl     | n/a                 | To adjust the pH to 2.5 |
| ddH₂O   | n/a                 | Up to 100 mL |
| Total   | n/a                 | 100 mL |

Dissolve glycine with 80 mL of distilled water. Adjust the pH with HCl and bring the total volume to 100 mL. Store the solution at 4°C up to 6 months.

### STEP-BY-STEP METHOD DETAILS

#### Part 1: Preparation of testis extracts

**Starting materials**

- **Timing:** 3–4 h for collection of testes, homogenization of testis and extraction of cytosolic proteins
- **Timing:** 2–3 h for preparation of testis nuclear extracts

Since expression levels and stage specificities are different among the proteins of your interest, the number of testes required for a tandem immunoprecipitation experiment should be
empirically estimated. In our case, the expression pattern and the expression level of the protein of interest can be roughly estimated by immunostaining of 3xFLAG-HA tagged protein in the seminiferous tubule section of the knock-in mouse. We often collect numerous testes (40–100 testes from 20–50 juvenile mice) of different stages, and store frozen testes until sufficient number of testes are acquired for one immunoprecipitation experiment. Collected testes can be stored at −80°C until preparation of extracts. For storage in a freezer, it is favorable to detunicate the testes in PBS every time after collection, remove PBS as much as possible, and store them in a tube. The following procedure for the preparation of testis extracts is also applicable to conventional immunoprecipitation.

Collection of testes, homogenization of testis and extraction of cytosolic proteins.

1. To prepare testis extracts, remove testes from 20 juvenile male (postnatal day 10–21, spermatocytes are in the first wave of spermatogenesis) of mock control mice and 3xFLAG-HA-tagged knock-in mice, detunicate with fine tweezers in PBS (Figure 2A). Collect detunicated testes into a new 15 mL tube.

Optional: Snap freeze the collected testes in liquid nitrogen, and store at −80°C until the preparation of extracts as described in step 2.

2. Resuspend the testes in 10 mL of buffer A at 4°C. The volume of buffer A should be proportionally scaled up according to the number of testes used. Testes from wild type non-tagged mice should be used for negative control.

If you use frozen testes, resuspend the frozen testes directly in buffer A at 4°C, and leave for 5 min.

Note: The volume of buffer A for one testis should be empirically determined depending on the expression level of the protein of your interest and the size of the testis (the number of spermatocytes/testis). We suggest starting from 40 testes/10 mL.

Optional: Pass the seminiferous tubules through 1 mm syringe to remove interstitial cells of the testis (Figure 2B). Collect the clumps of seminiferous tubules with forceps and place them in a new 10 mL of buffer A at 4°C.

3. Homogenize the testes with Potter-type tissue grinder pestle and tube (30 mL) by 5–10 strokes at 4°C. (Figure 2C, Methods video S1). Take 10 μL of homogenate on a slide glass to confirm the cells are disrupted.

4. Hold a cell strainer (100 μm) on the top of a 50 mL tube. Take homogenized testis extract by pipet and filtrate it through the cell strainer to remove debris (Figure 2D, Methods video S1). If the cell strainer is stuck, change it with a new one.

5. Transfer homogenized testis extracts to Open-Top Thickwall Polycarbonate ultra-centrifuge tubes. Centrifuge testis extracts by ultra-centrifugation at 100,000 g for 30 min at 4°C with TLA100.3 Fixed-Angle Titanium Rotor (Figures 2E and 2F).

6. Take the supernatant into a new tube at 4°C. This is the extract mainly containing the cytosol. Protein concentration should be around the range of 3–5 mg/mL by Bradford assay.

Note: Keep 40 μL (0.4% of the starting volume) of cytosolic extract as the input sample of immunoprecipitation, if you need to check it later by western blot.

Optional: The insoluble pellet can be stored at −80°C until the preparation of nuclear extracts.
Preparation of testis nuclear extracts

7. Resuspend the insoluble pellet with 5 mL of buffer B at 4°C, and then transfer into a new tube.

Sonicate the pellet briefly (Duty cycle: constant, output control: 1), and incubate at 4°C for 30 min. For sonication, use sonifier 250 with 1/8" Tapered Microtip (Branson) or equivalent sonicator (Figure 2G, Methods video S1).
Note: The volume of buffer B for one testis should be empirically determined depending on the expression level of the protein of your interest and the size of the testis (the number of spermatocytes/testis). Described here is 5 mL for insoluble pellet from 40 testes. But if you need higher protein concentration, less than 5 mL (within a range of 2 ~ 5 mL) can be used.

8. Transfer homogenized testis extracts to Open-Top Thickwall Polycarbonate ultra-centrifuge tubes. Centrifuge the solubilized extracts by ultra-centrifugation at 100,000 g for 30 min at 4°C.

9. Take supernatant (total 5 mL) into a new tube at 4°C. This is the nuclear extract solubilized by high salt. Protein concentration will be around the range of 1–3 mg/mL by Bradford assay depending on the age of the starting materials.

Note: Keep a small amount (20 µL: 0.4% of the starting volume) of nuclear extract at −80°C as the input sample of immunoprecipitation, if you need to check it later by western blot.

Part 2: Tandem immuno-affinity precipitation of the tagged proteins from testis extracts

The following protocol describes immunoprecipitation of the cytosolic proteins and the nuclear proteins. For immunoprecipitation of the cytosolic proteins obtained at step 5, use buffer A, and follow the procedure as described below.

For immunoprecipitation of the nuclear proteins obtained at step 8, dilute the extract with equal amount of buffer C (5 mL) to bring the salt concentration down to 200 mM KCl, then use buffer D and follow the same procedure as described below. For immunoprecipitation of the nuclear proteins at high salt condition, go to immunoprecipitation procedure without dilution, use buffer B and follow the same procedure as described below. Under this stringent condition, while the hydrophobic interaction is retained, the electrostatic protein-protein interactions are lost causing certain dissociation of interacting proteins. Thus, the salt condition should be empirically optimized for the protein of your interest.

Anti-FLAG immuno-affinity purification

© Timing: 2–4 h

10. Equilibrate 100 µL of anti-FLAG M2 magnetic beads with buffer A by taking 100 µL of anti-FLAG M2 magnetic beads into a tube, place it on a magnetic stand, remove the supernatant, and suspend the anti-FLAG M2 magnetic beads in 1 mL of buffer A. Repeat the wash twice. After the final wash, suspend the magnetic beads in 100 µL of buffer A.

Note: You may use commercially available anti-FLAG M2 agarose beads.

11. Add 100 µL of anti-FLAG M2 magnetic beads suspension to the 10 mL extract.
12. Incubate for 1–3 h at 4°C with rotation.
13. Collect 1.5 mL of the mixture of the extract and anti-FLAG M2 magnetic beads into a new 1.5 mL tube. Place on a magnetic stand and remove the unbound supernatant. Repeat for the whole mixture of the extract and anti-FLAG M2 magnetic beads by collecting 1.5 mL each time into the same tube.

Note: Keep the unbound supernatant into a different tube for later confirmation.

14. Wash anti-FLAG M2 magnetic beads with 1 mL of buffer A for 4 times on a magnetic stand. Leave the tubes on the magnetic stand for 1 min at each wash. After the final wash, keep the tubes on the magnetic stand.
15. Remove the washing buffer and suspend the magnetic beads in 200 μL of buffer A containing 3xFLAG peptide (final 100 μg/mL) at 4°C. Elute bead-bound immunoprecipitates with 200 μL of buffer A containing 3xFLAG peptide at 4°C with rotation for 5 min.

16. Place the tube on a magnetic stand. Transfer the supernatant into a new tube.

**Note:** For later verification, store 20 μL of the eluted samples at –80°C.

**Note:** Repeat elution with another 200 μL of buffer A containing 3xFLAG peptide and combine the eluates. This additional elution increases the yield of anti-FLAG IP products.

**Anti-HA immuno-affinity purification**

@ **Timing:** 2–4 h

17. Equilibrate 100 μL of anti-HA magnetic beads with buffer A by taking 100 μL of anti-HA magnetic beads into a tube, place it on a magnetic stand, remove the supernatant and suspend the anti-HA magnetic beads in 1 mL of buffer A. Repeat the wash twice. After the final wash, suspend the magnetic beads in 100 μL of buffer A.

**Note:** You may use commercially available anti-HA agarose beads.

18. Add 50 μL of anti-HA magnetic beads suspension to the FLAG-purified immunoprecipitates from mock control and 3xFLAG-HA-tagged knock-in mice.

19. Incubate for 1–3 h at 4°C with rotation.

20. Wash anti-HA magnetic beads with buffer A for 4 times on a magnetic stand. After the final wash, keep the tubes on the magnetic stand.

**Note:** Keep the unbound supernatant into a different tube for later confirmation.

21. Remove the supernatant and suspend the magnetic beads in 40 μL of acid elution buffer. Elute bead-bound immunoprecipitates with 40 μL of acid elution buffer by gently tapping the tube for 2 min at 20°C–25°C (room temperature).

22. Place the tube on a magnetic stand. Remove the supernatant and transfer it into a new tube, and neutralize with 4 μL of 1 M Tris-HCl (pH 8.0).

△ **CRITICAL:** Final elution of beads-bound immunoprecipitates should be done with acid elution buffer rather than boiling. In our experience, boiling the beads-bound immunoprecipitates in SDS-sample buffer causes significant dissociation of IgG from the beads, which leads to relatively higher detection of peptides derived from IgG in MS analyses, impeding the detection of peptides from the immunoprecipitates.

No avoid inclusion of IgG, elution with HA peptide can also be applied instead of acid elution.

IID **Pause point:** The immunoprecipitated samples can be stored at –80°C until the following MS analysis.

**Optional:** Run SDS-PAGE using 4 μL of the final immunoprecipitates together with the input samples. Western blotting with anti-FLAG or anti-HA antibody should be performed to examine whether 3xFLAG-HA-tagged proteins are immunoprecipitated (Figure 1B).

**Part 3: MS shotgun analysis of the immunoprecipitates**

Proteins in the immunoprecipitates will be identified by LC-MS/MS analysis. For shotgun screening of interacting proteins to the protein of your interest, whole immunoprecipitates in the gel are
digested by trypsin, and the derived peptides are analyzed by LC-MS/MS according to previously established method (Shevchenko et al., 2006). For desalting purpose, the immunoprecipitates first should be run on a gel by 0.5–1 cm from the well. The immunoprecipitated samples contained in the excised gel pieces undergo reduction of S-S bonds and alkylation, followed by trypsin digestion in the gel. The eluted peptides from the gel are subjected to LC-MS/MS analysis. For LC-MS/MS analysis at better resolution, the immunoprecipitates are fully separated on a gel, and stained with Coomassie-blue or silver. Then, the gel pieces containing particular protein bands of your interest can be excised.

Alternatively, whole immunoprecipitates after acid elution can be directly digested in solution for MS shotgun analysis. However, please note that residual detergents derived from the IP buffer could raise significant noises at the base line in the later MS spectrum. For In-liquid digestion, buffer B without detergents should be used at the final washing step (step 20).

**Sample preparation for MS shotgun analysis**

**Timing:** 2–3 days

1. Boil the immunoprecipitated samples in NuPAGE LDS Sample Buffer containing Sample Reducing Agent at 90°C for 5 min. Run the samples on NuPAGE 4%–12% Bis-Tris gel in MOPS-SDS buffer with antioxidant at 200 V for 3–5 min. The front of blue dye should migrate by 0.5–1 cm from the top. In this way, proteins ranged from high to low molecular weight are stacked in a narrow space of the gel.

2. Immerse the gel in pure water for 10 min with rotation. Stain the gel in Simply Blue solution for > 2 h.

   **Pause point:** The Simply Blue stained gel can be stored overnight at room temperature or at 4°C.

3. Discard Simply Blue solution and wash the gel in pure water.

4. Excise the gel by approximately 0.8 cm × 0.5 cm immediately below the wells (Figure 2H). Then cut the gel slices into approximately 1–1.5 mm sized cubes for efficient in-gel trypsin digestion, and transfer them into a 1.5 mL Protein LoBind micro-tube (Figure 2H, right).

   **Optional:** For identification of a protein of a particular band size rather than shotgun analysis, run the immunoprecipitated samples through NuPAGE 4%–12% Bis-Tris gel, and stain with Silver Quest according to manufacturer’s instruction. The gel pieces containing silver-stained bands at the size of your interest can be cut out for MS analysis (Figure 2I).

5. Destain the protein bands in gel pieces with 300 µL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10–15 min by shaking the micro-tubes at room temperature in the Maximizer MBR-022UP at the speed of 900 rpm.

   **Note:** Unless otherwise specified, all the steps of in-gel digestion are performed by shaking at 900 rpm in the Maximizer MBR-022UP.

6. Remove the supernatant and discard.

7. Destain the protein bands in gel pieces with 300 µL of 40 mM ammonium bicarbonate, 20% acetonitrile for 10–15 min.

8. Remove the supernatant and discard.

9. Repeat 28–31 once more (Figure 2J).

10. Dehydrate the gel pieces with 150 µL of acetonitrile for 10 min. Remove the supernatant, and discard.
33. Finally, dehydrate the gel pieces with a centrifugal evaporator for 15 min.
34. After dehydration, reduce the proteins in the gel pieces (cubes) with 200 μL of 10 mM DTT in 25 mM ammonium bicarbonate for 1 h at 56°C. Remove the supernatant and discard.
35. Wash the gel pieces (cubes) with 100–150 μL of 25 mM ammonium bicarbonate for 10 min. Remove the supernatant and discard.
36. Alkylate proteins in the gel pieces (cubes) with 100–150 μL of 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 45 min with protection from light. Remove the supernatant and discard.
37. Wash the gel pieces with 100–150 μL of 25 mM ammonium bicarbonate for 10 min. Remove the supernatant and discard.
38. Alkylate proteins in the gel pieces (cubes) with 100–150 μL of 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 45 min with protection from light. Remove the supernatant and discard.
39. Wash the gel pieces with 100–150 μL of 25 mM ammonium bicarbonate for 10 min. Remove the supernatant and discard.
40. Alkylate proteins in the gel pieces (cubes) with 100–150 μL of 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 45 min with protection from light. Remove the supernatant and discard.
41. Dehydrate the gel pieces with 300 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min. Remove the supernatant and discard.
42. Dehydrate the gel pieces with 300 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min. Remove the supernatant and discard.
43. Dehydrate the gel pieces with 300 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min. Remove the supernatant and discard.
44. Dehydrate the gel pieces with 300 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min. Remove the supernatant and discard.
45. Dehydrate the gel pieces with 300 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min. Remove the supernatant and discard.
46. Dehydrate the gel pieces with 300 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min. Remove the supernatant and discard.
47. Dehydrate the gel pieces with 300 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min. Remove the supernatant and discard.
48. Dehydrate the gel pieces with 300 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min. Remove the supernatant and discard.
49. Dehydrate the gel pieces with 300 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 10 min. Remove the supernatant and discard.
50. Finally, dehydrate the gel pieces with a centrifugal evaporator for 15 min.
51. After dehydration, digest the proteins in the gel pieces (cubes) with 20 ng/μL trypsin and lysyl endopeptidase in a buffer containing 40 mM ammonium bicarbonate, pH 8.0, overnight at 37°C, by shaking at the speed of 650 rpm.

Note: Wait until the gel pieces (cubes) expand with the solution of trypsin/lysyl endopeptidase on ice for about 1 hr. Then cover the gel pieces (cubes) with 40 mM ammonium bicarbonate.

42. Extract the digested peptides from the gel pieces (cubes) with 50–100 μL of 50% acetonitrile, 0.1% formic acid for 30–60 min at room temperature in the Maximizer at the shaking speed of 1,300 rpm. Remove the supernatant and transfer it into a fresh micro-tube.
43. Re-extract the digested peptides from the gel pieces (cubes) with 50–100 μL of 70% acetonitrile, 0.1% formic acid for 30–60 min at room temperature in the Maximizer at the shaking speed of 1,300 rpm. Remove the supernatant, transfer, and combine into the micro-tube of step 42.
44. Concentrate the combined supernatant to 15 μL with a centrifugal evaporator.

Pause point: Until LC-MS/MS analysis, the concentrated samples can be stored at −20°C for 2–3 days.

Mass spectrometry shotgun analysis

© Timing: 2–4 days

45. For LC-MS/MS analysis on Advance UHPLC system coupled to Q Exactive mass spectrometer, it is required before the analysis to perform mass calibration for positive ion mode by automatic calibration procedure with LTQ Velos ESI Positive Ion Calibration Solution.

Note: The following procedure is the condition optimized for the above equipment. If you are using other equipment, please optimize accordingly.

46. Settings for MS and MS/MS acquisitions:
   a. MS range is set to m/z 350–2,000, resolution 70,000, positive ion mode.
   b. MS/MS spectra are acquired by the following settings: Top-10 DDA (data-dependent acquisition), resolution 17,500, high-energy collision dissociation value 27.
   c. ESI spray: Advance Captive Spray Ionization probe (AMR/Bruker), 1,400 V, 250°C, 500 nL/min.
47. Setting for Advance UHPLC system and autosampler (PAL HTS-xt, AMR/CLC Analytic AG):
a. The sample in autosampler is injected into a pre-column though the multi-valve. The injected sample is trapped in the pre-column (L-column 2 ODS, 5 μm, 0.3 × 5 mm, cartridge trap column, CERI), then desalted and concentrated.

Note: The solvent for equilibration and desalting consists of 2% acetonitrile and 0.1% trifluoroacetic acid. Therefore, the samples are diluted 2-fold with the solvent of 2% acetonitrile, 0.1% trifluoroacetic acid.

b. After the sample is desalted and concentrated, change the position of the multi-valve to connect it to the line of separation column.

c. Separation column (analytical column): micro-ODS column (L-column 2 ODS, 3 μm, 0.1 × 150 mm, CERI), at 40°C with flow rate 500 nL/min. Mobile phase A (LCMS grade water, 0.1% formic acid), mobile phase B (LCMS grade acetonitrile, 0.1% formic acid).

Note: The gradient should be chosen according to the sample. We tested several gradient conditions: For example, 5%–45%B/0–20 min, 5%–45%B/0–40 min, 5%–45%B/0–60 min, and so on. In particular samples described above, we suggest the following condition: 5%–45%B/0–60 min, 45%–95%B/60–70 min.

d. Perform continuous injection with the auto-sampler controlled by Xcalibur/Tune (Thermo Fisher Scientific).

48. Analyze the raw LC-MS/MS data against the UniprotKB (Swissprot + TrEMBL) and NCBI non-redundant protein/translated nucleotide database restricted to Mus musculus using Proteome Discoverer version 1.4 (Thermo Fisher) with the Mascot search engine version 2.7 (Matrix Science).

49. Set the parameters for the analysis as follows:
   a. Fixed modification: carbamidomethylation of Cys.
   b. Variable modification: oxidation of Met, deamidation of Asn, and deamidation of Gln.

   Note: Whether addition of deamidation of Asn and deamidation of Gln is optional.

   c. Precursor mass tolerance: 10 ppm.
   d. Fragment ion mass tolerance: 0.02 Da.

50. Estimate the false discovery rate (FDR) with a decoy database comprised of either randomized or reversed sequences from the target database. Evaluate false positives by Percolator algorithm. Filter the search results with 1% global FDR for high confidence level.

EXPECTED OUTCOMES

Tandem 3× FLAG-HA immunoprecipitation specifically enriches the protein of your interest from cytosolic and nuclear fractions. Although the sensitivity of protein detection depends on MS instruments, roughly 1–10 ng order of protein can be unambiguously identified by our LC-MS/MS analysis. On a silver staining gel (Figure 2I), you may see the most abundant band that does not represent any specific protein. Abundant bands are usually common contaminants affecting IP-MS protocols. Further, you may be aware that even potential interactors do not necessarily represent intense bands in a stoichiometric manner. Bands that are faintly visible on the gel could also represent specific but transient interactors. Since some non-specific proteins are detected in IP-MS analysis, you should carefully assess which are the bona fide interactors to the bait protein by repeating the IP-MS experiments at least three times to consider the reproducibility and the specificity. Further, it is important to perform the same procedure for negative control immunoprecipitation using the extracts of non-tagged protein to filter out non-specific precipitates. Parallel comparison of the MS data between two immunoprecipitation samples derived from mice expressing 3xFLAG-HA
tagged protein and non-tagged control mice (mock control) makes it possible to identify the candidate proteins that specifically interact with the 3xFLAG-HA-fusion protein with higher accuracy. Most of such proteins can be identified by subtracting the MS data of the control non-tagged mice from those of the knock-in mice. Finally, biological interpretation of the potential interactors should be verified by other means of experiments.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analyze the raw LC-MS/MS data against the UniprotKB (SwissProt + TrEMBL) database restricted to *Mus musculus* (latest data can be downloaded from the UniprotKB web site) using Proteome Discoverer version 1.4.1.14 (Thermo Fisher) with Mascot search engine version 2.7.0. After filtering the dataset with 1% FDR (false discovery rate), Proteome Discoverer shows simple dataset that consists of quantitative value of Mascot score, PSMs (peptide-spectrum matches shown by Total Spectrum Count) and Area (top3-average area of total ion chromatogram). PSMs values require 2 or higher for identification of proteins. Before comparing the proteins identified in control-IP and FLAG-HA IP, some contaminant proteins derived from experimental processes, in particular enzymatic digestion (for example keratin), should be excluded. It is also useful to subtract the proteins commonly identified in control-IP from those identified in FLAG-HA IP to validate the protein list. In some cases, we perform verification of the above protein dataset by other proteome software, such as Scaffold 4.8.4 and 5.1.2 (Proteome software) which are equipped with other functions (for example, statistical test by fisher’s extract test, or quantitative scatterplot by volcano plot).

LIMITATIONS

Extraction and solubilization of nuclear proteins rely on higher salt concentration in our protocol. Since Protein-protein interactions are salt sensitive, it is possible that electrostatic interaction is disrupted by high salt concentration, and certain dissociation of interacting proteins may occur. Thus, our protocol may not necessarily detect all the protein-protein interactions. It is required to characterize the proteins of your interest for salt solubility by fractionating with different salt concentrations. It is also possible that a certain level of cytosolic proteins are included in the nuclear fraction. The protocol provides rough enrichment of the nuclear fraction from mouse testes tissue without isolation of the nuclei. Thus, the condition should be empirically optimized for the protein of your interest.

TROUBLESHOOTING

**Problem 1**

Detection of even the 3x FLAG-HA fusion protein is quite low in MS analysis of the immunoprecipitates.

**Potential solution**

This happens when the expression of the 3x FLAG-HA fusion is low or limited to a specific cell stage. Scaling up the starting materials and pre-fractionation enrichment of the protein should be considered.

Another potential reason could be that the solubility of the 3x FLAG-HA tagged protein of your interest is low in the fraction. Optimization for protein solubilization should be considered (steps 1–9). Other potential reason could be that the 3x FLAG-HA fusion may affect the intrinsic function of the protein of your interest, leading to a certain defect to the cells. Alternatively, the accessibility of the FLAG and HA antibodies to 3x FLAG-HA tags could be structurally interfered. In such cases, it should be considered which of the N- or C-terminal fusion of the 3x FLAG-HA tag is appropriate. We consider this issue by pilot 3x FLAG-HA immunoprecipitations using N- and C-terminal versions of the fusion proteins transiently expressed in cultured cells.
First, you should consider whether the low efficiency of extraction and/or immunoprecipitation was due to technical reasons (steps 1–22) or intrinsic nature of the protein, such as stability, solubility or expression level. To diagnose the problem, you should check the immunoprecipitation efficiency of the 3xFLAG-HA-tagged protein by western blot (Figure 1B). By western blot of the samples taken at the collection steps as described above, you can verify whether immunoprecipitation has properly worked. With the sample volume suggested in the table below, you can roughly estimate the enrichment efficiency of the 3xFLAG-HA-tagged protein.

### Problem 2
Detection of peptides derived from non-specific proteins are high even in the negative control mock IP.

**Potential solution**
Although tandem 3× FLAG-HA immunoprecipitation specifically enriches the protein of your interest, the surface of beads itself nonspecifically absorbs abundant proteins at a certain level. This causes the detection of 3× FLAG-HA fusion protein being relatively lower than other abundant proteins. To overcome the problem, you may block the magnetic beads with 3% BSA in bufferA before immunoprecipitation. Alternative reason could be high protein concentration of the extracts, which raise non-specific absorption of abundant proteins by the beads. Protein extracts should be diluted at a concentration between 1 to 5 mg/mL before starting immunoprecipitation (steps 6 and 9).

### Problem 3
Numerous peptides derived from IgG masks MS data of other proteins, even though diagnostic analysis indicates that the 3× FLAG-HA fusion protein is detected.

**Potential solution**
This happens when a significant amount of IgG have been eluted from FLAG-beads and/or HA-beads. If this is the case, you will find intense bands around 50 kDa and 25 kDa by western blot, which are derived from IgG heavy chain and light chain, respectively.

One potential reason for this could be the elution of immunoprecipitates from the FLAG- and/or HA-beads not by acid elution but by boiling (steps 21 and 22). Although anti-FLAG- and anti-HA-IgG are crosslinked to the surface of beads, reduction of S-S bonds by the reducing reagent causes dissociation of IgG light chains from heavy chains. Boiling the immunoprecipitates should be avoided. Inclusion of residual level of magnetic beads into the gel potentially causes the same problem. Pay attention not to include residual magnetic beads during the transfer of acid eluted samples into a new tube. Further, since commercially available FLAG-beads and/or HA-beads still have un-crosslinked IgG, be sure to pre-wash the magnetic beads with acid solution before equilibration.

To avoid inclusion of IgG in LC-MS/MS analysis, run the sample on SDS page longer and separate the bands containing IgG. Alternatively, you can elute the samples by HA peptide for the final elution at step23.

| Step the samples are taken at | Sample                      | Sample volume | Volume taken for analysis | Relative to HA-IP eluted (%) | Storage condition |
|-----------------------------|-----------------------------|---------------|---------------------------|-----------------------------|-----------------|
| Part 1 step 6              | Cytosolic extract (input)   | 10 mL         | 40 μL                     | 4%                          | −80°C           |
| Part 1 step 9              | Nuclear extract (input)     | 5 mL          | 20 μL                     | 4%                          | −80°C           |
| Part 2 step 13             | FLAG IP unbound            | 5 mL          | 20 μL                     | 4%                          | −80°C           |
| Part 2 step 16             | FLAG IP eluted             | 200 μL        | 20 μL                     | 100%                        | −80°C           |
| Part 2 step 20             | HA IP unbound              | 200 μL        | 20 μL                     | 100%                        | −80°C           |
| Part 2 step 22             | HA IP eluted               | 40 μL         | 4 μL                      | 100%                        | −80°C           |
Problem 4
Cytoplasmic extracts become highly viscous.

Potential solution
You will find this problem when extracts are difficult to suck by pipet. This happens when DNA comes out during the initial step of homogenization in low salt buffer A (steps 1–4). One potential reason could be that cellular and nuclear membranes are already disrupted before homogenization. Detergents disrupts cellular and nuclear membranes. When testes are stored in a freezer, do not include buffer containing detergents such as Triton X100 or Tween20. Upon thawing the cells, leakage of DNA easily occurs from broken membranes leading high viscosity of extracts. In such cases, discard the sample.

Problem 5
Seemingly non-specifically bound proteins in the 3XFLAG-HA immunoprecipitation are detected, which are not reproducible.

Potential solution
This may happen if the protein concentration is high in the extracts and protein molecules non-specifically interact with each other. Protein extracts should be diluted at a concentration between 1 to 5 mg/mL before starting immunoprecipitation (steps 6 and 9).

Alternatively, this may also happen when protein precipitates or aggregates are formed during immunoprecipitation, and they are non-specifically absorbed by the beads (steps 11–16, 18–20). Some proteins that have been solubilized in buffer may precipitate once you store the extracts in a freezer before immunoprecipitation. If you start immunoprecipitation from frozen extracts, centrifuge the extracts to remove any insoluble materials before using.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kei-ichiro Ishiguro (ishiguro@kumamoto-u.ac.jp).

Materials availability
Mouse lines used in this study have been deposited to Center for Animal Resources and Development (CARD): Stra8-3xFLAG-HA-p2A-GFP knock-in mouse (ID 2555), Fbxo47-3xFLAG-HA knock-in mouse (ID 2972), and Skp1-3xFLAG-HA knock-in mouse (ID 2638). All unique/stable reagents relevant to this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability
All relevant data of mass analyses are present in the papers and the supplemental information (Ishiguro et al., 2020) (Tanno et al., 2022). The original raw data are deposited in public depository Mendeley Data https://data.mendeley.com/datasets/ct84bbswv5/2.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101452.

ACKNOWLEDGMENTS
We would like to thank Ryuki Shimada for technical assistance for the photos and movie. This work was supported in part by KAKENHI grants (#19H05743, #20H03265, #20K21504, #16H06276, and #22H04922 to K.I.) from MEXT Japan, a grant from AMED PRIME (21gm6310021h0001 to K.I.), a grant from Takeda Science Foundation (to K.I.), and the program of the Joint Usage/Research for
AUTHOR CONTRIBUTIONS

N. Tanno and K.I. performed the immunoprecipitation experiments. N. Tani performed MS analyses. K.I. supervised the experiments, conducted the study, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11, 1475–1489. https://doi.org/10.1093/nar/11.5.1475.

Fujiwara, Y., Horisawa-Takada, Y., Inoue, E., Tani, N., Shibuya, H., Fujimura, S., Kariyazono, R., Sakata, T., Ohta, K., Araki, K., et al. (2020). Meiotic cohesins mediate initial loading of HORMAD1 to the chromosomes and coordinate SC formation during meiotic prophase. PLoS Genet. 16, e1009048. https://doi.org/10.1371/journal.pgen.1009048.

Horisawa-Takada, Y., Kodera, C., Takemoto, K., Sakashita, A., Horisawa, K., Maeda, R., Shimada, R., Usuki, S., Fujimura, S., Tani, N., et al. (2021). Meiosis-specific ZFP541 repressor complex promotes developmental progression of meiotic prophase towards completion during mouse spermatogenesis. Nat. Commun. 12, 3184. https://doi.org/10.1038/s41467-020-19728-4.

Ikura, T., Ogryzko, V.V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000). Involvement of the Tip60 histone acetylase complex in DNA repair and apoptosis. Cell 102, 463–473. https://doi.org/10.1016/s0092-8674(00)00051-9.

Ishiguro, K., Kim, J., Fujiyama-Nakamura, S., Kato, S., and Watanabe, Y. (2011). A new meiosis-specific cohesin complex implicated in the cohesin code for homologous pairing. EMBO Rep. 12, 267–275. https://doi.org/10.1038/embor.2011.2.

Ishiguro, K., Kim, J., Shibuya, H., Hernandez-Hernandez, A., Suzuki, A., Fukagawa, T., Shioi, G., Kyo, H., Li, X.C., Schimenti, J., et al. (2014). Meiosis-specific cohesin mediates homolog recognition in mouse spermatocytes. Genes Dev. 28, 594–607. https://doi.org/10.1101/gad.237313.113.

Ishiguro, K.I., Matsuura, K., Tani, N., Takeda, N., Usuki, S., Yamane, M., Sugimoto, M., Fujimura, S., Hosokawa, M., Chuma, S., et al. (2020). MEIOSIN directs the switch from mitosis to meiosis in mammalian germ cells. Dev. Cell 52, 429–445.e10. https://doi.org/10.1016/j.devcel.2020.01.010.

Kim, J., Ishiguro, K., Nambu, A., Akiyoshi, B., Yokobayashi, S., Kagami, A., Ishiguro, T., Pendas, A.M., Takeda, N., Sakakibara, Y., et al. (2015). Meikin is a conserved regulator of meiosis-I-specific kinetochore function. Nature 517, 466–471. https://doi.org/10.1038/nature14097.

Nakatani, Y., and Ogryzko, V. (2003). Immunopurification of mammalian protein complexes. Methods Enzymol. 370, 430–444. https://doi.org/10.1016/S0076-6879(03)70037-8.

Odajima, J., Saini, S., Jung, P., Ndisa-Colday, Y., Picco, S., Geng, Y., Marco, E., Michowski, W., Wang, Y.E., DeCaprio, J.A., et al. (2016). Proteomic landscape of tissue-specific cyclin E functions in vivo. PLoS Genet. 12, e1006429. https://doi.org/10.1371/journal.pgen.1006429.

Shevchenko, A., Tomas, H., Havli, J., Olsen, J.V., and Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1, 2856–2860. https://doi.org/10.1038/nprot.2006.48.

Takemoto, K., Tani, N., Takada-Horisawa, Y., Fujimura, S., Tanno, N., Yamane, M., Okamura, K., Sugimoto, M., Araki, K., and Ishiguro, K.-i. (2020). Meiosis-specific C19orf57/4930432K21Rik/BRME1 modulates localization of RADS1 and DMC1 to DSBs in mouse meiotic recombination. Cell Rep. 31, 107686. https://doi.org/10.1016/j.celrep.2020.107686.

Tanno, N., Takemoto, K., Takada-Horisawa, Y., Shimada, R., Fujimura, S., Tani, N., Takeda, N., Araki, K., and Ishiguro, K.I. (2022). FBXO47 is essential for preventing the synaptonemal complex from premature disassembly in mouse male meiosis. iScience 25, 104008. https://doi.org/10.1016/j.isci.2022.104008.

Yoshimi, K., Kunihiro, Y., Kaneko, T., Nagashora, H., Voigt, B., and Mashimo, T. (2016). ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. Nat. Commun. 7, 10431. https://doi.org/10.1038/ncomms10431.