Use of *in vivo* biotinylation to study protein–protein and protein–DNA interactions in mouse embryonic stem cells

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In gene regulation, proteins function as members of protein complexes to recognize chromosomal target DNA loci. In dissecting the pluripotent state in mouse embryonic stem (mES) cells, we have used *in vivo* biotinylation of critical transcription factors for affinity purification of protein complexes and chromatin immunoprecipitation (ChIP)-on-chip for target identification, respectively. Here, we describe detailed procedures for such studies to dissect protein–protein and protein–DNA interactions in mES cells. Specifically, the following three procedures will be described: (i) *in vivo* biotinylation system setup in mES cells; (ii) affinity purification of multiprotein complexes by one-step streptavidin capture and tandem anti-FLAG/streptavidin affinity purification; (iii) biotin-mediated ChIP (bioChIP). The system setup takes ~50 d to complete, and it takes another ~15 d and ~3 d to perform affinity purification of protein complexes and bioChIP, respectively.

INTRODUCTION

Embryonic stem (ES) cells are pluripotent and have great potential in cell replacement therapies. An understanding of pluripotency at the molecular level should provide clues into ways to direct differentiation along specific lineages or reprogram somatic cells to an ES cell phenotype. Vital cellular functions require the coordinated action of a large number of proteins that assemble into an array of multiprotein complexes of distinct composition and structure to execute transcriptional regulation of target genes. The analyses of protein complexes and intricate protein–protein interaction and protein–DNA transcriptional regulatory networks are key to understanding these complex biological systems. ES cells are an excellent system for such biochemical studies because they are relatively homogenous and easily expandable. Purification of pluripotency protein complexes and delineation of the protein interaction network, and mapping chromosomal binding loci of critical ES cell factors, will provide information on how pluripotency factors regulate target gene expression, uncover new factors in self-renewal signaling pathways, and ultimately lead to a better understanding of the molecular mechanisms by which ES cells self-renew and maintain pluripotency.

Methods for studying protein–protein and protein–DNA interactions

Mammalian protein complexes have been studied by combining protein affinity purification with mass spectrometry (MS) and bioinformatics[1]. Proteins and other macromolecules of interest can be purified from crude extracts or other complex mixtures by a variety of methods. Affinity purification makes use of specific binding interactions between molecules and generally involves the following steps: incubation of crude sample extracts with an immobilized affinity support material; washing away nonbound sample components from the solid support; and elution (dissociation and recovery) of the target molecule together with its associated proteins by altering the buffer conditions so that the binding interaction weakens or no longer occurs. Prominent among affinity purification strategies are single-step streptavidin capture of *in vivo* biotinylated proteins and tandem affinity purification involving one traditional affinity tag (e.g., FLAG) coupled with a biotinylation peptide (FLAGBIO)[2,3] (see below for details).

Protein–DNA interactions have been studied traditionally *in vitro* using EMSA[4,5] (electrophoretic mobility shift assay) and SELEX[6] (systematic evolution of ligands by exponential enrichment). In contrast, ChIP is a method[7,8] commonly used to determine site-specific occupancy of genomic DNA by selected proteins *in vivo*. This technique gives a picture of the protein–DNA interactions that occur inside the nucleus of living cells or tissues used. The principle underpinning this assay is that DNA-binding proteins (including transcription factors) in living cells can be cross-linked to the DNA that they are binding. After shearing or cutting the genomic DNA, the resulting protein–DNA complexes can be immunoprecipitated from the cellular lysates using an antibody that is specific to the DNA-binding protein of interest. Subsequently, the purified DNA can be hybridized to tiled oligonucleotide microarrays (ChIP-on-chip or ChIP-chip)[9] to find where the protein binds on a genome-wide scale. Alternatively, ChIP-Sequencing (ChIP-Seq)[10] has recently emerged as a new technology that can localize protein-binding sites in a high-throughput, cost-effective manner.

Advantages and disadvantages of the *in vivo* biotinylation method

In vivo biotinylation is based upon a short 'biotinylation peptide'[11] fused to a protein of interest (e.g., a transcription factor; bioTF) that serves as an *in vivo* substrate mimic for *Escherichia coli* biotin holoenzyme synthetase (*BirA*), an enzyme that performs highly selective biotinylation of the fusion protein (bioTF). In mammalian

cells, plasmid expression vectors carrying the bioTF and *birA* genes can be used to obtain high-level production of soluble bioTF and *BirA* proteins, and under appropriate culture conditions, the bioTF protein produced by this system is completely biotinylated.

Biotinylation offers a number of advantages over traditional immunoaffinity approaches to protein complex purification. First, the high affinity of biotin for streptavidin (10^{-15} M kDa) allows efficient purification of the biotinylated protein and associated proteins. Second, for ChIP applications, the high biotin–streptavidin affinity allows very high stringency washing conditions (2% (vol/vol) sodium dodecyl sulfate (SDS)), thus reducing background binding that may be observed with other affinity tags or native antibodies. Third, there are few naturally biotinylated proteins, thus reducing the chance for cross-reaction. Fourth, the approach obviates the need for generation of protein-specific antibodies, which often may cross-react with other cellular proteins. In cases where it has been examined, biotinylation of a tagged transcription factor has not been found to significantly alter its protein interactions, DNA-binding properties *in vivo*, and subnuclear distribution[2]. Therefore, it offers a unique methodology to study protein–protein and protein–DNA interactions simultaneously.

However, potential limitations do exist: first, additional time and effort are required to establish cell lines for *in vivo* biotinylation compared with direct antibody-based immunoprecipitation; second, similar to any other overexpression system, ectopic expression of a protein significantly beyond endogenous levels may result in spurious protein complexes and increase nonspecific DNA binding. Therefore, it is important to select cell lines expressing the lowest level of the biotinylated proteins required for analysis (typically below the level of the endogenous target protein) (see Step 23 of PROCEDURE for details).

With all these factors in mind, we have tested the utility of *in vivo* biotinylation of transcription factors in mES cells[3,12]. First, we established an *in vivo* biotinylation system for *BirA*-mediated biotinylation of critical pluripotency factors in mES cells. Second, we developed and optimized an approach for single streptavidin and tandem anti-FLAG immunoaffinity–streptavidin purification of pluripotency protein complexes involving streptavidin capture of biotinylated proteins (called bioSAIP)[3]. Third, we demonstrated the feasibility of *in vivo* biotinylation for mapping global/chromosomal targets of many different transcription factors (called bioChIP-chip)[12].

Here, we provide a detailed protocol for affinity purification of protein complexes (bioSAIP) and bioChIP with a single *in vivo* biotinylation system. Although we have performed all of our studies in mES cells, this approach should be readily applicable to other cellular systems with established, expandable tissue culture conditions, and this is particularly true for bioChIP where starting material is much less demanding than bioSAIP. **Figure 1** shows schematic representations of the two plasmid vectors used in this protocol, and **Figure 2** gives an overview of the main steps of the protocol. **Figure 3** shows a sample timeline for the procedures. Detailed protocols for MS-based identification of protein complexes[1] and array[9] or sequencing[10] technologies to identify DNA targets of the ChIP material have been published by others and will not be described in this protocol.

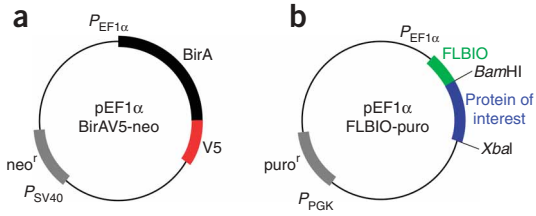

Figure 1 | Plasmids used in this study. (**a**) pEF1α*BirA*V5-neo. cDNA encoding the bacterial *BirA* enzyme is fused C-terminally to V5 tag that is used for the detection of *BirA* expression in western blot analysis using anti-V5 antibody. The vector contains an expression cassette for neomycin resistance gene (neo^r) regulated by the SV40 promoter (P_{SV40}). (**b**) pEF1αFlagbio(FLBIO)-puro. The restriction sites (*Bam*HI and *Xba*I) for cloning cDNA into the pEF1αFLBIO-puro vector are shown. The vector also contains an expression cassette for puromycin resistance gene (puro^r) under PGK promoter (P_{PGK}). Both *BirA*V5 and FLBIO-tagged gene products are transcribed from a constitutive promoter EF1α ($P_{EF1\alpha}$). Plasmids are available on request.

Experimental design

Cell types and sample preparation. Two cell lines are generated in the study. ES cells lines stably expressing *BirA* are established first to serve as control cells for background signals during affinity purification of protein complexes and bioChIP. These *BirA*-expressing cell lines are then used for subsequent introduction of FLBIO plasmid encoding a FLAG epitope tag, a *BirA* recognition/substrate peptide sequence (MSGLNDIFEAQKIEWHEGAPSSR) and the protein of interest (see **Fig. 1**). Establishing of *BirA*-only ES cell lines first (using G418 selection) allows future introduction of different FLBIO-tagged genes (using puromycin selection); thus, multiple FLBIO-tagged cell lines can be established, which all

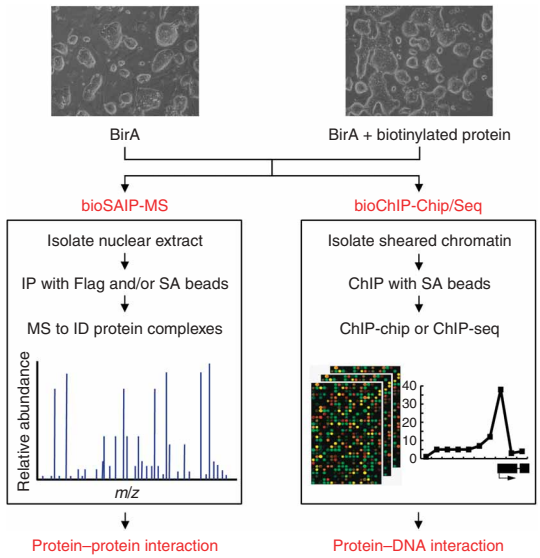

Figure 2 | Strategies for studying protein–protein and protein–DNA interactions in mouse ES cells. The ES cells expressing *BirA* alone (as control) and *BirA* plus biotinylated transcription factors (bioTF) can be used for isolation of protein complexes using streptavidin (SA) immunoprecipitation (IP) coupled with LC-MS/MS (dubbed bioSAIP-MS) and construction of a protein–protein interaction network; meanwhile, the same ES cells can be subjected to *in vivo* biotinylation-mediated ChIP and microarray or sequencing (dubbed bioChIP-chip/bioChIP-Seq) to identify protein–DNA interactions and construct a transcriptional regulatory network.

express the same amount of *BirA*. Nuclear extracts are prepared simultaneously from *BirA*-expressing cells with and without tagged genes of interest, and affinity purification (one-step or tandem) is carried out simultaneously.

bioChIP reference sample. Protein occupancy 'at' its target loci identified by ChIP is measured by relative enrichments over an unenriched reference sample. Most ChIP protocols use input genomic DNA or a mock ChIP sample as a reference for the ChIP reaction[13]. As there are few known endogenous proteins as non-specific substrates of the exogenous bacterial *BirA* enzyme[2], bioChIP material from the cells expressing only *BirA* is used as a reference here.

Cross-linking and sonication optimization. Cross-linking time and sonication conditions need to be optimized for all ChIP-related experiments. We have found that incubation with formaldehyde (1%, vol/vol) for 5 min is applicable for most bioChIP reactions of transcription factors in various human and mouse cells. We found that 4–6 sessions of 30 pulses (1 s on and 1 s off) using a Branson Sonifier at 50% amplitude achieves DNA fragments of the desired size range (0.2–0.5 kb) from mouse ES cells.

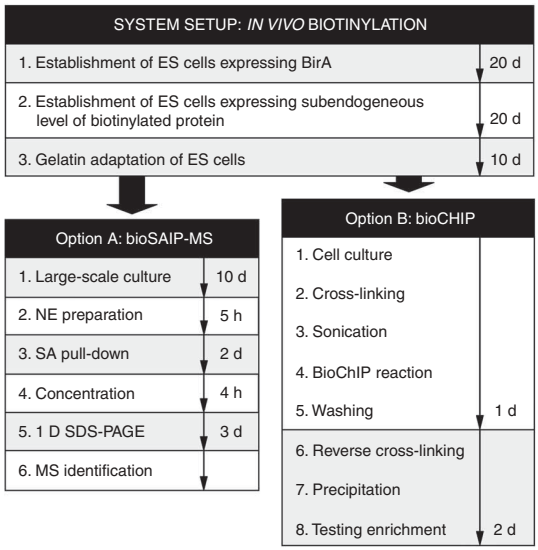

Figure 3 | An overview of the protocol. The whole protocol is divided into three parts (system setup, option A and option B). Major steps and timeline within each procedure are shown.

MATERIALS

REAGENTS

- J1 ES cells (ATCC, cat. no. SCRC-1010)
- ES media (see REAGENT SETUP)
- Mouse embryonic fibroblast (MEF) cells (Open Biosystems, cat. no. MES3948) or irradiated embryonic feeders (IEFs) (see REAGENT SETUP)
- Nucleoside mix (100×; Chemicon, cat. no. ES-008-D)
- Recombinant leukemia inhibitory factor (LIF; Chemicon)
- Penicillin/streptomycin (GIBCO, cat. no. 15070-063)
- Fetal calf serum (Hyclone, cat. no. SH30071.03)
- pEF1α*BirA*V5-neo (**Fig. 1a**) (available upon request)
- pEF1αFlagbiotin (FLBIO)-puro plasmid (**Fig. 1b**) (available upon request)
- G418 (GIBCO, cat. no. 11811): it is also known as geneticin and is an aminoglycoside antibiotic that blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. Resistance to G418 is conferred to mammalian cells genetically engineered to express a protein product encoded by the neomycin phosphotransferase gene (i.e., neomycin-resistance gene)
- Gelatin (Bacto, DIFCO, cat. no. 0143-15-1)
- Puromycin (Sigma, cat. no. P8833)
- 0.05% (wt/vol) trypsin (Mediatech, cat. no. 25-052-CI)
- 0.25% (wt/vol) trypsin (Mediatech, cat. no. 25-053-CI)
- 2× freezing media (see REAGENT SETUP)
- RIPA (radioimmunoprecipitation assay) buffer (Boston BioProducts, cat. no. BP-115)
- Anti-V5–horseradish peroxidase (HRP) (Invitrogen, cat. no. 46-0708)
- Streptavidin–HRP (Amersham, cat. no. RPN1231)
- Nuclear extract buffer A (see REAGENT SETUP)
- Nuclear extract buffer B (see REAGENT SETUP)
- Protease inhibitor cocktail (Sigma Mammalian Protease Inhibitor cocktail, cat. no. P8340)
- Trypan Blue solution (GIBCO, cat. no. 15250-061)
- Bradford assay: protein concentration Bio-Rad Dye kit (Bio-Rad, cat. no. 500-0006)
- IP350 buffer with different NP-40 concentrations (see REAGENT SETUP)
- Protein G-agarose (Roche, cat. no. 11-243-233001)
- FLAG M2-agarose beads (Sigma, cat. no. A2220-5ML)
- FLAG peptide (Sigma, cat. no. F-3290)
- Streptavidin-agarose beads (Invitrogen, cat. no. 15942-050)
- Colloidal Coomassie Stain (Invitrogen, cat. nos. 46-7015 and 46-7016)
- Dynabeads MyOne Streptavidin T1 (Invitrogen, cat. no. 656-01)
- ChIP buffer (see REAGENT SETUP)
- Washing buffer 1 (see REAGENT SETUP)
- Washing buffer 2 (see REAGENT SETUP)
- Washing buffer 3 (see REAGENT SETUP)
- TE (Tris-EDTA) buffer (see REAGENT SETUP)
- RNaseA (1 mg ml^{-1}; Ambion, cat. no. 2271)
- Glycogen (20 mg ml^{-1}; Roche, cat. no. 901393)
- Proteinase K (20 mg ml^{-1}; NEB, cat. no. P81029)
- Water (nuclease free; Ambion, cat. no. am9937)
- Phenol–chloroform–isoamyl alcohol (PCI) solution (Invitrogen UltraPure phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol/vol)
- 3-M NaOAc, pH 5.5 (Sigma, cat. no. S7899)
- Phase Lock gel, heavy, 2 ml (5 Prime, cat. no. 2302830)
- SYBR green mix (Bio-Rad iQ SYBR Green Supermix, cat. no. 170-8882)

EQUIPMENT

- 37 °C water bath
- 65 °C water bath
- Heating block (preset at 100 °C)
- NanoDrop 1000 (Thermo Fisher Scientific)
- 10-cm tissue culture plate (Falcon, cat. no. 35-3003)
- 15-cm tissue culture plate (Corning, cat. no. 430599)
- Hemocytometer
- Culture incubator (37 °C, 5% CO_2)
- Nalgene high-speed centrifuge tube (Nalgene, cat. no. 3114-0050)
- Electroporator (Bio-Rad Gene Pulser II)
- 0.4-cm-gap cuvette for electroporator (Bio-Rad, cat. no. 165-2008)
- 24-well plate, flat-bottomed (Falcon, cat. no. 35-3047)
- 48-well plate, flat-bottomed (Falcon, cat. no. 35-3078)
- 96-well plate, U-shape bottomed (Corning, cat. no. 3799)
- Beckman Coulter Allegra X-22R Centrifuge
- Beckman J-6M/E Centrifuge with JS 4.2 rotor
- Beckman Coulter Avanti J-25 Centrifuge with JA-25.50 rotor
- 250-ml conical plastic bottles (Corning, cat. no. 430776)
- 15-ml conical tubes (Corning, cat. no. 430791)
- 50-ml conical tubes (Corning, cat. no. 430829)
- Rotating wheel (Scientific Equipment Products, cat. no. 60448)
- Glass Dounce homogenizer (40-ml size) with type B pestle (Wheaton, cat. no. 432-1273)
- Glass Dounce homogenizer (15-ml size) with type B pestle (Wheaton, cat. no. 432-1272)
- YM-10 Centricon (10,000 MWCO (molecular weight cut-off); Amicon Bioseparations, cat. no. 4205)

- 1.5-ml screw cap tube (Fisher Scientific, cat. no. 056697)
- Pasteur pipette (VWR, cat. no. 14672-380)
- Bio-Rad iCycler (Bio-Rad)
- Bio-Rad Protean II xi Basic Unit with casting stand (Bio-Rad, cat. no. 165-1834)
- Magnet Dynal MPC (Invitrogen Dynal MPC-S, cat. no. 120.20D)
- Nutator (VWR)
- Branson sonifier (Branson)

REAGENT SETUP

ES media Dulbecco's modified Eagle's medium, 15% (vol/vol) fetal calf serum (FCS), 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM nonessential amino acid, 1% (vol/vol) nucleoside mix, 1,000 U ml^{-1} recombinant LIF, 50 U ml^{-1} penicillin/streptomycin. Store at 4 °C for a month. ▲ CRITICAL Each batch of FCS needs to be prescreened to ensure that it supports optimal ES cell growth.

Gelatin Dissolve 5 g of gelatin in 500 ml of distilled water and autoclave (1% stock). Store at room temperature (25 °C) indefinitely. Before use, dilute 1:10 (to make 0.1% working solution) with sterile dH_2O and filter through 0.45-μm filter apparatus.

MEF cells Mouse embryonic fibroblast cells are used when culturing mouse ES cells. They provide a substrate for the ES cells to grow on and secrete many factors necessary for ES cells to maintain pluripotency. Feeders are MEF cells that have been mitotically inactivated by treatment with mitomycin C or by γ-irradiation. A unique quad-resistant DR4 feeder cell line can be purchased (see REAGENTS) or prepared from DR4 mouse embryos (JAX mice strain STOCK Tg(DR4)1Jae/J; The Jackson Laboratory) as described previously[14]. Briefly, E14.5 embryos were dissected out and cultured for embryonic fibroblast growth. The established fibroblast cell culture was then expanded and inactivated by γ-irradiation. These cells are referred to as IEFs in this article. Normally 3 million IEF cells are frozen down as 1× stock, which can be used to seed a full plate of 96-, 48-, 24-, 12- and 6-well tissue culture plate or one 10-cm tissue culture dish.

2× freezing media Mix 20% (vol/vol) DMSO and 80% (vol/vol) FCS and pass the mix through a 0.25-μm filter to sterilize. Store at 4 °C for a month.

Nuclear extract buffer A Mix 20 mM HEPES, 10 mM KCl, 1 mM EDTA, 0.1 mM Na_3VO_4, 0.2% (vol/vol) Nonidet P40 (NP-40), 10% (vol/vol) glycerol and add fresh 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (1:1,000). Store at 4 °C for several months without protease inhibitors.

Nuclear extract buffer B Mix 20 mM HEPES, 10 mM KCl, 1 mM EDTA, 0.1 mM Na_3VO_4, 350 mM NaCl and 20% (vol/vol) glycerol, and add fresh 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (1:1,000). Store at 4 °C for several months without protease inhibitors.

IP350 buffer (0.5% (vol/vol) NP-40) Mix 350 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.5% (vol/vol) NP-40, 1 mM EDTA and 10% (vol/vol) glycerol (vol/vol), and add fresh 1 mM DTT, 0.2 mM PMSF and protease inhibitor cocktail (1:1,000). Store at 4 °C for several months without protease inhibitors.

IP350 buffer (0.3% (vol/vol) NP-40) Mix 350 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.3% (vol/vol) NP-40, 1 mM EDTA and 10% (vol/vol) glycerol (vol/vol), and add fresh 1 mM DTT, 0.2 mM PMSF and protease inhibitor cocktail (1:1,000). Store at 4 °C for several months without protease inhibitors.

2× SDS sample buffer Mix 1% (vol/vol) glycerol, 3% (vol/vol) SDS, 0.5 M Tris-HCl (pH 6.8) and 0.004% (vol/vol) bromophenol blue.

SDS ChIP buffer Mix 0.1% (vol/vol) SDS, 1% (vol/vol) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 150 mM NaCl, and add fresh protease inhibitor cocktail (1:1,000). Store at 4 °C for several months without protease inhibitors.

Washing buffer 1 2% (vol/vol) SDS. Store at room temperature for several months.

Washing buffer 2 Mix 0.1% (vol/vol) deoxycholate, 1% (vol/vol) Triton X-100, 1 mM EDTA, 50 mM HEPES (pH 7.5) and 500 mM NaCl. Store at room temperature for several months.

Washing buffer 3 Mix 250 mM LiCl, 0.5% (vol/vol) NP-40, 0.5% (vol/vol) deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.1). Store at room temperature for several months.

TE buffer Mix 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Store at room temperature for several months.

SDS elution buffer Mix 1% (vol/vol) SDS, 10 mM EDTA and 50 mM Tris-HCl (pH 8.1). Store at room temperature for several months.

Harvest ES cells by trypsinization Embryonic stem cells grow as a monolayer in gelatin-coated vessels. To harvest cells, ES medium is removed by aspiration and ES cells are rinsed once with 0.05% trypsin. Then, a sufficient amount of 0.25% trypsin is added to cover the ES cells followed by incubation at 37 °C for 3–5 min. The ES cells become detached from the vessels and can be collected after neutralization of the trypsin with 3× vol of ES cell medium (pipetting up and down to mix them).

Determination of protein concentration We use Bio-Rad Dye kit to perform the Bradford assay as described previously[15] to determine protein concentration of nuclear extract.

Primer design for bioChIP-PCR Primer pairs can be designed from genomic regions ~150 bp in length that contain predicted or previously known target loci of tested protein; aim to generate an amplified product 100–125 bp in size. Multiple negative control primer pairs can be designed from known nontarget genomic regions. We use the Web-based Primer3 program with the following parameters: optimal Tm value 60 (±1) °C and primer length 23(±3) bp.

EQUIPMENT SETUP

Electroporation For J1 ES cells, use 0.4-cm-gap cuvette, 25 μF, 450 V, and time constant for each electroporation should read around 00.6–00.8 msec.

Gelatin-coated tissue culture plates Add a sufficient amount of 0.1% gelatin (see REAGENT SETUP) to each well of the culture plates, let stand for 20 min to 1 h. Aspirate the gelatin solution and air-dry the plates. Store the gelatin-coated plates at room temperature indefinitely.

Preparation of 48-well culturing and 96-well trypsin plates The following setup allows direct multichannel pipetting/transfer of cells between the two plates. Coat a flat 48-well plate with 0.1% (vol/vol) gelatin and seed with a layer of mouse IEF (1×) with 200 μl of ES medium in every well. For a 96-well plate (U-shaped bottomed), add 35 μl of 0.05% trypsin to wells 1, 3, 4, 6, 7, 9, 10 and 12 of alternate rows (there is no need to gelatinize the plate). The picked clones will first be trypsinized in the 96-well plate and then transferred using a P200 12-channel pipette into the corresponding 48-well plate for culturing.

Preparation of a 96-well plate for cell stock Add 65 μl of cold 2× freezing media (see REAGENT SETUP) to wells 1, 3, 4, 6, 7, 9, 10 and 12 in alternate rows of a U-shape-bottomed 96-well plate. This allows 48 clones to be frozen down directly from the 48-well culturing plate mentioned above.

Preparation of ES cell culturing IEF plates Coat the plates (24-well, 12-well, 10-cm) with 0.1% (vol/vol) gelatin (see REAGENT SETUP) and seed with a layer of mouse IEF (1×) with an appropriate amount of ES medium per well (e.g., 1 ml per well for 24-well plate, 2 ml per well for 12-well plate and 10 ml per plate for 10-cm plate). ▲ CRITICAL The plates should be prepared 1 d before they are required to allow IEF cells to firmly attach to the wells.

Preparation of protein G, FLAG (M2) and streptavidin agarose This should be carried out just before the agarose beads are required. Equilibrate protein G, FLAG (M2) or streptavidin-agarose beads (100 μl of partial slurry per 10 mg protein) in two 50-ml tubes containing 15 ml of cold IP350 (0.3% vol/vol NP-40) buffer. Rotate in a cold room for 5 min, centrifuge at 300*g* for 4 min at 4 °C and decant buffer. Repeat once.

Preparation of large denaturing polyacrylamide gels Use Bio-Rad Protean II xi Basic Unit with casting stand. Pour lower running gel (10%) first, seal with ethanol for polymerization and then pour upper stacking gel (4%), taking care not to introduce bubbles in the comb wells. Pull out the bottom gel blocker carefully before use.

PROCEDURE

In vivo biotinylation system setup: establishment of ES cell lines expressing *BirA* ● TIMING 20 d

1| Using standard cell culture procedures, thaw J1 ES cells (or ES cells of your choice) on a 10-cm IEF plate (see EQUIPMENT SETUP). Incubate cells at 37 °C and 5% CO_2 till they reach ~70% confluence; this can take a few days to a week depending on the starting cell numbers. Also, prepare four 10-cm IEF plates (see EQUIPMENT SETUP) for use in Step 6.

2| Harvest cells by trypsinization as described in REAGENT SETUP.

3| Wash harvested cells twice with PBS, and resuspend at 1.3 × 10^7 ml^{-1} in PBS.

4| Add 20–30 μg of pEF1α*BirA*V5-neo DNA in TE to 0.75 ml of cell suspension (or 10^7 cells) for each electroporation. Note that linearization of the plasmid before electroporation is not necessary.

5| Perform electroporation as described in EQUIPMENT SETUP. Incubate for 5 min on ice.

6| Mix electroporated cells with enough medium to bring the final cell suspension volume to 10 ml, and transfer them to the 10-cm IEF plates prepared in Step 1. Rock plates gently to mix. Then incubate at 37 °C and 5% CO_2.

7| On the second day (24 h after initial plating after the electroporation), add G418 (final concentration 300 μg ml^{-1}) directly to cultures. Rock plates gently to mix the drug completely with medium and return to incubator.

8| Feed the cells with fresh G418 drug and medium daily for the next 7–9 d. Swirl plates gently to resuspend and remove dead cells and debris by aspiration. Replace with 10 ml of freshly made drug/medium mix to each plate. After 3 or 4 d of this treatment, most of the ES cells will appear dead. By day 5 or 6, each of the surviving cells will form a colony and be ready for picking by day 8 or 9.

9| A day before picking, prepare a flat 48-well IEF plate and a 96-well trypsin plate as described in EQUIPMENT SETUP.

10| On the day of picking, wash each dish from Step 8 with 10 ml of PBS. Replace the wash with 5 ml of fresh PBS to prevent the cells form drying out during picking.

11| Pick individual colonies with a P200 set at 10 μl and transfer each colony into a trypsin-containing well of the U-shape-bottomed 96-well plate prepared in Step 9.

12| After 48 clones or 30 min (whichever is first), incubate the plate at 37 °C for 10 min.

13| Using a multichannel pipette, transfer 150 μl of ES media from the 48-well feeder plate (from Step 9) to trypsin plate containing the colonies (from Step 12). Pipette the solution up and down and then transfer the entire cell suspension back to feeder plate. Return to the incubator and culture the cells in 48-well plate overnight.

14| Replace the old medium with fresh ES media (300 μg ml^{-1} G418) every day.

15| When the majority (>70%) of wells are ready (i.e., cells reach near or over 70% confluence), wash with PBS, add 35 μl of 0.05% trypsin and incubate at 37 °C for 10 min. Meanwhile, prepare a 96-well plate for cell stock as described in EQUIPMENT SETUP.

16| Using a 12-channel pipette, add 65 μl of media to each well, mix by pipetting up and down and transfer 65 μl to the U-shape-bottomed 96-well plate containing 65 μl of cold 2× freezing media (20% (vol/vol) DMSO + 80% (vol/vol) FCS) prepared above. Wrap the plate in parafilm and store at −80 °C until use at Step 20.

17| Add 200 μl of media to the remaining cells in the feeder plate, and return the plate to the incubator. Replace the old media with fresh media daily for 3–4 d.

18| When the medium in most wells is yellow or cells reach near-confluence, aspirate the medium and add 200 μl of RIPA buffer to each well to make total lysate. Carry out a standard western blot analysis with the lysate (20 μg) using anti-V5–HRP. As *BirA* is V5-tagged, you should expect to see a band near 35 kDa in the positive clones.

19| Prepare a 24-well IEF feeder plate (see EQUIPMENT SETUP).

20| The next day, thaw positive clones (as determined by western blotting, Step 18) by adding 100 μl of warm media to frozen wells (from Step 16). Mix and transfer the thawed cell suspension to 24-well plate prepared in Step 19; repeat until all the remaining cells are thawed and transferred.

21| Let clones grow in selection media (with 300 μg ml^{-1} G418) until they are 70% confluent; this takes ~1 week.

22| Freeze 60% of the cells as frozen stocks and grow the remaining 40% in 10-cm feeder plates until 70% confluent.
■ **PAUSE POINT** Alternatively, all the cells can be frozen and stored at −80 °C (for days) or in liquid nitrogen LN_2 (months to years) for later use.

***In vivo* biotinylation system setup: establishment of ES cell lines expressing subendogenous levels of biotinylated proteins** ● **TIMING 20 d**

23| Repeat Steps 2–22 to electroporate *BirA*-containing cells (from Step 22) with pEF1αFlagbio-tagged plasmid containing a specific gene of interest; use media containing 300 μg ml^{-1} G418 and 1–2 μg ml^{-1} puromycin to select cells expressing both

pEF1α*BirA*V5-neo and pEF1αFLBIO(gene)-puro plasmids (see **Fig. 1**). The presence of the biotinylated protein of interest can be confirmed by western blot analysis using streptavidin–HRP conjugate (to detect the biotin tag) and a native antibody against the protein of interest (to detect both the biotinylated and nonbiotinylated versions of the protein).
▲ CRITICAL STEP For the streptavidin–HRP incubation step, do not include milk in the buffer, as it contains high levels of free biotin, which will compete for the streptavidin–HRP and result in a false-negative signal on the blot.

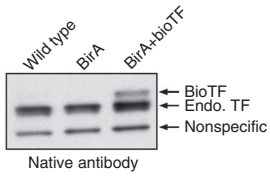

Figure 4 | An example of western blot analysis using native antibody against both endogenous and biotinylated protein. The subendogenous level of the biotinylated transcription factor (bioTF) and the expression level of the endogenous protein are indicated.

▲ CRITICAL STEP Western blot analysis with the native antibody allows detection of relative expression level of biotinylated protein versus endogenous protein. Only the clones with subendogenous expression levels should be selected for affinity purification (see an example in **Fig. 4**). The selection of subendogenous expression levels of tagged protein ensures minimal interference with endogenous protein complexes by the tagged protein and thus allows for affinity purification of the bona fide interacting partners.
? TROUBLESHOOTING

***In vivo* biotinylation system setup: gelatin adaptation of ES cells expressing *BirA* control and *BirA* + biotinylated protein ● TIMING 10 d**

24| Thaw and expand ES cells expressing *BirA* control (from Step 22) and *BirA* plus biotinylated protein (from Step 23) in a 12-well plate with feeders until they are 70% confluent.

25| Harvest cells by trypsinization as described in REAGENT SETUP and replate the cells in one well of a six-well gelatin-coated plate—this is the G1 (gelatin no. 1) well.
▲ CRITICAL STEP Gelatin adaptation to make ES cells feeder-independent is important for the following two reasons: (i) it eliminates contamination with feeder cells in downstream purification; (ii) it greatly reduces the experimental cost incurred by the large-scale culture of ES cells required for affinity purification of protein complexes. Be aware that not all ES cells are amenable to gelatin adaptation and feeder-independent growth, so it is critical to select ES cell lines that can be gelatin-adapted (e.g., J1 ES cells) or that can be grown without feeders (e.g., E14 ES cells).

26| When the G1 well is approximately 60–80% confluent, split 1:6 to new wells of a six-well gelatin-coated plate—these are the G2 wells (total six wells).

27| When the G2 wells are approximately 60–80% confluent, harvest cells from five wells and freeze in 1 ml of 10% DMSO/90% FCS as G2 stock vials (1× six-well cells per vial). Split the remaining G2 well to all six wells (1:6 split) of a new six-well gelatin-coated plate—these are the G3 wells.

28| When the G3 wells are approximately 60–80% confluent, freeze cells from five wells in five vials with 1 ml of 10% DMSO/90% FCS as six-well G3 stock. The remaining wells of both *BirA* control and *BirA* + biotinylated protein–expressing cells can be advanced to Step 29.
■ PAUSE POINT Alternatively, all the cells can be frozen and stored at −80 °C (short term) or in liquid nitrogen LN_2 (long term) for later use.

29| To perform affinity purification of protein complexes, proceed as described in option A. Simultaneously, bioChIP can be performed as described in option B.

(A) Affinity purification of protein complexes ● TIMING 14 d

(i) Begin with 2× 10-cm dishes of culture each for *BirA* control cells and cells expressing *BirA* and biotinylated protein (gelatin-adapted cells from Step 28) as described in Experimental design. Grow cells under G418 (300 μg ml^{-1}) and G418 (300 μg ml^{-1}) + puromycin (1–2 μg ml^{-1}) selection, respectively.
(ii) When cells reach 80–90% confluence, split the cells with 1:2 ratio into 15-cm culture dishes. Now you have 4× 15-cm dishes each.
(iii) When cells reach 80–90% confluence again, split 1:5 into 15-cm dishes each. Use 20 dishes each for large-scale affinity purification (drug selection can be omitted during the scale-up culture to reduce the cost).
(iv) When cells reach 70–80% confluence, add 10 ml of fresh medium to each dish. By the next day, the medium should have turned very yellow and the cells should be close to 90–100% confluence and are ready to be harvested as described below.
(v) Trypsinize cells: rinse plate with 5 ml of 0.05% trypsin, then add 7 ml of 0.25% trypsin per dish and incubate the plate at 37 °C for 5 min. It is recommended that ten dishes should be processed at a time.

(vi) Add 10 ml of fresh medium to the cells from Step A(v) to neutralize the trypsin by mixing, and transfer cells to 250-ml conical plastic bottles. Rinse the plates with an additional 10 ml of medium, collect the residual cells and combine with the cells in the bottle. RPMI (Roswell Park Memorial Institute) or low-glucose Dulbecco's modified Eagle's medium with 10% (vol/vol) FCS can be used instead of ES cell culture medium to neutralize trypsin. LIF is not required in the neutralization medium.

(vii) Centrifuge the cells in 250-ml conical plastic bottles for 15 min using a JS 4.2 rotor at 4 °C and 2,400*g*.

(viii) Carefully decant the supernatant, resuspend the cell pellet with 50 ml of ice-cold PBS and transfer to 50-ml conical tubes. Count cell numbers using a hemocytometer.

(ix) Centrifuge the 50-ml conical tubes at 2,400*g* for 10 min at 4 °C in a JS 4.2 rotor.

(x) Remove the supernatant carefully. Estimate the packed cell volume (PCV). Resuspend in ~5× PCV of ice-cold PBS. Centrifuge again at 2,400*g* for 10 min at 4 °C in a JS 4.2 rotor.

(xi) Remove supernatant, and rapidly resuspend with ~5× PCV of ice-cold nuclear extract buffer A with 1 M DTT (1 μl ml^{-1})/0.2 M PMSF (5 μl ml^{-1}) and 1:1,000 protease inhibitor cocktail (add fresh).

(xii) Centrifuge at 2,400*g* for 5 min at 4 °C in a JS 4.2 rotor.

(xiii) Aspirate the supernatant carefully. Add ~3× PCV of ice-cold nuclear extract buffer A and additives described in Step A(xi) (except that 1:100 protease inhibitor cocktail should be used). Incubate on ice for 10 min to swell cells.

(xiv) Transfer by pouring into glass Dounce homogenizer (40-ml size) with type B pestle prechilled on ice (prerinse with nuclear extract buffer A). Homogenize up and down 10 times, slowly.

▲ **CRITICAL STEP** If using a single Dounce homogenizer, prepare the *BirA* control sample first and wash out well afterward to avoid cross-contaminating samples. Transfer homogenate to 50-ml conical tubes.

▲ **CRITICAL STEP** Check for cell lysis by examining of small aliquot with Trypan Blue stain as described in the manufacturer's instruction under microscope (>80% of cells should be lysed).

(xv) Centrifuge at 4,300*g* for 15 min at 4 °C in a JS 4.2 rotor.

(xvi) Carefully remove and discard the supernatant using a drawn-out glass Pasteur pipette. Add to the nuclei pellet 3 ml (per 1 × 10^9 starting number of cells) of ice-cold nuclear extract buffer B with 1 M DTT (1 μl ml^{-1}), 0.2 M PMSF (5 μl ml^{-1}) and protease inhibitor cocktail 1:1,000 (add fresh).

(xvii) Dislodge the pellet and transfer to a glass Dounce homogenizer (15-ml size) with type B pestle (prechilled and rinsed with nuclear extract buffer B). Homogenize up and down ten times, slowly. Transfer homogenate to Nalgene centrifuge tube (prerinse with nuclear extract buffer B) and rotate for 30 min on a rotating wheel in the cold room (4 °C).

(xviii) Centrifuge at 25,000*g* in a JA-25.50 rotor at 4 °C for 30 min to remove insoluble material. Meanwhile, prepare for the Bradford assay according to the manufacturer's instructions (see REAGENT SETUP).

(xix) Carefully transfer the supernatant (contains nuclear extract (NE)) to 50-ml conical tubes.

(xx) Determine the protein concentration of each NE using the Bradford assay (we use the Bio-Rad Dye kit).

? TROUBLESHOOTING

(xxi) Use equal amounts of NE (as determined by the Bradford assay) from the *BirA* (control) sample and *BirA* + biotinylated protein sample. Add an appropriate amount of cold IP350 buffer (0.3% or 0.5% (vol/vol) NP-40) to each sample to adjust the final NE concentration to be ~2 mg ml^{-1} containing ~0.25% (vol/vol) NP-40. Add 1 M DTT (1 μl ml^{-1})/0.2 M PMSF (5 μl ml^{-1}) and protease inhibitor cocktail 1:1,000 to the final IP350 buffer.

▲ **CRITICAL STEP** A sufficient amount of the starting NE in an appropriate volume of IP350 buffer is important to obtain enough final material for MS analysis. A good starting point is 50–100 mg of NE in the final 25–50 ml of IP350 buffer.

(xxii) Prepare Protein G-agarose as described in EQUIPMENT SETUP. Preclear supernatant with Protein G-agarose (100 μl of partial slurry per 10 mg of protein) for 1–2 h at 4 °C with continuous mixing.

(xxiii) Spin samples at 300*g* for 5 min at 4 °C. Transfer precleared supernatant to new tubes. Proceed to **Box 1** for tandem affinity purification or **Box 2** for one-step streptavidin affinity purification. The tandem affinity purification yields higher confidence but fewer candidate proteins due to its high stringency and prolonged incubation; some transient or weak interacting proteins may be lost during tandem affinity purification. By contrast, the one-step streptavidin affinity purification is quicker and yields more candidate proteins but has relatively higher background.

(xxiv) Transfer all eluate (should be clear blue) into chamber of YM-10 Centricon (10,000 MWCO) device. If just thawed from the freezer, warm samples in a 37 °C water bath for 5 min to dissolve SDS.

(xxv) Centrifuge in the Avanti J25 centrifuge using JA-25.50 fixed angle rotor at 25 °C at 5,000*g* until as much filtrate runs through the chamber as possible (approximately 2–3 h). After centrifugation, there should be about 100 μl of residual concentrated material.

▲ **CRITICAL STEP** This centrifuge step has to be carried out at room temperature to avoid precipitation of the SDS in the samples. You will need to use blue rubber adaptors to hold the Centricon device.

BOX 1 | TANDEM AFFINITY PURIFICATION OF PROTEIN COMPLEXES

1. Prepare FLAG M2-agarose, as described in EQUIPMENT SETUP.
2. Carefully transfer the precleared nuclear extract prepared from Step 29A(xxiii) to pre-equilibrated FLAG M2 resin. Divide into more 50-ml tubes if necessary to ensure complete mixing during immunoprecipitation. Place on end-over-end rotating wheel at 4 °C overnight.
3. Centrifuge the tubes from overnight incubation at 300*g* for 4 min at 4 °C. Remove supernatant (nonbound material).

? TROUBLESHOOTING

4. Wash FLAG-agarose beads four times with 20 ml of ice-cold IP350 (0.3% (vol/vol) NP-40) buffer. Place on rotating wheel at 4 °C for 15 min per wash. Centrifuge at 300*g* for 4 min at 4 °C between washes.
5. After the final wash, remove most of supernatant. Use IP350 (0.3% (vol/vol) NP-40) buffer to transfer beads to a 15-ml tube. Pool beads if multiple tubes were used for the incubation and washes from previous steps. Centrifuge at 300*g* for 4 min at 4 °C to pellet beads. Remove the supernatant carefully.
6. Elute beads four times with 10 ml of IP350 with 0.1 mg ml^{-1} FLAG peptide. Place sample tubes on rotating wheel for 1–1.5 h at 4 °C during each elution. Centrifuge at 300*g* for 4 min at 4 °C, and carefully transfer supernatant (contains eluted proteins) to a new 50-ml conical tube. Combine total 40 ml of each sample. Discard beads.
7. Proceed with the streptavidin purification as described in **Box 2**.

(xxvi) Remove filtrate chamber first (acceptable to discard). Attach collection vial and invert quickly. Recentrifuge inverted chamber (with collection vial) at 800*g* for 2 min at 25 °C. Transfer to a 1.5-ml Eppendorf tube.

■ **PAUSE POINT** Samples can be quick frozen in LN_2 and stored at −80 °C for months.

? TROUBLESHOOTING

(xxvii) Prepare a large 10% denaturing polyacrylamide gel, as described in EQUIPMENT SETUP.

(xxviii) Heat samples at 95–100 °C for 5 min (if just thawed from −80 °C) and spin briefly. Load samples and run the gel for ~1 h at 120 V in stacking gel and run samples ~2.5 cm into separating (lower) gel (it takes ~1 h at 120 V). Fill any empty well with the same volume of 1× protein sample buffer to prevent 'smiling' effect.

(xxix) Disassemble gel apparatus and stain gel overnight with Colloidal Coomassie Stain.

(xxx) Destain gel with multiple rinses of HPLC-grade water. Destain until background is very clear (can take 1–2 d) on rocker platform at room temperature.

▲ **CRITICAL STEP** Wear gloves when handling dishes and gels, and keep dish covered all the time with Saran Wrap to avoid contamination.

(xxxi) Cut out the whole lane and separate into three or four slices for MS (a sample gel from one-step streptavidin purification is shown in **Fig. 5**). Using scalpel or razor blade, cut into ~1-mm cubes and transfer to clear 1.5-ml Eppendorf tubes. Add a small amount of HPLC-grade water so that gel slices do not dry out.

▲ **CRITICAL STEP** Do not spin tubes to avoid samples leaching out of gels.

■ **PAUSE POINT** Can store cut-out gel slices at 4 °C for days (at −20 °C for months).

BOX 2 | ONE-STEP STREPTAVIDIN PURIFICATION OF PROTEIN COMPLEXES

1. Prepare streptavidin agarose, as described in EQUIPMENT SETUP.
2. Add the precleared NE from Step 29A(xxiii) (one-step purification) or the FLAG-eluate (40 ml each) from Step 6 in **Box 1** (tandem purification) into equilibrated streptavidin-agarose and place on end-over-end rotating wheel at 4 °C overnight.
3. Centrifuge tubes from overnight incubation at 300*g* for 4 min at 4 °C. Remove supernatant (nonbound material).
4. Wash streptavidin-agarose beads four times with 20 ml of ice-cold IP350 (0.3% (vol/vol) NP-40) buffer. Place on rotating wheel for 15 min at 4 °C per wash. Centrifuge as described in Step 3 of **Box 2**.
5. After the final wash, remove most of the supernatant. Use cutoff pipette tip and remaining IP350 buffer to transfer beads into a 1.5-ml screw-cap tube. Pool beads, if multiple tubes were used for the incubation and washes. Wash out original tubes and pipette tips with IP350 buffer and pool with sample. Centrifuge using tabletop microcentrifuge at 300*g* for 2 min at 4 °C to pellet beads.
6. Remove as much supernatant as possible from the centrifuged beads using a drawn-out Pasteur pipette. Add 500 μl of 2× SDS sample buffer. Vortex gently and heat at 95–100 °C for 5 min and vortex again. Allow to cool to room temperature. Centrifuge at the maximum speed for 1 min at room temperature to repellet beads. Carefully transfer the supernatant to a new screw-cap tube. Add 500 μl of 1× SDS sample buffer to residual beads and vortex gently. Recentrifuge at maximum speed at room temperature for 1 min.
7. Pool sample with the 1st eluate (**Box 2**, Step 6) in screw-cap tube. Repeat washing of beads one more time with 400 μl of 1× SDS sample buffer and combine with samples (should now have 1.4 ml total).
8. Centrifuge at a maximum speed for 2 min at room temperature (to pellet any residual agarose beads that were carried over).

Transfer the sample into new 2-ml screw-cap cryotubes. ▲ **CRITICAL STEP** Carryover of residual agarose beads will block the Centricon in Steps 29A(xxiv–xxvi).

■ **PAUSE POINT** Can quick-freeze in LN_2 at this point and store at −80 °C for months.

(xxxii) MS identification of protein complexes. Analyze sample at MS facility with the ability to perform liquid chromatography coupled with tandem MS (LC-MS/MS) for protein identification. The detailed protocol for in-gel digestion for mass spectrometric characterization of proteins has been described previously[16].
▲ CRITICAL STEP An experienced, reliable MS facility is critical to ensure the success from this expensive and time-consuming experiment. The same experiment should be repeated two or three times to ensure identification of bona fide protein–protein interactions with high confidence.
? TROUBLESHOOTING

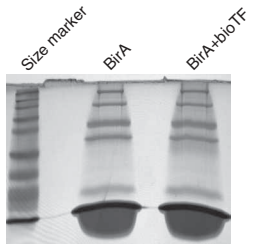

Figure 5 | A typical gel picture after SDS-PAGE fractionation of bioSAIP samples. The distinct bands present in both *BirA* and *BirA*+bioTF samples are common endogenously biotinylated proteins. The banding patterns of the control and real samples may look similar due to the limited separation of samples. Nevertheless, MS is sensitive enough to distinguish protein components between the samples (the positive detection of peptide sequences of bioTF in the *BirA*+bioTF sample, but not *BirA* control, is a good indication of success).

(B) bioChIP: Cross-linking and sonication ● TIMING **3 d**

(i) Use ~5 × 10^7 mouse ES cells (one or two 10-cm dish with ~70% confluence) expressing both *BirA* and biotinylated protein (from Step 28) for each bioChIP reaction. Use approximately the same number of ES cells that are expressing only *BirA* enzyme as a reference sample. Two parallel bioChIP reactions, one for the actual sample and the other for the reference, need to be performed.
▲ CRITICAL STEP Cells need to be maintained under proper drug selection as described above in system setup (Step 23).

(ii) Cross-link the cells by the addition of formaldehyde (final concentration 1%, vol/vol) directly to a culture plate without changing the medium.

(iii) Shake the plate containing media and formaldehyde using a shaker with horizontal shaking platform at 50 r.p.m. for 5 min at room temperature.
▲ CRITICAL STEP Incubation time must be optimized. For cross-linking optimization, multiple sets of samples with different cross-linking time periods need to be tested. In general, less than 10 min of cross-linking is desirable for mammalian ChIP reaction.

(iv) Add glycine (125 mM final concentration) to a culture plate to terminate cross-linking. Shake the plate with horizontal shaker at 50 r.p.m. for 5 min at room temperature.

(v) Remove culture media completely by aspiration and rinse the cells with cold PBS (4 °C) containing 1:1,000 protease inhibitor cocktail (add fresh). Remove PBS by aspiration. Do not dry the plates.

(vi) Rinse cells quickly with 3 ml of 0.05% trypsin and remove trypsin by aspiration.

(vii) To detach ES cells from the culture plate, add 3 ml of 0.25% trypsin and incubate the plate at room temperature for 5 min. Alternatively, a scraper can be used to detach the cells from the culture plates instead of trypsin.

(viii) Pipette up and down to detach the cells completely from the culture plate. Apply additional pipetting to make single cell suspension. Transfer the harvested cells to a 15-ml conical tube.

(ix) Add 3 ml of ES medium containing serum to a culture dish and collect the rest of the cells by pipetting. Transfer cells to the 15-ml conical tube described in Step B(viii) and spin at 2,400*g* for 5 min at 4 °C.

(x) Remove supernatant by careful aspiration to avoid losing the cell pellet. Add 10 ml of cold PBS containing protease inhibitors and wash cells with gentle pipetting.

(xi) Collect cell pellet again by centrifugation as described in Step B(ix) and discard supernatant carefully. Wash with PBS, as described in Step B(x), one more time and remove supernatant by aspiration.

(xii) Without adding PBS, spin the tube one more time at 2,400*g* for 5 min at 4 °C and remove all supernatant by careful pipetting.
■ PAUSE POINT Sample pellet can be stored at −80 °C at this point and will be stable for months.

(xiii) Resuspend the pellet with 1 ml of ChIP buffer in a 15-ml conical tube. The tube needs to be kept on ice during sonication. Applying more ChIP buffer helps to shear chromatin; however, too much dilution increases the volume of reaction, which may require increased volume of streptavidin beads in Step B(xx). In contrast to other ChIP protocols, nuclear extraction is not necessary because of the strong affinity between biotin and streptavidin in addition to stringent washing condition.

(xiv) Set up the tube for sonication with a height-adjustable table. Make sure that the probe of sonicator is centered in a 15-ml conical tube and does not contact the side or bottom of the tube during sonication.

(xv) Apply sonication for 4–6 sessions of 30 pulses (1 s on and 1 s off) using a Branson sonifier at 50% amplitude. Let the tube sit on ice for 3 min to chill between each session of sonication.
▲ CRITICAL STEP Sonication time must be optimized.

(xvi) Transfer cell lysate to a 1.5-ml Eppendorf tube. Spin at 12,000*g* for 10 min at 4 °C using tabletop centrifuge to pellet cell debris.

(xvii) Transfer clear (or slightly yellow) supernatant to a new 1.5-ml Eppendorf tube. Keep the tube on ice.

(xviii) Aliquot 50 µl of sample and boil using a heat block or PCR machine for 15 min.

(xix) Visualize the average DNA fragment size on 1% (wt/vol) agarose gel using ~10 µl of boiled sample. The average size of smeared DNA fragments should be in the range 0.2–0.5 kb. If the average size of sheared DNA fragments is too large, apply additional sonication sessions in Step B(xv) and repeat Step B(xv–xix).

▲ **CRITICAL STEP** This boiling method is a simple quick way of testing DNA fragment size. To test true sonication efficiency, overnight reverse-cross-linking and proteinase K treatment is necessary (Step B(xxx–xl)).

■ **PAUSE POINT** At this point, the sample can be kept at −80 °C and will be stable for months.

? TROUBLESHOOTING

(xx) Add 50 µl of streptavidin-magnetic bead slurry to an empty 1.5-ml Eppendorf tube. Make the tube sit on Dynal MPC magnet for 2 min and remove the supernatant by pipetting, taking care not to disrupt the streptavidin beads.

(xxi) Add 1 ml of ChIP buffer to the tube and wash the bead well by gentle pipetting. Place the tube on Dynal MPC magnet for 2 min, and collect beads as described in Step B(xx). Perform this wash twice.

(xxii) Transfer the chromatin sample from Step B(xvii) to the tube containing streptavidin beads prepared in Step B(xxi). Mix the sample and streptavidin beads carefully with gentle pipetting.

(xxiii) Incubate the tube on the rotator at 4 °C for 3 h. Overnight incubation is also applicable. We have found that a minimum of 3 h incubation is enough to perform successful immunoprecipitation reaction. Longer incubation times can also be used.

(xxiv) Quickly spin (1–2 s) the tube containing sample and streptavidin beads from Step B(xxiii). Mix the sample and beads well by gentle pipetting and transfer to a fresh tube placed on the Dynal MPC. Wait for 2 min to collect beads to the side of an Eppendorf tube. Remove the supernatant with a pipette, taking care not to disrupt the beads.

▲ **CRITICAL STEP** All of the following washing steps should be carried out at room temperature; each wash should be performed quickly to prevent the beads from drying out.

(xxv) Add 1 ml of washing buffer 1 to the tube and gently resuspend beads. Place the tube on a Nutator for 8 min.

(xxvi) Pulse-spin the tube. Collect the beads using Dynal MPC for 2 min as described in Step B(xxiv) and remove the washing buffer. Transfer the sample to a new tube, perform a second washing with washing buffer 1, collect the beads again and remove washing buffer 1, as described in Step B(xxiv).

(xxvii) Wash once with 1 ml of washing buffer 2, as described in Step B(xxiv).

(xxviii) Wash once with 1 ml of washing buffer 3, as described in Step B(xxiv).

(xxix) Wash twice with 1 ml of TE. Transfer the sample to a new tube during the second wash. Remove TE completely.

(xxx) Add 300 µl of elution buffer to a sample tube from Step B(xxix) and gently resuspend beads by pipetting. Transfer elution buffer with beads to a 1.5-ml sterilized screw cap tube. Incubate the sample at 65 °C overnight. Using screw cap tubes minimizes evaporation of elution buffer during overnight reverse cross-linking. Unlike other ChIP protocols, elution and reverse cross-linking steps are combined.

(xxxi) Pulse-spin the tubes and place in the Dynal MPC. Wait for 3 min and collect and transfer the supernatant by pipetting to a new tube.

(xxxii) Place the new tube in the Dynal MPC and wait for 3 min to completely remove any remaining beads. Transfer the supernatant to a new tube.

(xxxiii) Add 1 volume (300 µl) of TE to the sample and 3 µl of 0.5 µg µl^{-1} RNaseA. Incubate at 37 °C for 30 min.

(xxxiv) Add 2 µl of 20 mg ml^{-1} glycogen and 3 µl of 20 mg ml^{-1} proteinase K. Mix well and incubate at 37 °C for 2 h.

(xxxv) Transfer sample to a 2-ml Phase Lock gel tube (heavy) and add 1 volume (600 µl) of PCI solution (25:24:1, vol/vol/vol). Mix well and spin at 12,000*g* for 3 min. Transfer aqueous phase to two new tubes (300 µl each).

(xxxvi) Add 2.5 volumes (750 µl) of 100% ethanol and 1/10 volume (30 µl) of 3M NaOAc (pH 5.2) to the tubes containing 300 µl of sample each. Incubate tubes at −80 °C for 15 min and spin at 12,000*g* for 20 min at 4 °C.

(xxxvii) Remove the supernatant and add 500 µl of 70% ethanol, taking care not to disrupt the pellet. Spin again at 12,000*g* for 10 min at 4 °C.

(xxxviii) Remove the supernatant again by careful pipetting and briefly spin the tubes again. Remove the supernatant completely without touching pellet by careful pipetting.

(xxxix) Let the pellets air-dry for 3 min.

▲ **CRITICAL STEP** Do not overdry the pellets to avoid flaking and loss of pellets.

(xl) Add 25 µl of water (nuclease free) to each tube and resuspend well. Combine two tubes and measure DNA concentration with NanoDrop 1000. After testing enrichment of bioChIP material (see **Box 3** and **Fig. 6**), the sample can be used for further quantitative bioChIP-PCR, bioChIP-chip or bioChIP-sequencing experiments.

■ **PAUSE POINT** The bioChIP sample can be stored at −80 °C for months. Otherwise keep the sample at −20 °C (for weeks).

BOX 3 | TESTING ENRICHMENT AND GLOBAL MAPPING OF PROTEIN–DNA INTERACTION

1. Design primer pairs for testing enrichment of bioChIP samples, as described in Experimental design.
2. Perform quantitative PCR using a Bio-Rad iCycler in a 25-μl SYBR Green reaction with ~2% of bioChIP sample with PCR parameters of: 1 cycle at 95 °C for 3 min and 40 cycles of 95 °C for 20 s, 60 °C for 30 s and 68 °C for 30 s, as described previously[12].
3. Determine the amount of each amplification product, as described previously[17]. Calculate fold enrichment by comparing amplified product from bioChIP sample and reference bioChIP sample from *BirA* containing ES cells with a negative control primer set. If there is a previously known target locus for the test protein but the exact binding position is not identified, designing tiling PCR sets will be helpful to test the quality of bioChIP material. Some of the test loci with lower enrichment may serve as negative controls. Actual target loci must show strong enrichment (approximately more than fivefold enrichment from multiple tests). We observe ten- to hundredfold enrichment from many target loci. Examples are shown in **Figure 6**.
4. Once the quality of bioChIP reaction is confirmed by bioChIP-PCR, bioChIP samples are ready to be processed for microarray experiments for the global mapping of protein–DNA interaction, as described previously[13]. Alternatively, sequencing-based methods can be applied[10,18].

● TIMING

As shown in **Figure 3**, the *in vivo* biotinylation system setup is the most time-consuming process, which needs to be planned carefully and executed in a timely manner. You may be able to shorten the setup process by establishing the *BirA*-only ES cell line and *BirA* + Flagbiotag ES cell lines simultaneously. In this case, you will select for the former line with G418 only after pEF1α*BirA*V5-neo electroporation and the latter line with both G418 and puromycin after co-electroporation with pEF1α*BirA*V5-neo plasmid and pEF1αFLBIO-tagged gene of interest plasmid. Options A and B of Step 29 can be performed independently once the system is set up. In general, we try to prepare nuclear extract, determine protein concentration and set up Flag M2-agarose or streptavidin-agarose incubation all in the same day, to avoid potential disruption of multiprotein complexes after prolonged storage and/or freeze–thaw cycles.

ANTICIPATED RESULTS

For *in vivo* biotinylation system setup, we usually screen 24–48 G418-resistant clones for *BirA*V5 expression (using anti-V5–HRP antibody); over half of the clones should show varying amounts of *BirA* expression. We have found that low, medium and high *BirA* expressing cells all mediate efficient biotinylation of the Flagbiotin (FLBIO)-tagged gene product. We screened a similar number of *BirA* + Flagbiotag clones, and one-quarter to one-third of the clones should be positive for Flagbiotin tag (using streptavidin–HRP antibody).

For one-step streptavidin capture of protein complexes, we usually get some background binding of endogenously biotinylated proteins in the *BirA* control cells. In *BirA* + Flagbiotag cells, we get most of the endogenously biotinylated proteins together with potential interacting partners of the biotin-tagged protein. For tandem affinity purification, we usually get only very minimal background binding signals in *BirA* control cells and low amount of endogenously biotinylated proteins in *BirA* + Flagbiotag cells. However, the total number of potential interacting partners in the latter cells is also greatly reduced. Numbers vary according to individual proteins and range from a few to more than ten candidates. These candidate proteins are usually of high confidence, although further validation by reverse tagging followed by affinity purification and/or co-immunoprecipitation is required. The detailed information on the *BirA* control background signals and the endogenously biotinylated proteins have been described elsewhere[2,3].

For bioChIP, we usually get approximately 20–30 ng μl^{-1} of final ChIP DNA from both bioChIP and reference reactions (total 50 μl). In total, 2% of bioChIP material is used in a bioChIP-PCR to test the quality of bioChIP sample. In most of the cases, we observed fold enrichment between 5 and 100 from the target loci.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

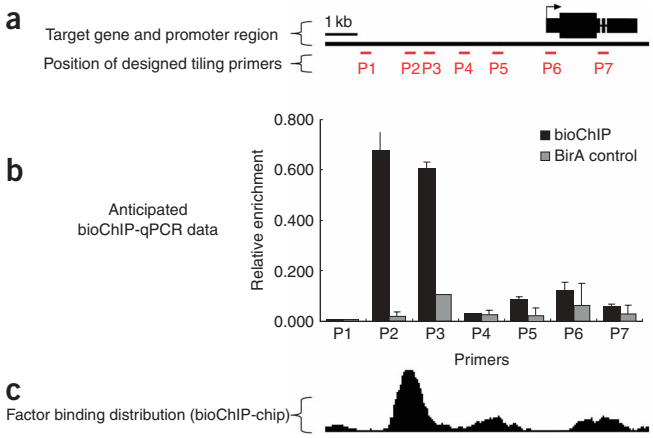

Figure 6 | An example of qRT-PCR confirmation of bioChIP sample. (**a**) Where there is limited or no previous information about the exact binding loci of protein of interest, tiling PCR primer sets (P1–7, marked in red) for the predicted target gene (**a**) will be useful to test the quality of bioChIP material and target locus. (**b**) Representative bioChIP-qPCR data. Some of the tested loci showing lower enrichment (e.g., P1 or P4) may serve as negative controls. True target loci must show strong enrichment over the negative control(s) (e.g., P2 and P3). (**c**) A representative bioChIP-chip data for the anticipated target gene is shown.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
23	Weak or no positive signals for the biotinylated proteins	Milk contains high levels of biotin, which blocks the interaction between streptavidin and the biotinylated protein	Western blot analysis with streptavidin–HRP should be done without milk
		No true positive clones	Screen more clones
29A(xx)	Low yield of nuclear extract from both control *BirA* and *Bir-A*+Flagbiotin-tagged samples	Cell lysis is not complete	Make sure that cell lysis is complete at Step 29A(xiv)
		Low starting cell numbers	Optimize cell culture and increase the amount of starting material
Box 1	Protein precipitates after overnight incubation	The salt concentration is not optimal	Should be 350 μM for J1 ES cell nuclear extract. For other ES cells, the optimal salt concentration will need to be determined
		The NE concentration at Step 29A(xxi) is too high	Dilute the NE concentration to $\sim$2 mg ml^{-1}
29A(xxvi)	The final volume does not go down to 100 μl	Residual resin in the eluate, which clogs the Centricon device	Briefly centrifuge the eluate, and recover the supernatant carefully
			Extend the centrifuge time
29A(xxxii)	High nonspecific binding present in both BirA only and BirA+ Flag-biotin-tagged samples	The NE concentration at Step 29A(xxi) is too high	Dilute the NE concentration to $\sim$2 mg ml^{-1}
		Percentage of NP-40 in IP350 is too low	Increase NP-40 in IP350
29B(xix)	Average size of sheared DNA is too big	Sonication is not efficient	Apply additional sessions of sonication
		Foaming occurs during sonication	Spin down sample tube between each sonication session to avoid foaming
		Too many cells in a small volume of ChIP buffer are used	Add additional ChIP buffer

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