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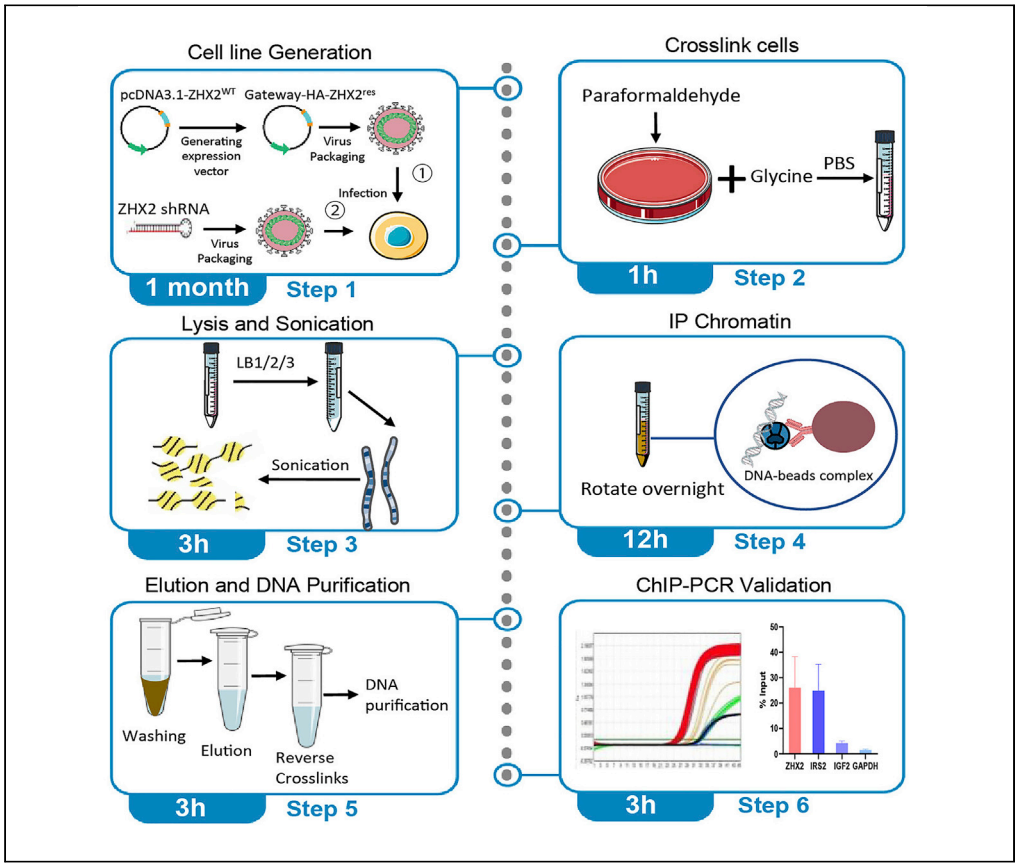
Optimized protocols for chromatin immunoprecipitation of exogenously expressed epitope-tagged proteins



Chromatin immunoprecipitation (ChIP) assay is widely used for investigating the interaction between DNA and DNA-binding proteins such as transcription factors, co-factors, or chromatin-associated proteins. However, a successful ChIP assay largely depends on the quality of a ChIP-grade primary antibody. In cases where specific antibodies are unavailable or with low binding affinity, here, we describe a tailored protocol to achieve robust and reproducible chromatin binding by expressing an exogenous epitope-tagged protein in cells, followed by ChIP assays using a tag-specific antibody.

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

Wentong Fang, Chengheng Liao, Qing Zhang

chengheng.liao@utsouthwestern.edu (C.L.)
qing.zhang@utsouthwestern.edu (Q.Z.)

Highlights

Protocol to express an exogenous protein and depleting its native counterpart in cells

An alternative ChIP strategy when ChIP-grade antibody of a protein is unavailable

Optimized ChIP protocol by using a tag-specific antibody

Optimized ChIP conditions were provided

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Optimized protocols for chromatin immunoprecipitation of exogenously expressed epitope-tagged proteins

Wentong Fang,[1,4] Chengheng Liao,[2,4,5,*] and Qing Zhang[2,3,6,*]

[1]Department of Pharmacy, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China

[2]Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[3]Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[4]These authors contributed equally

[5]Technical contact: chengheng.liao@utsouthwestern.edu

[6]Lead contact

*Correspondence: chengheng.liao@utsouthwestern.edu (C.L.), qing.zhang@utsouthwestern.edu (Q.Z.)
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SUMMARY

Chromatin immunoprecipitation (ChIP) assay is widely used for investigating the interaction between DNA and DNA-binding proteins such as transcription factors, co-factors, or chromatin-associated proteins. However, a successful ChIP assay largely depends on the quality of a ChIP-grade primary antibody. In cases where specific antibodies are unavailable or with low binding affinity, here, we describe a tailored protocol to achieve robust and reproducible chromatin binding by expressing an exogenous epitope-tagged protein in cells, followed by ChIP assays using a tag-specific antibody.

For complete details on the use and execution of this protocol, please refer to Fang et al. (2021)[1] and Kidder et al. (2011).[2]

BEFORE YOU BEGIN

Experimental considerations

The following protocol can be applied to construct cells stably expressing exogenous tagged protein and then ChIP is performed using a tag-specific antibody.[2] The rationale of this optimized protocol is to provide an alternative strategy to characterize the DNA-binding profile of a protein of interest in case the ChIP-grade antibodies against the native protein are not available or perform poorly for immunoprecipitation. The gene coding the protein of interest with several mutations in the recognition sequences of short hairpin RNA (shRNA) or single guide RNA (sgRNA) that is resistant to their recognition could first be transduced into the cells by lentivirus infection. Afterward, the endogenous protein of interest will be eliminated by genetically depleting (knock down or knock out) its coding gene from cells either using shRNA interference or CRISPR-Cas9 gene editing strategy. This leads to the generation of stable exogenously expressed protein with certain epitope-tag(s), and the elimination of the competitive binding to the chromatin by the endogenous protein of interest that could affect the outcome of subsequent ChIP analysis.

In this protocol, we take Zinc Fingers and Homeoboxes 2 (ZHX2) as an example to perform the ChIP assay to illustrate this process. ZHX2 is a transcriptional factor that plays a critical oncogenic function in multiple cancers,[1,3–6] We first combined site-directed mutagenesis, GATEWAY cloning, and lentivirus infection strategies to construct a HA-tagged ZHX2^{res} (shRNA #45-resistant) expressed cell line, which could stably express exogenous HA-tagged ZHX2^{res} proteins. We then used the ZHX2 shRNA #45 to deplete its endogenous gene expression to generate a cell line only express

exogenous HA-Tagged ZHX2^{res} protein. We used this cell line to perform the ChIP assay with a ChIP-grade HA antibody, and followed by ChIP-PCR analysis or next generation sequencing. We also provided optimized conditions of the ChIP to investigate the DNA binding profile of ZHX2 protein.

Design primers

Timing: 1 h:0 min

1. Design primers for shRNA resistant site-directed mutation.

The pcDNA3.1 inserted with wild-type ZHX2 (pcDNA3.1-ZHX2^{wt}) was used as gene template for site-directed mutagenesis. The target region of ZHX2 shRNA #45[3] is: CCGTAGCAAGGAAAGC AACAA. We mutated above-mentioned sequence to CCGTTGCTAGGTAAGCTACAA (mutation sites were underlined) in pcDNA3.1-ZHX2^{wt} to generate a shRNA #45 resistant ZHX2 gene, hereafter pcDNA3.1-ZHX2^{res}. The mutagenic oligonucleotide primers used in this step should be designed individually according to the targeted mutation. Both forward and reverse primers should contain the targeted mutation and anneal to the same sequence on opposite strands of the plasmid. The optional primers should be 25–45 bases and with a melting temperature (Tm) of $\geq$78°C. The optional primers should have a GC percent more than 40% and terminate with C or G base. Here, we used QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, cat# 200516) to conduct the site-directed mutagenesis, other primer design considerations can follow the guidelines of the manufacturer's protocol. All the primers for site-directed mutation were listed in key resources table. Bold sequence denotes the shRNA targeted sequence with flanker sequences in italic added at both ends. Desired mutation sites were underlined.

⚠ CRITICAL: The mutated bases are all the synonymous mutations of the original bases and should not cause any amino acid sequence change. The desired mutations should be in the middle of the primer and with 10–15 bases of fully complementary sequence on both sides.

2. Design primers for ZHX2^{res} GATEWAY cloning.

ZHX2^{res} clone primers containing *att*B sequence were designed by Vector NTI Advance software and listed in key resources table. Bold sequence denotes ZHX2^{res} clone primers, italic sequence denotes the *attB* sequence added at the 5′ end of clone primer.

Prepare solutions

Timing: 4 h:0 min

3. Prepare the buffers listed below, making sure to adjust the pH and/or temperature if indicated. See materials and equipment for buffer recipes.
   a. Protease inhibitor solution (100 × stock, −20°C);
   b. Block solution (4°C);
   c. Lysis buffer 1 (LB1, 4°C);
   d. Lysis buffer 2 (LB2, 4°C);
   e. Lysis buffer 3 (LB3, 4°C);
   f. RIPA wash buffer (4°C);
   g. Elution buffer (4°C);
   h. 100 mM PMSF/Isopropanol (−20°C);

⚠ CRITICAL: All solutions should be filtered by 0.20–0.45 μm filter unit.

Prepare equipment

Timing: 30 min

4. All centrifugation prior to the elution step will be performed at 4°C, so make sure that centrifuges have been precooled.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Dynabeads™ Protein G	Invitrogen	cat# 10007D;
HA-Tag (C29F4) Rabbit mAb	Cell Signaling	cat# 3724S; RRID: AB_1549585
Bacterial and virus strains		
XL10-Gold ultracompetent cell	Agilent	cat# 200317
DH5α competent cell	Thermo Fisher	cat# 12297016
Chemicals, peptides, and recombinant proteins		
16% paraformaldehyde	Electron Microscopy Sciences	cat# 15170
cOmplete™ Protease Inhibitor Cocktail Tablets	Roche Applied Bioscience	cat# 04693116001
Triton X-100	Sigma-Aldrich	cat# T8787 CAS: 9036-19-5
Quick Change XL Site-Directed Mutagenesis Kit	Agilent Technologies	cat# 200516
Gateway BP Clonase II Enzyme mix	Invitrogen	cat# 11789020
Gateway LR Clonase Enzyme mix	Invitrogen	cat# 11791019,
HEPES	Sigma	cat# H3375
Sodium deoxycholate	Sigma-Aldrich	cat# 30750 CAS: 4418-26-2
Lithium chloride (LiCl) solution 8 M	Sigma	cat# L7026
RNase A	Thermo Fisher	cat# EN0531
Proteinase K	Thermo Fisher	cat# AM2546
BSA	Sigma-Aldrich	cat# V900933
TE buffer	Sigma-Aldrich	cat# 93283
FastStart SYBR Green	Roche	cat# 4673484001
Qiagen PCR purification kit	QIAGEN	cat# 28106
KOD Hot Start DNA Polymerase	MilliporeSigma	cat# 71086-5
QIAquick Gel Extraction Kit	QIAGEN	cat# 28704
QIAGEN Plasmid Mini Kit (100)	QIAGEN	cat# 12125
Lipofectamine 3000	Invitrogen	cat# 2369247
PMSF	Sigma-Aldrich	cat# P7626-250MG CAS: 329-98-6
β-Mercaptoethanol	Merck millipore	cat# 444203 CAS: 60-24-2
Experimental models: Cell lines		
MDA-MB-231	ATCC	ATCC HTB-26
HEK293T	ATCC	CRL-3216
Oligonucleotides		
Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
shRNA #45 resistant ZHX2 (ZHX2^{res})	*CTACGATG*CCGTTGCTA GGTAAGCTACAA*AACCCATG*	*CATGGGTT*TTGTAGCTTACC TAGCAACGG*CATCGTAG*
ZHX2 *att*B	*GGGGACAACTTTGTACA AAAAAGTTGGCATG*GCTA GCAAACGAAAATCTAC	*GGGGACAACTTTGTACAAGAA AGTTGGGTA*GGCCTGGCCAGC CTCTGCAGG
ZHX2-ChIP	TTGGCCGGGTTTGTGATT	CCTCTCTGTATCCGCTCATTTC
IRS2-ChIP	GCTTGGTCGGTTGTCCT	CGTCGATGGCGATGTAGTT
IGF2-ChIP	GCTTGGTCGGTTGTCCT	CGTCGATGGCGATGTAGTT
GAPDH-ChIP	CGCCCCCGGTTTCTATAAAT	GGCGACGCAAAAGAAGATG

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
Plasmid: pcDNA3.1-HA-ZHX2^{WT}, pcDNA3.1-HA-ZHX2^{Res}, plenti-Gateway-HA-ZHX2^{Res}	Constructed using standard molecular biology techniques	
Plasmid: pLKO-ZHX2 shRNA #45	Broad Institute TRC shRNA library	Clone ID: TRCN0000017745
Plasmid: pcDNA3.1	Addgene	Plasmid #52535
Plasmid: pDONR 223	Invitrogen	Plasmid #12536017
Plasmid:plenti-UBC-HA-gate-pGK-HYG	Addgene	Plasmid #107396
Plasmid:psPAX2	Addgene	Plasmid #12260
Plasmid:pMD2.G	Addgene	Plasmid #12259
Software and algorithms		
Vector NTI Advance software	Invitrogen	12605050
Other		
Bioruptor UCD-200	Diagenode	UCD-200
ThermoMixer F2.0	Eppendorf	5387000072
Digital waving rotator	Thermo Fisher	88882004
Safe Imager 2.0 Blue Light Transilluminator	Invitrogen	G6600
0.45 μm filters	Merck millipore	SLHVR33RB
DynaMag™-2 Magnet	Invitrogen	12321D
The QuantStudio Dx Real-Time PCR Instrument	Thermo Fisher	4480299

MATERIALS AND EQUIPMENT

Below are recipes to prepare solutions needed for this protocol:

Protease Inhibitor Solution (100 × stock)		
Reagent	Final concentration	Amount
PBS	N/A	1 × PBS 0.5 mL
cOmplete™ Protease Inhibitor Cocktail Tablets	N/A	1 tablet
The buffer can be stored at −20°C for six months.		

Block Solution		
Reagent	Final concentration	Amount
PBS	N/A	1 × PBS 500 mL
BSA	0.5%	BSA 2.5g
The buffer can be stored at 4°C for six months.		

Lysis Buffer 1 (LB1)		
Reagent	Final concentration	Amount
Hepes-KOH, pH 7.5	50 mM	1 M Hepes-KOH (pH 7.5) 5 mL
NaCl	140 mM	5 M NaCl 2.8 mL
EDTA	1 mM	0.5 M EDTA 0.2 mL
Glycerol	10%	50% glycerol 20 mL
NP-40	0.5%	10% NP-40 5 mL
Triton x-100	0.25%	10% Triton X-100 2.5 mL
ddH2O	N/A	ddH2O 64.5 mL
Add protease inhibitor solution (1% v/v) before use. The buffer can be stored at 4°C for six months.		

Lysis Buffer 2 (LB2)		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0	10 mM	1 M Tris-HCl (pH 8.0) 1 mL
NaCl	200 mM	5 M NaCl 4 mL
EDTA	1 mM	0.5 M EDTA 0.2 mL
EGTA	0.5 mM	0.5 M EGTA 0.1 mL
ddH2O	N/A	ddH2O 94.7 mL
Add protease inhibitor solution (1% v/v) before use. The buffer can be stored at 4°C for six months.		

Lysis Buffer 3 (LB3)		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0	10 mM	1 M Tris-HCl (pH 8.0) 1 mL
NaCl	100 mM	5 M NaCl 2 mL
EDTA	1 mM	0.5 M EDTA 0.2 mL
EGTA	0.5 mM	0.5 M EGTA 0.1 mL
Na-Deoxycholate	0.1%	10% Na-Deoxycholate 1 mL
N-Lauroylsarcosine	0.5%	20% N-lauroylsarcosine 2.5 mL
Triton X-100	1.0%	10% Triton X-100 10 mL
ddH2O	N/A	ddH2O 83.2 mL
Add protease inhibitor solution (1% v/v) before use. The buffer can be stored at 4°C for six months.		

RIPA Wash Buffer		
Reagent	Final concentration	Amount
Hepes-KOH, pH 7.6	50 mM	1 M Hepes-KOH(pH 7.6) 12.5 mL
LiCl	500 mM	1 M LiCl 125 mL
EDTA	1 mM	0.5 M EDTA 0.5 mL
NP-40	1%	10% NP-40 25 mL
Na-Deoxycholate	0.7%	10% Na-Deoxycholate 17.5 mL
ddH2O	N/A	ddH2O 69.5 mL
The buffer can be stored at 4°C for six months.		

Elution Buffer		
Reagent	Final concentration	Amount
Tris-HCL, pH 8.0	50 mM	1 M Tris-HCL, pH 8.0 5 mL
EDTA	10 mM	0.5 M EDTA 2 mL
SDS	1%	10% SDS 10 mL
ddH2O	N/A	ddH2O 83 mL
The buffer can be stored at 4°C for six months.		

100 mM PMSF/Isopropanol		
Reagent	Final concentration	Amount
PMSF	100 mM	17.4 mg
Isoproponal	N/A	1 mL
The buffer can be stored at −20°C for six months.		

STEP-BY-STEP METHOD DETAILS

Prepare plasmid with shRNA resistant gene

Timing: about 4 days

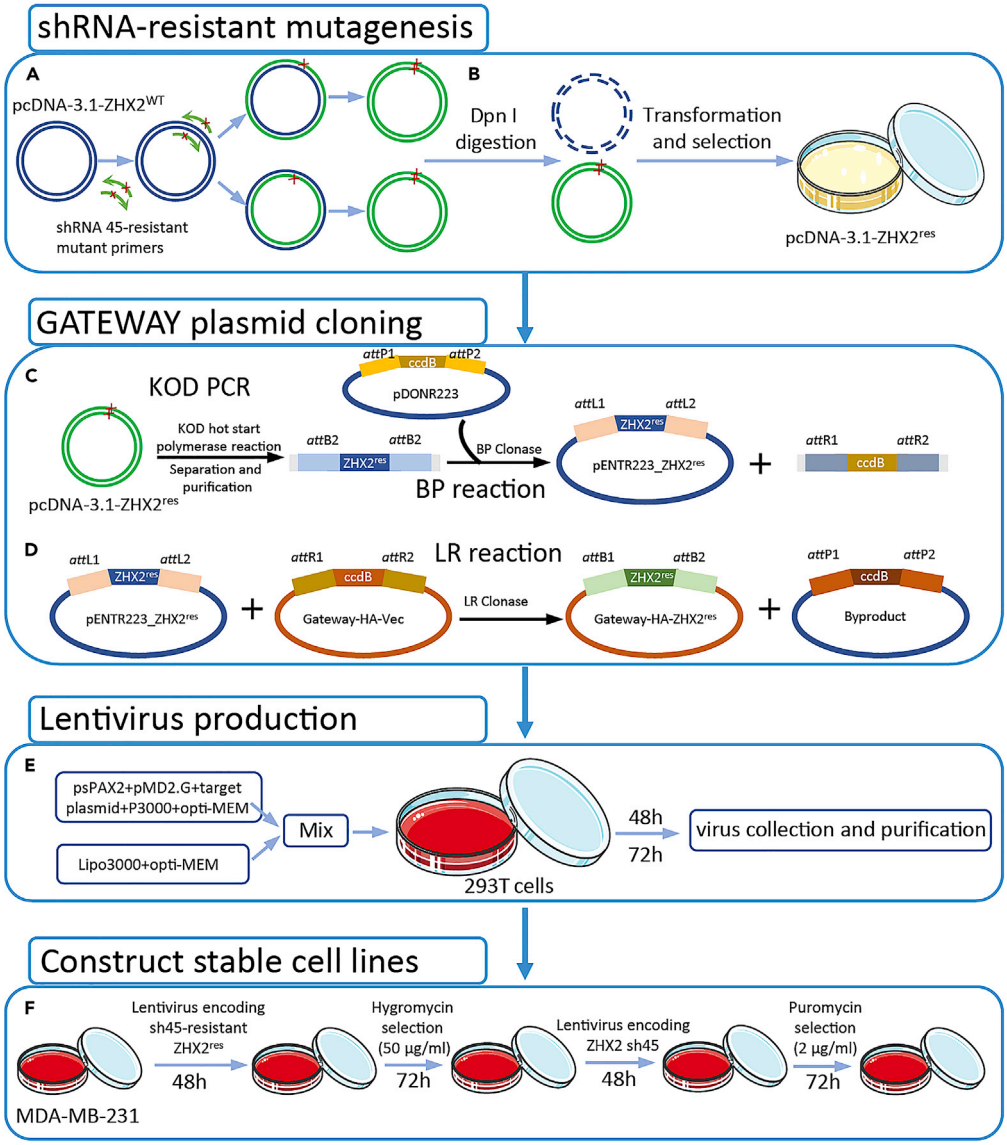

Figure 1. Flowchart of construction of cell lines expressing exogenous epitope-tagged proteins

(A) Prepare shRNA resistant gene by site-directed mutagenesis.

(B) Selection of desired mutation by Dpn I digestion, transformation, and antibiotic selection.

(C) Construction of pENTER223-ZHX2mut by KOD PCR and BP reaction.

(D) Construction of Gateway-HA-ZHX2mut by LR reaction.

(E) Steps of lentivirus production.

(F) Construction of cell lines stably exprressing exogenously HA-ZHX2mut protein and depletion of the endogenous ZHX2 protein.

1. We mutated specific sites (2124 A to T, 2127 A to T, 2131 A to T, 2136 A to T; numbers denote base position from the first letter) in the coding region of ZHX2 where shRNA #45 targeting using pcDNA3.1-ZHX2^{wt} as template to generate pcDNA3.1-ZHX2^{res} (Figure 1A). Site-directed mutagenesis was performed using the Quick Change XL Site-Directed Mutagenesis Kit according to manufacturer's instruction.
   a. Mutant strand synthesis reaction.

Prepare the mutagenesis PCR reaction(s) on the ice as indicated below:

Reagent	Amount
10 × reaction buffer	5 μL
dsDNA template (5 ng/μL)	2 μL
ZHX2^{res} forward primer (100 ng/μL)	1.25 μL
ZHX2^{res} reverse primer (100 ng/μL)	1.25 μL
dNTP mix	1 μL
QuikSolution	3 μL
ddH2O	36.5 μL

Then add 1 μL of PfuUltra HF DNA polymerase (2.5 U/μL) to the above reactions. Cycle each reaction using the following cycling parameters:

Segment	Temperature	Time	Cycles
1	95°C	1 min	1
2	95°C	50 s	18
	60°C	50 s	
	68°C	1 min / kb of plasmid length	
3	68°C	7 min	1

Note: Use a plasmid with small size backbone such as pcDNA will increase the success rate of the site-directed mutagenesis.

b. Dpn I digestion of the amplification products.
Add 1 μL Dpn I restriction enzyme (10 U/μL) directly to each amplification reaction and mix thoroughly. Incubate the reactions at 37°C for 1 h to digest the nonmutated supercoiled dsDNA (Figure 1B).

Note: Dpn I is a Type IIM restriction enzyme which digests methylated DNA. pcDNA3.1-ZHX2^{wt} was prepared from *E. coli* dam^{+} strain and had G^{m}ATC sequence which could be cleaved by Dpn I. The new synthetic pcDNA3.1-ZHX2^{res} did not have G^{m}ATC sequence and could not be cleaved by Dpn I.

2. Transformation of XL10-Gold ultracompetent cells. Add 2 μL β-Mercaptoethanol to 45 μL XL10-Gold ultracompetent cells and mix for 10 min on ice. Add 2 μL of the Dpn I-treated DNA to the ultracompetent cells and mix 30 min on ice. Then heat in 42°C water bath for 30 s and quickly back on ice for 2 min. Add 0.5 mL LB solution, and incubate the tubes at 37°C for 1 h with shaking at 225–250 rpm.
3. Selection of targeted plasmid. Spread cells on LB–ampicillin agar plates, and incubate the transformation plates at 37°C for about 16 h.
4. Purify plasmid and confirm sequence. Transfer desired single clone to LB–ampicillin buffer, and incubate at 37°C for more than 16 h with shaking at 225–250 rpm. Collected the products and extracted plasmid with Qiagen plasmid DNA purification kits. Sequencing the plasmid after mutation to confirm the DNA sequence. Here generated the pcDNA3.1-ZHX2^{res} plasmid from the above steps.

Prepare plasmid for virus production

Timing: about 1 week

5. This part is to generate a lentivirus-based expression plasmid from the pcDNA3.1-ZHX2^{res} using the GATEWAY cloning technique. Based on the GATEWAY cloning protocol,[7] we first perform a KOD hot start polymerase reaction using the *att*B-primers to generate an *att*B-flanked PCR product. Here we used the above generated pcDNA3.1-ZHX2^{res} plasmid as a template to generate *att*B-ZHX2^{res} (Figure 1C).
   a. KOD hot start polymerase reaction.
      Standard reaction setup

Reagent	Amount
10× Buffer for KOD Hot Start DNA Polymerase	5 μL
25 mM MgSO4	3 μL
dNTPs (2 mM each)	5 μL
PCR Grade Water	32 μL
Forward primer (10 μM)	1.5 μL
Reverse primer (10 μM)	1.5 μL
pcDNA3.1-ZHX2^{res} (10 ng/μL)	1 μL
KOD Hot Start DNA Polymerase (1 U/μL)	1 μL

Cycle each reaction using the following cycling parameters:

Segment	Temperature	Time	Cycles
1	95°C	2 min	1
2	95°C	20 s	21
	60°C	10 s	
	70°C	20 s /kb	

   b. Purify *att*B-PCR product.
      Separate the *att*B-PCR product by 1% agarose DNA gel electrophoresis, and confirm the location of *att*B-PCR product by using Safe Imager 2.0 Blue Light Transilluminator. The brightest band at ~ 2.5 kb was the desired product of *att*B-ZHX2^{res} (Figure 2). Cut off the targeted product and purified it by a QIAquick Gel Extraction Kit.
6. Perform BP recombination reaction between *att*B-PCR product (attB-ZHX2^{res}) and a donor vector pDONR223 by using Gateway BP Clonase II Enzyme mix (see key resources table) to generate an entry clone[8] (Figure 1C).
   a. Add the following components to a 1.5 mL microcentrifuge tube at room temperature (21°C–22°C) and mix:

Reagent	Final concentration	Amount
*att*B-ZHX2^{res} (150 ng/μL)	15 ng/μL	2 μL
pDONR 223 (150 ng/μL)	15 ng/μL	2 μL
5× BP Clonase™ reaction buffer	1 ×	4 μL
TE Buffer, pH 8.0	N/A	8 μL
BP Clonase™ enzyme mix	N/A	4 μL

Mix above reaction well by vertexing followed by centrifuge briefly. Incubate reaction at 25°C for 1 h. Add 2 μL of 2 μg/μL Proteinase K solution and incubate at 37°C for 10 min.

   b. Transformation. Transform 1 μL of each BP reaction into 15 μL of DH5α. Incubate tube on ice for 30 min. Heat-shock cells by incubating at 42°C for 30 s and quickly put the tube back on ice for 2 min. Add 500 μL of LB broth medium and incubate at 37°C for 1 h with shaking. Plate 50 μL of each transformation onto LB–spectinomycin agar plates.
   c. Transfer desired single clone to LB–spectinomycin buffer, and incubate at 37°C for more than 16 h with shaking at 225–250 rpm.

d. Collect bacteria and extracted plasmid (pENTR223-ZHX2^{res}) with Qiagen plasmid DNA purification kits. Sequencing the plasmid to confirm the DNA sequence.

7. Perform an LR recombination reaction between pENTR223-ZHX2^{res} and Gateway-HA-Vec (plenti-UBC-HA-gate-pGK-HYG) by using Gateway LR Clonase Enzyme mix (see key resources table) to generate an expression clone (Gateway-HA-ZHX2^{res}) (Figure 1D).
   a. Add the following components to a 1.5 mL tube at room temperature (21°C–22°C) and mix:

Reagent	Final concentration	Amount
pENTR223-ZHX2^{res} (100 ng/reaction)	10 ng/μL	2 μL
Gateway-HA-vec (plenti-Ubcp-FLAG-HA-gateway vector, 150 ng/μL)	15 ng/μL	1 μL
TE buffer, pH 8.0	N/A	5 μL
LR Clonase ™ II enzyme mix	N/A	2 μL

Mix above reaction well by vertexing followed by centrifuge briefly. Incubate reactions at 25°C for 1 h. Add 1 μL of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 min.

   b. Transformation. Transform 1 μL of each LR reaction into 15 μL of DH5α. Incubate on ice for 30 min. Heat-shock cells by incubating at 42°C for 30 s. Add 500 μL of LB broth medium and incubate at 37°C for 1 h with shaking. Plate 50 μL of each transformation onto LB–kanamycin agar plates.
   c. Transfer desired single clone to LB–kanamycin buffer, and incubate at 37°C for more than 16 h with shaking at 225–250 rpm.
   d. Collect bacteria and extracted plasmid (Gateway-HA-ZHX2^{res}) with Qiagen plasmid DNA purification kits. Sequencing the plasmid to confirm the DNA sequence.

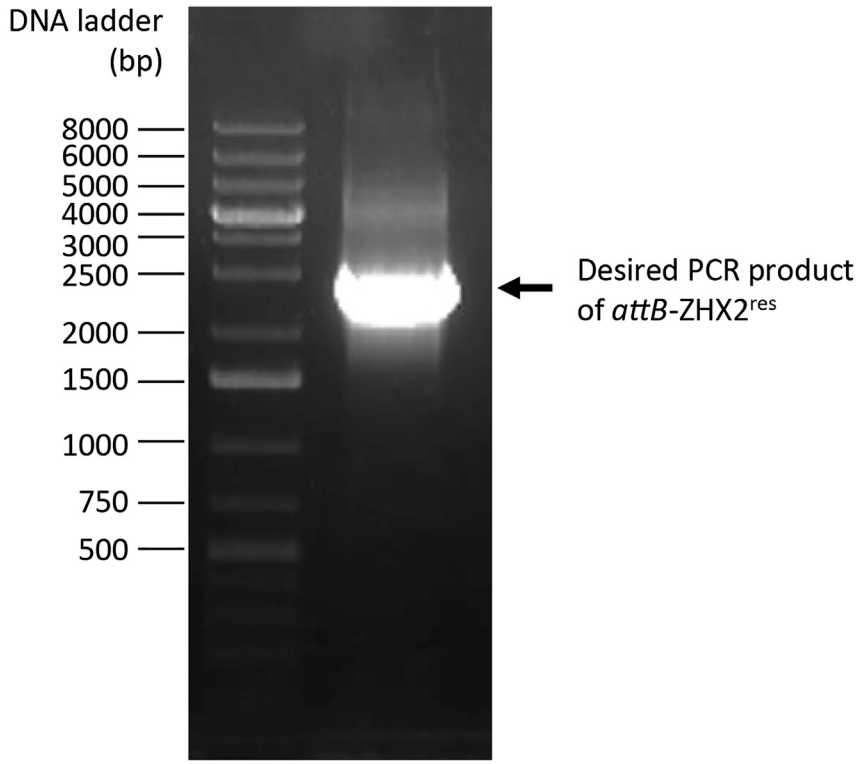

Figure 2. Separate the PCR product by 1% agarose DNA gel electrophoresis

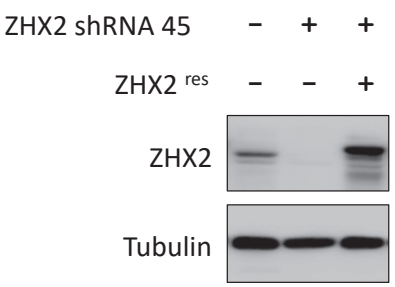

Figure 3. Immunoblot of cell lysates of MDA-MB-231 infected with lentivirus encoding shRNA #45-resistant ZHX2 (ZHX2^{res}) or control vector (EV), then followed by ZHX2 shRNA #45 or Ctrl lentivirus infection

Construct cell lines expressed exogenous HA-tagged proteins

Timing: about 1 month

8. Virus production.

Gateway-HA-ZHX2^{res} plasmid was constructed as described above. Lentiviral ZHX2 shRNA (pLKO-ZHX2 shRNA #45) was obtained from Broad Institute TRC shRNA library (Clone ID: TRCN0000017745). HEK293T packaging cell lines were used for lentiviral amplification. Lentiviral infection was carried out as previously described.[9,10] Briefly, viruses were collected at 48 h and 72 h post-transfection. Viruses were purified by passing through 0.45 μm filters (Figure 1E).

Alternatives: Here we used Lipofectamine 3000 reagent to assemble plasmid mixture and package the virus, other transfection reagents could be used as a substitute.

9. Construct stable cell lines.

The triple-negative breast cancer cell line MDA-MB-231 were firstly infected with lentivirus encoding Gateway-HA-ZHX2^{res} for 48 h in the presence of 8 μg/mL polybrene. Then the cells were selected by hygromycin (50 μg/mL) for at least 72 h. Afterward, MDA-MB-231 were infected with lentivirus encoding ZHX2 sh45 using the same virus infection condition for 48 h and selected by puromycin (2 μg/mL) for at least 72 h (Figure 1F).

Note: 1. The order of two lentivirus infection could be switched depending on the essentiality of gene of interest (see troubleshooting 3). 2. Confirmation of the expression of exogenous protein and depletion of endogenous protein should be performed to ensure the desired cell line. Here we used western blot to confirm the expression of exogenous/endogenous ZHX2 (Figure 3). 3. We suggest to using validated shRNA/sgRNA sequence to eliminate potential off-target effect. In our case, previous studies have validated the function and absence of off-target effect of exogenous ZHX2 by phenotypic rescue experiments.[1,3]

⚠ CRITICAL: It is highly recommended to set up a control group when performing antibiotic selections and the selection time depends on 100% death of the parental cell after selection. We observe 72 h of selection is sufficient to kill MDA-MB-231. Different cell line needs different antibiotic concentrations; therefore, an antibiotic kill curve should be performed.

Pause point: Optional stop step. The constructed MDA-MB-231 could be resuspended in cryoprotectant and frozen at −80°C.

10. Cell expansion for ChIP assay.

Cells were passaged several rounds and proliferated to ~1 × 10^8 cells (about 20 × 15 cm dishes for MDA-MB-231) to get enough starting materials for ChIP assay.

Crosslink cells

Timing: about 1 h

11. Crosslink cells for 10 min with 1% paraformaldehyde in media (add 1 mL 16% paraformaldehyde per 15 mL DMEM media to each 15 cm dish) at room temperature (21°C–22°C);
12. Add 800 μL 2.5 M glycine to the media (finally concentration: 125 μM) in order to quench unreacted paraformaldehyde with glycine, then rotate 5 min at 50 rpm by Digital Waving Rotator at room temperature (21°C–22°C);
13. Remove supernatant and wash cells with 15 mL cold 1 × PBS one time;
14. Resuspend cells with 5 mL cold PBS + protease inhibitors. Scrape cells and transfer to a new 15 mL tube. Centrifuge cells at 225 g for 5 min. Wash cells for one time by using 5 mL cold PBS + protease inhibitor;
15. Pellet cells by centrifugation at 225 g for 5 min;

⚠ CRITICAL: 1. Crosslink 10 min (max. 20 min) at room temperature (21°C–22°C), shake to mix at 50 rpm by using a Digital Waving Rotator. Long crosslink may hurt the sonication effect. 2. Add protease inhibitors to PBS just before use.

Pause point: Optional stop step, cell pellets can be stored at −80°C.

Lysis and sonication

Timing: 3 h

16. Resuspend cells in 10 mL LB1 + protease inhibitor, rotating 10 min at 50 rpm using a Digital Waving Rotator at 4°C, then centrifuge in 1,350 g for 5 min at 4°C;
17. Resuspend cells in 10 mL LB2 + protease inhibitor, rotating 10 min at 50 rpm using a Digital Waving Rotator at 4°C, then centrifuge in 1,350 g for 5 min at 4°C;
18. Resuspend cells in 6 mL LB3 + protease inhibitors, pass through a syringe with 27 gauge needle three times to break up cell clumps;
19. Split into aliquots evenly for sonication (1.5 mL per 15 mL conical tube), add 10 μL of 100 mM PMSF/Isopropanol to eliminate foams;
20. Sonicate 30 s, stop 30 s, 90 cycles by Bioruptor UCD-200, high power, cooled by circulating water system;

Alternatives: Other sonication systems with similar levels of function are acceptable.

21. Centrifuge 18,000 g for 15 min at 4°C;
22. Save 30 μL sonication supernatant as sonication check samples in −80°C; save 30 μL sonication supernatant as ChIP input in −80°C; save residual sonication supernatant for IP chromatin;

Note: Stop 5 min every 15 cycles of sonication.

Pause point: Optional stop step, cell lysate can store at −80°C.

Check the effect of sonication

Timing: 5 h

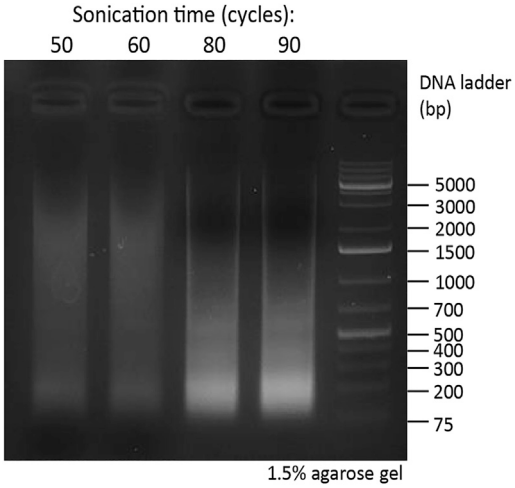

Figure 4. Monitor DNA shearing effect by 1.5% agarose DNA gel electrophoresis

23. Add 170 μL elution buffer and 2 μL 500 μg/mL RNase A to above 30 μL sonication check sample, do reverse crosslink at 37°C for 1 h (keep vortex at 300 rpm by ThermoMixer F2.0);
24. Add 2 μL of 20 mg/mL Protease K to digest protein for 2 h at 55°C (vortex at 300 rpm by ThermoMixer F2.0);
25. Spin down 1 min for 16,000 g at room temperature (21°C–22°C);
26. Transfer 200 μL supernatant to new Eppendorf tube;
27. Recover DNA by Qiagen PCR purification kit;
28. Monitor DNA shearing effect by 1.5% agarose DNA gel electrophoresis;

Note: The optimal size range of chromatin for ChIP-Seq analysis should be between 150 and 300 bp. DNA fragments within this size range, which are equivalent to mono- and di-nucleosomal chromatin fragments, provide high resolution of binding sites, and they work well for next generation sequencing platforms. In our case, which showed in Figure 4, the distribution of DNA in 1.5% DNA gel electrophoresis is between 150–900 kb, but most fragments located in 150–300 kb range.

⚠ CRITICAL: It is important to handle all the samples with the same sonication treatment. Make sure the fragmented chromatin size at the same level for all samples to avoid potential ChIP bias by loss/gain of chromatin sites by unequal shearing.

IP chromatin

Timing: 24 h

29. Wash 100 μL Dynabeads Protein G with 1 mL block solution three times, gently mix, collect beads by a magnetic stand, remove supernatant, resuspend beads in 500 μL block solution;
30. Add 10 μL HA antibody to 500 μL resuspended beads, rotate 30 rpm at 4°C for 2 h using a Digital Waving Rotator to form HA antibody-beads conjugates (Dynabeads coupled to HA antibodies), wash three times with 1 mL block solution, collect HA antibody-beads conjugates by magnetic stand, remove supernatant, and resuspend in 100 μL block solution;
31. Add 100 μL resuspended beads to sonication supernatant (6 mL);
32. Rotate overnight (about 16 h) by Digital Waving Rotator 30 rpm at 4°C;
33. Wash the DNA-HA-beads complex with 1 mL RIPA wash buffer 8 times on magnetic stand;
34. Wash the beads with 1 mL TE buffer (1 mL TE buffer + 50 mM NaCl), spin 3 min at 960 g in 4°C, and carefully remove residual TE buffer with a pipet.

Note: 1. Transfer materials to a new clean Eppendorf tube during rinsing process; 2. Use centrifuge to collect DNA-HA-beads complex after washing by TE buffer.

Elute, reverse crosslinks, and digest RNA and protein

Timing: 3 h

35. Add 210 μL elution buffer and 2 μL 500 μg/mL RNase A to HA beads; Do reverse crosslink on ThermoMixer F2.0 at 37°C for 1 h (keep rotation at 300 rpm);
36. Add 2 μL of 20 mg/mL Protease K to digest protein 2 h on ThermoMixer F2.0 at 55°C (keep rotation at 300 rpm);
37. Spin down 1 min at 16,000 g in room temperature (21°C–22°C);
38. Transfer 200 μL supernatant to a new 1.5 mL Eppendorf tube;
39. Recover DNA by Qiagen PCR purification kit according to the handbook except the last step. At the last step, elute DNA with 100 μL ddH_2O rather than the EB buffer provided in the kit;
40. Determine the concentration of purified DNA by Qubit dsDNA HS kits and Qubit 2.0 Fluorometer[11];
41. Take 5 μL of ChIPed, purified DNA for real-time PCR analysis. Store remaining 95 μL at −20°C.

Note: Elute DNA with 100 μL ddH_2O at the last step of purification by Qiagen PCR purification kit.

Pause point: Optional stop step, the eluted DNA products can store at −20°C.

ChIP-PCR analysis

Timing: 3 h

42. ChIP-PCR is commonly used to analyze protein binding to a known subset of target loci in the genome. In ChIP-PCR, enriched DNA fragments are identified and quantified using common qPCR reagents and protocols according to manufacturer's instructions. We diluted ChIP Input and ChIP sample in 1:100 separately, and made PCR reaction master mix as follow:

Reagent	Amount
DNA template	10 μL
2× SYBR Mix	12.5 μL
Forward primer (5 μM)	0.5 μL
Reverse primer (5 μM)	0.5 μL
ddH_2O	1.5 μL

43. Real time PCR was done by the QuantStudio Dx Real-Time PCR Instrument. PCR cycling conditions as follow:

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	60 s	1
Denaturation	95°C	10 s	40
Annealing	55°C	20 s	
Extension	72°C	20 s	
Denaturation	95°C	15 s	1
Annealing	60°C	60 s	
Extension	95°C	15 s	
Hold	4°C	forever	

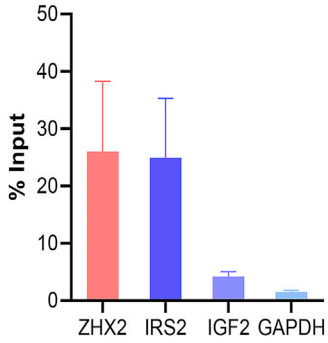

Figure 5. Real time PCR results of ChIP sample and ChIP Input

EXPECTED OUTCOMES

Steps 8–10: A successful construction of desired cell lines should observe a significant depletion of the endogenous protein of interest and an equivalent expression of exogenous epitope-tagged proteins as resolved by western blot (Figure 3).

Steps 23–28: The expected length of sonicated DNA fragments should between 150–900 bp. It is highly recommended that the sonication should be performed multiple times to determine the optimal sonication time. In our experiment, the DNA shearing effect was monitored by 1.5% agarose DNA gel electrophoresis and had significantly enrichment around 200 bp (Figure 4).

Steps 42–43: The expected outcome should be the relative enrichment of a site expected to be bound by the protein of interest (positive control) in comparison to a site that is not expected to be bound (negative control). The Ct value of Real-time PCR analysis is an important indicator. Calculate the percentage of input value by dividing the value of each IP by its corresponding input. In our ChIP-PCR, the binding sites of ZHX2 protein, such as ZHX2, IRS2, and IGF2, were significantly enriched compared to GAPDH (negative control) (Figure 5).

QUANTIFICATION AND STATISTICAL ANALYSIS

Ct values of quantitative PCR results were provided by the software of the real-time PCR machine. The fraction of the input chromatin saved in this study was 0.50%. IP efficiency could be calculated by using the Percent Input Method, and the equation shown below:

$$\text{Percent Input} = 0.5\% \times 2^{(Ct\ (ChIP\ Input) - Ct\ (ChIP\ Sample))}$$

LIMITATIONS

Although this protocol has been successfully applied for ChIP-seq analysis of exogenous HA-tagged ZHX2 in MDA-MB-231 breast cancer cells, it has not yet been tested in other systems and thus has potential limitations. We highly recommend that users make some optimizations for each step for their specific experiments. To this end, several critical considerations and potential caveats by using our protocols were raised.

Critical considerations: (1) selection of expression system and epitope tags for epitope-tagged protein (see troubleshooting 2); (2) altered viability and/or gene regulation due to lack of expression of the endogenous protein (see troubleshooting 3); (3) genomic DNA characterization of transduced cell lines and immunocharacterization of the epitope-tagged protein. Since generating an epitope-tagged protein expressed cell line is the key element of this protocol, three points above should be carefully considered, and we have provided some potential solutions to address them. According to the guideline of ENCODE consortium,[12] we performed RT-qPCR to confirm the presence of the intended integrated sequence at the intended site of integration in the genome, and we used western blot analysis to confirm

the immunocharacterization of the epitope-tagged protein. These validation steps are essential and highly recommended for successfully recapitulating the protocol.

Potential caveats: (1) potential artefacts of chromatin interactions that caused by dysregulated and/or high expression of the exogenous epitope-tagged protein; (2) potential disruption and /or dysregulation of nearby endogenous genes from vector integration; (3) a protein of interest that only binds chromatin very transiently. Due to the nature of expressing an epitope-tagged protein or the biological property of the protein of interest, these potential caveats somehow are difficult to overcome and beyond the overall scope of this protocol. We acknowledge that these are the common issues when expressing an epitope-tagged protein in cells. We also acknowledge the fact that in some cases even using validated antibodies with a tagged-cell line does not necessarily mean great ChIP results, in the case of a protein that has a weak binding affinity or very transient binding fashion with chromatin."

For caveat point #1, the artifacts that may brought by an epitope-tagged protein could be evaluated in the following experiments. For example, if a high throughput sequencing was performed after ChIP assay, read counts of chromatin regions that typically don't interact with native proteins would be detected with exogenously expressed proteins. Further validation experiments need to be performed such as by examining these exogenously regulatory signaling pathways with endogenous proteins.

For caveat point #2, with regarding to potential disruption and /or dysregulation of nearby endogenous genes from vector integration, further experiments can be designed to check expression of these nearby endogenous genes by RT-PCR or western blots on whether they will be affected by vector integration.

For caveat point #3, with regarding to "a protein of interest that only binds chromatin very transiently", potentially a combination of crosslinkers can be used to trap protein and DNA interactions. For example, formaldehyde is normally used for crosslinking, which could be combined with other linkers, such as EGS (ethylene glycol bis (succinimidyl succinate)) or DSG (disuccinimidyl glutarate).[13]

TROUBLESHOOTING

Problem 1

No colonies were formed in site-directed mutagenesis after transformation.

Potential solution

We found this was a common issue when performing site-directed mutagenesis. This may be due to many facts including: 1. poorly designed primers; 2. too large plasmid backbone; 3. Inappropriate template concentration in the PCR reaction; 4. Inappropriate bacteria transformation. All these potential facts need to be considered especial for 1 and 2. Re-check primers to make sure they have sufficient length of complementary flanking sequences at both ends. Using a plasmid with a large backbone will decreases the efficiency of mutagenesis PCR reactions. We suggest using a plasmid with small size backbone such as pcDNAs to increase the success rate of the site-directed mutagenesis.

Problem 2

Unexpected expression level (e.g., too high / low) of the exogenous epitope-tagged protein.

Potential solution

It is recommended to match the expression level of the exogenous expressed protein of interest to its endogenous counterpart. This could be realized by selecting of a proper expression system to provide low or high expression of the epitope-tagged protein. Usually,

mammalian expression vectors that has different promotors will lead to various protein expression levels. Therefore, choosing a vector with strong promotor (such as CMV) or relative weak promotor (such as Ubc) should be considered ahead based on the level of endogenous protein. In addition, there are inducible expression vectors that allow for controllable gene expression in mammalian cells with Tetracycline.[14] An optimized concentration of Tetracycline could finely determine the desired protein expression level. We suggest performing a titration of Tetracycline treatment and exam the protein expression to get a desired expression level. On the other hand, selection of a proper epitope tag is also important that may affect the expression of protein and following ChIP experiment. The effects that may be elicited from a tag including the type and the direction of fusion (N- or C-terminal of the protein). Different tag has various size and binding affinity. We suggest using a relatively small tag than a big one to avoid adding extra molecular weight to the protein.

We considered following aspects when we chose the expression vector:

- Expression system. In this study, mammalian expression system should be chosen.
- Epitope tag. The expression vector may include a purification tag, such as HA, His, FLAG, etc., for the subsequent research. In any case, whether a C-terminal or N-terminal tag is needed depends on the specific needs of experiments. Generally, affinity tags such as 6×His, GST (Glutathione-S-transferase), and MBP (Maltose Binding Protein) are widely used in affinity purification. Peptide tags such as HA (Hemagglutinin), Myc, FLAG, and V5 are popularly used for detection in western blot, immunocytochemistry, and co-immunoprecipitation.
- Applications of the target protein. We wanted to stably express exogenously protein and delete endogenous protein; hence, two different expression vectors should be chosen.
- Strength of the promoter. A proper promoter is strong enough to allow high product accumulation within host cells and can minimize any unfavorable impacts like toxicity on cell growth.
- Selectable marker. Two different selectable markers should be used to select desirable cells.
- Vector Size. Vectors must be relatively small molecules for the convenience of manipulation.

Problem 3

Altered cell viability due to genetic manipulation of the gene of interest.

Potential solution

If a gene is important for cell survival or proliferation, manipulation of such gene by genetic knock down/out or overexpression will cause altered (increased or decreased) cell viability. Usually, decreased cell viability will cause difficulty in generating stable cell lines during antibiotic selection. In this case, the order of two lentivirus infections will be critical since theoretically they have opposite phenotypic outcomes. Therefore, the order of two lentivirus infections largely depends on the function of gene of interest. In our study, we first generated ZHX2 overexpression then depleted endogenous ZHX2 using shRNA because we found ZHX2 is essential for breast cancer cell growth. For this consideration, users should have an idea about the biological function of the gene of interest and choose a proper sequential virus infection order. We do not recommend infecting with two viruses and performing different antibiotics selection simultaneously, which will cause high selection pressure for the cells.

Problem 4

The length of the sonicated DNA is not within the optimal size range.

Potential solution

In the study of Kidder et al.,[2] the recommended size range of chromatin for ChIP-Seq analysis was 150–300 bp. DNA fragments within this size range, which are equivalent to mono- and di-nucleosomal chromatin fragments, provide high resolution of binding sites, and they work well for next

generation sequencing platforms. The number of cycles of sonication required to run number of tests prior to perform the immunoprecipitation process.[15] In our study, the distribution of DNA in 1.5% DNA gel electrophoresis is between 75–900 kb, but most fragments were concentrated in 150–300 kb with 80/90 cycles of sonication (Figure 4). We performed next generation sequencing and ChIP-PCR with the samples with 90 cycles sonication and obtained good results, which suggests a relative flexibility about size of the DNA fragments. In addtion, all the samples should have the same fragmented chromatin size in order to avoid potential ChIP bias by loss/gain of chromatin sites by unequal shearing. It should be noted that sonication condition may vary with the type/model of the sonicator, cell type, cell concentration, crosslink time, etc.[15,16] Several attempt should be made to confirm the optimal time and model of sonication.

Problem 5

Inconsistent shearing of chromatin.

Potential solution

- Stop 5 min every 15 cycles of sonication, every cycle sonicates 30 s, stop 30 s;
- Keep the same paraformaldehyde concentration and fix time;
- The volume of cell lysis in 15 mL tube should no more than 1.5 mL;
- Keep the same concentration and volume of cell lysis in every conical before sonication;
- Sonicate samples in ice water, replace ice every 15 cycles in order to keep the temperature;
- Different cell types and different chromatin states also affect sonication requirements. Cells should be crosslinked when they reached 80–90% confluence.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Qing Zhang (Qing.Zhang@UTSouthwestern.edu) or Chengheng Liao (Chengheng.Liao@UTSouthwestern.edu).

Materials availability

This study did not generate novel reagents. Plasmids generated in this study are available upon request.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

W.F. optimized the protocol and wrote the original manuscript. C.L. and Q.Z. edited the manuscript and supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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