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Lentiviral Fluorescent Protein Expression Vectors for Biotinylation Proteomics

Irene Riz¹, Teresa S. Hawley², and Robert G. Hawley¹

¹Department of Anatomy and Regenerative Biology, The George Washington University, Washington, DC 20037, USA

²Flow Cytometry Core Facility, The George Washington University, Washington, DC 20037, USA

Abstract

In vivo biotinylation tagging, based on a method in which a protein of interest is tagged with a peptide that is biotinylated *in vivo* by coexpression of *E.coli* BirA biotin ligase, has been successfully used for isolation of protein-protein and protein-DNA complexes in mammalian cells. We describe a modification of this methodology in which cells stably expressing the tagged gene of interest and the BirA gene can be selected by fluorescence activated cell sorting (FACS). We recently implemented this approach to isolate and characterize proteins associated with TLX1, a homeodomain transcription factor with leukemogenic function. The modified technique utilizes two components: a lentiviral vector coexpressing the gene of interest containing a biotinylation tag on a bicistronic transcript together with a downstream yellow fluorescent protein gene; and a second lentiviral vector encoding a fusion protein composed of bacterial BirA linked to the green fluorescent protein. This FACS-based binary *in vivo* biotinylation tagging system allows precise control over the levels of BirA-mediated biotinylation as well as the expression of the gene of interest, which is especially important if high-level expression negatively impacts cell growth or viability.

Keywords

In vivo biotinylation tagging; *E.coli* BirA biotin ligase; simian immunodeficiency virus-based lentiviral vectors; fluorescent protein reporters; TLX1 homeodomain transcription factor

1. Introduction

A variety of biochemical methods exist for studying protein-protein interactions in mammalian cells (1,2), including those based on fluorescence activated cell sorting (FACS) (3) (*see also* the chapter by Szöllösi *et al.*, this volume). Strouboulis and colleagues developed a method in which a recombinant protein is tagged with a peptide that is biotinylated *in vivo* by the coexpressed *E.coli* BirA biotin ligase (4). A strength of this approach is the very high affinity interaction between biotinylated substrates and streptavidin ($K_d = 10^{-15}$), which allows high stringencies to be employed during purification (5). The biotinylation tagging method described here has been successfully used for the single-step purification (6) of a number of transcription factor complexes in nuclear extracts of mammalian cells (4,7–12).

In the initial version of the method (4), expression cassettes encoding the tagged protein of interest and BirA were subcloned into separate plasmids that also carried a selectable drug resistance gene, the neomycin phosphotransferase gene and the puromycin *N*-acetyltransferase gene, respectively. Typically, target cells would be transfected with the BirA plasmid by physical methods such as electroporation, stable clones obtained by selection for puromycin resistance, and then screened for BirA expression by Northern or Western blot analysis. An appropriate BirA-expressing clone would then be transfected with the vector encoding the tagged protein of interest and stable cells expressing the tagged protein biotinylated by BirA obtained by selection for cells that were resistant to the neomycin analog geneticin as well as to puromycin. A drawback of this experimental design is that the optimal concentration of drugs used for selection depends on the cell line and needs to be determined a priori. Moreover, selection for drug resistance of the transfected plasmids does not necessarily ensure coexpression of BirA or the tagged gene of interest.

Hoang and colleagues reported a modification of this methodology in which the tagged gene of interest was coexpressed from a lentiviral vector on a bicistronic transcript that also contained the BirA gene (9). In addition, the BirA gene was expressed as a fusion protein with the green fluorescent protein (BirA-GFP), allowing stably transduced cells coexpressing the tagged gene of interest and BirA to be isolated by FACS. As described below, we have further modified the FACS-based strategy of Hoang and colleagues. The expression system we have developed consists of two components: a lentiviral vector coexpressing the gene of interest containing a biotinylation tag together with a downstream yellow fluorescent protein (YFP) gene via an encephalomyocarditis virus internal ribosome entry site (IRES); and a second lentiviral vector expressing GFP-BirA (12).

Our work is focused on *TLX1* (*T-cell leukemia homeobox 1*, previously known as *HOX11* or *TCL3*), an evolutionarily conserved member of the dispersed NKL (NK-Like or NK-Linked) subclass of homeobox genes (13,14). The murine ortholog of human *TLX1* is essential for splenogenesis and required for the development of certain neurons (15,16). Although *TLX1* is not expressed in the hematopoietic system, its inappropriate activation is a recurrent event in human T cell acute lymphoblastic leukemia (T-ALL) (17). The manner in which deregulated *TLX1* expression induces neoplastic conversion remains to be fully elucidated (18,19). Several lines of evidence indicate that *TLX1* functions as a transcriptional regulator that can either activate or repress gene expression via direct or indirect modes of action (20–28). In this regard, *TLX1* has been reported to form protein-protein interactions with other transcription factors as well as with a number of transcriptional coregulators and chromatin-modifying enzymes. Among the molecules that have been identified are: CTF1, a ubiquitous transcription factor that associates with TFIIB and the basal transcription machinery (29); MEIS and PBX members of the TALE (three amino acid loop extension) superclass of homeodomain proteins (30,31); the acetyltransferase coactivator CREB-binding protein (26); the serine/threonine phosphatases PP1 and PP2A (25,32); and the eukaryotic initiation factor 4E (eIF4E) (33).

We recently implemented the *in vivo* biotinylation tagging approach to isolate and characterize the various *TLX1* protein complexes in T-ALL cells (12). Our initial attempts to coexpress biotinylation-tagged *TLX1* and GFP-BirA from a bicistronic lentiviral vector were unsuccessful because the cells did not tolerate high levels of *TLX1*. Since BirA was expressed on the same transcript, it was selected against. As a result, much higher expression levels of BirA were achieved with the empty GFP-BirA vector, making it difficult to obtain similar levels of endogenous biotinylated proteins for comparisons between the experimental and control samples. To circumvent these problems, we designed a two-component expression system using the simian immunodeficiency virus (SIV) lentiviral vector backbone pCL20cSLFR MSCV-GFP (34). In pCL20cSLFR MSCV-GFP,

which was constructed from the nonpathogenic SIV_{mac1A11} isolate, the GFP gene is expressed from an internal promoter derived from the long terminal repeat (LTR) of the murine stem cell virus (MSCV), which is highly active in most mammalian cell types (35). A biotinylation tagging vector expressing a COOH-terminal tagged TLX1 (TLX1^{bio}) under the control of the MSCV LTR was created by inserting the TLX1 coding region in frame upstream of the coding sequences for the BirA target peptide linked to an IRES-YFP cassette, generating pCL20cSLFR MSCV-TLX1^{bio}-IRES-YFP (component 1). A BirA expression vector was similarly constructed by replacing the GFP gene of pCL20cSLFR MSCV-GFP with GFP-BirA, generating pCL20cSLFR MSCV-GFP-BirA (component 2) (Fig. 1A).

In pilot studies, SupT1 cells, which are negative for *TLX1* expression but which are arrested at the same stage of T-cell differentiation as TLX1⁺ T-ALL cells, were transduced with recombinant CL20cSLFR MSCV-TLX1^{bio}-IRES-YFP lentiviral vector particles and/or recombinant CL20cSLFR MSCV-GFP-BirA lentiviral vector particles and stable cell lines obtained by sorting for YFP and/or GFP fluorescence (Fig. 1B). SupT1 cells expressing GFP-BirA alone served as control for binding of any biotinylated endogenous proteins to the streptavidin beads. Using single-step affinity capture on streptavidin beads, followed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, we identified the Groucho/transducin-like Enhancer of split (Gro/TLE) family member TLE1 as an *in vivo* binding partner of TLX1 (12) (Fig. 2A). Gro/TLE proteins are regulated by multiple signaling cascades and serve as corepressors for different families of transcription factors (36,37). The transcription factors that interact with Gro/TLE corepressors contain short peptide sequences related to either WRPW or to FXIXXIL (where X is any amino acid), the latter referred to as the Engrailed homology 1 (Eh1) motif, a repression domain first identified in the *Drosophila* Engrailed homeodomain protein (38). We demonstrated that TLX1 interacts with TLE1 *in vitro* and *in vivo* through a 7 amino acid sequence encompassing amino acids 19 to 25 (FGIDQIL) that exhibits similarity to an Eh1 motif (Fig. 2B). Moreover, we found that this motif was required for optimal regulation of expression of two TLX1 target genes, *Aldh1a1* and *Fhl1* (21,22,27). As shown in Fig. 2A, a previously reported *in vitro* TLX1 interacting protein, eIF4E (33), is also efficiently precipitated by streptavidin affinity capture of *in vivo* TLX1^{bio} protein complexes. Thus, we believe that our adaptation of the FACS-based *in vivo* biotinylation tagging system will provide a powerful tool for characterization of transcription factor and other protein-protein complexes in mammalian cells.

2. Materials

2.1. Biotinylation Tagging Vectors

1. Starting material plasmids: the SIV_{mac1A11}-based pCL20cSLFR MSCV-GFP lentiviral vector was provided by Arthur Nienhuis (St. Jude Children's Research Hospital, Memphis, TN) (34); coding sequences for a 23-amino acid biotinylation peptide tag (*see* Note 1) and the GFP-BirA fusion gene were obtained from the pTRIP/BPceter/IRES/EGFPBirA plasmid (provided by John Strouboulis, Erasmus Medical Center, Rotterdam, The Netherlands) (9).
2. SIV lentiviral vectors: pCL20cSLFR MSCV-TLX1^{bio}-IRES-YFP expressing the TLX1 homeodomain transcription factor containing a COOH-terminal biotinylation

¹More recently, we have constructed an NH₂-terminal biotin-tagged TLX1 (bioTLX1) modeled after the bioNanog homeodomain protein and other biotin-tagged transcription factors described by Orkin and colleagues (46,47), which is based on the 15 amino acid AviTagTM sequence (GLNDIFEAQKIEWHE) that is very efficiently biotinylated *in vivo* by BirA (Avidity, LLC, Aurora, CO; www.avidity.com).

peptide tag linked to a downstream yellow fluorescent protein (YFP) reporter on a bicistronic transcript (12); pCL20cSLFR MSCV-GFP-BirA expressing a fusion protein consisting of a green fluorescent protein (GFP) reporter and the bacterial BirA biotin ligase (GFP-BirA) (12). The pCL20cSLFR MSCV-IRES-YFP and pCL20cSLFR MSCV-GFP-BirA plasmids can be obtained from the authors upon request.

3. Restriction and modifying enzymes for PCR amplification and subcloning of genes of interest.
4. Plasmid DNA purification kits: QIAquick Gel Extraction Kit, QIAGEN Plasmid Maxi Kit (Qiagen, Valencia, CA).

2.2. Production and Titration of Vector Particles

2.2.1. Transient Transfection

1. 293T/17 (293T) human embryonic kidney cell line (CRL-11268; American Type Culture Collection (ATCC), Manassas, VA) (*see* Note 2).
2. 293T cell culture medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4.5 g/L glucose, 2 mM L-glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, 10% heat-inactivated fetal bovine serum (FBS). Store at 4°C and warm up to 37°C before use.
3. Phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS): 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, 8.1 mM Na₂HPO₄ (adjust pH to 7.4 if necessary, using 1 N HCl or 1 N NaOH).
4. Solution of trypsin and ethylenediamine tetraacetic acid (trypsin-EDTA): 0.05% trypsin, 0.53 mM EDTA in PBS.
5. pCL20cSLFR MSCV-IRES-YFP biotinylation tagging SIV lentiviral vector plasmid DNA containing the gene of interest; pCL20cSLFR MSCV-GFP-BirA SIV lentiviral vector plasmid DNA.
6. SIV lentiviral packaging plasmid DNAs (e.g., pCAG-SIVgprre and pCAG4-RTR-SIV; provided Arthur Nienhuis) (34).
7. Vesicular stomatitis virus G (VSV-G) glycoprotein envelope plasmid DNA (e.g., pMD.G; (39,40)).
8. 2.5 M CaCl₂: Dissolve 183.7 g CaCl₂ dihydrate (tissue culture grade) in deionized water. Bring the volume up to 500 mL and filter-sterilize using a 0.22-µm nitrocellulose filter. Store at -20°C.
9. 2X N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES)-buffered saline (2X HBS): 50 mM HEPES (H4034; Sigma-Aldrich Corp., St. Louis, MO), 280 mM NaCl, 1.5 mM Na₂HPO₄. Titrate to pH 7.05 with 5 N NaOH. Filter sterilize using a 0.22-µm nitrocellulose filter. Store as single use aliquots at -20°C.

²The 293T/17 cell line is a clone of the 293T (293tsA1609neo) human embryonic kidney cell line (48), which was selected for high transfectability and capability of producing high-titer vector stocks. The 293T cell line is a derivative of 293 cells into which the simian virus 40 (SV40) large T antigen gene was inserted. 293 cells express the adenovirus serotype 5 E1A 12S and 13S gene products, which strongly transactivate transcription from expression vectors containing the human CMV enhancer-promoter (49). Expression of the SV40 large T antigen by 293T cells may stimulate extrachromosomal replication of plasmids containing the SV40 origin of replication during transient transfection.

2.2.2. Collection and Concentration of Vector Particles

1. 0.45- μ m pore-size filter units: Stericup 150-mL unit, low protein binding Durapore (PVDF) unit (Millipore Corp., Bedford, MA).
2. Ultracentrifuge, polycarbonate 70-mL ultracentrifuge bottles and caps.

2.2.3. Titration of Vector Particles

1. HT1080 human fibrosarcoma cells (CCL-121; ATCC).
2. HT1080 cell culture medium: same as 293T cell culture medium (*see Subheading 2.2.1.*).
3. Polybrene (hexadimethrine bromide, H9268; Sigma-Aldrich Corp.) stock solution (1000X): 8 mg/mL in sterile, deionized water. Aliquot and store at -20°C .
4. Analytical flow cytometer.

2.3. Generation of Cells Stably Expressing Biotin-Tagged Proteins

1. Replication-defective SIV lentiviral vector particles (e.g., CL20cSLFR MSCV-TLX1^{bio}-IRES-YFP and CL20cSLFR MSCV-GFP-BirA).
2. Target cells (e.g., SupT1 human T-ALL cell line, CRL-1942; ATCC).
3. Suspension cell culture medium: Iscove's modified Dulbecco's medium (IMDM) supplemented with 2 mM L-glutamine, 50 IU/mL penicillin, 50 μ g/mL streptomycin, 10% heat-inactivated FBS.
4. Polybrene stock solution (*see Subheading 2.2.3.*).
5. Fluorescence-activated cell sorter (*see Note 3*).

2.4. Cell Fractionation

1. Phenylmethylsulfonyl fluoride (PMSF): 0.5 M in dimethylsulfoxide. Store at -20°C .
2. Dithiothreitol (DTT): 0.5 M in deionized water. Store at -20°C .
3. Protease inhibitors (Protease inhibitor cocktail tablets, 11836153001; Roche Diagnostics Corp., Indianapolis, IN).
4. Phosphatase inhibitors (PhosSTOP phosphatase inhibitor cocktail tablets, 04906837001; Roche).
5. Hypotonic wash buffer: 10 mM HEPES, pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT.
6. NP40 nuclear extraction (NP40 NE) buffer: 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl_2 , 20 mM KCl, 0.25 M NaCl, 0.1% NP40, 5 mM EDTA, 1 mM PMSF, 0.5 mM DTT, protease inhibitors (1 tablet for 5 mL), phosphatase inhibitors (1 tablet for 5 mL).
7. Other solutions: PBS; 0.4% trypan blue in normal saline.
8. (Optional) Sonifier (e.g., Branson Sonifier 250 with microtip).

³Standard detection filters supplied with most commercial flow cytometers are unable to distinguish between GFP and YFP signals. Suppliers of custom optical filters include Omega Optical Inc. (Brattleboro, VT), Chroma Technology Corp. (Rockingham, VT), and Semrock (Rochester, NY).

2.5. Streptavidin Affinity Precipitation and Mass Spectrometry Analysis

1. Streptavidin sepharose beads (17-5113-01; GE Healthcare, Piscataway, NJ).
2. Chicken albumin: 20 mg/mL in deionized water. Store at -20°C .
3. HENG buffer: 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl_2 , 0.25 mM EDTA.
4. NP40 T buffer: 70 mM Tris-HCl, pH 8.0, 25% glycerol, 1.5 mM MgCl_2 , 20 mM KCl, 0.25 M NaCl, 0.1% NP40, 5 mM EDTA, 1 mM PMSF, 0.5 mM DTT.
5. Tris-glycine SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels, and associated running and loading buffers.
6. SDS-PAGE staining reagents (Colloidal blue staining kit, LC6025; Invitrogen, Carlsbad, CA).
7. In-gel tryptic digestion reagents (Trypsin profile IGD kit, PP0100; Sigma Aldrich Corp.).
8. ZipTip (0.6 μL C_{18} resin, ZTC18S096; Millipore Corp.).
9. Elution buffer: 50% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) in Milli-Q[®] grade water.
10. α -cyano-4-hydroxycinnamic acid (α -cyano-CHCA, C8982; Sigma-Aldrich Corp.): 10 mg/mL in 50% ACN, 0.05% TFA.
11. Mass spectrometer.

3. Methods

3.1. Biotinylation Tagging Vectors

1. Subclone the gene of interest linked to a biotinylation peptide tag upstream of the IRES-YFP cassette in the pCL20cSLFR MSCV-IRES-YFP SIV lentiviral vector (*see* Notes 1 and 4).
2. Extract plasmid DNA using plasmid DNA purification kits.

3.2. Production and Titration of Vector Particles

293T (293 human embryonic kidney cells expressing simian virus 40 [SV40] large tumor [T] antigen) cells are highly transfectable such that transient cotransfection with the self-inactivating SIV lentiviral vectors, packaging plasmids and envelope plasmids yields high-titer, replication-defective vector particles (41). Lentiviral vectors pseudotyped with the VSV-G glycoprotein have a broad host-cell range and can be utilized to transduce all cell types (39,40).

3.2.1. Transient Transfection

1. Propagate 293T cells in 293T cell culture medium at 37°C in a humidified atmosphere with 5% CO_2 .
2. Passage the cells every 3 to 4 days by splitting them 1/4 to 1/8. Remove the medium and rinse the cells with PBS. Remove the PBS and add enough trypsin-

⁴Common recombinant DNA techniques are used for plasmid DNA preparations, restriction enzyme digestions, PCR amplifications and subclonings. Detailed protocols for each technique can be obtained from commercial sources, as well as from various standard molecular biology manuals. Accordingly, these techniques have not been described here.

EDTA to cover the cells. Incubate the plate at room temperature until the cells round up and detach. Add an equal volume of cell culture medium to inactivate the trypsin. Collect the cells and centrifuge for 5 min at 375g. Resuspend the cells in fresh cell culture medium for plating.

3. Transfect 293T cells with plasmid DNA using the calcium phosphate precipitation method (42). On the day before transfection, plate 293T cells in 7 mL of cell culture medium at a density of 5×10^6 cells per 100-mm tissue culture dish.
4. Prepare a mixture of 20 μ g of plasmid DNA composed as follows: 10 μ g of pCL20cSLFR MSCV-IRES-YFP biotinylation tagging SIV lentiviral vector containing the gene of interest (or pCL20cSLFR MSCV-GFP-BirA SIV lentiviral vector), 6 μ g of pCAG-SIVgprre plus 2 μ g of pCAG4-RTR-SIV packaging plasmids, and 2 μ g of the pMD.G VSV-G glycoprotein envelope plasmid (34) (*see* Note 5). Bring the volume up to 400 μ L with sterile, deionized water. Add 100 μ L of 2.5 M CaCl_2 and mix. Add the DNA/ CaCl_2 solution dropwise to 500 μ L of 2X HBS in a 15-mL conical tube. Use a second pipettor and a 2-mL pipet to bubble the 2X HBS as the DNA/ CaCl_2 solution is added. Vortex immediately for 5 sec and incubate at room temperature for 20 min. Add the 1 mL of DNA/calcium phosphate mixture directly to each 100-mm dish while swirling (*see* Note 6). Incubate the cells at 37°C overnight (16 h). Change the medium and culture for 24–48 h (this method usually results in transfection of 50–80% of the cells).

3.2.2. Collection and Concentration of Vector Particles—Collect the culture medium containing vector particles 24–48 h after medium change. Centrifuge at 2000g for 10 min to remove cellular debris and filter through a 0.45 μ m pore-size filter (depending on the volume, use a 150-mL filter unit or a small filter unit attached to a 5-mL syringe). Use directly for transductions or aliquot and store at -80°C (*see* Note 7).

Several procedures have been developed to concentrate lentiviral vector particles (43). The choice of concentration protocol depends on the envelope selected to pseudotype the particle and the quantities of particles to be produced. The stability of the VSV-G envelope protein allows generation of high-titer lentiviral vector particles by ultracentrifugation, as described here.

1. Ultracentrifuge the vector particles at 50,000g and 4°C for 90 min.

⁵The pCL20cSLFR MSCV-GFP SIV-based vector system is modeled after ‘third generation’ lentiviral vector systems that have incorporated features to enhance safety and improve the efficiency of vector particle production (50). Specifically, several viral accessory proteins have been eliminated from the pCAG-SIVgprre gag-pol packaging construct, and the LTRs have been modified to contain the human CMV enhancer-promoter and to eliminate Tat dependence during vector production (5' LTR) and to be self-inactivating (3' LTR) upon integration. Because both the expression of the pCL20cSLFR MSCV-GFP SIV lentiviral vector backbone as well as the *gag* and *pol* genes encoded by the pCAG-SIVgprre packaging construct are dependent on trans complementation by the Rev protein, use of this system requires cotransfection with a rev expression construct (e.g., pCAG4-RTR-SIV).

⁶Vector titer depends on the vector backbone design, the size and nature of inserted sequences as well as the efficiency of transfection. To achieve optimal transfection efficiency, use an exponentially growing culture (50–70% confluent) of 293T cells for transfection and make sure that the cells form a uniform monolayer upon plating. Transfection efficiency is also affected by the quality of the plasmid DNA used. Commercially available plasmid DNA purification kits usually yield highly purified endotoxin-free supercoiled plasmid DNA (traditionally obtained by purifying on two separate cesium chloride gradients). Sterilize the plasmid DNA by ethanol precipitation, resuspend the air-dried pellet in sterile, deionized water, and determine its concentration and quality by spectrophotometric analysis and gel electrophoresis. Pay special attention to the pH of the 2X HBS solution, which should be between 7.05 and 7.12. Upon addition of the transfection mixture to the cells, a fine precipitate should develop within a few minutes.

⁷pH and temperature fluctuations as well as freeze-and-thaw frequency could have an impact on the stability of vector particles, and hence, vector titer (51). Minimize pH changes by adding HEPES at a final concentration of 10 mM to buffer the cell culture medium. Once collected, vector particles should be kept on ice at all times. For future use, they should be stored in aliquots at -80°C . Avoid repeated freezing and thawing.

2. Discard supernatant. Using gentle pipetting, resuspend the pellet in ~100 μ L of medium appropriate for the downstream application. To facilitate complete resuspension, vortex gently overnight at 4°C (*see* Note 8).
3. To remove cellular debris, centrifuge concentrated vector particles at 10,000g and 4°C for 5 min in a microcentrifuge. Collect the supernatant containing the vector particles, aliquot and freeze at -80°C (*see* Note 9).

3.2.3. Titration of Vector Particles—The HT1080 human fibrosarcoma cell line can be used to determine the titer of the lentiviral vector particles.

1. Propagate HT1080 cells in HT1080 cell culture medium at 37°C in a humidified atmosphere with 5% CO₂.
2. 4–6 h prior to titrating the vector particles, plate 2.5×10^5 HT1080 cells into each well of a 6-well tissue culture dish.
3. Prepare serial dilutions of each vector preparation (e.g., 10^0 , 10^{-1} , and 10^{-2} for unconcentrated vector particles; 10^{-2} , 10^{-3} , and 10^{-4} for concentrated vector particles) using cell culture medium, in a final volume of 1 mL per dilution. Add 1 μ L of polybrene stock solution to each dilution. Remove the medium from the cells and add 1 mL of each dilution to each well. Incubate at 37°C for 4 h.
4. Remove the vector particles after the 4-h transduction and replace with 2 mL of fresh cell culture medium. Return to the CO₂ incubator and incubate at 37°C.
5. After 48 h, determine the relative end-point vector titer (in transducing units per mL [TU/mL]) by flow cytometric analysis. Evaluate GFP or YFP expression on an analytical flow cytometer equipped with 488 nm excitation wavelength and a 530/30-nm bandpass (BP) filter (44).
6. To determine vector titer, use the following equation: Vector titer = number of HT1080 cells $\times$ % fluorescent protein-positive cells $\times$ dilution factor (*see* Note 10).

3.3. Generation of Cells Stably Expressing Biotin-Tagged Proteins

The following protocol is used to transduce suspension cell lines with the recombinant biotinylation tagging lentiviral vectors. For adherent cells, the protocol described in **Subheading 3.2.3.** for titration of vector particles is used.

1. Propagate cells (e.g., SupT1) in suspension cell culture medium at 37°C in a humidified atmosphere with 5% CO₂.
2. Prepare an appropriate dilution of vector particles in 1 mL of cell culture medium containing 1 μ L of polybrene stock solution. Resuspend $1\text{--}2 \times 10^5$ cells in the diluted vector particles in a 14-mL conical polystyrene centrifuge tube.
3. Centrifuge the mixture of cells and vector particles at 600g and 15°C for 2 h. Resuspend the cell pellet in 0.5 mL of fresh cell culture medium and incubate

⁸Small (~2 to 5-mm-diameter) pellets should be visible after concentration by centrifugation. Expect a 50–75% recovery following vector concentration.

⁹The vector particle preparation can be further purified if necessary by centrifuging through a sucrose cushion (52).

¹⁰When reporter proteins are evaluated by flow cytometry, it is advantageous to wait at least 5 days before analyzing the transduced cells. This will minimize the contribution of false positive signals due to pseudotransduction, which is the direct transfer of reporter protein (adhered to vector particles or incorporated into vector particles) to the target cells; this is particularly problematic for VSV-G glycoprotein-pseudotyped vectors (53,54). Note that it has also been shown that transgenes can be efficiently transiently expressed from unintegrated lentiviral vectors during this timeframe (10–14 days) (55).

overnight. Repeat the above centrifugation-enhanced transduction once a day for 2 days.

4. Culture for 10 days. Isolate target cells stably expressing the appropriate fluorescent protein(s) on a fluorescence-activated cell sorter equipped with 488 nm excitation wavelength (*see* Note 11). Evaluate GFP and YFP fluorescence with a 510/20-nm BP filter and a 550/30-nm BP filter, respectively, separated by a 525-nm shortpass dichroic mirror. To determine correct settings for fluorescence compensation, use cells expressing GFP or YFP alone as single color controls (44).

3.4. Cell Fractionation

The protocol described below is a variation of the Dignam procedure for preparation of nuclear extracts for human tissue culture cells (45) that has been modified for T-ALL cell lines (e.g., SupT1). The standard procedure is based on a starting cell number of 2×10^8 cells and can be adjusted to accommodate 2×10^9 cells by increasing the buffer volumes at **steps 2 and 3** by a factor of 4. All solutions are kept on ice; prior to cell harvesting, freshly thawed PMSF and DTT, and freshly prepared protease and phosphatase inhibitors are added to the prechilled buffers. Centrifugations are performed at 4°C.

1. Harvest the cells by centrifuging at 600g for 10 min.
2. Wash once in 10 mL of PBS and centrifuge at 600g for 10 min.
3. Wash once in 10 mL of hypotonic wash buffer and centrifuge at 600g for 5 min.
4. Remove supernatant immediately and discard; resuspend the pellet in 3 mL of hypotonic wash buffer.
5. Subject the cells to hypotonic shock by incubating for 30 min.
6. Check the efficiency of cell lysis under a microscope by staining an aliquot with trypan blue.
7. Centrifuge homogenate at 3,300g for 15 min.
8. Remove supernatant (mostly composed of cytosolic and loosely-associated nuclear proteins) and adjust salt concentration by adding an equal volume of NP40 NE buffer. Freeze sample at -80°C or keep on ice until streptavidin affinity precipitation (*see* Subheading 3.5.).
9. Resuspend the pellet in 3 mL of NP40 NE buffer.
10. Incubate for 30 min on ice to extract nuclear proteins; during the incubation period, shear DNA either by mild sonication (e.g., for 20 sec using a Branson sonifier 250 set at constant duty and microtip output control limited to 3) or by passing the material through a 25-gauge needle.
11. Freeze sample at -80°C or keep on ice until streptavidin affinity precipitation (*see* Subheading 3.5.).

3.5. Streptavidin Affinity Precipitation and Mass Spectrometry Analysis

Streptavidin affinity precipitation is performed essentially as described (6) (*see* Note 12).

¹¹When the target cells already express the protein of interest, sort YFP/GFP-positive cells that express a subendogenous level of the biotin-tagged protein so as to avoid perturbing any existing protein-protein network interactions. To further minimize competition between endogenous and biotin-tagged proteins under these circumstances, we have recently adapted the shRNA-expressing pLKO.1-puro lentiviral vector (56), by substituting the puromycin *N*-acetyltransferase gene with the cyan fluorescent protein (CFP) gene, so that simultaneous knockdown of endogenous transcripts can be achieved by sorting for CFP-positive cells.

1. Wash 120 μ L of streptavidin sepharose beads (for 2 samples) with 1 mL of HENG buffer. Centrifuge at 10,000g for 20 sec. Resuspend the beads in 500 μ L of HENG buffer containing 200 μ g/ml of chicken albumin and incubate on a rotating platform for 1 h at room temperature. Centrifuge and resuspend the beads in 65 μ L of HENG buffer.
2. Centrifuge the samples at 25,000g for 30 min. To decrease nonspecific binding, transfer supernatants to new tubes even if there is no visible insoluble material.
3. Add 30 μ L of streptavidin bead suspension per sample and incubate on a rotating platform for 1 h at 4°C.
4. Wash the sample twice by resuspending in 1 mL of NP40 T buffer and centrifuging at 10,000g for 20 sec.
5. Wash the sample three times by resuspending in 1 mL of NP40 T buffer, incubating on a rotating platform at room temperature for 5 min, and centrifuging at 10,000g for 20 sec.
6. Resuspend the material in 2X loading buffer and fractionate by SDS-PAGE.
7. Stain gel with colloidal blue. Excise discretely stained bands that are evident only in the presence of biotinylated protein of interest.
8. Subject each band to in-gel tryptic digestion. Desalt and concentrate resultant peptides through a ZipTip, and elute in 5 μ L of elution buffer.
9. Load 1 μ L of peptides and 1 μ L of α -cyano-CHCA matrix onto a target plate.
10. Conduct peptide mass fingerprinting using MALDI-TOF mass spectrometry. Perform protein database searches using Mascot software (www.matrixscience.com).
11. Confirm the identity of interacting proteins revealed by mass spectrometry using Western blot analysis.

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References

1. Kocher T, Superti-Furga G. Mass spectrometry-based functional proteomics: from molecular machines to protein networks. *Nat Methods*. 2007; 4:807–15. [PubMed: 17901870]

¹²While single-step purification to generate protein complexes sufficiently clean to be analyzed by mass spectrometry is a strength of the *in vivo* biotinylation approach (6), it might be advantageous to perform tandem-affinity purifications in some experiments. Elegant studies carried out by Orkin and colleagues in murine embryonic stem cells to elaborate the transcriptional interaction network involving nine transcription factors, including the Nanog homeoprotein, have validated and demonstrated the utility of *in vivo* biotinylation of tagged proteins and streptavidin affinity capture to identify downstream targets on a global scale by ChIP-on-chip (46,47). In those studies, both single-step capture on streptavidin beads as well as tandem purification with anti-Flag immunoprecipitation followed by capture with streptavidin were carried out. For example, candidate Nanog-interacting proteins fell into three groups: the first group included proteins present in at least two of three independent one-step purifications and tandem purification; the second group included proteins that may be part of unstable or transient complexes that dissociate during tandem purification; and the third group contained proteins that were 'masked' in one-step purifications but were recovered in the tandem procedure (46). For this reason, we have also included NH₂-terminal Flag tags on our recombinant proteins (12,23,26), which can be used for this purpose.

2. Suter B, Kittanakom S, Stagljar I. Two-hybrid technologies in proteomics research. *Curr Opin Biotechnol.* 2008; 19:316–23. [PubMed: 18619540]
3. Lievens S, Van der Heyden J, Vertenten E, Plum J, Vandekerckhove J, Tavernier J. Design of a fluorescence-activated cell sorting-based Mammalian protein-protein interaction trap. *Methods Mol Biol.* 2004; 263:293–310. [PubMed: 14976373]
4. de Boer E, Rodriguez P, Bonte E, Krijgsveld J, Katsantoni E, Heck A, et al. Efficient biotinylation and single-step purification of tagged transcription factors in mammalian cells and transgenic mice. *Proc Natl Acad Sci USA.* 2003; 100:7480–5. [PubMed: 12802011]
5. Savage, MD.; Mattson, G.; Desai, S.; Nielander, GW.; Morgensen, S.; Conklin, EJ. *Avidin-Biotin Chemistry: A Handbook.* Pierce Chemical Co; Rockford, IL: 1992.
6. Rodriguez P, Braun H, Kolodziej KE, de BE, Campbell J, Bonte E, et al. Isolation of transcription factor complexes by in vivo biotinylation tagging and direct binding to streptavidin beads. *Methods Mol Biol.* 2006; 338:305–23. [PubMed: 16888367]
7. Rodriguez P, Bonte E, Krijgsveld J, Kolodziej KE, Guyot B, Heck AJ, et al. GATA-1 forms distinct activating and repressive complexes in erythroid cells. *EMBO J.* 2005; 24:2354–66. [PubMed: 15920471]
8. Schuh AH, Tipping AJ, Clark AJ, Hamlett I, Guyot B, Iborra FJ, et al. ETO-2 associates with SCL in erythroid cells and megakaryocytes and provides repressor functions in erythropoiesis. *Mol Cell Biol.* 2005; 25:10235–50. [PubMed: 16287841]
9. Goardon N, Lambert JA, Rodriguez P, Nissaire P, Herblot S, Thibault P, et al. ETO2 coordinates cellular proliferation and differentiation during erythropoiesis. *EMBO J.* 2006; 25:357–66. [PubMed: 16407974]
10. Meier N, Krpic S, Rodriguez P, Strouboulis J, Monti M, Krijgsveld J, et al. Novel binding partners of Ldb1 are required for haematopoietic development. *Development.* 2006; 133:4913–23. [PubMed: 17108004]
11. Sanchez C, Sanchez I, Demmers JA, Rodriguez P, Strouboulis J, Vidal M. Proteomic analysis of Ring1B/Rnf2 interactors identifies a novel complex with the Fbxl10/Jmjd1B histone demethylase and the BcoR corepressor. *Mol Cell Proteomics.* 2007; 6:820–34. [PubMed: 17296600]
12. Riz I, Lee HJ, Baxter KK, Behnam R, Hawley TS, Hawley RG. Transcriptional activation by TLX1/HOX11 involves Gro/TLE corepressors. *Biochem Biophys Res Commun.* 2009; 380:361–5. [PubMed: 19250647]
13. Owens BM, Hawley RG. HOX and non-HOX homeobox genes in leukemic hematopoiesis. *Stem Cells.* 2002; 20:364–79. [PubMed: 12351808]
14. Holland PW, Booth HA, Bruford EA. Classification and nomenclature of all human homeobox genes. *BMC Biol.* 2007; 5:47. [PubMed: 17963489]
15. Roberts CW, Shutter JR, Korsmeyer SJ. *Hox11* controls the genesis of the spleen. *Nature.* 1994; 368:747–9. [PubMed: 7908720]
16. Qian Y, Shirasawa S, Chen CL, Cheng L, Ma Q. Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes *Rnx/Tlx-3* and *Tlx-1*. *Genes Dev.* 2002; 16:1220–33. [PubMed: 12023301]
17. Aifantis I, Raetz E, Buonamici S. Molecular pathogenesis of T-cell leukaemia and lymphoma. *Nat Rev Immunol.* 2008; 8:380–90. [PubMed: 18421304]
18. Hawley RG, Fong AZC, Lu M, Hawley TS. The HOX11 homeobox-containing gene of human leukemia immortalizes murine hematopoietic precursors. *Oncogene.* 1994; 9:1–12. [PubMed: 7905617]
19. Hawley RG, Fong AZC, Reis MD, Zhang N, Lu M, Hawley TS. Transforming function of the *HOX11/TCL3* homeobox gene. *Cancer Res.* 1997; 57:337–45. [PubMed: 9000579]
20. Dear TN, Sanchez-Garcia I, Rabbitts TH. The HOX11 gene encodes a DNA-binding nuclear transcription factor belonging to a distinct family of homeobox genes. *Proc Natl Acad Sci USA.* 1993; 90:4431–35. [PubMed: 8099440]
21. Masson N, Greene WK, Rabbitts TH. Optimal activation of an endogenous gene by HOX11 requires the NH₂-terminal 50 amino acids. *Molecular and Cellular Biology.* 1998; 18:3502–8. [PubMed: 9584190]

22. Greene WK, Bahn S, Masson N, Rabbitts TH. The T-cell oncogenic protein HOX11 activates *Aldh1* expression in NIH 3T3 cells but represses its expression in mouse spleen development. *Mol Cell Biol*. 1998; 18:7030–7. [PubMed: 9819390]
23. Owens BM, Zhu YX, Suen TC, Wang PX, Greenblatt JF, Goss PE, et al. Specific homeodomain-DNA interactions are required for HOX11-mediated transformation. *Blood*. 2003; 101:4966–74. [PubMed: 12586625]
24. Hoffmann K, Dixon DN, Greene WK, Ford J, Taplin R, Kees UR. A microarray model system identifies potential new target genes of the proto-oncogene HOX11. *Genes Chromosomes Cancer*. 2004; 41:309–20. [PubMed: 15384172]
25. Riz I, Hawley RG. G₁/S transcriptional networks modulated by the *HOX11/TLX1* oncogene of T-cell acute lymphoblastic leukemia. *Oncogene*. 2005; 24:5561–75. [PubMed: 15897879]
26. Riz I, Akimov SS, Eaker SS, Baxter KK, Lee HJ, Marino-Ramirez L, et al. TLX1/HOX11-induced hematopoietic differentiation blockade. *Oncogene*. 2007; 26:4115–23. [PubMed: 17213805]
27. Rice KL, Kees UR, Greene WK. Transcriptional regulation of FHL1 by TLX1/HOX11 is dosage, cell-type and promoter context-dependent. *Biochem Biophys Res Commun*. 2008; 367:707–13. [PubMed: 18073142]
28. Riz I, Hawley TS, Johnston H, Hawley RG. Role of *TLX1* in T-cell acute lymphoblastic leukaemia pathogenesis. *Br J Haematol*. 2009; 145:140–3. [PubMed: 19133982]
29. Zhang N, Shen W, Hawley RG, Lu M. HOX11 interacts with CTF1 and mediates hematopoietic precursor cell immortalization. *Oncogene*. 1999; 18:2273–9. [PubMed: 10327073]
30. Allen TD, Zhu YX, Hawley TS, Hawley RG. TALE homeoproteins as HOX11-interacting partners in T-cell leukemia. *Leuk Lymphoma*. 2000; 39:241–56. [PubMed: 11342305]
31. Milech N, Gottardo NG, Ford J, D'Souza D, Greene WK, Kees UR, et al. MEIS proteins as partners of the TLX1/HOX11 oncoprotein. *Leuk Res*. 2010; 34:358–63. [PubMed: 19559479]
32. Kawabe T, Muslin AJ, Korsmeyer SJ. HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint. *Nature*. 1997; 385:454–8. [PubMed: 9009195]
33. Topisirovic I, Culjkovic B, Cohen N, Perez JM, Skrabanek L, Borden KL. The proline-rich homeodomain protein, PRH, is a tissue-specific inhibitor of eIF4E-dependent cyclin D1 mRNA transport and growth. *EMBO J*. 2003; 22:689–703. [PubMed: 12554669]
34. Hanawa H, Hematti P, Keyvanfar K, Metzger ME, Krouse A, Donahue RE, et al. Efficient gene transfer into rhesus repopulating hematopoietic stem cells using a simian immunodeficiency virus-based lentiviral vector system. *Blood*. 2004; 103:4062–9. [PubMed: 14976042]
35. Hawley RG, Lieu FHL, Fong AZC, Hawley TS. Versatile retroviral vectors for potential use in gene therapy. *Gene Ther*. 1994; 1:136–8. [PubMed: 7584069]
36. Buscariet M, Stifani S. The 'Marx' of Groucho on development and disease. *Trends Cell Biol*. 2007; 17:353–61. [PubMed: 17643306]
37. Cinnamon E, Paroush Z. Context-dependent regulation of Groucho/TLE-mediated repression. *Curr Opin Genet Dev*. 2008; 18:435–40. [PubMed: 18721877]
38. Smith ST, Jaynes JB. A conserved region of engrailed, shared among all *en-*, *gsc-*, *Nk1-*, *Nk2-* and *msh-* class homeoproteins, mediates active transcriptional repression in vivo. *Development*. 1996; 122:3141–50. [PubMed: 8898227]
39. Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci USA*. 1993; 90:8033–7. [PubMed: 8396259]
40. Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996; 272:263–7. [PubMed: 8602510]
41. Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci U S A*. 1993; 90:8392–6. [PubMed: 7690960]
42. Hawley TS, Sabourin LA, Hawley RG. Comparative analysis of retroviral vector expression in mouse embryonal carcinoma cells. *Plasmid*. 1989; 22:120–31. [PubMed: 2560217]
43. Ramezani A, Hawley RG. Generation of HIV-1-based lentiviral vector particles. *Curr Protoc Mol Biol*. 2002; 16.22:1–15.

44. Hawley TS, Herbert DJ, Eaker SS, Hawley RG. Multiparameter flow cytometry of fluorescent protein reporters. *Methods Mol Biol.* 2004; 263:219–38. [PubMed: 14976369]
45. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 1983; 11:1475–89. [PubMed: 6828386]
46. Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, et al. A protein interaction network for pluripotency of embryonic stem cells. *Nature.* 2006; 444:364–8. [PubMed: 17093407]
47. Kim J, Chu J, Shen X, Wang J, Orkin SH. An extended transcriptional network for pluripotency of embryonic stem cells. *Cell.* 2008; 132:1049–61. [PubMed: 18358816]
48. DuBridge RB, Tang P, Hsia HC, Leong PM, Miller JH, Calos MP. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol Cell Biol.* 1987; 7:379–87. [PubMed: 3031469]
49. Gorman CM, Gies D, McCray G, Huang M. The human cytomegalovirus major immediate early promoter can be trans-activated by adenovirus early proteins. *Virology.* 1989; 171:377–85. [PubMed: 2548325]
50. Ramezani A, Hawley RG. Overview of the HIV-1 lentiviral vector system. *Curr Protoc Mol Biol.* 2002; 16.21:1–15.
51. Higashikawa F, Chang L. Kinetic analyses of stability of simple and complex retroviral vectors. *Virology.* 2001; 280:124–31. [PubMed: 11162826]
52. Tiscornia G, Singer O, Verma IM. Production and purification of lentiviral vectors. *Nat Protoc.* 2006; 1:241–5. [PubMed: 17406239]
53. Liu ML, Winther BL, Kay MA. Pseudotransduction of hepatocytes by using concentrated pseudotyped vesicular stomatitis virus G glycoprotein (VSV-G)-Moloney murine leukemia virus-derived retrovirus vectors: comparison of VSV-G and amphotropic vectors for hepatic gene transfer. *J Virol.* 1996; 70:2497–2502. [PubMed: 8642678]
54. Gallardo HF, Tan C, Ory D, Sadelain M. Recombinant retroviruses pseudotyped with the vesicular stomatitis virus G glycoprotein mediate both stable gene transfer and pseudotransduction in human peripheral blood lymphocytes. *Blood.* 1997; 90:952–7. [PubMed: 9242523]
55. Nightingale SJ, Hollis RP, Pepper KA, Petersen D, Yu XJ, Yang C, et al. Transient gene expression by nonintegrating lentiviral vectors. *Mol Ther.* 2006; 13:1121–32. [PubMed: 16556511]
56. Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepper AM, Hinkle G, et al. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell.* 2006; 124:1283–98. [PubMed: 16564017]

Fig. 1. FACS-based binary *in vivo* biotinylation tagging system. **(A)** Schematic representation of the self-inactivating SIV lentiviral vectors used to produce biotinylated TLX1. *Top*, pCL20cSLFR MSCV-TLX1^{bio}-IRES-YFP biotinylation tagging vector. *Bottom*, pCL20cSLFR MSCV-GFP-BirA vector encoding a GFP-BirA fusion protein. Abbreviations: CMV, cytomegalovirus immediate early enhancer-promoter; R, direct repeat; U5, unique 5' region of long terminal repeat; MSCV, murine stem cell virus promoter; TLX1^{bio}, TLX1 homeodomain transcription factor with COOH-terminal biotinylation peptide tag (the version indicated is a homeodomain deletion mutant); IRES, internal ribosome entry site; YFP, yellow fluorescent protein; GFP-BirA, green fluorescent protein-BirA biotin ligase fusion protein; ΔU3, deleted unique 3' region of long terminal repeat; polyA, polyadenylation signal. **(B)** Bivariate histograms depicting SupT1 cells stably expressing TLX1^{bio}-IRES-YFP and/or GFP-BirA. Cell sorting and data analysis were performed using a FACSAria instrument equipped with FACSDiva software (BD Biosciences, San Jose, CA) as previously described (44).

Fig. 2.

Example of TLX1-interacting proteins detected in extracts of SupT1 cells coexpressing TLX1^{bio} and BirA biotin ligase. **(A)** Western blot analysis for TLE1 and eIF4E after streptavidin pulldown of extracts from GFP-BirA-expressing SupT1 cells coexpressing TLX1^{bio}-IRES-YFP. Antibodies used: anti-TLX1 (C-18), anti-TLE1 (M-101) and anti-eIF4E (P-2) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). TLE1 was initially identified as a TLX1-interacting protein by mass spectrometry (12). Mass spectrometry analysis was performed using a Kratos Axima CFR/Plus instrument equipped with Kompact software (Shimadzu Biotech, Columbia, MD) in reflectron mode. **(B)** Schematic diagram of TLX1 indicating: a 7 amino acid sequence (FGIDQIL) encompassing amino acids 19 to 25 exhibiting similarity to an Engrailed homology 1 (Eh1) motif (FXIXXIL, where X is any amino acid) that mediates interactions involving Gro/TLE corepressors (38); a 7 amino acid sequence (YGLGCLV) encompassing amino acids 45 to 51 representative of a consensus eukaryotic initiation factor 4E (eIF4E)-binding motif (YXXXXLΦ, where X is any amino acid and Φ is a hydrophobic residue) (33); and, the homeodomain (HD), which is deleted in the version of TLX1^{bio} used in these experiments (12).