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Inducible LAP-tagged Stable Cell Lines for Investigating Protein Function, Spatiotemporal Localization and Protein Interaction Networks

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Abstract

Multi-protein complexes, rather than single proteins acting in isolation, often govern molecular pathways regulating cellular homeostasis. Based on this principle, the purification of critical proteins required for the functioning of these pathways along with their native interacting partners has not only allowed the mapping of the protein constituents of these pathways, but has also provided a deeper understanding of how these proteins coordinate to regulate these pathways. Within this context, understanding a protein's spatiotemporal localization and its protein-protein interaction network can aid in defining its role within a pathway, as well as how its misregulation may lead to disease pathogenesis. To address this need, several approaches for protein purification such as tandem affinity purification (TAP) and localization and affinity purification (LAP) have been designed and used successfully. Nevertheless, in order to apply these approaches to pathway-scale proteomic analyses, these strategies must be supplemented with modern technological developments in cloning and mammalian stable cell line generation. Here, we describe a method for generating LAP-tagged human inducible stable cell lines for investigating protein subcellular localization and protein-protein interaction networks. This approach has been successfully applied to the dissection of multiple cellular pathways including cell division and is compatible with high-throughput proteomic analyses.

Video Link

The video component of this article can be found at http://www.jove.com/video/54870/

Introduction

To investigate the cellular function of an uncharacterized protein it is important to determine its *in vivo* spatiotemporal subcellular localization and its interacting protein partners. Traditionally, single and tandem epitope tags fused to the N or C-terminus of a protein of interest have been used to facilitate protein localization and protein interaction studies. For example, the tandem affinity purification (TAP) technology has enabled the isolation of native protein complexes, even those that are in low abundance, in both yeast and mammalian cell lines[1,2]. The localization and affinity purification (LAP) technology, is a more recent development that modifies the TAP procedure to include a localization component through the introduction of the green fluorescent protein (GFP) as one of the epitope tags[3]. This approach has given researchers a deeper understanding of a protein's subcellular localization in living cells while also retaining the ability to perform TAP complex purifications to map protein-protein interaction networks.

However, there are many issues associated with the use of TAP/LAP technologies that has hampered their widespread use in mammalian cells. For example, the length of time that is necessary to generate a stable cell line expressing a TAP/LAP tagged protein of interest; which typically relies on cloning the gene of interest into a viral vector and selecting single cell stable integrants with the desired expression level. Additionally, many cellular pathways are sensitive to constitutive protein overexpression (even at low levels) and can arrest cells or trigger cell death over time making the generation of a TAP/LAP stable cell line impossible. These and other constraints have impeded LAP/TAP methodologies from becoming high-throughput systems for protein localization and protein complex elucidation. Therefore, there has been considerable interest in the development of an inducible high-throughput LAP-tagging system for mammalian cells that takes advantage of current innovations in cloning and cell line technologies.

Here we present a protocol for generating stable cell lines with Doxycycline/Tetracycline (Dox/Tet) inducible LAP-tagged proteins of interest that applies advances in both cloning and mammalian cell line technologies. This approach streamlines the acquisition of data with regards to LAP-tagged protein subcellular localization, protein complex purification and identification of interacting proteins[4]. Although affinity proteomics utilizes a wide range of techniques for protein complex elucidation[5], our approach is beneficial for expediting the identification of these complexes and their native interaction networks and is amenable to high-throughput protein tagging that is necessary to investigate complex biological pathways that contain a multitude of protein constituents. Key to this approach are advancements in cloning strategies that enable high fidelity and expedited cloning of target genes into an array of vectors for gene expression *in vitro*, in various organisms like bacteria and baculovirus,

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and in mammalian cells[6,7]. Additionally, the ORFeome collaboration has cloned thousands of sequence validated open reading frames in vectors that incorporate these advances in cloning, which are available to the scientific community[8-11]. In our system, the pGLAP1 LAP-tagging vector enables the simultaneous cloning of a large number of clones, which facilitates high-throughput LAP-tagging. This expedited cloning procedure is coupled to a streamlined approach for generating cell lines with LAP-tagged genes of interest inserted at a single pre-determined genomic locus. This makes use of cell lines that contain a single flippase recognition target (FRT) site within their genome, which is the site of integration for LAP-tagged genes. These cell lines also express the tetracycline repressor (TetR) that binds to Tet operators ($TetO_2$) upstream of the LAP-tagged genes and silences their expression in the absence of Dox/Tet. This allows for Dox/Tet inducible expression of the LAP-tagged protein at any given time. Having the capability of inducible LAP-tagged protein expression is critical, since many cellular pathways are sensitive to the levels of critical proteins governing the pathway and can arrest cell growth or trigger cell death when these proteins are constitutively overexpressed, even at low levels, making the generation of non-inducible LAP-tagged stable cell lines impossible[12].

Protocol

NOTE: An overview of the generation of inducible LAP-tagged stable cell lines for any protein of interest is illustrated in **Figure 1** and the overview of LAP-tagged protein expression, purification and preparation for mass proteomic analyses is illustrated in **Figure 3**.

1. Cloning the Open Reading Frame (ORF) of the Gene of Interest into the LAP-tag Vector

1. Cloning the ORF of the Gene of Interest into the Shuttle Vector.
    1. Use polymerase chain reaction (PCR) to amplify the ORF of interest with the appropriate attB1 and attB2 sites within the primers for either N-terminal fusion or C-terminal fusion. See **Table 1** for primer sequences and **Table 2** for PCR conditions.
    2. Gel purify the PCR products by resolving them on a 1% agarose gel. Excise the amplified band that is the correct size from the gel and extract it from the gel slice using a DNA gel extraction kit as per manufacturer's instructions.
    3. Incubate the purified attB containing PCR products with an attP containing shuttle vector and the recombinase that allows the PCR products to recombine into the vector as per manufacturer's instructions (see M**aterials Table**).
    NOTE: Empty shuttle vectors and LAP-tagging vectors contain the *ccd*B gene and have to be propagated in *ccd*B resistant bacterial cells (see **Materials Table**). However the *ccdB* gene is recombined out when an ORF is inserted into these vectors, hence use standard DH5α *E. coli* cells when propagating vectors with cloned ORFs.
    4. Transform DH5α *E. coli* cells with 1 µl of the reaction product and plate the transformed cells onto a Luria broth (LB) agar plate with 50 mg/ml Kanamycin[13].
    5. Select the Kanamycin resistant colonies.
    6. Grow the selected colonies in LB media with 50 mg/ml Kanamycin, make a DNA mini-prep and confirm gene integration by DNA sequencing using the sequencing primers listed in **Table 3**.

2. Transferring the Gene of Interest from the Shuttle Vector into the LAP-tag Vector.
    1. Incubate the shuttle vector containing the sequence verified gene of interest ORF with the LAP-tag vector (pGLAP1 for N-terminal fusion) and the recombinase that mediates the transfer of the gene of interest from the shuttle vector into the LAP-tag vector as per manufacturer's instructions (see Materials).
    NOTE: A series of LAP/TAP vectors that can be used based on the desired promoter, epitope-tag, and N or C-terminal tagging can be found in **Table 4**.
    2. Transform DH5α *E. coli* cells with 1 µl of the reaction product and plate the transformed cells onto an LB agar plate with 100 mg/ml Ampicillin[13].
    3. Select the Ampicillin resistant colonies.
    4. Grow the selected colonies in LB media with 100 mg/ml Ampicillin, make a DNA mini-prep, and confirm gene integration by DNA sequencing using the sequencing primers listed in **Table 5**.

2. Generation of an Inducible Stable Cell Line that Expresses the LAP-tagged Gene of Interest

1. Select the cell line best suited for the project of interest. Alternatively, create a host cell line from any existing cell line that constitutively expresses the TetR and contains a FRT site that allows the LAP-tagged gene of interest to be stably integrated into the genome (see Materials).
NOTE: This protocol uses a HEK293 cell line that contains the TetR and an FRT site, which is grown in -Tet DMEM/F12 media [made with 10% fetal bovine serum (FBS) that is devoid of Tetracycline (-Tet)] [4].
2. Determine the minimum concentration of Hygromycin required to kill the host cell line within 1 to 2 weeks after drug addition. The concentration can vary between host cell lines, with most ranging between 100 µg/ml and 800 µg/ml.
NOTE: HEK293 cells grown in -Tet DMEM/F12 media with 100 µg/ml Hygromycin at 37 °C and 5% CO_2 will die within 1-2 weeks.
3. Co-transfect the vector that expresses the flippase recombinase (mediates the integration of the LAP-tagged gene of interest into the FRT site within the genome of the cell) with the LAP-tagged vector into HEK293 cells using a transfection reagent as per manufacturer's instructions. Use a ratio of 4:1 of recombinase vector to LAP vector[14].
NOTE: The optimal ratio is dependent on the host cell line and method of transfection, and may require a titration. A ratio of 4:1 works well for most cell lines. Include a mock-transfected plate as a negative control.
4. One day post-transfection, replace the -Tet DMEM/F12 media with fresh media.
5. Two days post-transfection, split cells to 25% confluence. Allow cells ~5 hr to attach, then add Hygromycin-containing -Tet DMEM/F12 media at the concentration predetermined in step 2.2. For HEK293 cells use 100 µg/ml Hygromycin.

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NOTE: The FRT containing HEK293 cell line also contains the TetR that is associated with Blasticidin resistance, therefore 5 µg/ml Blasticidin is used during the stable cell line selection process to select for the TetR and to minimize leaky expression.

6. Replace Hygromycin-containing -Tet DMEM/F12 media as needed until distinct cell foci appear that resemble opaque spots against the transparent plate.
7. Add 20 µl of trypsin on top of each cell foci and pipet up and down 2 times with a 200 µl pipet tip. Place cells in a 24 well plate and expand the cells by continual growth in Hygromycin-containing -Tet DMEM/F12 media.
8. Screen cells for inducible LAP-tagged protein expression by fixed-cell or live-cell fluorescence microscopy and/or Western blotting for the GFP tag within the LAP-tag[15].

3. Purification of LAP-tagged Protein Complexes

NOTE: The following LAP-tagged protein purification protocol details recommendations on conditions and volumes used for a typical LAP-tagged protein purification based on previous experience. However, caution should be exercised to ensure that empirical optimization is carried out for any protein complex and protein expression level of interest to provide the best results.

1. Cell Growth and Cell Harvesting.
    1. Expand the validated LAP-tagged cell line for TAP isolation of protein complexes, by continually passaging all HEK293 cells into larger plates and/or roller bottles in -Tet DMEM/F12 media at 37 °C and 5% CO_2.
    2. For Tet/Dox inducible cell lines, induce for 10-15 hr at a concentration of 0.2 µg/ml Tet/Dox when the cells reach ~70% confluency, before harvesting cells.
    NOTE: The concentration and induction time should be determined for each protein, a titration of 0.1-1 µg/ml Tet/Dox for 10-15 hr is recommended.
    3. Harvest cells by agitation or trypsinization and pellet cells at 875 x g for 5-10 min.

2. Coupling Anti-GFP Antibody to Protein A Beads
    1. Use 40 µg of antibody for immunoprecipitation from a lysate prepared from a 0.5 ml cell pellet, packed cell volume (PCV).
    NOTE: The amount of antibody needed will depend on the abundance of the LAP-tagged protein, among other factors, and will require optimization. A titration of 10-40 µg is recommended.
    2. Equilibrate 160 µl packed volume (PV) Protein A beads into PBST (PBS + 0.1% Tween-20) in a 1.5 ml tube. Wash 3 times with 1 ml of PBST.
    NOTE: All washes herein are carried out by centrifuging the beads at 5,000 x g for 10 sec.
    3. Resuspend beads in 500 µl PBST and add 80 µg of affinity-purified rabbit anti-GFP antibody to each tube of 160 µl beads. Mix for 1 hr at room temperature (RT).
    4. Wash beads 2 times with 1 ml of PBST. Then, wash beads 2 times with 1 ml of 0.2 M sodium borate, pH 9 (20 ml 0.2 M sodium borate + 15 ml 0.2 M boric acid). After the final wash, add 900 µl of the 0.2 M sodium borate, pH 9 to bring the final volume to 1 ml.
    5. Add 100 µl of 220 mM dimethylpimelimidate (DMP) to a final concentration of 20 mM. Rotate the tubes gently at RT for 30 min. For 220 mM DMP, resuspend contents of one 50 mg bottle in 877 µl of 0.2 M sodium borate, pH 9 and add immediately to the bead suspension.
    6. After incubation with DMP, wash beads 1 time with 1 ml of 0.2 M ethanolamine, 0.2 M NaCl pH 8.5 to inactivate the residual crosslinker. Resuspend beads in 1 ml of the same buffer and rotate for 1 hr at RT. Pellet beads and resuspend beads in 500 µl of 0.2 M ethanolamine, 0.2 M NaCl pH 8.5. Beads are stable for several months at 4 °C.

3. Preparation of Buffers for Cell Lysis and Complex Purification
    1. Prepare LAPX buffers (where X is the desired salt concentration [mM KCl] of the LAP buffer; 300 mM for cell lysis, 200 mM for most bead washes, and 100 mM for washing beads prior to eluting proteins) by making a pH 7.4 solution containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), X mM KCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM $MgCl_2$, and 10% glycerol.
    NOTE: The components of this buffer are used to approximate the environment in living cells. HEPES is used as a buffer in the pH rage of 7.2-8.2. KCl is a salt used to maintain the ionic strength of the buffer. EGTA is a chelating agent that binds calcium ions to reduce the levels of calcium compared to magnesium. Glycerol and $MgCl_2$ are used to improve the stability of proteins.
    2. Prepare $LAPX^N$ buffer by adding 0.05% nonyl phenoxypolyethoxylethanol to the LAPX buffer.
    NOTE: Nonyl phenoxypolyethoxylethanol is a mild detergent that solubilizes proteins, but preserves protein-protein interactions, thus a higher concentration is used during the extraction process and it is then lowered during the binding and washing steps.

4. Preparation of Cell Lysates
    1. Resuspend 500 µl of PCV into 2.5 ml of LAP300 with 0.5 mM dithiothreitol (DTT) and protease inhibitors. Add 90 µl of 10% nonyl phenoxypolyethoxylethanol (0.3% final) and mix by inversion.
    2. Place on ice for 10 min. Centrifuge at 21,000 x g for 10 min. Collect this low speed supernatant (LSS). Take a 10 µl sample of the LSS for gel analysis.
    3. Transfer LSS to a TLA100.3 tube and spin at 100,000 x g for 1 hr at 4 °C. Collect this high speed supernatant (HSS), in a tube and place on ice. Take a 10 µl sample of the HSS for gel analysis.
    NOTE: Avoid taking the top most lipid layer and the bottom most cell debris layer. The lipid layer should be removed by vacuum aspiration prior to collecting the HSS.

5. First Affinity Capture: Binding to Anti-GFP Beads
    1. Pre-elute antibody coupled beads (use 160 µl of beads per 0.5 ml cell pellet (PCV)) by washing them 3 times with 1 ml of elution buffer [3.5 M $MgCl_2$ with 20 mM Tris, pH 7.4] to get rid of uncoupled antibodies and reduce background. Do quickly. Do not leave beads in high salt for a long time.
    2. Wash beads 3 times with 1 ml of $LAP200^N$. Mix HSS extract with antibody beads for 1 hr at 4 °C. Centrifuge at 21,000 x g for 10 min. Take a 10 µl sample of the supernatant (*i.e.*, the flow through (FT)) for gel analysis.

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3. Wash beads 3 times with 1 ml of LAP200^N with 0.5 mM DTT and protease inhibitors. Wash beads 2 times (5 min each) with 1 ml of LAP200^N with 0.5 mM DTT and protease inhibitors. Wash quickly 2 times with 1 ml of LAP200^N with 0.5 mM DTT and no protease inhibitors before adding the tobacco etch virus (TEV) protease.

6. TEV Cleavage
    1. Dilute 10 µg TEV protease in 1 ml of LAP200^N and rotate tubes at 4 °C overnight.
       NOTE: This step can be optimized for any LAP-tagged protein to be completed in a few hours by adjusting the concentration of TEV protease, which can aid the preservation of LAP-tagged protein complexes.
    2. Pellet beads and transfer supernatant to a fresh tube. Rinse beads twice with 160 µl LAP200^N with 0.5 mM DTT and protease inhibitors (triple concentration) to remove any residual protein. Take a 10 µl sample of the supernatant for gel analysis.

7. Second Affinity Capture: Binding to S Protein Agarose
    1. Wash 1 tube of 80 µl S protein agarose slurry (40 µl packed resin) 3 times with 1 ml of LAP200^N.
       NOTE: S protein binding to the S-tag will reconstitute an active RNase and an alternative second epitope tag should be considered for RNA containing protein complexes.
    2. Add TEV eluted supernatant to S protein agarose beads and rock for 3 hr at 4 °C. Pellet beads and wash 3 times with 1 ml of LAP200^N with 0.5 mM DTT and protease inhibitors. Wash beads 2 times with 1 ml of LAP100.

8. Protein Elution
    1. Elute proteins off S protein agarose by adding 50 µl of 4x Laemmli sample buffer and heat at 97 °C for 10 min.
       NOTE: Proteins can also be eluted from the beads with elution buffer (3.5 M $MgCl_2$ with 20 mM Tris, pH 7.4).

4. Identify Interacting Proteins by Mass Spectrometry Analysis

1. Test the quality of the purification by analyzing the collected samples by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), silver staining the gel (see **Materials Table**) and by immunoblotting the eluates and probing with anti-GFP antibodies to ensure that the LAP-tagged purification worked[16], see **Figure 4**.
2. To identify stoichiometric and substoichiometric co-purifying species, take the final elution sample and separate it by SDS-PAGE. Stain the gel with a mass spectrometry compatible protein stain. Excise the most prominent bands and the space in between them from the gel and process them for analysis by mass spectrometry separately[16].
   NOTE: There are numerous approaches for separating the final purification eluates and preparing them for mass spectrometry[5]. For example, LAP-purified complexes can be eluted from S-protein beads by using high salt (3.5 M $MgCl_2$) and the entire protein population en masse can be analyzed by mass spectrometry[17]. Alternatively, final eluates can be separated for 1 mm by SDS-PAGE and a single 1 mm band can be excised and analyzed. This clears the complex mixture of any beads or particulate matter that will interfere with the analysis.

Representative Results

To highlight the utility of this system, the open reading frame (ORF) of the Tau microtubule binding protein was cloned into the shuttle vector by amplifying the Tau ORF with primers containing attB1 and attB2 sites (**Table 1**) and incubating the PCR products with the shuttle vector and a recombinase that mediates the insertion of the PCR products into the shuttle vector. The reaction products were used to transform DH5α bacteria[13] and plasmid DNA from Kanamycin resistant colonies was sequenced to ensure Tau insertion. A sequence validated shuttle-Tau vector was then used to transfer the Tau ORF into the pGLAP1 vector, which fused Tau in frame with the LAP (EGFP-TEV-S-Protein) tag, by incubating the shuttle-Tau vector with the pGLAP1 vector and the recombinase that mediates the transfer of the ORF from the shuttle vector to pGLAP1. The reaction products were used to transform DH5α bacteria[13] and plasmid DNA from Ampicillin resistant colonies was sequenced to ensure that the LAP-Tau fusion was in frame. Sequence validated pGLAP1-LAP-Tau was then co-transfected with a vector that expresses the flippase recombination enzyme into HEK293 cells that contained a single flippase recognition target (FRT) site within their genome, which is the site of integration for LAP-tagged genes[14]. This cell line also expressed the TetR that binds to Tet operators upstream of the LAP-tagged genes and silences their expression in the absence of Tet/Dox. Stable integrants were selected with -Tet DMEM/F12 media with 100 µg/ml Hygromycin for 5 days. Individual Hygromycin resistant cell foci were harvested by adding 20 µl of trypsin on top and pipetting up and down 2 times. Cells were placed in a 24 well plate and expanded by continual growth in -Tet DMEM/F12 media.

To verify that the Hygromycin resistant cells were capable of expressing LAP-Tau, HEK293 LAP-Tau cells were induced with 0.1 µg/ml Dox for 15 hr and protein extracts were prepared from non-induced and Dox-induced cells. These extracts were separated by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted for GFP and Tubulin as loading control. As seen in **Figure 4A**, LAP-Tau (visualized with anti-GFP antibodies) was only expressed in the presence of Dox. To validate that LAP-Tau was properly localized to the mitotic microtubule spindle during mitosis, as had been previously shown for endogenous Tau[18], HEK293 LAP-Tau cells were induced with 0.1 µg/ml Dox for 15 hr and cells were fixed with 4% paraformaldehyde and co-stained for DNA (Hoechst 33342) and microtubules (anti-Tubulin antibodies). Consistently, LAP-Tau was localized to the mitotic spindle during metaphase of mitosis (**Figure 4B**). To verify that LAP-Tau and its interacting proteins could be purified with this system, HEK293 LAP-Tau cells were grown in roller bottles to ~70% confluency, induced with 0.1 µg/ml Dox for 15 hr, harvested by agitation, lysed with LAP300 buffer, and LAP-Tau was purified using the above protocol. Eluates from the LAP-Tau purification were resolved by SDS-PAGE and the gel was silver stained. **Figure 4C** shows the LAP-Tau purification, marked with an asterisk is LAP-Tau and several other bands indicative of Tau interacting proteins can be seen.

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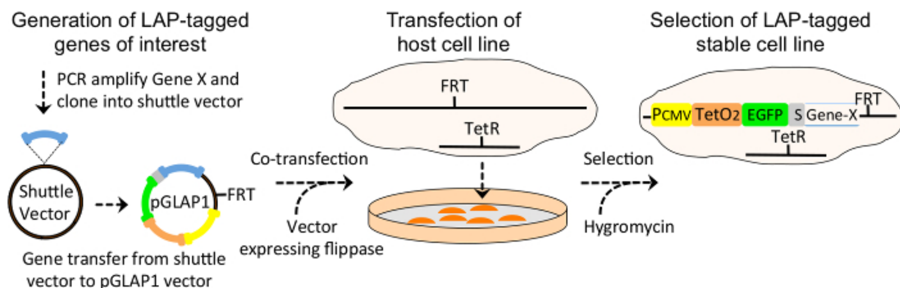

Figure 1: Overview of the Generation of LAP-tagged Inducible Stable Cell Lines for any Protein of Interest. The open reading frame (ORF) of genes of interest are amplified with attB1 and attB2 sites flanking the 5' and 3' end sequences, respectively (primer sequences are given in **Table 1**) and cloned into the shuttle vector. Sequence verified shuttle vectors with the gene of interest are then used to transfer the gene of interest into the pGLAP1 vector. The sequence verified pGLAP1 vector with the gene of interest is then co-transfected with the vector containing the flippase recombinase into the desired cell line that contains a single flippase recognition target (FRT) site within their genome, which is the site of integration for LAP-tagged genes. These cell lines also express the Tet repressor (TetR) that binds to Tet operators ($TetO_2$) upstream of the LAP-tagged genes and silences their expression in the absence of Tet/Dox. The LAP-tagged gene of interest is then recombined into the FRT site and stable integrants are selected with Hygromycin. Please click here to view a larger version of this figure.

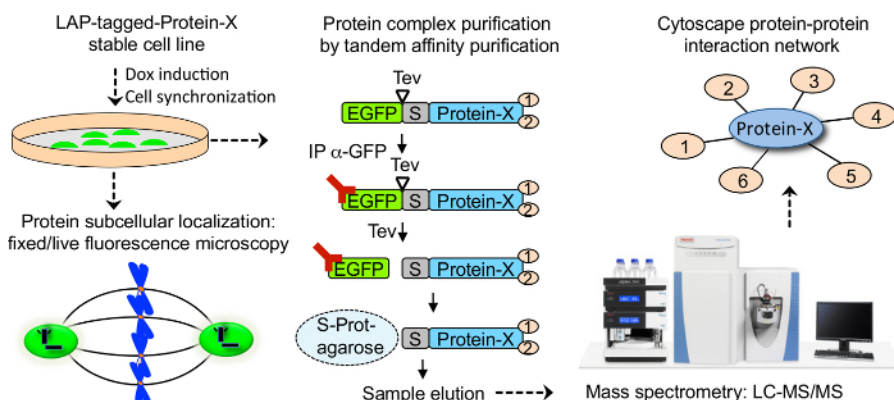

Figure 2: Overview of the Applications for LAP-tagged Stable Cell Lines. LAP-tagged inducible stable cell lines are induced to express the LAP-tagged protein of interest by Dox addition and can be synchronized at various stages of the cell cycle or can be stimulated with chemicals or ligands to activate any desired signaling pathway. The subcellular localization of the LAP-tagged protein of interest can be analyzed by live cell or fixed cell imaging. LAP-tagged proteins can also be tandem affinity purified and their interacting proteins can be identified by liquid chromatography tandem mass spectrometry (LC-MS/MS). Finally, Cytoscape can be used to generate a protein-protein interaction network of the bait protein. Dox indicates Doxycycline, IP indicates immunoprecipitate, EGFP indicates enhanced green fluorescent protein, Tev indicates TEV protease cleavage site, and S indicates S-tag. Please click here to view a larger version of this figure.

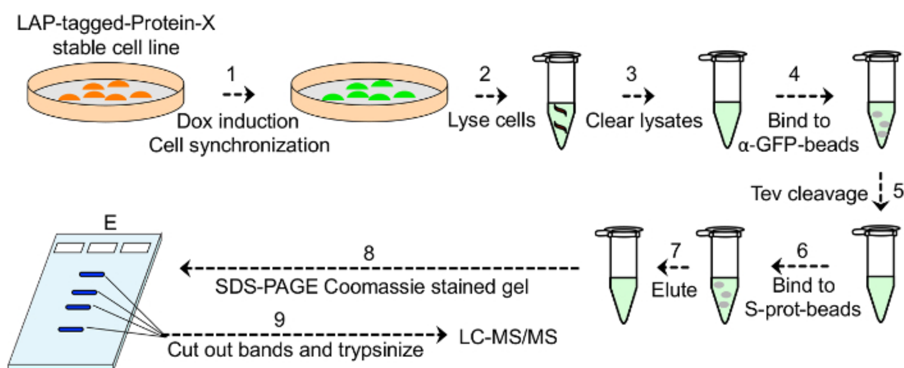

Figure 3: Overview of LAP-tagged Protein Expression, Purification and Preparation for Mass Spectrometry. The protocol has 9 steps: 1) growth and induction of LAP-tagged protein expression, 2) cell harvesting and lysis, 3) the preparation of lysates, 4) the binding of lysates to anti-GFP beads, 5) TEV protease cleavage of the GFP-tag, 6) the binding of lysates to S-protein beads, 7) the elution of the bait protein and interacting proteins, and 8-9) the preparation of samples for mass spectrometry-based proteomic analyses. Please click here to view a larger version of this figure.

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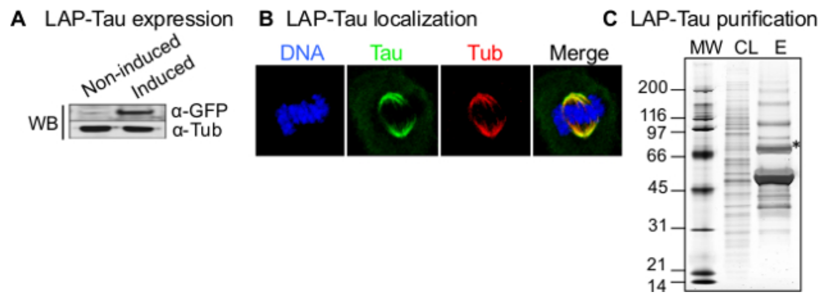

Figure 4: Verification of LAP-Tau expression. (**A**) Western blot (WB) analysis of protein samples from non-induced and Dox induced LAP-Tau HEK293 cells probed with anti-GFP and anti-Tubulin antibodies to detect the LAP-tagged Tau protein and the Tubulin loading control, respectively. Note that LAP-Tau is only expressed when the cells are induced with Dox. (**B**) Mitotic cells expressing LAP-Tau were fixed and co-stained for DNA (Hoechst 33342) and Tubulin (Tub) with anti-tubulin antibodies and the subcellular localization of LAP-tau was analyzed by fluorescence microcopy. Note that LAP-Tau localizes to the mitotic spindle and spindle poles during mitosis. (**C**) Silver stained gel of the LAP-Tau purification. MW indicates molecular weight, CL indicates cleared lysates, and E indicates final eluates. Samples were run on a 4-20% SDS-PAGE and the gel was silver stained to visualize the purified proteins. Note that a band corresponding to LAP-Tau is marked with an asterisk and several other bands corresponding to co-purifying proteins can be seen. Please click here to view a larger version of this figure.

N-terminal fusion	
Forward	5'-**GGGGACAAGT TTGTACAAAAAAGCAGGCT**TCATG-(>18gsn)-3'
Reverse	5'-**GGGGACCACTTTGTACAAGAAAGCTGGGT**T TTATCA-(>18gsn)-3'
C-terminal fusion	
Forward	5'-**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCACC-(>18gsn)-3'
Reverse	5'-**GGGGACCACTTTGTACAAGAAAGCTGGGT**G-(>18gsn)-3'

Table 1: Forward and Reverse Primers for Amplifying ORFs or Interest for Insertion into the Shuttle Vector. The attB sites are highlighted in bold letters, gsn denotes that more than 18 gene specific nucleotides are added to the primer.

Step		**Temperature**	**Time**
Initial denaturation		94 °C	2 min
PCR Amplification Cycles (35)	Denature	94 °C	30 sec
	Anneal	55 °C (depending on the primer Tm)	30 sec
	Extend	72 °C	1 min/kb
Hold		4 °C	indefinitely

Table 2: PCR Conditions for Amplification of the ORFs of Interest.

Vector	**Forward Sequencing Primer**	**Reverse Sequencing Primer**
Shuttle Vector	5'-TGTAAAACGACGGCCAGT-3'	5'-CAGGAAACAGCTATGAC-3'

Table 3: Forward and Reverse Sequencing Primers for the Shuttle Vector.

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ID	Structure	Parental	Promoter	Bac Res	Mam Res	Tet reg?
pGLAP1	N-term EGFP-TEV-S peptide	pcDNA5/FRT/TO	CMV	Amp	Hyg	Yes
pGLAP2	N-term Flag-TEV-S peptide	pcDNA5/FRT/TO	CMV	Amp	Hyg	Yes
pGLAP3	N-term EGFP-TEV-S peptide; C-term V5	pEF5/FRT-V5	EF1a	Amp	Hyg	No
pGLAP4	N-term Flag-TEV-S peptide; ; C-term V5	pEF5/FRT-V5	EF1a	Amp	Hyg	No
pGLAP5	C-term S peptide-PreProt x2-EGFP	pEF5/FRT-V5	EF1a	Amp	Hyg	No

Table 4: List of Available LAP/TAP Vectors with Variable Promoters, Epitope-tags, and Dox Inducible Expression Capabilities for N or C-terminal Protein Tagging. Vectors are commercially available. Bac Res indicates bacterial resistance marker, Mam Res indicates mammalian cell resistance marker, Tet reg? indicates whether expression is Tet/Dox regulatable.

Vector	Forward Sequencing Primer	Reverse Sequencing Primer
pGLAP1	5'-ATCACTCTCGGCATGGACGAGCTGTACAAG-3'	5'-TGGCTGGCAACTAGAAGGCACAGTCGAGGC-3'
pGLAP2	5'-CGAACGCCAGCACATGGACAGGG-3'	5'-TGGCTGGCAACTAGAAGGCACAGTCGAGGC-3'
pGLAP3	5'-AGAAACCGCTGCTGCTAA-3'	5'-TAGAAGGCACAGTCGAGG-3'
pGLAP4	5'-AGACCCAAGCTGGCTAGGTAAGC-3'	5'-TAGAAGGCACAGTCGAGG-3'
pGLAP5	5'-CGTAATACGACTCACTATAG-3'	5'-TCCAGGGTCAAGGAAGGCACGG-3'

Table 5: Forward and Reverse Sequencing Primers for pGLAP Vectors.

Discussion

The outlined protocol describes the cloning of genes of interest into the LAP-tagging vector, the generation of inducible LAP-tagged stable cell lines, and the purification of LAP-tagged protein complexes for proteomic analyses. With respect to other LAP/TAP-tagging approaches, this protocol has been streamlined to be compatible with high-throughput approaches to map protein localization and protein-protein interactions within any cellular pathway. This approach has been widely applied to the functional characterization of proteins critical for cell cycle progression, mitotic spindle assembly, spindle pole homeostasis, and ciliogenesis to name a few and has aided the understanding of how misregulation of these proteins can lead to human diseases[15,16,19,20]. For example, our group recently utilized this system to define the function and regulation of the STARD9 mitotic kinesin (a candidate cancer target) in spindle assembly[15,21], to define a new molecular link between the Tctex1d2 dynein light chain and short rib polydactyly syndromes (SRPS)[19], and to define a new molecular link to understanding how mutation of the Mid2 ubiquitin ligase can lead to X-linked intellectual disabilities[16]. Other laboratories have also successfully applied this method, including one that determined that Tctn1, a regulator of mouse Hedgehog signaling, was a part of a ciliopathy-associated protein complex that regulated ciliary membrane composition and ciliogenesis in a tissue-dependent manner[22,23]. Therefore, this protocol can be broadly applied to the dissection of any cellular pathway.

A critical step in this protocol is the selection of LAP-tagged stable cell lines that are Hygromycin resistant. Special care should be taken to ensure that all cells in the control plate are dead before selecting foci in the experimental plate for amplification. Hygromycin can also be added during routine cell culturing of LAP-tagged stable cell lines to further ensure that all cells maintain the LAP-tagged gene of interest at the FRT site. We caution that not all LAP-tagged proteins will be functional and that it is important to have assays in place that can be used to test protein function. Examples of assays used to test protein function include the rescue of siRNA-induced phenotypes and *in vitro* activity assays. To address any potential problems with the addition of a large LAP-tag, we have previously generated TAP-tag vectors compatible with this system that contain smaller tags, like FLAG, which are less likely to inhibit the function and localization of the protein of interest[4]. In addition, LAP-tagging vectors exist for generating C-terminal LAP-tagged proteins or C-terminal TAP-tagged proteins that are compatible with this system, which can be used in cases where a LAP/TAP tag is not tolerated at the N-terminus of a protein. Additionally, the salt and detergent concentrations of the purification buffers ($LAPX^N$) can be modified to increase or decrease the purification stringency if none or too many interactions are observed. Similarly, the tandem affinity purification procedure is more stringent than single purification procedures and weak interactors may be lost, thus a single purification scheme can be used when few or no interactors are identified.

It is important to note that other GFP epitope tagging approaches exist that allow large scale GFP protein tagging for protein localization and purification studies[24,25]. These include the BAC TransgenOmics approach that utilizes bacterial artificial chromosomes to express GFP-tagged genes of interest from their native environment that contains all the regulatory elements, which mimics endogenous gene expression[24]. More recently, CAS9/single-guided RNA (sgRNA) ribonucleoprotein complexes (RNPs) have been used to endogenously tag genes of interest with a split-GFP system that allows the expression of GFP-tagged genes from their endogenous genomic loci[25]. Although both of these approaches enable the expression of tagged proteins under endogenous conditions, compared to the LAP-tagging protocol described here, they do not allow for inducible and tunable expression of the tagged genes of interest. Additionally, they have yet to be applied to tandem epitope tagging for TAP.

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It is also important to note that other tagging systems can also be modified to become compatible with the system described here for generating inducible epitope-tagged stable cell lines. For example, proximity-dependent biotin identification (BioID) has garnered considerable attention due to its ability to define spatial and temporal relationships among interacting proteins[26]. This technique exploits protein fusions to a promiscuous strain of the *Escherichia coli* biotin ligase BirA, which biotinylates any protein within a ~10 nm radius of the enzyme. The biotinylated proteins are then affinity purified using biotin-affinity capture and analyzed for composition by mass spectrometry. BirA will biotinylate any protein in close proximity, even transiently, which makes it especially suited for detecting weaker interacting partners within a complex[27]. Additionally, the purification scheme does not necessitate that endogenous protein-protein interactions remain intact and can be carried out under denaturing conditions, thus reducing the rate of false positives. Within our current protocol, the substitution of the pGLAP1 vector by a BirA-tagging vector could transform this system from identifying protein-protein interactions based on affinity to detecting them based on proximity. Such a system would be highly advantageous for detecting transient protein interactions as is the case between many enzyme-substrate interactions and for mapping the spatiotemporal protein-protein interactions within defined structures as has been carried out for the centrosome and cilia[26,28].

Disclosures

The authors have nothing to disclose.

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References

1. Rigaut, G. *et al.* A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol.* **17**, 1030-1032 (1999).
2. Puig, O. *et al.* The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods.* **24**, 218-229 (2001).
3. Cheeseman, I. M., & Desai, A. A combined approach for the localization and tandem affinity purification of protein complexes from metazoans. *Sci STKE.* **2005**, pl1 (2005).
4. Torres, J. Z., Miller, J. J., & Jackson, P. K. High-throughput generation of tagged stable cell lines for proteomic analysis. *Proteomics.* **9**, 2888-2891 (2009).
5. LaCava, J. *et al.* Affinity proteomics to study endogenous protein complexes: pointers, pitfalls, preferences and perspectives. *Biotechniques.* **58**, 103-119 (2015).
6. Landy, A. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. *Annu Rev Biochem.* **58**, 913-949 (1989).
7. Hartley, J. L., Temple, G. F., & Brasch, M. A. DNA cloning using in vitro site-specific recombination. *Genome Res.* **10**, 1788-1795 (2000).
8. The ORFeome Collaboration: a genome-scale human ORF-clone resource. *Nat Methods.* **13**, 191-192 (2016).
9. Brasch, M. A., Hartley, J. L., & Vidal, M. ORFeome cloning and systems biology: standardized mass production of the parts from the parts-list. *Genome Res.* **14**, 2001-2009 (2004).
10. Lamesch, P. *et al.* hORFeome v3.1: a resource of human open reading frames representing over 10,000 human genes. *Genomics.* **89**, 307-315 (2007).
11. Rual, J. F. *et al.* Human ORFeome version 1.1: a platform for reverse proteomics. *Genome Res.* **14**, 2128-2135 (2004).
12. Fiering, S., Kim, C. G., Epner, E. M., & Groudine, M. An "in-out" strategy using gene targeting and FLP recombinase for the functional dissection of complex DNA regulatory elements: analysis of the beta-globin locus control region. *Proc Natl Acad Sci U S A.* **90**, 8469-8473 (1993).
13. Sambrook, J., Fritsch, E. F., & Maniatis, T. *Molecular cloning : a laboratory manual.* 2nd ed. edn, Cold Spring Harbor Laboratory, (1989).
14. Uyttersprot, N., Costagliola, S., & Miot, F. A new tool for efficient transfection of dog and human thyrocytes in primary culture. *Mol Cell Endocrinol.* **142**, 35-39 (1998).
15. Senese, S. *et al.* A unique insertion in STARD9's motor domain regulates its stability. *Mol Biol Cell.* **26**, 440-452 (2015).
16. Gholkar, A. A. *et al.* The X-Linked-Intellectual-Disability-Associated Ubiquitin Ligase Mid2 Interacts with Astrin and Regulates Astrin Levels to Promote Cell Division. *Cell Rep.* **14**, 180-188 (2016).
17. Graumann, J. *et al.* Applicability of tandem affinity purification MudPIT to pathway proteomics in yeast. *Mol Cell Proteomics.* **3**, 226-237 (2004).
18. Connolly, J. A., Kalnins, V. I., Cleveland, D. W., & Kirschner, M. W. Immunoflourescent staining of cytoplasmic and spindle microtubules in mouse fibroblasts with antibody to tau protein. *Proc Natl Acad Sci U S A.* **74**, 2437-2440 (1977).
19. Gholkar, A. A. *et al.* Tctex1d2 associates with short-rib polydactyly syndrome proteins and is required for ciliogenesis. *Cell Cycle.* **14**, 1116-1125 (2015).
20. Cheung, K. *et al.* Proteomic Analysis of the Mammalian Katanin Family of Microtubule-severing Enzymes Defines Katanin p80 subunit B-like 1 (KATNBL1) as a Regulator of Mammalian Katanin Microtubule-severing. *Mol Cell Proteomics.* **15**, 1658-1669 (2016).
21. Torres, J. Z. *et al.* The STARD9/Kif16a Kinesin Associates with Mitotic Microtubules and Regulates Spindle Pole Assembly. *Cell.* **147**, 1309-1323 (2011).
22. Garcia-Gonzalo, F. R. *et al.* A transition zone complex regulates mammalian ciliogenesis and ciliary membrane composition. *Nat Genet.* **43**, 776-784 (2011).
23. Chih, B. *et al.* A ciliopathy complex at the transition zone protects the cilia as a privileged membrane domain. *Nat Cell Biol.* **14**, 61-72 (2012).
24. Leonetti, M. D., Sekine, S., Kamiyama, D., Weissman, J. S., & Huang, B. A scalable strategy for high-throughput GFP tagging of endogenous human proteins. *Proc Natl Acad Sci U S A.* **113**, E3501-3508 (2016).

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25. Poser, I. *et al.* BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals. *Nat Methods.* **5**, 409-415 (2008).
26. Firat-Karalar, E. N., & Stearns, T. Probing mammalian centrosome structure using BioID proximity-dependent biotinylation. *Methods Cell Biol.* **129**, 153-170 (2015).
27. Roux, K. J., Kim, D. I., & Burke, B. BioID: a screen for protein-protein interactions. *Curr Protoc Protein Sci.* **74**, Unit 19 23 (2013).
28. Gupta, G. D. *et al.* A Dynamic Protein Interaction Landscape of the Human Centrosome-Cilium Interface. *Cell.* **163**, 1484-1499 (2015).

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