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Knocking out multiple genes in cultured primary neurons to study tubulin posttranslational modifications

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Running head: Knocking out multiple genes in neurons to study tubulin PTMs

Abstract:

Microtubules, as integral part of the eukaryotic cytoskeleton, exert numerous essential functions in cells. A mechanism to control these diverse functions are the posttranslational modifications of tubulin. Despite known for decades, relatively little insight into the cellular functions of these modifications has been gained so far. The discovery of tubulin-modifying enzymes and a growing number of available knockout mice now allows working with primary cells from those mouse models to address biological functions and molecular mechanisms behind those modifications. However, a number of those mouse models show either lethality or sterility, making it difficult to impossible to obtain a sufficient number of animals for a systematic study with primary cells. Moreover, many of those modifications are controlled by several redundant enzymes, and it is often necessary to knock out several enzymes in parallel to obtain a significant change in a given tubulin modification. Here we describe a method to generate primary cells with combinatorial knockout genotypes using conditional knockout mice. The conditional alleles are converted into knockout in the cultured primary cells by transduction with a lentivirus encoding cre-recombinase. This approach has allowed us to knock out the two main brain deglutamylases in mouse primary neurons, which leads to strongly increased polyglutamylation in these cells. Our method can be applied to measure different cellular processes, such as axonal transport, for which it can be combined with the expression of different fluorescent reporters to label intracellular proteins. Using a panel of conditional knockout mice, our method can further be applied to study the functions of a variety of tubulin modifications that require simultaneous knockout of multiple genes.

Key words:

Primary cells, primary hippocampal neurons, microtubules, tubulin posttranslational modifications, polyglutamylation, mouse models.

1. Introduction

Microtubules, together with actin and intermediate filaments constitute the cytoskeleton of each eukaryotic cell. Microtubules assemble from evolutionarily conserved α - and β -tubulin heterodimers in a highly dynamic manner, and are involved in a plethora of cellular processes: they build meiotic and mitotic spindles during cell division, drive directed cell migration, determine cell shape, and serve as tracks for intracellular transport. Microtubules are particularly crucial in neurons, where they play a pivotal role in determination of neuronal polarity, dendritic arborization, axon branching and in the transport of cellular cargoes from the cell body to synaptic terminals and *vice versa* [1,2]. A key question is how microtubules are able to adapt their properties to participate in those highly diverse events. A mechanism to control microtubule properties that could potentially regulate these different functions are tubulin posttranslational modifications (PTMs), such as acetylation, detyrosination or polyglutamylation, which are all highly enriched in neurons [3].

Though the presence of multiple PTMs on brain tubulin was identified decades ago, their biochemical and functional characterisation became possible only after the enzymes catalysing them had been discovered (reviewed in [4]). Overexpression or knockdown of those enzymes allowed performing experiments at the cellular level thus providing first insights into the functions of tubulin PTMs (reviewed in [5,6]). In parallel, the development of a variety of mouse models lacking modifying enzymes delivered novel insights into the physiological roles of tubulin PTMs [7-15]. Approaches allowing to combine these mouse models with cell-based assays can bolster the discovery of underlying mechanisms in physiologically relevant cell types. For instance, culturing primary neurons from mouse models with altered modification levels has shed light on the role of tubulin PTMs in several neuronal functions [11,16,7,8,17,18].

Culturing primary cells with altered modification levels can be straightforward for some mouse models, and very difficult for others. Mice with no sterility phenotypes can be obtained from homozygous breeding pairs, which is the case for the two enzymes involved in tubulin acetylation, α TAT1/Mec-17 [19,20] and HDAC6 [21]. Sterile mouse strains, or mice with perinatal lethality, such as the knockout for tubulin tyrosine ligase (TTL) [11] need to be bred from heterozygous progenitors, which subsequently requires single-embryo cell cultures followed by genotyping. More difficult still are the combinatorial knockout strains, in particular with one or more of the transgenes leading to sterility. These are almost impossible to use for generating sufficient amounts of cells, as the breeding schemes are very complex,

and the relevant offspring is statistically too rare to perform systematic cell culture studies. Here we propose a method to overcome this problem, which we have developed to study the role of tubulin polyglutamylation in neurons.

Polyglutamylation is generated by multiple enzymes belonging to the family of tubulin tyrosine ligase like proteins (TTL) [22,23] and removed by enzymes from the cytosolic carboxy peptidase (CCP) family [10,24]. Several of these enzymes are involved in controlling polyglutamylation levels in neurons: TTL1 and TTL7 are the major glutamylases [8,22], and CCP1 and CCP6 are the main deglutamylases [10,7]. To strongly reduce polyglutamylation in the brain, both TTL1 and TTL7 need to be depleted concomitantly, while to increase polyglutamylation, the inactivation of CCP1 and CCP6 is required in at least some parts of the brain, such as hippocampus [7]. In both cases, one of the two knockout mouse strains (CCP1 and TTL1) is male sterile, and mice can thus only be bred heterozygous, which strongly reduces the probability of generating double-knockout mice.

To establish a reliable and sensitive cell biological approach for studying the impact of polyglutamylation on neuronal functions, we developed a cell-culture based method to achieve increased polyglutamylation in primary hippocampal neurons, which can be easily extended to study the effects of increased or decreased PTMs other than polyglutamylation.

There are two possible strategies allowing to obtain primary neurons with perturbed tubulin PTM levels: overexpressing or silencing the corresponding enzyme(s). To obtain increased polyglutamylation levels one can overexpress glutamylating enzymes, or deplete the main deglutamylating enzymes, either by RNA interference (RNAi) or by gene knockout.

Overexpression of glutamylases has many drawbacks, the most important being that the amount of enzyme is far from being physiologically relevant, and could thus result in artefacts. For instance, all glutamylases overexpressed in HeLa cells promiscuously glutamylated α - and β -tubulin, while normally these enzymes are specific to either α - or β -tubulin [23]. Moreover, in neurons, the most relevant enzyme is the glutamylase TTL1, which could so far not be expressed as an active enzyme in cells due to the necessity to form a complex with at least four other proteins [22]. Finally, some tubulin-modifying enzymes have been observed to bind microtubules when overexpressed (our unpublished observations), which might alter microtubule dynamics and could impair the movement of organelles on the microtubules, independently of the PTM generated by the enzyme.

We thus strongly favour the strategy to remove the major deglutamylating enzymes, which in neurons are CCP1 and CCP6 [10,7]. We decided against RNAi, as it rarely generates a complete loss of the target transcript, which in the case of the low-expressing tubulin-modifying enzymes would be very hard to control. Thus, our method of choice was to culture primary neurons from knockout mouse strains. The breeding scheme we developed to obtain *ccp1^{-/-}ccp6^{-/-}* animals was very difficult for a number of reasons, such as the male infertility of *ccp1^{-/-}* mice and a general lack of fitness of the double-knockout mice (which is why we used *Ccp6^{flox/flox} nestin-cre* to breed the double-knockout animals [7]). The theoretical turnout of 1/16 of this breeding scheme was not appropriate for systematic studies using primary neuronal cultures, therefore we generated a mouse model in which both, *CCP1* and *CCP6*, can be conditionally knocked out (*Ccp1^{flox/flox}Ccp6^{flox/flox}*). These mice behave like wild type and thus can be easily bred to give homozygous embryos, which are the source of primary hippocampal neurons. Once neurons are cultured, they are transduced with a lentivirus expressing cre recombinase to convert both flox alleles into knockout alleles, leading to the removal of deglutamylases, which results in tubulin hyperglutamylation. In parallel, the neurons from the same culture are transduced with a control virus, and can be used as an internal control with normal polyglutamylation levels. To verify the effect of the cre recombinase alone, the equivalent experiment is performed using neurons from wild type embryos transduced with the same GFP- and GFP-cre viruses.

Thus, we describe a novel, physiologically relevant cell-based assay to attain excessive polyglutamylation and its possible applications. Our cell-culture based method is not limited to studying microtubules or neurons, but has broader applications in the field of cell biology. As far as microtubule PTMs are concerned, our method can be expanded to study the impact of reduced polyglutamylation (using *Ttll1^{flox/flox}Ttll7^{flox/flox}* neurons) or reduced detyrosination (with *Vash1^{flox/flox}Vash2^{flox/flox}* neurons), all of which require the depletion of several genes.

2. Materials

2.1 Cloning of Cre-recombinase or Reporter Genes into Lentiviral Vector

1. pTRIP vector with CMV promoter, pTRIP-CMV-GFP-2A (modified from Addgene #102611)
2. Template for PCR reaction (brain cDNA or plasmid containing gene of interest)
3. KAPA HiFi HotStart 2× ReadyMix (Kapa Biosystems)
4. Specific primers
NLS-cre-FS-2A: 5'- tccactagtgtcgacATGCCCAAGAAGAAGAGGAAGGTG-3'
NLS-cre-FS-2A: 5'- catgttttcttaggtTAATCGCCATCTTCCAGCAGGC-3'
(See **Note 1**)
5. PCR machine
6. DNase/RNase-Free Distilled Water
7. T4 DNA polymerase
8. XhoI
9. NheI
10. Buffer 2.1 (New England BioLabs)
11. 1× Tris-Borate-EDTA (TBE) buffer: add 100 ml of 10× TBE to 900 ml of distilled water.
12. 0.8% Agarose gel: add 4 g of agarose and 300 ml of 1× TBE in a 500-ml glass bottle. Heat the mix in the microwave oven until the agarose is dissolved and make up the volume to 500 ml with 1× TBE. Add 20 µl of 10 mg/ml Ethidium Bromide solution and store in incubator set at 55°C for not more than two weeks. Prepare the gel tray and comb of desired size, pour the agarose and let polymerize at room temperature for at least 15 min.
13. 1 kb DNA Ladder
14. Gel extraction kit
15. 1.5-ml tubes
16. Chemically competent *E. Coli* strain Dh5alpha
17. Bacterial incubator at 37°C.
18. LB-agar plates containing 100 µg/ml of Ampicillin
19. LB medium without antibiotic used for recovery of bacteria
20. LB medium with 100 µg/ml of ampicillin
21. 14-ml round-bottom tubes

22. Bacterial incubator with shaking set at 37°C.

2.2 *Lentivirus Production*

1. X-Lenti 293T (Takara) (see **Note 2**).
2. Cell culture incubator at 37°C with 5% CO₂.
3. Tissue culture treated 75-cm² flasks.
4. Phosphate-buffered saline (PBS).
5. Trypsin.
6. Plain medium: Dulbecco's Modified Eagle Media (DMEM) without any supplements.
7. Complete culture medium: Supplement DMEM with a final concentration of 10% FBS (vol/vol), 200 mM L-Glutamine, and 1× Penicillin-Streptomycin. Store at 4°C.
8. Tissue culture treated 6-well plates.
9. Tissue culture treated 24-well plates.
10. Transfection reagent: TransIT-293 (Mirus Bio LLC) (see **Note 3**).
11. Expression and packaging plasmids: pTRIP lentiviral vector containing gene of interest under the CMV or CMV-enhanced chicken beta-actin (CAG) promoter and packaging plasmids including psPAX2 (a gift from D. Trono, Addgene plasmid #12260) and pCMV-VSVG (a gift from B. Weinberg, Addgene plasmid #8454). (See **Note 4**).
12. Virus collection medium: Neurobasal medium-PS: supplement Neurobasal medium with a final concentration of 1× Penicillin-Streptomycin. Store at 4°C. (See **Note 5**).
13. 15-ml and 50-ml screw-cap tubes
14. 0.45 µm filters and 20-ml syringes
15. 1.5- and 2-ml tubes

2.3 *Mouse Primary Hippocampal Neuron Cultures and Lentiviral Transduction*

1. DNase/RNase-Free Distilled Water
2. 1 mg/ml poly-D-Lysine: add 50 ml of DNase/RNase-Free distilled water to 50 mg of poly-D-Lysine bottle. Aliquot by 5 ml or 10 ml in sterile 15-ml tubes and store at -20°C.
3. Autoclaved distilled water
4. Tissue culture treated 75-cm² flasks
5. Dissection medium: add 10 ml of 10× HBSS and 1 ml of 1 M HEPES solution to 89 ml of DNase/RNase-Free distilled water.
6. Dissection tools
7. Tissue culture-treated 100-mm dishes

8. Tissue culture-treated 60-mm dishes
9. 15-ml screw-cap tubes
10. 10× Trypsin (2.5%), no phenol red. Aliquot by 500 µl in 1.5-ml tubes and store at -20°C. Avoid repeated freeze-thaw cycles.
11. Plating medium: add 5 ml of filter sterilized heat inactivated horse serum, 1.3 ml of glucose and 1 ml of 100× Penicillin-Streptomycin to 93.7 ml of Minimum Essential Medium. Prepare fresh before use.
12. 70-µm cell strainers
13. KOVA™ Glasstic™ Slide 10 with Grids (Fisher Scientific)
14. 35-mm glass bottom dishes
15. Tissue culture treated 6-well plates
16. Tissue culture treated 24-well plates
17. 12-mm, No.1.5H, round cover slips
18. Tissue culture treated 12-well plates
19. B27 supplement (Thermo Fisher). Aliquot by 1 ml in 1.5-ml tubes and store at -20°C. Avoid repeated cycles of freeze-thawing.
20. Neurobasal-PS: supplement Neurobasal medium with a final concentration of 1X Penicillin-Streptomycin. Store at 4°C.
21. B27 medium: add 2 ml of 50× B27 solution and 1 ml of Glutamax™ supplement to 97 ml of neurobasal medium-PS. Prepare fresh for each experiment.
22. 2× B27 medium: add 2 ml of 50× B27 solution and 1 ml of Glutamax™ to 47 ml of neurobasal-PS medium. Prepare fresh for each experiment.

2.4 Genotyping

1. 2% Agarose: add 10 g of agarose and 300 ml of 1× TBE in a 500-ml glass bottle. Heat the mix in the microwave oven until the agarose is dissolved and make up the volume to 500 ml with 1× TBE. Add 20 µl of 10 mg/ml Ethidium Bromide solution and store in incubator set at 55°C for not more than two weeks. Prepare the gel tray and comb of desired size, pour the agarose and let polymerize at room temperature for at least 15 min.
2. 100 bp DNA Ladder.
3. 10 mg/ml Proteinase K: Dissolve 100 mg of proteinase K in 10 ml of autoclaved distilled water. Aliquot and store at -20°C indefinitely.
4. 1 M Tris-HCl pH 8: dissolve 12.1 g of Tris in 100 ml autoclaved distilled water and adjust to pH 8 with HCl. Filter sterilize and store at room temperature.

5. 5 M NaCl: dissolve 29.2 g of NaCl in 100 ml autoclaved distilled water. Filter sterilize and store at room temperature.
6. 0.5 M EDTA pH 8: dissolve 14.6 g of EDTA in 100 ml autoclaved distilled water and adjust to pH 8 with KOH. Filter sterilize and store at room temperature.
7. Tail lysis buffer: 0.1 M Tris-HCl pH 8, 0.2 M NaCl, 5 mM EDTA and 0.4% SDS. Mix 50 ml of 1 M Tris-HCl pH 8, 20 ml of 5 M NaCl, 5 ml of 0.5 M EDTA pH 8 and 10 ml of 20% SDS and complete the volume to 500 ml with distilled water.
8. Specific primers.
9. For CCP1, primer mix 1: mix primers (5'-TTAAGCAGTGGCTGCCGGAGTGC-3') and (5'-GTCTACAGCCACGTGCTCAGCAAAGG-3') at 1:1 and primer mix 2: Mix primers (5'-GGTCATCTTGTAACAGTAGGGAAATCAAGCC-3') and (5'-GGAAAGCATAGCATCATGTAAAACCTCCCTCC-3') at 1:1.
10. For CCP6, primer mix 1: mix primers (5'-GAATGGCAATGAGATCACCCTCTCCAGC-3') and (5'-CTGTTGGGTGTCTGAGGCAAACACTTCC-3') at 1:1 and primer mix 2: Mix primers (5'-AGGTGTTTCAGTGTAAGGAATCTGGTCCC-3') and (5'-CTGTTGGGTGTCTGAGGCAAACACTTCC-3') at 1:1.
11. GoTaq[®] DNA polymerase (Promega), use according to manufacturer's instructions.
12. Electrophoresis cell: Mini-PROTEAN[®] cell with a PowerPac[™] power supply (Bio-Rad).

2.5 *SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting*

1. 5× Laemmli sample buffer: 400 mM Tris-HCl pH 6.8, 450 mM DTT, 10% (wt/vol) SDS and 50% (vol/vol) glycerol. Preheat 16 ml of 1 M Tris-HCl pH 6.8 to app. 40°C, add 2.6 g of DTT and 4 g of SDS. Stir the solution gently using a magnetic stirrer and add 20 ml of 100% glycerol. Continue stirring until the solution is homogenous and add a pinch of Bromophenol blue to reach the desired intensity of colour. Aliquot and store at -20°C indefinitely.
2. Acrylamide/bis-acrylamide stock solution: 40% Acrylamide supplemented with 1.04% (wt/vol) Bis N,N'-Methylene-Bis-Acrylamide. Store at 4°C for up to 6 months.
3. 4× resolving gel buffer: 1.5 M Tris-HCl pH 8.8 and 0.4% SDS. Dissolve 36.3 g of Tris in autoclaved distilled water and adjust to pH 8.8 with HCl for a final volume of 396 ml. Then, add 4 ml of 20% SDS stock solution. Filter-sterilize and store at room temperature indefinitely. (See **Note 6**).

4. 4× stacking gel buffer: 0.5 M Tris-HCl pH 6.8 and 0.4% SDS. Dissolve 12.1 g of Tris in autoclaved distilled water and adjust to pH 6.8 with HCl for a final volume of 196 ml. Then, add 4 ml of 20% SDS stock solution. Filter-sterilize and store at room temperature indefinitely.
5. N, N, N', N'-Tetramethylethylenediamine (TEMED).
6. 10% Ammonium persulfate (APS): Dissolve 1 g of APS in 10 ml of autoclaved distilled water. Aliquot and store at -20°C indefinitely.
7. SDS-PAGE electrophoresis equipment (Bio-Rad)
8. Electrophoresis cell: Mini-PROTEAN® cell with a PowerPac™ power supply
9. Gel running buffer: 50 mM Tris-HCl, 384 mM glycine and 0.1% SDS. Dissolve 6 g of Tris and 28.75 g of glycine in autoclaved distilled water for a final volume of 995 ml. Then, add 5 ml of 20% SDS stock solution.
10. Trans-Blot® Turbo™ Mini Nitrocellulose Transfer Packs (Biorad)
11. Trans-Blot® Turbo™ Transfer System (Biorad)
12. Tris-buffered saline – Tween 20 (TBST) solution: 20 mM Tris-HCl pH 7.4, 140 mM NaCl and 0.1% Tween 20. Dissolve 2.42 g of Tris and 8.18 g of NaCl in autoclaved distilled water and adjust to pH 7.4 with HCl for a final volume of 999 ml. Add 1 ml of Tween 20 to the buffer and mix well. (See **Note 7**).
13. TBST containing 5% (wt/vol) fat-free dry milk: Add 0.5 g of milk to 10 ml TBST.
14. Antibodies: 12G10, anti- α -tubulin antibody (used at 1/500 (vol/vol), developed by J. Frankel and M. Nelson, obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by the University of Iowa). PolyE, anti-polyglutamylated tubulin antibody (used at 1/20,000 (vol/vol); AdipoGen). Anti-GFP antibody (used at 1/5,000 (vol/vol); Acris Antibodies).
15. Clarity™ Western ECL Substrate (Biorad)
16. Western blot developer or imager

3. Methods

Here, we describe a method to obtain primary neurons in which we can induce the knockout of tubulin-modifying enzymes thus obtaining strong alterations in the levels of specific tubulin PTMs. As an example, we describe the protocol to generate hyperglutamylation in primary hippocampal neurons, which can be used to study the role of polyglutamylation in several neuronal functions, such as axonal outgrowth and branching, neuronal connectivity, or axonal transport.

Briefly, we culture hippocampal neurons from *Ccp1^{lox/lox}Ccp6^{lox/lox}* E17.5 (embryonic day 17.5) mouse embryos and transduce them either with a GFP- or GFP-2A-*cre*-expressing lentivirus at DIV 0 (days in vitro). The use of lentivirus-mediated gene transfer allows a nearly complete transduction of all cells in the culture dish, and appears to not exert additional stress on the neurons, as we observe no neuronal death.

Cre-recombinase was cloned into the pTRIP vector under the CMV or the CMV-enhanced chicken beta-actin (CAG) promoter using one step sequence and ligation independent cloning (SLIC) method [26]. Considering the possibility that direct GFP-tagging of Cre protein might affect its recombinase activity, we cloned Cre behind a self-cleavable 2A peptide sequence [27] present in frame with the GFP protein sequence in the vector (**Fig. 1a**). In cells, the cre-recombinase is cleaved from GFP, which we confirmed by immunoblotting (**Fig. 2b**).

Transduction of *Ccp1^{lox/lox}Ccp6^{lox/lox}* neurons with GFP-2A-cre resulted in ‘defloxing’ of both alleles of the two genes (**Fig. 4a**), and a concomitant increase in tubulin polyglutamylation (**Fig. 4b**). In parallel, wild type neurons were transduced with the same GFP- and GFP-cre viruses to confirm that the cre-recombinase alone has no effect on polyglutamylation levels (**Fig. 4c**).

Thus, obtained neurons can be used to determine the impact of increased polyglutamylation on different neuronal processes including neuronal differentiation and intracellular transport, by staining organelles using commercially available dyes such as Mitotracker and Lysotracker. Furthermore, this protocol combined with lentivirus-based expression of reporter genes can extend the applications of this method to study the trafficking of other vesicles such as endosomes (Rab5, Rab7 and LAMP1), BDNF vesicles (BDNF), autophagosomes (LC3), or synaptic vesicles (Synaptotagmin, Synaptophysin). Alternatively, the system can be used to study microtubule dynamics by the use of end-binding protein- EB3 fused to GFP.

3.1 Cloning of Cre-Recombinase and Reporter Genes into a Lentiviral Vector

3.1.1. Cloning of Cre-recombinase into the pTRIP vector - generation of the pTRIP-CMV-GFP-2A-Cre plasmid (Fig. 1).

1. Linearize the pTRIP vector by restriction digestion with XhoI (Fig. 1a). Digest 3 µg of plasmid DNA for ~2.5 h with 0.5 µl of XhoI enzyme (20 U/µl) in a 50 µl reaction. Run the whole sample on a 0.8% agarose gel until the dye front runs out. (See **Note 8**)
2. Purify the digested vector from the gel using the gel purification kit. Elute the vector DNA in 50 µl of elution buffer.
3. Amplify the cre-recombinase gene using the primers containing at least 15 bp of homology to the ends of the vector. In a PCR tube, mix 25 µl of 2× KAPA ready mix, 0.2 µl of each primer (100 µM), PCR substrate and make up the volume to 50 µl with DNase/RNase-Free distilled water and perform the PCR reaction. Use the following PCR conditions: an initial step of denaturation at 95°C for 3 min; 15 cycles of amplification (95°C for 30 sec, annealing of primers at 65°C for 30 sec, and extension at 72°C for ~1.5 min). (See **Note 9**).
4. Run the whole sample on a 0.8% agarose gel and purify the PCR product from the gel using the gel purification kit. Elute the PCR product in 40 µl of elution buffer.
5. Mix 7 µl of insert or elution buffer (negative control), 2 µl of digested vector and 1 µl of buffer 2.1 in to a 1.5-ml tube, centrifuge briefly and place on ice (Fig. 1b).
6. Add 0.3 µl of T4 DNA polymerase (3 U/µl) to each tube and mix by firmly tapping the tube (Fig. 1b).
7. Centrifuge briefly and leave the reaction mix at room temperature for 2.5 min to generate 5' over-hangs (Fig. 1b). (See **Note 10**).
8. After the incubation, immediately transfer the tubes on ice to stop the activity of T4 DNA polymerase and leave it for ~5 min to facilitate the annealing of single-stranded over-hangs (Fig. 1b).
9. Add 100 µl of chemically competent *E. coli* to the DNA reaction and mix by gentle tapping at the bottom of the tube.
10. Leave the *E. coli* with the reaction mix on ice for 30 min, provide heat shock at 42°C for 45 s, bring back the tube on ice for 10 min. Add 1 ml of LB medium without antibiotics and let bacteria recover at 37°C for at least 1 h.
11. After the incubation, pellet the *E. coli* cells at room temperature, 1,000 RCF for 3 min, resuspend in ~100 µl of LB medium, plate the entire cell suspension on a LB-agar-ampicillin plate and incubate at 37°C for 16 h.

12. Next day, inoculate 5 to 10 colonies, each in a 14-ml round bottom tube with ~ 5 ml of LB medium containing ampicillin. (See **Note 11**).
13. Extract the plasmid DNA and test the clones by restriction digestion with appropriate enzymes. Finally, verify the positive clones by sequencing.

3.1.2. Cloning of reporter genes into pTRIP lentivectors - generation of the pTRIP-CMV-reporter-GFP-2A and pTRIP-CMV-reporter-GFP-2A-Cre plasmids.

1. Linearize pTRIP-CMV-GFP-2A and pTRIP-CMV-GFP-2A-Cre lentivectors by restriction digestion with NheI (Fig. 1a). Digest 3 µg of plasmid DNA for ~2.5 h with 0.5 µl of NheI enzyme (20 U/µl) in a 50-µl reaction. Gel-purify the digested vector in 50 µl of elution buffer. (See **Note 12**).
2. Amplify reporter gene using the primers containing at least 15 bp of homology to the ends of the vector. In a PCR tube, mix 25 µl of 2× KAPA ready mix, 0.2 µl of each primer (100 µM), PCR substrate and make up the volume to 50 µl with DNase/RNase-Free distilled water.
3. Then follow the same procedure as in section 3.1.1, from step 3 onwards.

3.2 Lentivirus Production and Transduction (Fig. 2a) (See Note 13)

Grow X-lenti 293T cells in a 75 cm² flask to near confluence. It is critical not to let the cells become over-confluent as it will affect the efficiency of virus production. From a confluent 75-cm² flask, ~10×10⁶ cells can be obtained. Pre-warm Trypsin, PBS, and DMEM complete culture medium to 37°C.

3.2.1. Day -1: Seeding cells for transfection

1. Remove the medium from the 75 cm² flask and gently wash the cells with 5 ml of pre-warmed PBS to remove any excess medium left in the flask.
2. Add 1 ml of Trypsin to the flask and leave in the incubator for not more than 1 min. Collect all the trypsinized cells in culture medium.
3. Pellet down the cells by centrifugation at 150 RCF for 2 min.
4. Re-suspend the cells gently in 1 ml of complete culture medium. It is important to dissociate the cells adequately, as leaving cell clumps will reduce the transfection efficiency in later steps.
5. Count and dilute the cells to 0.15×10⁶ cells per ml in complete culture medium.
6. Add 3 ml of the cell suspension (0.45×10⁶ cells) per well of a 6-well plate and leave the plate in the cell culture incubator (37°C, 5% CO₂).

3.2.2. Day 0: Transfection of plasmid DNA

Pre-warm the DMEM complete culture medium and plain DMEM to 37°C. The following protocol gives volumes for transfection of one well of a 6-well plate for each lentiviral vector, GFP and GFP-2A-Cre.

1. Mix 1.6 µg of lentiviral plasmid of interest with 0.4 µg of pCMV-VSVG and 1 µg of psPAX2 in a 1.5-ml tube.
2. Mix the TransIT-293 transfection reagent by gently inverting the tube. Add 8 µl of TransIT-293 to 200 µl of plain DMEM medium in a 1.5-ml tube.
3. Mix the diluted TransIT-293 well by vortexing for ~10 s and spin shortly to collect all the liquid in the bottom of the tube.
4. Add 200 µl of the diluted TransIT-293 reagent to 3 µg of plasmid DNA mix. (See **Note 14**).
5. Mix the tube gently by inverting and leave under the hood for 15-20 min to allow the formation of TransIT-293-DNA transfection complexes.
6. During the incubation time, replace the medium of the cells with 2 ml of fresh, pre-warmed complete culture medium.

7. After the incubation time, gently tap the tube containing the TransIT-293-DNA transfection mix and add 200 μ l of the mix dropwise into each well.

3.2.2. Day 1: Replace culture medium with virus collection medium

1. After 16 h of transfection, aspirate the complete culture medium and gently add 3 ml of prewarmed virus collection medium on the cells. To avoid keeping cells for too long outside the cell culture incubator, treat one plate at the time. (See **Note 15**).

3.2.3. Day 2: Virus collection

After 24-30 h after medium change, collect the medium containing the lentivirus (see **Note 16**).

1. Collect the medium containing the lentivirus in a 15-ml or 50-ml screw-cap tube.
2. Filter the medium through a 0.45 μ m syringe filter to remove cell debris. (See **Note 17**).
3. Use the virus fresh, or gently aliquot the virus-containing medium by desired volumes in 1.5-ml tubes and store at -80°C .

3.2.4. Testing the virus

The amount of virus to add on to the neurons has to be determined by testing the lentivirus on mammalian cell lines such as U-2 OS.

1. Prepare a cell suspension of 50,000 cells/ml, plate 500 μ l of the cell suspension per well of a 24-well plate and leave the plate in the incubator.
2. On the next day, thaw the lentivirus from -80°C directly in the hand or under the hood. (See **Note 18**).
3. Gently add different volumes (50 μ l, 100 μ l, 200 μ l, 500 μ l) of lentivirus, having removed the equivalent amount of media from the wells. Leave the cells with the virus in the incubator for the next 24-48 h.
4. Based on the GFP expression, visually estimate the number of cells transduced and the intensity of GFP-fluorescence for the different volumes used. Accordingly, determine the desired amount of virus to be added to the neurons to achieve maximum transduction efficiency. Note that the same criteria of choosing the virus volume should be applied to both GFP and GFP-2A-Cre lentiviruses. (See **Note 19**).

3.2.5. Testing the cleavage of Cre-recombinase and GFP

Once the virus volume to be used is determined, test the post-translational cleavage of Cre-recombinase and GFP by immunoblotting.

1. Prepare a cell suspension of 50,000 cells/ml, and add 2 ml of this suspension per well of a 6-well plate and leave it in the incubator.

2. Next day, thaw the required amount of lentivirus and transduce the cells.
3. After 48 h of transduction, wash the cells at least two times with fresh medium or PBS to remove any left-over lentivirus.
4. Collect cells from each well in 500 μ l of 2 \times Laemmli buffer and boil for 5 min.
5. Perform an immunoblot with an anti-GFP antibody. If the GFP and Cre are cleaved, GFP band will appear at \sim 27 kDa, else the fusion protein will migrate at \sim 65 kDa. In case you test the construct containing another reporter gene (vesicular marker or EB3 protein), take into the account the size of this protein.

3.3. Mouse Primary Hippocampal Neuron Cultures and Lentiviral Transduction

Primary mouse hippocampal neurons are cultured from embryos at 17.5 days of gestation. In our hands, a single hippocampus gives app. 0.3×10^6 neurons. For each experiment in which untransduced, GFP- and GFP-cre-transduced neurons will be analysed, app. 2.2×10^6 cells (4 embryos) will be necessary: 6 wells of a 12-well plate (40,000 cells per well) for genotyping, 6 wells of a 6-well plate (80,000 cells per well) for immunoblotting, 6 glass-bottom dishes (80,000 cells per well) for imaging and two 6-well plates (80,000 cells per well) for conditioned medium. If immunostaining analysis of the cells is also required, the necessary number of neurons should be seeded (20,000 cells per 12-mm diameter coverslip). Accordingly, coat the required number of culture plates in forehand. (See **Note 20**).

3.3.1. Poly-D-Lysine coating (See Note 21).

1. Thaw frozen aliquots of poly-D-Lysine directly in the hands.
2. Add 500 μ l of 1 mg/ml poly-D-Lysine solution to the 35-mm glass bottom dishes ensuring it covers only the glass surface of the dish. For coating each well of a 12-well and 6-well dishes, 500 and 800 μ l of poly-D-Lysine is used, respectively. For coating glass coverslips, put them one-by-one in 24-well plate wells and add 80 μ l of poly-D-Lysine to cover the surface of coverslips.
3. Leave the dishes in the cell culture incubator for at least 4 h or overnight.
4. Remove the poly-D-Lysine and wash the dishes by adding \sim 2 ml of autoclaved distilled water to the dishes and plate wells. Add 1 ml of distilled water to coverslips in 24-well plates. Repeat the wash three times.
5. Finally, remove any remaining droplets of water by using the vacuum pump.
6. Leave the dishes open under the cell culture hood until they are completely dry. Use the poly-D-Lysine-coated dishes immediately or store at 4°C, sealed with parafilm until further use.

3.3.2. Neuronal cell culture

Before proceeding, prepare freshly anticipated amounts of dissection medium, plating medium, 1 \times B27 medium and 2 \times B27 medium in tissue culture flasks. Keep the plating medium and B27 medium in the cell culture incubator for 2 h to allow it to equilibrate its temperature and CO₂ content. Thaw adequate amount of 10 \times trypsin aliquots and filter-sterilized horse serum. For up to 5 embryos, we typically prepare 100 ml of both dissection and plating media, 30 ml of 1 \times B27 media, 20 ml of 2 \times B27 medium, 500 μ l of trypsin and 5 ml of horse serum.

1. Sacrifice a 17.5-days-pregnant dam by cervical dislocation and collect the embryos in a 100-mm dish containing ~5 ml of dissection medium.
2. Rapidly decapitate the embryos, dissect the brains and separate the two hemispheres (Fig. 3a). Collect all the hemispheres in a 60-mm dish containing ~3 ml of dissection medium.
3. With the help of a stereo microscope, carefully remove the meninges off the hemisphere and dissect out the hippocampus (Fig. 3a). Collect the hippocampi in a 15-ml tube containing 4.5 ml of dissection medium. (See **Note 22**).
4. Add 0.5 ml of 2.5% trypsin (10×) to the tube containing hippocampi, mix gently and place it in the incubator or water bath at 37°C for 20-25 min. During the incubation time, gently mix the tube regularly by inverting.
5. Meanwhile, fire-polish the edges of two glass Pasteur-pipettes using Bunsen burner to make their sizes successively smaller (Fig. 3b). Label the treated pipettes according to their size and fix sterile rubber bulbs at their ends.
6. Coat the inner walls of the glass pipettes with horse serum by pipetting it up and down several times, to avoid the sticking of cells to the pipette walls while triturating.
7. After the trypsinization, use the largest triturating pipette to carefully remove the trypsin from the 15-ml tubes. (See **Note 23**).
8. Wash off the excess trypsin from the tissue by incubating with ~ 5 ml of plating medium for 5 min. Repeat the wash once more and resuspend the hippocampi in ~ 1 ml of plating medium.
9. Gently aspirate and expulse hippocampi 6 to 10 times using the largest triturating pipette, avoiding formation of air bubbles. Repeat the procedure using the smaller pipette. (See **Note 24**).
10. Pass the cell suspension through a 70-µm cell strainer to remove any tissue clumps. (See **Note 25**).
11. Count cells and prepare a cell suspension at 40,000 cells/ml. Add 500 µl of the cell suspension on each glass coverslip (for immunostaining), 1 ml in each 12-well plate well (for genotyping), 2 ml in each 6-well plate well (for immunoblotting and for conditioned medium) and in each 35-mm glass bottom dish (for live-cell imaging). The day of seeding is considered as DIV 0 (*days in vitro 0*). (See **Note 26**).
12. Two to three hours after plating, change the medium for the Neurobasal-B27. For the neurons for conditioned medium, simply replace the plating medium by 1× B27 medium. For neurons to be transduced with the lentiviral particles, see next chapter.

3.3.3. Transduction of neurons

In this protocol we assume that the volume of the virus added to the neurons constitutes 50% of the total volume of the culture medium. As the virus-containing-medium (Neurobasal) does not contain the supplements necessary for neurons to grow, it is essential to add twice the final amount of these supplements to the medium (2× B27 medium) used to complement the virus-containing medium volume.

1. Prepare fresh (see chapter 3.2), or thaw the required amount of the desired lentiviruses (for example GFP- and GFP-2A-Cre encoding lentiviruses) under the hood.
2. In the meantime, remove the plating medium and add 0.5 volume of 2× B27 medium to the neurons. For example, for a 35-mm glass-bottom dish add 1 ml of 2× B27 medium.
3. Once the virus is completely thawed, add 0.5 volume of the lentivirus-containing medium (for a 35-mm glass bottom dish, add 1 ml of lentivirus). For untransduced samples, add Neurobasal-PS medium. Rapidly return the cells to the cell culture incubator.

3.4 Genotyping

Prior to the analysis of the transduced neurons, confirm the genotype of neurons in order to verify the activity of the cre-recombinase.

1. Add 100 µl of the lysis buffer containing proteinase K to each well of 12-well dish and lyse the neurons using a p200 micropipette.
2. Incubate the lysate at 55°C for at least 4 h or overnight.
3. Boil the samples at 95°C for 10 min.
4. In order to determine the genotype of CCP1 and CCP6, perform PCR reactions using primer mix 1 and primer mix 2 for both genes.
5. Set up separate PCR reactions for each primer mix (25 µl): Add 18 µl of water, 5 µl of 5× buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of GoTaq® DNA polymerase (1 U/µl), 0.1 µl of primer mix and 1 µl of DNA sample.
Use the following PCR conditions: an initial step of denaturation at 96°C for 5 min; 35 cycles of amplification (96°C for 30 sec, annealing of primers at 63.5°C for 30 sec, and extension at 72°C for ~30 sec).
6. Run 10 µl of the PCR product on the 2% agarose gel. (See **Note 27**).
7. For CCP1, primer mix 1 amplifies ~400 bp fragment in the flox allele (and no product in the knockout allele) and the primer mix 2, ~600 bp fragment in the knockout allele (and no product in the flox allele). For CCP6, primer mix 1 amplifies ~440 bp

fragment in the flox allele (and no product in the knockout allele) and the primer mix 2, ~800 bp fragment in the knockout allele (and no product in the flox allele) (**Fig. 4a**). Note, that the primer mix 1 still amplifies a faint band in both CCP1 and CCP6, suggesting a residual presence of flox alleles, which could come from untransduced cells.

3.5 SDS-polyacrylamide gel electrophoresis and immunoblotting

Prior to the analysis of the transduced neurons, collect the samples to later verify the effect of the depletion of CCP1 and CCP6 on the levels of tubulin polyglutamylation in transduced neurons by immunoblot (**Fig. 4b**).

1. Add 200 μ l of the Laemmli sample buffer to each well of 6-well dish and lyse the neurons using a p200 micropipette. (See **Note 28**).
2. Boil the samples at 95°C for 5 min and spin down briefly.
3. Set up the gel casting apparatus according to manufacturer's description. For one 1-mm-thick 10% acrylamide gel, mix 1.25 ml of acrylamide/bis solution, 1.25 ml of 4 \times resolving buffer and 2.5 ml of distilled water in a 15-ml tube. Add 40 μ l of 10% APS and 5 μ l of TEMED, mix gently by inverting the tube and immediately pipette the solution into the preassembled casting apparatus. Add a small layer of distilled water (~300 μ l) on the gel solution and leave it undisturbed for at least 30 min.
4. Remove the overlaid water layer by inverting the gel and drain off the remaining water droplets with a tissue paper.
5. Prepare the stacking gel: for one 1 mm-thick resolving gel, mix 175 μ l of acrylamide/bis solution, 406 μ l of 4 \times stacking buffer and 1044 μ l of distilled water. Add 10 μ l of 10% APS and 4 μ l of TEMED, mix gently by inverting the tube and immediately pipette the solution onto the polymerized resolving gel. Carefully insert a comb into the stacking gel and allow it to polymerize for 30 min.
6. Assemble the electrophoresis tank and fill the chamber with the gel running buffer. Remove the comb and wash the wells with the buffer.
7. Boil the samples at 95°C for 5 min, spin down briefly, load the samples on the gel and separate the proteins at 200 V until the dye front runs out from the gel. (See **Note 29**).
8. Transfer the separated proteins from the gel to a nitrocellulose membrane using the Biorad Trans-Blot[®] Turbo system, according to manufacturer's instructions.
9. Block the membrane in TBST containing 5% skimmed milk for 1 h, with gentle shaking, at room temperature.

10. Incubate the membrane with primary antibodies (12G10, PolyE or anti- GFP) diluted in TBST containing 2.5% skimmed milk for 1 h, with gentle shaking, at room temperature.
11. Wash the membrane 3×5 min in TBST with vigorous shaking.
12. Incubate the membrane with secondary, HRP-conjugated antibodies (anti-mouse for 12G10; anti-rabbit for poly E and anti-GFP) diluted in TBST buffer for 1 h, with gentle shaking, at room temperature.
13. Wash the membrane 3×5 min in TBST with vigorous shaking.
14. Reveal the chemiluminescence signal on the membrane by incubating it with the ECL solution according to manufacturer's instructions and using the available equipment (either chemical or digital imager)

4. Notes

1. The primer sequence written in lower case shares homology with the ends of the target vector, pTRIP-CMV-GFP-2A
2. Always use cells which have been in culture for no more than 5-8 passages, as using cells after multiple passages may reduce the viral titre. It is very important to ensure that X-lenti 293T cells are mycoplasma free, as mycoplasma contamination can affect the virus production efficiency.
3. We have used other transfection reagents such as jetPEI for virus production and found that the virus titre produced is lower than when using TransIT-293.
4. We used pTRIP vector with CAG and CMV promoters for the experiments. In our experience, there is no difference in their expression levels in neurons at young age. However, if transduction to be performed at a differentiated stage, we suggest to use vectors with CAG promoter, as it expressed better than CMV promoter in mature neurons.
5. The choice of medium used for virus-production depends on the medium preferred by the cells you intend to transduce. In this case, we used Neurobasal medium as our target cells were neurons.
6. If there is a need to separate alpha-and beta-tubulins on the SDS-PAGE, we recommend using a different gel composition [25].
7. As Tween 20 is highly viscous, add it to the TBS solution using a cut p1000 pipette tip and rinsing the tip by repeated pipetting
8. It is crucial to run the gel for a sufficiently long time, as it will aid in separating the cut from the uncut vector, reducing the contamination with uncut vector during purification of digested vector.
9. Extension time needs to be adjusted based on the length of the amplicon and speed of the polymerase. In our case, we consider that polymerase requires ~1 min to amplify ~1 kb of DNA template
10. The timing of the reaction mix is to be strictly followed. Leaving the reaction mix for less or more time will reduce the efficiency of SLIC.
11. Usually, when the SLIC reaction is efficient, we get ~80 colonies in the positive plate and ~5 colonies in negative control plate. If the SLIC efficiency is poor, you will get less colonies in the positive plate. If the vector is not properly digested, you will get many colonies in the negative control plate.

12. NheI is selected when the reporter gene is to be C-terminally GFP-tagged. Use BsrGI enzyme when the reporter gene is to be N-terminally GFP-tagged.
13. Rules and regulations regarding working with lentiviral particles are specific to each country. Please enquire about and follow the rules that apply in your country.
14. Always add the diluted TransIT-293 into DNA mix, and not the other way around.
15. Usually we add 3 ml of virus collection medium per well of a 6-well plate. It is possible to obtain a more concentrated virus by adding a lower volume of medium. The minimal volume possible is 1.5 ml.
16. Avoid exceeding this time, as it might reduce the competence of produced lentivirus.
17. Do not use 0.2 μ m filter, as it might damage the lentivirus.
18. As viruses are very sensitive to mechanical stress, do not accelerate thawing by pipetting or shaking.
19. As a general rule, transduction of primary cell cultures is difficult compared to cell lines. Therefore, the intensity of GFP-fluorescence in U-2 OS cells and neurons might be different. If the GFP intensities are very different, then we suggest to use wild type neurons for testing and determining the amount of virus to be added.
20. For cleaning coverslips, place a maximum of 200 coverslips in a 100-ml glass bottle. Wash them by shaking for at least 30 min in 3 M HCl, 2 \times 10 min in distilled water, 3 \times 10 min in 95% EtOH and 2 \times 10 min in distilled water. Let the coverslips dry under the hood or in an oven.
21. Proper coating of the surface with poly-D-Lysine is crucial for attachment and development of the neurons. If the dishes are not coated properly, it will cause the neurons to clump or not adhere well.
22. Success of primary neuron culture depends on the time of dissection (from sacrificing the dam to collecting all the hippocampi) and should take no longer than 90 min.
23. Be careful as the hippocampi tend to be aspirated by the suction created by pipetting. If it is difficult to remove the trypsin without the hippocampi, proceed immediately to washes. The tendency of hippocampi to be aspirated with the pipette suction reduces after washes with plating medium.
24. Do not exceed the number of trituration passages through the Pasteur pipettes, even if pieces of tissue are still visible at the end of the procedure.
25. Some labs use 40 μ m cell strainers to pass the cell suspension. In our hands, 70- μ m cell strainers worked best without affecting the survival of neurons.

26. For live-cell imaging and immunostaining, it is important to plate neurons at a low-density. Neurons seeded at high-density will have overlapping axons and dendrites, making it difficult to use them for analysis.
27. Usually, 10 μ l of PCR product is sufficient to visualize the amplification. If there is an unexpected problem with the gel, make a fresh gel and run another 10 μ l from the remaining PCR product.
28. Depending on the day of the experiment, the sample might need to be further diluted before immunoblotting in order to not overload the gel with protein.
29. When analysing the differences in levels of tubulin modifications, we strongly recommend to load low amounts of protein on the gel [25].

Fig. 1. Strategy used to clone the cre-recombinase and reporter genes into the pTRIP

lentiviral vector. (a) Vector map of the pTRIP-CMV-EGFP-2A lentiviral plasmid. To allow the cleavage of cre recombinase and GFP, cre was inserted into the XhoI site. For further insertion of additional reporter genes, the backbone can be opened with NheI to generate a C-terminally GFP-tagged fusion protein, or with BsrGI to generate a N-terminally GFP-tagged fusion protein. (b) Flowchart of the sequence- and ligation-independent cloning (SLIC) method (adapted from [26]).

Fig. 2. Production and functional analysis of the lentiviral particles.

(a) Flowchart of the production of lentiviral particles. X-lenti 293T cells are transfected with the envelope vector (pCMV-VSV-G), packaging vector (psPAX2) and the backbone pTRIP vector containing the gene of interest. On the day following the transfection, the medium is replaced by the desired virus-collection medium, which is collected 24-36 h later, filtered and aliquoted. The medium can either be directly used to transduce cells, or frozen for later use. Note that the medium changes colour to more orange during virus production. (b) Immunoblots showing the efficiency of the cleavage of the P2A site between the GFP and the cre recombinase. Extracts from U-2OS cells transduced with lentivirus encoding either GFP, or GFP-2A-cre-recombinase are immunoblotted with anti-GFP antibody. In both cases, only a single band of ~28 kDa, corresponding to GFP, is detected. The absence of a higher-molecular-weight band, corresponding to the fusion protein GFP-cre-recombinase, demonstrates that all GFP-cre fusion protein was cut. Total α -tubulin (detected by the 12G10 antibody) is used as a loading control.

Fig. 3. Mouse primary hippocampal neuron culture and the experimental design.

(a) Representative pictures of successive steps of hippocampus dissection from an E17.5 embryonic brain. Scale bar: 2 mm. (b) Representative images of unpolished, fire-polished and reduced-diameter Pasteur pipettes used to dissociate hippocampi. Scale bar: 2 mm. (c) Schematic representation of the culture dishes needed for one experiment including non-transduced, GFP- and GFP-2A-cre-transduced neurons.

Fig. 4. Analysis of efficiency of the cre-mediated recombination in primary hippocampal neurons.

(a) An agarose gel showing the genotypes of $Ccp1^{lox/lox}Ccp\delta^{lox/lox}$ neurons transduced with GFP- and GFP-2A-cre viruses. Note that in the presence of GFP alone, only flox alleles are detected, while with GFP and cre recombinase the major band comes from the

KO alleles. Note the presence of a faint band (corresponding to the flox alleles) in the presence of the cre recombinase, which could come from the incomplete transduction of neuronal population. **(b)** Immunoblot from *Ccp1^{flox/flox}Ccp6^{flox/flox}* neurons after transduction with GFP- or GFP-2A-cre viruses. In the cells transduced with GFP-2A-cre virus, knockout of *Ccp1* and *Ccp6* result in a strong increase of tubulin polyglutamylation levels (polyE antibody). Equal loading is verified using the total α -tubulin antibody (12G10). **(c)** Immunoblot showing that the cre-recombinase expressed in wild-type neurons has no effect on tubulin polyglutamylation levels (polyE antibody). Equal loading is verified using the total α -tubulin antibody (12G10).

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