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CRISPR-based RNA proximity proteomics (CBRPP)

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We use this protocol and it's working

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Construction of inducibly expressing dPspCas13b-BioID2 cell line.

2m

- 1 Construction of inducibly expressing dPspCas13b-BioID2 cell line. The two consecutive manipulation steps are necessary to generate Tet-on cell line with inducible expression of dPspCas13b-BioID2. The first step is generation of cells stably expressing reverse tetracycline-controlled transactivator (rtTA). HEK293T cells were infected at ~50% confluency by lentiviruses containing pLVX-TetO3G(rtTA)-hygr vector for 48 h, followed by selection with 50 ug/ml hygromycin in growth medium for 7 days, and hygromycin resistant clones were selected. Several clones were picked and tested for rtTA expression by immunoblotting. After testing for all molecular and cell biological parameters of interest, the 'best' rtTA-positive clone was expanded and stored. 1m
- 2 The next step is generation of Tet-on cell lines with inducible expression of dPspCas13b-BioID2. The 'best' rtTA-positive clone was infected by lentiviruses containing target plasmid (Inducible-dPspCas13b-BioID2) for 48 h, followed by selection with 1 ug/ml puromycin in growth medium for 7 days. The puromycin resistant clones were harvested by limiting dilution in cell pools. Several individual cell clones were picked, expanded and screened by immunoblotting for doxycycline-inducible expression of dPspCas13b-BioID2. Finally, clones of interest were expanded, re-tested and stored. 1m

Designing crRNAs and validating their targeting

1w

- 3 Designing crRNAs targeting different regions of an endogenous RNA of interest and non-targeting crRNAs. 3d
- 4 Validating crRNAs' targeting by knockdown with an active PspCas13b or by RNA immunoprecipitation (RIP)-qPCR with dPspCas13b-BioID2. The optimal crRNAs were selected for the following experiments. 4d

CBRPP labeling

3d

- 5 Inducibly expressing dPspCas13b-BioID2 HEK293T cells were plated in 10 cm dishes at 70% confluency 18 h prior to transfection. Cells were transfected with 20ug crRNA plasmid per dish (including targeting crRNAs and non-targeting crRNAs). After 6 hours of transfection, the culture medium was replaced with new media containing 0.1 ug/ml doxycycline. Biotin was added to the culture medium at a final concentration of 50uM after 15 h of transfection. After 18 hours of biotin, the medium was removed and cells were washed three times with ice cold PBS. Cells were scraped and transferred to 1.5 ml tubes with ice cold PBS, spun at 3600 rpm for 5 minutes, flash frozen in liquid nitrogen and stored at -80°C. 3d



Streptavidin magnetic beads enrichment of biotinylated proteins and identification by MS

4d

- 6 Cell pellets as described above were lysed in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, protease cocktail [TargetMol], and 1 mM PMSF) at 4°C for 10 minutes. The lysates were cleared by centrifugation at 12,000 g for 10 min at 4 °C. 50ul of each lysate supernatant was reserved for detection of biotinylation activity by western blotting. Streptavidin magnetic beads were washed twice with RIPA lysis buffer and then mixed with lysates supernatant together with rotation overnight at 4 °C. On day 2, the beads were subsequently washed twice with 1 mL of RIPA lysis buffer, once with 1 mL of 1 M KCl, once with 1 mL of 0.1 M Na₂CO₃, once with 1 mL of 2 M urea in 10 mM Tris-HCl (pH 8.0), and twice with 1 mL RIPA lysis buffer. Finally, biotinylated proteins were eluted by boiling the beads in 150 µL of elution buffer (55 mM pH 8.0 Tris-HCl, 0.1% SDS, 6.66mM DTT, 0.66 mM biotin) for 10 minutes and sent for mass spectrometry.

4d