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Cell lysis and gel electrophoresis for protein analysis of HeLa cells (Provisional unformatted)

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ABSTRACT

Here, we present multiple protocols used for biochemical analysis of protein expression and association. First, we used a simple lysis technique to determine the efficiency of an siRNA knockdown. Then, we modified two previously published methods for assaying co-precipitation of p62 and NEMO with magnetic beads conjugated to a GFP-trap molecule. In the first, we pulled down EGFP-NEMO in control or mitochondrial-damaged conditions, and in the second, we pulled down EGFP-Ubiquitin in p62-/- cells with expression of wild-type p62 or a dysfunctional mutant. Since p62 is known to form multimers, we used specialized buffers to preserve those putative interactions. We were able to reproduce results published previously by pulling down EGFP-Ubiquitin in p62-expressing cells. However, interestingly, we did not find evidence that NEMO interacts with p62 in the soluble fraction, or via ubiquitin chains generated in basal conditions. These studies demonstrated that NEMO recruitment to damaged mitochondria occurs in specific circumstances, and NEMO co-localization with p62 is also dependent on multiple factors.

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1 - This protocol was developed to analyze protein expression and enrichment in cell culture, including HeLa-M cells and HeLa p62-/- cells

2 - Option 2 was modified from a protocol used in Turco et al, Molec. Cell, 2019

3 - Option 3 was modificed from a protocol used in Wurzer et al, eLife, 2015 Materials

4 -

5 5 mL capped tubes (Millipore Sigma, EP022364120)

6 - Cell scrapers

7 - Liquid nitrogen

8 -

9 5 mL Lo-Bind capped tubes (Eppendorf, 022431081)

- 10% acrylamide gels with desired number of wells (make or purchase) Reagents For all Options

- 1X Phosphate buffered saline (PBS)

- 2% bovine serum albumin (BSA) (Fisher Scientific, BP1605-100) for Option 2

- 10% sodium dodecyl sulfate solution (SDS) (Invitrogen, 15553-035)

- Protease and phosphatase inhibitors

o Leupeptin (10 mg/mL = 1000X)

o DTT (1M = 1000X)

o Pepstatin A (1 mg/mL = 1000X)

o TAME (10 mg/mL = 1000X)

o PMSF (100mM = 100X)

- 4X Denaturing buffer (DB) [900 uL (4% SDS, 50% glycerol, 125 mM Tris HCl, pH 6.8, 0.2% w/v Orange G) + 100 uL beta-mercaptoethanol (BME)]

- Methanol

- 4X Running buffer (RB)

- 1X Tris buffered saline (TBS)

- 1X TBS with 0.1% Tween (BIO-RAD, 1706531) (TBST)

- Running Buffer (RB) (250 mL 4X RB + 750 mL water + 10 mL 10% SDS)

- Transfer buffer (125 mL 4X RB + 775 mL water +

mL methanol + 500 uL 10% SDS + 560 uL BME)

- PVDF membranes

- REVERT 700 Total Protein Stain (LICOR, 926-11021)

- REVERT Wash Buffer (6.7% w/v glacial acetic acid, 30% v/v methanol in water)

- REVERT Reversal Buffer (0.1 M NaOH, 30% v/v methanol in water)

- Desired primary antibodies

- LICOR secondary antibodies such as donkey anti-mouse 800 (926-32212) and donkey anti-rabbit 680 (926-68073)

- TrueBlack buffer (Biotium, 23013B-1L)

- EveryBlot buffer (BIO-RAD, 12010020) Specialized buffers and other reagents

- RIPA buffer (Option 1) Reagent stock concentration final concentration volume of stock (for 10 mL) Tris-HCl (pH 8.0)

M

mM

uL EDTA

mM

mM

42 uL EGTA

43 mM

44 mM

45 uL Triton X-100 10% 1%

46 uL DOC 5%

47 50%

48 uL SDS 10%

49 10%

50 uL NaCl

51 M

52 mM

53 uL Water - -

54 mL

55 - Lysis Buffer-A (Option 2) Reagent Final conc For 10 mL

56 M HEPES/KOH

57 mM pH 7.5

58 uL

59 M Sorbitol

60 mM

61 5 mL

62 mM EGTA

63 5 mM

64 uL

65 M Mg-Acetate

66 mM

67 uL ddH2O

68 92 mL

69 - PBS with 0.1% TWEEN (PBST) for Option 2

70 - Wash Buffer (Option 3) Reagent Final conc For 20 mL

71 M Tris-Cl

72 mM, pH 7.4

73 uL 100% Glycerol 10%

74 mL

75 M NaCl

76 mM

77 uL ddH2O

78 06 mL

79 - Lysis Buffer-B (Option 3) Reagent Final conc For 5 mL Master buffer

80 mM, pH 8.0

81 975 mL 100% NP-40 (IGEPAL)

82 5%

83 uL

- Pierce BCA Assay (ThermoFisher, 23225) for Option 1

- GFP-Trap Magnetic Particles (Chromotek M-270) for immuno-precipitations Equipment

- vacuum apparatus

- end-on-end rotating apparatus

- refrigerated centrifuge

- magnetic rack (for GFP-Trap particle precipitation)

- rockers at room temperature (R.T.) and at 4C

- Plate reader (such as BioTex Synergy Mx)

- Odyssey CLx membrane scanner (LI-COR, 9140)

- ImageStudio software (LI-COR)

94 - Heat source to 95 C

95 - Gel electrophoresis apparatus (BIO-RAD)

96 - Membrane transfer apparatus (BIO-RAD)

97 - Excel Before start

98 - The start point for this protocol is after cells grown on 3.5 cm, or 10 cm dishes have been transfected with relevant constructs for 18-24 hours and treated with appropriate small molecules or vehicles

99 - For 3.5 cm dishes, follow transfection procedures enumerated in imaging protocols.

100 - Chill all reagents on ice

101 - Add protease and phosphatase inhibitors to 1X to each lysis buffer immediately before use Step-by-step Wash cells

102 - Aspirate media from dishes

103 - Wash samples quickly x2 with ice cold PBS

104 o Note: Can stop after washes by scraping cells with the second wash of PBS into 1.5 mL tube, spin down at 2.4G, 3min, 4 degrees. Aspirate PBS and snap-freeze tubes in liquid

nitrogen. If frozen, add respective lysis buffer and inhibitors and let thaw on ice 10 min before proceeding Option 1: Standard lysis with RIPA Note: We used this protocol to assess depletion of p62 in HeLa-M cells after siRNA treatment and imaging NEMO recruitment. Samples were collected from 35 mm imaging dishes.

105 - Add

106 uL RIPA + inhibitors to dish and scrape cells into 1.5 uL tube, OR add buffer to thawed sample and resuspend by pipetting

107 - Rotate resuspended sample on end-over-end machine at 4 degrees for 20 min

108 - Spin at top speed (17 G), 4 degrees, 20 min

109 - Remove supernatant as Lysis and keep on ice or store at -80 C

110 - Measure protein concentration with Pierce BCA assay by adding 25 uL sample or BSA standard to each well in duplicate and 200 uL Reagent A+B. Incubate 37 C for 30 min then measure absorbance on a plate reader

111 o Note: it is likely necessary to dilute samples 1:4 or more to measure within the range of the assay.

112 - Add 1/3 volume of 4X DB to remaining Lysis or a measured fraction of sample and heat 95 C for 5 min

113 - Proceed to gel electrophoresis Option 2: Co-IP EGFP-NEMO for p62 association assay in AntA/OligA or vehicle conditions

114 - The samples for this assay are: A) EGFP-vector + veh, B) EGFP-vector + AntA/OligA, C) EGFP-NEMO + veh, and D) EGFP-NEMO + AntA/OligA. Start with 3x 10 cm dish per condition

for one biological replicate

115 - 18-24 hr before collection, transfect 3 ug Parkin and 1.5 ug EGFP-NEMO (or vector), to 70-80% confluent cells on each dish

116 - it is best to plan collection times such that each is collected and snap frozen ~10 min apart. Then all snap frozen samples can be processed simultaneously from that step Lysis with heavy fraction isolation

117 - Scrape and combine like samples into 800 uL PBS, transfer to 1.5 mL tube

118 - Spin

119 min, 2 G, at 4 C

120 - Aspirate PBS and snap freeze in liquid nitrogen

121 - Add

122 uL LB-A with inhibitors to each frozen sample

123 - Pipet to resuspend

124 - Incubate on ice 10 min

125 - centrifuge at 1000g, 5 min, 4 degrees

126 - Transfer sup. (Cytosol) to a new tube

127 - Centrifuge again at top speed, 10 min, 4 degrees

128 - Save pellet as Heavy Fraction if desired and resuspend in 50 uL 1X DB Prep IP beads

129 - Mix GFP-Trap beads by swirling and gently pipetting to break up accumulations

130 - aliquot 25 uL bead slurry into LoBind tubes (one for each condition)

131 - Remove supernatant from beads by inserting the tube in a magnetic rack and carefully aspirating clear solution

132 - Resuspend beads in 500 uL cold PBST

133 - Insert to magnetic rack and remove PBST wash

134 - Block beads in 2% BSA (in water) for 1 hr at 4 degrees with end-over-end rotation

135 - wash 2X with 500 uL r.t. PBST

136 - wash 1X with 500 uL LB-A

137 - Allow magnets to collect beads for up to 5 min Bead capture

138 - Add

139 uL Cytosol to bead tube, rotate end-over-end gently for 1 hr, 4 degrees (save the rest of Cytosol for Input)

140 - Remove supernatant by magnet (up to 5 min) and save as Flow Through

141 - Wash 3X with 500 uL R.T. PBS (no TWEEN) (can save these washes)

142 - for the last wash, transfer beads and wash to a new tube

143 o Note: optional if using Lo-bind tubes

144 - Remove sup from last wash and resuspend in ~90 uL 1X DB with rough mixing (vortex, pipetting), then heat 95 C for 5 min

145 - Magnetize 5 min and save sup as Eluate

146 - Add 1/3 volume of 4X DB to Input and Flow Through and heat 95 C for 5 min

147 - Proceed to gel electrophoresis Option 3: Co-IP GFP-Ubiquitin for p62 and NEMO association assay

148 - The samples for this assay are in p62-/- Hela cells: A) EGFP-vector + mCherry-vector, B) EGFP-vector + mCherry-p62, C) EGFP-Ubiquitin + mCherry-vector, D) EGFP-Ubiqutin + mCherry-p62, and E) EGFP-Ubiquitin + mCherry-p62-ΔPB1. Start with 2x 10 cm dish per condition for one biological replicate

149 - 18-24 hr before collection, transfect 3 ug Parkin and EGFP-Ubiquitin (or vector), 1.5 ug NEMO, and 2-2.5 ug mCherry-p62 (or vector) to 70-80% confluent cells in each dish

150 - Note: it is best to plan collection times such that each is collected and snap frozen ~10 min apart. Then all snap frozen samples can be processed simultaneously from that step Lysis

151 · Combine samples by scraping cells into 600 uL LB-B with inhibitors and transfer to 1.5 mL tube

152 · Incubate on ice, 15 min

153 · Centrifuge at 16,100 g (16.1 G), 13 min, 4 degrees

154 · Transfer sup. to a new tube as Lysis Prep IP beads

155 · Mix GFP-Trap beads by swirling or gently pipetting to break up accumulations and aliquot 10 uL into LoBind tubes (one for each condition)

156 · Remove supernatant from beads by inserting the tube in a magnetic rack and carefully

aspirating clear solution

157 · Resuspend beads in 500 uL r.t. Wash Buffer

158 · Insert to magnetic rack and remove wash

159 · Repeat for a total of 2 washes

160 · wash 1X with 500 uL LB-B

161 · Allow magnets to collect beads for up to 5 min Bead capture

162 · Add 500 uL Lysed sample to bead tube, rotate gently for 1 hr, R.T.

163 · save the rest of the Lysis as Input

164 · Remove sup by magnet (up to 5 min) and save as Flow Through

165 · Wash 3X with 500 uL R.T. Wash buffer (can save these washes)

166 · for the last wash, transfer beads and wash to a new tube (optional if you're using Lo-bind tubes)

· Remove sup from last wash and resuspend in ~90 uL 1X DB with rough mixing (vortex, pipetting), then heat block 10 min

· Magnetize 5 min to collect beads and save sup as Eluate

· Add 1/3 volume of 4X DB to Input and Flow Through and heat 95 C for 5 min

· Proceed to gel electrophoresis Gel electrophoresis and immune-blotting

- This protocol was developed for use with the LI-COR system for protein detection Set-up

- Set up electrophoresis cell with 10% gels by manufacturer's instructions

- Fill cell with RB and flush wells with a plastic transfer pipet.

- Invert samples by hand to mix, and ensure all samples are at the bottom of tubes by briefly centrifuging

- Load wells with equal amounts of protein (Option 1) or equal volumes (Options 2 and

and molecular weight standard (4-5 uL).

o Note: We load 15-25 uL eluate and 10 uL Input

178 - For empty lanes, load approx. equal volume of 1X DB. Running

179 - Run samples through stacking gel (85 V, 20-40 min)

180 - Run samples through 10% gel (125 V, until front has reached bottom of gel, usually ~70 min) Transfer

181 - Remove gels from electrophoresis cell and construct transfer cassettes with PVDF membranes according to manufacturer's instructions

182 - Place the cassettes in the transfer cell and fill cell with Transfer buffer and icepack

183 - place the cell in a basin

184 - Fill basin with ice around cell

185 - Run transfer for 60-70 min, 100 V Membrane processing and total protein stain

186 - Dry membrane between filter paper in the dark for at least 45 min

187 o Note: this is most important for small proteins

188 o Note: can be a stopping point for several days

189 - Rehydrate membrane in MetOH

190 - Wash in ddWater

191 - Wash in 1X TBS 2 min

192 - Stain total protein, 5 min, R.T., with REVERT 700 Total Protein Stain

193 - Wash membrane 2x 30 sec with REVERT wash buffer

194 - Image total protein on LICOR

195 o Note: can cut the membrane based on total stain if desired

196 - Wash off total stain with REVERT Reversal (up to 10 min, R.T.)

197 - Rinse in ddWater Immuno-labeling

198 - Block membranes in EveryBlot buffer, 5 min, R.T. with rocking

199 - Incubate in vacuum packs with primary antibodies in EveryBlot overnight at 4 C. See materials and methods for concentrations of antibodies used.

200 - Wash with TBST 4x 5 min

201 - Incubate with secondary antibody 1:20,000 in TrueBlack antibody diluent with 0.2% TWEEN and 1:1000 10% SDS for up to 1 hr

202 - Wash with TBST 4x 5 min

203 - Wash 1X with TBS to clear TWEEN

204 - Wash 1X with water

205 - Image Quantification

206 - For quantification of knockdown (Option 1)

207 o Use ImageStudio software to draw rectangles around total protein in each lane of Total Protein image and subtract background

208 o Then add rectangles to outline p62 bands and subtract background

209 o Transfer intensity measurements to Excel

210 o Calculate p62 expression relative to total protein for each experiment

211 - For quantification of p62 enrichment from GFP-NEMO immuno-precipitation

212 o Use ImageStudio to add rectangles around input/cytosolic p62 and eluted p62

213 o Transfer intensity measurements with background subtracted to Excel

214 o Calculate p62 eluted relative to input amounts

215 o Calculate elution/input quantity relative one condition

216 § Note: we found it necessary to perform this secondary normalization due to variability across replicates. In our case, we normalized results from each condition to the EGFP-NEMO + AntA/OligA results