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Supplemental Information

Cdh1-APC Regulates Protein Synthesis and Stress Granules in Neurons through an FMRP-Dependent Mechanism

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Table S1. Cdh1 interactome, Related to Figure 1

Table of proteins identified in the Cdh1 interactome following immunoaffinity chromatography and mass spectrometry analysis of FLAG-Cdh1 transfected cells. Proteins are listed from highest enrichment in the samples to lowest enrichment. N indicates nontransfected cells and C indicates FLAG-Cdh1 transfected cells. + indicates lysates co-incubated with FLAG peptide to control for nonspecific binding. Proteins that are known stress granule proteins based on Jain et al. (2016) and Markmiller et al. (2018) are indicated.

Table S2. DAVID Gene Ontology Analysis, Related to Figure 1

Proteins with at least a four-fold difference in Cdh1-transfected cells compared to control cells were entered into the DAVID Functional Annotation Bioinformatics Microarray Analysis tool (https://david.ncifcrf.gov) to classify proteins based on biological processes.

Figure S1

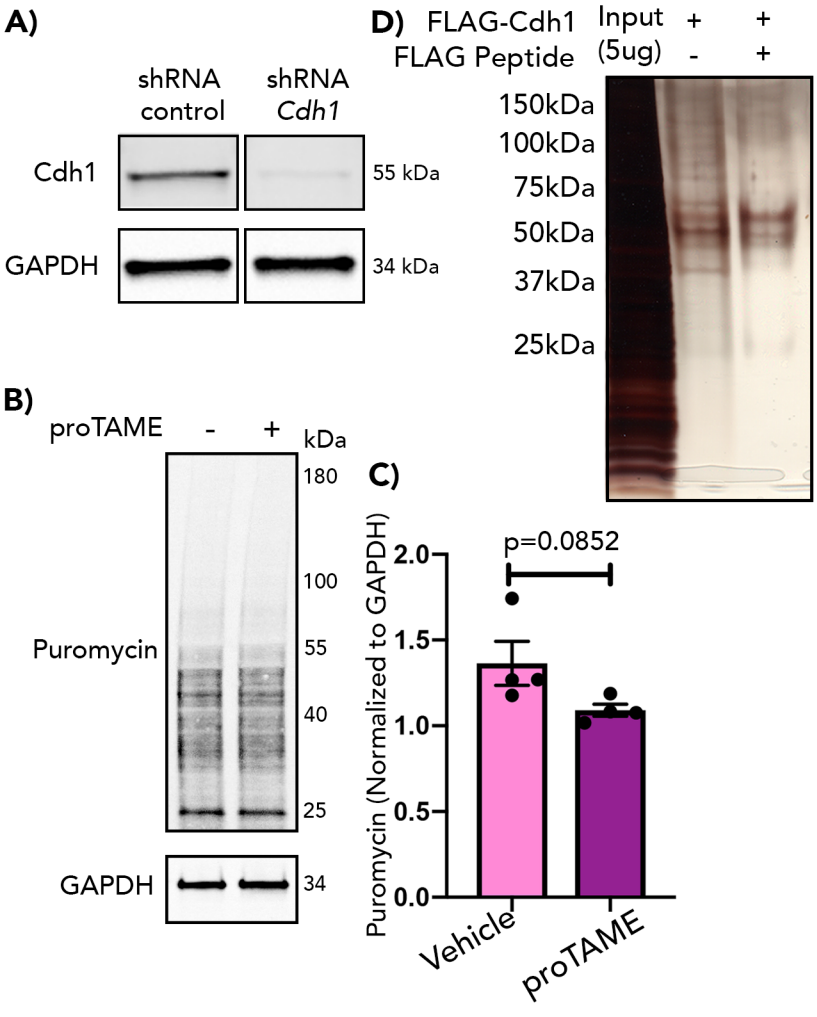
A)
shRNA control
shRNA Cdh1
Cdh1
55 kDa
GAPDH
34 kDa
B)
proTAME - +
kDa
180
100
55
40
25
Puromycin
GAPDH
34
C)
p=0.0852
Puromycin (Normalized to GAPDH)
2.0
1.5
1.0
0.5
0.0
Vehicle
proTAME
D)
FLAG-Cdh1 Input + +
FLAG Peptide (5ug) - +
150kDa
100kDa
75kDa
50kDa
37kDa
25kDa

Figure S1. Confirmation of Cdh1 knockdown and regulation of protein synthesis, Related to Figure 1

A) N2A cells were transfected with shRNA targeting *Cdh1* (*Fzr1*) or a control shRNA from Dharmacon. 72 hours after transfection, cells were lysed and immunoblotted to assess knockdown efficiency. Upon confirmation of knockdown, the Dharmacon plasmids were sent to the Emory Viral Vector core for lentiviral production. **B)** DIV 14 cortical neurons were treated with proTAME (12μM) or vehicle (DMSO) for 4 hours. 75 minutes prior to lysis, neurons underwent puromycylation (10μg/mL). **C)** Quantification of puromycin normalized to GAPDH for **B**); n=4**.** Significance calculated by Student's t test. Data are represented as mean +/- SEM. **D)** Silver stain from FLAG-Cdh1 IP in Figure 1E shows an enrichment of putative Cdh1 interacting proteins from sample without FLAG peptide co-incubation

Figure S2

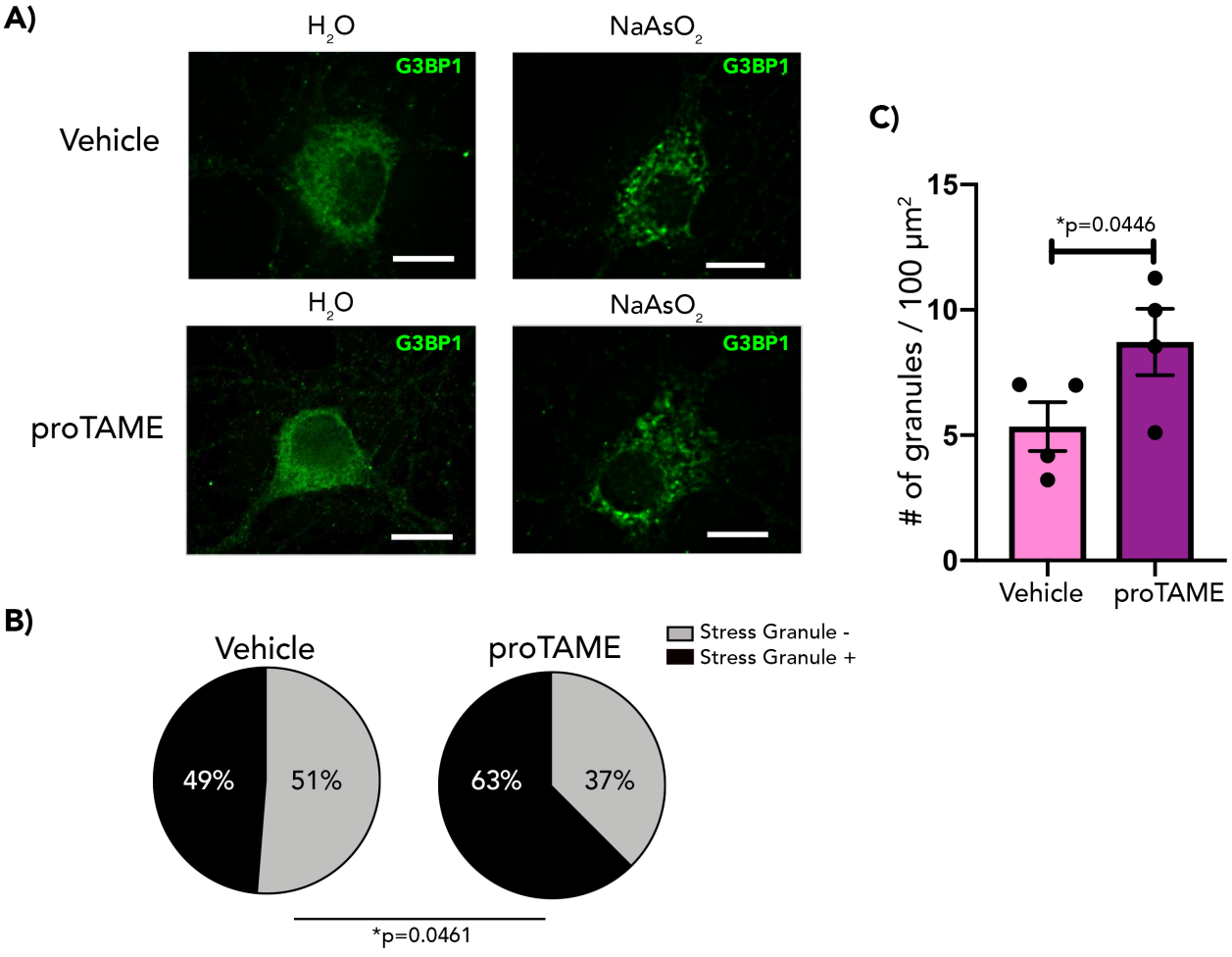
A)
H2O
NaAsO2
G3BP1
Vehicle
proTAME
B)
Vehicle
proTAME
Stress Granule -
Stress Granule +
49%
51%
63%
37%
*p=0.0461
C)
# of granules / 100 µm2
15
10
5
0
*p=0.0446
Vehicle
proTAME

Figure S2. proTAME treatment increases stress granule formation, Related to Figure 2

A) DIV 14 cortical neurons were treated with vehicle (DMSO) or proTAME for 3 hours 15 minutes. Neurons were then treated with sodium arsenite ($NaAsO_2$) (0.5mM) or water for 45 minutes hour prior to fixation. Immunofluorescence was done with antibodies against G3BP. Scale bar indicates 10μM. **B)** User-blind scoring of neurons that were stress granule positive or stress granule negative following arsenite treatment. N=60 neurons for both conditions **C)** Number of granules within the soma. Data are represented as mean +/- SEM.

Statistical significance was calculated by Z test (**B**) or by Student's t-test (**C**).

Figure S3

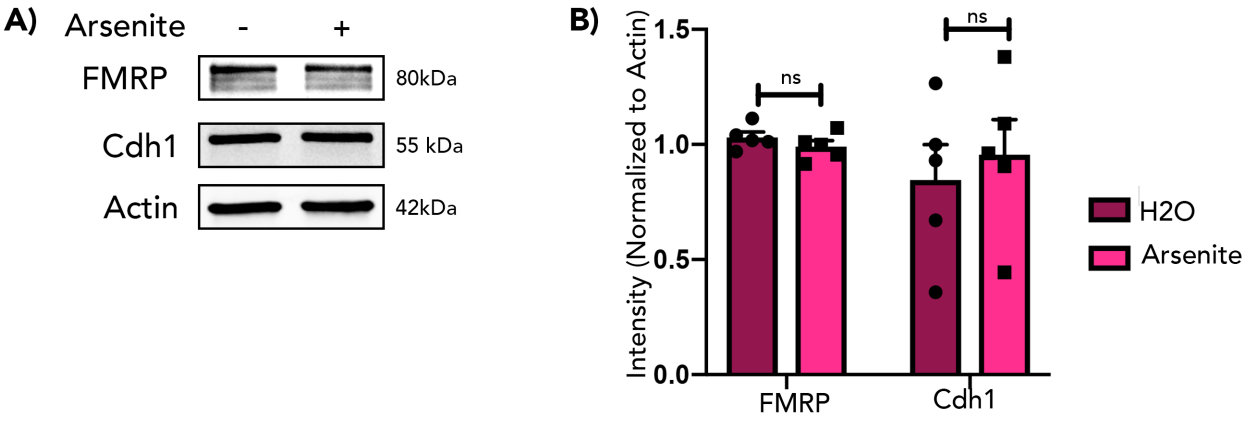
A)
Arsenite
-
+
FMRP
80kDa
Cdh1
55 kDa
Actin
42kDa
B)
Intensity (Normalized to Actin)
1.5
1.0
0.5
0.0
ns
ns
FMRP
Cdh1
H2O
Arsenite

Figure S3. **Sodium arsenite treatment does not affect FMRP or Cdh1 expression, Related to Figure 4**

A) DIV 14 cortical neurons were treated with sodium arsenite for 45 minutes and lysed and immunoblotted for FMRP, Cdh1, and actin. **B)** Quantification of FMRP and Cdh1 normalized to actin. Data are represented as mean +/- SEM. Statistical significance was calculated by two-way ANOVA.

Figure S4

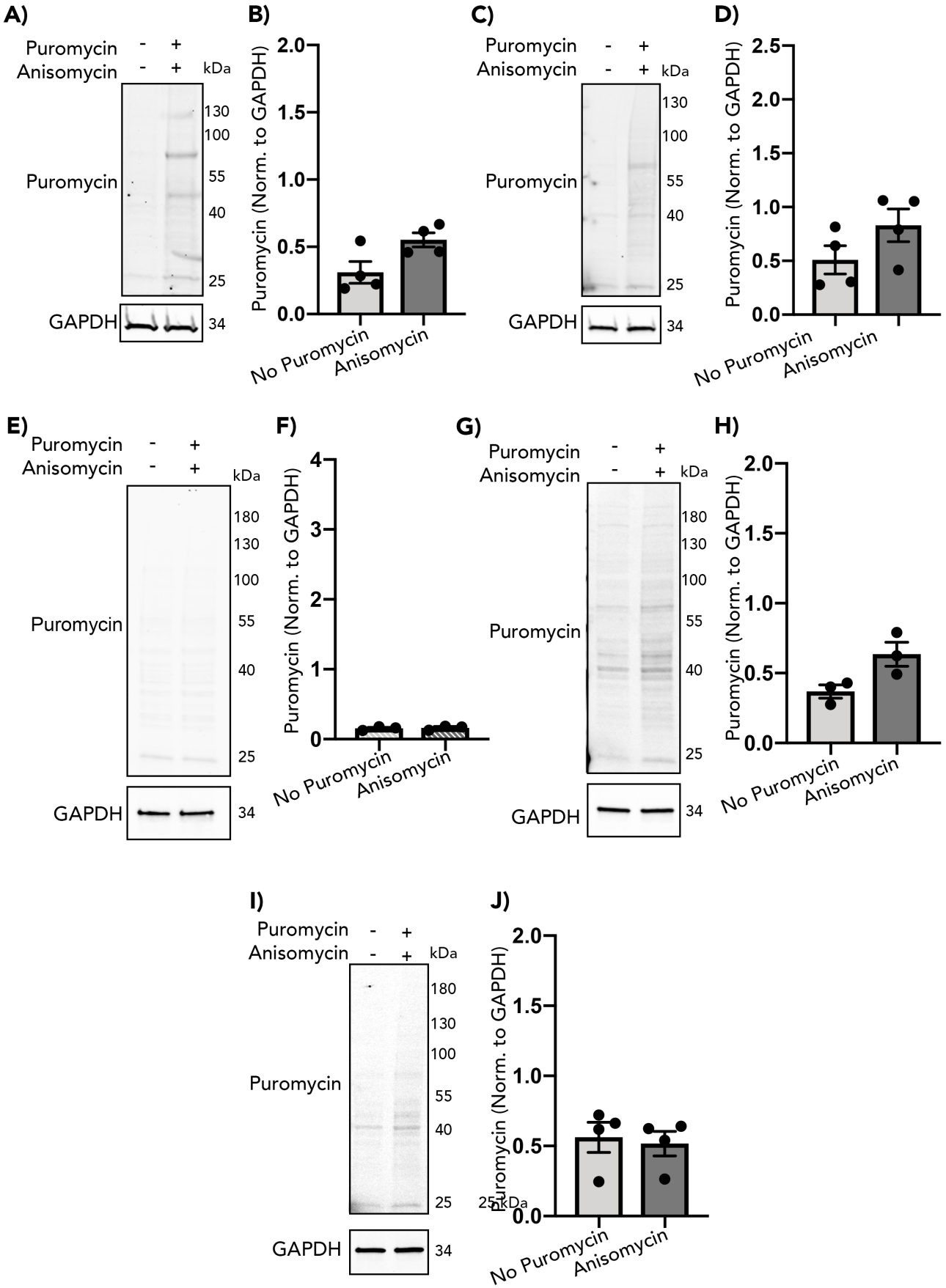
A)
Puromycin - +
Anisomycin - + kDa
130
100
Puromycin
55
40
25
GAPDH
34
B)
Puromycin (Norm. to GAPDH)
2.0
1.5
1.0
0.5
0.0
No Puromycin
Anisomycin
C)
Puromycin - +
Anisomycin - + kDa
130
100
Puromycin
55
40
25
GAPDH
34
D)
Puromycin (Norm. to GAPDH)
2.5
2.0
1.5
1.0
0.5
0.0
No Puromycin
Anisomycin
E)
Puromycin - +
Anisomycin - + kDa
180
130
100
Puromycin
55
40
25
GAPDH
34
F)
Puromycin (Norm. to GAPDH)
4
3
2
1
0
No Puromycin
Anisomycin
G)
Puromycin - +
Anisomycin - + kDa
180
130
100
Puromycin
55
40
25
GAPDH
34
H)
Puromycin (Norm. to GAPDH)
2.0
1.5
1.0
0.5
0.0
No Puromycin
Anisomycin
I)
Puromycin - +
Anisomycin - + kDa
180
130
100
Puromycin
55
40
25
GAPDH
34
J)
Puromycin (Norm. to GAPDH)
2.0
1.5
1.0
0.5
0.0
25 kDa
No Puromycin
Anisomycin

Figure S4. Controls for puromycin labeling, Related to Figures 1, 2, and S1

A,B) No puromycin and anisomycin (40μM) controls for Figure 1A. **C,D)** No puromycin and anisomycin (40μM) controls for Figure 1C. **E,F)** No puromycin and anisomycin (40μM) controls for Figure 2A. **G,H)** No puromycin and anisomycin (40μM) controls for Figure 2C. **I,J)** No puromycin and anisomycin (40μM) controls for Figure S1B.

Supplemental Information

Transparent Methods

Key Resources Table

Reagent/Resource	Source	Identifier
Antibodies		
Mouse anti-β-Actin	Thermo Fisher	AM4302
Rabbit anti-Caprin	Proteintech	15112-1-AP
Mouse anti-FLAG	Sigma	F1804
Rabbit anti-FLAG	Bethyl	A190-102A
Rabbit anti-FMR1 C terminal	Sigma	F4055
Rabbit anti-FMRP 7G-1	DSHB	7G1-1-S
Mouse anti-FMRP	BioLegend	834701
Rabbit anti-FXR1	Proteintech	13194-1-AP
Rabbit anti-GAPDH	Cell Signaling	2118S
Rabbit anti-G3BP1	Proteintech	13057-2-AP
Rabbit anti-hnRNP-U	Proteintech	14599-1-AP
Rabbit anti-HuR	Proteintech	11910-1-AP
Guinea Pig anti-MAP2	Synaptic Systems	188 004
Rabbit anti-myc	Bethyl	A190
Mouse anti-puromycin	Millipore	MABE343
Mouse anti-puromycin	Kerafast	EQ0001
Mouse anti-RPS3	Proteintech	66046-1-IG
Mouse anti-tubulin	Sigma-Aldrich	T6199
Mouse anti-Ubiquitin (FK2)	Millipore	04-263

Alexa Fluor 488 Donkey anti-Rabbit IgG (H+L)	Life Technologies	A21206
Donkey Anti-Mouse IgG Cy3	Jackson ImmunoResearch	715-165-150
Donkey Anti-Rabbit IgG Cy3	Jackson ImmunoResearch	711-165-152
Alexa Fluor 647 Goat anti-Guinea Pig IgG (H+L)	Thermo Fisher Scientific	A21450
Donkey anti-Rabbit IgG IR 800CW	LiCor	926-32213
Donkey anti-Mouse IgG IR 680LT	LiCor	926-68022
Chemicals, Peptides, and Recombinant Proteins		
Apcin	Sigma-Aldrich	SML 1503
Sodium arsenite	Millipore-Sigma	S7400
proTAME	Cayman Chemicals	25835
MG132	Sigma-Aldrich	M7449
3x FLAG Peptide	Sigma-Aldrich	F4799
Experimental Models: Cell Lines		
Neuro2A Cells	ATCC	CCL-131
Recombinant DNA		
mCherry-myc	Emory Integrated Genomics Core	n/a
mCherry-Cdh1-myc	Emory Integrated Genomics Core	n/a

Myc-DDK	Origene	PS10001
Myc-DDK-*Fzr1*	Origene	MR207910
mCMV-TurboRFP shRNA control	Dharmacon	VSC11715
mCMV-TurboRFP shRNA *Cdh1*	Dharmacon	V3SVMM08_13411185
Software and Algorithms		
Prism	GraphPad Software	Version 8
Image J	NIH	

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Gary Bassell (gary.bassell@emory.edu).

Experimental model and subject details

Neuro2A Cells: Neuro2A (N2A) cells, a mouse neuroblastoma cell line, were obtained from ATCC and were cultured in DMEM media supplemented with 10% fetal bovine serum and 10 mM Hepes, (Invitrogen) at 37° C in 5% CO_2.

Primary cortical neuronal cultures: Primary cortical neurons were prepared from C57BL/6J mouse embryos (Charles River) of either sex on embryonic day 17.

Supplemental Information

For Figure 4, *Fmr1*^{HET} females (backcrossed on C57BL/6J background) were crossed with WT C57BL/6J males (Jackson Laboratory) to generate litters of pups with mixed genotypes (*Fmr1*-KO, *Fmr1*^{HET}, or wild-type). Cerebral cortices were dissected and cultured from genotyped WT and *Fmr1*-KO pups on P0-P3.

Cortices were dissociated using trypsin (Thermo Fisher Scientific) and then mechanically dissociated in Minimum Essential Medium (MEM; Fisher) supplemented with 10% Fetal Bovine Serum (Hyclone). Neurons were plated on dishes or coverslips previously coated with poly-l-lysine (Sigma) and then cultured in standard growth medium (glial conditioned neurobasal (Fisher) supplemented with glutamax (Gibco) and B27 (Invitrogen)). Culture medium was exchanged once a week until experiments were performed; cultures were maintained in an incubator regulated at 37° C in 5% CO_2. Animal protocols were approved by the institutional Animal Care and Use Committee at Emory University.

Methods Details

Transfection/Transduction

N2A cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer instructions 24 hours prior to experimental use.

Neurons were transduced with lentivirus at DIV 7 and were utilized at DIV 14-16 for experimental use.

Pharmacology

Supplemental Information

Apcin (2μM) (Sigma-Aldrich) or DMSO vehicle treatment was carried out for 16-18 hours. proTAME (12μM) (Cayman chemicals) or DMSO vehicle treatment was carried out for 4 hours. To induce stress granule formation, sodium arsenite (Millipore Sigma) (0.5mM) or water treatment was carried out for 45 minutes. Treatments were done at 37°C.

Puromycylation

Following transduction and/or drug treatment, neurons were washed with warm neurobasal media containing B27 and glutamax with or without 10µg/mL puromycin (Sigma-Aldrich) for 75 minutes at 37°C. The protein synthesis inhibitor anisomycin (Sigma-Aldrich) was used as a control at 40µM. Cells were then washed ice cold 1x PBS and lysed in buffer A with 0.5% Triton-X100. Lysates were sonicated and incubated on ice for 30 minutes. Lysate protein concentration was then measured by BCA assay (ThermoFisher Scientific) and then used for western blotting.

Transfected N2As were washed with warm DMEM and incubated with or without 10µg/mL puromycin (Sigma-Aldrich) for 45 minutes at 37°C. Cells were lysed and utilized for western blotting.

FLAG Immunoprecipitation

For FLAG immunoprecipitation, was performed as described by Gokhale et al. (Gokhale et al., 2012). N2A cells were transfected with FLAG-Cdh1 (Origene MR207910) with PolyMag Neo (OZ Biosciences) for 24 hours, as per manufacturer instructions. Cells were rinsed twice with 1x PBS and lysed in buffer A (150 mM NaCl, 10mM HEPES, 1mM EGTA, and 0.1mM $MgCl_2$, pH 7.4) with 0.5% Triton-X100 and Complete anti-protease (Roche). Cells were sonicated followed by incubation on ice for 30 minutes. Cell lysates were centrifuged at 16,100 x g for 15 minutes.

Clarified supernatant was collected and protein concentration was measured by Bradford Assay (Bio-Rad). 500ug of protein extract was added to 30uL of Dynabeads (Invitrogen 11031) and incubated for 3 hours at 4° C. In some assays, immunoprecipitation was performed in the presence of antigenic 3x-FLAG peptide (340uM, Sigma F4799) as a control. After incubation, beads were washed 6 times with buffer A with 0.1% Triton X-100. Proteins were eluted from the beads with 2-hour incubation on ice with buffer A and 340uM 3x-FLAG-antigenic peptide. Samples were resolved by SDS-PAGE and contents analyzed by immunoblot or silver stain. For proteomic analysis, proteins eluted from the beads were combined and concentrated by TCA precipitation.

Endogenous Cdh1 Immunoprecipitation

Embryonic brain tissues were collected from E17 (C57BL/6) mouse embryos. Brain tissues were lysed in buffer A (150mM NaCl, 10mM HEPES, 1mM EGTA, and 0.1mM MgCl2, pH 7.4) with 0.5% TX-100 and Halt Protease and Phosphatase inhibitor (Thermo Fisher 78441) by sonication. After incubation on ice for 30 minutes, tissue lysates were centrifuged at 12,000 x g for 30 minutes. Clarified supernatants were collected and protein concentrations were measured by BCA assay (Thermo Fisher 23227). Protein lysates were diluted to 5ug/ul by lyse buffer and 100ul of protein lysates were mixed with 400ul of buffer A as immunoprecipitation input. 20uL of ProteinG Dynabeads (Thermo Fisher 10-003-D) were washed by buffer A and then incubated with either 2ug rabbit anti-FZR1 (Cdh1) (Proteintech 16368-1) antibody or 2ug normal rabbit IgG (Millipore 12-370) for 2 hours. After incubation, beads were washed by buffer A, and then incubated with immunoprecipitation input at 4C overnight. After incubation, beads were washed sequentially by buffer A with 0.1% Triton X-100, buffer A with 50mM NaCl and 0.25% Triton X-100, and buffer A with 100mM NaCl and 0.5% Triton X-100. Proteins were eluted from the beads with 50ul of

0.2mM glycine, pH2.6. Samples were run on a 4-20% Criterion TGX gel (Bio-Rad 4561093) and transferred onto Nitrocellular membrane. Blots were blocked with 5% milk in 1x PBST for 1hr at room temperature, and then incubated overnight at 4C with primary antibody diluted in blocking buffer. Blots were incubated with HRP conjugated secondary antibodies (ABclonal AS014 and AS003) diluted in blocking buffer for 1hr at room temperature. Three 10-minute washes with PBST were performed before and after addition of secondary antibodies. Blots were developed using Tanon High-sig ECL kit (ABclonal 180-501) and Amersham Hyperfilm (GE Lifesciences).

Western Blotting

For FLAG immunoprecipitation experiments, samples were run on a 4-20% Criterion TGX gel (Bio-Rad) and transferred onto PVDF membrane. Blots were blocked with 5% milk in 1x TBS-0.5%. TritonX-100 for 30 minutes at room temperature. Blots were incubated overnight at 4°C in primary antibody diluted in 3% BSA, 0.5% Sodium Azide, and 1x PBS. Blots were incubated in HRP secondary antibodies (Sigma) diluted in 5% milk in 1x TBS-0.5% TritonX-100 for 40 minutes at room temperature. Three 5-minute washes with TBS-0.5% TritonX-100 were performed before and after addition of secondary antibodies. Blots were developed using Western Lightning ECL Pro (Perkin Elmer) and Amersham Hyperfilm (GE Lifesciences).

For puromycin labeling experiments, 20µg of protein per sample were resolved by SDS-PAGE on a 4-20% Mini-Protean TGX protein gel (Bio-Rad) and transferred to a nitrocellulose membrane. Blots were blocked in Odyssey Blocking Buffer (LI-COR), and incubated overnight at 4°C in primary antibodies diluted in a 1:1 mix of blocking buffer and PBS-Tween-0.1%. Blots were incubated in secondary antibodies (LI-COR) diluted in PBS-Tween-0.1% for 1 hour at room

temperature. Three 10-minute washes with PBS-Tween-0.1% were performed before and after addition of secondary antibodies. Blots were viewed on a Bio-Rad ChemiDoc MP. Protein levels were assessed by quantitative densitometry using ImageJ.

Proteomics Analysis

Samples were analyzed for interactome analysis by MS Bioworks (http://www.MSBioworks.com). Proteomics samples were separated on a 10% Bis-Tris Novex mini-gel (Invitrogen) using the MES buffer system. The gel was stained with coomassie and excised into ten equally sized segments. Gel segments were processed using a robot (Progest, DigiLab). First, gel segments were washed with 25mM ammonium bicarbonate followed by acetonitrile. They were then reduced with 10mM dithiothreitol at 60°C followed by alkylation with 50mM iodoacetamide at RT. Then, segments were digested with trypsin (Promega) at 37°C for 4 hours. They were then quenched with formic acid and the supernatant was analyzed directly without further processing. The gel digests were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75µm analytical column at 350nL/min; both columns were packed with Luna C18 resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 FWHM resolution and 17,500 FWHM resolution, respectively. The fifteen most abundant ions were selected for MS/MS. Data were searched using a local copy of Mascot.

Bioinformatic Analysis

Supplemental Information

Gene ontology analysis was performed with Database Annotation, Visualization and Integrated Disovery (DAVID, https://david.ncifcrf.gov). Cytoscape with Enrichment Map plugin for visualizing DAVID outputs was used to represent the Biological Processes enriched within the Cdh1 interactome.

Immunofluorescence:

Cells were fixed in 4% paraformaldehyde for 15 minutes, washed three times for 10 minutes in PBS. Cells were blocked for 1 hour in blocking solution consisting of 5% normal donkey serum, 0.1% bovine serum albumin, and 0.1% Triton-X 100 in PBS. Cells were incubated overnight in primary antibodies diluted in blocking solution. The next day, cells were washed 3 times for 10 minutes in PBS. They were incubated in secondary antibodies in blocking solution for one hour at room temperature. Cells were washed 3 times for 10 minutes. Coverslips with the cells were dipped in ultrapure water and then mounted using Prolong Gold Antifade mounting media (Invitrogen). Cells were imaged using a Keyence BZ-X810 or a Nikon Eclipse TE300 widefield fluorescence microscope with a 60X objective. Z-series were acquired at 0.2μm steps and image stacks were deconvolved using AutoQuant X3 software and a 3-D blind algorithm.

Quantification and statistical analysis

Statistical analyses and graphs were prepared in GraphPad Prism (v.8). All data are expressed as mean +/- SEM. Replicates are reported in the figure legends or directly on the figures. For all experiments, α was set as 0.05. See figure legends for specific statistical analyses.

Quantification of Stress Granules

Supplemental Information

Coverslips for immunocytochemistry were blinded during imaging and quantification. Imaged neurons were classified as being stress granule positive or negative based on G3BP1 staining. Diffuse G3BP1 staining was classified as stress granule negative whereas cells with punctate G3BP1 staining were classified as stress granule positive. The total number of stress granule positive or negative neurons for each condition were then input into GrapPad Prism and a z-test was ran on the data.

Number of stress granules in the soma of each cell were quantified using Image J plugin TrackMate (Tinevez et al., 2017) with the Laplacian of Gaussian detector (threshold set from 5-15). Total area of the cell body was quantified using Image J plugin Mitomorphology macro (Dagda et al., 2009) with threshold set as 130-180. The number of granules were normalized to the cell body area.

Supplemental References

Dagda, R.K., Cherra, S.J., 3rd, Kulich, S.M., Tandon, A., Park, D., and Chu, C.T. (2009). Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. J Biol Chem *284*, 13843-13855.

Gokhale, A., Larimore, J., Werner, E., So, L., Moreno-De-Luca, A., Lese-Martin, C., Lupashin, V.V., Smith, Y., and Faundez, V. (2012). Quantitative proteomic and genetic analyses of the schizophrenia susceptibility factor dysbindin identify novel roles of the biogenesis of lysosome-related organelles complex 1. J Neurosci *32*, 3697-3711.

Tinevez, J.Y., Perry, N., Schindelin, J., Hoopes, G.M., Reynolds, G.D., Laplantine, E., Bednarek, S.Y., Shorte, S.L., and Eliceiri, K.W. (2017). TrackMate: An open and extensible platform for single-particle tracking. Methods *115*, 80-90.