# Cell-to-cell transmission of C9orf72 poly-(Gly-Ala) triggers key features of ALS/FTD

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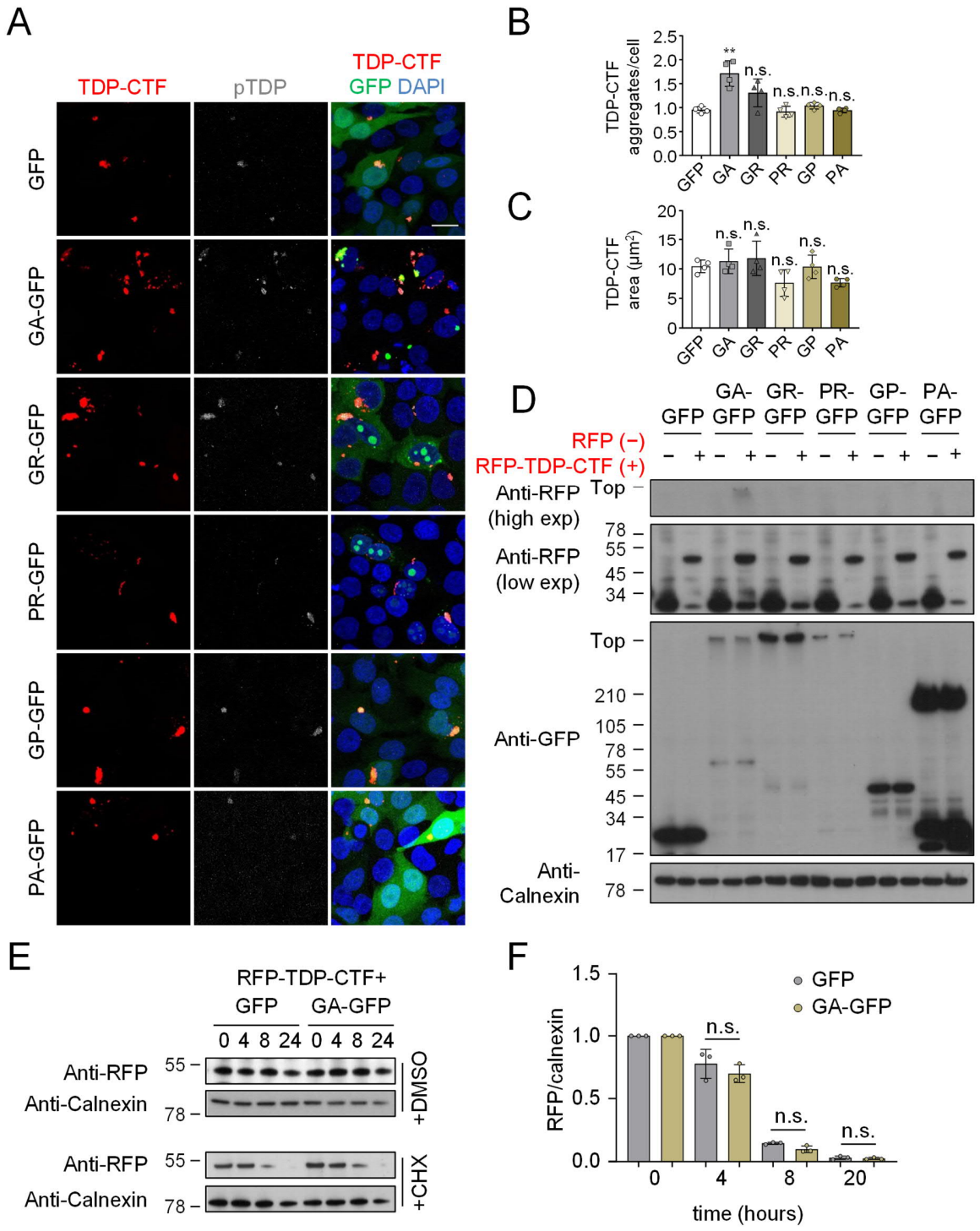
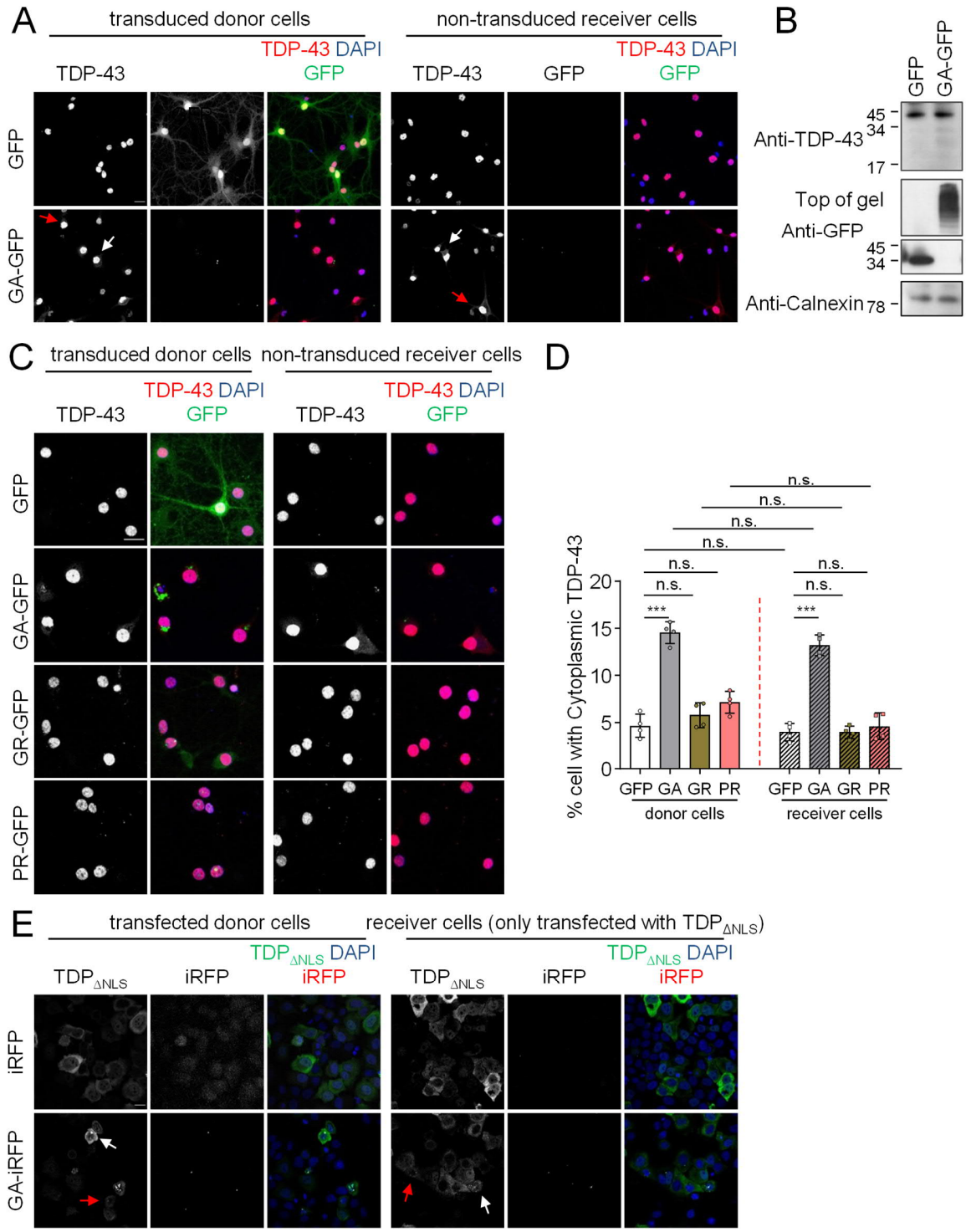



Appendix Figure S1. Poly-GA promotes TDP-43-CTF aggregation.

(A-C) HeLa cells were co-transfected with GFP or GFP-tagged DPR species and the RFP-tagged TDP-43 C-terminal fragment (TDP-CTF, amino acids 220-414) and analyzed for TDP-CTF aggregation. $GA_{175}$-GFP, GFP-$GR_{149}$, $PR_{175}$-GFP, GFP-$GP_{47}$ and $PA_{175}$-GFP were used. (A) Immunofluorescence shows enhanced TDP-CTF aggregation upon poly-GA expression. (B) Automated quantification of aggregate number per

cell and (C) aggregate average area in $\mu m^2$. n=4 biological replicates. In total 395 GFP, 412 $GA_{175}$-GFP, 385 GFP-$GR_{149}$, 387 $PR_{175}$-GFP, 381 GFP-$GP_{47}$, and 414 $PA_{175}$-GFP cells with TDP-CTF aggregates were analyzed. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ** denotes $p<0.01$. (D) Immunoblotting of HeLa cells co-transfected with GFP-DPRs and RFP-TDP-CTF or RFP control. Note that $GA_{175}$-GFP co-expression results in TDP-43 accumulation and high-molecular weight aggregation at the top of the gel. Control cells were co-transfected with GFP-DPRs and RFP. (E-F) HeLa cells were co-transfected with RFP-TDP-CTF and GFP or $GA_{175}$-GFP. 24h after transfection, cells were treated with vehicle or 150 µg/ml cycloheximide (+CHX) for 0, 4, 8, and 24h. Protein turn-over was measured by immunoblotting of cell lysates. (F) Quantification of RFP-TDP-CTF protein levels normalized to calnexin. n=4 biological replicates. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.



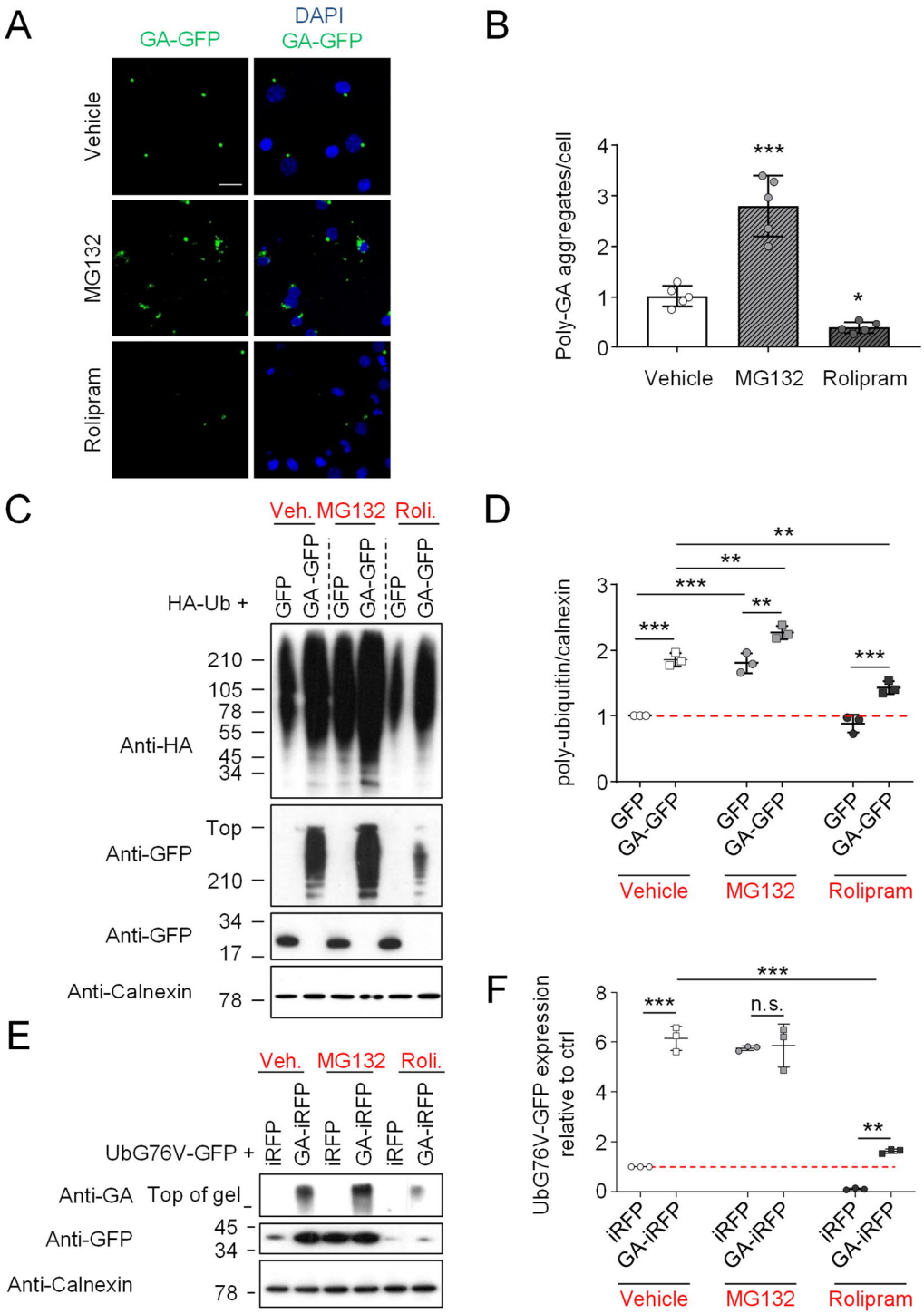

Appendix Figure S2. Cell-to-cell transmission of poly-GA causes cytoplasmic mislocalization of TDP-43.

(A) Additional images with larger fields of view from the experiments in Fig. 1B. Primary hippocampal neurons were transduced with GFP or $GA_{175}$-GFP (DIV4+4) and co-cultured with naïve primary neurons for 4 days. Endogenous TDP-43 and poly-GA aggregates in donor and receiver coverslips were analyzed by immunofluorescence. Cytoplasmic TDP-43 immunostaining is elevated not only in poly-GA transduced neurons, but also in the non-transduced receiver cells. White and red arrows indicate cells with

cytoplasmic TDP-43 in GFP positive and negative cells, respectively. In (B) HeLa cells were transfected with GFP or $GA_{175}$-GFP for 48h and immunoblotted for TDP-43. Note that there is no TDP-43 cleavage in $GA_{175}$-GFP expressing cells.

(C) Primary hippocampal neurons were transduced with GFP, $GA_{175}$-GFP, GFP-$GR_{149}$, and $PR_{175}$-GFP (DIV4+4) and co-cultured with naïve primary neurons for 4 days. Endogenous TDP-43 and poly-GA,-GR,-PR aggregates in donor and receiver coverslips were analyzed by immunofluorescence. (D) Automated quantification of cells with cytoplasmic TDP-43 in GFP or $GA_{175}$-GFP, GFP-$GR_{149}$, $PR_{175}$-GFP, transduced (donor), non-transduced (receiver) neurons. n=4 biological replicates. In total 334 donor GFP, 300 donor $GA_{175}$-GFP, 315 donor GFP-$GR_{149}$, 307 donor $PR_{175}$-GFP,322 receiver GFP and 302 receiver $GA_{175}$-GFP, 319 receiver GFP-$GR_{149}$, 294 receiver $PR_{175}$-GFP cells were analyzed. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. *** denotes $p<0.001$.

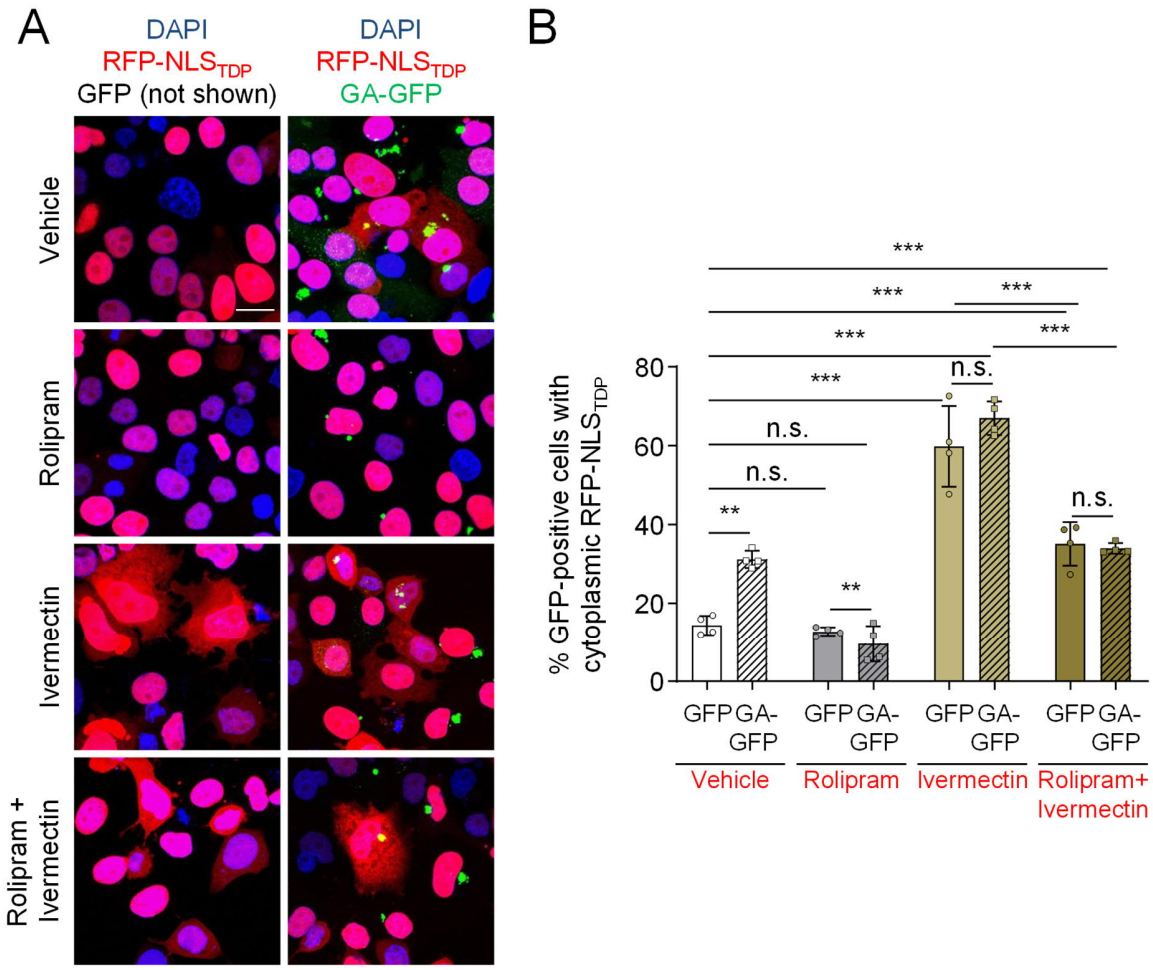
(E) Additional images with larger fields of view from the experiments in Fig. 1D. Immunofluorescence of co-culture model in HeLa cells transfected with iRFP or $GA_{175}$-iRFP in the donor compartment and TDP-$43_{\Delta NLS}$-GFP in donor and receiver compartment. White and red arrows indicate cells with cytoplasmic TDP-43 in GFP positive and negative cells, respectively.




Appendix Figure S3. Rolipram reduces poly-GA aggregate number and enhances proteasomal activity.

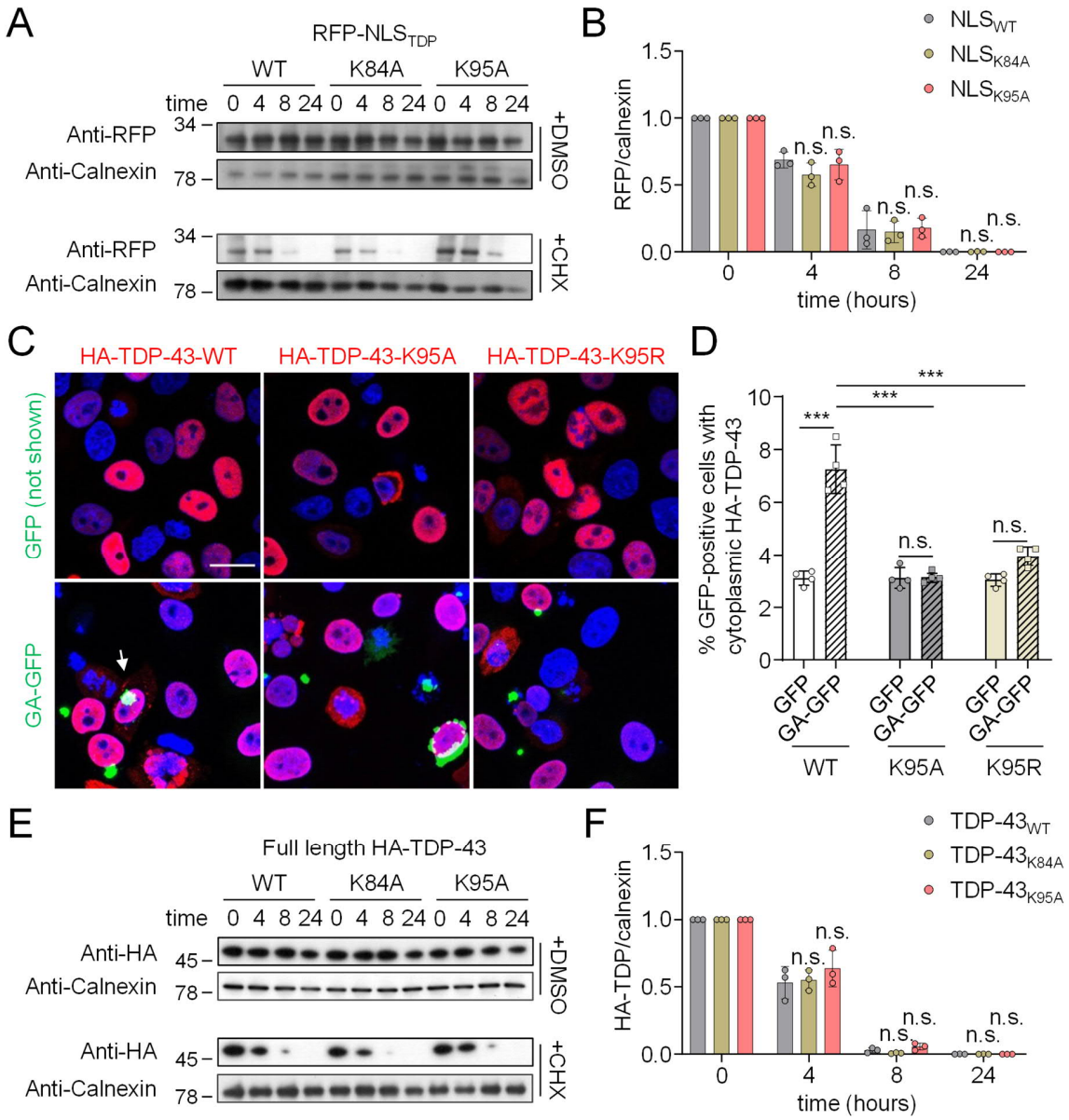
(A) Immunofluorescence of primary hippocampal neurons transduced with GFP or $GA_{175}$-GFP and treated with MG132 (10 µM), rolipram (30 µM) or vehicle control for 16h. (B) Automated analysis of ratio of poly-GA aggregate number to cell number. n=5 biological replicates. In total 261 cells treated with vehicle, 302 cells with MG132 and 351 cells with rolipram treatments were analyzed. Scatter plot with

bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. * denotes p<0.05, and *** denotes p<0.001. Scale bar denotes 20 µm. (C, D) Immunoblots of HeLa cells that were co-transfected with HA-Ubiquitin and GFP or $GA_{175}$-GFP and treated as above, and furthermore analyzed by densitometry. Scatter dot plot, mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ** denotes p<0.01, and *** denotes p<0.001. (E,F) HeLa cells were co-transfected with $Ub_{G76V}$-GFP reporter and iRFP or $GA_{175}$-iRFP and treated as above. (D) Immunoblots of n=3 biological replicates were (E) quantified by densitometry. Scatter dot plot, mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ** denotes p<0.01, and *** denotes p<0.001.




Appendix Figure S4. Rolipram treatment promotes degradation of cytoplasmic TDP-43 RFP-NLS reporter.

(A) HeLa cells were co-transfected with RFP-NLS$_{TDP}$ reporter and GFP or GA$_{175}$-GFP. 24h after transfection, cells were treated with nuclear import inhibitor ivermectin (10 µM), proteasome activator rolipram (30 µM), or both, and analyzed by immunofluorescence. (B) Automated quantification shows cytoplasmic mislocalization of RFP-NLS reporter upon ivermectin treatment that is partially rescued by additional rolipram treatment. n=4 biological replicates. In total 337 GFP and 361 GA$_{175}$-GFP cells treated with vehicle, 383 GFP and 328 GA$_{175}$-GFP cells treated with rolipram, 337 GFP and 329 GA$_{175}$-GFP cells treated with ivermectin, 331 GFP and 340 GA$_{175}$-GFP cells treated with rolipram and ivermectin were analyzed. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ** denotes $p<0.01$, and *** denotes $p<0.001$.




Appendix Figure S5. TDP-43 K95 mutation blocks poly-GA induced cytoplasmic mislocalization of full length TDP-43 without affecting overall TDP-43 turnover.

(A, B) HeLa cells were transfected with RFP-NLS$_{TDP}$ wild-type, K95A or K95R. 24h after transfection, cells were treated with vehicle or 150 µg/ml cycloheximide (+CHX) for 0, 4, 8, and 24h. Protein stability was measured by immunoblotting of cell lysates. (B) Quantification of RFP-NLS$_{TDP}$ protein levels normalized to calnexin. n=4 biological replicates. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.

(C) HeLa cells were co-transfected with HA-tagged full-length TDP-43 wild-type, K95A or K95R together with GFP or GA$_{175}$-GFP and analyzed by immunofluorescence. (D) Automated quantification of cells with cytoplasmic HA-TDP-43 in GFP or GA$_{175}$-GFP transfected HeLa cells. n=4 biological replicates. In total 322 GFP and 314 GA$_{175}$-GFP cells with TDP-43 WT, 291 GFP and 310 GA$_{175}$-GFP cells with TDP-43 K95A, 363 GFP and 328 GA-GFP cells with TDP-43 K95R were analyzed. Scatter plot with bar-graphs of mean ± SD.

One-way ANOVA with Tukey's multiple comparisons test. *** denotes $p<0.001$. Scale bar denotes 20 µm.

(E, F) HeLa cells were transfected with full length TDP-43 wild-type, K95A or K95R. 24h after transfection, cells were treated with vehicle or 150 µg/ml cycloheximide (+CHX) for 0, 4, 8, and 24h. Protein stability was measured by immunoblotting of cell lysates. (F) Full length TDP-43 protein levels were quantified and normalized to calnexin. n=4 biological replicates. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.