Cell Reports, Volume *42*

Supplemental information

Poly(GR) interacts with key stress granule factors promoting its assembly into cytoplasmic inclusions

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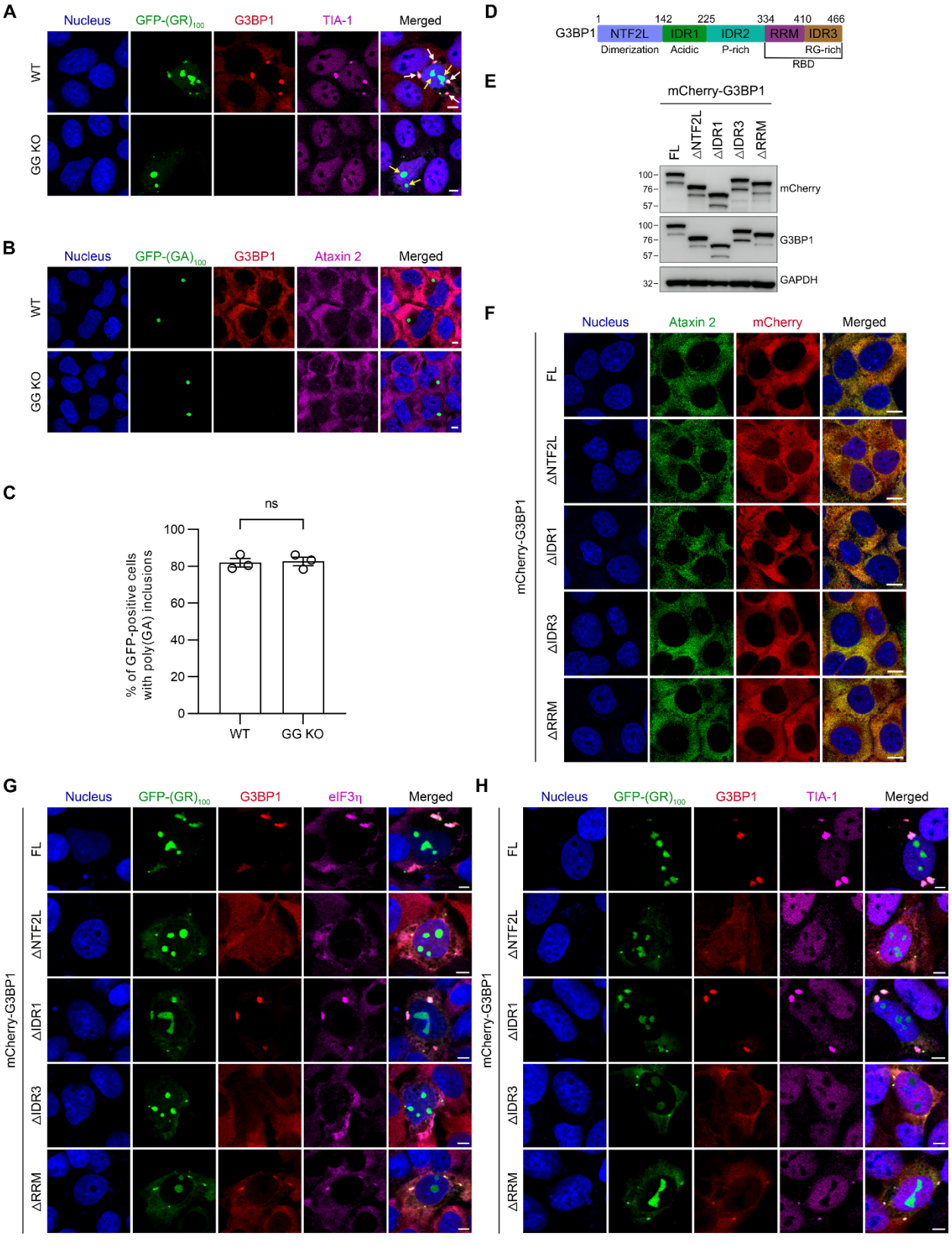

A
Nucleus
GFP-(GR)100
G3BP1
TIA-1
Merged
WT
GG KO
B
Nucleus
GFP-(GA)100
G3BP1
Ataxin 2
Merged
WT
GG KO
C
ns
% of GFP-positive cells with poly(GA) inclusions
100
80
60
40
20
0
WT
GG KO
D
1
142
225
334
410
466
G3BP1
NTF2L
IDR1
IDR2
RRM
IDR3
Dimerization
Acidic
P-rich
RG-rich
RBD
E
mCherry-G3BP1
FL
ΔNTF2L
ΔIDR1
ΔIDR3
ΔRRM
100
76
57
mCherry
G3BP1
32
GAPDH
F
Nucleus
Ataxin 2
mCherry
Merged
mCherry-G3BP1
FL
ΔNTF2L
ΔIDR1
ΔIDR3
ΔRRM
G
Nucleus
GFP-(GR)100
G3BP1
eIF3η
Merged
mCherry-G3BP1
FL
ΔNTF2L
ΔIDR1
ΔIDR3
ΔRRM
H
Nucleus
GFP-(GR)100
G3BP1
TIA-1
Merged
mCherry-G3BP1
FL
ΔNTF2L
ΔIDR1
ΔIDR3
ΔRRM

Figure S1. G3BP1/2 is crucial for poly(GR) inclusion formation, related to Figure 1. (**A**) Double-immunofluorescence staining for G3BP1 and TIA-1 in U2OS WT and G3BP1/2 knockout (GG KO) cells expressing GFP-$(GR)_{100}$ 48 h post-transfection. White arrows indicate cytoplasmic poly(GR) inclusions and yellow arrows indicate nucleolar poly(GR) accumulation. Scale bars, 5 μm. (**B**) Double-immunofluorescence staining for G3BP1 and ataxin 2 in WT and GG KO U2OS cells expressing GFP-$(GA)_{100}$ 48 h post-transfection. Scale bars, 5 μm. (**C**) Quantification of the percentage of GFP-positive cells containing poly(GA) inclusions in WT and GG KO U2OS cells (n = 3 independent experiments). (**D**) Schematic of G3BP1 protein domains. (**E**) Immunoblot analysis using the indicated antibodies in GG KO cells stably expressing mCherry-G3BP1 species to confirm expression of mCherry-G3BP1 proteins. GAPDH was used as control for protein loading. (**F**) Immunofluorescence staining for ataxin 2 in GG KO U2OS cells stably expressing mCherry-G3BP1 species under basal conditions. Scale bars, 5 μm. (**G**, **H**) Immunofluorescence staining for eIF3η (**G**) or TIA-1 (**H**) in GG KO U2OS cells expressing GFP-$(GR)_{100}$ and stably expressing mCherry-G3BP1 species. Scale bars, 5 μm. Data shown as mean ± SEM, ns (not significant) $P = 0.8337$, unpaired two-tailed t-test.

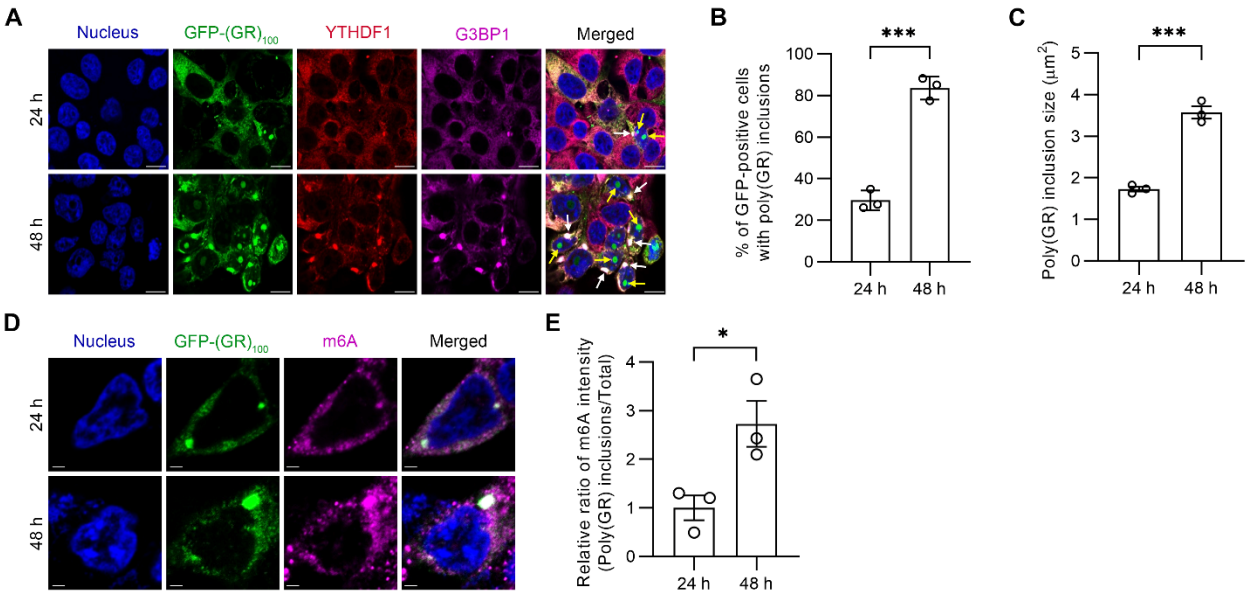
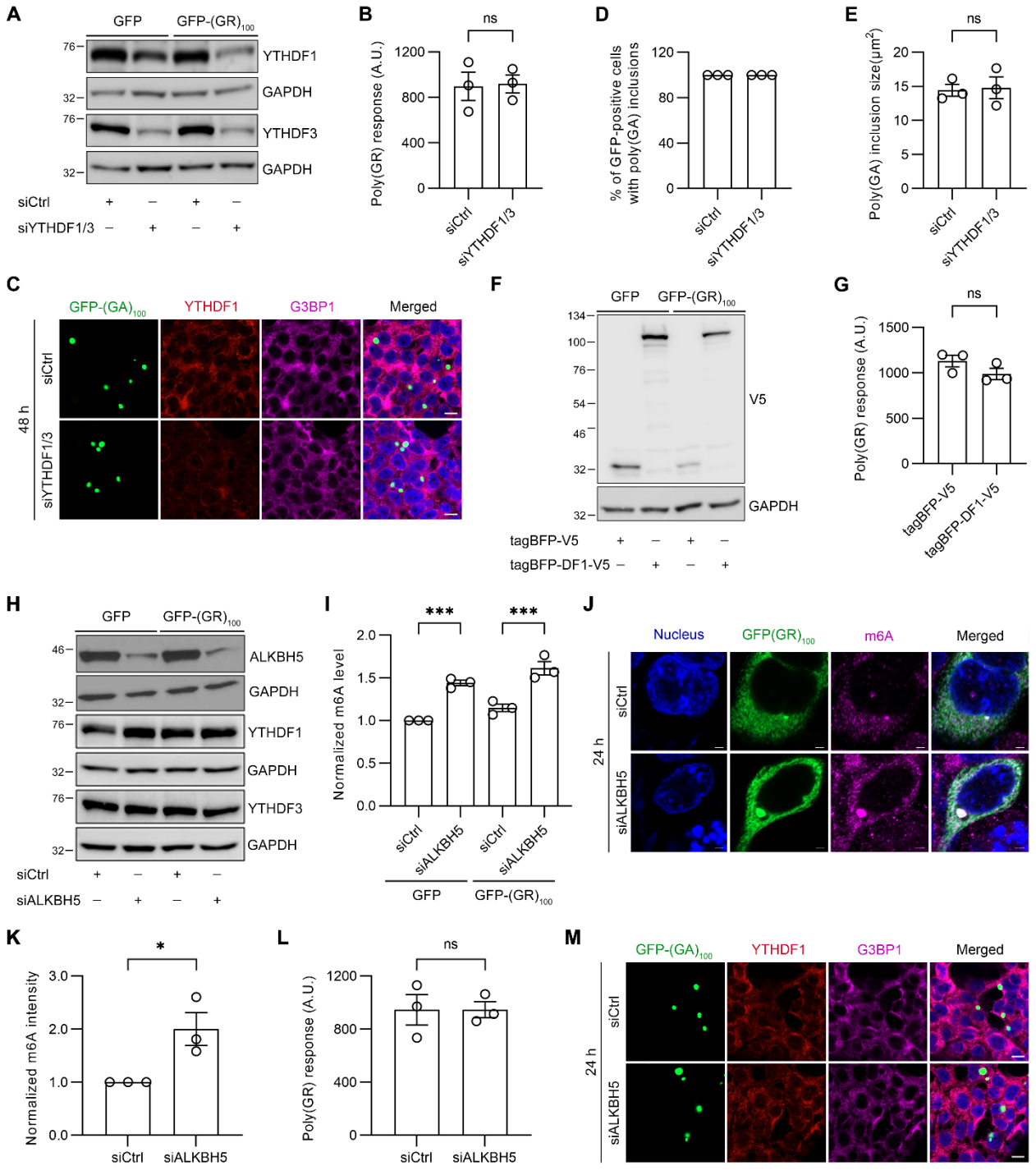


Figure S2. Time-dependent increase of cytoplasmic poly(GR) inclusions and enrichment of m6A modified RNAs in poly(GR) inclusions, related to Figure 2. (**A**) Double-immunofluorescence staining for YTHDF1 and G3BP in HEK293T cells expressing GFP-$(GR)_{100}$ for 24 or 48 h. White arrows indicate cytoplasmic poly(GR) inclusions and yellow arrows indicate nucleolar poly(GR) accumulation. Scale bars, 10 µm. (**B**) Quantification of the percentage of cells containing poly(GR) inclusions in GFP-$(GR)_{100}$-expressing HEK293T cells (n = 3 independent experiments). (**C**) Quantification of the size of poly(GR) inclusions in GFP-$(GR)_{100}$-expressing HEK293T cells (n = 3 independent experiments). (**D**) Double-immunofluorescence staining for GFP and m6A in HEK293T cells expressing GFP-$(GR)_{100}$ for 24 or 48 h; n = 3 independent experiments, scale bars, 2 µm. (**E**) Quantification of the relative ratio of oligo(dT) intensity [poly(GR) inclusions/Total] in GFP-$(GR)_{100}$-expressing HEK293T cells (n = 3 independent experiments). Data shown as mean ± SEM. In (B) *** $P = 0.0002$, unpaired two-tailed t-test. In (C), **** $P < 0.0001$, unpaired two-tailed t-test. In (E), * $P = 0.0322$, unpaired two-tailed t-test.


A
GFP
GFP-(GR)100
76
YTHDF1
32
GAPDH
76
YTHDF3
32
GAPDH
siCtrl + – + –
siYTHDF1/3 – + – +
B
ns
Poly(GR) response (A.U.)
1200
800
400
0
siCtrl
siYTHDF1/3
D
% of GFP-positive cells with poly(GA) inclusions
120
80
40
0
siCtrl
siYTHDF1/3
E
ns
Poly(GA) inclusion size(μm2)
20
15
10
5
0
siCtrl
siYTHDF1/3
C
GFP-(GA)100
YTHDF1
G3BP1
Merged
48 h
siCtrl
siYTHDF1/3
F
GFP
GFP-(GR)100
134
100
76
54
46
32
V5
32
GAPDH
tagBFP-V5 + – + –
tagBFP-DF1-V5 – + – +
G
ns
Poly(GR) response (A.U.)
1500
1000
500
0
tagBFP-V5
tagBFP-DF1-V5
H
GFP
GFP-(GR)100
46
ALKBH5
32
GAPDH
76
YTHDF1
32
GAPDH
76
YTHDF3
32
GAPDH
siCtrl + – + –
siALKBH5 – + – +
I
***
***
Normalized m6A level
2.0
1.5
1.0
0.5
0.0
siCtrl
siALKBH5
siCtrl
siALKBH5
GFP
GFP-(GR)100
J
Nucleus
GFP(GR)100
m6A
Merged
24 h
siCtrl
siALKBH5
K
*
Normalized m6A intensity
3.0
2.0
1.0
0.0
siCtrl
siALKBH5
L
ns
Poly(GR) response (A.U.)
1200
800
400
0
siCtrl
siALKBH5
M
GFP-(GA)100
YTHDF1
G3BP1
Merged
24 h
siCtrl
siALKBH5

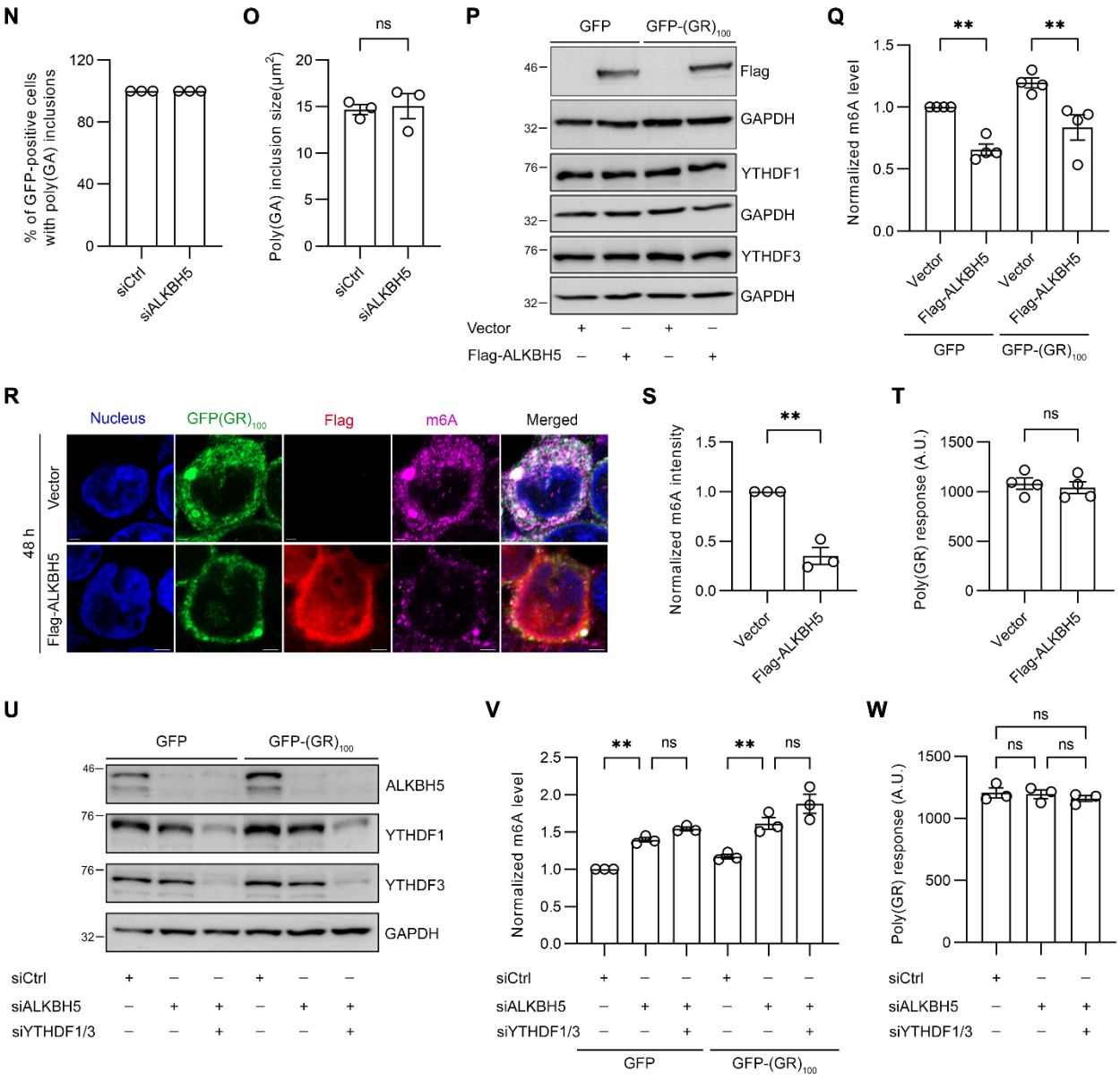


Figure S3. Modulating YTHDF proteins and m6A-modified RNAs does not affect poly(GR) expression, related to Figure 3. (**A**) Immunoblots of YTHDF1 or YTHDF3 in YTHDF1/3-depleted HEK293T cells expressing GFP or GFP-$(GR)_{100}$. GAPDH was used as a loading control. (**B**) MSD immunoassay for poly(GR) in YTHDF1/3-depleted HEK293T cells expressing GFP or GFP-$(GR)_{100}$ (n = 3 independent experiments). (**C**) Double-immunofluorescence staining for YTHDF1 and G3BP1 in YTHDF1/3-depleted HEK293T cells expressing GFP-$(GA)_{100}$ 48 h post-transfection. Scale bars, 10 μm. (**D**) Quantification of the percentage of GFP-positive cells containing poly(GA) inclusions in YTHDF1/3-depleted HEK293T cells expressing GFP-$(GA)_{100}$ (n = 3 independent experiments). (**E**) Quantification of the size of poly(GA) inclusions in YTHDF1/3-depleted HEK293T cells expressing GFP-$(GA)_{100}$ (n = 3 independent experiments). (**F**) Immunoblots of V5 in GFP or GFP-$(GR)_{100}$ overexpressing HEK293T cells co-expressing GFP or GFP-$(GR)_{100}$ and either tagBFP-V5 or tagBFP/V5 tagged wild-type YTHDF1 (tagBFP-

DF1-V5). GAPDH was used as a loading control. (**G**) MSD immunoassay for poly(GR) in GFP or GFP-$(GR)_{100}$ overexpressing HEK293T cells co-expressing either tagBFP-V5 or tagBFP-DF1-V5. (n = 3 independent experiments). (**H**) Immunoblot for ALKBH5, YTHDF1 and YTHDF3 in ALKBH5-depleted HEK293T cells expressing either GFP or GFP-$(GR)_{100}$. GAPDH was used for a loading control. (**I**) m6A-modified RNA quantification using ELISA in ALKBH5-depleted HEK293T cells expressing either GFP or GFP-$(GR)_{100}$ (n = 3 independent experiments). (**J**) Double-immunofluorescence staining for GFP and m6A in ALKBH5-depleted HEK293T cells expressing GFP-$(GR)_{100}$ 24 h post-transfection. Scale bars, 2 µm. (**K**) Quantification of the intensity for m6A in ALKBH5-depleted HEK293T cells expressing GFP-$(GR)_{100}$ 24 h post-transfection (n = 3 independent experiments). (**L**) MSD immunoassay for poly(GR) in ALKBH5-depleted HEK293T cells expressing either GFP or GFP-$(GR)_{100}$ (n = 3 independent experiments). (**M**) Double-immunofluorescence staining for YTHDF1 and G3BP1 in ALKBH5-depleted HEK293T cells expressing GFP-$(GA)_{100}$ 24 h post-transfection. Scale bars, 10 µm. (**N**) Quantification of the percentage of GFP-positive cells containing poly(GA) inclusions in ALKBH5-depleted HEK293T cells expressing GFP-$(GA)_{100}$ (n = 3 independent experiments). (**O**) Quantification of the size of poly(GA) inclusions in ALKBH5-depleted HEK293T cells expressing GFP-$(GA)_{100}$ (n = 3 independent experiments). (**P**) Immunoblots for Flag, YTHDF1 and YTHDF3 in Flag-ALKBH5-expressing HEK293T cells co-expressing either GFP or GFP-$(GR)_{100}$. GAPDH was used as a loading control. (**Q**) m6A-modified RNA quantification in Flag-ALKBH5-expressing HEK293T cells co-expressing either GFP or GFP-$(GR)_{100}$ by ELISA (n = 4 independent experiments). (**R**) Triple-immunofluorescence staining for GFP, Flag and m6A in Flag-ALKBH5-overexpressing HEK293T cells co-expressing GFP-$(GR)_{100}$ 48 h post-transfection. Scale bars, 2 µm. **(S)** Quantification of the intensity for m6A in Flag-ALKBH5 overexpressing HEK293T cells co-expressing GFP-$(GR)_{100}$ 48 h post-transfection (n = 3 independent experiments). (**T**) MSD immunoassay for poly(GR) in Flag-ALKBH5 expressing HEK293T cells co-expressing either GFP or GFP-$(GR)_{100}$ (n = 4 independent experiments). (**U**) Immunoblots for ALKBH5, YTHDF1 and YTHDF3 in ALKBH5-depleted and/or YTHDF1/3-depleted HEK293T cells overexpressing either GFP or GFP-$(GR)_{100}$. GAPDH was used for a loading control. (**V**) m6A-modified RNA quantification in ALKBH5-depleted and/or YTHDF1/3-depleted HEK293T cells overexpressing GFP-$(GR)_{100}$ by ELISA (n = 3 independent experiments). (**W**) MSD immunoassay for poly(GR) in ALKBH5-depleted and/or YTHDF1/3-depleted HEK293T cells overexpressing GFP-$(GR)_{100}$ (n = 3 independent experiments). Data shown as mean ± SEM. In (B), ns (not significant) $P = 0.8931$, unpaired two-tailed t-test. In (E), ns $P = 0.8497$, unpaired two-tailed t-test. In (G), ns $P = 0.1877$, unpaired two-tailed t-test. In (I), *** (left to right) $P = 0.0009$, and $P = 0.0006$, one-way ANOVA. Tukey's post hoc analysis. In (K), * $P = 0.0318$, unpaired two-tailed t-test. In (L), ns $P = 0.9962$, unpaired two-tailed t-test. In (O), ns $P = 0.8128$, unpaired two-tailed t-test. In (Q), ** (left to right) $P = 0.0070$ and $P = 0.0049$, one-way ANOVA, Tukey`s post hoc analysis. In (S), ** $P = 0.0016$, unpaired two-tailed t-test. In (T), ns $P = 0.6359$, unpaired two-tailed t-test. In (V), ** (left to right) $P = 0.0098$ and $P = 0.0038$; ns (left to right) $P = 0.4731$ and

$P = 0.1065$, one-way ANOVA, Tukey`s post hoc analysis. In (W), ns (left to right) $P = 0.9714$, $P = 0.6208$, and $P = 0.7507$, one-way ANOVA, Tukey`s post hoc analysis.

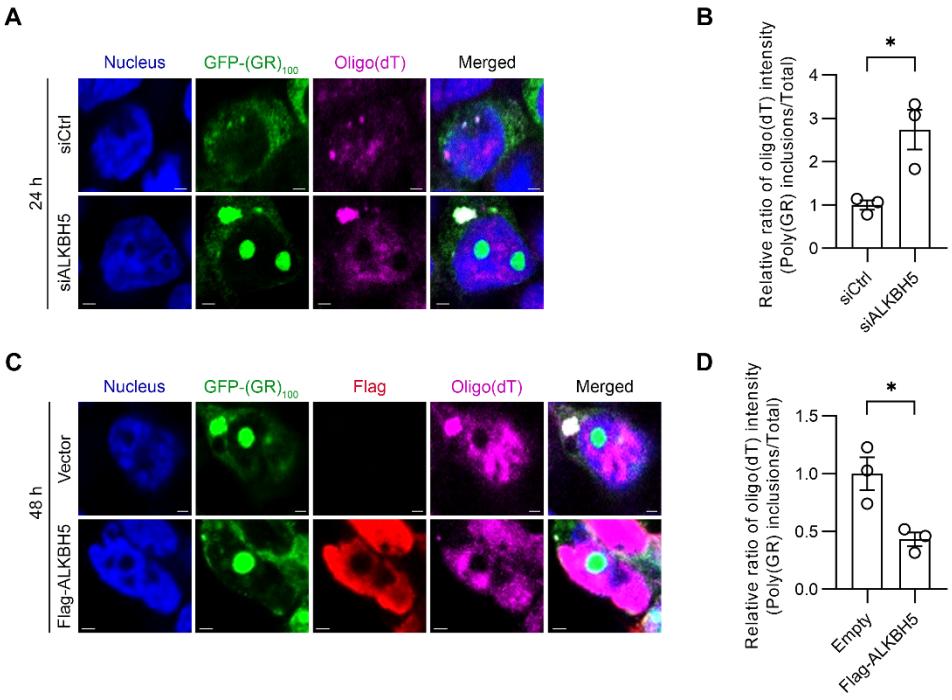


Figure S4. m6A modified RNAs facilitate mRNA incorporation in poly(GR) inclusions, related to Figure 4. (**A**) Immunofluorescence staining for oligo(dT) in ALKBH5-depleted HEK293T cells expressing GFP-$(GR)_{100}$ 24 h post-transfection. Scale bars, 2 µm. (**B**) Quantification of the relative ratio of oligo(dT) intensity [poly(GR) inclusions/Total] in ALKBH5-depleted HEK293T cells expressing GFP-$(GR)_{100}$ (n = 3 independent experiments). (**C**) Double-immunofluorescence staining for Flag and oligo(dT) in Flag-ALKBH5 and GFP-$(GR)_{100}$ co-expressing HEK293T cells 48 h post-transfection. Scale bars, 2 µm. (**D**) Quantification of the ratio of oligo(dT) intensity [poly(GR) inclusions/Total] in HEK293T GFP-$(GR)_{100}$-expressing cells transduced or not to express Flag-ALKBH5 (n = 3 independent experiments). Data shown as mean ± SEM. In (B), * $P = 0.0213$, unpaired two-tailed t-test. In (D), * $P = 0.0211$. unpaired two-tailed t-test.

Table S1. Characteristics of patients with c9FTLD, related to STAR Methods.

Case #	Pathological Diagnosis	Gender	Age at Onset	Age at Death	Disease Duration	*C9orf72* repeat expansion
1	FTLD	M	NA	63.74	NA	Yes
2	FTLD	M	72.19	76.19	4	Yes
3	FTLD	M	58.51	62.51	4	Yes
4	FTLD	F	60.36	61.36	1	Yes
5	FTLD	F	57.21	69.21	12	Yes
6	FTLD	F	59.29	70.29	11	Yes

FTLD, frontotemporal lobar degeneration

Table S2. Primers for site-directed mutagenesis of YTHDF1, related to STAR Methods.

Primers	Sequence
Forward	5’-TAGTACTCGCTGAGGGTGAAC-3’
Reverse	5’-CATGGCCTTCCGTGTTCCTA-3’