

Supplementary information

Early growth response 1 promotes RNA polymerase I-directed transcription and cancer growth by activating RRN3 expression

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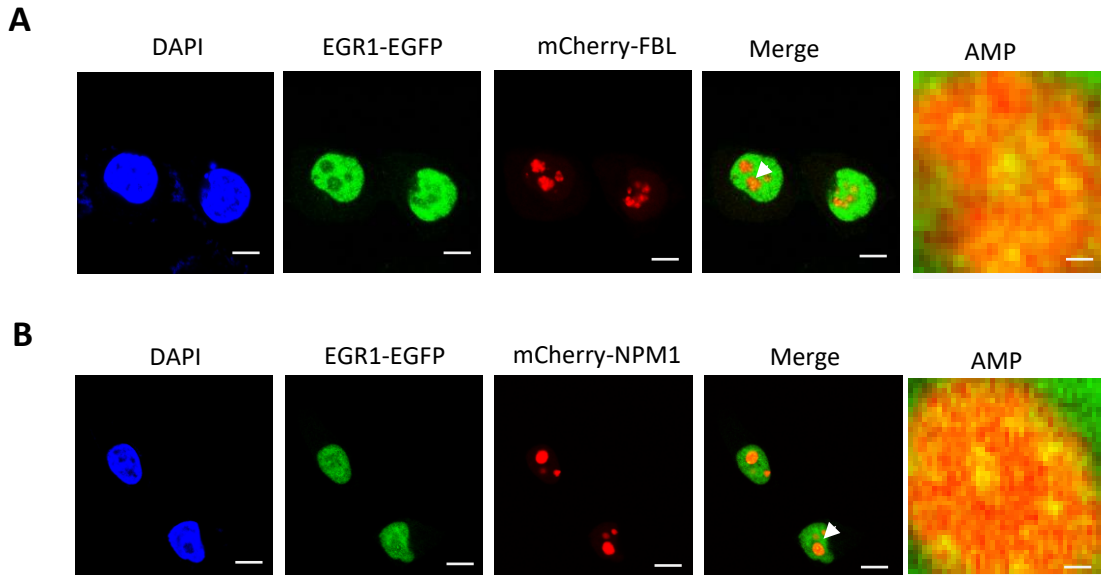
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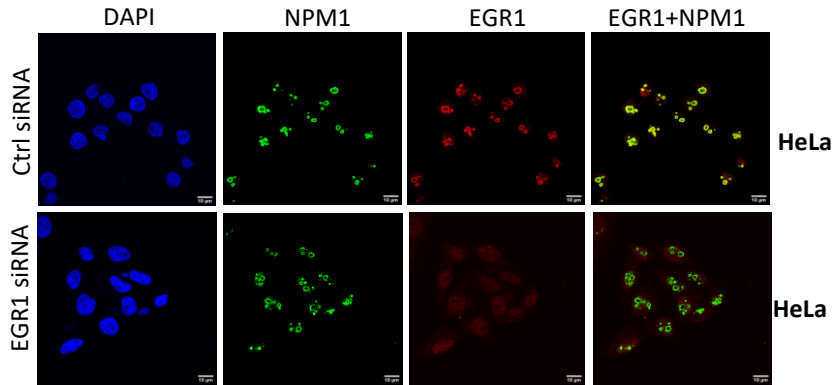
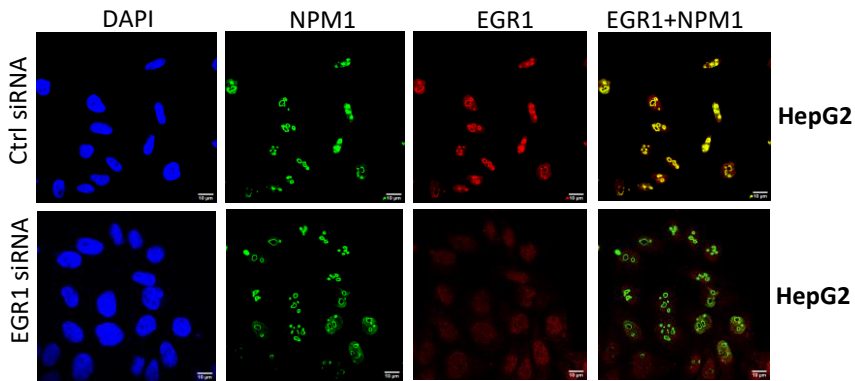
#: These authors contributed equally to this work

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Supplementary Figure I

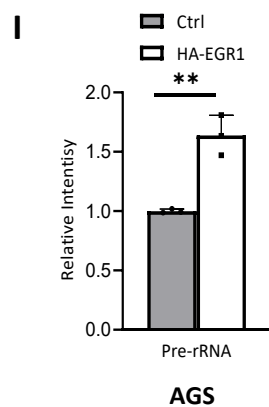
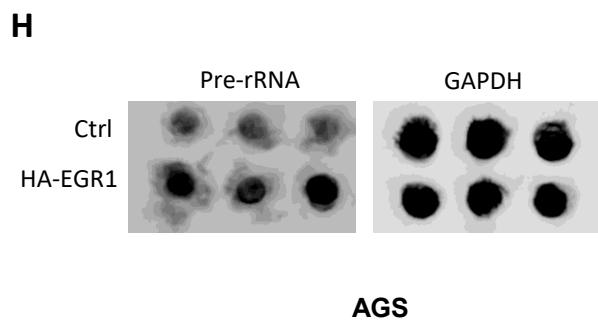
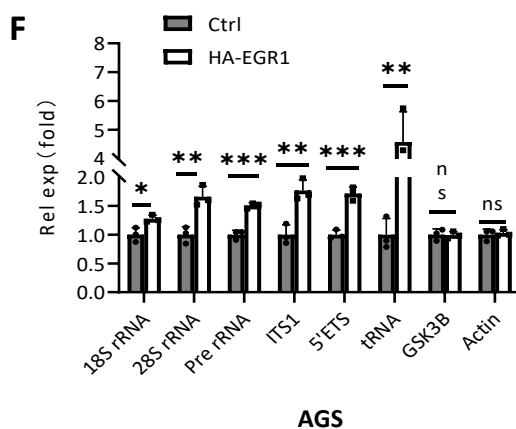
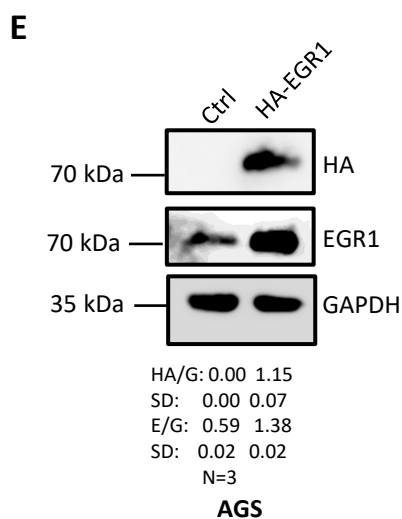
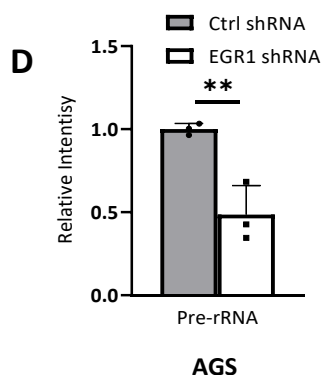
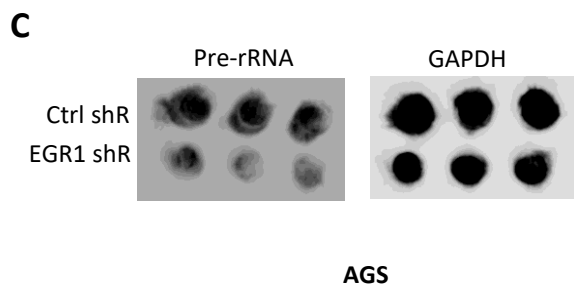
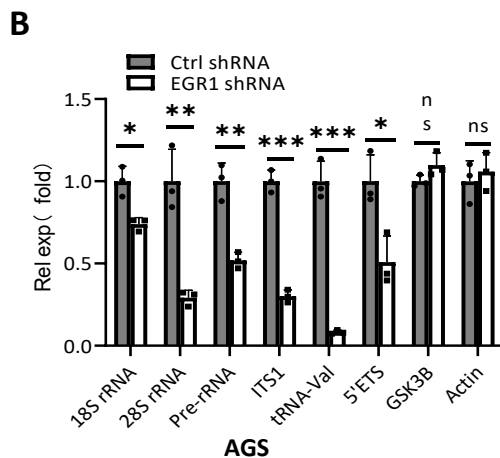
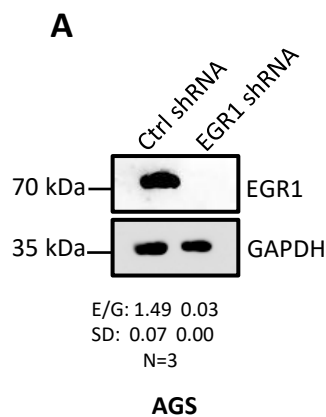


Supplementary Fig. 1 Co-localization analysis between EGR1-EGFP and mCherry-FBL (A) or mCherry-NPM1 (B) in HeLa cells. HeLa cells were co-transfected with the lentiviral vectors expressing EGR1-EGFP or mCherry-FBL (mCherry-NPM1) along with packaging vectors; after 48 hours, cells were seeded on coverslips in 12-well plates and cultured for 24 hours, followed by fixation and observing under a confocal fluorescence microscope. Images were captured with a 60x Olympus lens. The nucleolus pointed by white arrows in the image at the fourth column is amplified and presented on the last column (AMP). The scale bars in the first 4 images represent 2.5 μm ; the one in the last image represents 200 nm.

A**B**

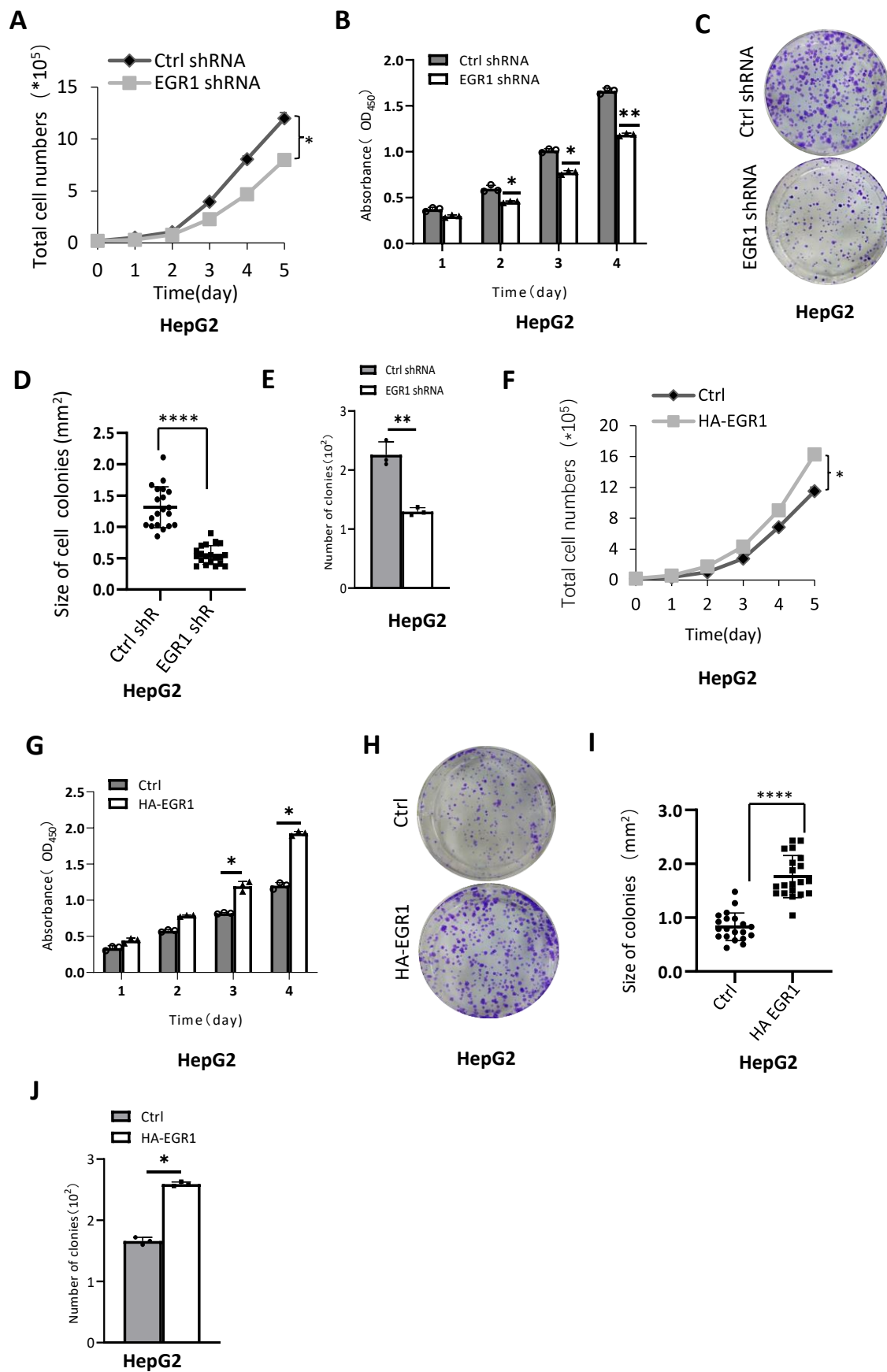
Supplementary Fig. 2 Analysis of immunofluorescence colocalization between EGR1 and Nucleophosmin (NPM1) in HeLa and HepG2 cells with or without EGR1 depletion. A)

Representative images for EGR1 and NPM1 IF staining using HeLa cells stably expressing control shRNA or EGR1 shRNA. B) Representative images for EGR1 and NPM1 IF staining using HepG2 cells stably expressing control shRNA or EGR1 shRNA. Scale bars in these images represent 10 μ m.



Supplementary Fig. 3 EGR1 positively regulates Pol I -directed transcription in AGS cells. A and B)

Effect of EGR1 silencing on the expression of Pol I products in AGS cells. Cell lines as indicated were used to analyze EGR1 expression by Western blot (A) and Pol I product expression by RT-qPCR (B). C and D) Northern dot blot showing effect of EGR1 silencing on rRNA expression in AGS cells. Dot intensity in the images (C) was quantified with ImagerJ and presented in D. E and F) Effect of EGR1 overexpression on the expression of Pol I products in AGS cells. Cell lines as indicated were used to analyze EGR1 expression by Western blot (E) and Pol I products by RT-qPCR (F). H and I) Northern dot blot showing effect of EGR1 overexpression on rRNA expression in AGS cells . Dot intensity in the images (H) was quantified with ImagerJ and presented in I. Each column in histograms represents the mean $\pm$ SD of three biological replicates (n=3). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. P values were obtained by Student's t -test, performed with the data from control and treatment groups.



Supplementary Fig. 4 EGR1 promotes the proliferation of HepG2 cells. **A and B)** Effect of EGR1 silencing on HepG2 cell proliferation. Cell proliferation analysis was performed by cell counting (A) and CCK-8 assays (B) using HepG2 cell lines with or without EGR1 silencing. **C -E)** The results of colony formation assays for HepG2 cell lines with or without EGR1 depletion. HepG2 cell colonies were stained and pictured with a camera (C), followed by statistically analysing the size (D) and number (E) of colonies. **F and G)** Effect of EGR1 overexpression on HepG2 cell proliferation. Cell proliferation analysis was performed by cell counting (F) and CCK-8 assays (G) using HepG2 cell lines with or without EGR1 overexpression. **H-J)** The results of colony formation assays for HepG2 cell lines with or without EGR1 overexpression. HepG2 cell colonies were stained and pictured with a camera (H), followed by statistically analysing the size (I) and number (J) of colonies. Each point/column in graphs represents the mean $\pm$ SD of three biological replicates (n=3). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. P values in A and F were obtained by two-way ANOVA, P values in B, D, E, G, I and J were obtained by Student's t -test, performed with the data from control and treatment groups.

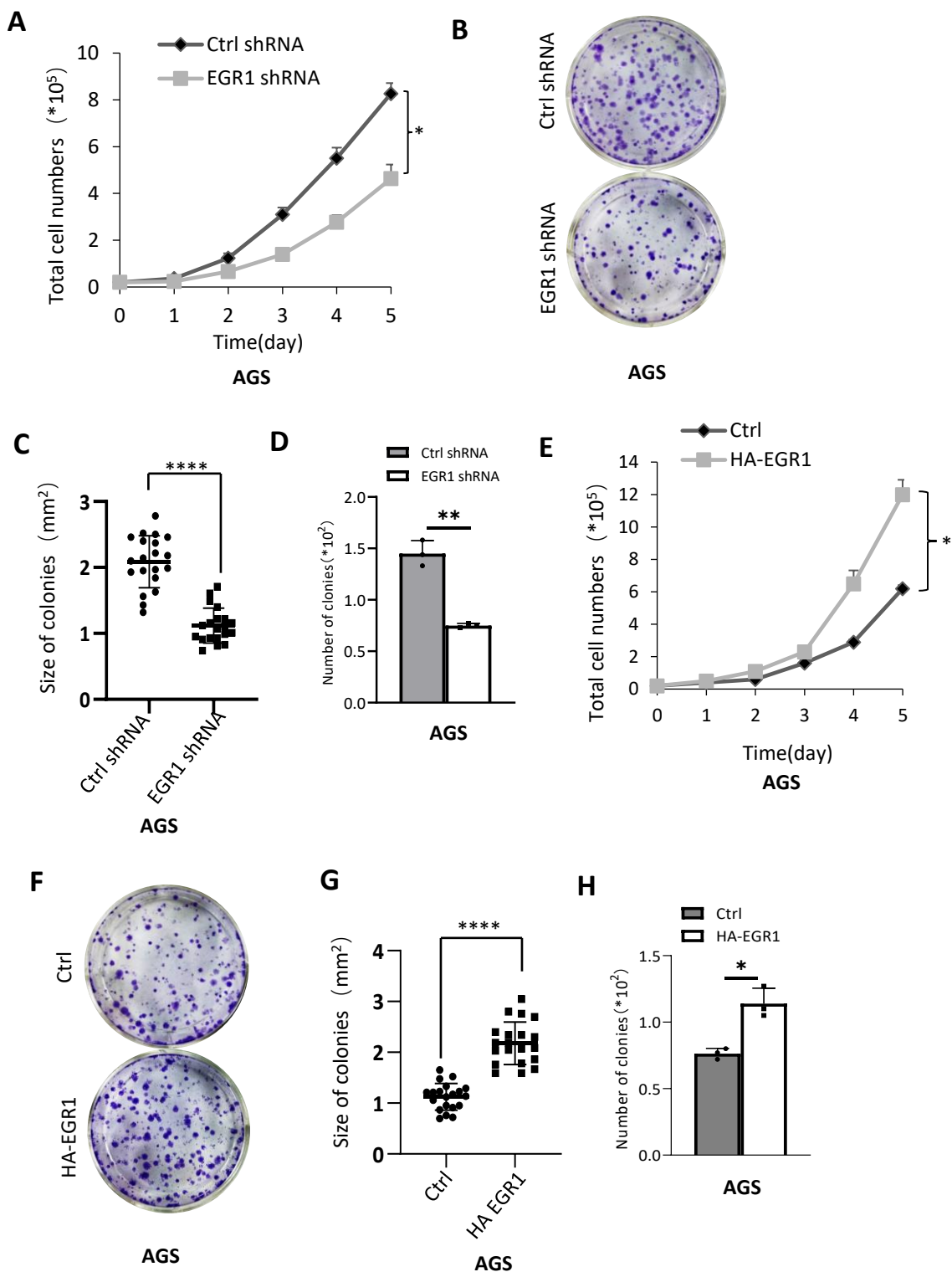
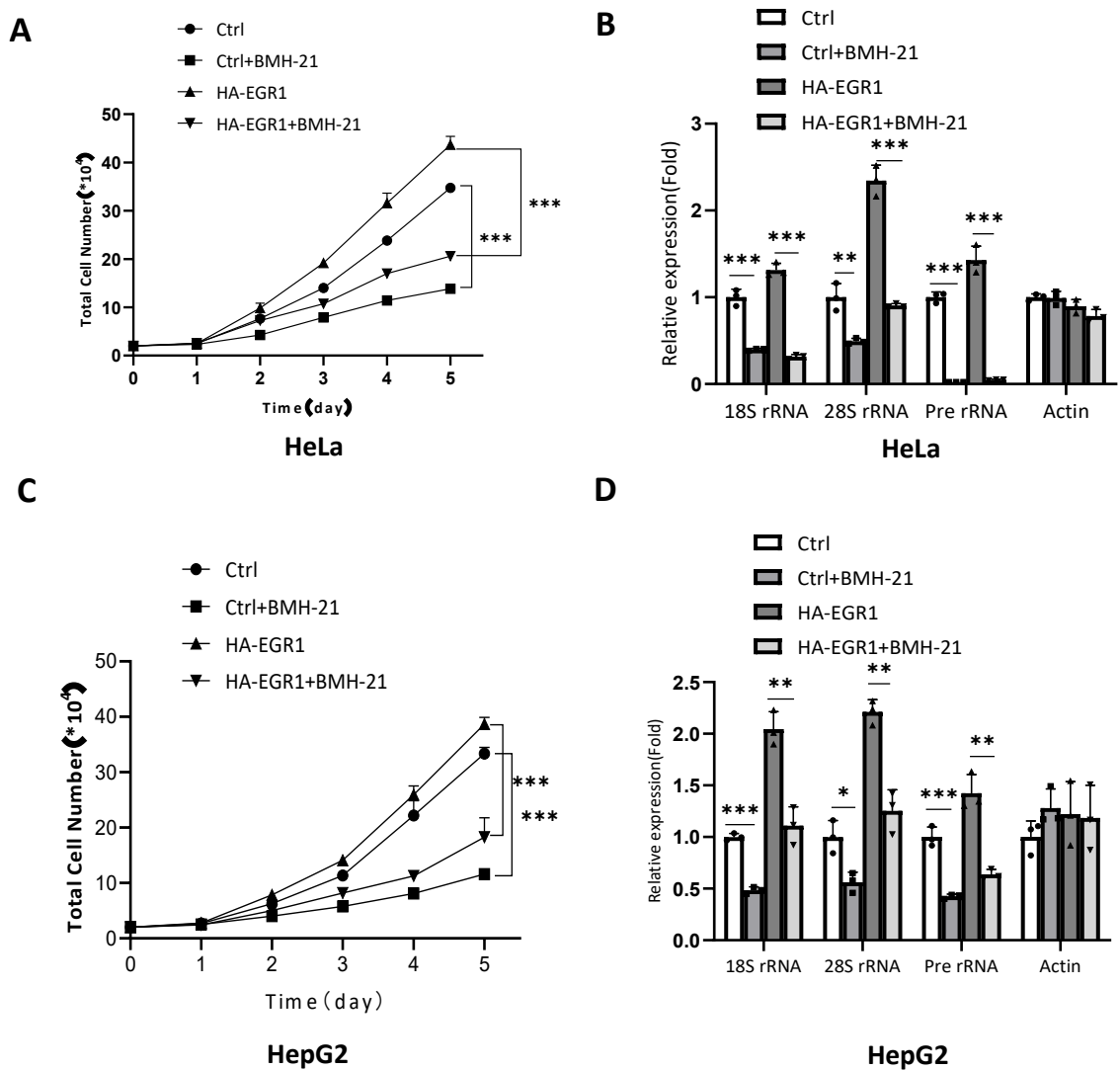
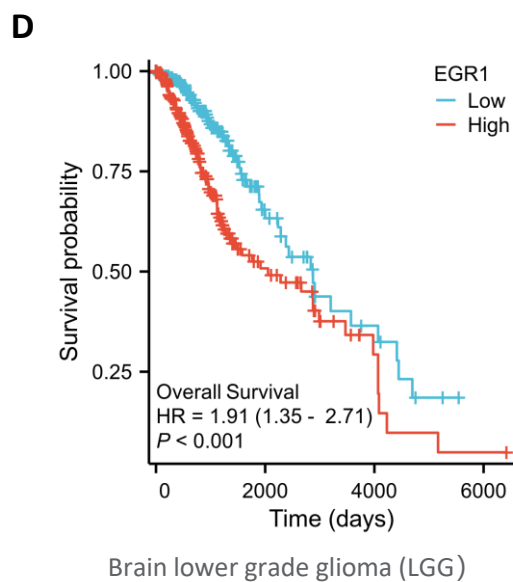
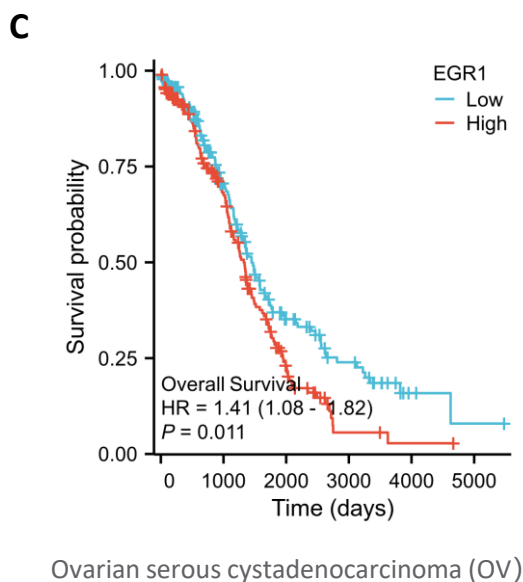
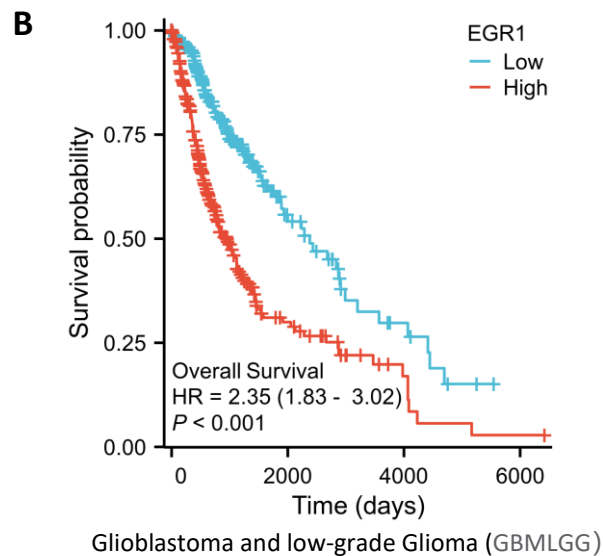
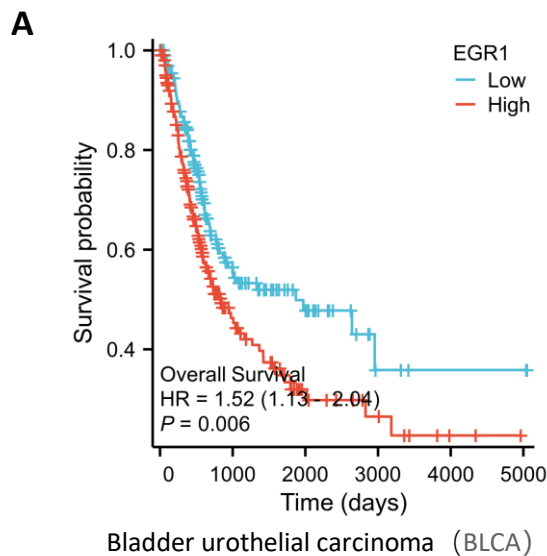


Figure S5. EGR1 promotes the proliferation activity of AGS cells. A) Effect of EGR1 silencing on AGS cell proliferation. Cell proliferation analysis was performed by cell counting using AGS cell lines with or without EGR1 silencing. **B-D)** The results of colony formation assays for AGS cell

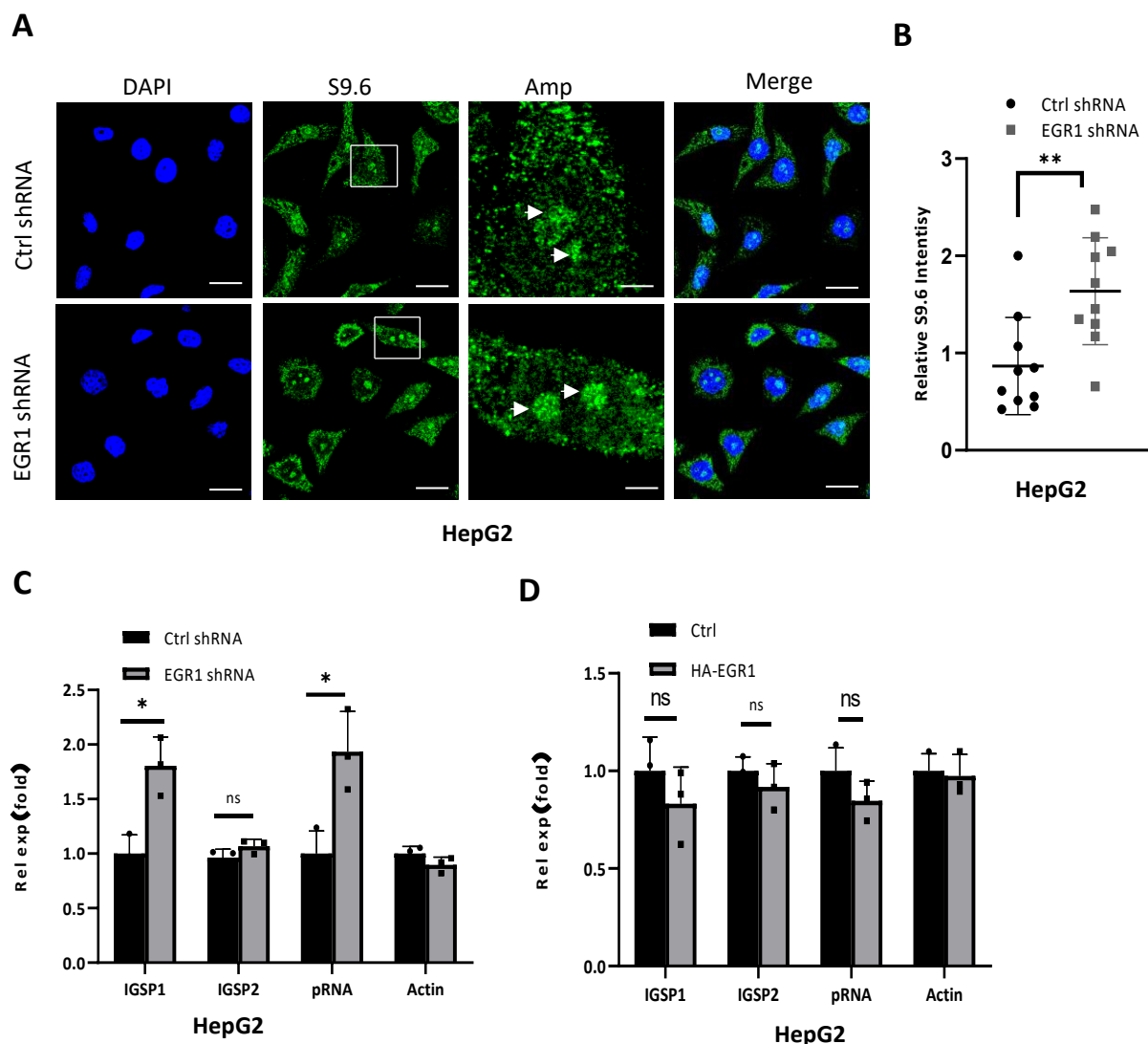
lines with or without EGR1 depletion. AGS cell colonies were stained and pictured with a camera (B), followed by statistically analysing the size (C) and number (D) of colonies. **E**) Effect of EGR1 overexpression on AGS cell proliferation. Cell proliferation analysis was performed by cell counting using AGS cell lines with or without EGR1 overexpression. **F-H**) The results of colony formation assays for AGS cell lines with or without EGR1 overexpression. AGS cell colonies were stained and pictured with a camera (F), followed by statistically analysing the size (G) and number (H) of colonies. Each point/column in graphs represents the mean $\pm$ SD of three biological replicates (n=3). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. P values in A and E were obtained by two-way ANOVA, P values in C, D, G, and H were obtained by Student's t -test, performed with the data from control and treatment groups.



Supplementary Fig. 6 Effect of BMH21 treatment on cell proliferation and Pol I products. A) Effect of BMH21 treatment on the proliferation of HeLa cells with or without EGR1 overexpression; **B)** Effect of BMH21 treatment on the synthesis of Pol I products in HeLa cells with or without EGR1 overexpression; **C)** Effect of BMH21 treatment on the proliferation of HepG2 cells with or without EGR1 overexpression; **D)** Effect of BMH21 treatment on the synthesis of Pol I products in HepG2 cells with or without EGR1 overexpression. *P* values in A and C were obtained by two-way ANOVA, *P* values in B and D were obtained by Student's *t*-test, performed with the data from control and treatment groups.

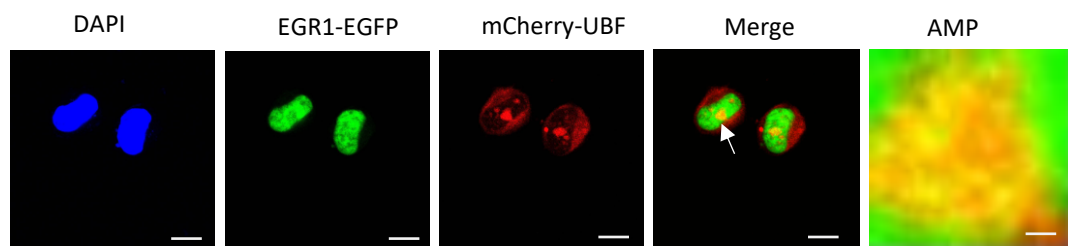


Supplementary Fig. 7 Kaplan-Meier Plots showing the relationship between EGR1 expression levels and survival probability and survival time based on the analysis of the RNA-seq data from the TCGA dataset for bladder urothelial carcinoma (BLCA, **A**), glioblastoma and low-grade Glioma (GBMLGG, **B**), ovarian serous cystadenocarcinoma (OV, **C**), and brain lower grade glioma (LGG, **D**).

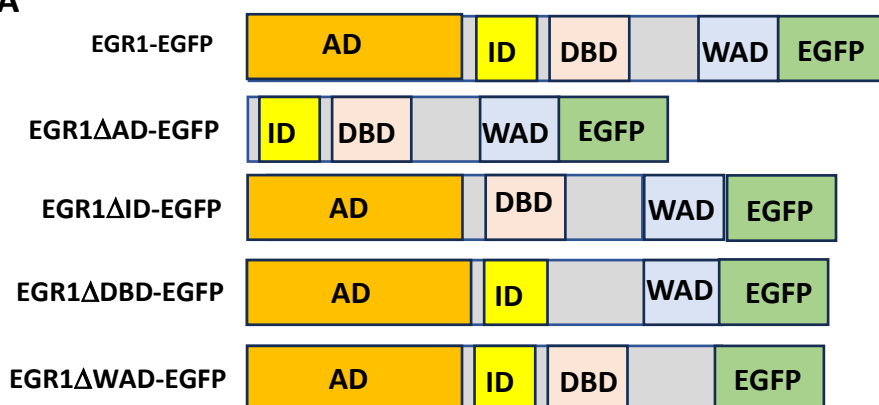
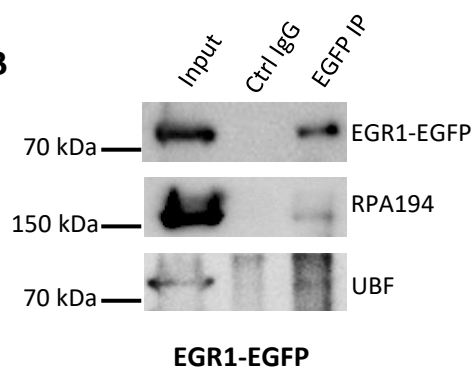
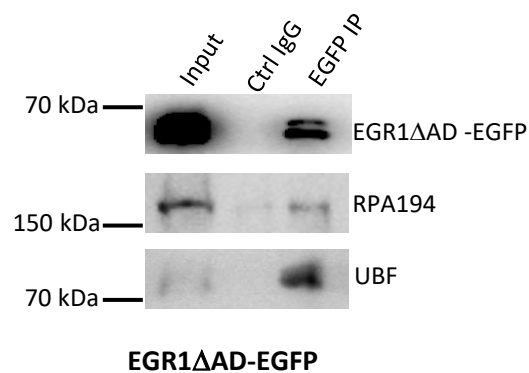
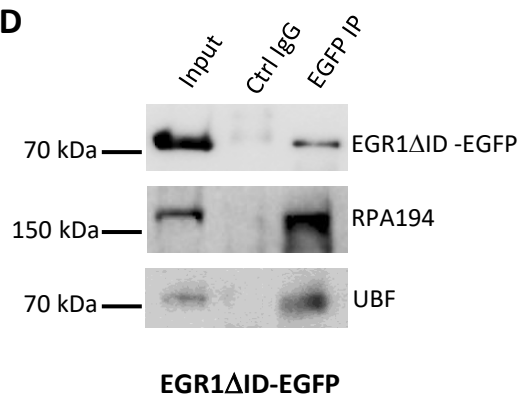
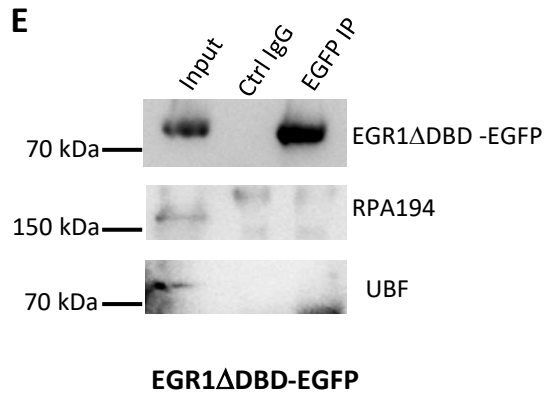
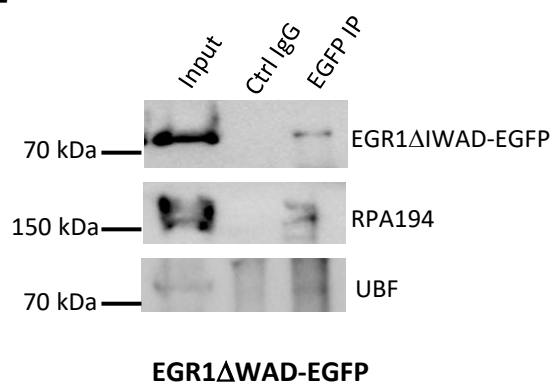


Supplementary Fig. 8 Effect of EGR1 silencing on R-loop formation and pRNA expression in HepG2 cells.

HepG2 cell lines as indicated were used IF assays using an anti-S9.6 antibody and imaged under a confocal microscope; representative images were presented in **A**, where a white frame enclosing cells in the second column were amplified and presented at the third column (AMP), followed by analysing fluorescence intensity for the nucleoli (white arrows pointed) in several HeLa cells (**B**). Scale bars in the images of the 1st, 2nd column 4th represents 10 μm ; and those in the 3rd column represent 2.5 μm . **C** and **D**) Effect of EGR1 silencing or overexpression on the expression of IGS RNA and rDNA promoter-associated RNA (pRNA) in HeLa cells. HepG2 cell lines with EGR1 silencing (**C**) or overexpression (**D**) were used to analyse the expression of IGS RNA fragments and pRNA by RT-qPCR. Each column in the histogram represents the mean $\pm$ SD of three biological replicates (n=3). *, $P < 0.05$; **, $P < 0.01$; P values were obtained by Student's t test for the data from the control and treatment groups.



Supplementary Fig. 9 Co-localization analysis between EGR1-EGFP and mCherry-UBF in HeLa cells. HeLa cells were co-transfected with the lentiviral vectors expressing EGR1-EGFP or mCherry along with packaging vectors; after 48 hours, cells were seeded on coverslips in 12-well plates and cultured for 24 hours, followed by fixation and observing under a confocal fluorescence microscope. Images were captured with a 60x Olympus lens. The nucleolus pointed by white arrow in the image at the fourth column is amplified and presented on the last column (AMP). The scale bars in the first 4 images represent 2.5 μm ; the one in the last image represents 200 nm.

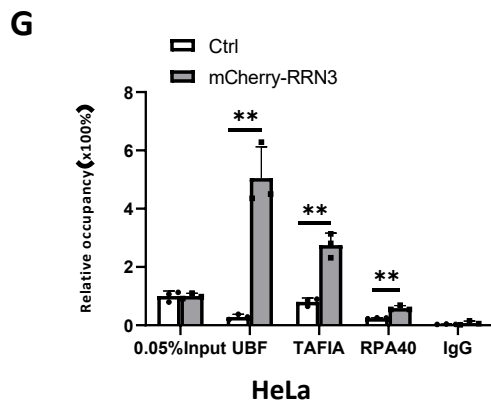
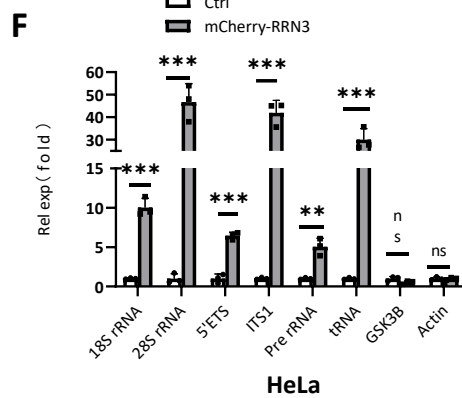
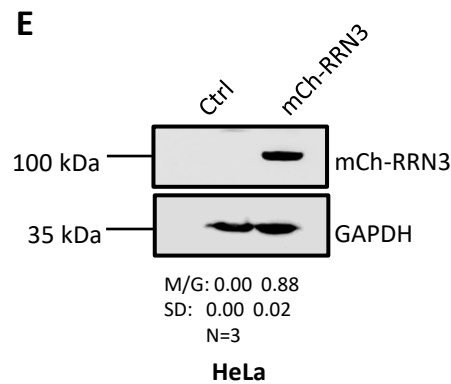
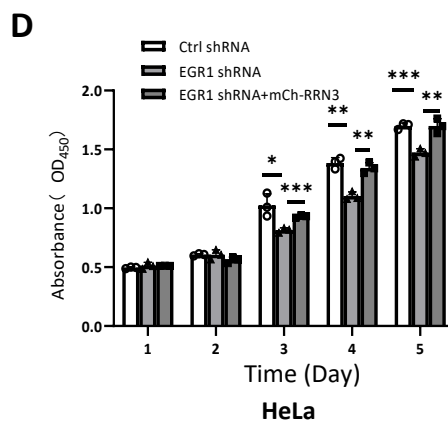
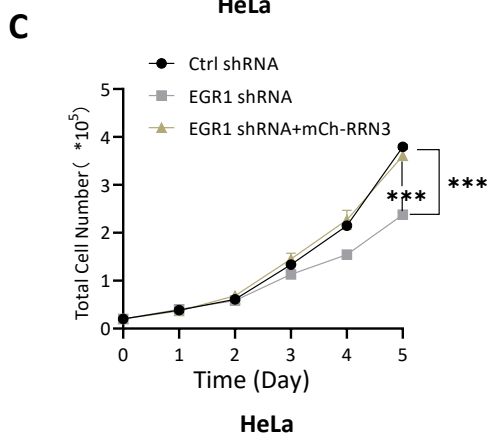
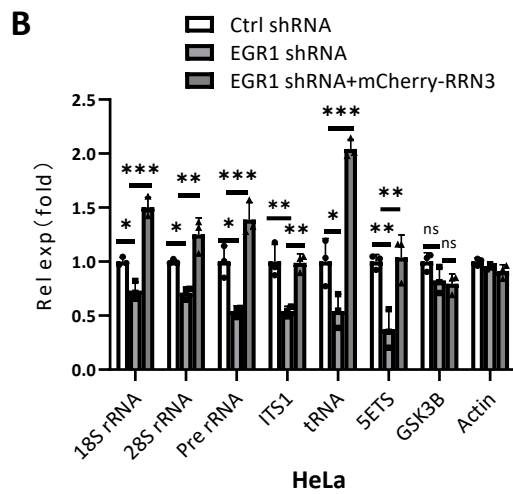
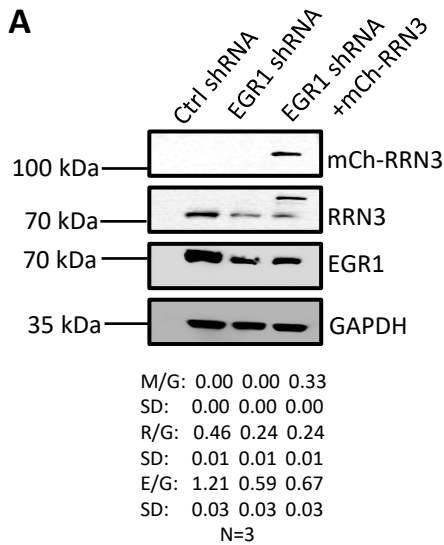
A**B****C****D****E****F**

Supplementary Fig. 10 EGR1 DNA binding domain is required for the co-immunoprecipitation between EGR1 and Pol I transcription machinery factors. **A)** A scheme showing deletion mutants of the EGR1-EGFP fusion protein. AD: activation domain; ID: inhibitory domain; DBD: DNA binding domain; WAD: weak activation domain; and Delta (Δ) represents deletion. **B)** Western blot images showing the results of the co-immunoprecipitation between EGR1-EGFP and RPA194 or UBF. **C)** Effect of EGR1 activation domain deletion on the co-immunoprecipitation between EGR1-EGFP and RPA194 or UBF. **D)** Effect of EGR1 inhibition domain deletion on the co-immunoprecipitation between EGR1-EGFP and RPA194 or UBF. **E)** Effect of EGR1 DNA binding domain deletion on the co-immunoprecipitation between EGR1-EGFP and RPA194 or UBF. **F)** Effect of EGR1 weak activation domain deletion on the co-immunoprecipitation between EGR1-EGFP and RPA194 or UBF. EGR1-EGFP and its deletion mutants-expressing lentiviral vectors were constructed and transfected into HeLa cells along with lentiviral packaging vectors, transfected cells were expanded, and nuclei were prepared with the transfected cells, the extract of the nuclei were used for immunoprecipitation using a EGFP antibody, the precipitated protein was used for immunoblotting analysis using the antibodies against EGFP, RPA194, and UBF, respectively. The Input in B-F is equivalent to 2.5% of the samples used for each IP assay.

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 G **CCGGCGGGCG**TGGTCGGTGACGCGACCTCATATCTTTCGCTCCGAGTCGGCAA
 TT TTGGGCCCGCCGGGTTATTA — +1

Supplementary Fig. 11 A diagram showing the sequence of human 45S rRNA gene promoter.

Red bases represent EGR1 putative binding sites (EGR1 canonical consensus sequences: 5'-GCG
 (T/G)GG GCG-3')



Supplementary Fig. 12 RRN3 reversed the inhibition of Pol I -directed transcription induced by EGR1

silencing. A) Representative images showing the overexpression of RRN3 in the HeLa cell line with EGR1 depletion. **B)** EGR1 overexpression reversed the inhibition of Pol I -directed transcription caused by EGR1 depletion. mCherry-RRN3 was expressed in the HeLa cell line with EGR1 silencing by a lentiviral transduction system, the resulting cells were used to analyze the expression of Pol I products by RT-qPCR. **C and D)** The expression of mCherry-RRN3 reversed the inhibition of proliferation activity for HeLa cells by EGR1 silencing. Cell counting (C) and CCK-8 (D) assays were performed using HeLa cell lines as indicated. **E and F)** RRN3 overexpression activated Pol I -directed transcription in HeLa cells. A HeLa cell line expressing mCherry-RRN3 and its corresponding control cell line were generated by a lentiviral transduction system. MCherry-RRN3 expression and Pol I products were analyzed by Western blot (E) and RT-qPCR (F), respectively. (G) ChIP assays results showing the effect of RRN3 overexpression on the occupancies of Pol I machinery factors at the rDNA promoter. Each column in histograms represents the mean $\pm$ SD of three biological replicates (n=3). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. P values were obtained by Student's t -test (B, D, and F) or two -way ANOVA (C).

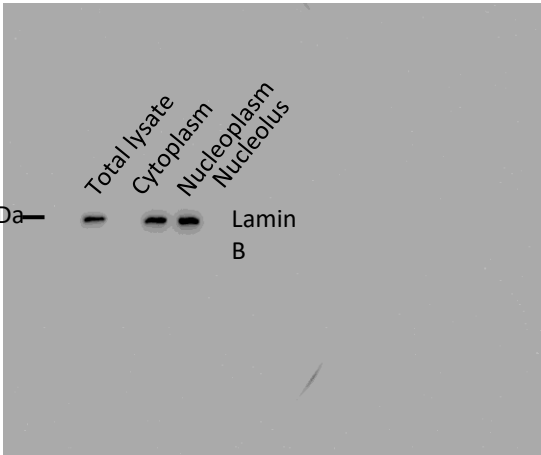
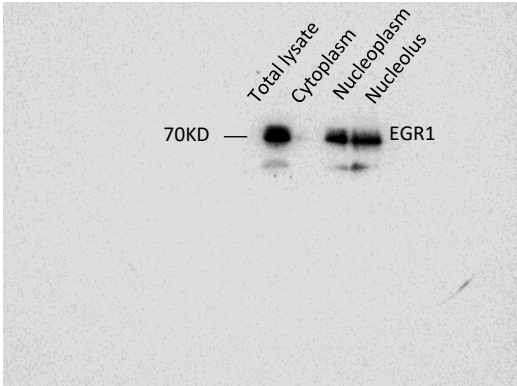
Supplementary table 1. The sources and dilution ratios of the antibodies used in the study

Protein Name	Company name	Catalogue No.	Dilution ratio for IF	Dilution ratio for IB
EGR1	Cell Signaling Technology (CST)	4154S		1:1000
EGR1	Abcam	ab194357	1:200	1:1000
Nucleophosmin (NPM1)	Abcam	ab183340	1:200	1:1000
UBF	Abcam	ab244287	1:200	1:1000
RRN3	Sant Cruz Biotech	SC-390464	1:50	1200
RP40	Abcam	ab196657	1:100	1:500
Alexa Fluor 488 Secondary AB (Rabbit IgG)	Thermo Scientific	A11034	1:500	
Alexa Fluor 488 Secondary AB (mouse IgG)	Thermo Scientific	A1101	1:500	
Alexa Fluor 594 Secondary AB (mouse IgG)	Thermo Scientific	A11005	1:500	
Alexa Fluor 594 Secondary AB (Rabbit IgG)	Therom Scientific	R37117	1:500	
S9.6	Sigma_Aldrich	MABE1095	1:200	
EGR1	Santa Cruz Biotech	SC-515830		1:200
TBP	Santa Cruz Biotech	SC-421		1:200
TAF1A	Santa Cruz Biotech	SC-393600		1:200
RPA194	Santa Cruz Biotech	SC-46699		1:200
GFP	ProteinTech	66002-1-Ig		1:20000
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Lamin B	Proteintech	12987-1-AP		1:1000

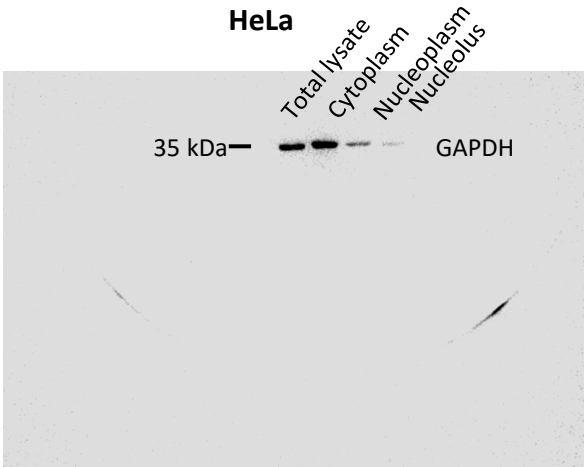
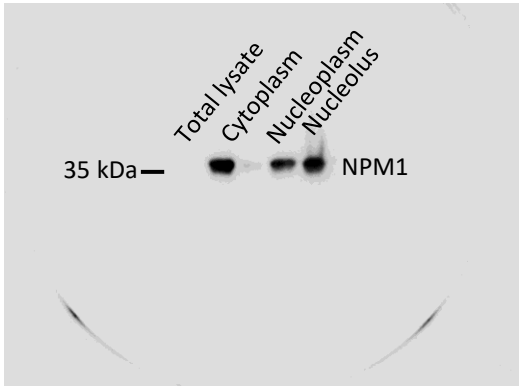
Supplementary Figure II

Fig. 1

E



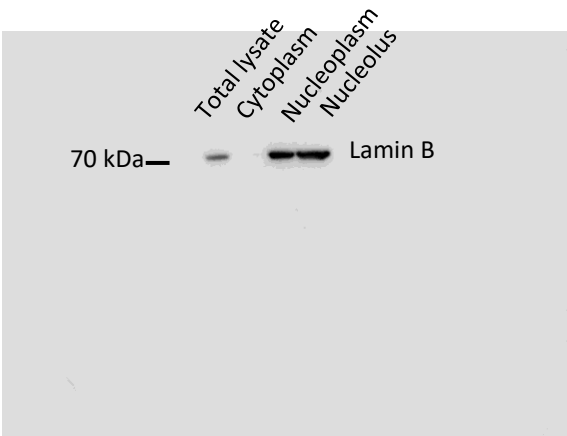
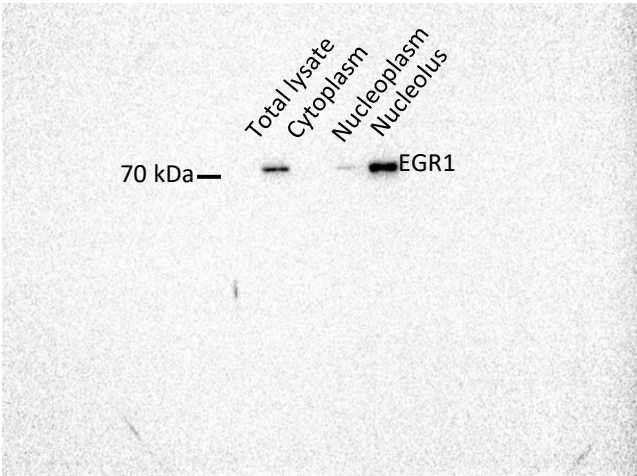
HeLa



HeLa

HeLa

F

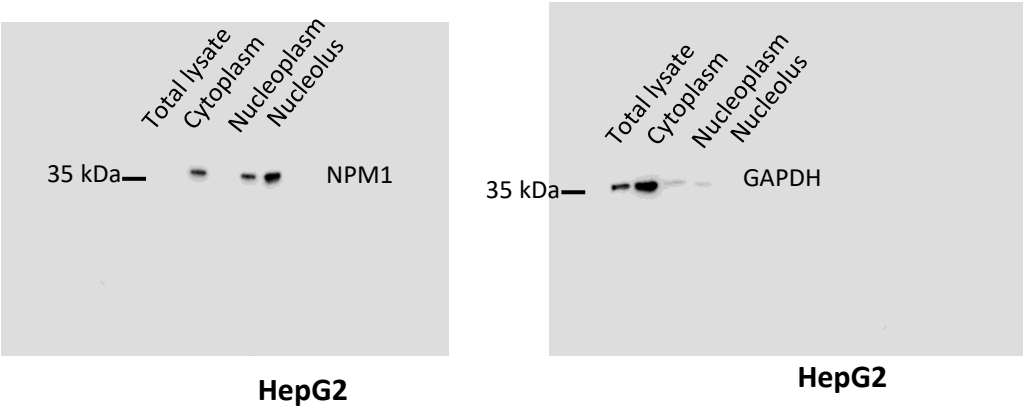


HepG2

HepG2

Fig. 1

F



G

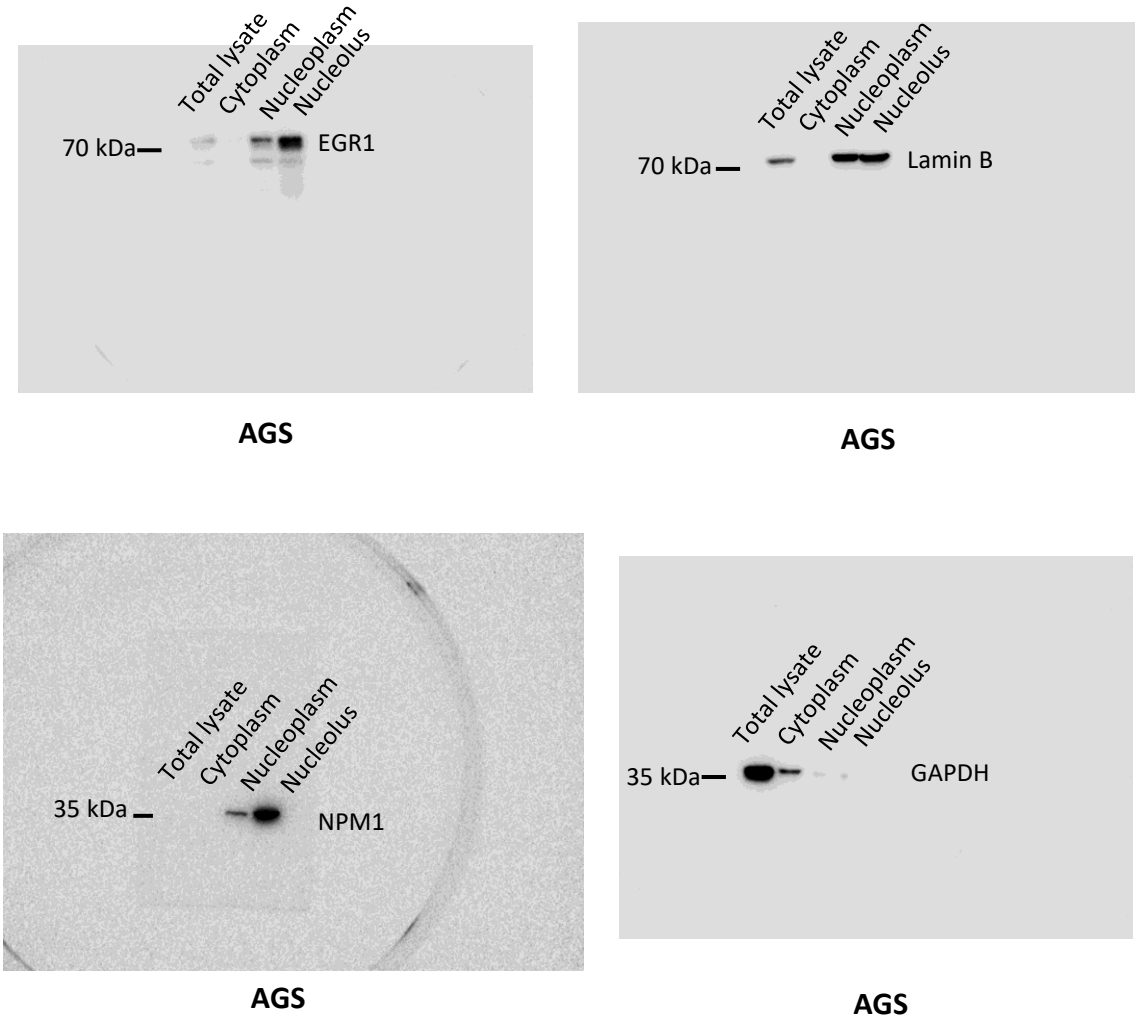
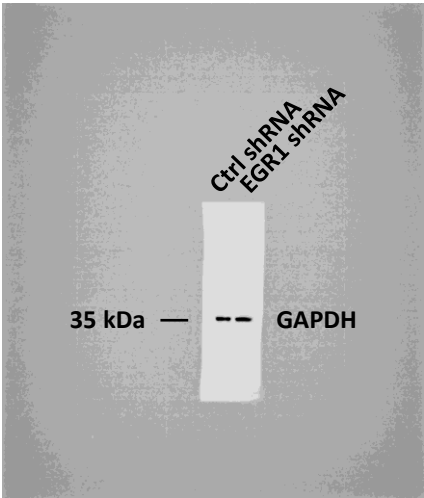
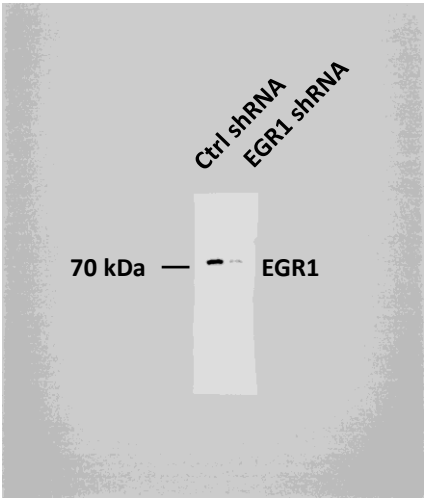


Fig.2

A



C

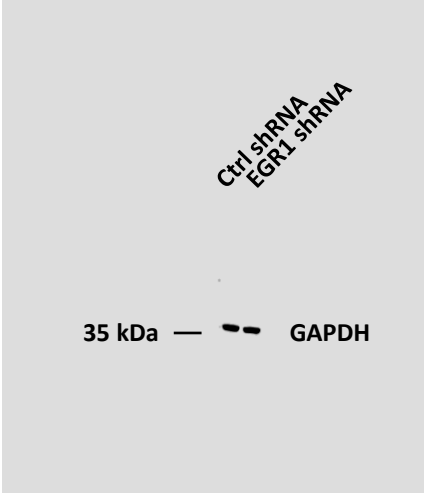
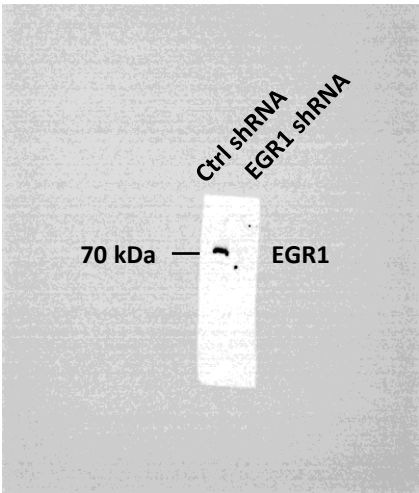
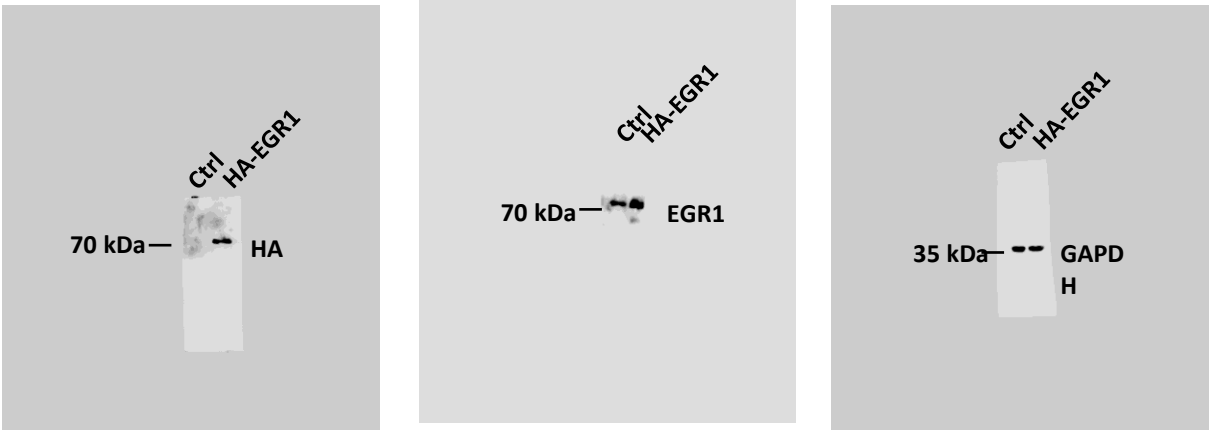


Fig. 3

A



C

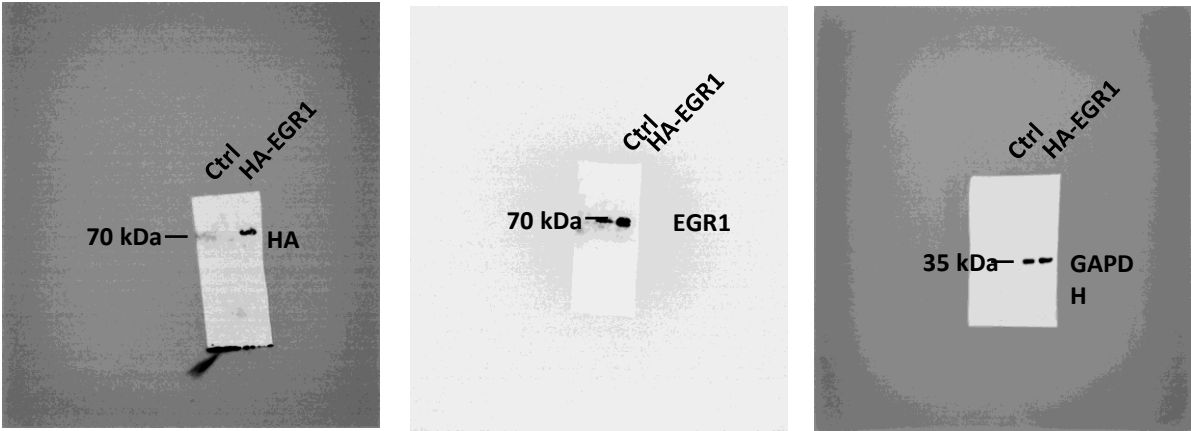
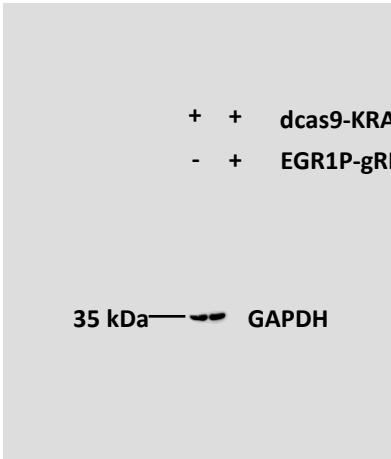
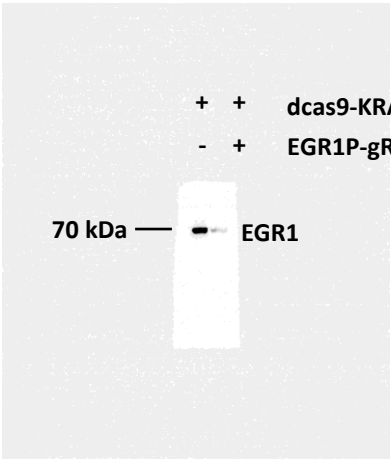


Fig. 4

C



G

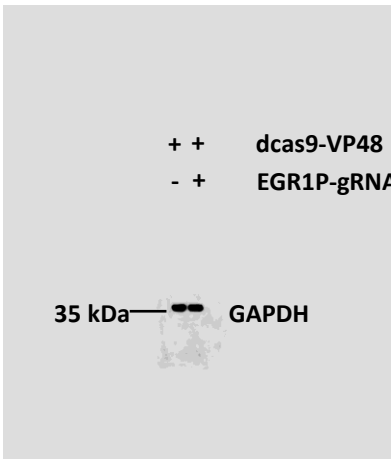
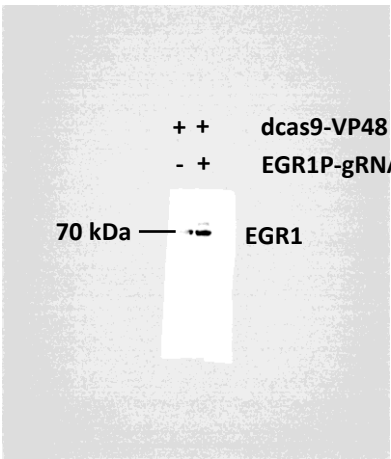
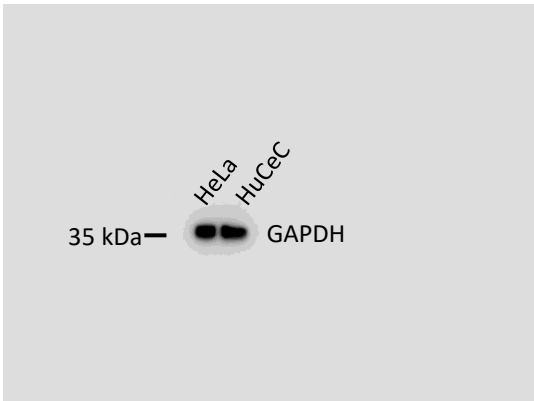
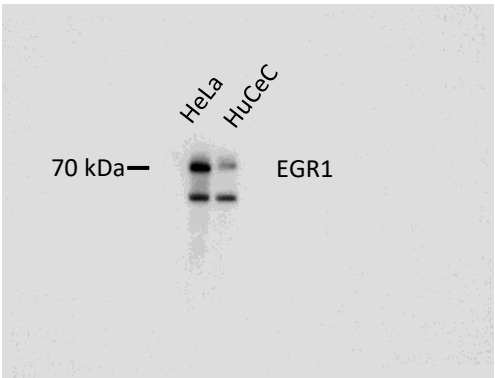


Fig. 5

B



D

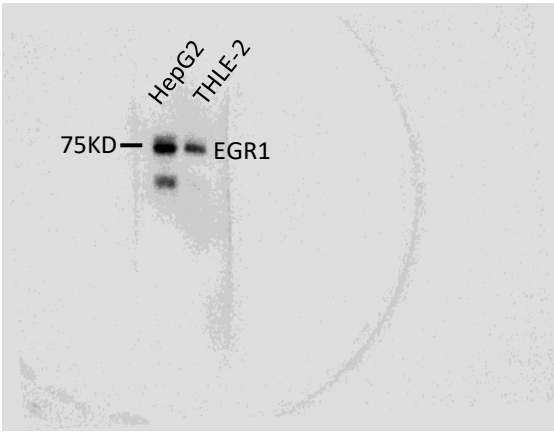


Fig. 6

H

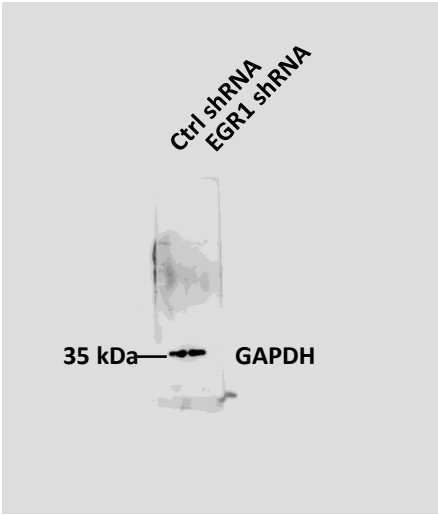
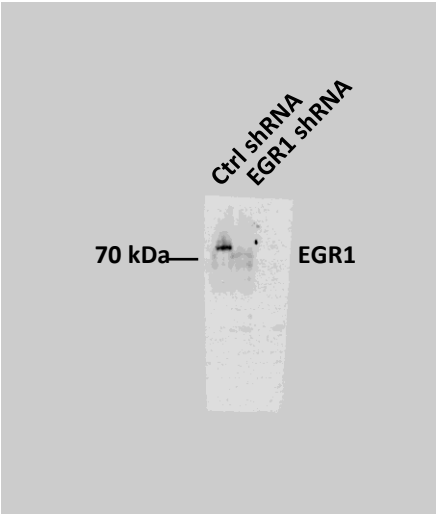
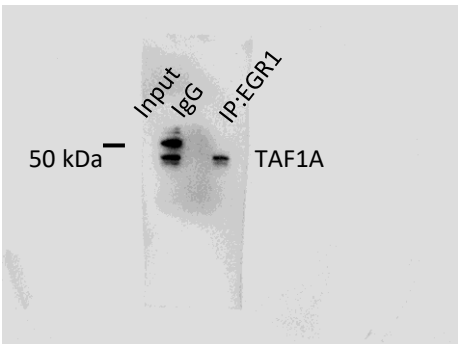
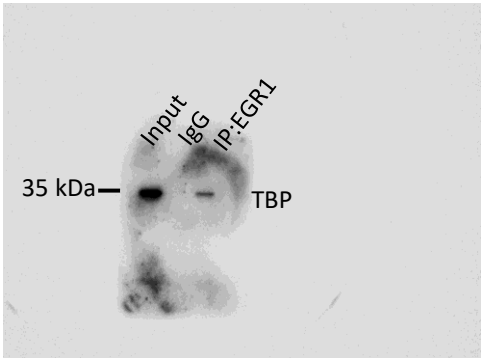
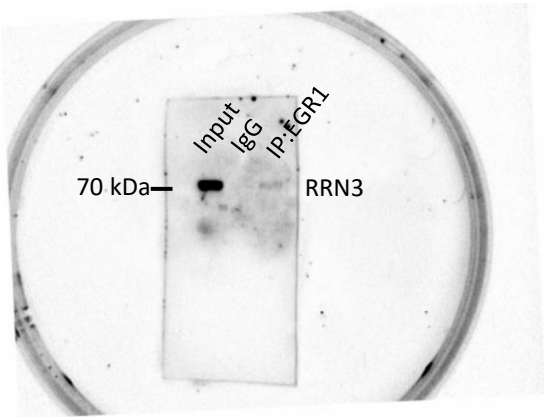
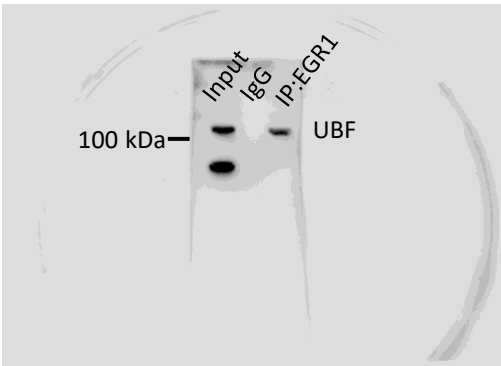
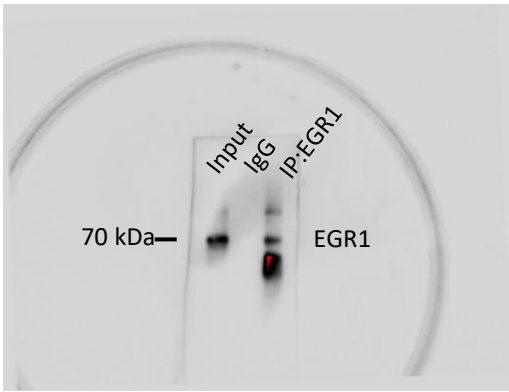


Fig. 8

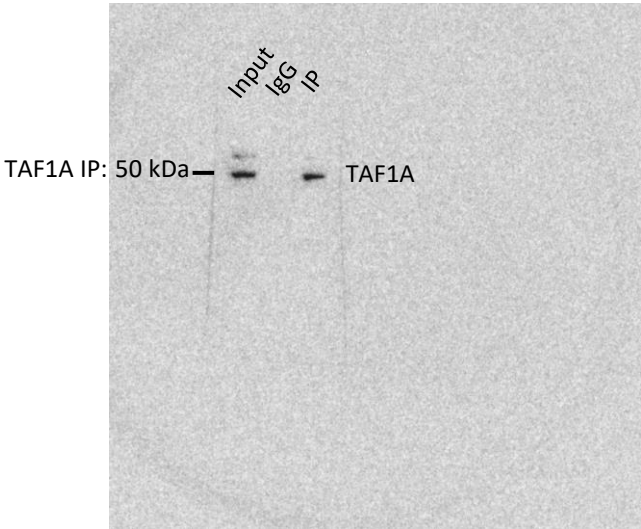
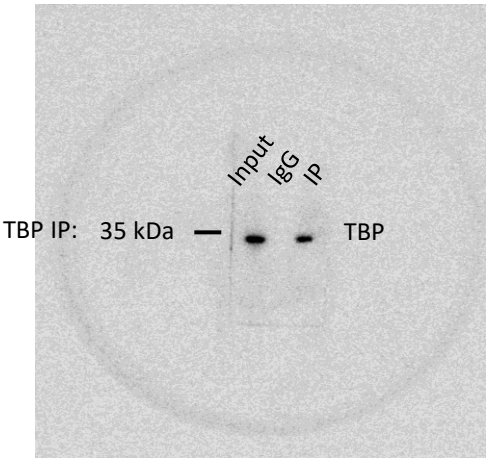
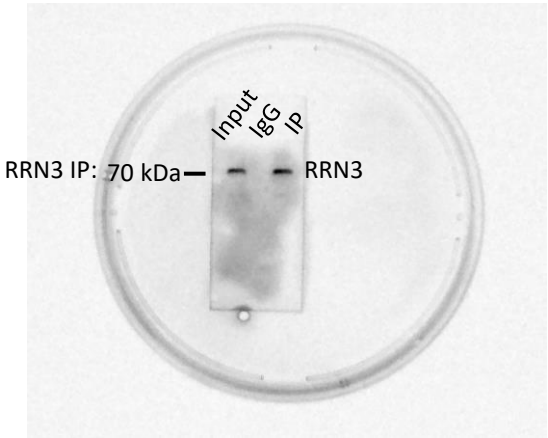
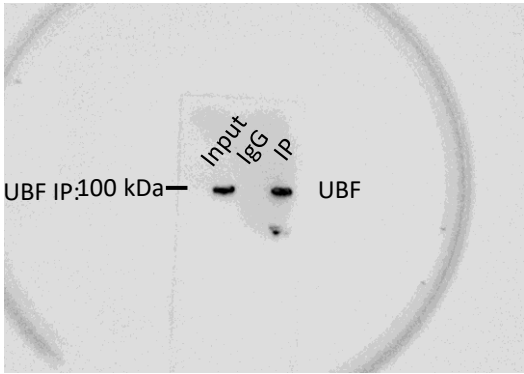
G



HeLa

Fig. 8

H



I

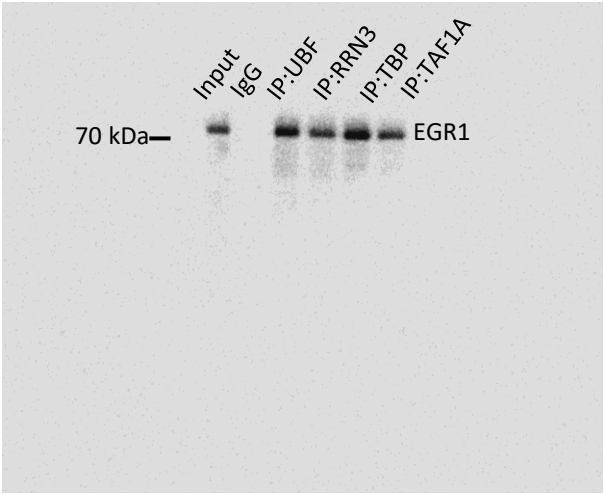
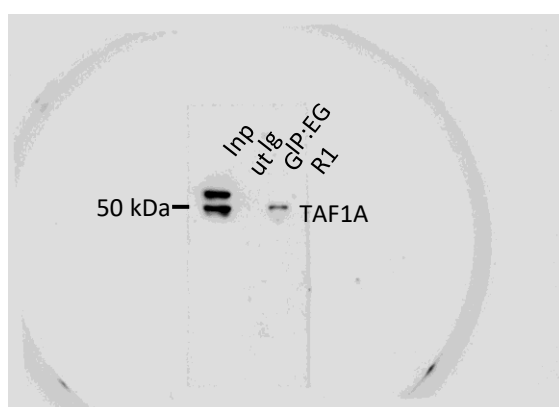
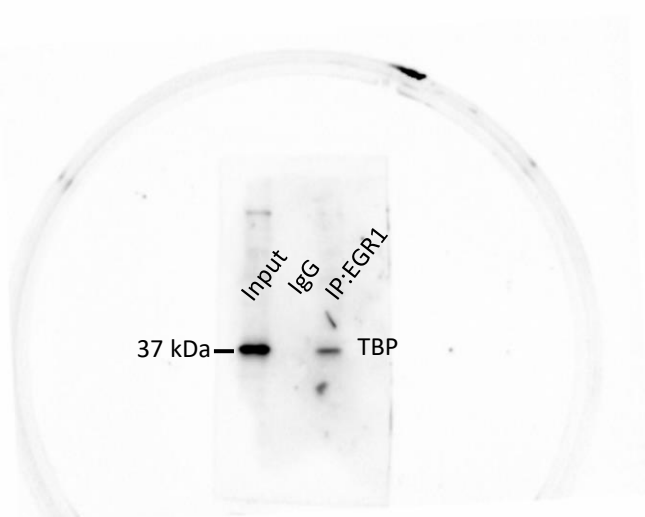
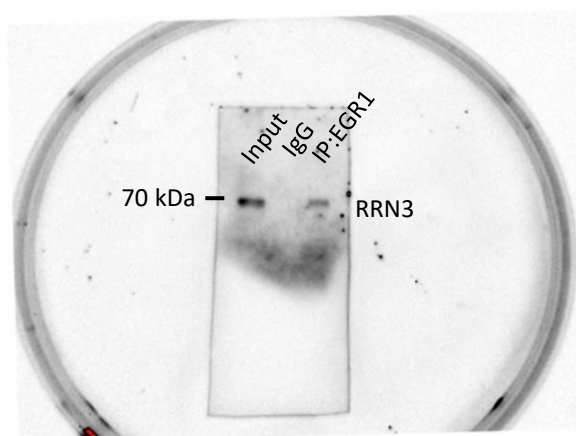
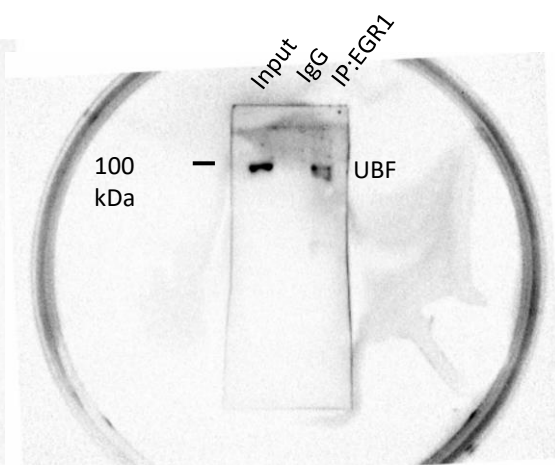
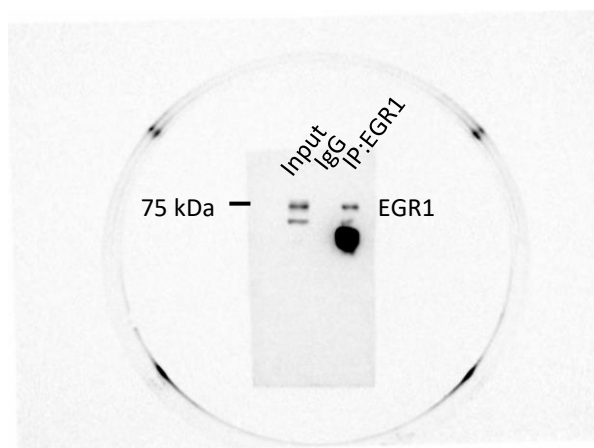


Fig. 8

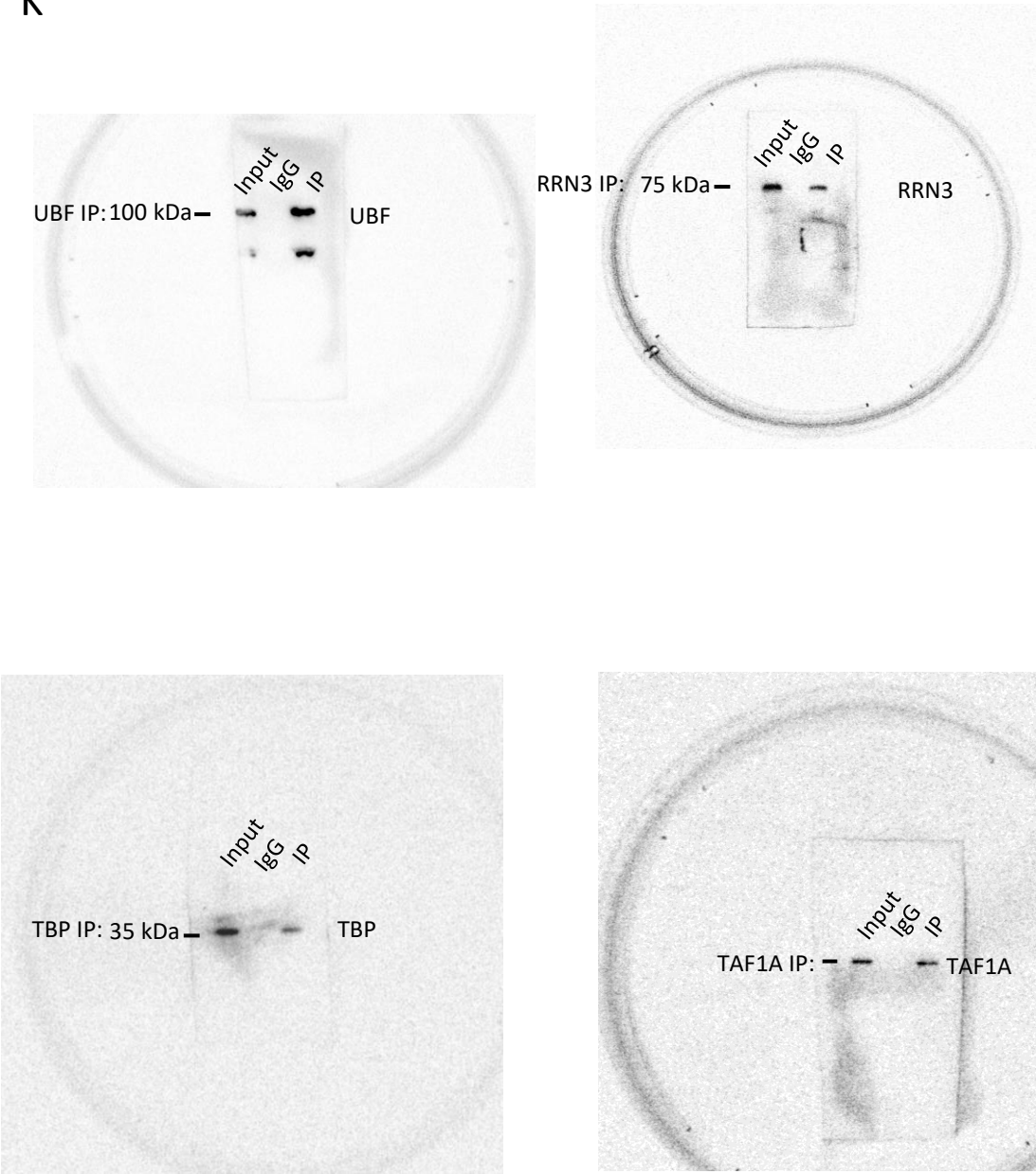
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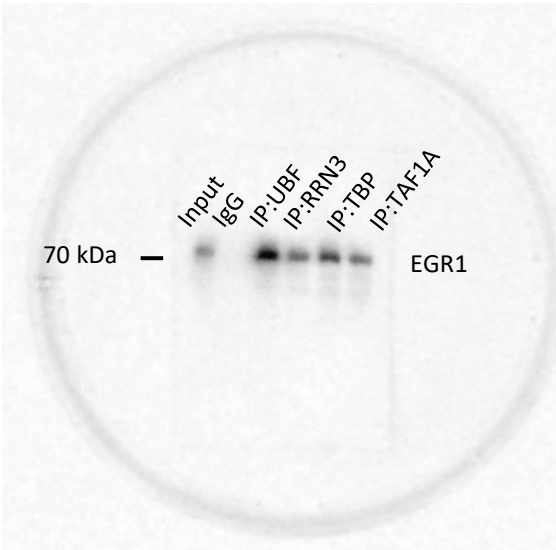
HepG2

Fig. 8

K



L

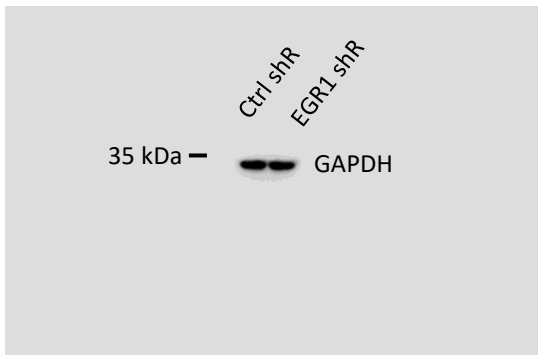
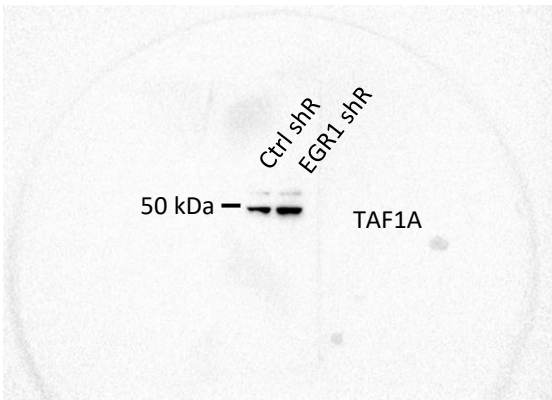
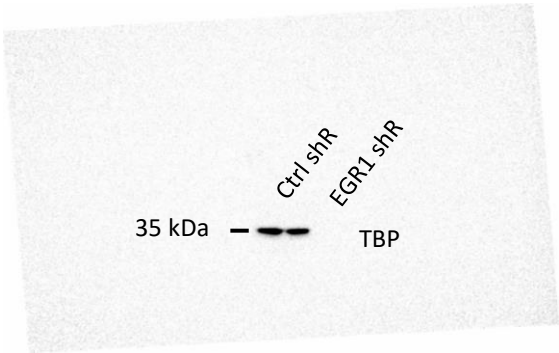
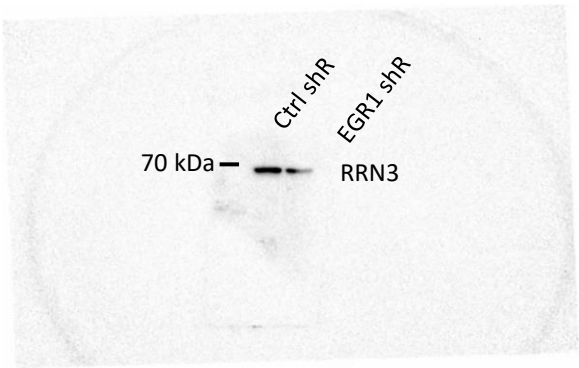
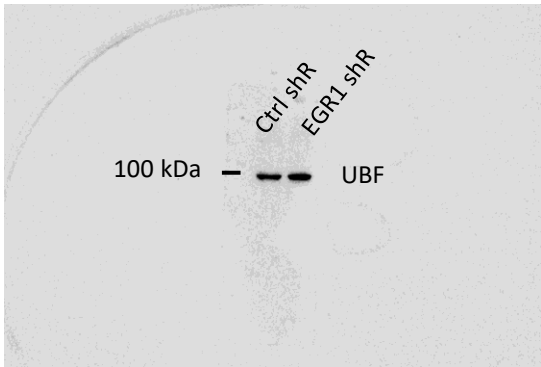
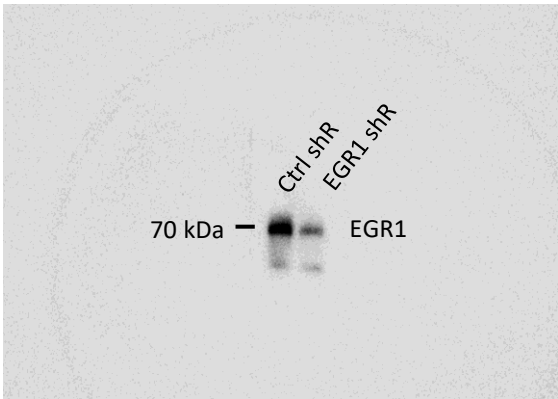


HepG2

HepG2

Fig. 9

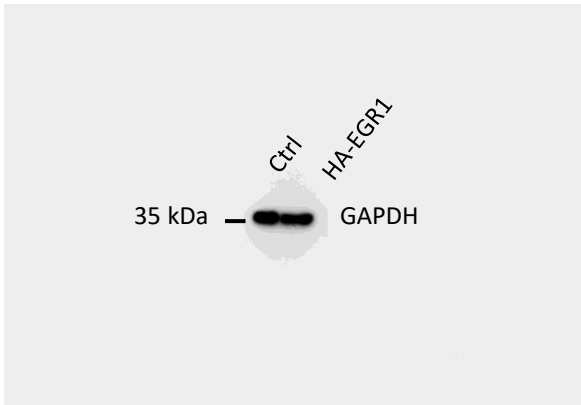
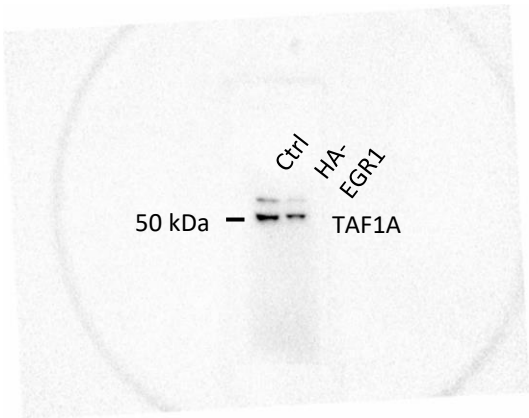
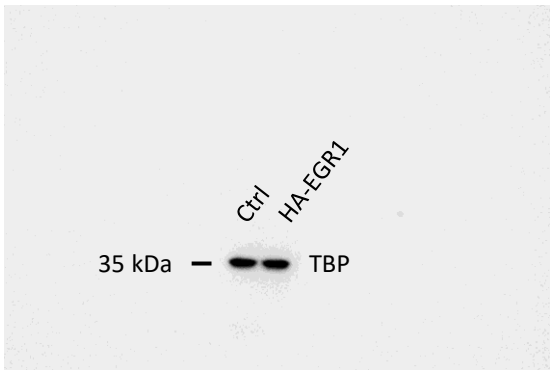
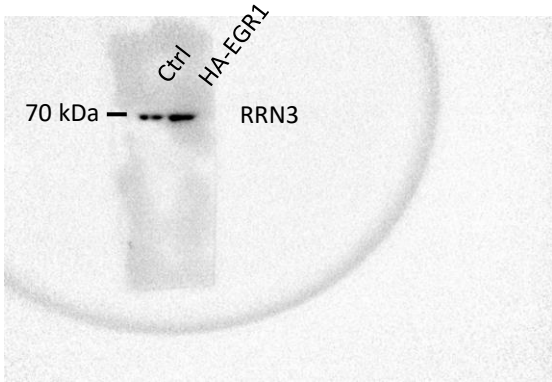
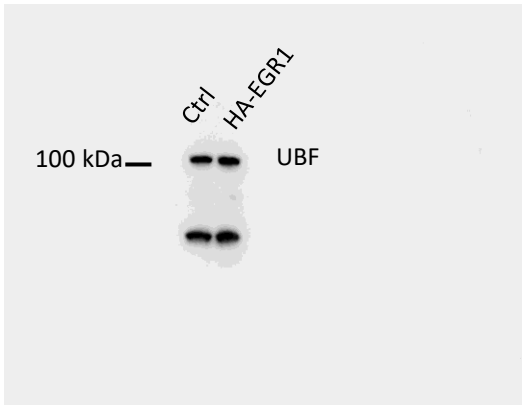
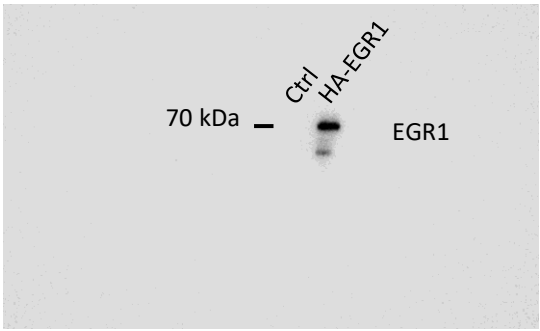
J



HeLa

Fig. 9

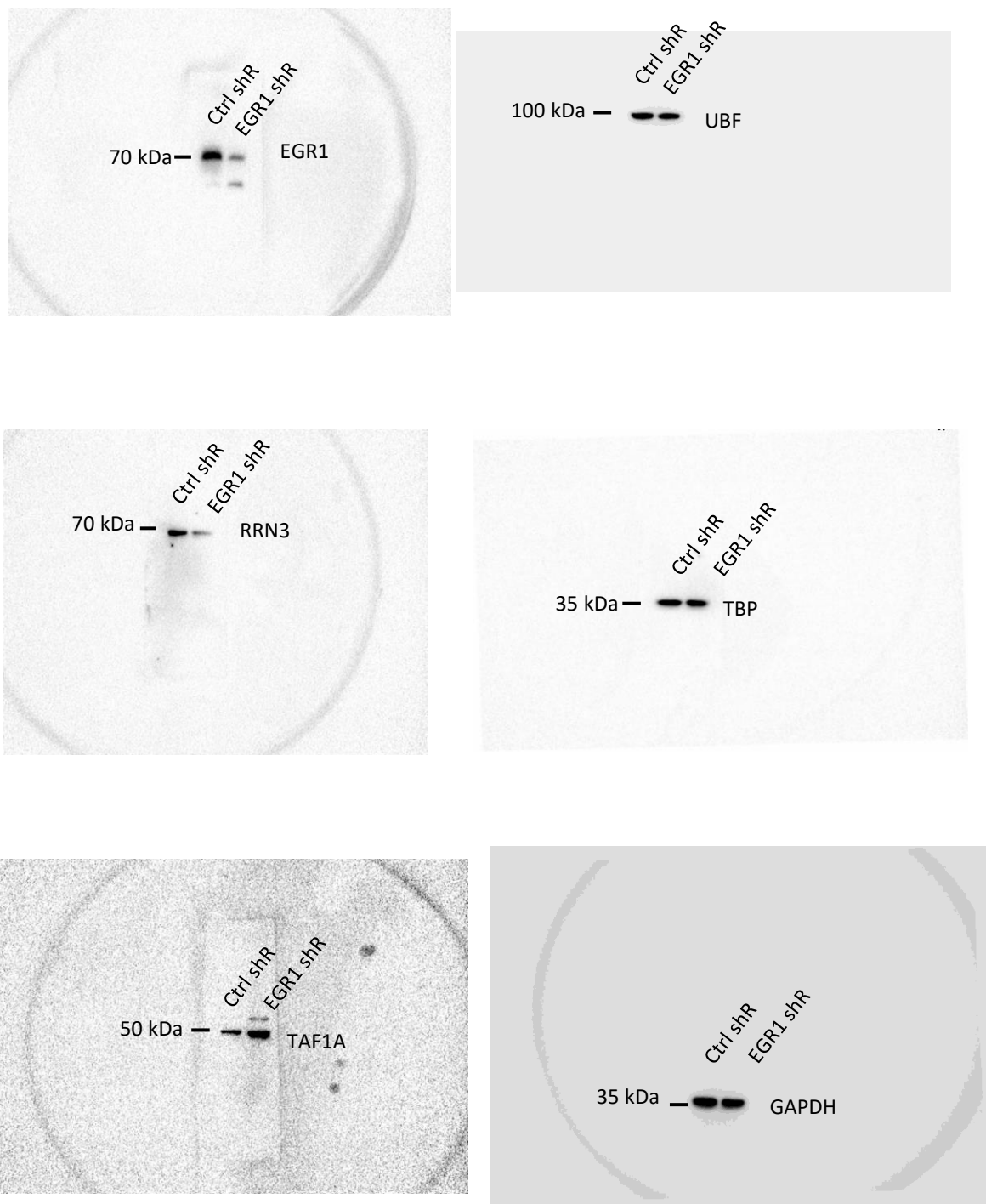
K



HeLa

Fig. 9

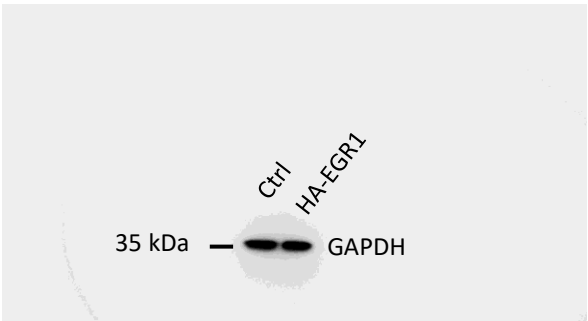
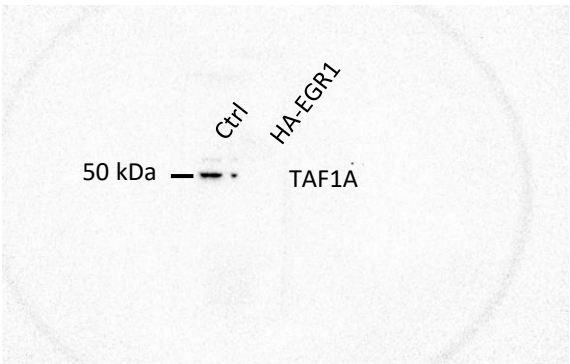
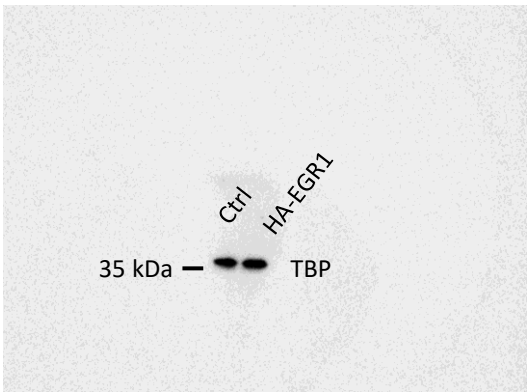
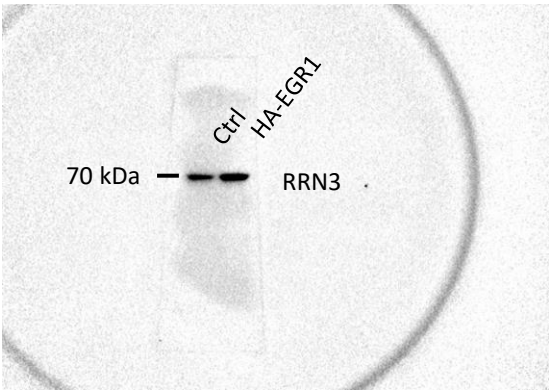
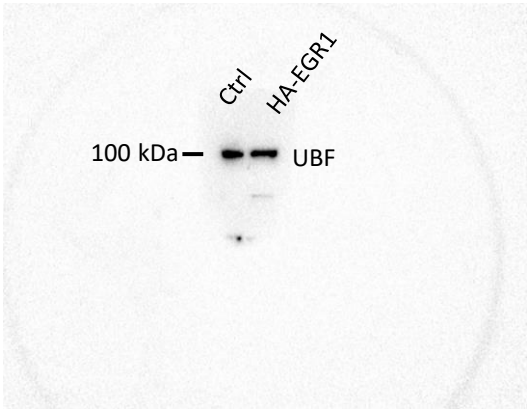
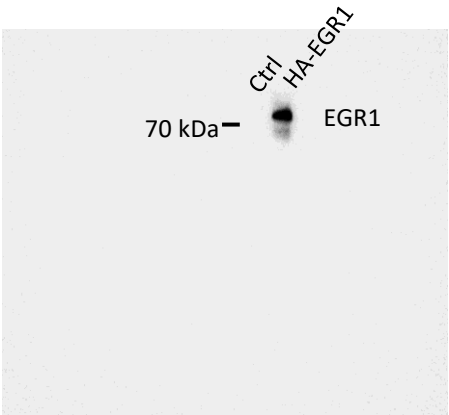
L



HepG2

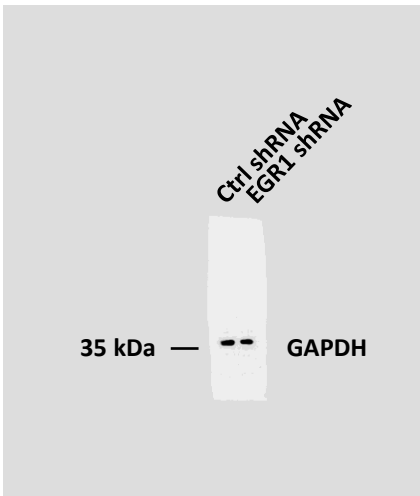
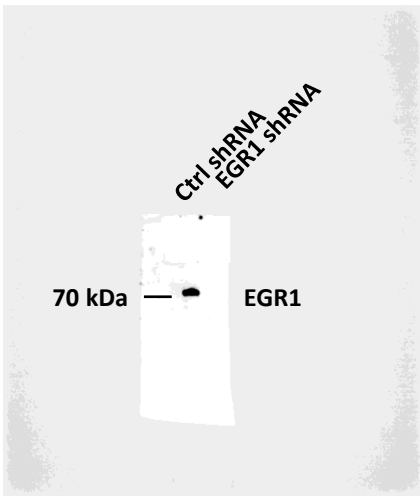
Fig. 9

M

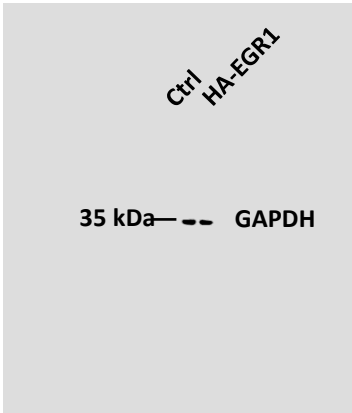
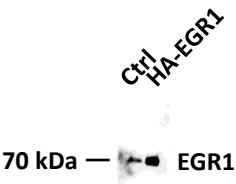
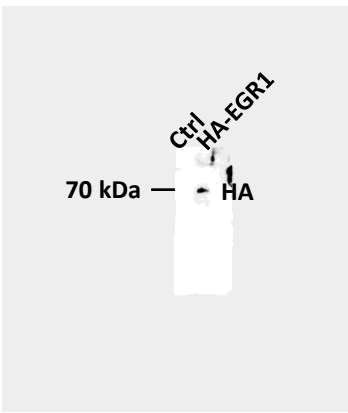


Supplementary Fig. 3

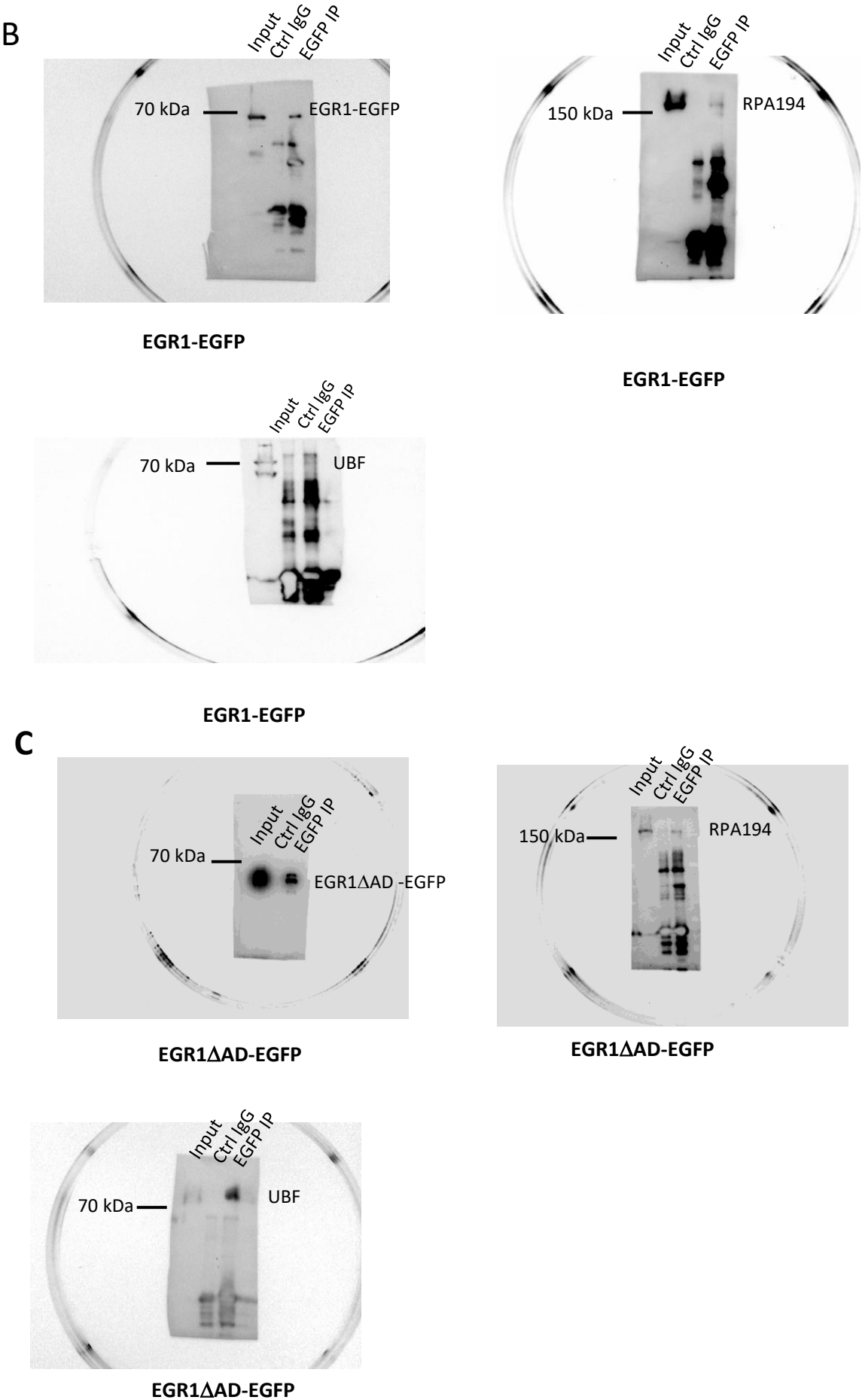
A



E

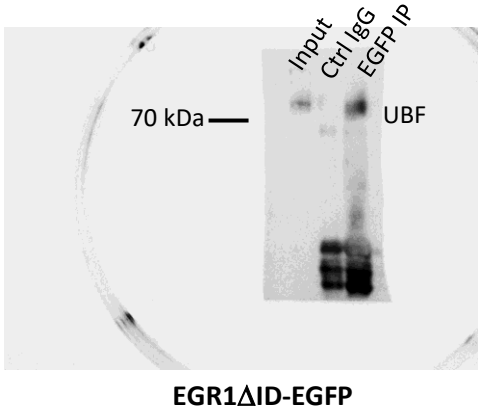
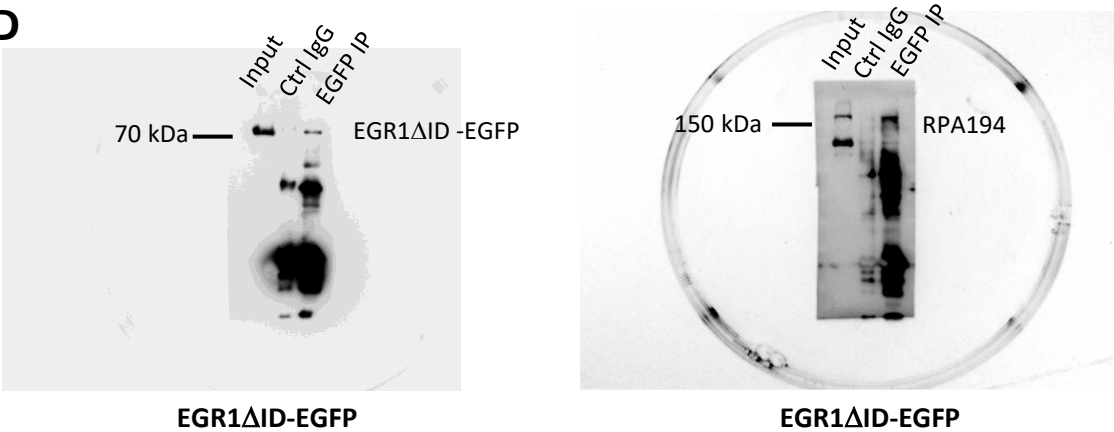


Supplementary Fig. 10

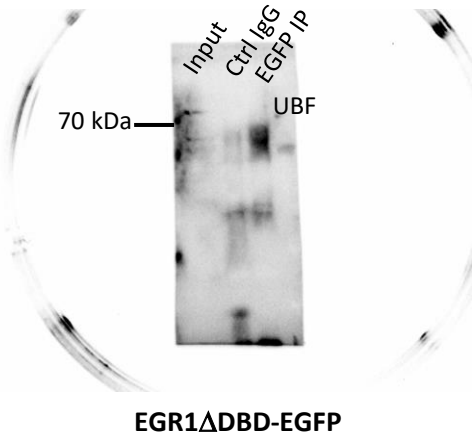
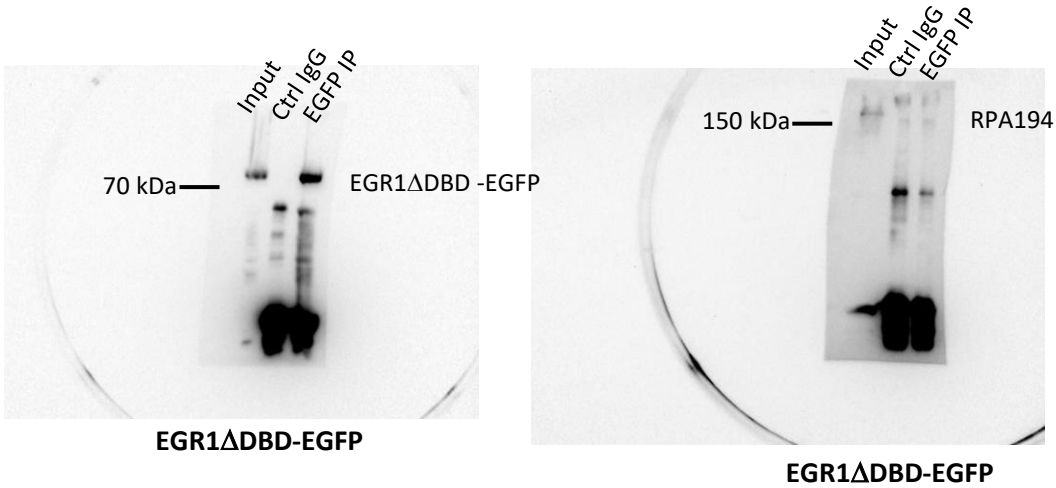


Supplementary Fig. 10

D

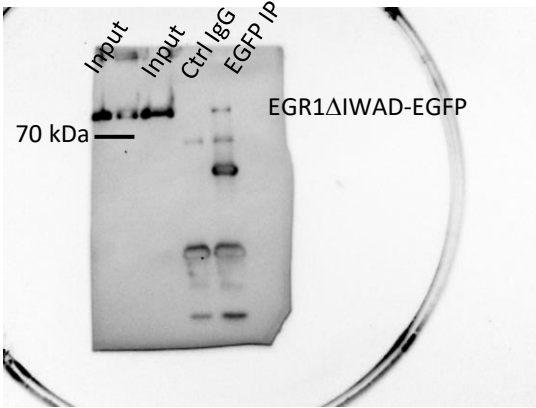


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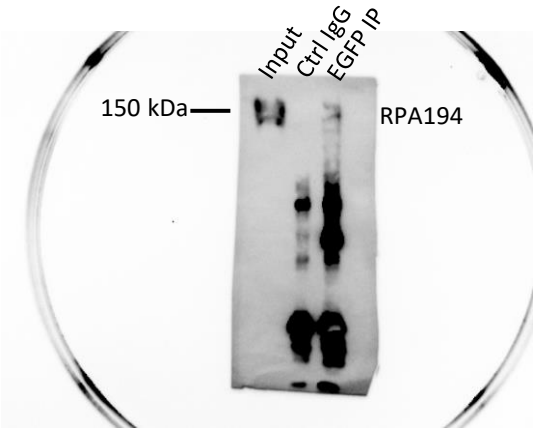


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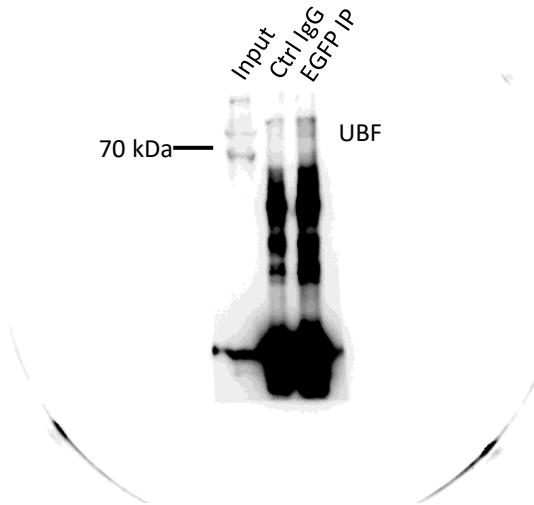
F



EGR1ΔWAD-EGFP



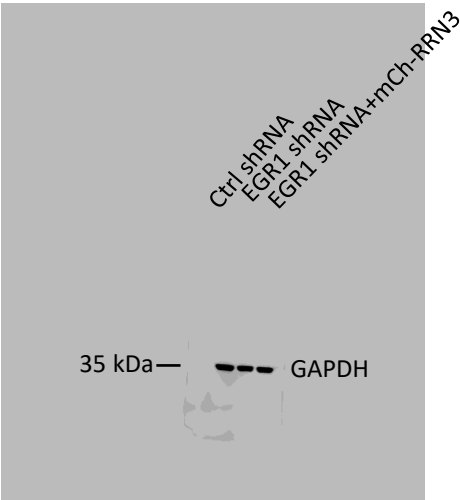
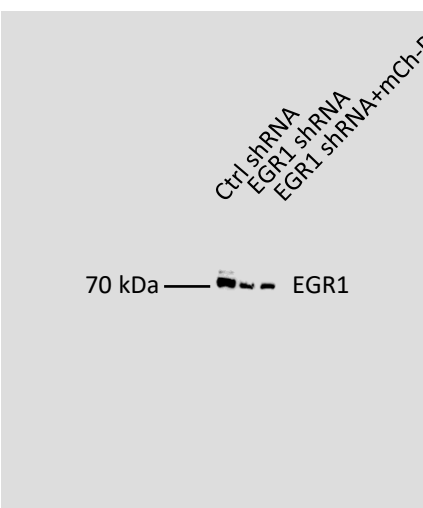
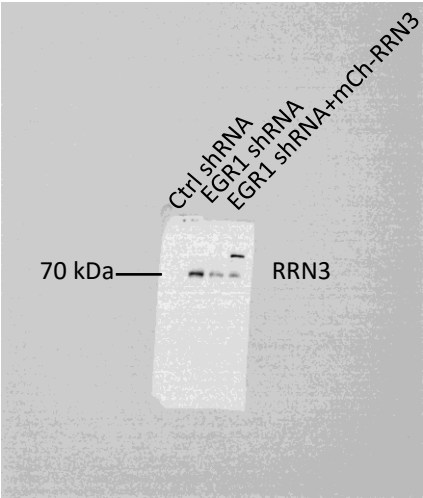
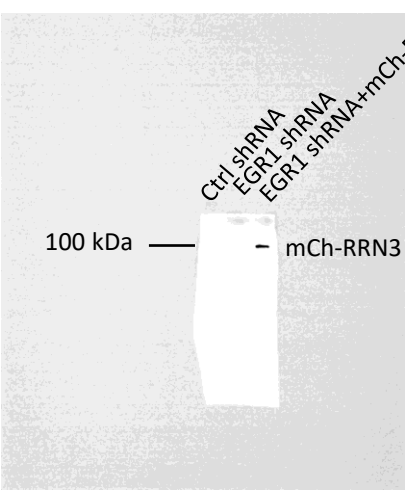
EGR1ΔWAD-EGFP



EGR1ΔWAD-EGFP

Supplementary Fig. 12

A



E

