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CGGBP1 phosphorylation constitutes a telomere-protection signal

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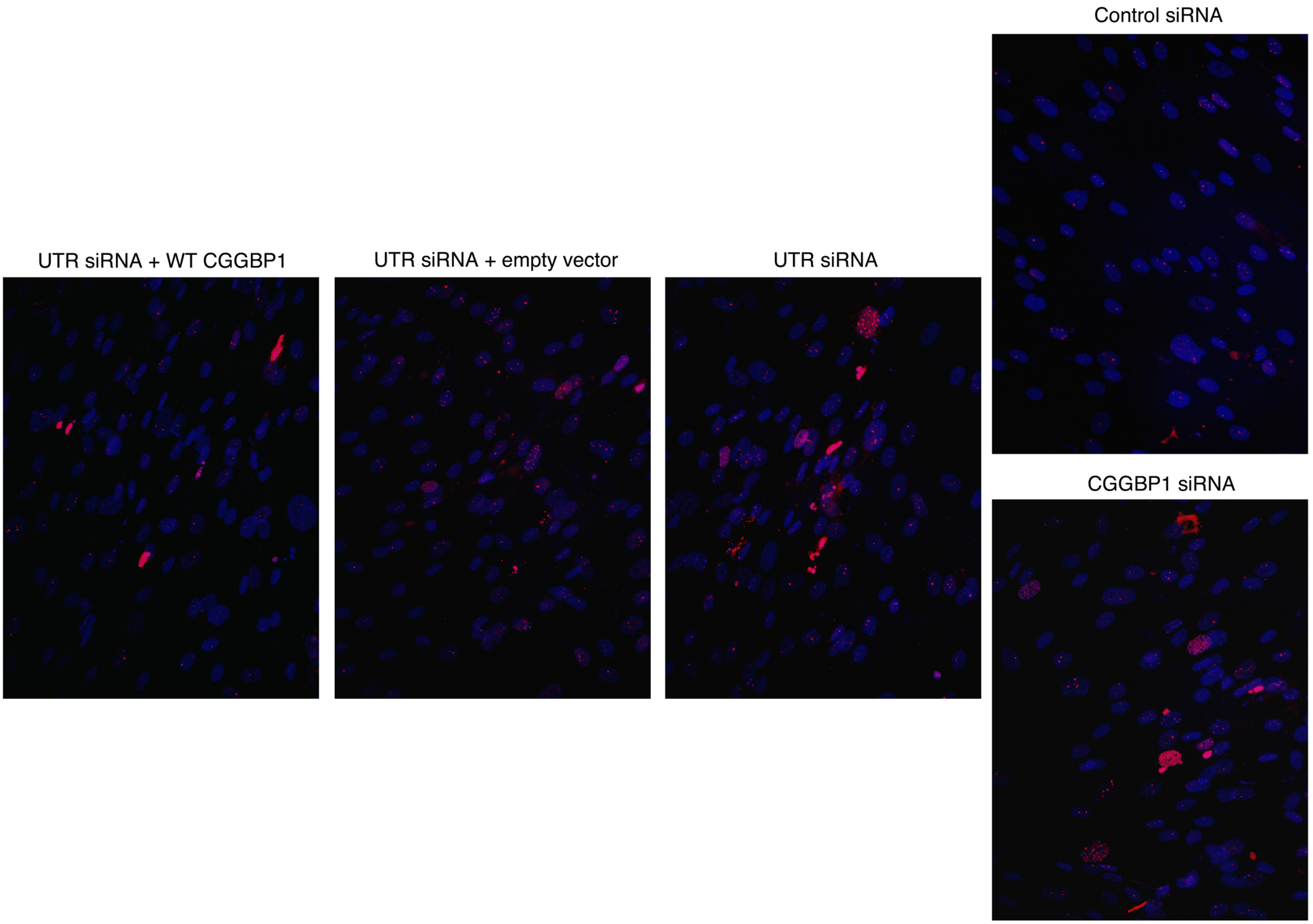
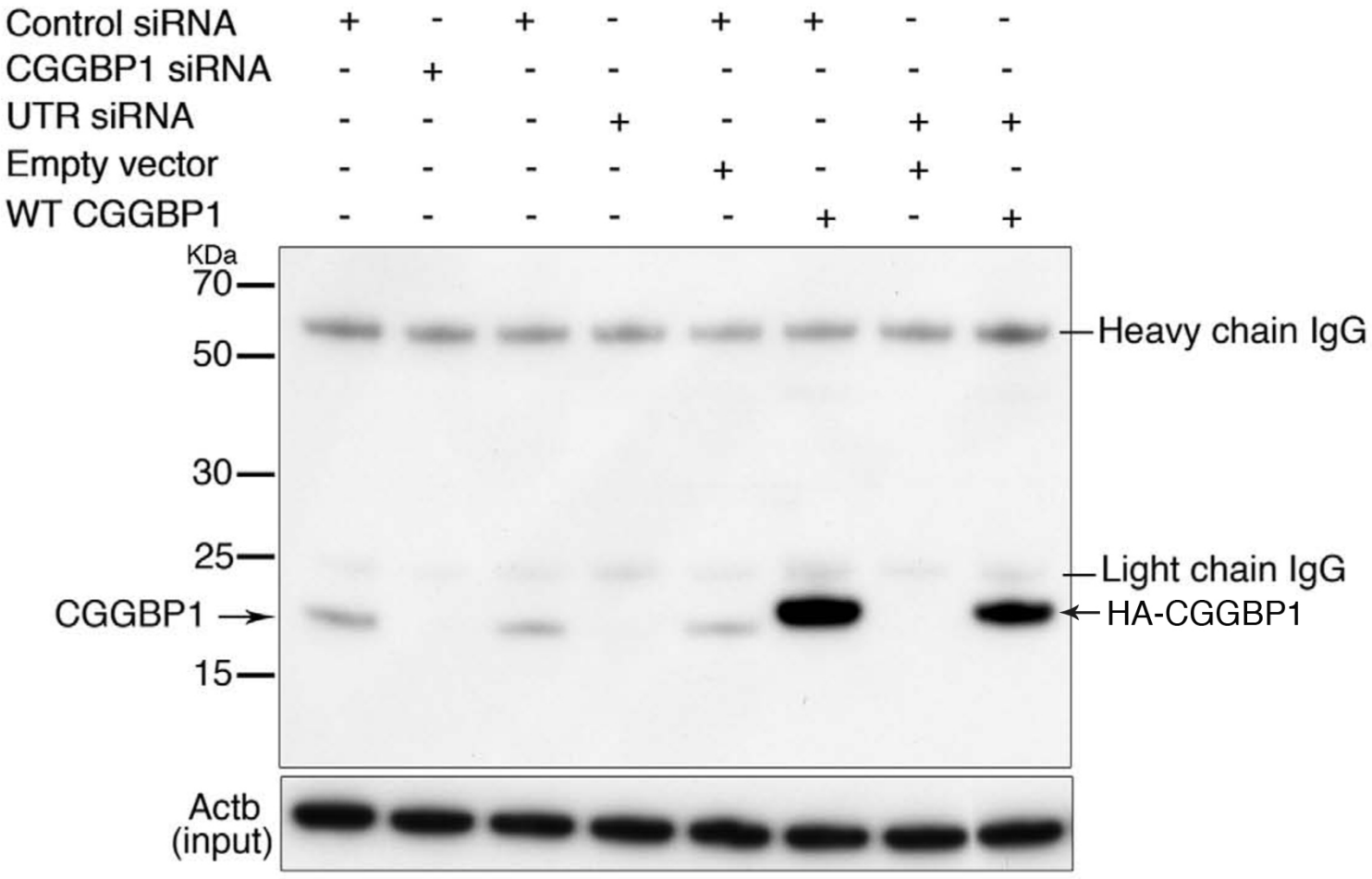
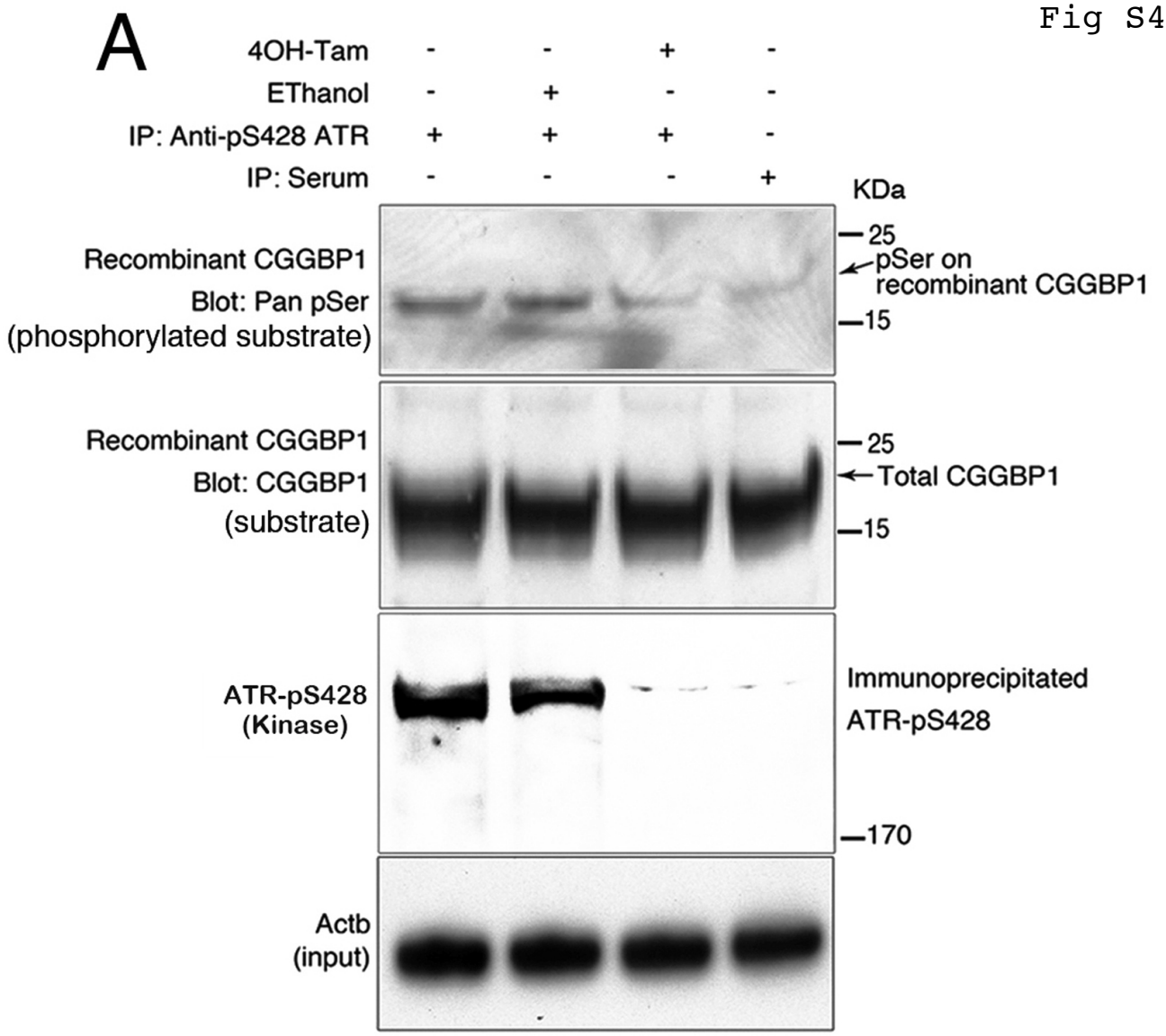

Control siRNA
UTR siRNA + WT CGGBP1
UTR siRNA + empty vector
UTR siRNA
CGGBP1 siRNA

Fig S1

Fig S2

Fig S3


Control siRNA + - + - + + - -
CGGBP1 siRNA - + - - - - - -
UTR siRNA - - - + - - + +
Empty vector - - - - + - + -
WT CGGBP1 - - - - - + - +
KDa
70
50
30
25
15
CGGBP1
Heavy chain IgG
Light chain IgG
HA-CGGBP1
Actb
(input)


A
4OH-Tam - - + -
EThanol - + - -
IP: Anti-pS428 ATR + + + -
IP: Serum - - - +
KDa
Recombinant CGGBP1
Blot: Pan pSer
(phosphorylated substrate)
25
pSer on
recombinant CGGBP1
15
Recombinant CGGBP1
Blot: CGGBP1
(substrate)
25
Total CGGBP1
15
ATR-pS428
(Kinase)
Immunoprecipitated
ATR-pS428
170
Actb
(input)

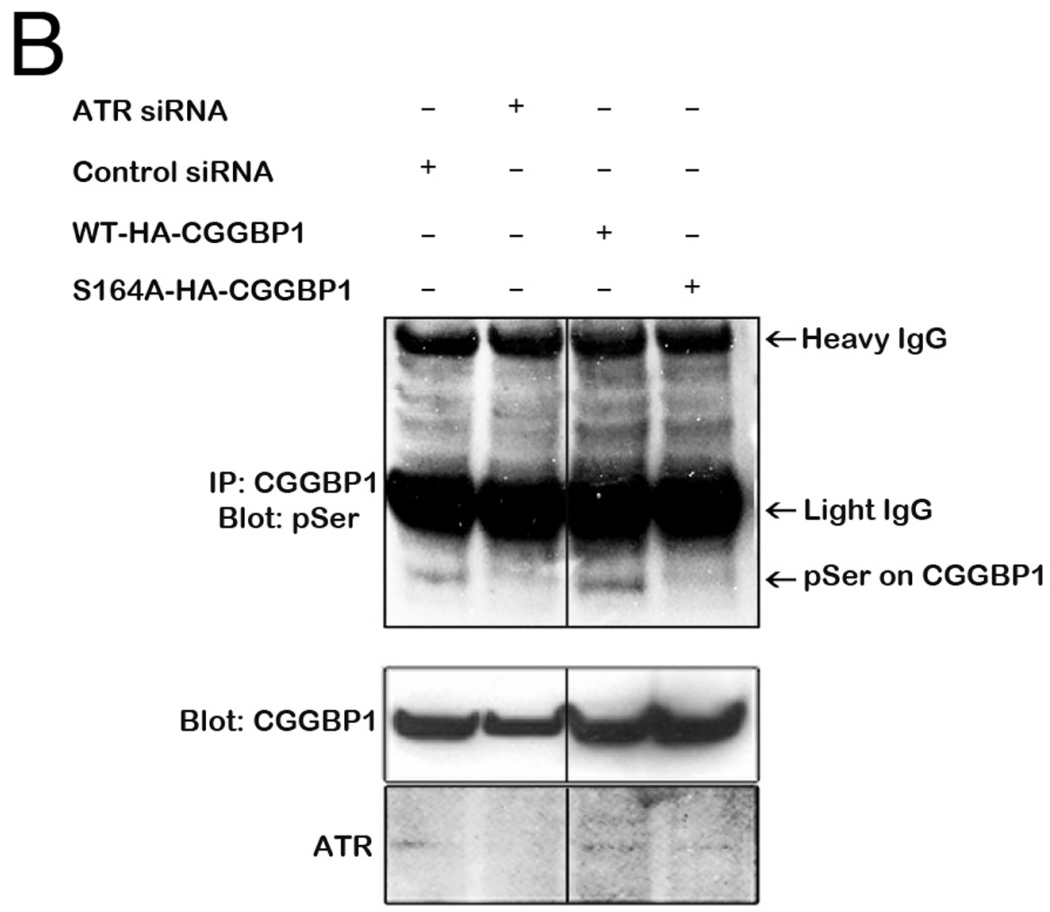
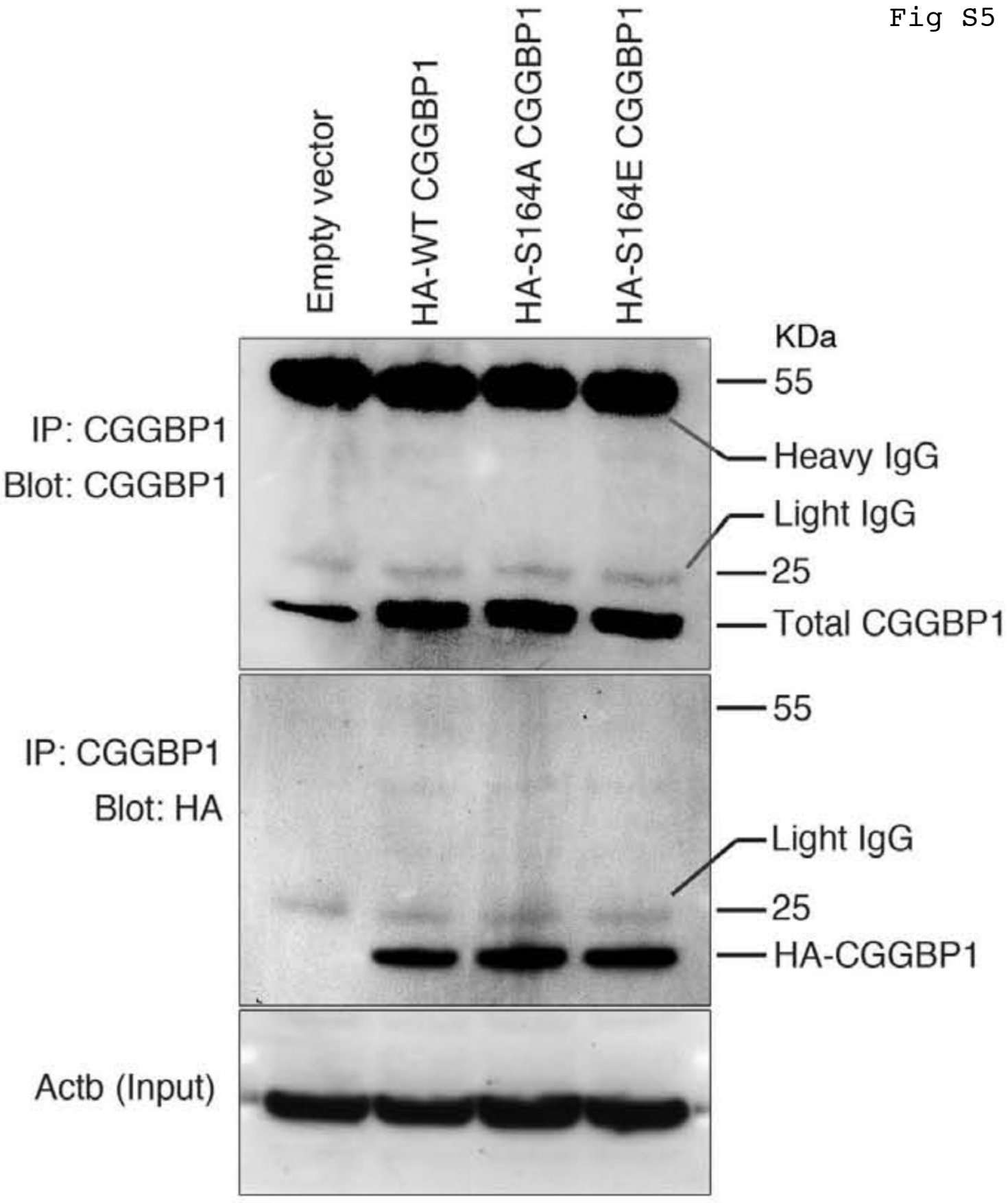

B
ATR siRNA – + – –
Control siRNA + – – –
WT-HA-CGGBP1 – – + –
S164A-HA-CGGBP1 – – – +
Heavy IgG
IP: CGGBP1
Blot: pSer
Light IgG
pSer on CGGBP1
Blot: CGGBP1
ATR

Fig S5


Empty vector
HA-WT CGGBP1
HA-S164A CGGBP1
HA-S164E CGGBP1
KDa
55
IP: CGGBP1
Blot: CGGBP1
Heavy IgG
Light IgG
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Total CGGBP1
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Blot: HA
Light IgG
25
HA-CGGBP1
Actb (Input)

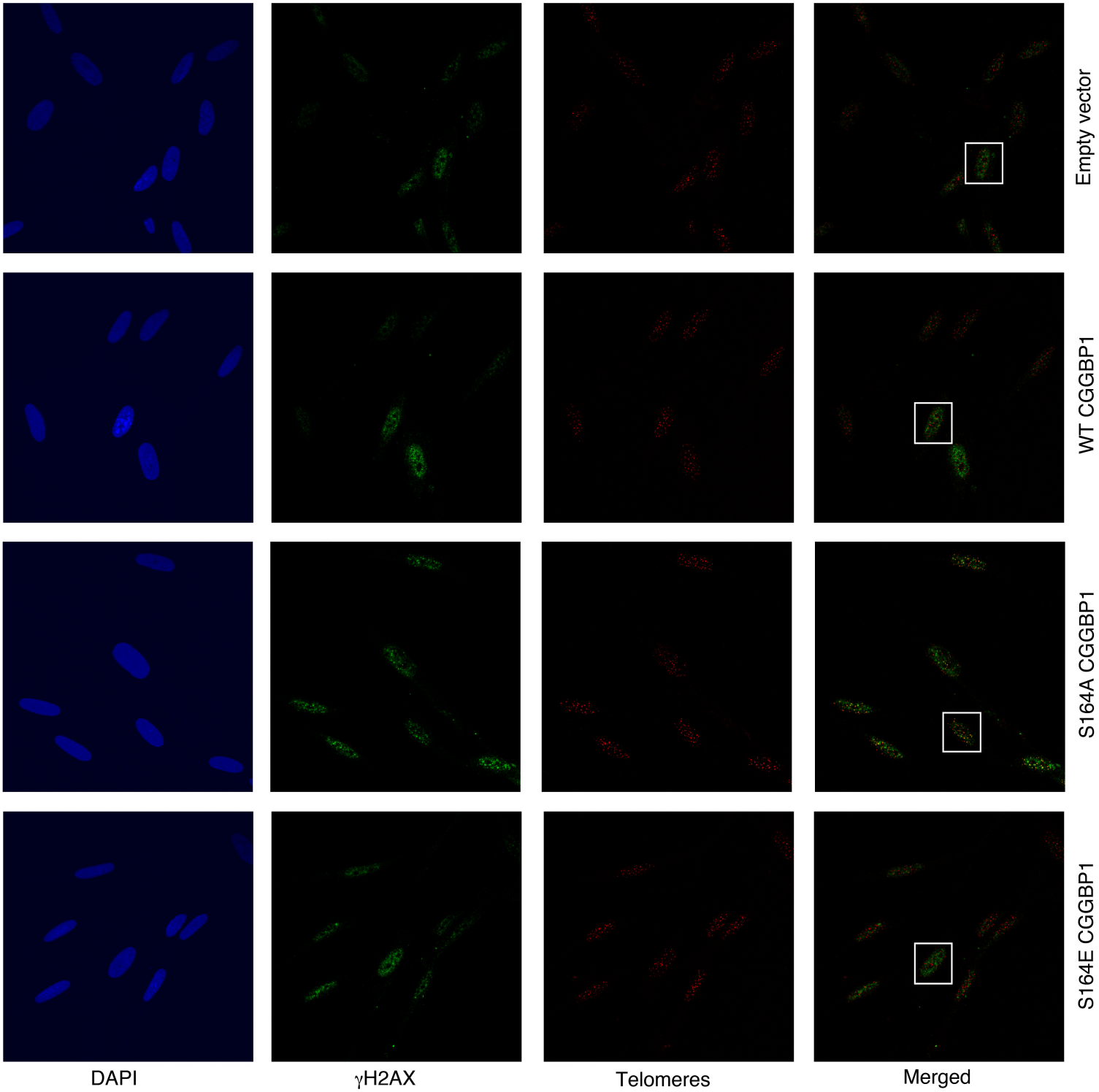
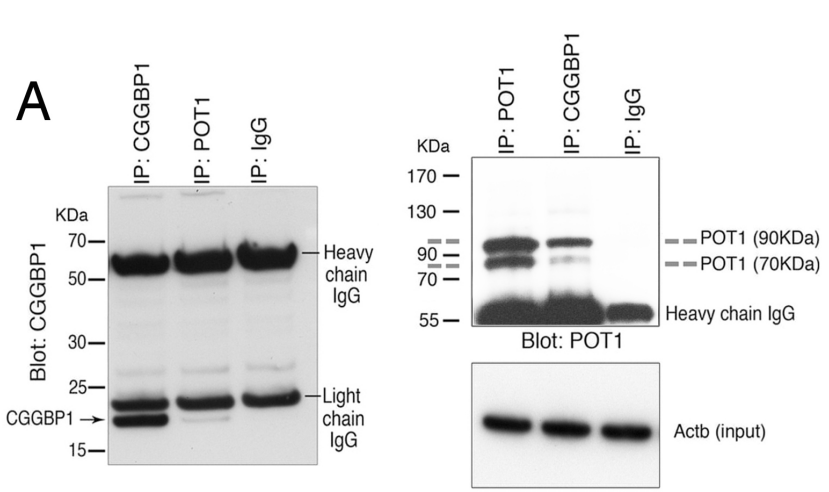
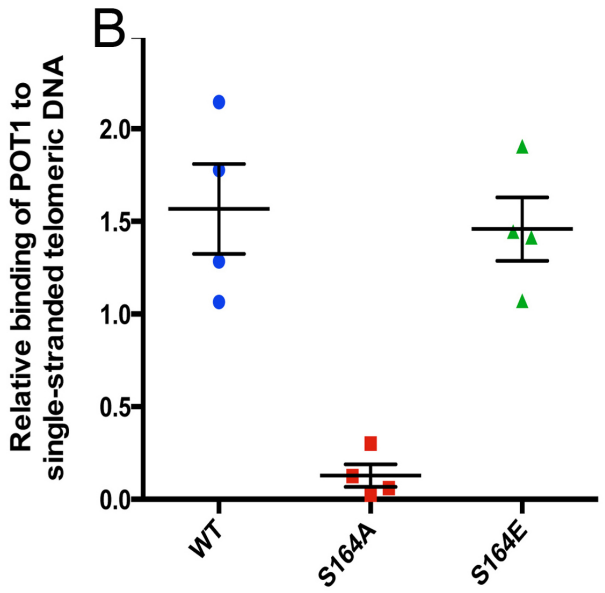
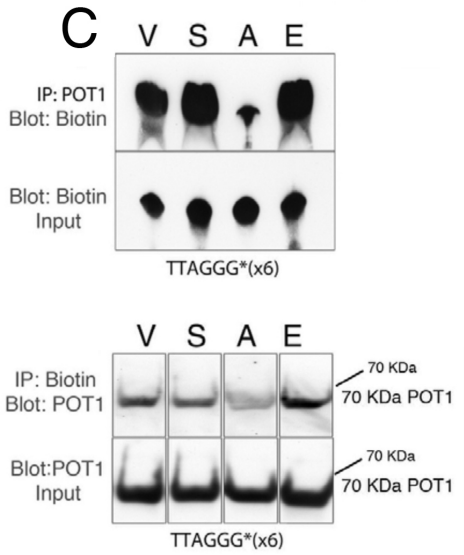
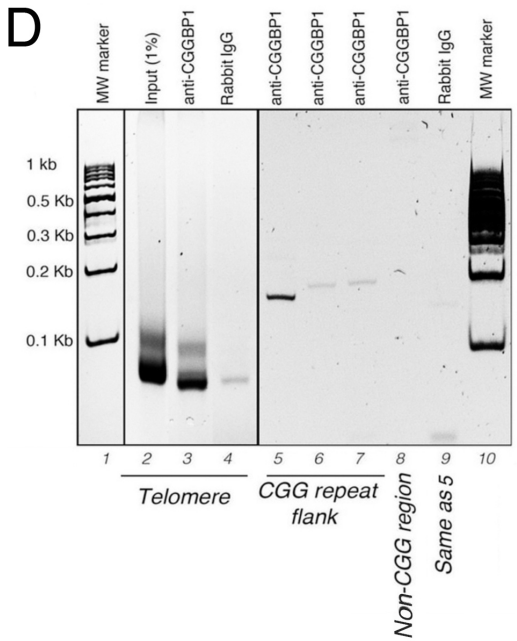


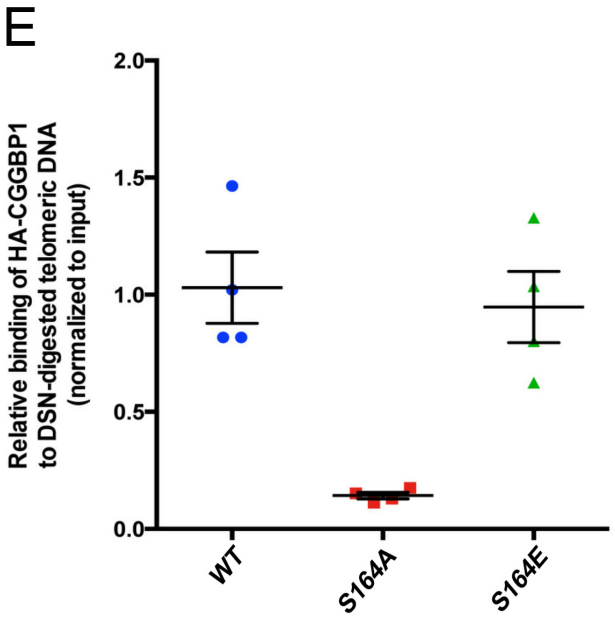
Fig S6
Empty vector
WT CGGBP1
S164A CGGBP1
S164E CGGBP1
DAPI
γH2AX
Telomeres
Merged


A
IP: CGGBP1
IP: POT1
IP: IgG
KDa
70
50
30
25
15
Blot: CGGBP1
CGGBP1
Heavy chain IgG
Light chain IgG
KDa
170
130
90
70
55
IP: POT1
IP: CGGBP1
IP: IgG
POT1 (90KDa)
POT1 (70KDa)
Heavy chain IgG
Blot: POT1
Actb (input)


B
Relative binding of POT1 to single-stranded telomeric DNA
2.0
1.5
1.0
0.5
0.0
WT
S164A
S164E


C
V S A E
IP: POT1
Blot: Biotin
Blot: Biotin
Input
TTAGGG*(x6)
V S A E
IP: Biotin
Blot: POT1
70 KDa
70 KDa POT1
Blot:POT1
Input
70 KDa
70 KDa POT1
TTAGGG*(x6)


D
MW marker
Input (1%)
anti-CGGBP1
Rabbit IgG
anti-CGGBP1
anti-CGGBP1
anti-CGGBP1
anti-CGGBP1
Rabbit IgG
MW marker
1 kb
0.5 Kb
0.3 Kb
0.2 Kb
0.1 Kb
1 2 3 4 5 6 7 8 9 10
Telomere
CGG repeat flank
Non-CGG region
Same as 5


E
Relative binding of HA-CGGBP1 to DSN-digested telomeric DNA (normalized to input)
2.0
1.5
1.0
0.5
0.0
WT
S164A
S164E

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Overview of micrographs showing γH2AX staining in 1064Sk cells with different siRNA and CGGBP1 overexpression regimens as indicated. Subsets of these are shown at a higher magnification in Fig. 1, B and D. For quantification of γH2AX spots, please refer to Fig. 1, A and C.

Supplementary Figure 2. Overviews of micrographs showing the overlap between γH2AX foci and telomere FISH signals in control or CGGBP1 siRNA treated 1064Sk cells. The demarcated insets are shown at a higher magnification in Fig. 1E.

Supplementary Figure 3. Western blot analysis of IP: CGGBP1, blot: CGGBP1 in 1064Sk cells treated with control, CGGBP1 or UTR siRNA in presence or absence of WT CGGBP1 overexpression. The endogenous CGGBP1 band shows up at approximately 20 KDa, just below the light chain IgG, visible as faint bands in each lane at 25 KDa. Both, CGGBP1 and UTR siRNA efficiently knocked down CGGBP1 to near undetectable levels at this level of exposure (lane 1 compared lane 2 and lane 3 compared to lane 4). The overexpression of HA-tagged WT CGGBP1 is strongly detected (lane 5 compared to lane 6). This overexpression of CGGBP1 is absent from the empty vector expressing cells (lane 5). The overexpressed CGGBP1, unlike the endogenous CGGBP1, is spared by the UTR siRNA (lane 7 compared to lane 8). The ACTB level for each sample in the input has been used to demonstrate the relative amounts of total proteins in the lysates subjected to the IPs. The numbers to the left of the gel indicate molecular weight in KDa. The assay was performed 96h post-transfection.

Supplementary Figure 4. ATR can phosphorylate CGGBP1 in vitro, affects CGGBP1 pSer levels in normal cells and S164A mutation reduces pSer levels associated with CGGBP1. **A:** Endogenously activated ATR (ATR-pS428) immunoprecipitated from mouse embryonic fibroblasts (kinases) induced serine phosphorylation of recombinant human CGGBP1 (substrate). ACTB represents amount of protein subjected to immunoprecipitation. Conditional deletion of ATR allele (4OH-Tam lane compared to ethanol lane) reduced ATR-pS428 as well as CGGBP1 phosphorylation levels. ATR regulates serine phosphorylation levels of CGGBP1 in 1064Sk cells and the known ATR target site at S164 is a major phosphorylation site on human CGGBP1. **B**: The amount of phosphoserine detected by a pan-phosphoserine antibody on endogenous CGGBP1 from 1064Sk cells (IP: CGGBP1, Blot: Pan-phosphoserine) was reduced by ATR siRNA (lane 1 compared lane 2), showing the regulatory effect of ATR levels on CGGBP1-serine phosphorylation. Phosphoserine levels on CGGBP1 were reduced by ATR siRNA and S164A mutation both. The line denotes that the total amount of input used for the first two lanes (confluent 10cm dish) was approximately 5 times that of the input used for the last two lanes (confluent well of a 6 well plate) and hence can not be quantitatively compared.

Supplementary Figure 5. Immunoprecipitation-western blot analysis of total CGGBP1 (endogenous plus overexpressed HA-tagged) and overexpressed CGGBP1 (HA-tagged) levels after 8 weeks post-selection to ascertain the exyent of CGGBP1 overexpression and any differences in overexpression of WT, S164A and S164E CGGBP1. The CGGBP1 (top) and HA (middle) blots were run after

immunoprecipitation with CGGBP1 antibody, whereas the input was used to measure ACTB levels (bottom).

Supplementary Figure 6. Overviews of micrographs showing the overlap between γH2AX foci and telomere FISH signals in empty vector, WT, S164A or S164E CGGBP1 overexpressing 1064Sk cells. The demarcated insets are shown at a higher magnification in Fig. 3H. Assays were performed 4 weeks post-transfection.

Supplementary Figure 7. CGGBP1 interacts with POT1 and S164 phosphorylation affects its DNA binding. **A:** Reciprocal co-immunoprecipitation and western blot analysis of endogenous CGGBP1 and POT1 in 1064Sk cells to detect interaction between them. Endogenous CGGBP1 and POT1 were co-immunoprecipitated with each other. An unexpected 90 KDa form of POT1 was also seen. The input (same for both sets of IP-westerns) was used to measure ACTB levels **B:** Graph showing ddCt analysis of qPCR performed on DSN-digested POT1-ChIP DNA to detect changes in POT1 binding to telomeric DNA upon WT or mutant CGGBP1 overexpression. In S164A cells POT1 binding to single stranded telomeric DNA was reduced significantly compared to WT or S164E samples (p value for both comparisons <0.01, n = 4 technical replicates of pooled duplicate assays). **C:** *In vitro* interaction assays between biotinylated single stranded telomeric oligos and endogenous POT1 were performed. Pull down using streptavidin-agarose beads precipitated only the 70 KDa form of POT1 as interacting with the telomeric probe. Amount of biotinylated telomeric probe pulled down with POT1 or amount of POT1 pulled down with streptavidin-agarose beads were both reduced in S164A sample compared to WT or S164E. (*V* = empty vector overexpression, *S* = S164S WT CGGBP1 overexpression, *A* = S164A overexpression and *E* = S164E overexpression) **D:** CGGBP1 also specifically gets recruited to telomeres. ChIP-PCR resolved on 6% acrylamide gel shows specific telomeric DNA pulldown using CGGBP1 antibody. Known positive control regions (CGG repeat flanking regions) and negative control regions were used to establish the specificity of the assay. **E:** Graph showing ddCt analysis of qPCR performed on DSN-digested HA-ChIP DNA to detect changes in overexpressed HA-tagged CGGBP1 binding to telomeric DNA upon S164A or S164E mutations. The S164A mutation reduced CGGBP1 binding to the single stranded telomeric DNA as compared to WT or S164E mutation (p value for both comparisons <0.01, n = 4 technical replicates of pooled duplicate assays).

METHODS

Cell culture

1064Sk normal human fibroblasts, passage (8-12), were cultured in Eagle's minimum essential medium (SIGMA) At passage (14-15), the cells were transfected with either WT or S164G CGGBP1-expressing constructs Four days post-transfection transfected cells were selected in 800 µg/ml G418 in the medium for 1 week and 300 µg/ml G418 in the medium later. To avoid the effects of variations between the clones, colonies were pooled and cultured. The pooling of colonies defined the first passage post-transfection. The numbers of passages post-transfection in which different experiments were carried out are as follows: 5-7 for the phospho-Serine levels measurement and ATR-CGGBP1 association, 5 for the start of the proliferation assay, 8 for cell cycle analysis by FACS, 10-15 for the measurement of the abscission time, 15-20 for β-Galactosidase activity assay, 10-15 for detection of chromatin bridges in dividing cells and endogenous DNA damage response activation, 15-20 for in vitro telomerase activity assay and telomere length measurement. Experiments on G-strand shortening and POT1-GCGBP1 interactions were done on transiently transfected cells 4 days post-transfection. Equal transfection efficiencies were established by no differences in the expression of the HA-tagged CGGBP1 levels.

Expression constructs and transfections

Full-length cDNA of CGGBP1 was amplified from RNA of 1064Sk human fibroblasts using primers TAT CCT TAC GAT GTA CCA GAC TAT GCT GAG CGA TTT GTA GTA ACA GC (with HA tag) at the 5′ end and TAT AGC GGC CGC TCA ACA ATC TTG TGA GTT GAG (with stop codon) at the 3′ end. A re-PCR using the same reverse primer but TGT AGG TAC CGC CAC CAT GGG ATA TCC TTA CGA TGT ACC AGA CTA TGC T as forward primer (with Kozak sequence and start codon upstream of HA tag), thus adding HA-tag and Kozak sequence into the CGGBP1 cDNA. Eluted PCR product was restricted with *Kpn*I and *Xho*I (New England Biolabs) endonucleases and the purified product was ligated into pcDNA3.1+ (Invitrogen), also restricted in the same way, using quick ligation reagents from New England Biolabs. The ligation mix was transformed into competent E.coli (Promega), selected on LB agar-ampicillin plates and plasmids were extracted and sequenced. Fugene (Roche) was used to transfect the plasmids and generate stably transfected cells, which were selected as a pool to avoid clonal variations using G418 in the culture media.

siRNA and transfections

CGGBP1 and ATR siRNA (Dharmacon) were separate pools of 4 different siRNA duplexes directed against CGGBP1 ORF and ATR ORF respectively (SmartPool, Dharmacon). The CGGBP1 UTR siRNA (Dharmacon) duplexes were custom made with Dharmacon's modifications (the target sequences were CCATTGTGATCAAGATAAA in the 3′UTR and ACGGAAAGTGCCAGGATTT in the 5′UTR of CGGBP1 mRNA having the RefSeq identification NM_001008390.1). Equimolar mixture of the two UTR siRNA was used as the UTR siRNA pool. Dharmafect 2 was used as the transfection reagent and about 50% confluent cultures were transfected after about 30 h of plating. The final siRNA concentration was 100 nM. Western blots or qRTPCRs were routinely performed to ascertain the efficacy of siRNA knockdown and has been described elsewhere in details[1].

Cell cycle analysis by flow cytometry

The method used for flow cytometry (BD Biosciences) measures the DNA content per cell. Briefly, the cells were washed twice in ice-cold 1xPBS trypsined, resuspended in culture medium with serum and filtered through a 40 micron strainer, and stained with Hoechst 33342 at 500 micrograms/ml. ModFit was used to analyze the results with automated settings for removal of multi-nuclear aggregates as well as fragmented nuclei from the dead cells.

Immunofluorescence assays

Immunofluorescence was performed by a previously described method[2].

Co-immunoprecipitation assays and western blotting

Phosphate-buffered RIPA, supplemented with protease and phosphatase inhibitor cocktails (SIGMA), was used to lyse cells on ice for 30 min and the lysates were centrifuged to clear them of insoluble fraction. Cleared lysates were incubated with with protein G sepharose beads for 1h for clearing. Then 2-4µg of antibody was added to the beads-free lysates and

samples were gently mixed overnight at 4°C. Protein G sepharose beads were used to pull down the antibody-protein complex conjugates by incubating the antibody-lysate mixture with beads for 2h at 4°C. Samples were centrifuged to precipitate the beads which were then washed with ice-cold phosphate buffered RIPA, mixed with the denaturing agent (Invitrogen) and heated at 85°C for 10 minutes for reducing conditions. All samples were simultaneously run on NuPage 4-12% Bis-Tris or 3-8% TA Gels and samples were maintained on ice unless mentioned otherwise.

γH2AX stainings and telomere FISH foci

γH2AX stainings were performed according to the standard immunofluorescence protocol as mentioned above. Following the last wash in PBST after incubation with secondary antibody, the coverslides (cells attached on them) were rinsed in 1xTBS and incubated for 2 minutes in 4% formaldehyde in 1xTBS. Further rinsing in 1xTBS was followed by denaturing of DNA using a pre-treatment solution (DAKO 5326) and rinsing again in 1xTBS. The slides were then dehydrated in ascending grades of ethanol maintained at -20C. Telomeric-PNA probe was laid on dry slides, covered with coverslides and enclosed in slide hybridization chambers (Cornig) with 10 ul of water spots at both ends. Closed chambers were incubated in waterbath at 80C for 5 minutes and room temperature in water for 16h. The coverslides were washed in rinse and wash buffers as directed (DAKO 5326), dehydrated and mounted with DAPI-containing mounting medium and sealed with nailpaint. Slides were stored at 4C and micrographs were obtained using a leica camera attached to Leica fluorescence microscope using Adobe Photoshop-import function.

DSN digestion and for single stranded telomeric DNA qPCR

DSN (Evrogen) digestion was performed as standerd manufacturer's instructions. 10units (excess) of the enzyme was used and duration of digestion was 1h. DSN digestion was done at 65C. ChIP DNA obtained from each sample was not digested or digested with 10 units of DSN. Subsequently, the samples were purified using Qiagen columns for gel-extraction and eluted in equal volumes of water. Samples were then denatured at 95C for 5 minutes and snap chilled on ice for 10 minutes. Equal volumes of samples were then used as templates in qPCR.

DAPI staining for DNA in chromatin bridges

Cells were fixed with methanol, permeabilized with PBS containing 0.2% triton-X-100 and processed for antibody incubations as needed. After washings, the cells were bathed in DAPI dissolved in PBS (300 nM) with gentle shaking for 15 min followed by thorough washings with PBS for 10 min with multiple changes of PBS. Finally, the mounting was done in a DAPI-containing aqua-based mounting medium.

Chromatin immunoprecipitation (ChIP) assay

Cells were collected by flow sorting 4 days post-transfection (1064Sk cells transfected with eGF-tagged CGGBP1 expressing vector, used for CGGBP1 and POT1 ChIP; 25000 cells were collected for each sample) and immediately fixed with 2% formaldehyde for 10 minutes at 37°C. CGGBP1 and POT1 vectors co-transfected HEK293T cells were fixed directly in plates at same final concentration of formaldehyde. The Upstate protocol, as we have used earlier[1] was followed with some variations. Lysis was performed as suggested and sonication of crosslinked chromatin was optimized to yield a sheared smear between 100 and 500 bp. After 1:8 dilution in ChIP dilution buffer, lysated were cleared with 50μl of Protein G Sepharose beads and then mixed with 4μg of CGGBP1 antibody and incubated overnight at 4°C. Protein G Sepharose beads were used to pull down the chromatin-antibody complexes, which were washed with Low Salt, Hight salt, Lithium Chloride and TE buffers before elution in 500ul of elution buffer for 45 minutes. Decrosslinking in presence of 20 μl of 5M NaCl overnight was followed by mixing of EDTA and Tris-HCl and proteinase K digestion was performed for 1h. Samples were purified using phenol chloroform extraction, ethanol precipitated in presence of 2 μg Glycogen, 1/10 v/v 3M Sodium Acetate and 2.5 volumes of 100% Ethanol overnight at -20°C. DNA pellets visualized by glycogen were dissolved in water and subjected to further treatments as described. qPCRs on purified DNA template was performed as previously described[3]. ChIP DNA was digested prior to the PCR with double

strand-specific nuclease to measure POT1 binding to the single stranded telomeric DNA. PCR was performed as described earlier[3].

Live cell time-lapse imaging

Cells were grown on glass slides custom made for a closed (no air and water flow through) chamber system designed for a confocal microscope (Zeiss). Glass slides with cells on were sealed with preconditioned cell culture medium, which was kept at 37°C and 5% CO2 overnight, flooding the cells and air bubbles were sucked out with syringes. At 37°C, controlled by a remote water bath fitted to the microscope stage, time-lapse microscopy was performed in bright field. To have minimal effect on cell viability, light exposure was limited only to 0.3 or 0.5 second exposures at 30 seconds intervals for the durations as mentioned.

Telomere length analysis

Telomere length analysis was performed as described elsewhere[3] with a change in the PCR conditions. A 3-step PCR with following conditions was used: 95C 10 minnutes, 95C 30 seconds, 58C, 30 seconds, 70C, 1 min, 45 cycles. PCR conditions for internal control ribosomal gene were unchanged from the original report[3].

β galactosidase activity assay

Cellular b-Galactosidase activity, a marker of mitotic exit, was measured by endogenous β-Gal activity detection, done as described elsewhere[4].

In Vitro DNA-Protein (POT1) immunoprecipitation

The protocol was modified from a previous report[5]. 10 pmoles of single stranded TTAGGC-oligonucleotide used in EMSA was incubated with different cell lysates (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1% aprotinin, and 1 mMphenylmethylsulfonyl fluoride) as indicated in the figure, on ice for 2 h in presence of 1ug/ml Poly (dI.dC). Immuno-precipitation for POT1 was performed using POT1 antibody (Proteintech, rabbit polyclonal) and the oligonucleotides attached to the immunoprecipitated POT1 were eluted by heating in presence of reducing agent. Samples were run on native 14% Urea-TBE gels and detected using streptavidin-HRP system as mentioned for EMSA. Reverse immunoprecipitations were performed using streptavidin-tagged agarose beads to capture the biotinylated oligos and the protein fraction thus pulled down was resolved on 3-8% Tris Acetate gel and probed with POT1 antibody. Prior to immunoprecipitations, 25 µl of the oligo-lysate mix was taken out and used to detect relative differences in the inputs.

***In vitro* kinase assay**

3-4 ATR inducible deletion mouse embryonic fibroblasts[6] were lysed in lysis buffer (50mM Tris, pH 7.5, 50mM Glycerophosphate, 150mM NaCl, 10% Glycerol, 1% Tween-20, 1mM) and lysates cleared by centrifugation. Cleared lysates were subjected to ATR-pSer-428 immunoprecipitation and beads-conjugated immunoprecipitates were resuspended in kinase-activity buffer (10mM HEPES, pH 7.5, 50mM Glycerophosphate, 50mM NaCl, 10mM $MgCl_2$, 10mM $MnCl_2$, 5 µM ATP, 1mM DTT) and used as source of kinase activity on 1ug of recombinant CGGBP1 in presence of 1mM ATP for 30 min at 30C. Samples were resolved on 4-12% Bis-Tris acrylamide gel and blotted for phospho-serine, stripped and blotted for CGGBP1.

Single telomere length analysis and telomere fusion analysis

We extracted DNA using standard proteinase K, RNase A, phenol/chloroform protocols. For telomere length analysis at the XpYp and 17p telomeres we used a modification of the single telomere length analysis (STELA) assay previously described [7-9]. Briefly genomic DNA was solubilised and quantified by Hoechst 33258 fluorometry (BioRad, Hercules, USA) and diluted to 10ng/µl in 10mM Tris-HCl pH 7.5. 10ng of DNA was further diluted to 250 pg/µl in a volume of 40µl containing 1µM Telorette2 linker and 1 mM Tris-HCl pH 7.5. Multiple PCRs (typically 6 reactions per sample) were carried out for each test DNA in 10µl volumes 250pg of DNA, 0.5µM of the telomere-adjacent and Teltail primers, 75mM Tris-HCl pH8.8, 20mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1.5mM $MgCl_2$, and 0.5 U of a 10:1 mixture of Taq (ABGene, Epsom, UK) and Pwo polymerase (Roche Molecular Biochemicals, Lewes, UK). The reactions were cycled with an MJ PTC-225 thermocycler (MJ research, Watertown, USA). The DNA fragments were resolved by 0.5% TAE agarose gel electrophoresis, and

detected by Southern hybridisations with random-primed a-^{33}P labelled (Perkin Elmer, USA) TTAGGG repeat probe together with a probe to detect the 1 kb (Stratagene, La Jolla, USA) and 2.5 kb (BioRad) molecular weight marker. The hybridised fragments were detected by phosphorimaging with a Typhoon Trio phosphorimager (GE, USA). The molecular weights of the DNA fragments were calculated using the Phoretix 1D quantifier (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

Telomere fusion was detected using single-molecule PCR as previously described [9, 10]. PCR reactions containing 56 ng of DNA were undertaken each containing the XpYpM, 17p6 and 21q1 PCR primers. Fusion molecules were detected and the frequencies quantified by Southern blotting and hybridisation with the 17p and XpYp telomere-adjacent probes as described previously [9, 10]. The oligonucleotides were as follows: Fusion PCR XpYpM: 5′-ACCAGGTTTTCCAGTGTGTT-3′, 17p6: 5′-GGCTGAACTATAGCCTCTGC-3′, 21q1: 5′-CTTGGTGTCGAGAGAGGTAG-3′, and the fusion detection probes: XpYpO:5′-CCTGTAACGCTGTTAGGTAC-3′, XpYpB2:5′-TCTGAAAGTGGACC(A/T)ATCAG-3′, 17p7:5′-CCTGGCATGGTATTGACATG-3′, 17p2:5′-GAGTCAATGATTCCATTCCTAGC-3′.

Western analysis

Western blot analyses were performed using standard methods. The gels used were NuPAGE 4-12% Bis-Tris run with MES or MOPS buffer or 3-8% Tris-Acetate gels run with Tris Acetate buffer (Invitrogen Life Sciences). Transfers were performed using iBlot dry transfer (Invitrogen Life Sciences). Membranes were incubated with blocking buffer (5% dry milk and 10% Fetal Bovine Serum in 1x standard Tris-buffered saline supplemented with 0.05% Tween-20 (TBST)) for 1-2h and then incubated with the primary antibody overnight at 4°C with gentle rocking. 30 minutes washing with 5 changes of TBST was followed by incubation with secondary antibody (1:5000 of HRP-conjugated donkey anti-rabbit or anti-mouse from GE Life Sciences or HRP-conjugated light chain specific anti-mouse and anti-rabbit antibodies from Jackson Labs, USA) diluted in blocking buffer for 1h at room temperature and subsequent washing with multiple changes of TBST for 45 minutes. Pierce ECL detection system was used to detect HRP-substrate chemiluminescent reaction and signals captured on hypersensitive X-ray films from GE Life Sciences. Films were scanned using an EPSON scanner in transparent mode. Western bands were quantified using NIH ImajeJ 1.44o software. Local backgrounds were individually subtracted from the area x signal values obtained for each band individually.

Proliferation assay

Cells were collected at passages as indicated (both floating cells from the medium, washed cells in PBS and trypsinized cells in mix of MEM medium and PBS; total volume 14 ml) and pelleted by centrifugation at 1500 rpm, 5 minutes and room temperature. After aspiration of 10 ml medium, cells were resuspended and passed through 40 μm cell strainers, 500 μl was diluted in 0.2 μm filtered PBS and particle count in 500 μl of this suspension was obtained using Beckman Coulter cell counter. Values obtained were used to calculate the amount of cells contained in the 14ml of sample. Values from 3 independent assays were averaged and plotted as average ± standard deviation. P values for significance were calculated using heteroscedastic two tailed T-test.

Chromatin-positive midbody frequency measurement

Control or ATR siRNA treated cells (mock, S164S, S164A or S164E overexpressing fibroblasts) were stained for AURKB and nuclei with DAPI 7 days post-transfection with one passage in between at 4 days post-transfection. DAPI-positive midbodies (exact overlap of chromatin with midbody signal or presence of unresolved chromatin or micronuclei between cells connected by a midbody) were calculated as percentage of total midbodies counted (n=100 in each case except 58 for S164A+control siRNA and 27 for S164A+ATR siRNA).

Antibodies

CGGBP1 (western and immunofluorescence): ABCAM Ab56412

CGGBP1 (immunoprecipitation): Proteintech 10716-1-AP

γH2AX: ABCAM Ab22551 and Cell Signalling 9718

TRF1 (immunofluorescence and western): ABCAM Ab47717
TRF1 (immunoprecipitation): Abnova H00007013-B01
TRF2 (western and immunoprecipitation): ABCAM Ab13579
POT1 (immunoprecipitation): Proteintech 10581-1-AP
POT1 (western): ABCAM Ab21382 (shown) and Ab47082 (not shown).
HA tag (western): ABCAM Ab18181
FLAG tag (immunoprecipitation and western): SIGMA F3165
ATR: ABCAM Ab2905
ATR (pSer428): Cell Signalling 2853
ATM (pSer1981): Cell Signalling 5883
CHK1 (pSer296): Cell Signalling 2349
CHK2 (pThr68): Cell Signalling 2661
CHK1: ABCAM 47574
CHK2: ABCAM 47433

Statistics

All calculations of average, standard deviation and significance calculation by T-test (heteroscedastic or homoscedastic and two-tailed) were performed using in-built tools in Microsoft Excel program, unless mentioned otherwise.

ChIP control primers

X.1.1.f 0-1kb	CAACTCCTTCAGCAACAGCA	NT_011669.17	5083550 - CAACTCCTTCAGCAACAGCA - 5083569	55°C	ChIP-qPCR
X.1.1.r 0-1kb	GACACCGACACTGCCTTACA	NT_011669.17	5083713 - GACACCGACACTGCCTTACA - 5083694	55°C	ChIP-qPCR
X.1.2.f 0-1kb	GGTACTCGAGGTCCCAAACA	NT_011669.17	5084861 - GGTACTCGAGGTCCCAAACA - 5084880	55°C	ChIP-qPCR
X.1.2.r 0-1kb	GCAGGCTGAAAGTGAAATCC	NT_011669.17	5085028 - GCAGGCTGAAAGTGAAATCC - 5085009	55°C	ChIP-qPCR
X.1.1.f 3-4kb	CTCTGATTCTTGGGGCTGAG	NT_011669.17	5081276 - CTCTGATTCTTGGGGCTGAG - 5081295	55°C	ChIP-qPCR
X.1.1.r 3-4kb	GGGAGGAAAAGGACGATAGG	NT_011669.17	5081503 - GGGAGGAAAAGGACGATAGG - 5081484	55°C	ChIP-qPCR
NCGX.1.2f	CAGGCTTCCAATGACCTGTT	NT_011669.17	5087118 - CAGGCTTCCAATGACCTGTT - 5087138	55°C	ChIP-qPCR
NCGX.1.2r	CACAAGCCCCATCACTTTTT	NT_011669.17	5087328 - CACAAGCCCCATCACTTTTT - 5087348	55°C	ChIP-qPCR

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