Supplementary Information

Supplementary Materials and Methods

Cell culture and treatments

LNCaP, HCT116, GM3657 and H1299 cells were maintained in RPMI-1640 medium (HyClone) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). HeLa cells were maintained in Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% FBS and antibiotics. Ionizing radiation (IR) and UV treatment were performed as previously described (Ou *et al.*, 2005).

Constructs

For mammalian expression of HA-tagged BTG3, the pXJ-HA vector was modified by insertion of a *Spe* I site into its original multiple cloning sites and the expression vectors of BTG3 FL and truncation mutants were then generated by cloning the polymerase chain reaction (PCR) products into the *Hind* III and *Spe* I sites of the pXJ-HA vector. The E2F1 expression plasmid pCMVE2F1 was a kind gift of Dr. Hsin-Fang Yang-Yen (Institute of Molecular Biology, Academia Sinica, Taiwan). E132 mutant was generated by PCR-based site-directed mutagenesis. E2F2 and E2F3 were amplified from LNCaP cells by RT-PCR and later cloned into pCDNA-HA.

For *in vitro* interaction experiments, the expression vector for GST-tagged FL BTG3 protein was generated by subcloning the wild-type *BTG3* cDNA in between the *EcoR* I and *Xho* I sites of the pGEX4T-1 vector (Amersham Biosciences); and the plasmids expressing BTG3 truncation mutants d1, d2 and d3 were produced by

subcloning the PCR products of the corresponding mutants in between the *Hind* III and *Kpn* I sites of pGEX4T-1. For expression of the d5 and d6 mutants, the *BamH* I site of the same vector was used instead. To produce His-E2F1, the *Pvu* II site of the pRSET vector (Invitrogen) was used for cloning the FL *E2F1*. A convenient *Bgl* II site inside the cDNA was then used to generate the *N* (1-283) and the *C* (284-437) constructs. For His-DP1, *DP1* was amplified by PCR from LNCaP cells and cloned in between the *EcoR* I and *BamH* I sites of the pRSET vector (Invitrogen).

To generate the *BTG3* luciferase reporter constructs, a 1088 bp promoter fragment containing the 5′ untranslated region of *BTG3* and a 902 bp fragment from the intron 2 region of *BTG3* were amplified from MRC5 cell genomic DNA using the following primers: BTG3-Promoter, 5′- AGCGAGCTCCTTGTATTCCTCCCGCTAGTTG-3′ and 5′- ACTCTCGAG TGTCCTGGCCGGGAACTGAG-3′; BTG3-Intron2, 5′-AGATGGGATCTTGCTGTGTTAC-3′ and 5′-AGCGCTAGCAAGCCTTCATCCAATGTCAGTT-3′. These PCR products were subsequently cloned into the region between *Sac* I and *Xho* I sites of the pGL2 or pGL3-promoter vectors (Promega). Mutations were introduced into the putative p53 binding site in intron 2 of *BTG3* by PCR-mediated mutagenesis and confirmed by DNA sequencing. To generate reporters containing only the putative p53 binding site, the following oligonucleotide pairs were synthesized and annealed: BTG3-p53WT, 5′-GATCGAATTCAAACATGCCCTGGCATGCTCA-3′ and 5′-GATCTGAGCATGCCAGGGCATGTTTGAATTC-3′; BTG3-p53Mut. 5′-GATCGAATTCAAAAATTCCCTGGAATTCTCA-3′ and 5′-GATCTGAGAATTCCAGGGAATTTTTGAATTC-3. The annealed

oligonucleotide pairs were cloned subsequently into the *Bgl* II-digested pGL3-promoter vector (Promega).

For the *ARF* luciferase reporter, an *ARF* promoter fragment from positions -735 to +75 was amplified by PCR from MRC5 genomic DNA and cloned into pGL2 (Promega) using the *Xho* I-*Hind* III sites.

Northern analysis and reverse transcription (RT)–PCR

Northern blot analyses were performed as described (Ou *et al*., 2005). The *BTG3* and *GAPDH* cDNA probes were amplified from the LNCaP cDNA library using the following primers: BTG3, 5′-AATCACTGGTATCCAGAA-3′ and 5′-GCAACAATGAATGCATTG-3′; GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.

In some experiments, RNA prepared by TRIzol extraction (Invitrogen) was reverse transcribed and then amplified by PCR. The primer pairs used are listed in Table S1.

Electrophoresis mobility shift assay (EMSA)

The DNA probe and competitors were prepared by annealing the complementary oligonucleotide pairs listed in Table S2. The probe was labeled by end-filling in the presence of [α-^{32}P] dCTP.

The EMSA gel shift assay was performed with recombinant His-p53 or His-E2F1 plus His-DP1 proteins and the ^{32}P-labeled probe in 20 μL of binding buffer (20 mM Hepes pH 7.9, 2 mM $MgCl_2$, 25 mM KCl, 10% glycerol, 0.025% NP-40, 0.1 mM EDTA, 1 mM DTT and 2 mM spermidine) containing 2 μg BSA and 100 ng of poly dI-dC at room temperature for 15 min. For competition experiments, 5-, 10-, or 50-fold excesses of unlabeled probe were added to the binding reaction mixtures. Protein-DNA complexes were separated on a 5% native polyacrylamide in 0.5 × TBE

at room temperature. For supershift assay, 1 μL of antibody against p53 (PAb122, NeoMarkers) or E2F1 (KH129, NeoMarkers) was added to the reaction before the addition of the probe.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as described (Ou *et al*., 2005). Primers used in the assays were listed in Table S3.

GST pull-down assay

Fifteen microliters of GST-Sepharose beads were incubated with GST or GST-fusion proteins in a final volume of 300 μL of sonication buffer (50 mM Na_2HPO_4 pH 8.0, 300 mM NaCl, 20% glycerol, 1 mM DTT and 1 mM PMSF). After rocking for 1 h at 4 ^oC, the beads were washed twice with 1 mL sonication buffer containing 1% Triton X-100 and once with 1mL Co-IP buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 1 mM PMSF). The beads were then rocked with purified His-tagged E2F1 for 1 h at room temperature in Co-IP buffer. After three washes with Co-IP buffer, the beads were boiled for 5 min in loading buffer and analyzed by SDS–PAGE followed by Western blotting.

Colony formation assay

HCT116 cells were plated on 35 mm dishes and transfected by Lipofectamine 2000 with plasmids encoding WT or mutant BTG3. Cells were trypsinized the next day and equal numbers of cells were plated in medium containing 0.4 μg /mL puromycin for 10 days. To enhance the visualization of colonies, cells were fixed briefly in 70% ethanol and stained with 1% crystal violet solution before counting.

H1 kinase assay

LNCaP or HCT116 cells were lysed in TEGN buffer (10 mM Tris pH 7.5, 1 mM EDTA, 420 mM NaCl, 10% glycerol and 0.5% NP40) containing a cocktail of protease inhibitors (Roche), 10 mM NaF, 10 mM β-glycerophosphate, 5 nM microcystin LR and 1 mM DTT; immunoprecipitation was carried out in the same buffer but with 200 mM NaCl and 15% glycerol in the presence of anti-Cyclin B (sc-245, Santa Cruz) and protein G beads (Pierce) at 4 ^{o}C for 2-3 h. After three washes in the same buffer and two washes in kinase buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$), kinase reaction was performed by the addition of 1 μg histone H1, 3 μCi [γ-^{32}P]ATP and unlabeled ATP to a final 50 μM at 30 ^{o}C for 30 min. The reaction products were subsequently analyzed by SDS-PAGE followed by autoradiography and immunoblotting.

Supplementary Figure Legends

Fig. S1. The p53 binding site in intron 2 of the *BTG3* gene contributes to the DNA damage response. (**A**) The 5′ promoter region of *BTG3* does not respond to DNA damage. Luciferase reporter assays were performed in LNCaP cells to examine the DNA damage response of the 1 kb promoter region (BTG3P) immediately upstream from the transcription start site. (**B**) The *BTG3* intron 2 confers the DNA damage response, which is abrogated by mutations in the putative p53 binding site. LNCaP cells were transfected with a luciferase reporter driven by a 900 bp fragment (BTG3I) derived from intron 2 of *BTG3*. A similar construct but with the putative p53 site mutated (BTG3Imut) was also examined side by side to demonstrate the importance of the p53 binding site within the intron. Mutated positions are underlined in Fig. 2A. (**C**) p53 levels in LNCaP cells shown in (B) taken at the heights of p53 induction (left panel) and at the time of harvest (right panel). Phosphorylation on Ser15 was also examined (pS15). (**D**) The intron 2 region can be activated by exogenously expressed p53 in p53-null H1299 cells.

Fig. S2. Specificity of BTG3 knockdown. (**A**) BTG3 siRNA only affected the expression of *BTG3* but not other *BTG* family members. LNCaP cells were transfected by btg3 siRNA, and RNA was isolated 40 h after transfection. RT-PCR was performed using primers specific to *BTG3, BTG1, BTG2, Tob, Tob2, ARF, or Actin*. Note that *ARF* expression was increased about 1.6 fold in BTG3-ablated cells. (**B**) Expression of p14/ARF protein was elevated in BTG3 knockdown LNCaP cells. LNCaP cells were irradiated with 8 Gy of X-ray and collected at the indicated time points. Protein lysates were analyzed by Western using anti-BTG3 or anti-p14/ARF (sc-8613, Santa Cruz).

Fig. S3. Effects of the BTG3 siRNA btg3-2 on the activity of E2F1 and checkpoint control. (**A, B**) Impaired G2M arrest in HCT116 in which BTG3 expression was downregulated by btg3-2 siRNA. Experiments were performed as described in Fig. 5B. Similar results were observed in two other experiments. The sequence targeted by btg3-2 is 5′-TTGAGAGGTTTGCTGAGAA-3′. (**C**) Comparison of BTG3 knockdown by siRNAs btg3 and btg3-2 in LNCaP cells. Shown are Western blots using specific indicated antibodies. Levels of CyclinE1 and Cyclin D1 were similarly affected when either siRNA was used. (**D**) The activity of E2F1 was increased when endogenous BTG3 was downregulated by btg3-2 siRNA. Experiments were performed as in Fig. 7K. Shown are averages of two independent duplicated experiments.

Fig. S4. Induction of CHK2 and ATM phosphorylation by DNA damage is normal in BTG3 knockdown cells. LNCaP cells transfected with control (lanes 1-5) or BTG3 targeting siRNA (lanes 6-10) were irradiated with 8 Gy of X-ray and analyzed for ATM or CHK2 phosphorylation by Western blotting using anti-CHK2 pT68 (Santa Cruz) or anti-ATM pS1981 (Cell Signaling).

Fig. S5. BTG3 ablation reduces cell survival with or without DNA damage. (**A**) Vector expressing BTG3-targeting shRNA effectively downregulated BTG3 in LNCaP cells. Cells were transfected with either an empty vector (pBabeH1, control) or a vector expression BTG3 shRNA (pBabe shBTG3). Lysates were prepared 48 h after tranfection, and analyzed by Western blotting using the indicated antibodies. (**B**) Clonogenic survival assay. LNCaP cells were transfected as described in (A) and replated after IR. Shown are cells survived after 10 d of puromycin selection.

Fig. S6. Cellular localization of HA-tagged BTG3 and endogenous E2F1. HCT116 cells were transiently transfected with HA-BTG3 and processed for confocal microscopy. Localization of BTG3 (green) and E2F1 (red) was detected by anti-HA and anti-E2F1, respectively.

Fig. S7. E2F2 and E2F3 interact directly with BTG3 *in vitro*. GST pull-down assay was performed using GST or GST-BTG3 fusion and recombinant, purified His-tagged E2F2 and E2F3 essentially as in Fig. 7B and 7D.

Fig. S8. (**A**) (**B**) Coomassie blue staining of bacteria-purified, His-tagged DP1, BTG3, and E2F1, full-length or its N- (1-283) or C- (283-437) terminal domain.

Fig. S1

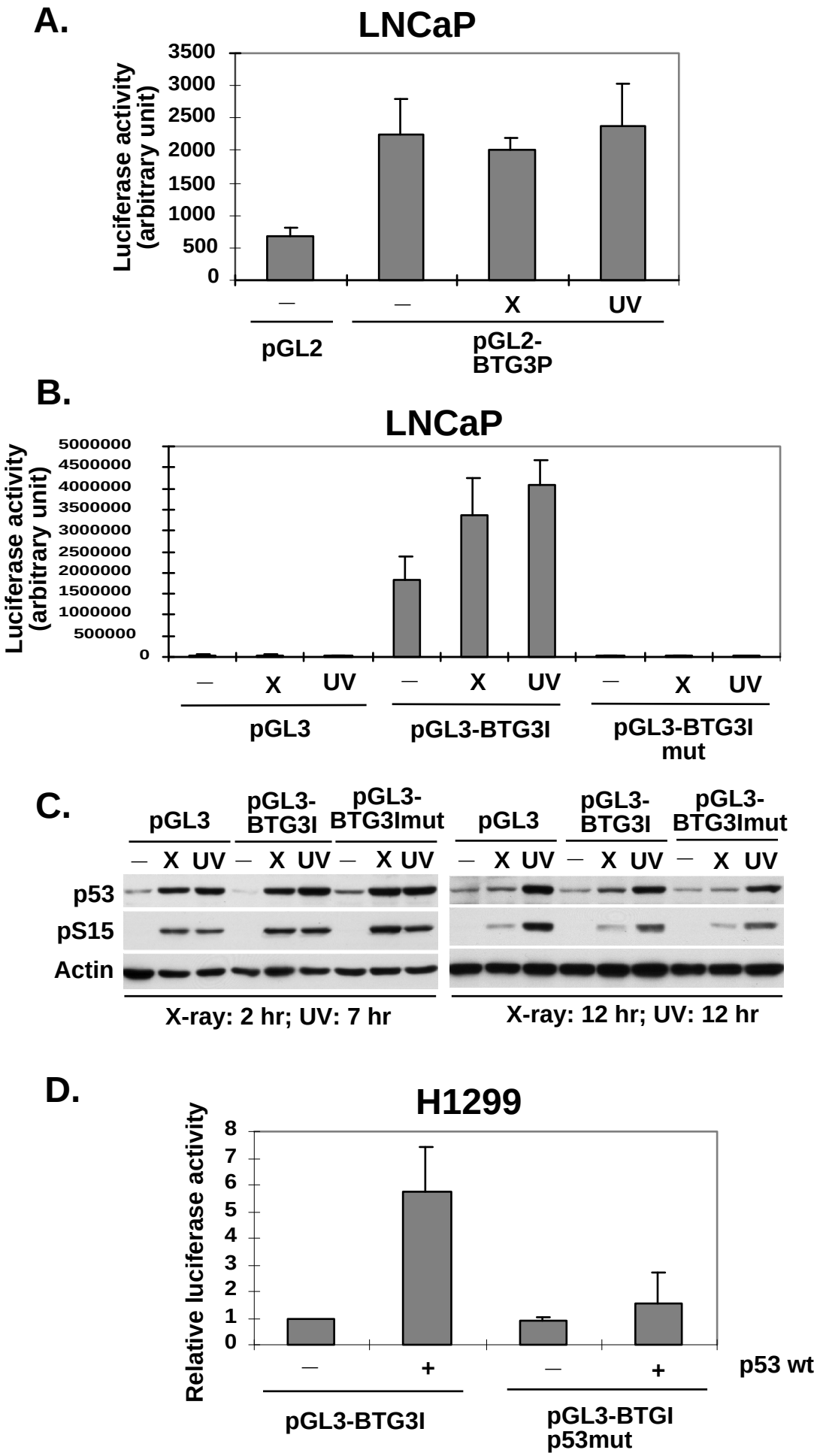
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Fig. S2

Ou_Fig. S2

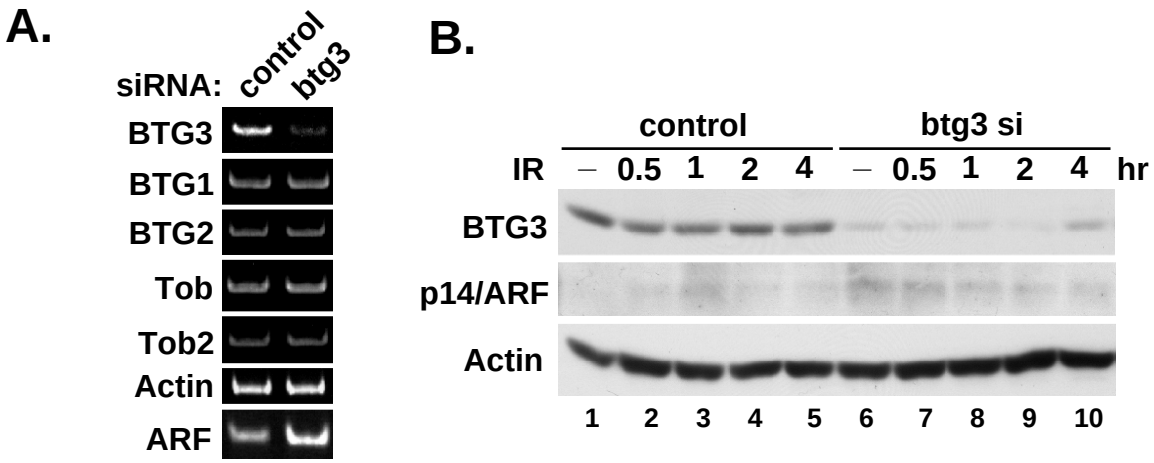

Fig. S3

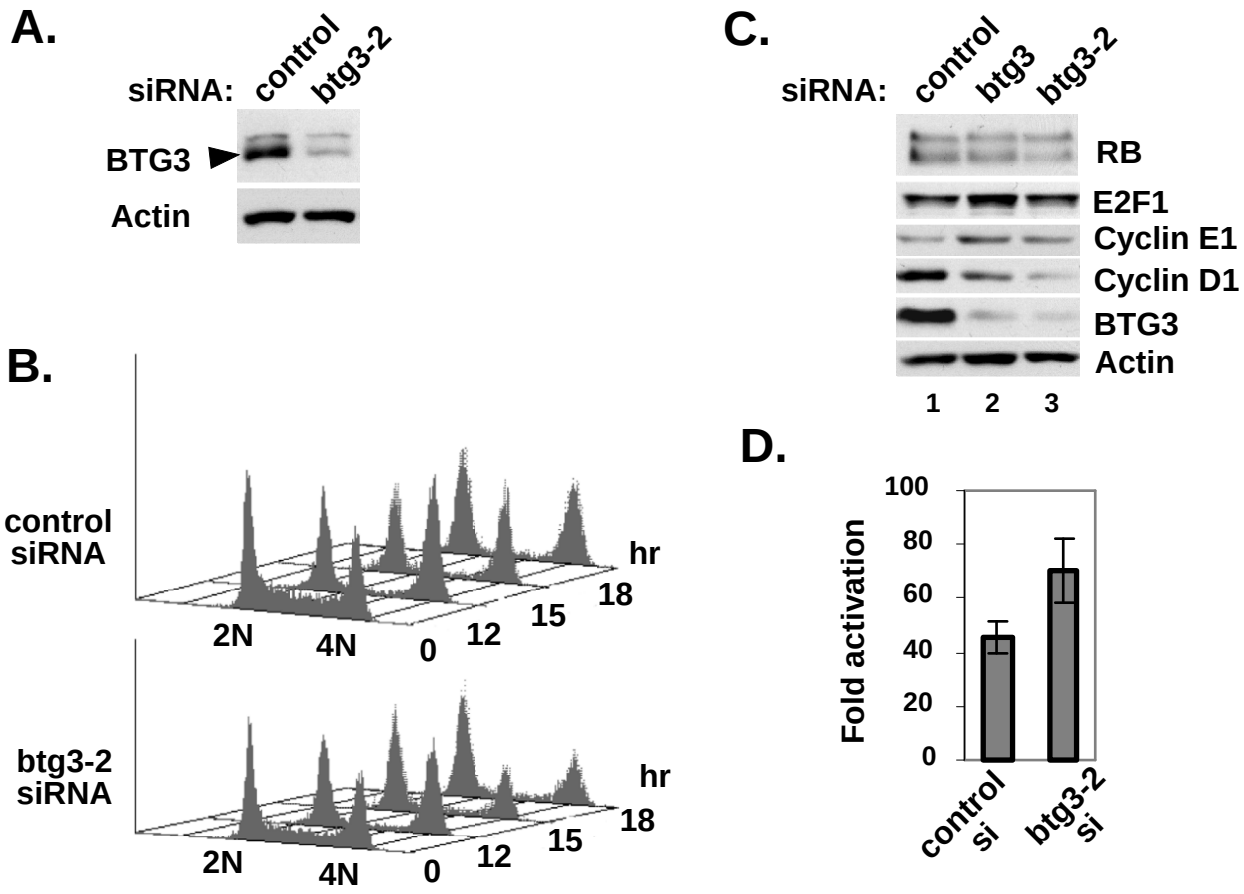
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Fig. S4

Ou_Fig. S4

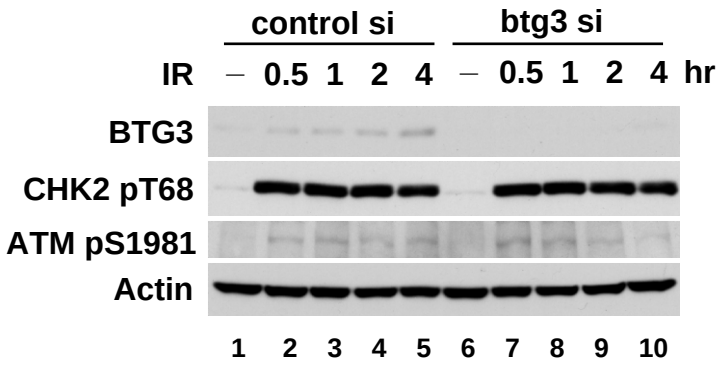

Fig. S5

Ou_Fig. S5

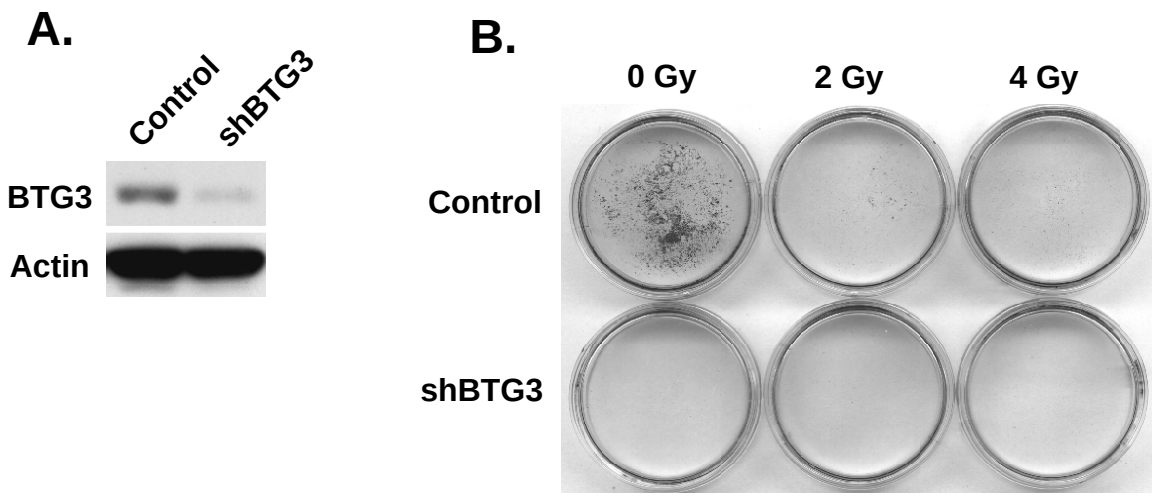

Fig. S6

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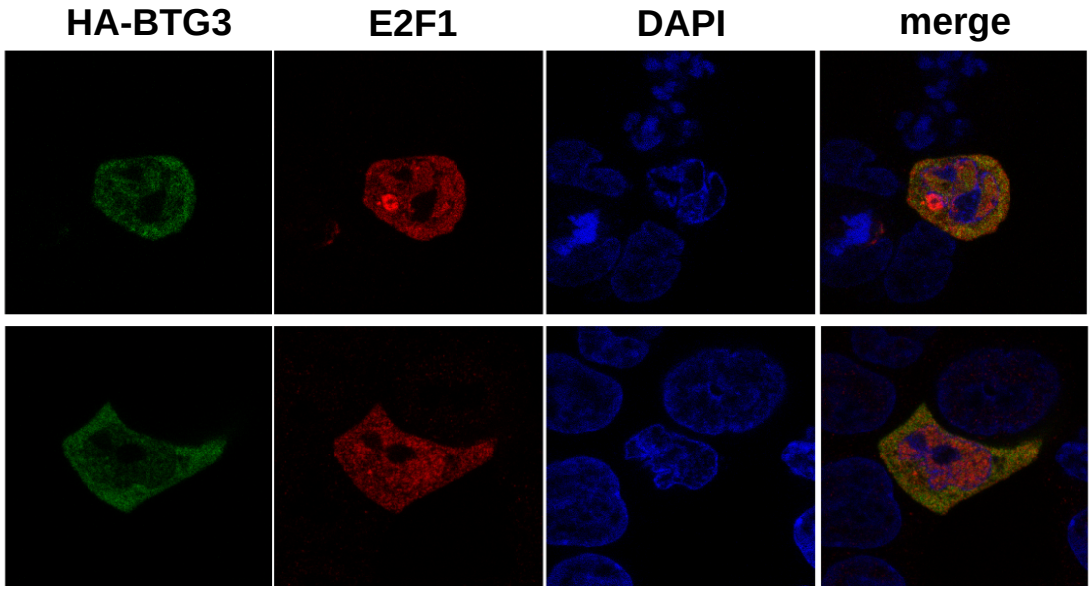
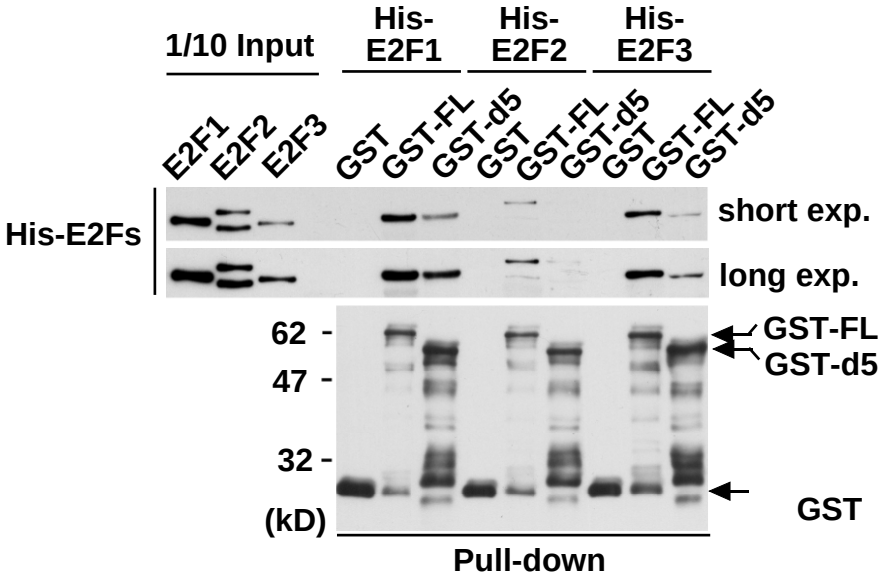


Fig. S7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GST-d5
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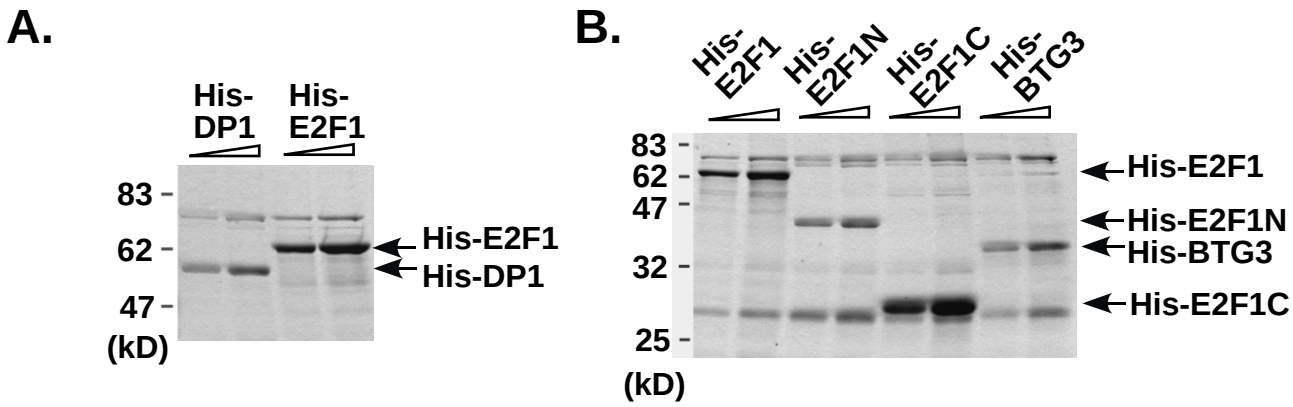
Pull-down

Fig. S8

Ou_Fig. S8



Supplementary Table

Table S1. Primer sequences used in RT-PCR

Gene	Primer Pairs (5′ to 3′)
ARF	CGCTCAGGGAAGGCG CCCTTTGGCACCAGAGGTGA
p21	AGCTGAGCCGCGACTGTGAT AGCAGAGCAGGTGAGGTGCC
BTG1	CCTATGAAGTGTCCTACAGA CAGATGATCCATCCACAGAC
BTG2	TCAGCCAGCCCCAGCTGC GCCTAGCTGGAGACTGCCAT
BTG3	TCTTCCAATGTGGCACCCTT ATGTCACTGGAATTGGGCGA
Tob	TGCAGGGTCAAGGTAGTAGT GTTAGCCATAACAGGCTGGA
Tob2	CAGCCTCTTCTTTGATGCGG GGAACTGCTGGCTGGGATAC

Table S2. Sequences of oligonucleotides used in the gel mobility shift assays (EMSA)

Binding Site	Annealed Oligonucleotides (5′ to 3′)
BTG3-p53WT	GATCGAATTCAAACATGCCCTGGCATGCTCA GATCTGAGCATGCCAGGGCATGTTTGAATTC
BTG3-p53Mut	GATCGAATTCAAAAATTCCCTGGAATTCTCA GATCTGAGAATTCCAGGGAATTTTTGAATTC
EREA	CTAGCTGAGCCGCCCGCGCGCGCCTCC TCGAGGAGGCGCGCGCGCGGGCGGCTCAG
DHFR	CTAGGCTAGCCAGCTGCTGCGATTTCGCGC GACTCGAGCGTCAAGTTTGGCGCGAAATCG - CAGCAGCTGGCTAGCCTAG

Table S3. Sequences of primer pairs used in ChIP assays

Gene	Primer Pairs (5′ to 3′)
BTG3 intron	CCACTGTTTTGGCACTGAGA CACATCCACAAAATGGGACA
BTG3 promoter	GAGGGTCTGCGCCAGGCTCA GGAGCGCAGCGAGCCTCGTC
p21	CATTGTTCCCAGCACTTCCTCTC AGAAAGCCAATCAGAGCCACAG
CDC6	GTAACTCTTCCACTGGATTG ATCCTTCTCACGTCTCTCAC
ARF	CGCTGAGGGTGGGAAGATGG AGATCTCGGAACGGCTCTGA
DHFR	CCAACCATGACAGCAGCGGG AGCACGCCGCGACCCTGCGT