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# Supplemental Material to:

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## Low ATM protein expression and depletion of p53 correlates with olaparib sensitivity in gastric cancer cell lines

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**Kubota et al, Supplementary Information**

**Supplementary Table 1:**

**Characteristics of the gastric cancer cell lines used in this study.**

NA, no data available; WT, wild type; mut, mutant; del, deleted. Mutations, where known, are indicated. *, from this study, Figure 2. + = robust expression/phosphorylation detected by western blot; (+) = weaker expression/phosphorylation detected by western blot.

| **Cell line** | **ATM gene status** | **ATM Protein Expression*** | **ATM 1981 phosphorylation*** | **p53 gene status** | **IR-induced P53-S15 phosphorylation*** |
|---|---|---|---|---|---|
| STKM-2 | NA | + | + | WT / WT[1] | + |
| KATOIII | NA | + | + | Del / Del [2] | - |
| AGS | No mutations found [3] | + | + | WT / WT[4] | + |
| NUGC4 | NA | (+) | (+) | WT / WT[5] | (+) |
| MKN1 | NA | + | + | Mut V143A / Del[2] | + |
| MKN28 | NA | + | + | Mut / Mut[6] | + |
| MKN45 | R2832H (also low ATM mRNA) [3] | (+) | (+) | WT / WT[2] | (+) |
| ISt-1 | NA | + | + | 94del[7] | + |

**Supplementary Table 2:**

**Sequences of primers used for RT-PCR**

P21- left primer: ggaagaccatgtggacctgt; right: aatctgtcatgctggtctgc

PUMA- left: ggagacaagaggagcagcag; right: gcacctaattgggctccatc

GADD45a- left primer: ccgaaaggatggataaggtg; right: gtcgacgttgagcagcttg

GUSB- left primer: CGTCCCACCTAGAATCTGCT; right: TTGCTCACAAAGGTCACAGG

Actin- left primer: GGGCATGGGTCAGAAGGAT; right: GTGGCCATCTCTTGCTCGA

**Supplementary Table 3:**

**Gene targeting sequences**

Thermo Scientific GIPZ Lentiviral shRNA Particles

Catalog number Gene target Sequence

V3LHS_404717 AGAAATGTTCTTGCAGTTA

V3LHS_333919 CACTACAACTACATGTGTA

V3LHS_333919 CGGCGCACAGAGGAAGAGA

**Supplementary Table 4:**

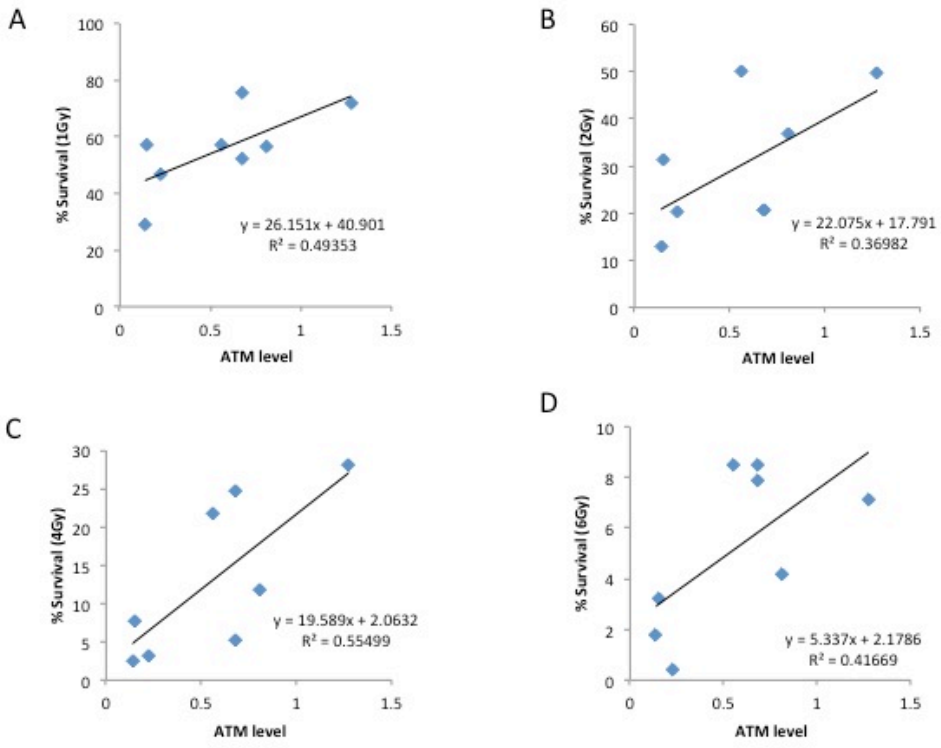
Summary of Spearman correlation co-efficients for protein expression and olaparib sensitivity from Supplementary Figures 2 and 3.

| Protein | Correlation | P value |
|---|---|---|
| ATM | 0.8143 | 0.0022 |
| 53BP1 | 0.4910 | n.d |
| Rad51 | 0.2309 | n.d |
| Chk2 | 0.2247 | n.d |
| Mre11 | 0.0578 | n.d |
| Nbs1 | 0.0054 | n.d |
| XRCC1 | 0.0049 | n.d |
| PNKP | 0.0023 | n.d |

**Supplementary Figures.**

**Supplementary Figure 1: Clonogenic survival of gastric cancer cell lines after treatment with IR.** Cells from the panel of gastric cancer cell lines shown in Figure 1 were irradiated with 1, 2, 4 or 6 Gy and survival was determined by the clonogenic survival assay. The Spearman correlation coefficient was calculated as in Figure 3.

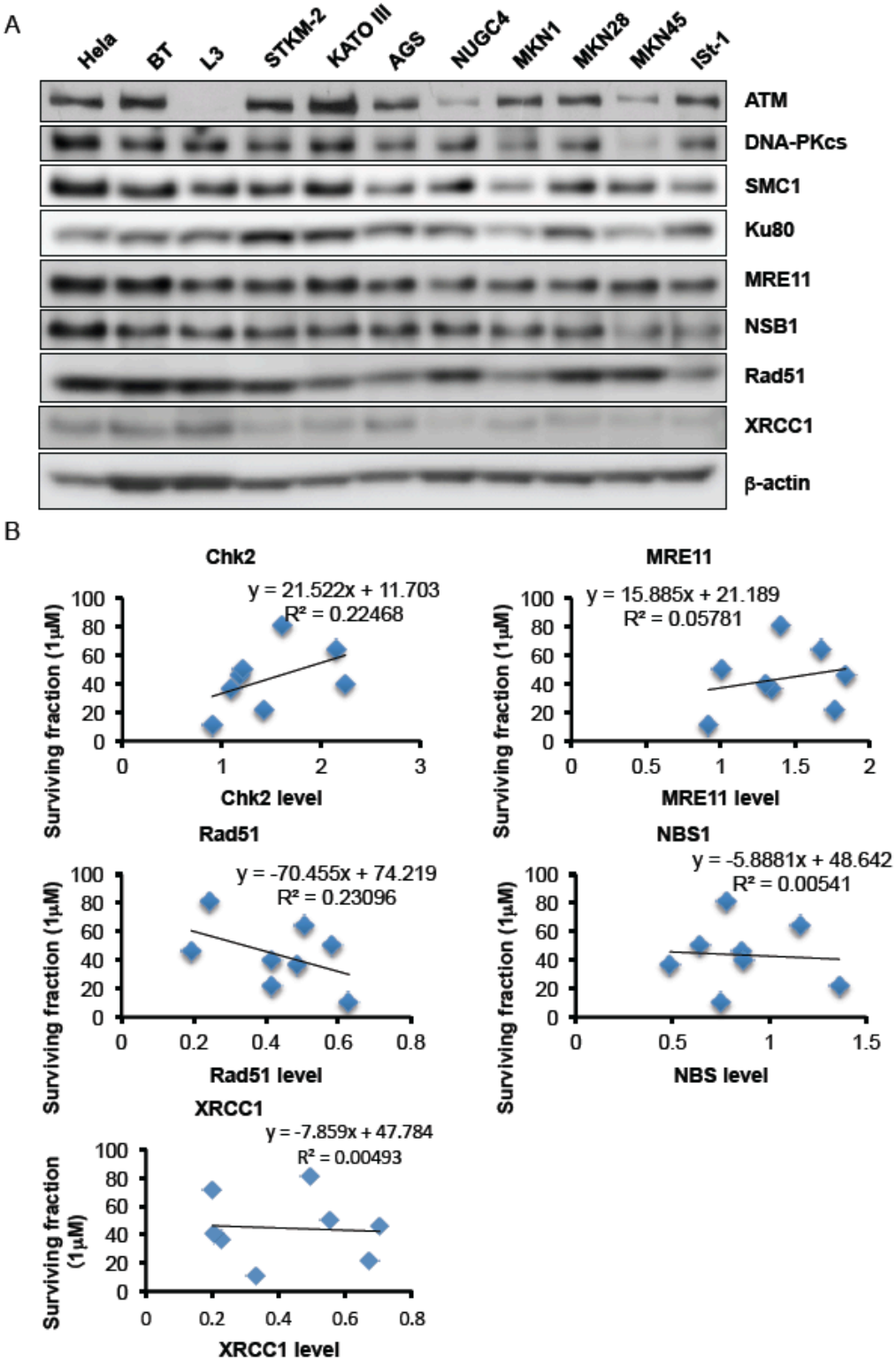
**Supplementary Figure 1**



**Supplementary Figure 2: Relative levels of Chk2, Mre11, Rad51, Nbs1 and XRCC1 in the gastric cancer cell lines STKM2, KATOIII, AGS, NUGC4, MKN1, MKN28, MKN45, and ISt-1 and correlation with olaparib sensitivity.**

(A) 25µg whole cell extract from the gastric cancer cell lines STKM2, KATOIII, AGS, NUGC4, MKN1, MKN28, MKN45, and ISt-1 as well as a control lymphoblastoid cell line C35ABR (BT), an A-T patient-derived lymphoblastoid cell line (L3), and Hela were run on SDS PAGE and western blots were probed with antibodies to ATM, DNA-PKcs, SMC1, Ku80, MRE11, NBS1, Rad51, XRCC1 and beta actin as indicated. After ECL, bands were visualized using a Fuji LAS 4000 imager. (B) Protein levels were quantitated, normalized to SMC1 expression and the correlation between expression level and sensitivity to olaparib (1 µM as determined by clonogenic survival assays) was calculated as described in Materials and Methods. The Spearman correlation coefficients are shown.

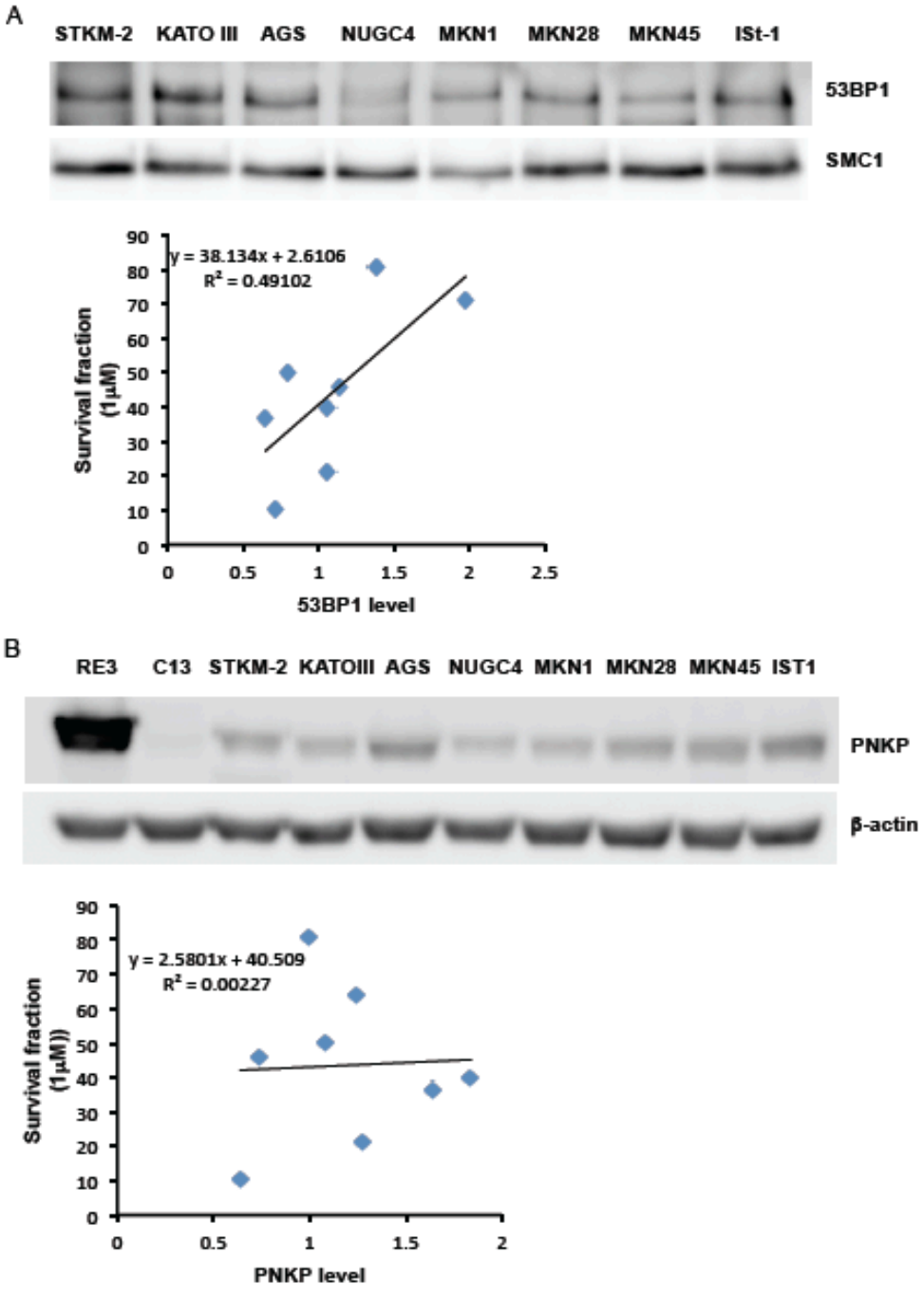
## Supplementary Figure 2



**Supplementary Figure 3. Relative levels of 53BP1 and PNKP in the gastric cancer cell lines STKM2, KATOIII, AGS, NUGC4, MKN1, MKN28, MKN45, and ISt-1 and correlation with olaparib sensitivity.**

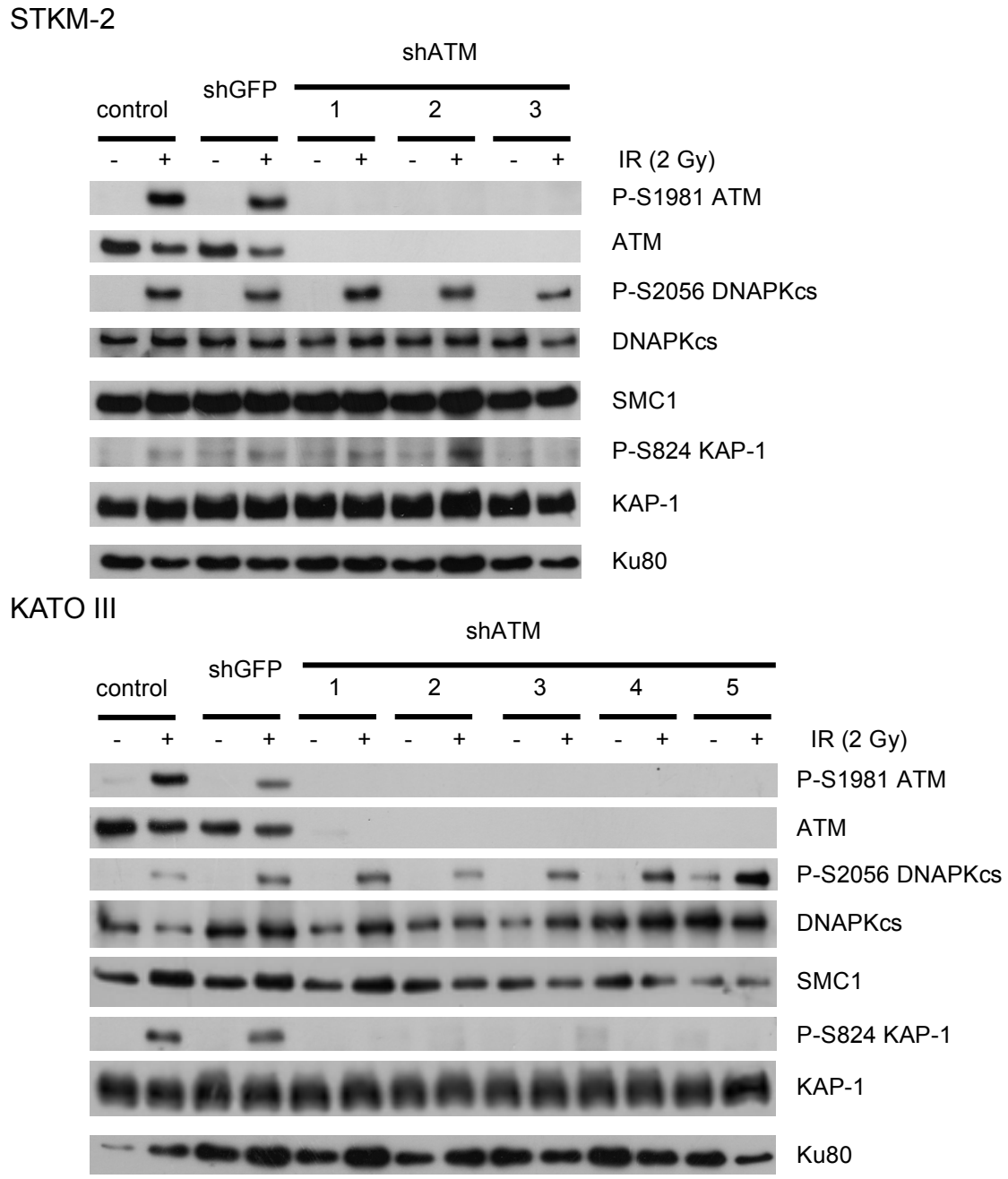
(A) 25μg of whole cell extract from each cell line was analyzed by SDS PAGE and western blots as described in Supplementary Figure 1. 53BP1 protein levels were quantitated, normalized to SMC1 expression and the correlation between the expression level and sensitivity to olaparib calculated as in Supplementary Figure 1. (B) Relative levels of PNKP in the gastric cancer cell lines compared to a human cell line in which endogenous PNKP had been depleted using shRNA (C13) and C13 cells in which shRNA resistant PNKP had been stably re-expressed (RE3)[8]. Western blots were probed with antibodies to PNKP and analyzed as in panel A.

**Supplementary Figure 3:**



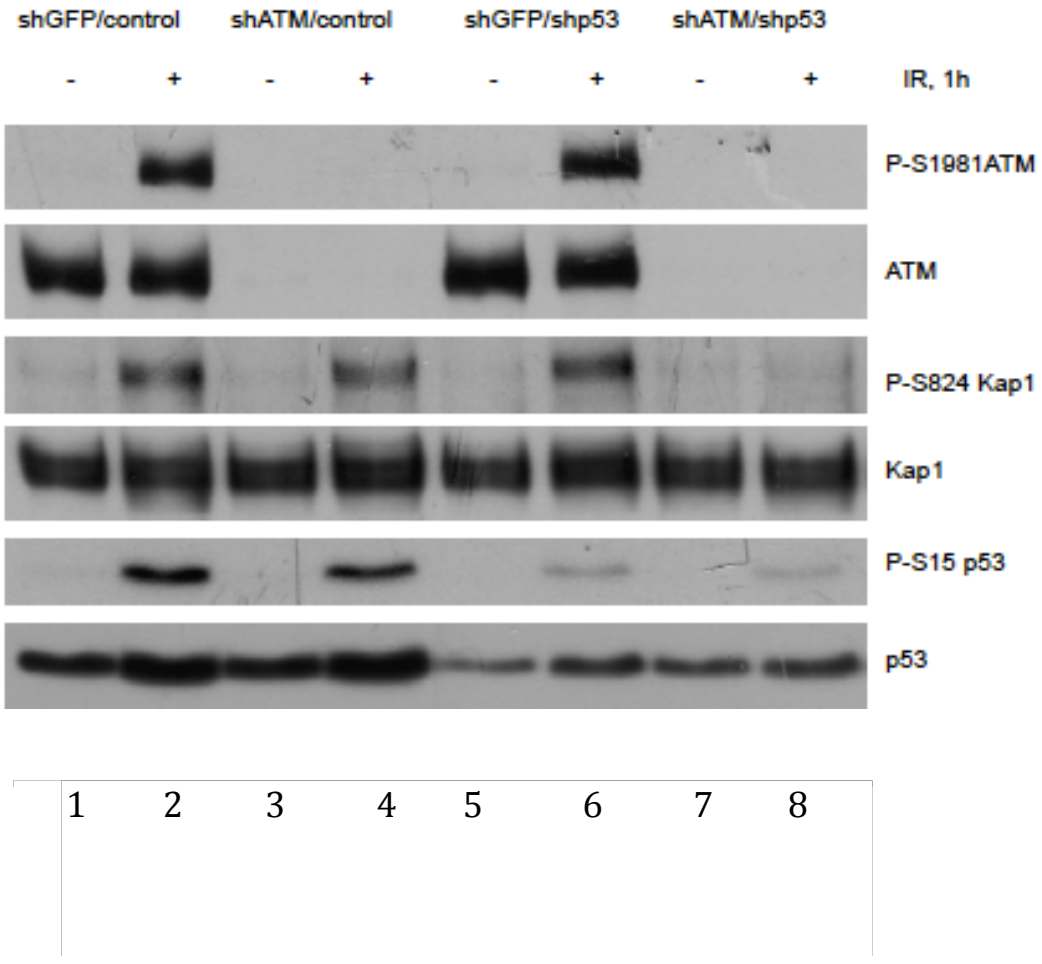
**Supplementary Figure 4. Characterization of shRNA knock down in STKM-2 (p53 proficient) and KATO III (p53 deficient) gastric cancer cell lines**. STKM-2 and KATO III cells were transfected with shRNA to GFP or shRNA to ATM. Individual clones were isolated and stable cell lines were generated and tested for expression of ATM by western blot. Cells were also tested for expression and activation of DNA-PKcs (DNA-PKcs-2056 phosphorylation), and KAP1 phosphorylation (S824) 1 hour after exposing to IR (2Gy).

**Supplementary Figure 4**



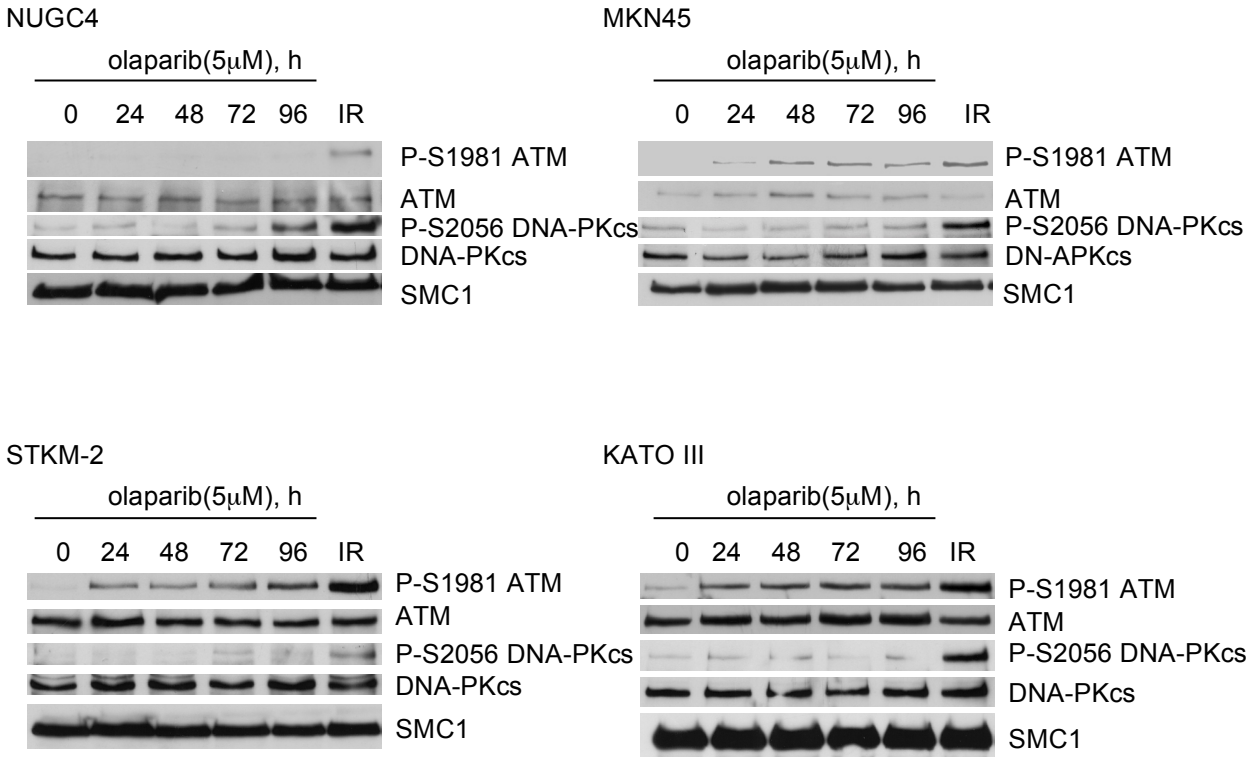
**Supplementary Figure 5. Characterization of shRNA knock down in STKM-2 expressing shATM.** Cells were infected with lentivirus vector expressing shp53. After transduction, cells were exposed to IR (2Gy, and harvested after 1h) or untreated and lysed with NET-N and sonication. Western blots were probed with the indicated antibodies

**Supplementary Figure 5**



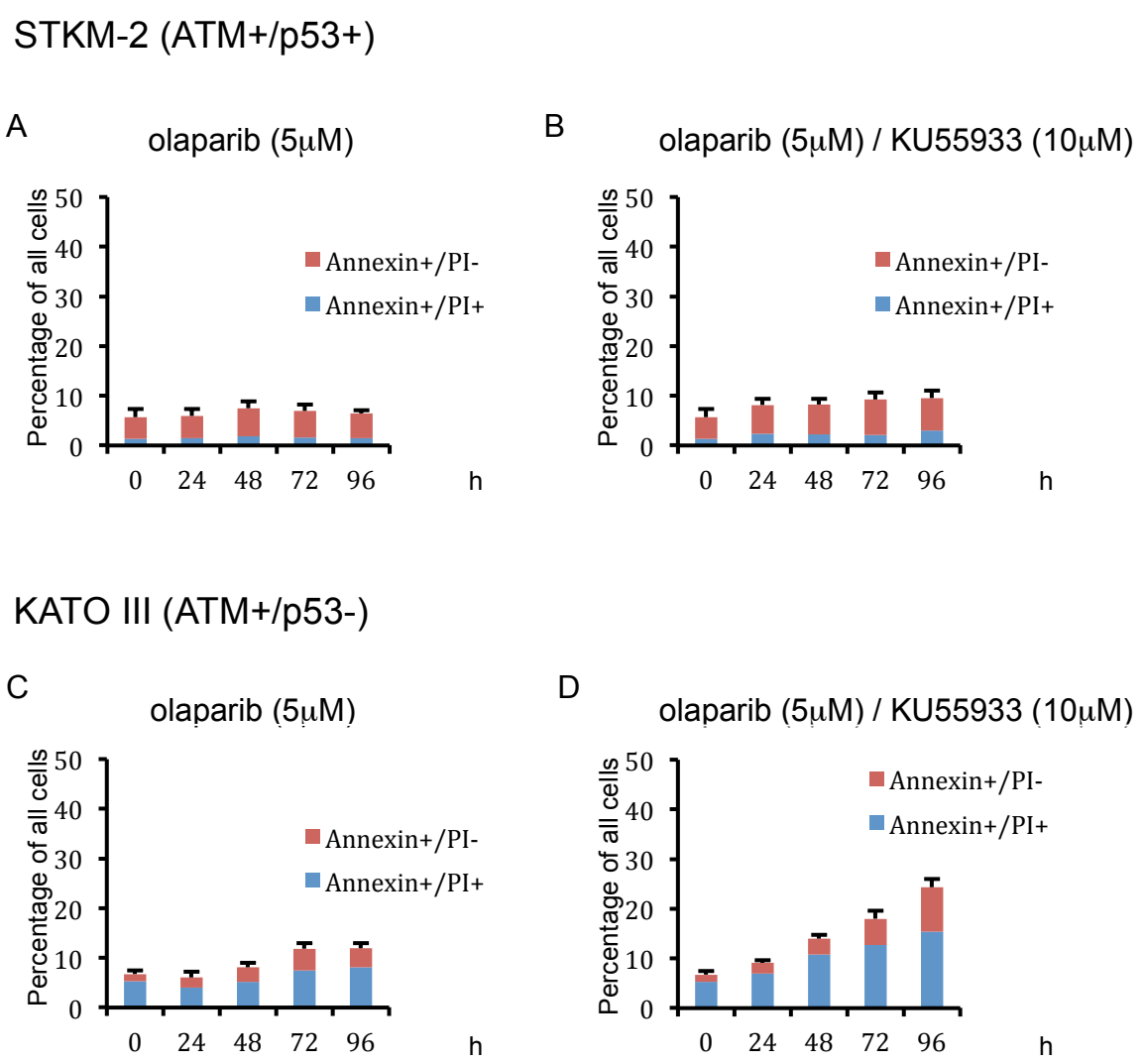
**Supplementary Figure 6. Olaparib induces autophosphorylation of ATM and DNA-PKcs in STMK2 and KATOIII cell lines.** Gastric cancer cell lines NUGC4, MKN45, STKM-2 and KATO III were treated with 5μM olaparib and harvested after 24, 48, 72, and 96 hours. Whole-cell extracts (25 μg total proteins) were analyzed by SDS PAGE and imminublotted for phosphorylation of ATM on Ser1981 (P-S1981), and phosphorylation of DNA-PKcs on Ser2056 (P-S2056). SMC1 is shown as a loading control. Cells were irradiated (2 Gy) and harvested after 1 hour as a positive control.

**Supplementary Figure 6**



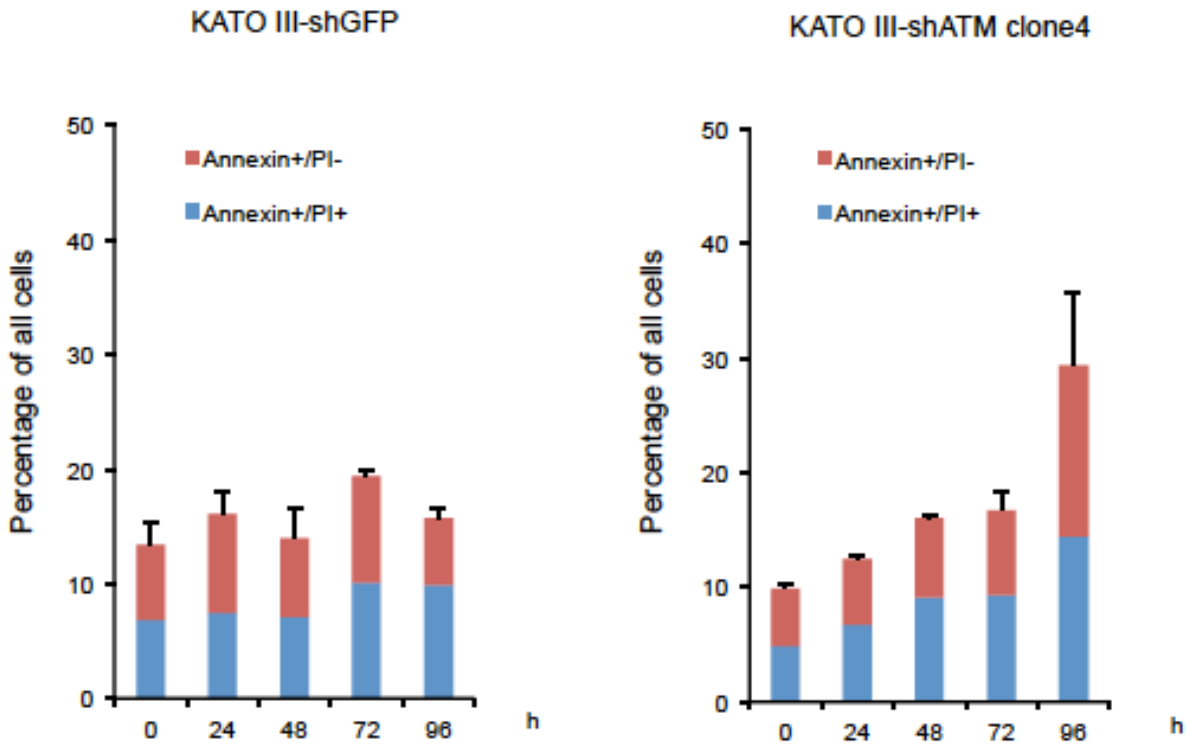
**Supplementary Figure 7. Olaparib induces apoptosis in KATO III but not STKM-2 cells.** Cells were treated with olaparib (5μM) with or without KU55933 (10μM). After 24, 48, 72, and 96 hours, cells were stained with Annexin V-FITC / PI and analyzed by FACS. Each point is in triplicate in three independent experiments. Error bars represent SEM.

**Supplementary Figure 7**



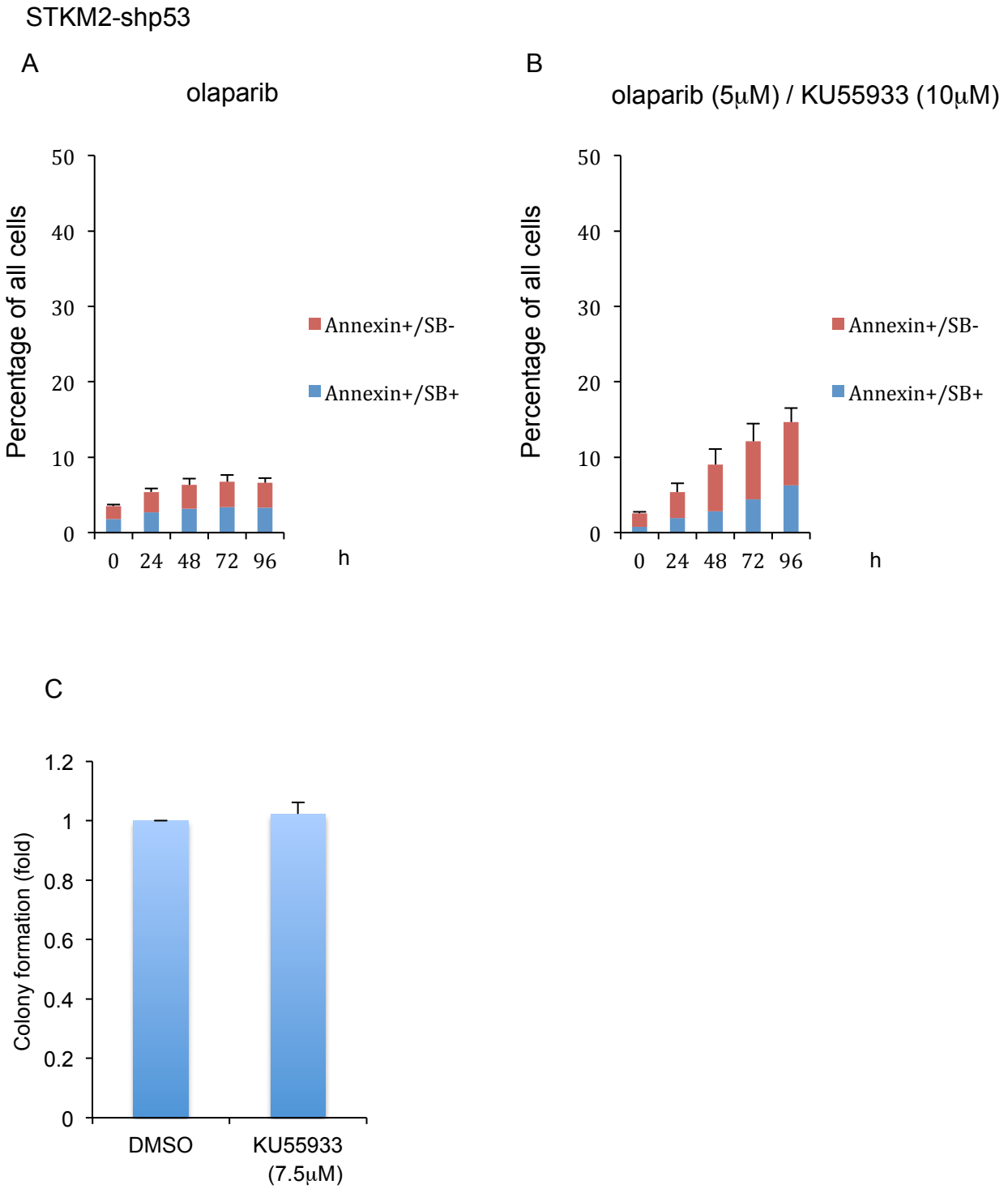
**Supplementary Figure 8: Olaparib induces apoptosis in KATOIII with stable knock down of ATM.** Cells were treated with olaparib (5μM). After 24, 48, 72, and 96 hours, cells were stained with Annexin V-FITC / PI and analyzed by FACS. Each point is in triplicate in three independent experiments. Error bars represent SEM. PI = propidium iodide.

**Supplementary Figure 8**



**Supplementary Figure 9. Olaparib in combination with the ATM inhibitor KU55933 induces apoptosis in STMK2 cells with stable knock down of p53.** (A, B) Cells were treated with olaparib (5 μM) with or without KU55933 (10 μM). After 24, 48, 72, and 96 hours, cells were stained with Annexin V-PE / SYTOX BLUE (SB) and analyzed by FACS. (C) Cells were treated with DMSO or 7.5 μM KU55933. After 10 days, cells were stained and the number of colonies was counted. Each point is in triplicate in three independent experiments. Error bars represent SEM. The result shows that ATM inhibitor alone does not induce loss of viability in STKM-2 cells.

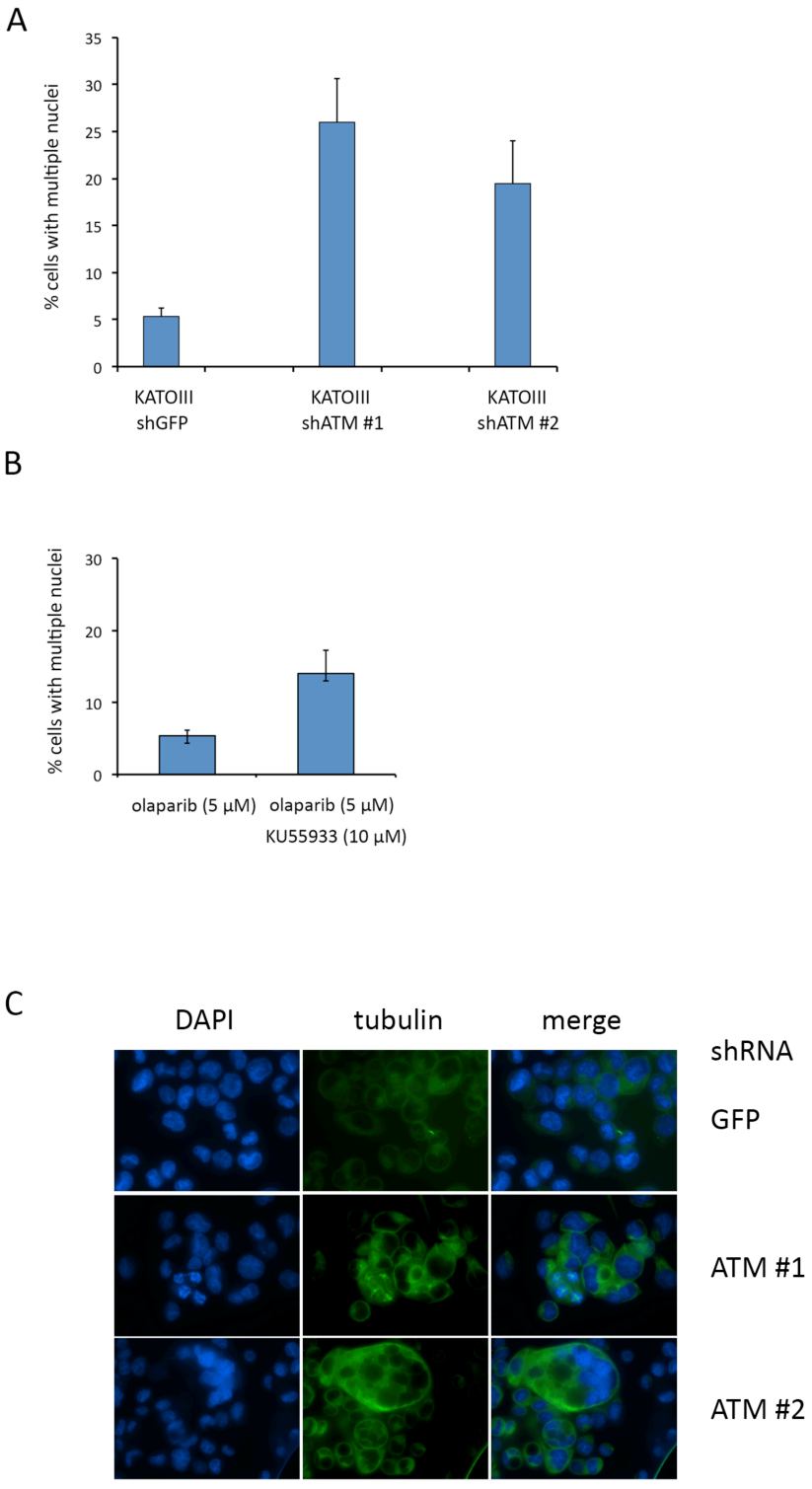
**Supplementary Figure 9**



**Supplementary Figure 10. Olaparib induces multinucleated in ATM depleted gastric cancer cells.**

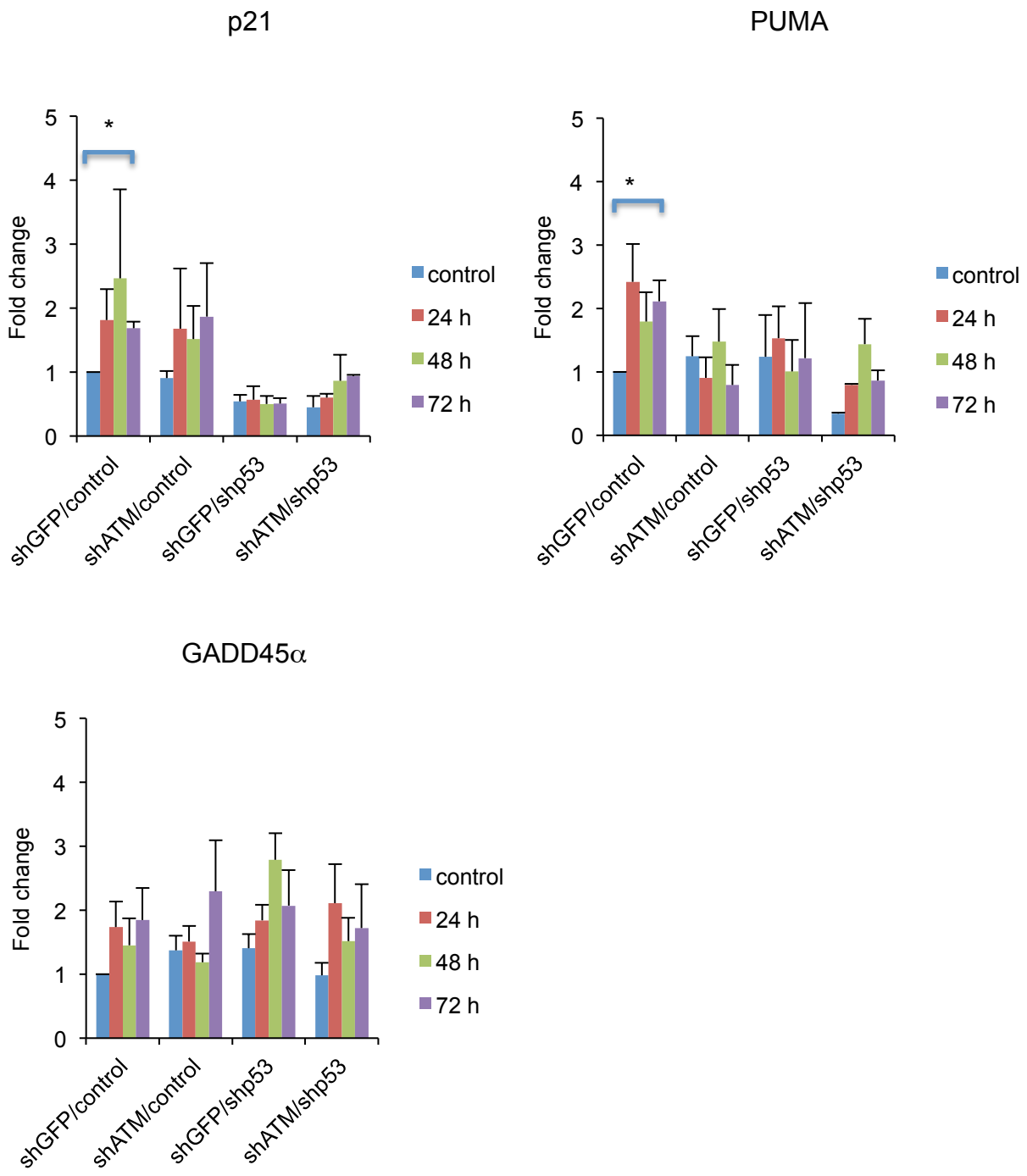
(A) Cells stably transfected with either shRNA to GFP or one of two shRNA's to ATM (indicated by 1 and 2) were treated with olaparib (5 μM) for 72h then stained with DAPI and FITC conjugated α-tubulin antibody. Briefly, samples were fixed with ethanol and acetone, then incubated with FITC conjugated anti alpha-tubulin antibody (Sigma-Aldrich). Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Images were obtained using a fluorescence microscope (Leica, Germany). Images were collected by a computer driven CCD camera and analyzed using VELOCITY software (PerkinElmer, MA, USA). At least 100 cells were scored per experiment and the number of cells with multiple nuclei were counted manually. Each point is in triplicate in three independent experiments. Error bars represent SEM. (B) KATOIII cells were incubated with olaparib (5 μM) or olaparib plus KU55933 (10 μM) for 72 hours as in panel A and multinucleate cells were scored as above. (C) Representative images of cells scored in panels A and B.

**Supplementary Figure 10**



**Supplementary Figure 11. Olaparib induces upregulation of p21 and PUMA in STKM2 cells.** ATM and/or p53 was depleted in STKM-2 cells using shRNA and stable cell lines were generated as described. Cells were treated with olaparib (5μM). After 24, 48, and 72 hours, mRNA was extracted, and the indicated genes were measured by quantitative RT-PCR. Each point is in triplicate in three independent experiments. Error bars represent SEM.

**Supplementary Figure 11**



**Supplementary references**

1. Endo S, Yamato K, Hirai S, Moriwaki T, Fukuda K, Suzuki H, Abei M, Nakagawa I, Hyodo I. Potent in vitro and in vivo antitumor effects of MDM2 inhibitor nutlin-3 in gastric cancer cells. Cancer Sci 2011; 102:605-13.

2. Yamada Y, Yoshida T, Hayashi K, Sekiya T, Yokota J, Hirohashi S, Nakatani K, Nakano H, Sugimura T, Terada M. p53 gene mutations in gastric cancer metastases and in gastric cancer cell lines derived from metastases. Cancer Res 1991; 51:5800-5.

3. Zhang L, Jia G, Li WM, Guo RF, Cui JT, Yang L, Lu YY. Alteration of the ATM gene occurs in gastric cancer cell lines and primary tumors associated with cellular response to DNA damage. Mutat Res 2004; 557:41-51.

4. Akama Y, Yasui W, Kuniyasu H, Yokozaki H, Akagi M, Tahara H, Ishikawa T, Tahara E. Genetic status and expression of the cyclin-dependent kinase inhibitors in human gastric carcinoma cell lines. Jpn J Cancer Res 1996; 87:824-30.

5. Nabeya Y, Loganzo F, Jr., Maslak P, Lai L, de Oliveira AR, Schwartz GK, Blundell ML, Altorki NK, Kelsen DP, Albino AP. The mutational status of p53 protein in gastric and esophageal adenocarcinoma cell lines predicts sensitivity to chemotherapeutic agents. Int J Cancer 1995; 64:37-46.

6. Zhou XM, Wong BC, Fan XM, Zhang HB, Lin MC, Kung HF, Fan DM, Lam SK. Non-steroidal anti-inflammatory drugs induce apoptosis in gastric cancer cells through up-regulation of bax and bak. Carcinogenesis 2001; 22:1393-7.

7. Cheng LL, Itahana Y, Lei ZD, Chia NY, Wu Y, Yu Y, Zhang SL, Thike AA, Pandey A, Rozen S, et al. TP53 genomic status regulates sensitivity of gastric cancer cells to the histone methylation inhibitor 3-deazaneplanocin A (DZNep). Clin Cancer Res 2012; 18:4201-12.

8. Zolner AE, Abdou I, Ye R, Mani RS, Fanta M, Yu Y, Douglas P, Tahbaz N, Fang S, Dobbs T, et al. Phosphorylation of polynucleotide kinase/ phosphatase by DNA-dependent protein kinase and ataxia-telangiectasia mutated regulates its association with sites of DNA damage. Nucleic Acids Res 2011; 39:9224-37.