Thompson et al 2015- Supplemental Information

Supplemental Data

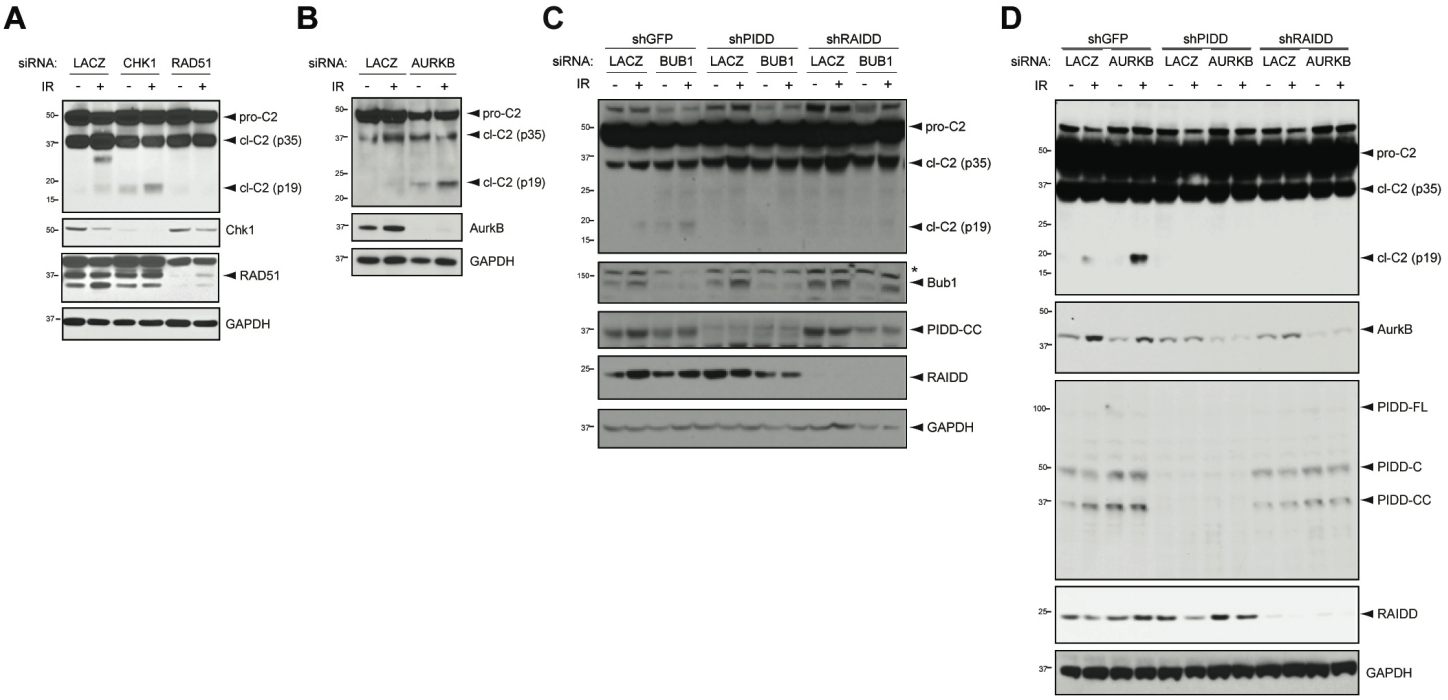

Figure S1 (related to Figure 1)

(A) HeLa cells transfected with the indicated siRNAs were treated 48 hours post transfection with or without 10 Gy IR and harvested 24 hr after IR. Lysates were analyzed by western blot. Pro-C2, procaspase 2; cl-C2 (p35), cleaved caspase 2; cl-C2 (p19), p19 fragment (mature cleavage product).

(B) HeLa cells transfected with the indicated siRNAs were treated 48 hours post transfection with or without 10 Gy IR and harvested 24 hr after IR. Lysates were analyzed by western blot.

(C) HeLa cells stably expressing the indicated shRNAs were transfected with siLacZ or siBub1 were treated 48 hours after transfection with or without IR (10 Gy) and harvested 24 hours after IR. Lysates were analyzed by western blot.

(D) HeLa cells stably expressing the indicated shRNAs were transfected with siLacZ or siAurKB were treated 48 hours after transfection with or without IR (10 Gy) and harvested 24 hours after IR. Lysates were analyzed by western blot.

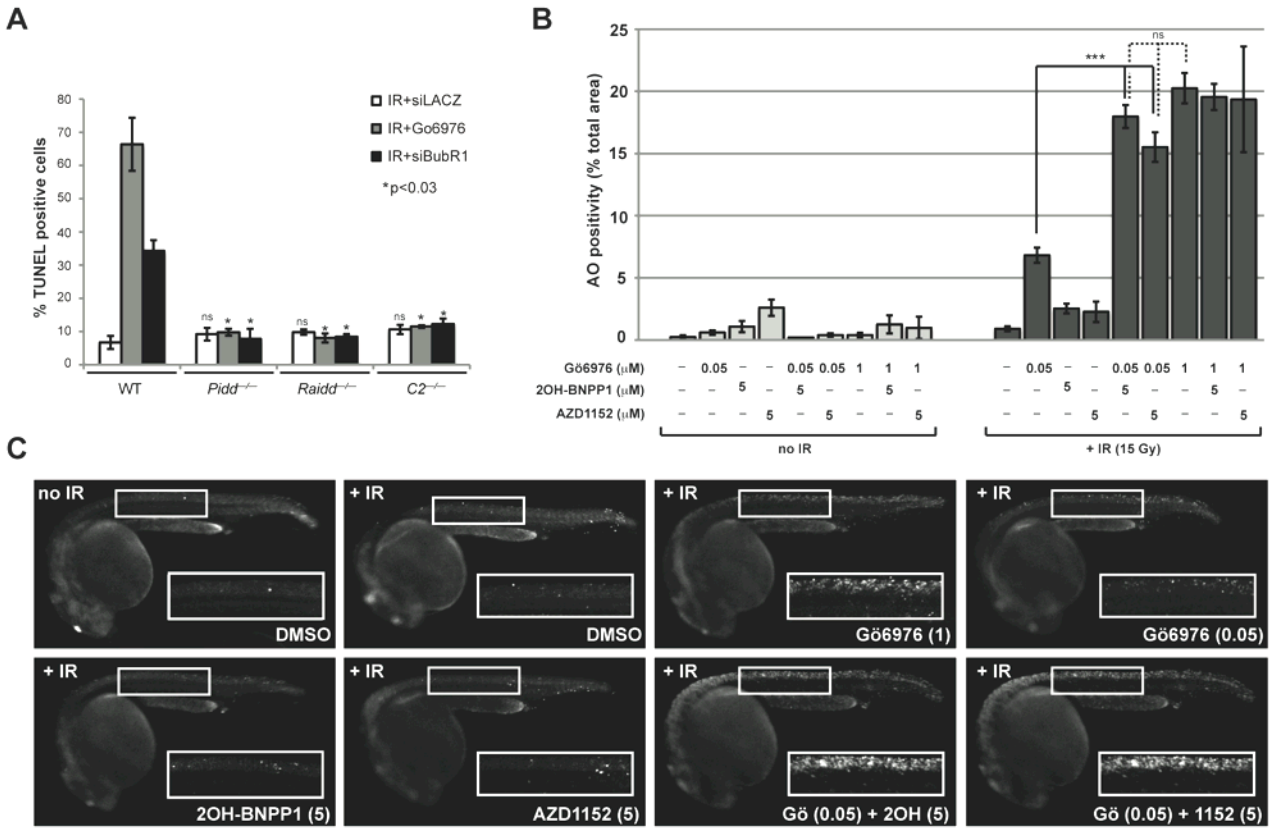

Figure S2 (related to Figure 2)

(A) SV40 transformed MEF cells of indicated genotypes were transfected wit indicated siRNAs or drugs and were treated 48 hours post transfection with IR and harvested 24 hours after IR. Cells were stained for TUNEL and analyzed by flow cytometry.

(B) Quantification of acridine-orange stains in (C). Data collected from three independent experiments (approximately 12 embryos per condition) are represented as means +/- SEM.

(C) 18 hours post-fertilization *p53*$^{M214K/M214K}$ zebrafish embryos were incubated with the indicated inhibitors (μM) with and without 10 Gy IR and stained with the cell death marker acridine orange (AO) after 7 hr.

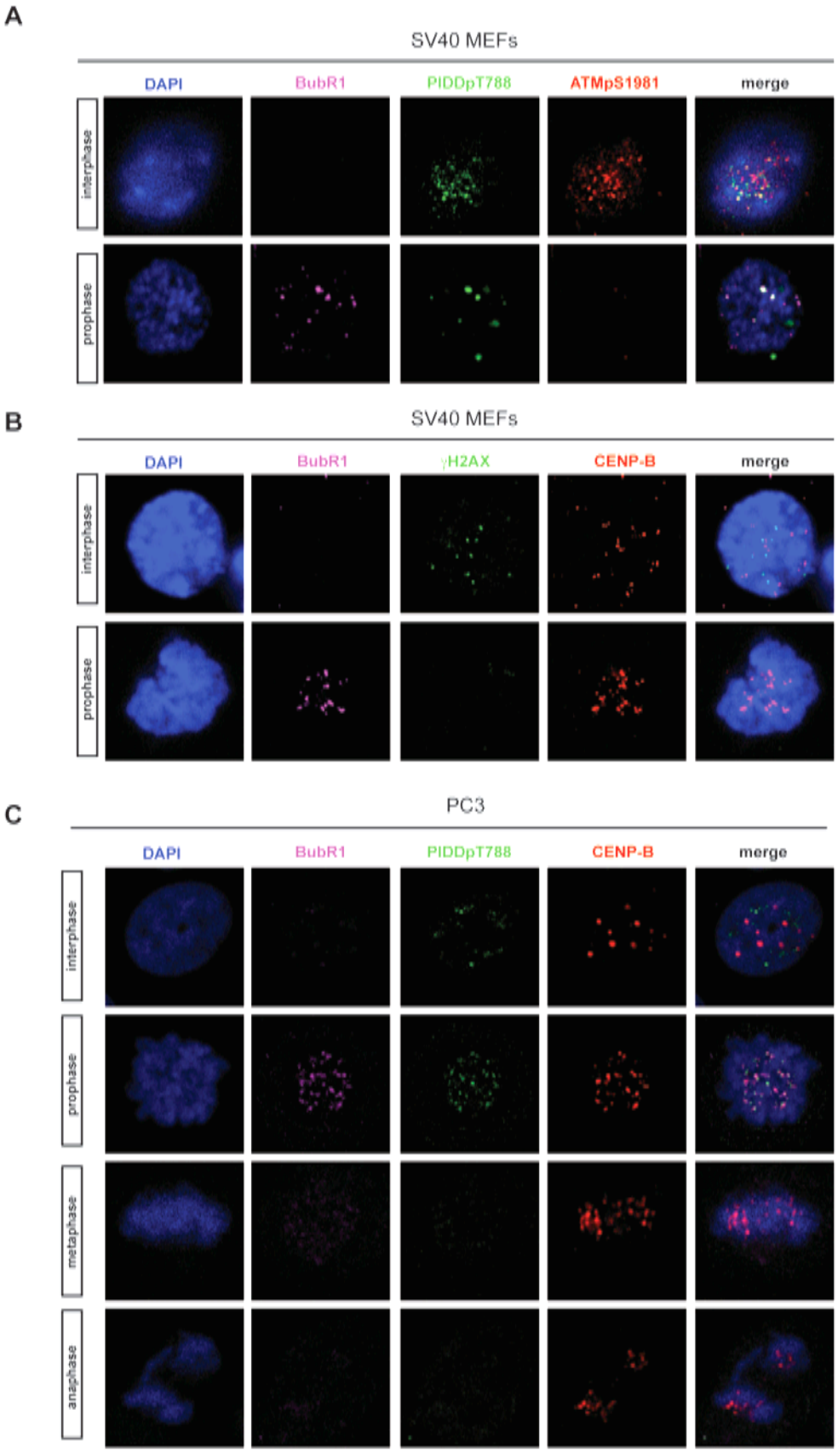

Figure S3 (related to Figure 3)

(A) SV40 transformed WT MEFs grown on coverslips were treated with Gö6976 (1 µM) and IR (10 Gy) and harvested 10 hr post IR. Coverslips were stained using the indicated antibodies and visualized by confocal microscopy (single 0.8 mm sections are shown).

(B) SV40 transformed WT MEFs grown on coverslips were treated with Gö6976 (1 µM) and IR (10 Gy) and harvested 10 hr post IR. Coverslips were stained using the indicated antibodies and visualized by confocal microscopy (single 0.8 mm sections are shown).

(C) HPV harboring PC3 cells grown on coverslips were treated with Gö6976 (1 µM) and IR (10 Gy) and harvested 10 hr post IR. Coverslips were stained using the indicated antibodies and visualized by confocal microscopy (single 0.8 mm sections are shown).

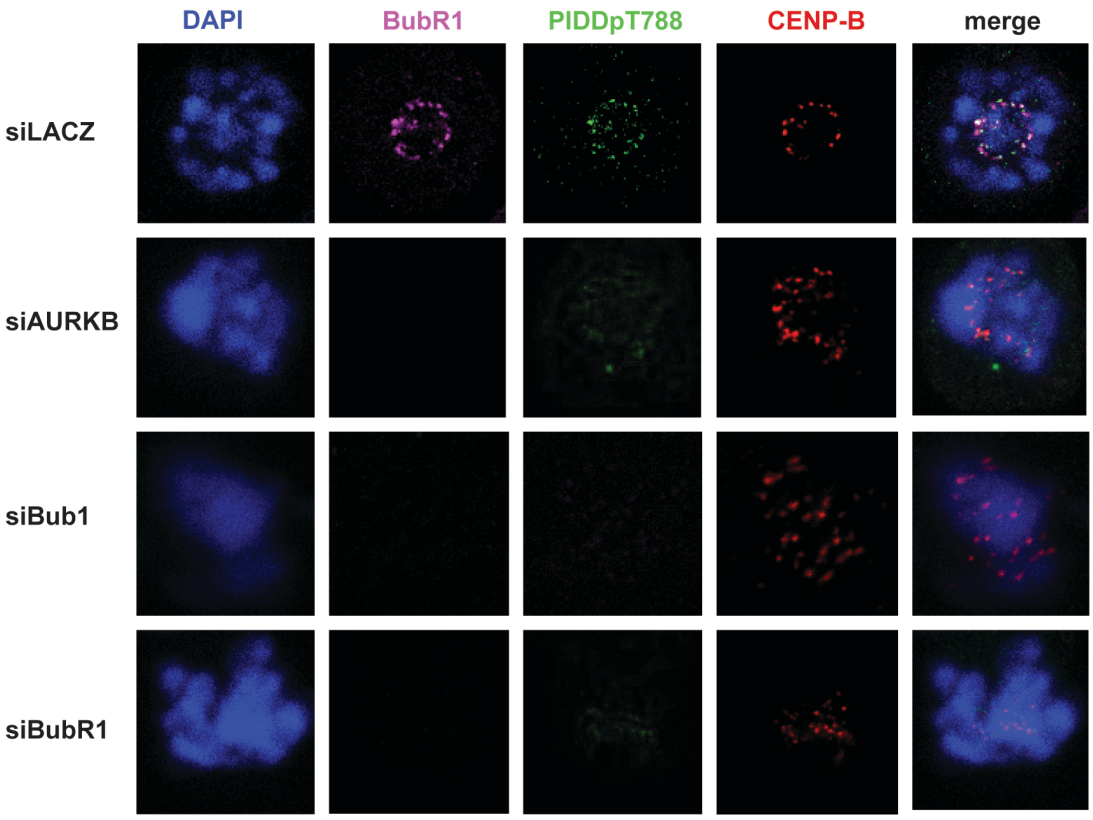

Figure S4 (related to Figure 4)

HPV harboring PC3 cells transfected with the indicated siRNAs and grown on coverslips were treated 48 hours post transfection with Gö6976 (1 µM) and IR (10 Gy) and harvested 10 hr post IR. Coverslips were stained using the indicated antibodies and visualized by confocal microscopy (single 0.8 mm sections are shown).

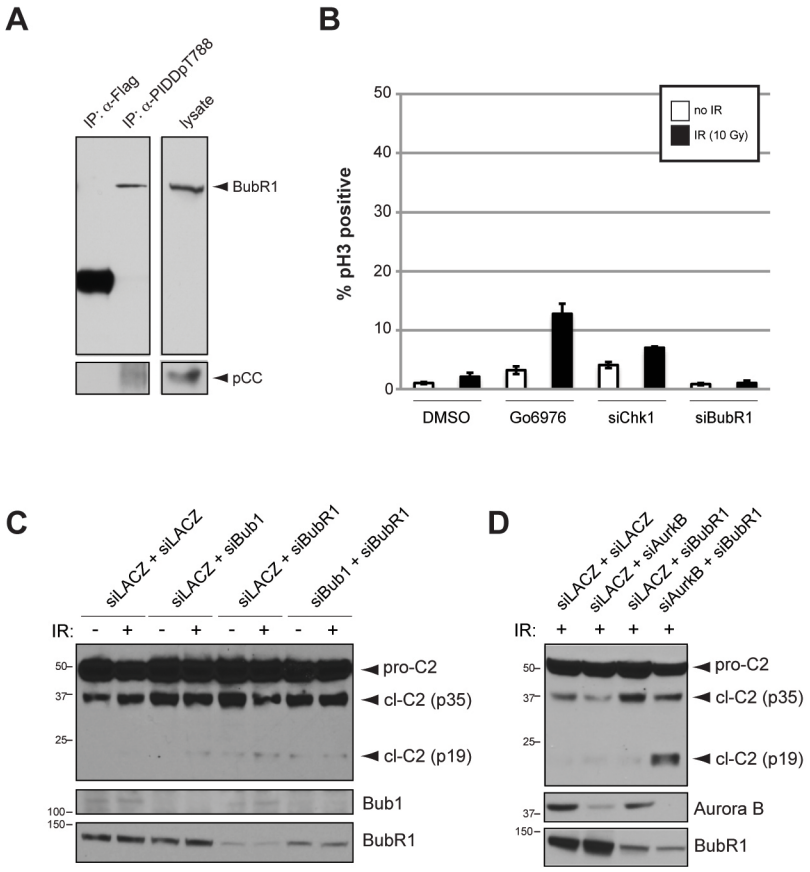

Figure S5 (related to Figure 5)

(A) HeLa cells treated with Gö6976 (1 μM) and IR (10 Gy) were harvested 10 hr post IR and immunoprecipitated using either Flag or pPIDD^{T788} antibodies. Immunoprecipitates were analyzed by western blot.

(B) HeLa cells transfected with the indicated siRNAs or with or without Gö6976 were treated with or without 10 Gy IR and harvested 24 hr after IR. Cells were stained for pHH3 and analyzed by flow cytometry.

(C) HeLa cells transfected with 10nM of each of the indicated siRNAs were treated with or without 10 Gy IR 48 hour post transfection and harvested 24 hr after IR. Lysates were analyzed by western blot.

(D) HeLa cells transfected with 10nM of each of the indicated siRNAs were treated with or without 10 Gy IR and harvested 24 hr after IR. Lysates were analyzed by western blot.

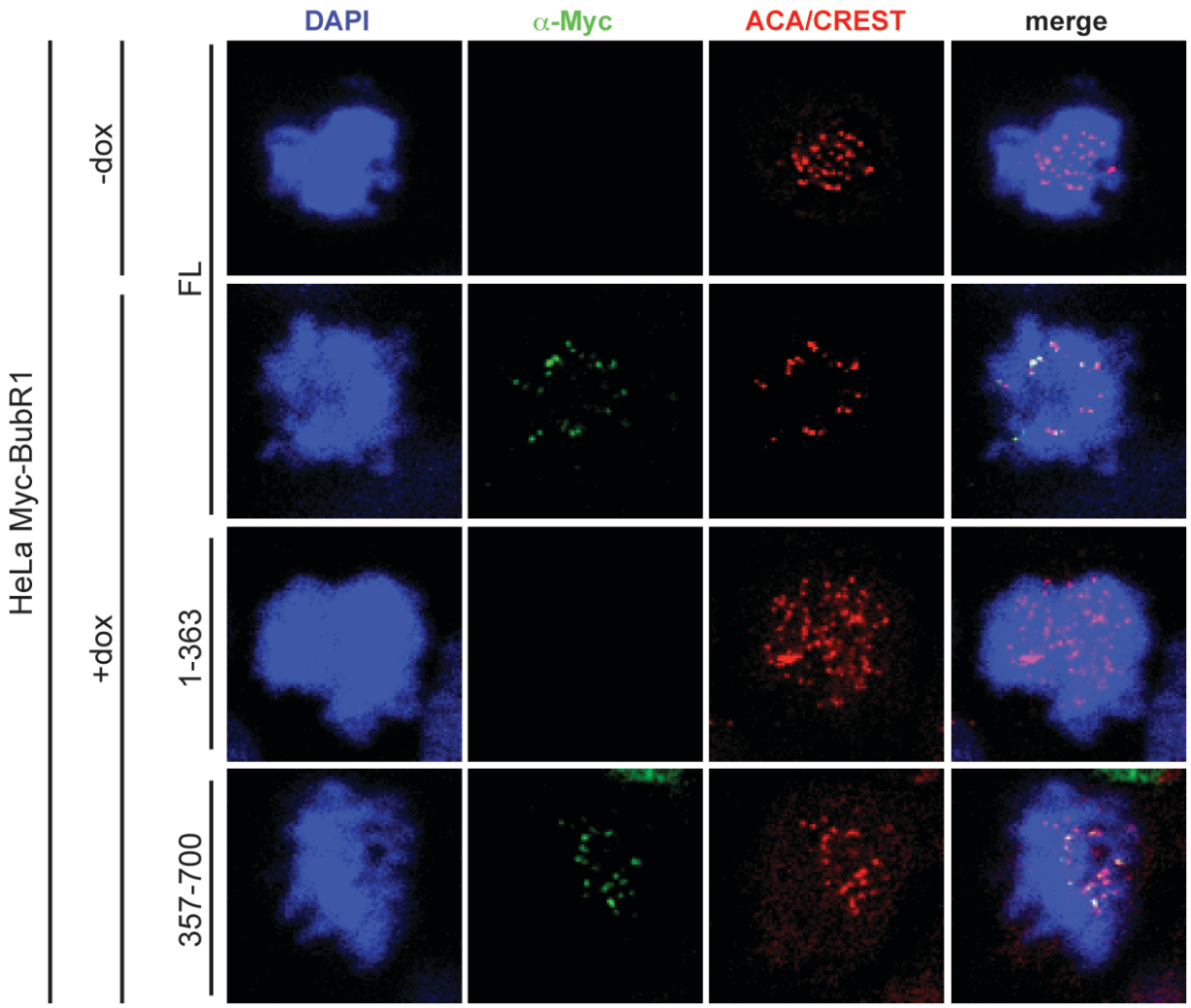

Figure S6 (related to Figure 5)

HeLa cells stably expressing inducible forms of the indicated BubR1 constructs were grown on coverslips, transfected with siRNA to the 3' UTR of endogenous BubR1, treated with or without Doxycycline (1 µg/ml) 24 hr post transfection and harvested 24 hr post Dox. Coverslips were stained using the indicated antibodies and visualized by confocal microscopy (single 0.8 mm sections are shown).

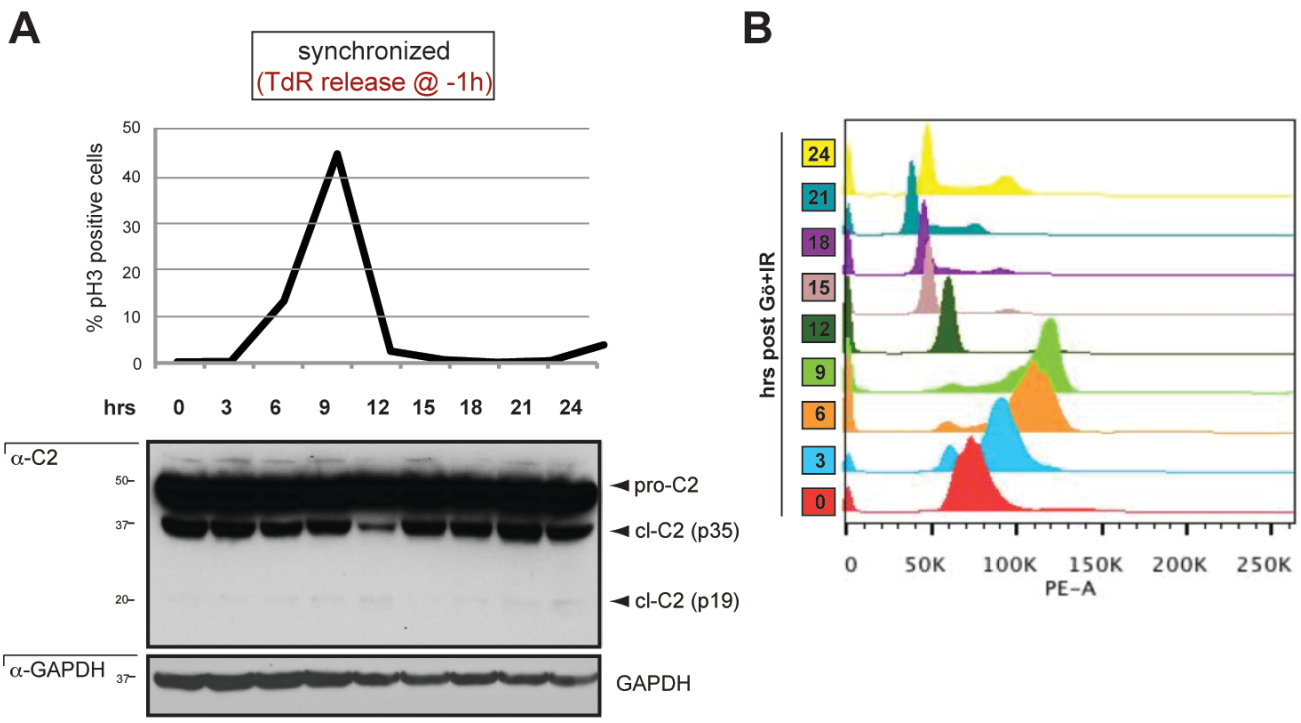

Figure S7 (related to Figure 7)

(A,B) HeLa cells were synchronized using the thymidine double block method then released and harvested every 3 hr post release. Half of each harvest was stained for propidium iodide and pHH3 and analyzed by flow cytometry (B) and half was lysed and analyzed by western blot (A).

Supplemental Experimental Procedures

RNAi

In HeLa cells, siRNA transfections were performed using X-tremeGENE siRNA transfection reagent (Roche) and unless otherwise stated 20nM siRNA according to the manufacturer's instructions. In MEF cells, siRNA transfections were performed using continuum transfection reagent (Gemini Bio-products) and 40nM siRNA according to the manufacturer's instructions. Cells were exposed to IR +/- Gö6976 at 48 hours post-transfection. Previously validated siRNAs were si*LACZ* (Sidi et al., 2008) and si*RAIDD* (RAIDD-2) (Tinel and Tschopp, 2004) (Qiagen).

siRNA to the 3' UTR of BubR1 was custom designed from Qiagen 5'AACTGTATGTGCTGTAATTTA-3' (Han et al., 2013). All other siRNAs were HP-validated siRNAs from Qiagen and were:
si*AURKB* (Hs_AURKB_5; 5'-AACGCGGCACTTCACAATTGA -3')
si*BUB1* (Hs_BUB1_5; 5'-CAGCTTGTGATAAAGAGTCAA-3')
si*BUBR1* (Hs_BUB1B_6; 5'-CAGATTTAGCACATTTACTAT-3')
si*BUBR1* (Mm_BUB1B_1 5'-AACGGGCATTTGAATCTGAAA -3')
si*MAD2* (Hs_MAD2L1_7; 5'-ATGGATATTTGTACTGTTTAA-3')
si*RAD51* (Hs_RAD51_7; 5'-AAGGGAATTAGTGAAGCCAAA-3')
si*LACZ* and si*CHK1* (Sidi et al., 2008; Wang et al., 2007) and si*RAIDD* (RAIDD-2) (Tinel and Tschopp, 2004) have been described.
Lentiviral shRNA transduction was performed in HeLa cells as previously descibed (Ando et al., 2012; Rodriguez-Barrueco et al., 2013). shGFP, shPIDD, shRAIDD and shCasp2 have been previously described (Ando et al., 2012). BubR1 shRNA was ordered from GE Biosciences: TRIPZ inducible Human BUB1B shRNA (V3THS_359184; 5'-ACATCTAGATCTTCTTCCG-3') When using inducible shBubR1 cell line, cells were treated with 1 µg/ml Doxycycline 24 hours prior to treatment.

Expression vectors and DNA transfections

Plasmid DNA was transfected into HeLa cells using X-tremeGENE HP (2:1) (Roche) according to the manufacturer's instructions. For DNA + siRNA cotransfections, as siRNA were transfected (see RNAi section) and plasmids were transfected 24 hours later. Where needed, transfected cells were treated with or without Gö6976 at 24-48 hr post-transfection and with or without IR (10 Gy) one hour later. Amount of cDNA transfected was kept constant by adding empty vector to a total amount of 6µg per 10cm plate. Original constructs were C-terminally Flag-tagged PIDD-FL, PIDD-N, PIDD-C, PIDD-CC, LRR, and CΔZU5, cloned in pcDNA5/FRT (Logette et al., 2011; Tinel et al., 2007), and N-terminally VSV-tagged RAIDD (VSV-RAIDD) cloned in pCR3 (Tinel and Tschopp, 2004). The LAP-GFP-BUBR1 wild type and deletion constructs cloned in pIC58 were a kind gift of Geert Kops (Suijkerbuijk et al., 2012). Stable, isogenic cell lines expressing MycGFP-BubR1 transgenes were a kind gift of Don Cleveland (Han et al., 2013). Expression of MycGFP-BubR1 transgenes was induced with 1µg/ml doxycycline. Empty pcDNA5/FRT and pcDNA3 vectors were generated by self-ligation after restriction enzyme digestion with PmeI. The Myc-BubR1^{WT} and Myc-BubR1^{E413K} mutant cloned in pCDNA.3 were kindly provided by Sabine Elowe (Elowe et al., 2010).

Morpholino Injections, Drug Treatment and Acridine Orange Labeling in Zebrafish

Embryos

Live *p53*$^{M214K/M214K}$ zebrafish embryos (Berghmans et al., 2005) were injected at the one-cell stage with 1 mM *std* MO (5'-CCTCTTACCTCAGTTACAATTTATA-3'), or *bubr1* MO (5'-GTCACTCACAAACTGGATCCAGAGT-3') targeted to the exon 3/intron 3 boundary of *bub1bb* (ZDB-GENE-030829-51). At 18 hours post-fertilization (hpf), embryos were irradiated (15 Gy) using a ^{137}Cs-irradiator. Following a 6 hr incubation at 28.5^{o}C, embryos were incubated in 5µg/mL AO in egg water for 20 min at room temperature and rinsed three times in egg water. Embryos were mounted in 2% methylcellulose and imaged on a Nikon SMZ 1500 fluorescence microscope. Analysis and quantification of spinal cord AO staining, as well as RT-PCR analysis of MO-mediated knockdown with primers GCTTCTTGAAAGAGCGGTCA (f) and CGCTGAGGCTCTTCATCATC (r), were performed as previously described (Sidi et al., 2008). MOs were from Gene Tools, LLC. For Aurora B and Bub1 inhibitor experiments, 17hpf embryos were dechorionated in pronase (2.0 mg/mL in egg water) for 7 min and rinsed three times in egg water. Embryos were then arrayed in 24-well plates and pre-treated with Go6976 (50nM or 1µM), 2OH-BMPP1 (5uM), or AZD1152 (5µM) for 1 hour before IR (15 Gy). Following a 6 hr incubation at 28.5^{o}C, embryos were washed, AO-stained and analyzed as above.

Immunoblotting and Antibodies

Whole-cell lysates were prepared in RIPA buffer or 1% NP-40 Buffer (Boston BioProducts). Lysate (25-200 µg) was added to NuPAGE LDS Sample Buffer (4X) (Invitrogen) and 5% 2-Mercaptoethanol (Sigma), and samples were incubated at 70°C for 10 min. Samples were run on a Tris-Acetate gel in MOPS or MES Running buffer (Invitrogen). After electrophoresis, samples were transferred for 2 hr (90V, 150-200 mA) to a Nitrocellulose membrane (Thermo Scientific) using a submerged transfer apparatus (Bio-Rad). Membranes were then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% tween (TBST) and probed overnight at 4°C with primary antibodies (see below). Membranes were then washed in TBST and probed with anti-rabbit, -rat, or -mouse (Cell Signaling Technology) HRP-linked antibodies at a 1:2000-4000 dilution for 1 hr, washed, and placed in SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology). The band of interest was then identified with photographic film. Antibodies used were: anti-caspase-2 mAb (clone 11B4, Millipore); anti-RAIDD mAb (clone 4B12, MBL, Nagoya, Japan); anti-PIDD pAb (AL233) and anti-PIDD mAb (Anto-1) were from Alexis Biochemicals; anti-mouse Pidd mAb (Lise-1) was from AdipoGen. Anti-Chk1 (G-4), Mad2 (FL-205), Rad51 (, were all from Santa Cruz Biotechnology. Anti-AurKB (3609), Bub1 (ab54893), anti BubR1 (mouse (8G1) and sheep) were from abcam. Anti-Histone H2A.X mAbs (Ser 139) were from Millipore (JBW301) and Cell Signaling Technology (20E3). Mouse anti-Flag (M2) was from Sigma, and rabbit anti-Flag (DYKDDDDK Tag/M2) was from Cell Signaling Technology. Anti-phospho-ATM (Ser 1981) (10H11.E12), Bub3 (3049), Myc-tag (9B11) and RIP1 (D94C12) were

from Cell Signaling Technology. Anti-VSV (A190-131-A) was from Bethyl and anti-GFP (7.1 13.1) was from Roche. The anti-phospho-T788 PIDD antibody [α-pPIDD (pT788)] has been described (Ando et al., 2012).

Antibodies for immunofluorescence

Antibody against	**Company**	**Fixation**	**Block**	**Antibody Dilution**
ACA/ CREST-Texas red conjugate	Antibodies Incorporated	1% PFA in PBS + 0.5% Triton-X100	1% BSA RT (with secondary)	1:40
pATM (Ser1891)	Rockland Antibodies	4% PFA	5% BSA 1hr 37°C	1:100
BubR1 (sheep)	Abcam	1% PFA in PBS + 0.5% Triton-X100	5% BSA 1hr 37°C	1:200
Cenp B (F4) (mouse)	Santa cruz	4% PFA or 1% PFA in PBS + 0.5% Triton-X100	5% BSA 1hr 37°C	1:200
γH2AX (JBW301) (mouse)	EMD Millipore	4% PFA	5% BSA 1hr 37°C	1:10,000
γH2AX (20E3) (rabbit)	Cell Signaling	4% PFA	5% BSA 1hr 37°C	1:500
Myc-tag (9B11)	Cell Signaling	1% PFA in PBS + 0.5% Triton-X100	1% BSA 1 hr 37°C	1:8000
NDC80 (9G3)	Abcam	1% PFA in PBS + 0.5% Triton-X100	1% BSA 1 hr 37°C	1:100
pPIDD T788	From immunizing rabbits with peptide CETGFL**pT$_{788}$**QSNLL (Invitrogen)	4% PFA or 1% PFA in PBS + 0.5% Triton-X100 or Methanol at -20°C	5% BSA 1hr 37°C	1:50

Coimmunoprecipitation

Lysates for immunoprecipitation were prepared in 1 or 0.1% NP-40 buffer (1 or 0.1% NP-40, 50 mM Tris-HCl [pH 8.0], 150-250 mM NaCl, 5mM EDTA, 1mM phenylmethylsulfonyl fluoride,

protease inhibitors cocktail [Complete Mini, Roche] and phosphatase inhibitor cocktail [PhosSTOP, Roche]). For endogenous IPs, whole-cell lysates (1-5 mg) were mixed with Protein-G magnetic beads (Invitrogen, 30 µl of a 50% slurry) and anti-PIDD (AL233), anti-PIDD (Anto-1) or anti-pPIDD^{T788} antibody (3-5 µg, 1 ml final volume) for 10 min to 3 hours at room temp on a rotating wheel. Beads were then washed three times with PBS-Tween20 (0.02%), resolved by SDS-PAGE, and probed with anti-PIDD (AL233 [1:200 dilution]), anti-PIDD (Anto-1), anti-BubR1 (8G1), anti-Bub1 (ab54893), anti-caspase-2 (11B4) and anti-RAIDD (4B12) detected with the corresponding secondary antibodies or mouse TrueBlot HRP-conjugated secondary antibodies [eBioscience]). For anti-Flag IPs, whole-cell lysates (0.15-2 mg) were mixed with 20 µl beads (50% slurry) and mouse anti-Flag (M2) antibody (3 µg) in 1% NP-40 buffer (500 µl final volume) for 10 min on a rotating wheel. Beads were then washed three times with PBS-T, resolved by SDS-PAGE, and analyzed by western blot.

TUNEL Assay

TUNEL assays were performed using the APO-BRDU kit (BD Biosciences) as described previously (Sidi et al., 2008).

Cell Survival Assay

HeLa cells were reverse transfected using X-tremeGENE siRNA transfection reagent (Roche) according to the manufacturer's instructions and seeded to 96 well plates at a density of 500 cells/ well. 24 hours post transfection they were treated with or without 10 Gy IR and incubated with alamar blue for 8 hours 3-4 days post IR or when the untreated control cells reached 80% confluence. Absorbance was measured at a wavelength of 570nm with a 600nm reference wavelength. Relative fluorescence (RFU) was calculated using cel free wells as a control reference.

Flow Cytometry

Cells were treated as stated, collected by tripsinization and fixed in ice cold 70% Ethanol overnight at -20°C. Cells were washed once in PBS, permeabilised for 5 mins in cold PBS containing 0.25% Triton-X-100. Following a further wash in wash buffer (PBS + 0.1% azide + 1% FBS) cells were incubated for 2 hours at 37°C with 1:100 pHH3 antibody (Cell signaling) in wash buffer. Cells were washed twice and incubated with alexa-488 labelled anti-rabbit secondary antibody (Invitrogen) for 30 mins at RT in the dark. Following two more washes, cells were incubated in propidium iodide + RNase A for 30 minutes in the dark prior to analysis on FACS Canto (BD biosciences).

Supplemental References

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