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Supplemental Data

The Cdc14B-Cdh1-Plk1 Axis Controls the G2 DNA-Damage-Response Checkpoint

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Supplemental Experimental Procedures

Cell culture

U2OS, HeLa, HeLa-S3, T98G, and HEK293T cells were cultured as described (Dorrello et al., 2006; Guardavaccaro et al., 2003). *ATM*+/+ (NHF1-hTERT) and *ATM*-/- (GM0252A-hTERT) fibroblasts were grown as described by (Heffernan *et al.*, 2002).

Antibodies

Mouse monoclonal antibodies were from Zymed/Invitrogen (anti-Cul1, anti-Plk1), Sigma (anti-FLAG, anti-Cdc27, anti-Cdh1), Santa Cruz Biotechnology (anti-Chk1, anti-Geminin, anti-Cdc25A), Boston Biochem (anti-UbcH10), BD Biosciences (anti-p27), Covance (anti-HA), and Abcam (anti-GFP). The mouse monoclonal antibodies against Claspin and USP28 were kind gifts from Thannos Halazonetis and Steve Elledge. Rabbit polyclonal antibodies were from Zymed/Invitrogen (anti-Cks1), Upstate (anti-phospho-Ser10 Histone H3, anti-phospho-Ser 139 Histone H2AX), Santa Cruz Biotechnology (anti-Wee1, Cdk2, phospho-Tyr15 Cdk1, Skp1, Bub1), and Cell Signaling (phospho-Ser317 Chk1, phospho-Thr68 Chk2). The rabbit polyclonal antibodies against TMPK and TK1 were a kind gift from Zee-Fen Chang. Rabbit polyclonal antibodies against Cdk1 (Carrano et al., 1999), cyclin A (Carrano and Pagano, 2001), and the mouse monoclonal antibody to cyclin E (Faha et al., 1993) were previously described.

In vitro ubiquitylation assay

Ubiquitylation assays were previously described (Bashir et al., 2004). Briefly, an anti-Cdc27 antibody was added to cell extracts and incubated for approximately 3 hours at 4°C. Protein G-agarose was then added and incubated for 45 minutes at 4°C on a rotating wheel. The beads were washed 4 times in Triton buffer and 4 times in QA buffer (10 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT). The resulting beads were used for two reactions of *in vitro* ubiquitylation. Ubiquitylation assays were performed in a volume of 10 µl containing 50 mM Tris pH 7.6, 5 mM MgCl2, 0.6 mM DTT, 2 mM ATP, 2 µl *in vitro* transcribed/translated unlabelled Cdh1, 50 ng/µl E1 (Boston Biochem), 100 ng/µl Ubc1, 100 ng/µl Ubc10, 2.5 µg/µl ubiquitin (Sigma), 1 µM ubiquitin aldehyde, and 1 µl ^{35}S-methionine-labelled *in vitro* transcribed/translated substrate [i.e. wild type NT-Claspin, NT-Claspin(ENL), CT-Claspin, Plk1, or cyclin B labeled *in vitro* transcribed/translated in rabbit reticulocyte lysate (RRL)]. The reactions were incubated at 30 °C for the indicated times and analyzed by SDS-PAGE and autoradiography. The RRLs containing the *in vitro* transcribed/translated substrates were treated with 5 mM NEM prior to ubiquitylation reactions, which transitorily inactivates ubiquitylating activities present in the RRL (Rodrigo-Brenni and Morgan, 2007). The assays shown in Figure S4D did not contain Cdh1.

GST Fusion Proteins and Pull-down Assay

GST-Cdh1 or GST-Claspin mutants were expressed in *E.coli* (BL-21) using the pGEX 4T2 vector (Amersham). For protein purification, bacteria were grown to an optical density of 600 nm in Luria-Bertani medium, induced at 37°C with 0.1 mM isopropyl-1-thio-D-galactopyranoside, and cultivated for 2h. Bacteria were then pelleted, resuspended in

NETN-buffer (100mM NaCl, 1mM EDTA, 50 mM Tris·HCl [pH 7.4], 0.5 % Nonidet P-40, 1mM phenylmethylsulfonyl fluoride, 5mM benzamidine), and sonicated. Insoluble material was removed by centrifugation. Thirty microliters of Glutathione-S-Sepharose 4b beads (Amersham) were added to the cleared lysate, incubated for 30 min at 4°C, and washed 3 times with NETN-buffer. GST pull-down assays were performed as described previously (Bassermann et al., 2005). Briefly, wild type and mutant Claspin proteins or Cdh1 were *in vitro* transcribed/translated and ^{35}S-radiolabeled using the TNT system (Promega). GST fusion proteins were added and incubated for one hour at 4 °C. Subsequently, protein complexes were washed thoroughly with NETN buffer, subjected to SDS-PAGE, and visualized by autoradiography.

Plasmids

cDNAs of wild type Claspin, Claspin point- and deletion-mutants, βTrcp1/2, Fbxw4, Fbxw5, Fbxw6, Fbxw7, Cdh1, and Cdc20 were cloned into pcDNA 3.1. pGFP-Cdc14A and pGFP-Cdc14B were kind gifts from Jiri Lukas. For retrovirus production, Cdc14B, Cdh1 WT, and Cdh1(4xA) were subcloned into the retroviral vector pMSCV while Plk1 WT and Plk1(R337A, L340A) were subcloned into the retroviral vector pBabe. All cDNAs were sequenced. Point mutants were generated using the QuikChange Site-directed Mutagenesis kit (Stratagene), and deletion mutants were prepared by standard PCR procedures.

siRNA oligos

The sequences of the oligonucleotides corresponding to βTrcp1/βTrcp2, Cdh1, Cdc14B, and Usp28 mRNAs were GUGGAAUUUGUGGAACAUC, UGAGAAGUCUCCCAGUCAG, GAUGCUACAUGGUUAUAUA, and CUGCAUUCACCUUAUCAUU, respectively. These dsRNA oligos have been previously validated: Cdh1 (Bashir et al., 2004; Brummelkamp et al., 2002; Donzelli et al., 2002; Ke and Chang, 2004), Usp28 (Zhang et al., 2006), βTrcp1/2 (Dorrello et al., 2006; Fong and Sun, 2002; Guardavaccaro, 2008; Guardavaccaro et al., 2003; Jin et al., 2003; Peschiaroli et al., 2006) and Cdc14B (Rodier et al., 2008). A dsRNA oligo to LacZ mRNA (CGUACGCGGAAUACUUCGA) served as control.

mRNA analysis

RNA was extracted using the RNeasy Kit (Qiagen). cDNA synthesis was performed using Superscript III (Invitrogen). Quantitative PCR analysis was performed according to standard procedures. Primer sequences were: 5'GTGCCATTGCAGTACATT3' and 5'AGCAGGCTATCAGAGTG3' (Cdc14B) and 5'CGCCGCTAGAGGTGAAATTC3' and 5'CTTTCGCTCTGGTCCGTCTT3' (18S rRNA).

Immunofluorescence microscopy

Direct and indirect immunofluorescence was performed as described (Frescas et al., 2007). Primary antibodies (anti-FLAG, Sigma; anti-GFP, Abcam) were used at a dilution of 1:1000.

***In vivo* labeling with orthophosphate**

U2OS cells expressing either wild type Cdh1 or Cdh1(4xA) were transfected with control or *Cdc14B* siRNA oligonucleotides and synchronized at G1/S using a double thymidine block. Seven hours post release, cells were washed twice in labeling medium [(phosphate-free DMEM, supplemented with 10% dialyzed serum (Hyclone)] and subsequently incubated for three hours in labeling medium, containing 0.5 μM doxorubicine and [^{32}P]-orthophosphate (0.5 mCi/ml, Perkin Elmer). Denatured cell extracts were subsequently prepared in 1% SDS. Prior to immunoprecipitation with anti-FLAG agarose (Sigma), cell extracts were diluted 10-fold with lysis buffer containing 1% Triton X-100.

Data mining

Gene expression data on Cdc14B, Cdh1 and Plk1 were retrieved from the Oncomine website (www.oncomine.org). Data was re-analyzed in GraphPad software to show expression levels of Cdc14B, Cdh1, and Plk1 for each cancer study. GraphPad software was used to determine *P* values. Additional details relating to these studies, including the pathological and clinical data, are available at Oncomine or via the individual journal websites. Kaplan-Meier survival curves of 219 brain cancer patients were obtained from the Repository of Molecular Brain Neoplasia Database Rembrandt website (caintegrator.nci.nih.gov/rembrandt). Kaplan-Meier survival plots were generated by grouping gliomas of all histological grades by the gene expression levels of Cdc14B, Cdh1 and Plk1. Additional details relating to these studies, including the pathological and clinical data of individual patients, are available on the Rembrandt website.

Normalization and quantification of protein levels

Protein concentrations of whole cell extracts (WCE) were performed using a Bio-Rad *DC* protein assay (Lowry assay) according to the manufacturers instructions. For each experiment, equal amounts of WCE (in general, 30 μg) were separated by SDS-PAGE and then analyzed by immunoblotting. Equal protein levels in each lane were confirmed by Ponceau S staining of the membrane and by immunoblotting a protein whose levels are not regulated by either DNA damage or during cell cycle progression (*e.g.*, Cul1 or Skp1). To make the assay as linear as possible, densitometric quantification of bands was performed using Quantity One software (Bio-Rad) on low-saturation exposures. To be able to directly compare protein levels of different gels and independent experiments, an equal WCE (*e.g.*, 30 μg of HeLa WCE) was loaded in each gel as a standard reference.

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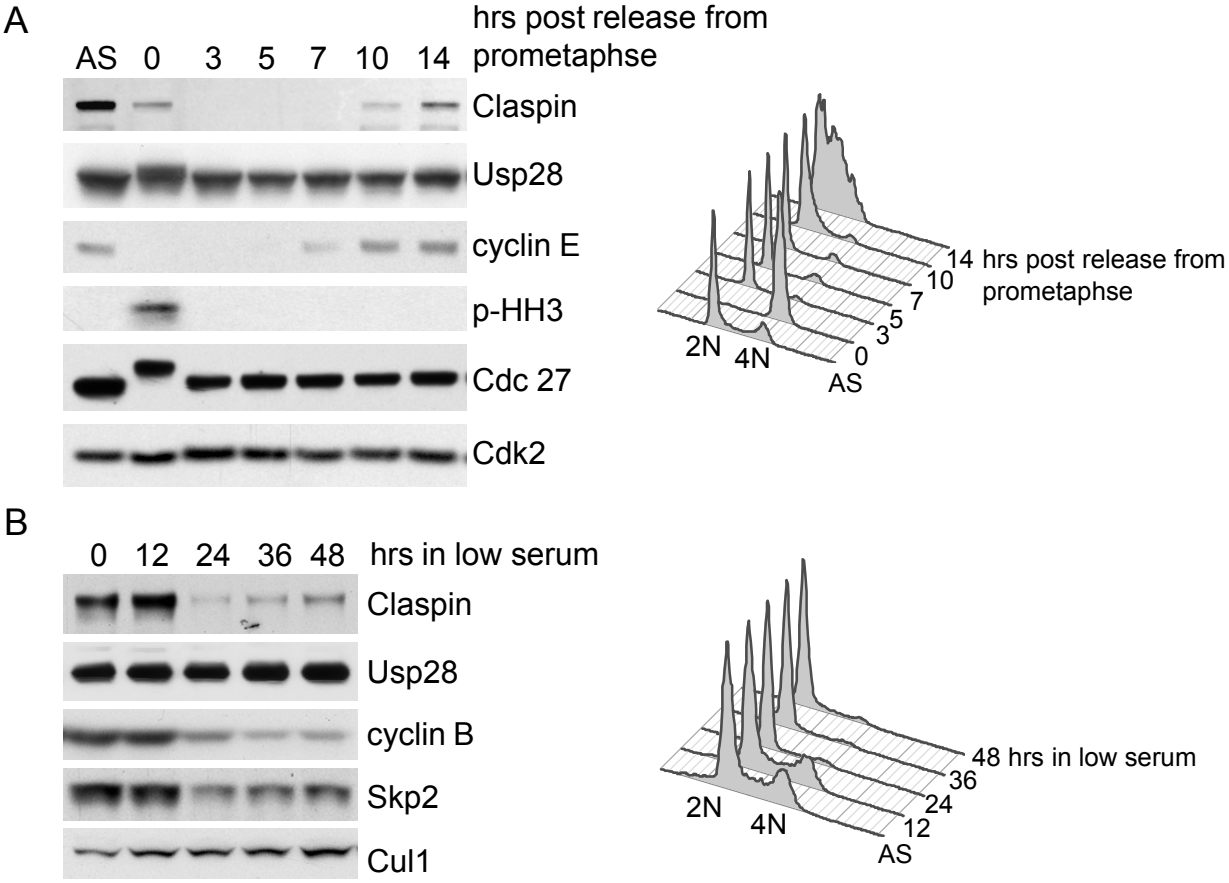
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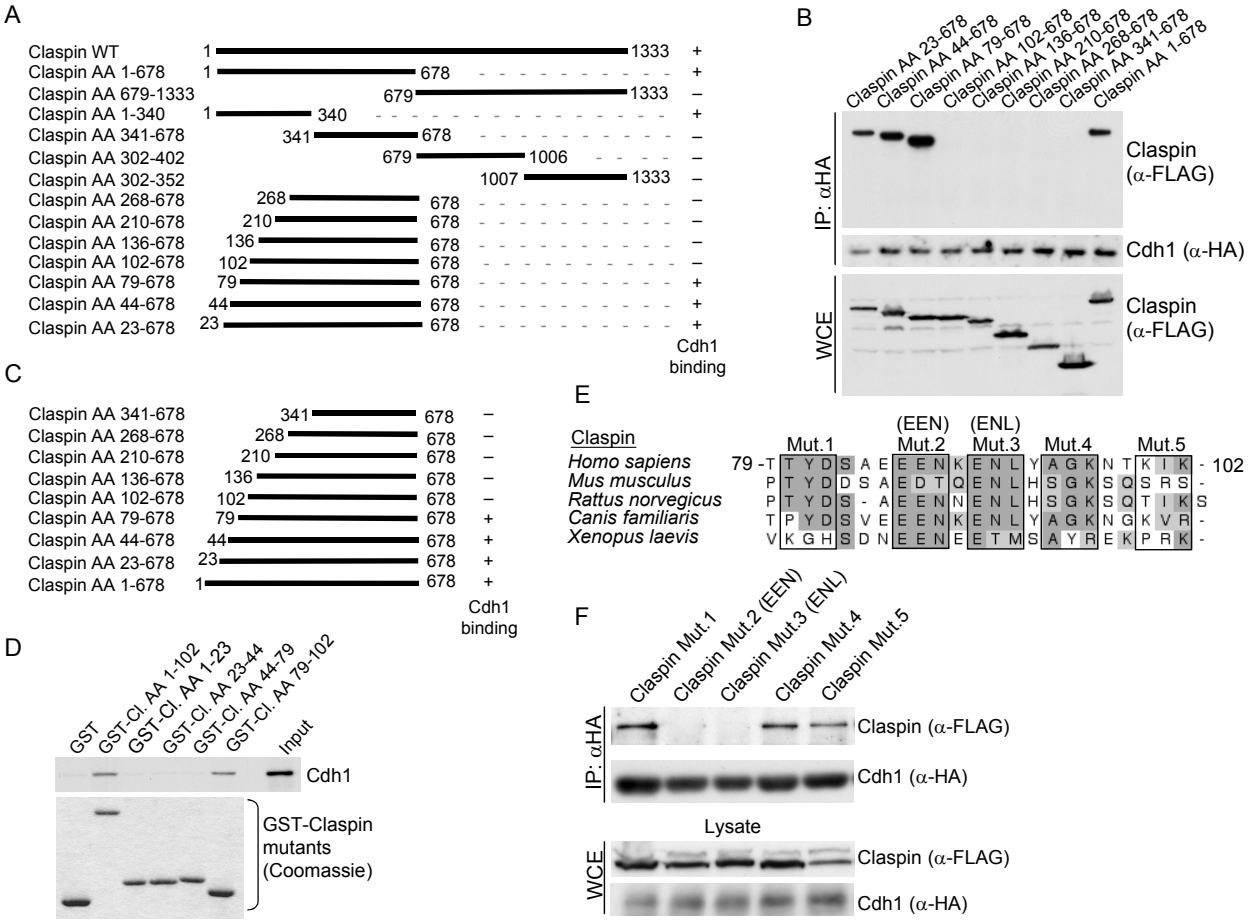
Supplementary Figure 1. $SCF^{\beta Trcp}$- independent degradation of Claspin in G1

(A) Left panels: U2OS cells were released from a prometaphase arrest (indicated as time 0) and collected at the indicated times. Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins. (AS, asynchronous cells). Right: Cell cycle profiles of the cells used in the experiment shown in the left panel as determined by flow cytometry.

(B) Left panels: T98G cells were switched to culture media containing 0.02% FBS to arrest them in G0/G1. Samples were collected at the indicated times after the beginning of the serum starvation and subjected to immunoblot analysis using antibodies to the indicated proteins. Right: Cell cycle profiles of the cells used in the experiment shown in the left panel as determined by flow cytometry.

(C) U2OS cells were transfected with siRNA oligos to both *βTrcp1* and *βTrcp2* mRNAs. Cells were collected after being synchronized in prometaphase (PM) or G1 phase, and cell extracts were analyzed by immunoblotting with antibodies to the indicated proteins.



Supplementary Figure 2. Mapping of the Cdh1-binding motif in Claspin

(A) The indicated deletion mutants were transcribed/translated *in vitro* and tested for their binding to GST-tagged Cdh1 using *in vitro* pull-down assays. Claspin mutants that were pulled-down by GST-Cdh1 were separated by SDS-PAGE and visualized by autoradiography (data not shown) and designated with the symbol (+).

(B) HEK293T cells were co-transfected with HA-tagged Cdh1 and the indicated FLAG-tagged deletion mutants of Claspin. Whole cell extracts (WCE, bottom panel) were immunoprecipitated (IP, upper two panels) with an anti-HA antibody, and the indicated proteins were detected by immunoblotting.

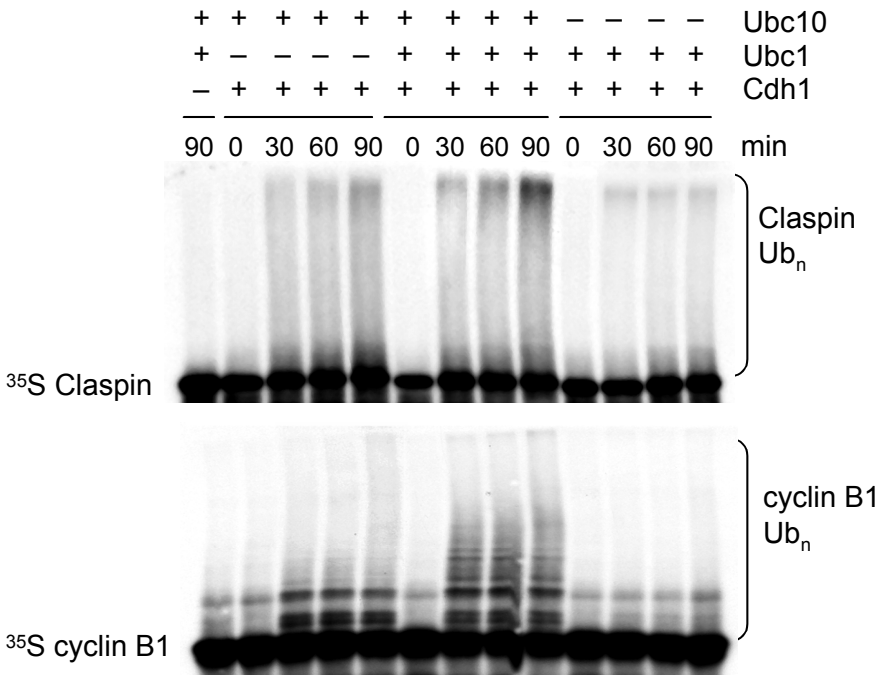
(C) Schematic representation of the *in vivo* binding data shown in (B).

(D) *In vitro* transcribed/translated, ^{35}S-labeled Cdh1 was assayed for *in vitro* binding to the indicated GST-tagged Claspin fragments. Bound Cdh1 was separated by SDS-PAGE and visualized by autoradiography.

(E) CLUSTALW alignment of Claspin orthologs with shading of conserved amino acids. Dark gray: identical residues; light gray: similar residues. Five groups of three amino acids mutated individually in Claspin are framed.

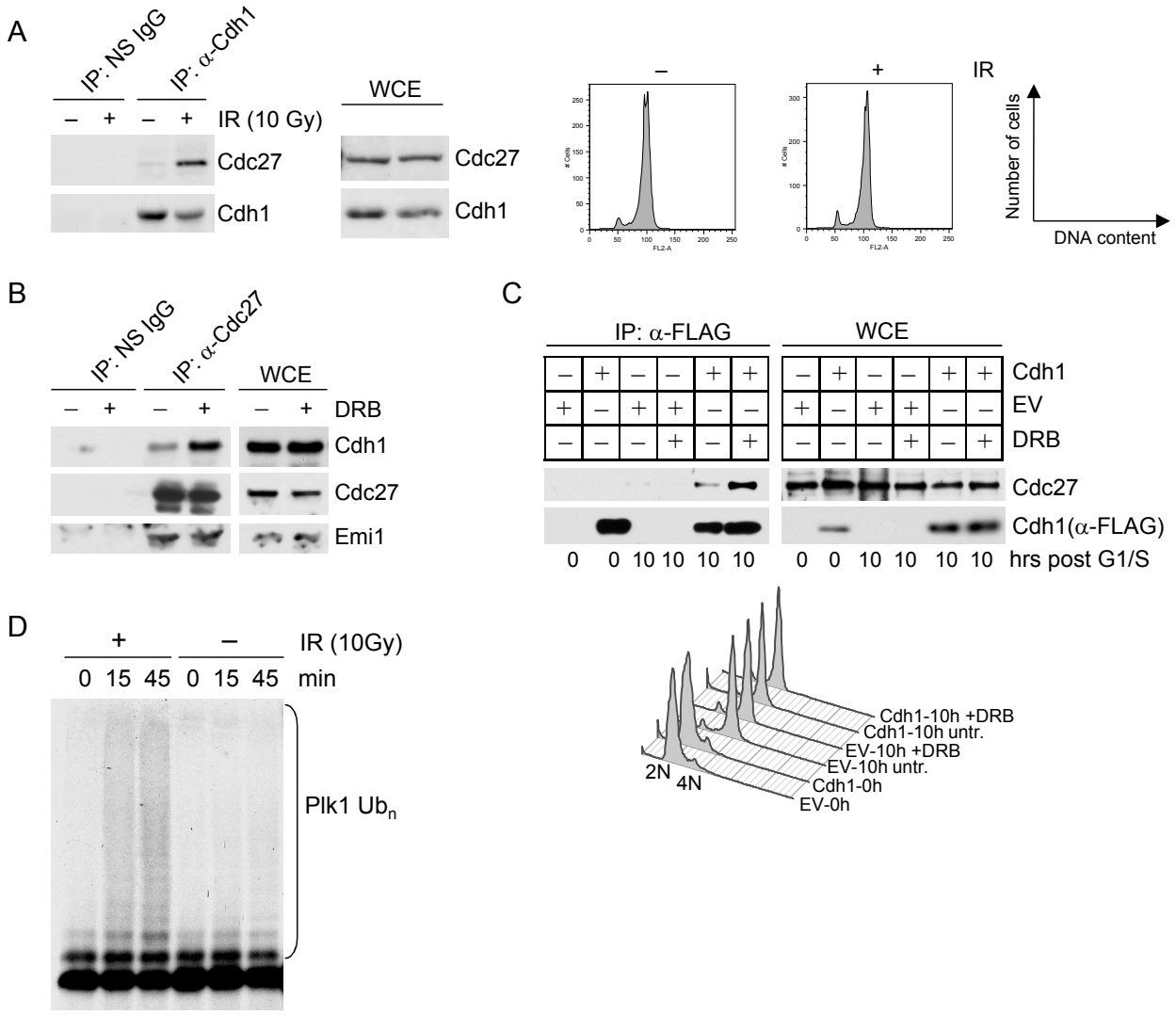
(F) HEK293T cells were cotransfected with HA-tagged Cdh1 and the indicated FLAG-tagged Claspin mutants. Whole cell extracts (WCE, bottom two panels) were immunoprecipitated (IP, upper two panels) with an anti-HA antibody, and the indicated proteins were detected by immunoblotting.

These data show that the Cdh1-binding site in Claspin is not a canonical degron for APC/C substrates (*i.e.* D-box or KEN box motifs). Non-standard degrons have also been described for Aurora A (the A-box) (Littlepage and Ruderman, 2002) and Orc1 (the O-box) (Araki et al., 2005).



Supplementary Figure 3. The N-terminus of Claspin is ubiquitylated in a Cdh1-dependent manner

In vitro ubiquitin ligation assay of ^{35}S-labeled, *in vitro* transcribed/translated Claspin N-terminus (NT, amino acids 1-678) or cyclin B (as a positive control) was conducted in the presence or absence of Cdh1 using different UBCs. Samples were incubated at 30°C and analyzed at the indicated times. The bracket on the right side marks a ladder of bands corresponding to polyubiquitylated proteins. This experiment shows that the ubiquitylation of Claspin is dependent on the presence of Cdh1 and is stimulated by Ubc1 (also called E2-25K), which promotes ubiquitylation of APC/C^{Cdh1} substrates by Ubc10 (Rodrigo-Brenni and Morgan, 2007).



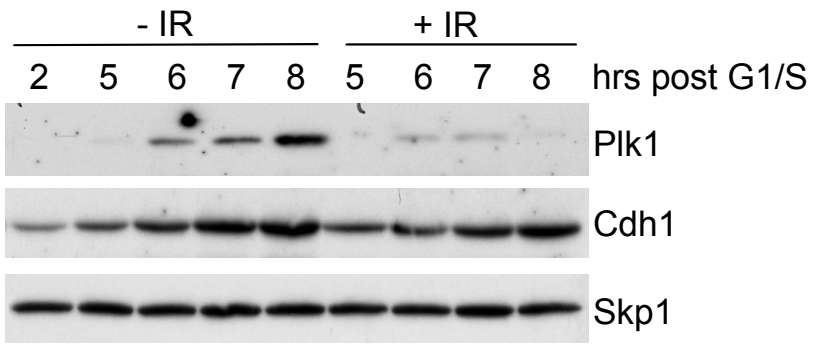
Supplementary Figure 4. Reassociation and activation of APC/C^{Cdh1} upon exposure to genotoxic stress in G2

(A) HeLa-S3 cells were synchronized at G1/S using a double thymidine block and then released to allow progression towards G2. At four hours post-release, cells were either left untreated or irradiated (IR) with 10 Gy before harvesting two hours thereafter. Subsequently, whole cell extracts (WCE) were immunoprecipitated (IP) with either a mouse anti-Cdh1 antibody or purified, non-specific mouse immunoglobulins (NS IgG), and the indicated proteins were analyzed by immunoblotting. DNA content analyses by FACS (right two panels) were performed to ascertain equal cell cycle distribution of the two samples.

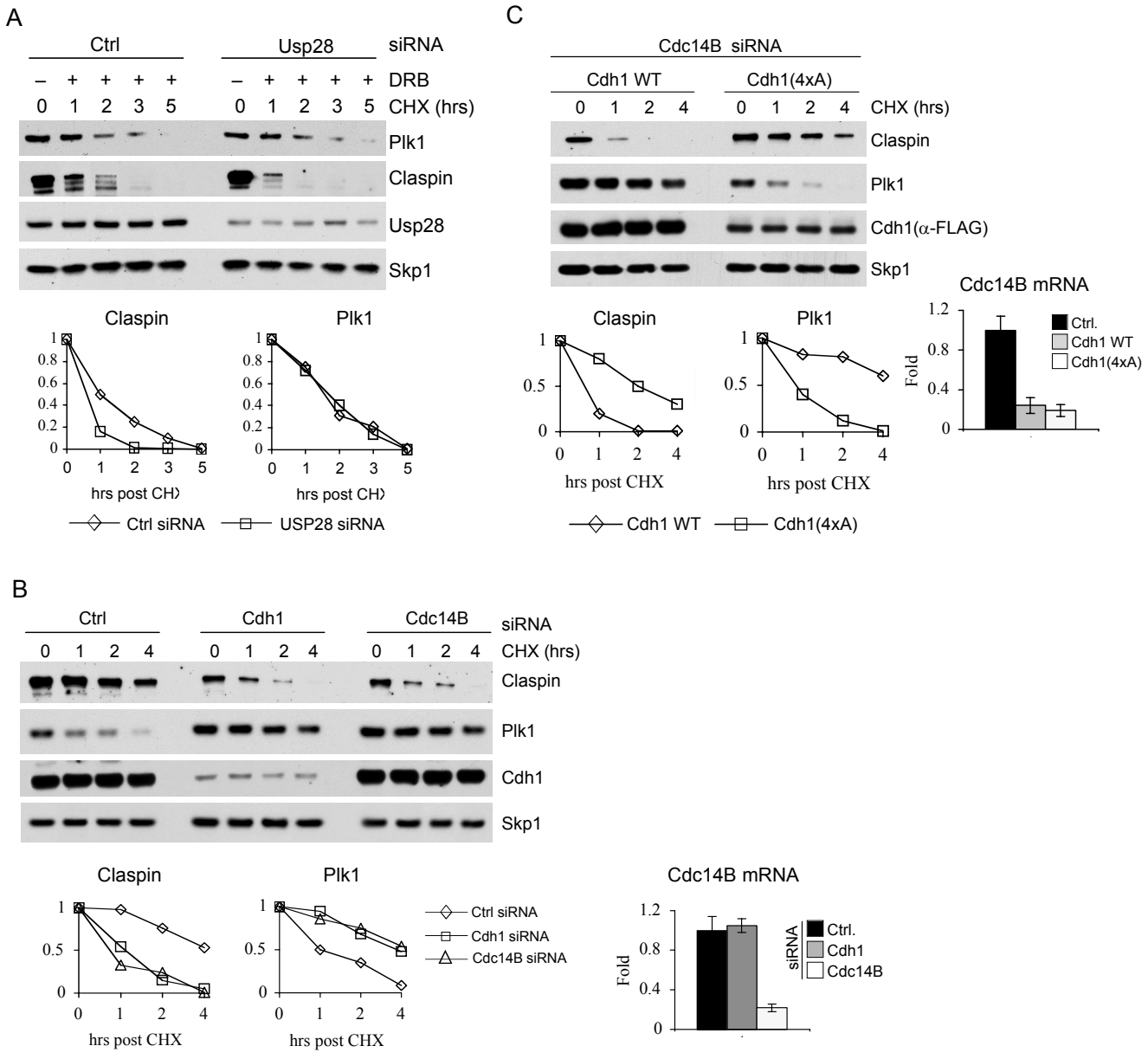
(B) U2OS cells were synchronized at G1/S and then released to allow progression towards G2. At seven hours post-release, cells were pulsed for one hour with either solvent (–) or doxorubicin (DRB) (+) and harvested two hours thereafter. Whole cell extracts (WCE) were immunoprecipitated with a monoclonal anti-Cdc27 antibody or non-specific, purified mouse immunoglobulins (NS IgG). Immunoprecipitates and extracts were immunoblotted with either anti-Cdh1 or anti-Cdc27 antibodies.

(C) U2OS cells infected with either an empty virus (EV) or retroviruses expressing FLAG-tagged Cdh1 were synchronized at G1/S and released as in (B). At seven hours post-release, cells were pulsed for one hour with either solvent or doxorubicin (DRB) and then harvested at the indicated times. Subsequently, whole cell extracts (WCE) were immunoprecipitated (IP) with anti-FLAG resin, and the indicated proteins were analyzed by immunoblotting. DNA content analyses measured by FACS (lower panel) show the cell cycle distribution of the samples.

(D) HeLa-S3 cells were synchronized at G1/S using a double thymidine block and then released to allow progression towards G2. At seven hours post release, cells were either left untreated or irradiated (IR) with 10 Gy before harvesting two hours thereafter. Subsequently, cell extracts were immunoprecipitated with an anti-Cdc27 antibody, and immunoprecipitates were used for *in vitro* ubiquitylation assays using *in vitro* transcribed/translated Plk1 as a substrate. Note that this ubiquitylation reaction relied only on the presence of Cdh1 co-immunoprecipitated with Cdc27 (*i.e.* in the absence of *in vitro* transcribed/translated unlabelled Cdh1, as in all other ubiquitylation assays shown herein) to allow a direct comparison of endogenous APC/C^{Cdh1} under the two different conditions.



Supplementary Figure 5. Decrease of Plk1 levels in response to DNA damage in G2 HeLa-S3 cells were either left untreated or irradiated (IR) with 10 Gy four hours after a release from a G1/S block induced by a double thymidine treatment. Whole cell extracts were harvested at the indicated times, resolved by SDS-PAGE, and immunoblotted with antibodies to the indicated proteins.

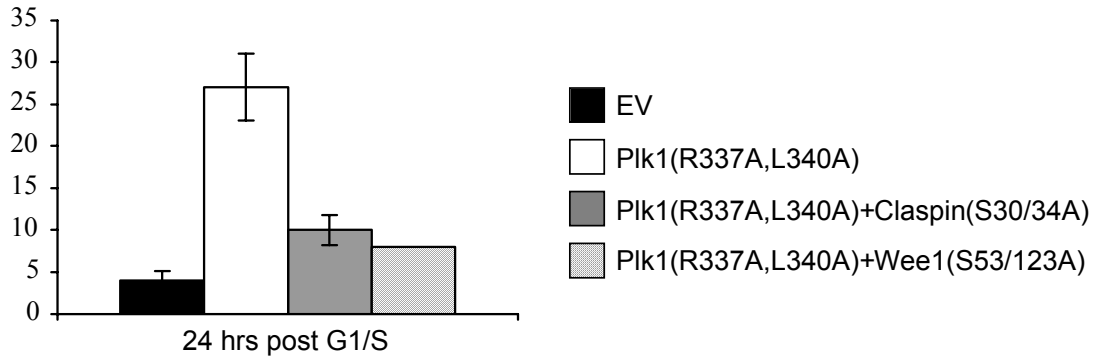


Supplementary Figure 6. Half-life analyses of Plk1 and Claspin under different conditions

(A) Usp28 downregulation destabilizes Claspin but not Plk1. U2OS cells were transfected with the indicated siRNA oligos and synchronized at G1/S using a double thymidine block. Cells were then released from the block to allow progression towards G2. Seven hours post release, doxorubicin (DRB) (+) and cycloheximide (CHX) were added to the cells, which were collected at the indicated times thereafter. Cell lysates were then immunoblotted with antibodies to the indicated proteins. The bottom panels show the quantification of the levels of Plk1 and Claspin shown in top panels.

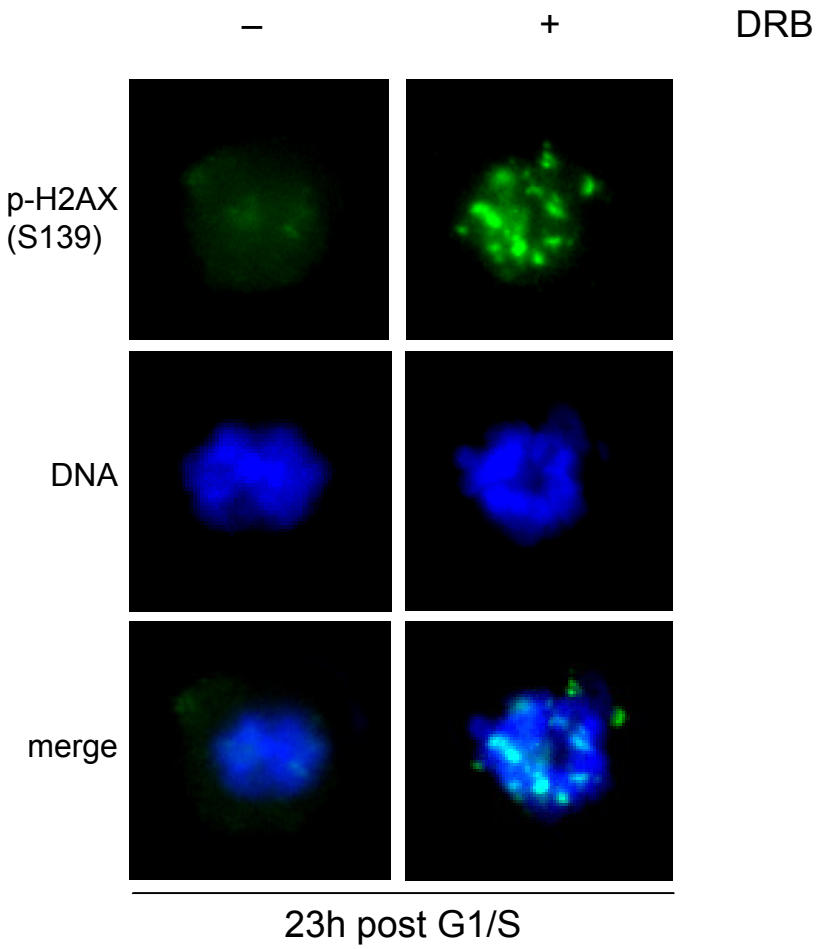
(B) Downregulation of either Cdh1 or Cdc14B results in the stabilization of Plk1 and in the destabilization of Claspin in DNA damaged cells. U2OS cells were transfected with the indicated siRNA oligos and synchronized at G1/S using a double thymidine block. Cells were then released from the block to allow progression towards G2. Seven hours post-release, cells were pulsed for one hour with either solvent (–) or doxorubicin (DRB) (+). Sixteen hours later, cycloheximide (CHX) was added to the culture for the indicated times prior to harvesting. Cell lysates were then immunoblotted with antibodies to the indicated proteins. The bottom panels show the quantification of the levels of Plk1 and Claspin shown in top panels. *Cdc14B* mRNA levels of cells used in top panels were analyzed at the "0 hrs" time point using real time PCR in triplicate measurements (± SD). The value given for the amount of *Cdc14B* mRNA present in cells treated with control oligos was set as 1.

(C) Expression of Cdh1(4xA) does not allow Plk1 stabilization after DNA damage, despite the downregulation of Cdc14B. U2OS cells were retrovirally infected with either wild type Cdh1 or Cdh1(4xA) mutant and subsequently transfected with siRNA oligos directed against *Cdc14B* mRNA. Cells were synchronized at G1/S using a double thymidine block and then released from the block to allow progression towards G2. Seven hours post release, cells were pulsed for one hour with either solvent (–) or doxorubicin (DRB) (+). Sixteen hours later, cycloheximide (CHX) was added to the culture for the indicated times prior to harvesting. Cell lysates were then immunoblotted with antibodies to the indicated proteins. Bottom panels show the quantification of the levels of Plk1 and Claspin shown in top panels. *Cdc14B* mRNA levels of cells used in top panels were analyzed at the "0 hrs" time point using real time PCR in triplicate measurements (± SD). The value given for the amount of *Cdc14B* mRNA present in the sample expressing wild type Cdh1 and treated with control oligos was set as 1.



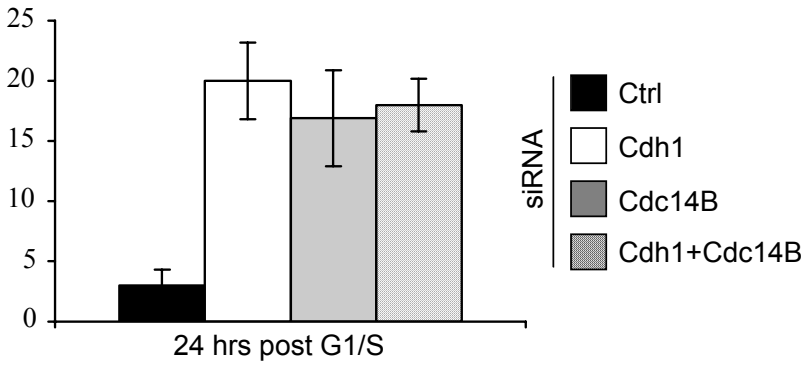
Supplementary Figure 7. Expression of either a stable Claspin mutant or a stable Wee1 mutant prevents Plk1 from bypassing the checkpoint

The experiment was performed as in Fig. 3E, except U2OS cells were also infected with retroviral constructs encoding both Plk1(R337A,L340A) and either a Claspin mutant lacking the βTrcp degron [Claspin(S30/34A)] (Peschiaroli et al., 2006) or a Wee1 mutant lacking the βTrcp degron [Wee1(S53/123A)] (Watanabe et al., 2004). The percentage of mitotic cells was monitored by immunodetection of Histone H3 phosphorylated on Ser10 using flow cytometry (n=3, ± SD for the first three bars and n=2 for the last one).



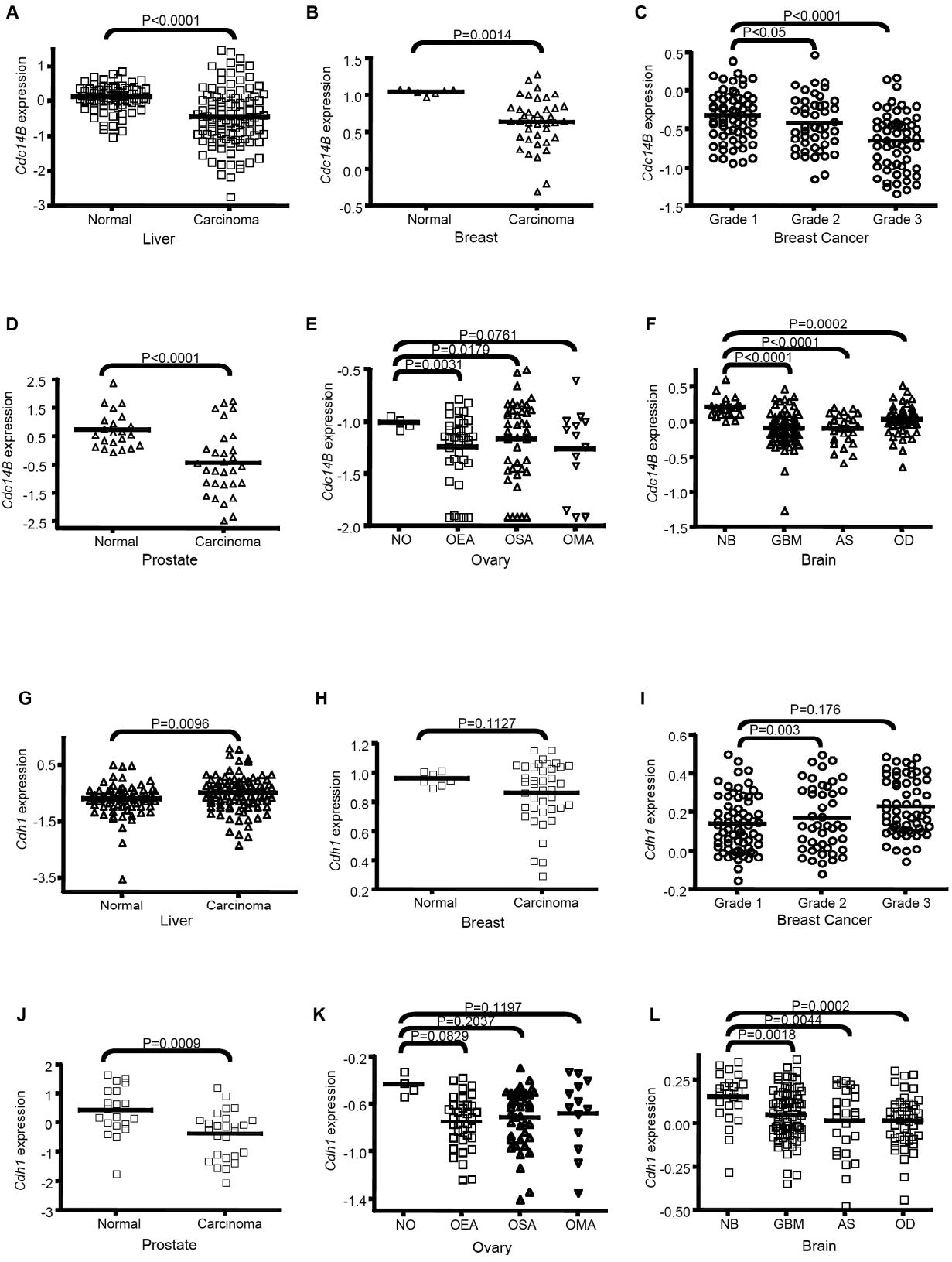
Supplementary Figure 8. Cells expressing stable Plk1 enter mitosis despite the presence of DNA damage

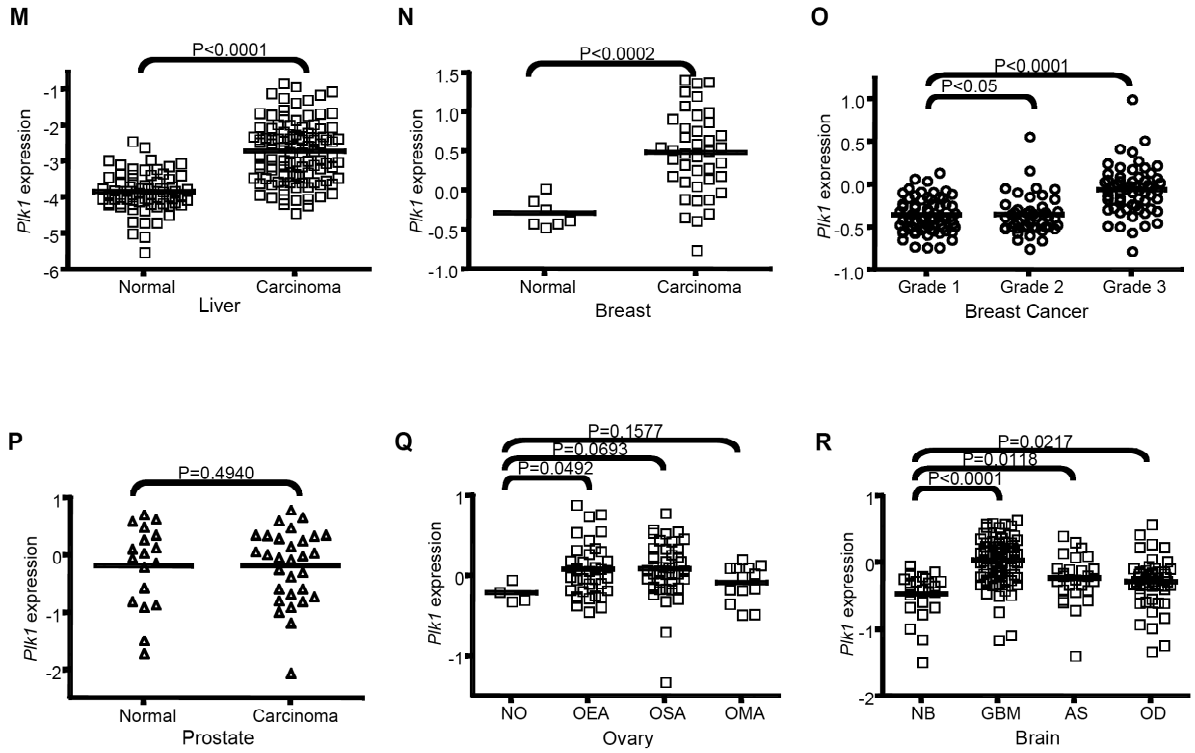
U2OS cells infected with a retrovirus encoding Plk1(R337A,L340A) were synchronized at G1/S using a double thymidine block. Cells were then released from the block to allow progression towards G2. At seven hours post release, cells were pulsed for one hour with either solvent (–) or doxorubicin (DRB) (+). Thereafter, cells were incubated in fresh medium containing nocodazole to trap cells in mitosis. Samples were collected 23 hours after release from G1/S and analyzed by indirect immunofluorescence with an antibody specific for phosphorylated H2AX and DAPI (to visualize condensed DNA). Chromosomes of all cells that entered mitosis after DRB treatment were positive for phospho-H2AX, showing that despite unrepaired DNA lesions cells expressing stable Plk1 reached mitosis.



Supplementary Figure 9. Co-silencing of Cdh1 and Cdc14B is not synergistic in bypassing the checkpoint

The experiment was performed as in Fig. 6C, except that U2OS cells were also transfected with siRNA oligos targeting both *Cdh1* and *Cdc14B*. The percentage of mitotic cells was monitored by immunodetection of Histone H3 phosphorylated on Ser10 using flow cytometry (n=3).


A
P<0.0001
Cdc14B expression
Normal
Carcinoma
Liver
B
P=0.0014
Cdc14B expression
Normal
Carcinoma
Breast
C
P<0.0001
P<0.05
Cdc14B expression
Grade 1
Grade 2
Grade 3
Breast Cancer
D
P<0.0001
Cdc14B expression
Normal
Carcinoma
Prostate
E
P=0.0761
P=0.0179
P=0.0031
Cdc14B expression
NO
OEA
OSA
OMA
Ovary
F
P=0.0002
P<0.0001
P<0.0001
Cdc14B expression
NB
GBM
AS
OD
Brain
G
P=0.0096
Cdh1 expression
Normal
Carcinoma
Liver
H
P=0.1127
Cdh1 expression
Normal
Carcinoma
Breast
I
P=0.176
P=0.003
Cdh1 expression
Grade 1
Grade 2
Grade 3
Breast Cancer
J
P=0.0009
Cdh1 expression
Normal
Carcinoma
Prostate
K
P=0.1197
P=0.2037
P=0.0829
Cdh1 expression
NO
OEA
OSA
OMA
Ovary
L
P=0.0002
P=0.0044
P=0.0018
Cdh1 expression
NB
GBM
AS
OD
Brain



Supplementary Figure 10. mRNA levels of *Cdc14B, Cdh1, and Plk1* in different human tumors

All data was provided by the *Oncomine* database. The associated *P* values are shown for each study.

(A, G, M) Data from (Chen et al., 2002) reanalyzed to show expression levels of *Cdc14B* (A*)*, *Cdh1* (G), and *Plk1* (M) in non-tumor liver (Normal) and hepatocellular carcinoma (Carcinoma).

(B, H, N) Data from (Richardson et al., 2006) reanalyzed to show expression levels of *Cdc14B* (B*)*, *Cdh1* (H), and *Plk1* (N) in normal breast (Normal) and breast carcinoma (Carcinoma).

(C, I, O) Data from (Sotiriou et al., 2006) reanalyzed to show expression levels of *Cdc14B* (C*)*, *Cdh1* (I), and *Plk1* (Q) in Grade 1, Grade 2, and Grade 3 breast cancers.

(D, J, P) Data from (Tomlins et al., 2007) reanalyzed to show expression levels of *Cdc14B* (D*)*, *Cdh1* (J), and *Plk1* (P) in benign prostate (Normal) and prostate carcinoma (Carcinoma).

(E, K, Q) Data from (Hendrix et al., 2006) reanalyzed to show expression levels of *Cdc14B* (E*)*, *Cdh1* (K), and *Plk1* (Q) in normal ovary (NO), ovarian endometrioid adenocarcinoma (OEA), ovarian serous adenocarcinoma (OSA), and ovarian mucinous adenocarcinoma (OMA).

(F, L, R) Data from (Sun et al., 2006) reanalyzed to show expression levels of *Cdc14B* (F), *Cdh1* (L), and *Plk1* (R) in normal brain (NB), glioblastoma multiform (GBM), astrocytoma (AS), and oligodendroglioma (OD).