Gain-of-function mutations of PPM1D/Wip1 impair the p53-dependent G1 checkpoint

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Introduction

Proliferating cells respond to genotoxic stress by activating a conserved DNA damage response (DDR) pathway that blocks cell cycle progression after genotoxic stress and represents an intrinsic barrier preventing cancer development. The serine/threonine phosphatase PPM1D/Wip1 inactivates p53 and promotes termination of the DDR pathway. Wip1 has been suggested to act as an oncogene in a subset of tumors that retain wild-type p53. In this paper, we have identified novel gain-of-function mutations in exon 6 of PPM1D that result in expression of C-terminally truncated Wip1. Remarkably, mutations in PPM1D are present not only in the tumors but also in other tissues of breast and colorectal cancer patients, indicating that they arise early in development or affect the germline. We show that mutations in PPM1D affect the DDR pathway and propose that they could predispose to cancer.

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and can thus promote further development of cancer. The most common example of such DDR defect is an inactivating somatic mutation in the TP53 gene that disables proper response to genotoxic stress, leads to genomic instability, and is found in about half of human tumors (Hollstein et al., 1991). On the other hand, tumors that retain wild-type p53 are likely to accumulate other genetic defects that would allow them to overcome the DDR barrier, providing a growth advantage in the presence of replicative stress. Importantly, amplification of the 17q23 locus carrying the PPM1D gene has been reported in various p53 wild-type tumors, pointing toward a role of Wip1 in cancer development, and Wip1 overexpression is associated with poor prognosis (Bulavin et al., 2002, 2004; Li et al., 2002; Saito-Ohara et al., 2003; Rauta et al., 2006; Castellino et al., 2008; Liang et al., 2012). The oncogenic behavior of Wip1 is further supported by mouse genetics showing that loss of Wip1 protects from cancer development (Bulavin et al., 2004; Nannenga et al., 2006). However, point mutations that affect Wip1 function have not been reported. Here, we have identified novel truncating mutations of Wip1 that show a gain-of-function effect and impair p53-dependent responses to genotoxic stress. Strikingly, mutations in the PPM1D gene were found also in breast and colorectal cancer patients, suggesting that such truncating mutations of Wip1 may predispose to a wider range of cancer types.

Results and discussion

Because amplification of the PPM1D gene occurs mainly in tumors that retain the wild-type p53, we have screened a panel of p53-proficient tumor cell lines to determine the expression level of Wip1 in tumors derived from various tissues (Bulavin et al., 2002; Rauta et al., 2006). Predictably, we could confirm high expression of Wip1 in MCF7 cells that are known to carry an extensive amplification of the PPM1D locus (Fig. 1 A; Pärssinen et al., 2008). All other tested cell lines expressed substantially lower amounts of full-length (FL) Wip1. Surprisingly, we noticed an abundant, faster migrating band, recognized by two distinct Wip1 antibodies in HCT116 and U2OS cells derived from colorectal adenocarcinoma and osteosarcoma, respectively (Fig. 1, A and B). Notably, antibodies recognizing an epitope corresponding to the amino acid residues 380–410 of Wip1 stained both bands, whereas an antibody directed against an epitope corresponding to the amino acid residues 500–550 of Wip1 recognized only the slower migrating band (Figs. 1 A and S1 A). In addition, both bands were depleted by three independent Wip1 siRNAs, indicating that the two protein bands correspond to various forms of Wip1 (Fig. S1 B). Consistent with this, sequencing of genomic DNA revealed heterozygous mutations (c.1349delT and c.1372C>T) within exon 6 of the PPM1D gene that caused truncation of the Wip1 protein (p.L450X in HCT116 and p.R458X in U2OS cells; Fig. 1 C). Expression of both the FL and truncated versions of Wip1 in U2OS and HCT116 cells was further confirmed by immunopurification of Wip1 and subsequent mass spectrometry (MS) analysis (Fig. S1, C and D). Importantly, epitope-tagged versions of truncated Wip1 proteins expressed from plasmid DNA showed electrophoretic mobility that closely resembled that of the aberrant endogenous Wip1 proteins (Fig. S1 E).

To understand whether truncation of Wip1 affects its function in the DDR, we asked whether the respective truncation mutants were capable of suppressing formation of ionizing radiation (IR)–induced foci (IRIF) as has been described for FL Wip1 (Macăreş et al., 2010). Both, Wip1-L450X and Wip1-R458X localized properly in the nucleus and were bound to the chromatin, suggesting that overall subcellular distribution of Wip1 is not affected by the exon 6 truncations (Fig. 2 A). As expected, overexpression of FL-Wip1 resulted in a reduction in IRIF formation, as determined by the number of 53BP1 foci induced after irradiation (Fig. 2 B). Similarly, overexpression of Wip1-L450X and Wip1-R458X (but not phosphatase-dead Wip1-D314A) also caused a dramatic reduction in IRIF formation, suggesting that the mutants retain normal phosphatase activity that opposes IRIF assembly (Fig. 2 B). In addition, expression of FL-Wip1, Wip1-L450X, and Wip1-R458X significantly decreased levels of radiation-induced phosphorylation of histone H2AX (γ-H2AX) and pSer15-p53 (both established markers of DDR and substrates of Wip1; Lu et al., 2005; Macăreş et al., 2010), suggesting that all tested Wip1 proteins are capable of silencing the DDR (Fig. 2 C). Wip1 is a monomeric phosphatase, and because all identified truncating mutations reside in the C-terminal region of Wip1 leaving the N-terminal catalytic domain intact, we hypothesized that the truncation mutants retain phosphatase activity. Indeed, immunopurified FL-Wip1, Wip1-L450X, and Wip1-R458X showed comparable phosphatase activity in vitro, and therefore, it is unlikely that mutation of PPM1D leads to production of a hyperactive Wip1 (Fig. 2 D).

Because mutations in oncogenes are expected to cause a gain-of-function effect, we wondered whether the C-terminal region coded by exon 6 reduces the stability of Wip1. Consistent with this notion, we found that both U2OS and HCT116 cells expressed ~10–20-fold more of the truncated Wip1 compared with the FL-Wip1 (Fig. 2 E). In contrast, no substantial differences between wild-type and mutated Wip1 expression were found at the mRNA level, indicating that these are not differentially regulated at the transcriptional level and that the high levels of the mutant Wip1 proteins reflect enhanced protein stability (Fig. S1 F). Indeed, the FL-Wip1 disappeared rapidly after treatment of cells with cycloheximide (half-life of 1–2 h), whereas both truncated mutants exhibited enhanced stability (half-life of >6 h; Fig. 3, A and B). In addition, treatment with the proteasomal inhibitor MG-132 reversed the effect of the cycloheximide on destabilization of the FL-Wip1 (Fig. 3 C). This suggests that the C-terminal domain of Wip1 somehow renders the FL protein unstable and that its turnover is regulated by the proteasome. To further corroborate this notion, we fused the C-terminal noncatalytic part of Wip1 (aa 380–605) to GFP and analyzed its effect on protein stability. Similar to what we observed for FL-Wip1, we found that the GFP fusion containing the C-terminal tail of Wip1 was less stable than GFP itself (Fig. 3 D). From this, we conclude that nonsense mutations in exon 6 of the PPM1D gene lead to increased protein levels of enzymatically active truncated Wip1 and thus result in increased total Wip1 activity in cells.
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Conclusion that expression of the truncated variant of Wip1 abrogates the G1 checkpoint (Fig. 4 B). As an alternative approach, we followed the progression from G1 to S phase by time-lapse analysis of living HCT116 cells expressing fluorescent markers to monitor cell cycle progression (Fig. 4 C). HCT116 cells treated with control siRNA were delayed in G1 after irradiation (Fig. 4 C), consistent with previous observations that degradation of Cdc25A and Cyclin D1 can delay S-phase entry in a p53-independent manner (Agami and Bernards, 2000; Mailand et al., 2000). However, the majority of control cells eventually entered S phase (Fig. 4 C). In contrast, HCT116 cells depleted of Wip1 mounted a lasting G1 checkpoint arrest (Fig. 4 C). In accordance with restoration of p53 function upon depletion of Wip1, we observed increased levels of p21 after exposure to IR in U2OS and HCT116 cells treated with Wip1 RNAi (Fig. 4, D and E).

Despite the fact that U2OS and HCT116 contain wild-type p53, they fail to arrest in G1 in response to IR and preferentially arrest in the G2 checkpoint that remains intact (Fig. S2, A and B). This is reminiscent of the behavior of cells lacking p53 or expressing mutant p53 and suggests that the p53 pathway is somehow compromised in U2OS and HCT116 cells. To test whether this may be caused by enhanced Wip1 levels and/or activity, we depleted Wip1 by RNAi and measured the ability of cells exposed to IR to arrest in G1. Indeed, we observed that U2OS cells did arrest in G1 after depletion of Wip1 and exposure to IR (Figs. 4 A and S2 C). Moreover, this arrest was fully dependent on p53 because codepletion of Wip1 and p53 or depletion of Wip1 in p53-negative cell lines SW480, DLD1, and HT29 did not restore any G1 checkpoint function (Fig. 4 A and not depicted). In addition, depletion of the truncated Wip1 (but not of the FL-Wip1) by isoform-specific RNAi was sufficient to rescue the G1 arrest in irradiated U2OS cells, thus further supporting the conclusion that expression of the truncated variant of Wip1 abrogates the G1 checkpoint (Fig. 4 B). As an alternative approach, we followed the progression from G1 to S phase by time-lapse analysis of living HCT116 cells expressing fluorescent markers to monitor cell cycle progression (Fig. 4 C). HCT116 cells treated with control siRNA were delayed in G1 after irradiation (Fig. 4 C), consistent with previous observations that degradation of Cdc25A and Cyclin D1 can delay S-phase entry in a p53-independent manner (Agami and Bernards, 2000; Mailand et al., 2000). However, the majority of control cells eventually entered S phase (Fig. 4 C). In contrast, HCT116 cells depleted of Wip1 mounted a lasting G1 checkpoint arrest (Fig. 4 C). In accordance with restoration of p53 function upon depletion of Wip1, we observed increased levels of p21 after exposure to IR in U2OS and HCT116 cells treated with Wip1 RNAi (Fig. 4, D and E).

We conclude that cells with mutations that enhance Wip1 protein stability are unable to engage p53 function, fail to arrest

Figure 1. The PPM1D gene is mutated in selected cancer cell lines. (A) Whole-cell lysates from indicated cell lines were probed with anti-Wip1 (Bethyl Laboratories, Inc.), anti-Wip1 (Santa Cruz Biotechnology, Inc. [SC]), and anti–14-3-3 (loading control) antibodies. Note the additional Wip1-reactive band around 60 kD in U2OS and HCT116 cells. PC3 cells are p53 negative and served as a control. (B) Wip1 was depleted in U2OS and HCT116 cells by siRNA, and lysates were probed with the indicated antibodies. (C) Sequencing chromatograms of PPM1D from genomic DNA isolated from U2OS and HCT116 cells. Numbering is based on NCBI GenBank reference sequence NT_010783.1.5. Mutations are indicated by arrowheads and underlined. WT, wild-type PPM1D; Mut, mutated PPM1D; c., nucleotide sequence; p., Wip1 peptide sequence.
truncated Wip1 may also directly cause accumulation of mutations through the described negative role of Wip1 in regulation of nucleotide excision repair (Nguyen et al., 2010). All these mechanisms may contribute to the elimination of the intrinsic DDR-mediated barrier against tumor development in cells carrying gain-of-function mutations of Wip1.

Finally, we wished to address the clinical relevance of the identified Wip1 mutations. We therefore performed mutational analysis of Wip1 in G1 after DNA damage, and progress to S phase. Thus, the genome integrity of cells expressing truncated Wip1 versions may be compromised by replication of the genome that contains unrepaired DNA lesions, including the highly pro-oncogenic DNA double-strand breaks. Increased expression of truncated Wip1 impairs the cellular responses to genotoxic stress also via a reduction in H2AX phosphorylation, which is an established substrate of Wip1. In addition, it is likely that high levels of truncated Wip1 may also directly cause accumulation of mutations through the described negative role of Wip1 in regulation of nucleotide excision repair (Nguyen et al., 2010). All these mechanisms may contribute to the elimination of the intrinsic DDR-mediated barrier against tumor development in cells carrying gain-of-function mutations of Wip1.

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Figure 3. **Truncated Wip1 mutants show increased protein stability.** (A) HCT116 and U2OS cells were treated for the indicated times with cycloheximide. Normalized cell lysates were probed using the monoclonal anti-Wip1 antibody (Santa Cruz Biotechnology, Inc. [SC]). (B) Signal intensity corresponding to the FL-Wip1 and truncated Wip1 from A was quantified using ImageJ software. The relative change in signal intensity is shown. Statistical significance was determined by unpaired two-tailed t test (n = 3; *, P < 0.05). (C) U2OS cells were treated with cycloheximide, proteasomal inhibitor MG132 or a combination of both for the indicated times and analyzed as in A. (D) U2OS cells were transfected with plasmid DNA coding for EGFP or EGFP fused to the C-terminal region of Wip1 (aa 380–605). Cells were treated for the indicated times with cycloheximide. Normalized cell lysates were probed with an anti-GFP antibody, and the signal intensity was quantified using ImageJ software. n = 3. Error bars indicate standard deviations. exp., exposure.
Figure 4. Mutated Wip1 impairs the p53-dependent G1 checkpoint. (A) U2OS cells were transfected by siRNA targeting luciferase, Wip1 and/or p53 and grown for 48 h. Cells were treated with BrdU and STLC, irradiated or not irradiated with 4 Gy, and grown for a further 16 h. Cells were analyzed by immunoblotting (top) or by flow cytometry (bottom) to determine the fraction of G1 cells (2n, BrdU negative). (B) U2OS cells were transfected by
analysis of the PPM1D gene in a panel of unselected colorectal cancer patients (n = 304) and a panel of high-risk patients with BRCA1/2-negative breast and ovarian cancer (n = 728) and identified four deleterious mutations in exon 6 (c.1372C>T and c.1602insT in patients with colorectal cancer and c.1601delT and c.1451T>G in patients with breast cancer) compared with no such mutations present in noncancer control samples (n = 450; Fig. 5 A and Table S1). All identified Wip1-truncating mutations (p.R458X, p.L484X, p.K535X, and p.F534X) and showed a striking similarity to mutations identified in the tumor cell lines. Functional analysis of all Wip1 mutants present in cancer patients confirmed that these truncations retain the enzymatic activity as well as the ability to bind to chromatin (Fig. 5, B and C; and not depicted). In addition, we analyzed protein levels of Wip1 in leukocytes in one of the mutant carriers and found that the truncated Wip1 is expressed at a much higher level than the FL-Wip1, thus phenocopying the situation in tumor cell lines (Fig. 5 D). Finally, we analyzed a noncancer mammary tissue and tumor tissue from one mutation carrier and identified a heterozygous mutation in the tumor tissue (Fig. 5 E).

Remarkably, all truncating mutations identified in the PPM1D gene in patients and cancer cell lines were heterozygous gain-of-function mutations, which is consistent with the role of Wip1 as an oncogene. Of note, alterations in cancer patients were identified in peripheral blood samples, excluding the possibility that these mutations arise in the developing tumor simply as a consequence of genetic instability. The targeting to a discrete hot-spot region in the exon 6 of the PPM1D oncogene—their gain-of-function character proven by in vitro experiments—the variable onset of cancer in affected individuals, and the versatile spectrum of cancer types appearing in mutation carriers and cancer cell lines all indicate that mutations in PPM1D may represent an unusual and novel genetic risk factor of general cancer predisposition not associated with a single specific cancer type.

Although the majority of hereditary cancers is caused by mutations in tumor suppressor genes, germline mutations in oncogenes are not unprecedented (Knudson, 2002). For example, germline mutations of oncogenic tyrosine kinases RET, MET, and KIT are linked with medullary thyroid carcinoma, hereditary papillary renal carcinoma, and hereditary gastrointestinal stromal tumor syndrome, respectively (Mulligan et al., 1993; Schmidt et al., 1997; Nishida et al., 1998). Whereas tumor development is substantially boosted by inactivation of the second allele of the tumor suppressor genes, monoallelic gain-of-function mutations are usually sufficient to activate oncogenes (Vogelstein and Kinzler, 2004). In agreement with this, identified mutations of PPM1D in the tumor cell lines were heterozygous, and both wild-type and truncated PPM1D alleles were expressed. We propose that the high expression level of truncated Wip1 impairs the p53-dependent genome surveillance system in mutation carriers, making their genomic DNA hypersensitive to various genotoxic insults. By this mechanism, mutations in other tumor-promoting genes may accumulate throughout the entire life span of the PPM1D mutation carriers and promote cancer development. The clinical significance of truncating PPM1D mutations in predisposition to breast and ovarian cancer was recently documented by an extensive case control study (Ruark et al., 2013). Further studies are needed to address the possibility that mutations in PPM1D may represent a hereditary cancer predisposition and that truncated Wip1 might be a suitable candidate for pharmacological intervention in cancer patients carrying PPM1D mutations.

Materials and methods

Antibodies

Antibodies used were rabbit anti-Wip1 (H300), mouse anti-Wip1 (F-10), anti-p53 (DO1), and anti-p21 (C19), and anti-S3BP1 (Santa Cruz Biotechnology, Inc.); anti-PPM1D (A300-664A, Bethyl Laboratories, Inc.); anti-pSer15p53, anti-p53, and anti-pSer139-H2AX (Cell Signaling Technology); anti-α-tubulin and anti-FLAG (Sigma-Aldrich); anti-Brdu (clone BU1/75; Abcam); and Alexa Fluor-conjugated secondary antibodies (Life Technologies).

Plasmids

DNA fragments coding for FL-tagged human FL or truncated (R458X, L450X, and F534X) Wip1 were generated by PCR and subcloned into BamHI–XbaI sites of the pcDNA4/TO plasmid. Alternatively, coding sequence for EGFP was inserted in HindIII site of pcDNA4/TO and FL, or truncated Wip1 was cloned in frame in BamHI–XbaI sites.

Cell culture

Human U2OS, HCT116, LoVo, MCF7, Hela, SH-SY5Y, and PC3 or non-tumor diploid retinal pigment epithelium cells were grown in DMEM (Gibco) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Tetracycline repressor-expressing U2OS-TR cells were grown in media containing tetracycline system-approved FCS, and protein expression was induced by tetracycline. A stable cell line expressing the fluorescent, ubiquitination-based cell cycle indicator (FUCCI) was engineered by transfection of HCT116 cells with pCSIEF-MCS-MKO2-2 (c10) [30–120] and pCSIEF-MCS-MAG-hGeminin[1–110] plasmids (Sakase-Sawano et al., 2008) followed by FACS sorting of double-positive cells from which a single clone was expanded. Transfections of plasmid DNA were performed using a standard calcium phosphate technique. ON-TARGETplus siRNAs targeting Wip1 (5'-GGCGAAGGGGUAGAUCUA-3'; 5'-CGAAUGGCUAAAGUCCGAA-3'; 5'-AGUAAAGUGCCGAAUCCGAA-3'; and 5'-AUAGGUGGACUGAUGCUC-3') and control siRNAs (Thermo Fisher Scientific) were transfected (5–10 nM) using RNAiMAX (Invitrogen). Alternatively, isoform-specific siRNAs targeting the FL-Wip1 (5'-AUAGCUCAGGAGAACUGCC-3') or the 458X-Wip1 (5'-AUAGCUCAGGAGAACUGCC-3') were used.

Immunofluorescence and microscopic analysis

Cells cultured on glass coverslips were left untreated or exposed to IR (dose 3–5 Gy as indicated) and fixed at the indicated times by 4% formaldehyde for 10 min at RT, permeabilized by ice-cold methanol, blocked with 3% BSA in PBS supplemented with 0.1% Tween 20, and incubated with the primary antibodies 60 min at RT. After washing, coverslips were
Figure 5. **Truncation mutations of Wip1 are present in cancer patients.** (A) Chromatograms of four truncating mutations identified by screening of the *PPM1D* gene in cancer patients. Mutations are indicated by arrowheads and underlined. WT, wild-type *PPM1D*; Mut, mutated *PPM1D*; c., nucleotide sequence; p., Wip1 peptide sequence. (B) Cells expressing EGFP–Wip1-FL, -R458X, -L484X, -F534X, or -K535X mutants were irradiated, and the number...
incubated with Alexa Fluor-conjugated secondary antibodies and mounted using Vectashield reagent (Vector Laboratories) supplemented with 1 μg/ml DAPI. Imaging was performed on a DeltaVision Imaging System using NA 1.4 objectives (Applied Precision). Automated image acquisition was performed using a high-content screening system (ScanR; Olympus; using charge-coupled device camera [IX81 and ORCA-2; Olympus]) equipped with a 40×/1.3 NA objective (RSM40XPTO, Olympus). Nuclei were identified based on the DAPI signal, and the average number of S3BP1 foci was determined using a spot detection module. At least 1,000 nuclei were counted per condition in three independent experiments. Cells transiently transfected with Fl- or mutant EGFP-Wip1 were gated according to the EGFP signal, and neighboring EGFP-negative cells were used as controls. Alternatively, HCT116-FUCCI cells were grown at 37°C in Lab-Tek II chamber slides in L15 media (Gibco) containing all supplements. Cells were irradiated or not irradiated, and videos were acquired using DeltaVision system equipped with a 10×/0.4 NA U-Plan S-Apochromat objective (Olympus), a camera (Coolsnap HQ2; Photometrics), Quad-Mcherry polychrome filter, and mCherry/EGFP emission filter sets. Nuclei negative for Geminin-mAG1 (corresponding to S) detectable over background in two consecutive frames (15 min).

**Immunoprecipitation and in vitro phosphatase assay**

U2OS cells were extracted by EBC buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, and 1 mM E6TA) followed by sonication (3 × 10 s) and spinning down (20,000 g for 10 min). Polyclonal anti-Wip1 (H300; 2 μg/ml) in ice-cold buffer (30 μl protein A/G resin [UltraLink; Thermo Fisher Scientific]) and with cell extracts for 4 h at 4°C. For specificity controls, cell extract was incubated with empty beads, and alternatively immobilized antibody was not incubated with cell extract. After extensive washing, beads were mixed with SDS sample buffer and boiled. Immunoprecipitates were probed with polyclonal anti-Wip1 (Bethyl Laboratories, Inc.) and monoclonal anti-Wip1 (Santa Cruz Biotechnology, Inc.). Alternatively, the gel was stained by Coomassie brilliant blue, and immunoprecipitated bands were subjected to MS analysis. Normalized cell extracts from cells expressing Fl or truncated FLAG-Wip1 were incubated with M2 agarose (Sigma-Aldrich). In vitro phosphatase assay was performed in a phosphatase buffer (40 mM Hepes, pH 7.4, 100 mM NaCl, 50 mM KCl, 1 mM EDTA, and 50 mM MgCl2) supplemented with 100 μM VEPP1pSQETF synthetic phosphopeptide (GenScript). Release of phosphate was measured after incubation at 30°C for 20 min using BIOMOL Green reagent (Enzo Life Sciences) and was described previously (Macère et al., 2010). Finally, beads containing all immunoprecipitated material were separated by SDS-PAGE and probed for Wip1.

**Subcellular fractionation**

Soluble and chromatin fractions were prepared as previously described (Macère et al., 2010). In brief, soluble cytosolic proteins were extracted from U2OS cells by incubating cells in buffer A (10 mM Hepes, pH 7.9, 10 mM KC1, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, and protease inhibitor cocktail) at 4°C for 10 min and spinning down at 20,000 g for 2 min. Soluble nuclear fraction was obtained by extraction of cell nuclei with an equal amount of buffer B (10 mM Hepes, pH 7.9, 3 mM EDTA, 0.2 mM EGTA, and 1 mM DTT) and spinning down at 20,000 g for 2 min. Both soluble fractions were mixed (1:1). Insoluble chromatin was washed with buffer B and finally resuspended in SDS sample buffer.

**Flow cytometry analysis**

Asynchronous cells were transfected with indicated siRNAs (20 nM) and incubated for 2 min. Both soluble fractions were mixed (1:1). Insoluble chromatin was washed with buffer B and finally resuspended in SDS sample buffer.

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Alexa Fluor-conjugated secondary antibodies. DNA was stained with propidium iodide. Flow cytometry was performed on a cytometer (FACSCalibur; BD), and single cells were analyzed with CellQuest software (BD). As STLC inhibits mitotic kinesin Eg5, cells that progress into mitosis remain arrested in mitosis. This allows identification of cells that were in G1 at the time of IR and remained arrested in the G1 checkpoint (2n DNA content, BrdU mm²). Cells that were in G1/S and progressed to G2 (4n DNA content, BrdU mm²) that were in G1/S and progressed to mitosis (4n DNA content, BrdU mm²), cells that were in G2 and remained arrested in the G2 checkpoint (4n DNA content, BrdU mm²), and cells that were in G2 and progressed to mitosis or were in mitosis at the start of the experiment (4n DNA content, BrdU mm²). The 4n BrdU-negative populations were used to exclude differences in cell cycle distribution at the time of irradiation. Wt populations were used for quantification of cells remaining in G1. Alternatively, cells were pulsed with 10 μM BrdU (15 min) before irradiation, and BrdU-positive cells were assayed for progression through the G2 phase by FACS analysis of the DNA content (Chen et al., 2001).

**Protein stability assay**

Cells treated for the indicated times with 50 μg/ml cycloheximide were lysed, and equal amounts of protein were separated on 4–12% Bis-Tris precast gels (NuPAGE; Life Technologies) and probed with the indicated antibodies. Where indicated, cells were treated with 5 μg/ml DMSO or MG-132. Unsaturated films were scanned at 600 dpi as 16-bit grayscale TIFF formatted images. The densitometry analysis was performed using Image software (National Institutes of Health). No image adjustments were made before the analysis. Signal intensities were normalized to the loading control from the same gel.

**MS**

Wip1 was immunoprecipitated from U2OS or Hela cells using a monoclonal anti-Wip1 antibody (Santa Cruz Biotechnology, Inc.; 2 μg/reaction) immobilized on protein A/G UltraLink resin. Samples were separated by SDS-PAGE and stained by protein stain (GelCode; Thermo Fisher Scientific). Proteins corresponding to both forms of Wip1 were digested in gel by trypsin (Promega) and analyzed by peptide mass fingerprinting (9.4T ApexQc; Bruker Daltonics). Mass spectra were analyzed and interpreted using DataAnalysis 4.0 and BioTools 3.2 software (Bruker Daltonics).

**Genomic DNA isolation**

Genomic DNA was isolated from peripheral blood of high-risk, BRCA1/2-negative, familial and/or early onset breast (n = 280) (avarian = 50 cancer patients, selected colorectal cancer patients (n = 304), and noncancer controls (n = 450) was described previously (Pohlreich et al., 2005; Kleib et al., 2009; Ticho et al., 2010). All patients and controls were Caucasians of the Czech descent that gave written informed consent with the genetic testing approved by local ethics committees. PCR amplifications covering all PPM1D exons with flanking intronic sequences were obtained using PCR master mix (High Resolution Melting; Roche) and a real-time PCR system (LightCycler 480; Roche). PCR amplification was performed using the following sets of primers: 5′-GGGAGCCGCCTG-TGTCTCC-3′ and 5′-GCCGCAAACAAGCCAGGGAAC-3′ (exon 1); 5′-GGTGGCATTGTAATCGACCAGTG-3′ and 5′-CTACGTAATAGCTG-3′ (exon 2); 5′-CCAGGATTTGTGCAATC-3′ and 5′-AGGATCCCAGTGAGGCA-3′ (exon 3); 5′-CTTTGCTGTGTT-CATCTTTAGCCTCC-3′ and 5′-TCCCAAATCCACCAAGGGGA-3′ (exon 4); 5′-GATAACGATGTAGTGTCAGAA-3′ and 5′-GCCCTACTACAAAAGCTG-3′ (exon 5); 5′-GGCTATCCACTCTCTCTG-3′ and 5′-TTTGTCATCCCAGGTGTTG-3′ (exon 6); and 5′-TTTTGTTTCTGTTTCTTCTC-3′ (exon 7). Aliquots of genomic DNA were subjected to PCR amplification using the exons 2–6 to a high-resolution melting analysis (Roche), and products were identified using bi-directional sequencing using a genetic analyzer (ABI 3130; Life Technologies). Direct sequencing analysis was performed for the analysis of exon 1. All identified PPM1D alterations were reconfirmed by sequencing of S3BP1 foci was analyzed as in Fig. 2 B. (C) FLAG–Wip1-FL, -R458X, -L484X, -F534X, or -K535X mutants were immunoprecipitated, and phosphatase activity was determined as in Fig. 2 D. (D) Wip1 expression in leukocytes from a healthy control or the BRCA1855 patient was analyzed by immunoblotting. The asterisk indicates a cross-reacting band in the blood sample. Note the increased expression level of the truncated Wip1 in leukocytes from cancer patient. (E) Mutation of PPM1D was analyzed in microdissected mammary noncancer tissue and in cancer tissue from the BRCA1855 patient. Error bars indicate standard deviations.
from an independent PCR amplification. The same method was used for the analysis of DNAs isolated from human tumor cell lines. Mutation analysis of amplicion covering exon 6 only was performed in the validation set of another 398 high-risk, BRCA1/2-negative breast cancer patients. Mutation analysis in the formalin-fixed, paraffin-embedded breast cancer specimen from a number BRCA1/855 patient was performed by sequencing after PCR amplification of exon 6 from DNA isolated from microdissected cancer and noncancer tissue by DNeasy Blood & Tissue kit (QIAGEN).

Online supplemental material

Fig. S1 shows validation of anti-Wip1 antibodies used in this study and data from MS analysis of truncated and FL forms of Wip1 purified from U2OS cells. Fig. S2 demonstrates that the G1 but not G2 checkpoint is affected in cells with truncated Wip1. Table S1 contains data from mutational analysis of the PPM1D gene and anamnetic data of PPM1D mutation carriers. Online supplemental material is available at http://www.jcgb.org/cgi/content/full/jcb.201210033/DC1.

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