The protein phosphatase 1 regulator PNUTS is a new component of the DNA damage response

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The function of protein phosphatase 1 nuclear-targeting subunit (PNUTS)—one of the most abundant nuclear-targeting subunits of protein phosphatase 1 (PP1c)—remains largely uncharacterized. We show that PNUTS depletion by small interfering RNA activates a G2 checkpoint in unperturbed cells and prolongs G2 checkpoint and Chk1 activation after ionizing-radiation-induced DNA damage. Overexpression of PNUTS—enhanced green fluorescent protein (EGFP)—which is rapidly and transiently recruited at DNA damage sites—inhibits G2 arrest. Finally, γ H2AX, p53-binding protein 1, replication protein A and Rad51 foci are present for a prolonged period and clonogenic survival is decreased in PNUTS-depleted cells after ionizing radiation treatment. We identify the PP1c regulatory subunit PNUTS as a new and integral component of the DNA damage response involved in DNA repair.

Keywords: DNA damage response; DNA repair; G2 checkpoint; PNUTS; PP1

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INTRODUCTION

Protein phosphatase 1 nuclear-targeting subunit (PNUTS, also known as p99 and R111) is predominantly localized to the interphase nucleus of mammalian cells (Jagiello *et al*, 1995; Kreivi *et al*, 1997; Allen *et al*, 1998; Landsverk *et al*, 2005). It binds to the α - and γ -isoforms of the catalytic subunit of PP1 (PP1c; Jagiello *et al*, 1995; Allen *et al*, 1998; Trinkle-Mulcahy *et al*, 2006) through a docking K³⁹⁷SVTW⁴⁰¹ (RVXF) motif. A W401A mutation or phosphorylation by protein kinase A within the RVXF motif disrupts binding to PP1c *in vitro* (Kreivi *et al*, 1997; Kim *et al*, 2003).

The function of PNUTS is largely uncharacterized. The upregulation of PNUTS occurs due to hypoxia, and overexpression or depletion of PNUTS causes apoptosis (Kim *et al*, 2003; Lee *et al*, 2007). In response to hypoxia (Udho *et al*, 2002) or chemotherapeutic agents (Krucher *et al*, 2006), PNUTS dissociates from PP1c, an event that correlates with retinoblastoma protein dephosphorylation and G₁ arrest. Recently, PNUTS was shown to interact with the telomeric protein telomere repeat factor 2 (Kim *et al*, 2009). As several telomere repeat factor 2-binding proteins are involved in DNA damage repair, PNUTS has been suggested to function in the DNA damage response (DDR).

The DDR coordinates DNA repair, cell cycle arrest and—depending on the severity of the insult—cell death after exposure to double-strand breaks (DSBs). It is essential for genome integrity and has been proposed to be an early barrier against cancer progression (Jackson & Bartek, 2009). A key signalling cascade in DDR is the ataxia telangiectasia and Rad3-related/Chk1 pathway that controls the S and G2 checkpoints and homologous recombination repair (Liu *et al*, 2000; Sorensen *et al*, 2005). A role for protein phosphatases, including PP1c, in DDR has recently emerged, with reports that PP1c is involved in DSB repair, cell survival and the DNA damage G2 checkpoint (den Elzen & O'Connell, 2004; Tang *et al*, 2008; Hamilton *et al*, 2009; Peng *et al*, 2010).

We have previously shown that PNUTS enhances mitotic chromosome decondensation in a cell-free system, suggesting that

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Fig 1 | Depletion of protein phosphatase 1 nuclear-targeting subunit induces a checkpoint-dependent delay at the G2-M transition. PNUTS-depleted (siPNUTS) and control HeLa^{H2B-EGFP} cells transfected with a scrambled oligonucleotide (Scr) monitored through mitosis by live cell imaging. (A) Chart showing average time spent in indicated stages of mitosis. Difference in prophase, $P < 0.0001$; differences in metaphase and telophase, $P < 0.05$. (B) Representative siRNA-transfected cells in prophase. Numbers indicate time in minutes before nuclear envelope breakdown. (C) Frequency distribution of prophase duration for Scr and siPNUTS cells. For control cells, median, 18 min; 95% CI, 18–21 min. For PNUTS-depleted cells, median, 48; 95% CI, 42–56 min. (D) Mean condensation (± s.d.; log scale) kinetics for 14 Scr cells and 15 siPNUTS cells. (E) siRNA-transfected HeLa cells grown in the presence of 1 µg/ml nocodazole alone (Noco) or in the presence of 2 mM caffeine or 2.5 µM SB218078 for 1 h. Mitotic cells were labelled with pH3S10 antibodies. Difference between Scr and siPNUTS for caffeine, $P < 0.05$ and for SB218078, $P < 0.005$. (F) Average median pH3S10 levels from three independent experiments as in (E). CI, confidence interval; PNUTS, protein phosphatase 1 nuclear-targeting subunit; siRNA, small interfering RNA.

it has a role in the regulation of chromosome structure (Landsverk *et al*, 2005). In this study, we examined the effect of PNUTS depletion on cell cycle progression through mitosis. We show a prolonged mitotic prophase caused by a checkpoint-dependent delay at the G2-M transition. Furthermore, we show an involvement of PNUTS in DDR, with a prolonged ionizing radiation (IR)-induced G2 checkpoint arrest, rapid and transient targeting of PNUTS to damage sites, prolonged presence of DNA damage markers and decreased cell survival after IR

treatment. Our results indicate a role for PNUTS in the regulation of DNA repair.

RESULTS AND DISCUSSION

Our earlier results suggested a role for PNUTS in the regulation of chromosome structure at mitosis (Landsverk *et al*, 2005). Consequently, we used time-lapse imaging of HeLa cells stably expressing histone H2B-enhanced green fluorescent protein (EGFP) to monitor the duration of mitotic phases after small

Table 1 | Average duration (in minutes) of mitotic phases in PNUTS knockdown and control cells

Mitotic phase	Scr	siPNUTS
Prophase	20.1 ± 6.8	60.1 ± 38.4
Prometaphase	11.6 ± 4.5	13.0 ± 5.1
Metaphase	14.8 ± 5.0	19.8 ± 8.1
Anaphase	11.3 ± 2.2	12.2 ± 1.8
Telophase	26.7 ± 4.6	31.0 ± 6.2

PNUTS, protein phosphatase 1 nuclear-targeting subunit; Scr, scrambled; siPNUTS, small interfering protein phosphatase 1 nuclear-targeting subunit.

interfering RNA (siRNA)-mediated PNUTS depletion. A marked lengthening of prophase—defined morphologically as the stage between the first visible signs of chromosome condensation and nuclear envelope breakdown—was observed, compared with controls. In PNUTS-depleted compared with control cells, average prophase duration increased threefold (60.1 ± 38.4 and 20.1 ± 6.8 min, respectively, $P < 0.0001$; Fig 1A,B; Table 1) and frequency distribution of prophase duration was much broader (median values of 48 min and 18 min, respectively; Fig 1C). To quantify chromatin condensation, we randomly selected subsets of cells for computerized image processing to measure the heterogeneity in the spatial distribution of fluorescently labelled chromatin (supplementary information online). Chromatin in PNUTS-depleted cells showed signs of condensation as early as 2.5 h before nuclear envelope breakdown, compared with 0.5 h in control cells (Fig 1D).

Prolonged prophase is characteristic of cells arrested at the G2–M transition (Pines & Rieder, 2001). To test this, we monitored mitotic entry after 1 h incubation with the G2 checkpoint inhibitor caffeine and the Chk1-inhibitor SB218078 (Jin *et al*, 2005). Remarkably, more PNUTS-depleted than control cells entered mitosis in the presence of caffeine or SB218078 (Fig 1E,F). Similar results were observed with an independent PNUTS siRNA oligonucleotide (supplementary Fig S1A,D online). Thus, PNUTS depletion activates a caffeine- and SB218078-sensitive G2 checkpoint in HeLa cells.

The above results led us to ask whether PNUTS might also be involved in the DNA-damage-induced G2 checkpoint. To assess this, we exposed siRNA-treated cells to IR treatment and monitored G2 accumulation after 24 h. In agreement with a previous study (De Leon *et al*, 2008), PNUTS-depleted and control cells showed similar cell cycle profiles in the absence of IR treatment (Fig 2A). However, IR-treated PNUTS-depleted cells showed enhanced accumulation in G2, compared with control cells (Fig 2A). This was accompanied by increased phosphorylation of Chk1 at Ser317 and Ser345 and of replication protein A (RPA; Fig 2B), consistent with increased activation of ataxia telangiectasia and Rad3-related/Chk1 in PNUTS-depleted cells. Similar effects were seen with an independent PNUTS siRNA oligonucleotide (supplementary Fig S1B,C online). Conversely, overexpression of PNUTS–EGFP inhibited accumulation in G2 phase (Fig 2C). To verify that the IR-treated PNUTS–EGFP-expressing cells were dividing, we added nocodazole to trap cells in mitosis (Fig 2C). The G2 arrest was quantified as the ratio

of cells in G2–M after IR treatment relative to IR + noco (averages were 37.8% for PNUTS–EGFP-expressing and 74.6% for GFP-negative cells; Fig 2D).

The decreased G2 accumulation in PNUTS–EGFP-expressing cells after IR treatment is reminiscent of that observed in G2-checkpoint-challenged HeLa cells expressing EGFP–PP1 α (den Elzen *et al*, 2004), suggesting a role for PNUTS in the DDR through activation of PP1c. To investigate this we expressed the PP1c-docking mutant PNUTS(W401A) (Kreivi *et al*, 1997; Kim *et al*, 2003) tagged to EGFP. First, we verified that PNUTS (W401A)–red fluorescent protein could not delocalize PP1 γ –EGFP, even when it was expressed at high levels (supplementary Fig S2 online), confirming that it is defective for PP1 targeting. PNUTS(W401A)–EGFP-expressing cells also showed a reduced G2 arrest 20 h after IR treatment ($P < 0.01$, Fig 2C,D), indicating that inhibition of G2 arrest by PNUTS overexpression is not affected by disruption of PP1c binding and is also not due to the delocalization of PP1c by PNUTS. This suggests that the role of PNUTS in the DDR is not restricted to the activation of PP1c.

As several proteins involved in the DDR are recruited at sites of DSBs, we assessed whether PNUTS is recruited at DNA damage sites. Although PNUTS–EGFP did not show visible recruitment at DNA damage sites after IR treatment (supplementary Fig S3C online), we observed a transient recruitment of PNUTS–EGFP at DNA damage sites induced by 405-nm microirradiation (Fig 3; supplementary Fig S3A,B online) in Hoechst 33258-sensitized cells (Ayoub *et al*, 2008). The PNUTS–EGFP fusion protein was recruited within a time duration of seconds to DNA damage sites and returned to original levels after approximately 5 min (Fig 3). Maximal intensity compared with initial intensity was observed within 1 min and was 1.3-fold for PNUTS–EGFP and 1.5-fold for PNUTS(W401A)–EGFP (Fig 3A,C,G; Table 2). The slightly stronger and prolonged recruitment of PNUTS(W401A)–EGFP could suggest that PP1c binding negatively influences the recruitment of PNUTS and participates in the removal of PNUTS from DNA damage sites. Notably, PNUTS–EGFP intensity returned to initial levels before maximal recruitment of GFP–53BP1 (Fig 3A,F,H; supplementary Fig S3B online; supplementary Movie S1 online). Fluorescence recovery after photobleaching experiments using a 488-nm laser for irradiation—which does not excite Hoechst 33258 or create DNA damage—did not result in PNUTS–EGFP recruitment, suggesting that recruitment was due to DNA damage (Fig 3B,D,G). Furthermore, a fluorescently tagged construct of another nuclear-targeting subunit of PP1c, nuclear inhibitor of PP1 (NIPP1)–EGFP, was not recruited (Fig 3E,H), demonstrating specific recruitment of PNUTS. Interestingly, the nonhomologous end-joining proteins Ku70, Ku80 and DNA-dependent protein kinase are also visibly recruited to laser-induced DNA damage, but do not form foci after IR treatment (Kim *et al*, 2005; Bekker-Jensen *et al*, 2006; Mari *et al*, 2006). One possibility is that PNUTS, similarly to these proteins, binds to DSBs or other DNA lesions at low abundance and this recruitment is therefore only visible after high amounts of localized DNA damage, as achieved by laser irradiation.

The above results are consistent with a role for PNUTS in the regulation of DNA repair, checkpoint signalling or both. To clarify this, we examined the DSB marker γ H2AX. From analysis by western blotting, PNUTS-depleted and control cells showed similar γ H2AX levels 2 h after IR treatment; however, γ H2AX

Fig 2 | Protein phosphatase 1 nuclear-targeting subunit negatively affects G2 arrest after ionizing radiation treatment. (A) Flow cytometry analysis of siRNA-transfected HeLa cells untreated (Exp) or 24 h after irradiation with 5, 7.5 or 10 Gy IR. (B) Western blot using indicated antibodies of siRNA-transfected cells collected after 2 and 3 days or treated with 10 Gy IR at 2 days and collected after 2, 6 and 24 h. (C) Flow analysis of gated GFP-negative, PNUTS-EGFP- or PNUTS(W401A)-EGFP-expressing cells either untreated (Exp) or 20 h after 5 Gy IR in the absence or presence of 1 µg/ml nocodazole (noco) added 2 h after IR treatment. IR treatment was carried out 4 h after transfection. G2/M gating is indicated. (D) Average relative percentage of cells in G2 from (C) determined as the ratio of cells in G2-M after IR over cells in G2/M after IR and nocodazole treatment. Data from two independent experiments. Differences between PNUTS-EGFP and EGFP-negative and between PNUTS(W401A)-EGFP and EGFP-negative cells were at the $P < 0.01$ level for both. EGFP, enhanced green fluorescent protein; IR, ionizing radiation; PNUTS, protein phosphatase 1 nuclear-targeting subunit; siRNA, small interfering RNA.

levels were higher in PNUTS-depleted cells 24 h after IR treatment (Fig 4A; supplementary Fig S1C online). Similar results were obtained from flow cytometry analysis 24 h after IR treatment for two independent PNUTS siRNA oligonucleotides (Fig 4B). Immunofluorescence analysis revealed that although all PNUTS-depleted and control cells contained more than 10 γ H2AX foci 1 h

after IR treatment, 74.2% of PNUTS-depleted compared with 26.1% of control cells contained more than 10 γ H2AX foci 24 h after IR treatment ($P < 0.05$; Fig 4C,D). Thus, removal of γ H2AX is delayed in PNUTS-depleted cells, suggesting that PNUTS is involved in the regulation of DNA repair or participates in dephosphorylation of γ H2AX.

Fig 3 | Protein phosphatase 1 nuclear-targeting subunit-enhanced green fluorescent protein is rapidly and transiently recruited to sites of double-strand breaks in the nucleus. (A–E) A single spot of (A,B) nuclear PNUTS–EGFP, (C,D) PNUTS(W401A)–EGFP, (F) GFP–53BP1 or (E) NIPP1–EGFP was bleached with a 500-ms pulse at 405 nm (A,C,E,F) or 488 nm (B,D), and fluorescence intensity in the bleached area was monitored over time. Graphs on the right-hand side show data points for individual cells. Scale bar, 10 μ m. (G,H) Superposition of recruitment dynamics of indicated constructs. Measured intensities were normalized to the values immediately before bleaching (set to 1) and plotted against time. Average results from several experiments as indicated in Table 2. 53BP1, p53-binding protein 1; EGFP, enhanced green fluorescent protein; NIPP1, nuclear inhibitor of PP1; PNUTS, protein phosphatase 1 nuclear-targeting subunit; wt, wild type.

Table 2 | FRAP analysis of PNUTS–EGFP, PNUTS(W401A)–EGFP, GFP–53BP1 and NIPP1–EGFP

Microirradiation (nm)	N	Time maximum intensity	Value maximum intensity	Intensity 2 min post-bleach
<i>PNUTS–EGFP</i>				
405	12	57 s 540 ms	1.296 ± 0.065	1.186 ± 0.059
488	6	ND	ND	0.980 ± 0.101
<i>PNUTS(W401A)–EGFP</i>				
405	15	60 s 828 ms	1.456 ± 0.135	1.358 ± 0.124
488	32	ND	ND	0.977 ± 0.018
<i>EGFP–53BP1</i>				
405	12	> 15 min	2.559 ± 0.284	1.221 ± 0.044
<i>NIPP1–EGFP</i>				
405	19	ND	ND	0.969 ± 0.039

Each data set was normalized to intensity immediately before bleach; mean values shown with given s.e.m. values.
53BP1, p53-binding protein 1; EGFP, enhanced green fluorescent protein; FRAP, fluorescence recovery after photobleaching; ND, not determined; NIPP1, nuclear inhibitor of PP1; WT, wild type.

To address whether PNUTS controls dephosphorylation of γ H2AX, we analysed γ H2AX levels in exponentially growing and IR-treated cells after overexpression of PNUTS–EGFP. As PNUTS overexpression did not result in a decrease in γ H2AX levels (Fig 4E), the PNUTS–PP1c holoenzyme probably does not dephosphorylate γ H2AX *in vivo*, although PP1c does so *in vitro* (Siino *et al*, 2002). We also analysed 53BP1 foci, a different marker for DSBs (Schultz *et al*, 2000). Consistent with delayed repair, PNUTS-depleted cells showed more 53BP1 foci 24 h after IR treatment than control cells (Fig 4F). PNUTS-depleted cells also showed increased Rad51 and RPA foci at 5 and 24 h after IR treatment (Fig 4F; data not shown), consistent with increased homologous recombination repair (Sorensen *et al*, 2005) and presence of single-strand DNA (Jackson & Bartek, 2009). These data strongly suggest that PNUTS regulates DNA repair rather than γ H2AX dephosphorylation.

Finally, we reasoned that if PNUTS regulates DNA repair, PNUTS-depleted cells should be hypersensitive to IR treatment in cell survival assays. The PNUTS siRNA oligonucleotides with PNUTS almost completely downregulated (siPNUTS 1; supplementary Fig S1D online) were unsuitable for clonogenic survival assays, as they showed reduced cloning efficiency, even in the absence of IR treatment (<10% cloning efficiency compared with control transfected cells). However, an increase in IR-induced death could be observed by measuring the percentage of sub-G1 cells in DNA histograms 72 h after 5-Gy IR treatment (supplementary Fig S4 online). More conclusively, a different PNUTS siRNA oligonucleotide (siPNUTS 3)—which resulted in only partial downregulation of PNUTS—showed decreased clonogenic survival after IR treatment in HeLa cells (Fig 4G). Thus, and consistent with defective DNA repair, PNUTS-depleted cells are hypersensitive to IR-induced cell death.

Specificity is provided to PP1c—a protein phosphatase with a broad range of substrates—through association with regulatory

subunits that target the holoenzyme to specific locations or substrates and control its activity (Moorhead *et al*, 2007; Bollen *et al*, 2009). Although PP1c has previously been implicated in the DDR (den Elzen *et al*, 2004; Tang *et al*, 2008; Hamilton *et al*, 2009), we identify here for the first time, to the best of our knowledge, that the regulatory subunit of PP1 PNUTS is an integral component of this pathway. The rapid and transient recruitment of PNUTS–EGFP to DSBs together with the long-term effects of PNUTS knockdown on DNA damage markers, Chk1 activation, G2 arrest and cell survival after IR treatment suggests that PNUTS regulates early events in the DDR, which affects major downstream targets involved in DNA repair.

METHODS

Cell culture, irradiation and drugs. Human epithelial HeLa, HeLa^{EGFP–PP1 γ} (Trinkle-Mulcahy *et al*, 2003) and HeLa^{H2B–EGFP} (Hirota *et al*, 2004) were grown in Dulbecco's Modified Eagle Medium containing 5% fetal calf serum (Invitrogen). Cells were irradiated using a ¹³⁷Cs source at a dose rate of 4.3 Gy/min or with an X-ray generator (Faxitron CP160, 160 kV, 6.3 mA) at a dose rate of 1 Gy/min. Caffeine and nocodazole were purchased from Sigma-Aldrich and SB218078 from Calbiochem.

DNA constructs and cloning. Wild-type PNUTS was cloned from HeLa complementary DNA into the enhanced green and monomeric red fluorescent protein vectors (Clontech) without the seven carboxy-terminal amino acids of PNUTS. The PNUTS (W401A)–EGFP mutant was produced by site-directed mutagenesis (Quickchange II, Stratagene). Mouse GFP–53BP1 was obtained from J. Lukas (Bekker-Jensen *et al*, 2006) and NIPP1–EGFP from L. Trinkle-Mulcahy (Trinkle-Mulcahy *et al*, 1999).

Western blotting, immunofluorescence and flow cytometry. Western blotting and immunofluorescence were performed as described previously (Landsverk *et al*, 2005). PNUTS antibody was provided by M. Bollen (Lesage *et al*, 2004), phospho-Chk1 (Ser317 and Ser345) and phospho-H3 (pH3S10) were obtained from Cell Signaling Technology, phospho-H2AX (γ H2AX clone JBW301) from Millipore, RPA (p34) from AH Diagnostics and γ -tubulin (GTU-88) from Sigma. Chk1 antibody (DCS310) has been described previously (Sorensen *et al*, 2005). Flow cytometry with double labelling was performed as described previously (Naderi *et al*, 2005) omitting the acid/pepsin and neutralization steps, and triple labelling is described in the supplementary information online.

Live cell imaging and laser irradiation. Procedures are described in the supplementary information online.

Clonogenic survival assay. Twenty-four hours after siRNA transfection, between 150 and 1,200 cells (depending on IR dose to yield 50–100 colonies per dish) were seeded on to 6-cm dishes, incubated for about 20 h, and treated with IR (0, 2, 4 and 6 Gy). After 14 days, cells were stained with methylene blue and colonies containing more than 50 cells were counted as survivors. Survival fractions after each IR dose were calculated as the average cloning efficiency ratio (from three parallel dishes in each experiment) of irradiated cells to non-irradiated cells.

Statistics. Statistical analysis was performed using unpaired Student's *t*-test and all experiments were performed three times or more, unless otherwise indicated. Error bars represent standard deviation values.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

Fig 4 | Protein phosphatase 1 nuclear-targeting subunit depletion prolongs the presence of DNA damage markers. (A) Western blot using indicated antibodies of siRNA-transfected cells collected after 2 and 3 days or treated with 10 Gy IR at 2 days and collected after 2, 6 and 24 h. (B) Average median γ H2AX levels from flow cytometry analysis of γ H2AX labelling compared with DNA content of siRNA-transfected cells 3 days after transfection or treated with 5 Gy IR and collected after 90 min or 24 h. (C) Immunofluorescence analysis of siRNA-transfected cells 3 days after transfection or collected at indicated times after 5 Gy IR using γ H2AX antibodies. Scale bar, 20 μ m. (D) Average percentage of cells with more than 10 γ H2AX foci from (C). (E) Flow cytometry analysis 24 h after transfection of PNUTS-EGFP or PNUTS(W401A)-EGFP in cells left untreated (Exp), or treated with 5 Gy IR 4 h after transfection (IR). Average median γ H2AX levels from gated EGFP-expressing or GFP-negative cells. (F) Percentage of cells with more than 10 53BP1, Rad51 or RPA foci 24 h after 5 Gy IR from immunofluorescence analysis as in (C). Average data from two (53BP1, RAD51) or three (RPA) experiments are shown. * $P < 0.05$, ** $P < 0.005$. (G) Clonogenic survival assays showing survival fraction of Scr and siPNUTS cells as a function of radiation dose (Gy). Average data from three experiments. 53BP1, p53-binding protein; EGFP, enhanced green fluorescent protein; IR, ionizing radiation; PNUTS, protein phosphatase 1 nuclear-targeting subunit; RPA, replication protein A; Scr, scrambled oligonucleotide; siRNA, small interfering RNA.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Allen PB, Kwon YG, Nairn AC, Greengard P (1998) Isolation and characterization of PNUTS, a putative protein phosphatase 1 nuclear targeting subunit. *J Biol Chem* **273**: 4089–4095
- Ayoub N, Jeyasekharan AD, Bernal JA, Venkitaraman AR (2008) HP1-beta mobilization promotes chromatin changes that initiate the DNA damage response. *Nature* **453**: 682–686
- Bekker-Jensen S, Lukas C, Kitagawa R, Melander F, Kastan MB, Bartek J, Lukas J (2006) Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. *J Cell Biol* **173**: 195–206
- Bollen M, Gerlich DW, Lesage B (2009) Mitotic phosphatases: from entry guards to exit guides. *Trends Cell Biol* **19**: 531–541
- De Leon G, Sherry TC, Krucher NA (2008) Reduced expression of PNUTS leads to activation of Rb-phosphatase- and caspase-mediated apoptosis. *Cancer Biol Ther* **7**: 833–841
- den Elzen NR, O'Connell MJ (2004) Recovery from DNA damage checkpoint arrest by PP1-mediated inhibition of Chk1. *EMBO J* **23**: 908–918
- den Elzen N, Kosoy A, Christopoulos H, O'Connell MJ (2004) Resisting arrest: recovery from checkpoint arrest through dephosphorylation of Chk1 by PP1. *Cell Cycle* **3**: 529–533
- Hamilton J, Grawenda AM, Bernhard EJ (2009) Phosphatase inhibition and cell survival after DNA damage induced by radiation. *Cancer Biol Ther* **8**: 1577–1586
- Hirota T, Gerlich D, Koch B, Ellenberg J, Peters JM (2004) Distinct functions of condensin I and II in mitotic chromosome assembly. *J Cell Sci* **117**: 6435–6445
- Jackson SP, Bartek J (2009) The DNA-damage response in human biology and disease. *Nature* **461**: 1071–1078
- Jagiello I, Beullens M, Stalmans W, Bollen M (1995) Subunit structure and regulation of protein phosphatase-1 in rat liver nuclei. *J Biol Chem* **270**: 17257–17263
- Jin ZH, Kurosu T, Yamaguchi M, Arai A, Miura O (2005) Hematopoietic cytokines enhance Chk1-dependent G2/M checkpoint activation by etoposide through the Akt/GSK3 pathway to inhibit apoptosis. *Oncogene* **24**: 1973–1981
- Kim H, Lee OH, Xin H, Chen LY, Qin J, Chae HK, Lin SY, Safari A, Liu D, Songyang Z (2009) TRF2 functions as a protein hub and regulates telomere maintenance by recognizing specific peptide motifs. *Nat Struct Mol Biol* **16**: 372–379
- Kim JS, Krasieva TB, Kurumizaka H, Chen DJ, Taylor AM, Yokomori K (2005) Independent and sequential recruitment of NHEJ and HR factors to DNA damage sites in mammalian cells. *J Cell Biol* **170**: 341–347
- Kim YM, Watanabe T, Allen PB, Kim YM, Lee SJ, Greengard P, Nairn AC, Kwon YG (2003) PNUTS, a protein phosphatase 1 (PP1) nuclear targeting subunit. Characterization of its PP1- and RNA-binding domains and regulation by phosphorylation. *J Biol Chem* **278**: 13819–13828
- Kreivi JP, Trinkle-Mulcahy L, Lyon CE, Morrice NA, Cohen P, Lamond AI (1997) Purification and characterisation of p99, a nuclear modulator of protein phosphatase 1 activity. *FEBS Lett* **420**: 57–62
- Krucher NA, Rubin E, Tedesco VC, Roberts MH, Sherry TC, De Leon G (2006) Dephosphorylation of Rb (Thr 821) in response to cell stress. *Exp Cell Res* **312**: 2757–2763
- Landsverk HB, Kirkhus M, Bollen M, Kuntziger T, Collas P (2005) PNUTS enhances *in vitro* chromosome decondensation in a PP1-dependent manner. *Biochem J* **390**: 709–717
- Lee SJ, Lim CJ, Min JK, Lee JK, Kim YM, Lee JY, Won MH, Kwon YG (2007) Protein phosphatase 1 nuclear targeting subunit is a hypoxia inducible gene: its role in post-translational modification of p53 and MDM2. *Cell Death Differ* **14**: 1106–1116
- Lesage B, Beullens M, Nuytten M, Van Eynde A, Keppens S, Himpens B, Bollen M (2004) Interactor-mediated nuclear translocation and retention of protein phosphatase-1. *J Biol Chem* **279**: 55978–55984
- Liu Q et al (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* **14**: 1448–1459
- Mari PO et al (2006) Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. *Proc Natl Acad Sci USA* **103**: 18597–18602
- Moorhead GB, Trinkle-Mulcahy L, Ulke-Lemee A (2007) Emerging roles of nuclear protein phosphatases. *Nat Rev Mol Cell Biol* **8**: 234–244
- Naderi S, Wang JY, Chen TT, Gutzkow KB, Blomhoff HK (2005) cAMP-mediated inhibition of DNA replication and S-phase progression: involvement of Rb, p21Cip1, and PCNA. *Mol Biol Cell* **16**: 1527–1542
- Peng A, Lewellyn AL, Schiemann WP, Maller JL (2010) Repo-man controls a protein phosphatase 1-dependent threshold for DNA damage checkpoint activation. *Curr Biol* **20**: 387–396
- Pines J, Rieder CL (2001) Re-staging mitosis: a contemporary view of mitotic progression. *Nat Cell Biol* **3**: E3–E6
- Schultz LB, Chehab NH, Malikzay A, Halazonetis TD (2000) p53-binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *J Cell Biol* **151**: 1381–1390
- Siino JS, Nazarov IB, Svetlova MP, Solovjeva LV, Adamson RH, Zolenskaya IA, Yau PM, Bradbury EM, Tomilin NV (2002) Photobleaching of GFP-labeled H2AX in chromatin: H2AX has low diffusional mobility in the nucleus. *Biochem Biophys Res Commun* **297**: 1318–1323
- Sorensen CS, Hansen LT, Dziegielewska J, Syljuasen RG, Lundin C, Bartek J, Helleday T (2005) The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nat Cell Biol* **7**: 195–201
- Tang X, Hui ZG, Cui XL, Garg R, Kastan MB, Xu B (2008) A novel ATM-dependent pathway regulates protein phosphatase 1 in response to DNA damage. *Mol Cell Biol* **28**: 2559–2566
- Trinkle-Mulcahy L, Ajuh P, Prescott A, Claverie-Martin F, Cohen S, Lamond AI, Cohen P (1999) Nuclear organisation of NIPP1, a regulatory subunit of protein phosphatase 1 that associates with pre-mRNA splicing factors. *J Cell Sci* **112**: 157–168
- Trinkle-Mulcahy L, Andrews PD, Wickramasinghe S, Sleeman J, Prescott A, Lam YW, Lyon C, Swedlow JR, Lamond AI (2003) Time-lapse imaging reveals dynamic relocalization of PP1gamma throughout the mammalian cell cycle. *Mol Biol Cell* **14**: 107–117
- Trinkle-Mulcahy L, Andersen J, Lam YW, Moorhead G, Mann M, Lamond AI (2006) Repo-Man recruits PP1-gamma to chromatin and is essential for cell viability. *J Cell Biol* **172**: 679–692
- Udho E, Tedesco VC, Zygmunt A, Krucher NA (2002) PNUTS (phosphatase nuclear targeting subunit) inhibits retinoblastoma-directed PP1 activity. *Biochem Biophys Res Commun* **297**: 463–467