Chk2-dependent phosphorylation of XRCC1 in the DNA damage response promotes base excision repair

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The DNA damage response (DDR) has an essential function in maintaining genomic stability. Ataxia telangiectasia-mutated (ATM)-checkpoint kinase 2 (Chk2) and ATM- and Rad3-related (ATR)-Chk1, triggered, respectively, by DNA double-strand breaks and blocked replication forks, are two major DDRs processing structurally complicated DNA damage. In contrast, damage repaired by base excision repair (BER) is structurally simple, but whether, and how, the DDR is involved in repairing this damage is unclear. Here, we demonstrated that ATM-Chk2 was activated in the early response to oxidative and alkylation damage, known to be repaired by BER. Furthermore, Chk2 formed a complex with XRCC1, the BER scaffold protein, and phosphorylated XRCC1 *in vivo* and *in vitro* at Thr²⁸⁴. A mutated XRCC1 lacking Thr²⁸⁴ phosphorylation was linked to increased accumulation of unrepaired BER intermediate, reduced DNA repair capacity, and higher sensitivity to alkylation damage. In addition, a phosphorylation-mimic form of XRCC1 showed increased interaction with glycosylases, but not other BER proteins. Our results are consistent with the phosphorylation of XRCC1 by ATM-Chk2 facilitating recruitment of downstream BER proteins to the initial damage recognition/excision step to promote BER.

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Introduction

The DNA damage response (DDR), that is, the response of the cell to genetic injury, is essential for maintaining the integrity

of the genome (Hoeijmakers, 2001; Friedberg *et al*, 2006). Failure of the DDR results in genomic instability and a predisposition to malignancy (Hoeijmakers, 2001). The DDR is carried out by DNA damage signalling mechanisms that are triggered and precisely regulated by checkpoint proteins (Kastan and Bartek, 2004; Sancar *et al*, 2004). DDR targeting of repair proteins to lesions for proper DNA repair is equally critical. Structurally complicated DNA damage, including double-strand breaks (DSBs), stalled replication forks, and bulky lesions, activates the checkpoint mechanisms regulated by p53 and the kinases ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) (Kennedy and D'Andrea, 2005; Stiff *et al*, 2006). Furthermore, there is mounting evidence that the proteins involved in repairing DNA DSBs and interstrand cross-link damage are regulated and targeted to damaged sites (Petrini, 2007; Wang, 2007). In contrast, the damage repaired by the base excision repair (BER) pathway is structurally simple and relatively small (Friedberg *et al*, 2006; Caldecott, 2007; Fortini and Dogliotti, 2007), and the nature of the DDR that senses this damage and leads to BER activation is still unclear.

The BER pathway consists of a number of coordinated sequential steps that detect and process the damage; the six core steps are (i) base removal, (ii) strand incision, (iii) incised strand processing to enable DNA synthesis, (iv) DNA synthesis to fill the gap, (v) flap removal, and (vi) ligation. In contrast to the other DNA repair pathways, BER depends on specific glycosylases that recognize and process different forms of DNA damage in the first step, initiating BER by releasing the damaged base (Sancar *et al*, 2004; Almeida and Sobol, 2007). Some glycosylases (the bifunctional glycosylases) have an associated apurinic/apyrimidinic (AP) lyase activity and further catalyse the cleavage of the sugar-phosphate chain and the excision of the abasic residue, leaving a single nucleotide gap. This gap is filled by DNA polymerase β (pol β) and the nick is sealed by the DNA ligase III/X-ray repair cross-complementing group 1 (XRCC1) complex. Other glycosylases (the monofunctional DNA glycosylases) have no associated lyase activity. When such enzymes initiate repair, the phosphodiester bond on the 5' side of the intact AP site is incised by AP endonuclease (APE1/APEX1). DNA pol β , DNA ligase III, and XRCC1 then complete the repair process, and the net result is the replacement of a single nucleotide (short-patch repair). In contrast, long-patch repair, which is involved in the removal of reduced abasic sites, requires further DNA synthesis, resulting in strand displacement and the generation of a damage-containing flap that is later removed by flap endonuclease. Strand displacement DNA synthesis is performed by DNA pol δ/ϵ , and DNA ligase I restores DNA integrity (Hung *et al*, 2005). The BER pathway is distinguished from other DNA repair pathways by the relatively short excision patch generated in double-stranded DNA after removal of the base lesion.

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To explore whether, and what, DDR signalling mechanisms are required to activate BER, we investigated possible involvement of established DDR pathways, and demonstrated that checkpoint kinase 2 (Chk2), which is usually activated by ATM once ATM senses DNA DSBs (Melchionna *et al*, 2000; Ismail *et al*, 2005), may have a function in regulating BER by phosphorylating XRCC1, the scaffold protein of BER (Caldecott, 2003). To explore possible functions of phosphorylated XRCC1, we examined whether site-specific phosphorylation of XRCC1 affects BER. Because BER is the predominant repair pathway responsible for the processing of a broad spectrum of ubiquitous small base lesions caused by alkylation and oxidative damage (Hoeijmakers, 2001; Slupphaug *et al*, 2003; Friedberg *et al*, 2006), the elucidation of the mechanisms underlying BER might result in a more comprehensive understanding of DDR.

Results

ATM-Chk2 activation is associated with base damage

To obtain initial information about the role of Chk2, we analysed checkpoint activation in human cells of different tissue origin (HeLa cells, MCF-7 cells, U2OS cells, and 293T cells) in response to different forms of insult to DNA, because Chk2 is central in transducing DDR signalling and is located downstream of ATM (Chaturvedi *et al*, 1999; Melchionna *et al*, 2000). Interestingly, as shown in Figure 1A, phosphorylation of Chk2 Thr⁶⁸, a site known to be phosphorylated in cells after ionizing radiation (IR)-induced DSB formation (Ahn *et al*, 2000; Melchionna *et al*, 2000), was induced not only by DNA DSB, but also by base damage, including DNA alkylation (treatment with methyl methanesulphonate; MMS) and oxidative damage (H₂O₂ treatment), which are known to be repaired by BER (Sancar *et al*, 2004). This finding of Chk2 activation was confirmed by immunofluorescence studies using a Chk2 Thr⁶⁸ phosphospecific antibody after treatment of U2OS cells with either MMS or H₂O₂ (Figure 1B). Chk2 activation might be linked to activation of ATM, the upstream kinase of Chk2, as a time-dependent increase in autophosphorylation of Ser¹⁹⁸¹ of ATM, a widely used biological marker to identify the active form of ATM (Bakkenist and Kastan, 2003), was also detected after MMS treatment, accompanied by Chk2 Thr⁶⁸ phosphorylation (Figure 1C). This finding lends support to the idea of a role of the activated ATM-Chk2 pathway in the response to DNA insults caused by the formation of small base lesions, such as DNA alkylation by MMS. Confirming this, ATM-targeting siRNA (siATM) caused a significant decrease in MMS-induced Chk2 Thr⁶⁸ phosphorylation, whereas ATR-targeting siRNA (siATR) had no effect (Figure 1D). We also examined whether TTK/hMps1 and DNA-PKcs, which have been implicated in IR- and UV-induced Chk2 Thr⁶⁸ phosphorylation (Li and Stern, 2005; Wei *et al*, 2005), caused MMS-induced Chk2 Thr⁶⁸ phosphorylation and found that was not the case (Figure 1D; Supplementary Figure S1).

The relationship between ATM-Chk2 and BER was further revealed by the knockdown of BER proteins. Although knockdown of XRCC1 did not significantly affect ATM-Chk2 activation by MMS, knockdown of MPG, a gene coding for a glycosylase that recognizes DNA alkylation caused by MMS and initiates BER, almost abolished the phosphorylation of ATM Ser¹⁹⁸¹ and Chk2 Thr⁶⁸ (Figure 1E; Supplementary

Figure S2A and B). This suggests that initial processing of base damage by glycosylases may be required for ATM-Chk2 activation.

It is possible that some less common alkylation and oxidative damage, such as N³-alkyladenine, or glycosylase-processed lesions (for example, single-strand break (SSB)) may block DNA replication in S phase, resulting in the collapse of the replication fork and the production of DSBs (Brem *et al*, 2008), and thus activate ATM-Chk2. To exclude this possibility, we first synchronized cells in the G1 phase by nocodazole arrest and then released them (Supplementary Figure S3A). Under this condition, activation of ATM-Chk2 by MMS was still observed as that seen in IR treatment (Supplementary Figure S3B), suggesting that the MMS-induced DDR we observed could occur without DNA replication.

Chk2 interacts with XRCC1 in vivo and in vitro

As Chk2 regulates DSB repair through its kinase activity (Zhang *et al*, 2004; Wang *et al*, 2006; Zhuang *et al*, 2006), it was of interest to know whether it regulated BER by the same mechanism and which BER proteins were Chk2 substrates. We hypothesized that XRCC1 might be the BER component interacting with Chk2, the rationale being: (a) that, as Chk2 was activated by DNA alkylation and oxidative damage (Figure 1), each of which is recognized by different glycosylases in BER (Slupphaug *et al*, 2003), it seems unlikely that Chk2 would phosphorylate a range of glycosylases to efficiently regulate BER, and (b) XRCC1, the major BER scaffold protein, has both upstream and downstream functions (Caldecott, 2003; Marsin *et al*, 2003), suggesting that it may be a suitable protein to interact with the checkpoint protein.

To investigate whether Chk2 kinase regulated BER function by interacting with XRCC1, we performed co-immunoprecipitation experiments with whole-cell extracts of 293T cells transfected with Myc-tagged Chk2 and His-tagged XRCC1. Using immunoprecipitation with anti-His antibodies followed by immunoblotting with anti-Myc antibodies, we found that XRCC1 was associated with Chk2 (Figure 2A). More importantly, an interaction was also detected between endogenous Chk2 and XRCC1 (Figure 2B and C; Supplementary Figure S4A). As a control, XRCC1 was not immunoprecipitated by Chk2 antibody from cells in which Chk2 expression was knocked down (Supplementary Figure S4B). The interaction was damage-inducible, as the amount of endogenous XRCC1 associated with Chk2 increased as the concentration of MMS used to treat the cells increased (Figure 2B, upper panel). This interaction between endogenous Chk2 and XRCC1 was also observed after H₂O₂ treatment (Figure 2C), further supporting the idea that Chk2 is associated with XRCC1 during BER. Interestingly, an IR-induced interaction between Chk2 and XRCC1 was also detected (Figure 2C). This is probably due to the fact that, in addition to DSB formation, IR induces oxidative base modification and SSBs (Friedberg *et al*, 2006; Jeggo and Lobrich, 2006), both of which are repaired by BER, thus providing additional support for the role of Chk2 in regulating XRCC1 in BER.

To determine whether the co-immunoprecipitation experiments reflected a direct interaction between Chk2 and XRCC1, *in vitro* binding assays were performed with purified recombinant GST-XRCC1 and His-Chk2 proteins, either full length or truncated (Figure 2D and F). The results were consistent with a direct physical interaction between Chk2

Figure 1 Induction of ATM and Chk2 checkpoint activation by alkylation- or oxidation-induced damage. (A) Immunoblots (IB) showing phosphorylation of ATM Ser¹⁹⁸¹ (ATM-pS1981) and Chk2 Thr⁶⁸ (Chk2-pT68) following exposure of HeLa, MCF-7, U2OS, or 293T cells to methyl methanesulphonate (MMS) (alkylation damage), H₂O₂ (oxidative damage), or ionizing radiation (IR). (B) Phosphorylation of endogenous Chk2 Thr⁶⁸ in U2OS cells after treatment with either MMS or H₂O₂, detected by immunofluorescence using a phosphospecific antibody (green in left panels). The right panels show DAPI-stained nuclei. (C) IB analysis showing time-dependent activation of ATM and Chk2 checkpoint, as evidenced by phosphorylation of ATM Ser¹⁹⁸¹ (ATM-pS1981) and Chk2 Thr⁶⁸ (Chk2-pT68), following exposure of HeLa cells (left panel) or MCF-7 cells (right panel) to MMS. (D) IB analysis showing that siRNAs targeting *ATM* or *Chk2*, but not *ATR* or *TTK*, block MMS-induced Chk2 activation in 293T cells, as evidenced by the decreased fraction of phosphorylated Chk2 Thr⁶⁸ in the total Chk2. (E) Decreased expression of the glycosylase MPG abrogates MMS-induced phosphorylation of ATM Ser¹⁹⁸¹ and Chk2 Thr⁶⁸. 293T cells were transfected with siRNA targeting either *MPG* or *XRCC1* prior to MMS treatment. Whole-cell lysates were then analysed by immunoblotting (IB) using indicated antibodies.

and XRCC1, with the B1D segment of XRCC1 (Figure 2E) interacting with the Chk2 N-terminal domain, including the SCD motif (an N-terminal domain rich in serine or threonine residues followed by the glutamine) and the FHA (forkhead-associated) domain (Figure 2G). This finding is mechanistically interesting, given that a BRCT (BRCA1 carboxyl-terminal) domain is located within B1D of XRCC1 and has been shown to mediate many protein–protein interactions involved in DNA damage and repair (Manke *et al*, 2003; Yu *et al*, 2003).

XRCC1 is a substrate of Chk2

To determine whether XRCC1 could be phosphorylated by Chk2, we performed *in vitro* kinase assays with His–Chk2 using GST-fused full-length or truncated XRCC1 as a substrate. Fusion proteins containing the NTL or B1D segment of XRCC1 were found to be phosphorylated by Chk2 (Figure 3A and B), suggesting that phosphorylation sites lie between amino acids 173 and 413 of XRCC1. The XRCC1 segment containing amino acids 1–196 (i.e. the NTD segment) was also phosphorylated to a lesser degree (Figure 3A and B). To precisely identify the residue(s) of XRCC1 phosphorylated by Chk2, we examined the XRCC1 sequence within the region

that was most probably phosphorylated, but found no evidence of putative Chk2 phosphorylation sites (O'Neill *et al*, 2002). We therefore decided to map the site(s) using mass spectrometry, and, as a result, several residues in the NTL segment, including Ser¹⁸⁴, Ser²¹⁰, and Thr²⁸⁴, were identified (Figure 3C). Ideally, mass spectrometry of XRCC1 immunoprecipitated from extracts before and after damage would also provide convincing evidence about the site that is phosphorylated in a damage-dependent manner, in cells. Using mass spectrometry, however, we were not able to unambiguously determine the phosphorylation sites in B1D. To further localize the major sites in the NTL segment, we performed an *in vitro* kinase assay using various GST–NTL mutants, each lacking a different putative phosphorylation site (S184A, S210A, or T284A), as substrates for Chk2. Thr²⁸⁴ was identified as the major site, as the Thr²⁸⁴ → Ala (T284A) mutation almost abolished phosphorylation (Supplementary Figure S5). On the basis of this finding, we generated GST-fused full-length XRCC1^{T284A} as a substrate for Chk2 in an *in vitro* kinase assay, and, as expected, XRCC1 Thr²⁸⁴ was required for XRCC1 phosphorylation by Chk2 (Figure 3D). Furthermore, to confirm the finding of XRCC1 Thr²⁸⁴ phosphorylation by Chk2, we generated a phosphospecific

Figure 2 Interaction between Chk2 and XRCC1. (A) *In vivo* association of Chk2 with XRCC1. 293T cells were co-transfected with Myc-tagged Chk2 and/or His-tagged XRCC1, then whole-cell extracts were subjected to immunoprecipitation (IP) with anti-His antibody and the immunoprecipitated proteins analysed by immunoblotting (IB) with anti-Myc or anti-His antibodies (upper panel). The lower panel shows IB of the whole-cell extract. (B) Association of Chk2 and XRCC1 is damage-inducible. 293T cells were either untreated or treated for 1 h with increasing concentrations of methyl methanesulphonate (MMS), then whole-cell lysates were subjected to IP with anti-Chk2 antibody and the immunoprecipitated proteins were analysed on IB using anti-XRCC1 or anti-Chk2 antibodies (upper panel). The lower panel shows IB of the whole-cell extract for Chk2, XRCC1, and phosphorylated p53 (p53-pS15), an indicator of DNA damage. (C) *In vivo* association of endogenous Chk2 with endogenous XRCC1 is induced by alkylation (MMS), oxidative damage (H₂O₂), or ionizing radiation (IR) (upper panel). Whole-cell lysates were analysed in the lower panel as in (B). (D) Constructs of GST-fused XRCC1 segments used in the *in vitro* binding assay. (E) Upper panel, purified Flag-tagged Chk2 produced in bacteria was incubated with purified GST-XRCC1 fragments, and the proteins pulled down by glutathione beads were analysed by IB with anti-Flag antibody. Lower panel, pull down of GST-XRCC1 fusion proteins was confirmed in a separate IB probed with anti-GST antibody. (F) The His-Chk2 constructs containing different segments used in the *in vitro* binding assay to detect direct interaction between Chk2 and XRCC1. (G) Upper panel, purified GST-BID produced in bacteria was incubated with the different purified His-Chk2 fragments and the GST pull-down proteins were analysed by IB using anti-His antibody. Lower panel, pull down of GST fusion proteins were confirmed in a separate IB probed with anti-GST antibody.

antibody against this residue, and found that XRCC1 Thr²⁸⁴ was phosphorylated by wild-type Chk2 (Chk2^{WT}), but not by a catalytically inactive (i.e. kinase dead) Chk2 mutant

(Chk2^{KD}) (Figure 3E). The T284A mutant was not recognized by this phosphospecific antibody, demonstrating its specificity (Figure 3E).

Figure 3 Localization of sites on XRCC1 phosphorylated by Chk2. (A) *In vitro* kinase assay using the different GST-XRCC1 segments and His-tagged wild-type Chk2. Top panel, *in vitro* Chk2-mediated phosphorylation of the different XRCC1 segments detected by autoradiography. Centre and bottom panels, input His-Chk2 or GST-XRCC1 segments were analysed by immunoblotting (IB) with anti-His or anti-GST antibodies, respectively. (B) Quantification of phosphorylation shown in the top panel of (A) after normalization to the amounts of proteins. (C) Identification of Thr²⁸⁴ of XRCC1 as the main site of phosphorylation by mass spectrometry. *In vitro* Chk2-phosphorylated GST-NTL was trypsinized and analysed by LC/MS/MS spectrometry. Three phospho-tryptic fragments of XRCC1 were identified to be derived from the tryptic peptide ²⁸¹TPAT*APVPA²⁹⁰ (T* represents the site of phosphorylation). The y6, y7, b3, and b4 product ions define the site of phosphorylation as Thr²⁸⁴. (D) *In vitro* kinase assay using Chk2 and either wild-type XRCC1 or the XRCC1 mutant with the Thr²⁸⁴ → Ala (T284A) mutation. (E) Top and third panels, *in vitro* kinase assay using wild-type Chk2 (Chk2^{WT}) or a Chk2 mutant lacking kinase activity (Chk2^{KD}) and either wild-type XRCC1 or XRCC1 T284A. Thr²⁸⁴ phosphorylation of XRCC1 (top panel) and autophosphorylation of Chk2 Thr68 (third panel) were detected using phosphospecific antibodies against Thr²⁸⁴ or Thr⁶⁸. Second and fourth panels, input XRCC1 and Chk2 used in the *in vitro* kinase assay.

XRCC1 Thr²⁸⁴ phosphorylation is induced by base damage

To confirm the finding of XRCC1 Thr²⁸⁴ phosphorylation by Chk2 *in vivo* and, more importantly, to examine whether this is of biological relevance, we used this phosphospecific antibody to demonstrate increased phosphorylation of endogenous XRCC1 when U2OS cells were treated with H₂O₂ (Figure 4A). Furthermore, this H₂O₂-induced site-specific phosphorylation of XRCC1 became undetectable when cells were pretreated with a Chk2-specific inhibitor (Figure 4A). Consistent with these findings, Thr²⁸⁴ phosphorylation was detected *in vivo* when XRCC1-defective EM9 cells stably transfected with wild-type XRCC1 (XRCC1^{WT}), but not the XRCC1 T284A mutant (XRCC1^{T284A}), were subjected to MMS or H₂O₂ treatment (Figure 4B; Supplementary Figure S6).

These results indicate that XRCC1 Thr²⁸⁴ phosphorylation by Chk2 is induced by either MMS or H₂O₂ in cells. In addition, we also performed immunoprecipitation using the anti-phospho-Thr²⁸⁴ XRCC1 antibody to demonstrate that XRCC1 was immunoprecipitated when cells were treated with MMS and that the amount of XRCC1 immunoprecipitated was reduced when Chk2 expression was downregulated by siRNA (Figure 4C).

Interaction of XRCC1 with glycosylases is enhanced by Thr²⁸⁴ phospho-mimicking mutation

The requirement for glycosylases to activate the ATM-Chk2 checkpoint (Figure 1E) leading to XRCC1 phosphorylation (Figures 1–4) raises the question of how the glycosylases and phosphorylated XRCC1 interact with each other to mediate

Figure 4 XRCC1 Thr²⁸⁴ phosphorylation *in vivo* in response to MMS or H₂O₂ treatment. (A) Phosphorylation of endogenous XRCC1 at Thr²⁸⁴ after 10 min treatment of U2OS cells (pretreated with either DMSO- or a Chk2-specific inhibitor, Chk2 inhibitor II) with 2 mM H₂O₂, detected by immunofluorescence (IF) using phosphospecific antibody against Thr²⁸⁴. (B) Phosphorylation of XRCC1 Thr²⁸⁴ in XRCC1-defective EM9 cells transfected with vector or wild-type XRCC1 after treatment with MMS (0.3 mg/ml) or H₂O₂ (2 mM), detected by IF using antibody against XRCC1 (red) or phosphospecific antibody against XRCC1 Thr²⁸⁴ (green). Nuclei were stained with DAPI (blue). (C) Phosphorylation of XRCC1 at Thr²⁸⁴ was induced by MMS in a Chk2-dependent manner. Whole-cell lysates prepared from 293T cells were subjected to IP with anti-phospho-Thr²⁸⁴ XRCC1 antibody and the immunoprecipitated proteins were analysed by immunoblotting (IB) using anti-XRCC1 antibody. The amount of XRCC1 immunoprecipitated was reduced when Chk2 expression was ablated by siRNA.

BER. The evidence that XRCC1 is a scaffold protein and can interact with each BER component (Caldecott, 2003; Campalans *et al*, 2005) suggests that phosphorylation of XRCC1 may facilitate its interaction either with glycosylases or with other BER downstream proteins and thus promote BER. In co-immunoprecipitation assays, the XRCC1 mutant mimicking phosphorylation by Chk2 (T284D) showed a higher affinity for the glycosylases MPG and UNG2 (a glycosylase that removes uracil near replication forks and in non-replicating DNA) than either wild-type XRCC1 or the XRCC1 mutant lacking the phosphorylation site (T284A) (Figure 5A and B). In contrast, the downstream BER proteins pol β and PARP1 interacted equally with wild-type XRCC1 and the T284D and T284A mutants (Figure 5C and D).

Because glycosylases have a function in DNA lesion recognition/excision to initiate BER, the finding that phosphorylation of XRCC1 Thr²⁸⁴ enhances the interaction between XRCC1 and glycosylases suggests a mechanism in which XRCC1 phosphorylation promotes the recruitment of XRCC1 to the initial DNA lesion recognized/excised by glycosylases. In line with this notion, an MMS-induced increase in chromatin-bound endogenous XRCC1 was significantly decreased when either MPG or Chk2 expression was knocked down by siRNA (Figure 5E). Moreover, more wild-type XRCC1 and the T284D mutant were associated with chromatin than the T284A mutant after MMS treatment, thus lending further support to the model (Figure 5F). Note that XRCC1 was associated with chromatin even before MMS treatment; such association appeared to be independent of MPG and Chk2 (Figure 5E and F).

XRCC1 Thr²⁸⁴ phosphorylation promotes BER and cell survival after base damage, and the interaction between Chk2 and XRCC1 is associated with cancer risk

To demonstrate that the interaction between glycosylases and phosphorylated XRCC1 is of functional importance in regulating BER, XRCC1-defective EM9 cells were stably transfected with wild-type or mutant XRCC1. As shown in Figure 6A, these cells expressed comparable amounts of XRCC1. The ability of these cells to perform BER was assessed by the

accumulation of SSBs, an unrepaired intermediate of BER generated by APE1, after exposure to MMS. To this end, we used an NAD(P)H depletion assay (Figure 6B), which has been successfully used to measure the extent of SSBs (Nakamura *et al*, 2003). We first demonstrated that the assay was indeed dependent on XRCC1 and PARP1 (Supplementary Figure S7). When stable clones harbouring XRCC1s with different phosphorylation statuses were treated with higher doses (0.4 and 0.6 mg/ml) of MMS, BER activity associated with SSB repair was restored by wild-type XRCC1 or the mutant mimicking phosphorylation by Chk2 (XRCC1^{T284D}), but not by the mutant lacking the site phosphorylated by Chk2 (i.e. XRCC1^{T284A}) (Figure 6C). Consistent with this, when a comet assay (Figure 6D; Supplementary Figure S8) was performed, though the BER was generally restored, the T284A-expressing cells were significantly less effective in BER when compared with the wild-type and T284D-expressing cells, showing a higher tail moment after MMS treatment. These findings prompted us to examine the overall effect of BER on cell survival in response to MMS treatment using the above stable clones of cells. As expected, XRCC1-defective cells were hypersensitive to MMS, and this sensitivity was complemented by the expression of XRCC1 (Figure 6E). However, notable differences in MMS sensitivity between these cells were observed. The T284D-expressing cells were significantly more resistant to MMS treatment than those expressing wild-type XRCC1 ($P < 0.05$), and the T284A-expressing cells were significantly more sensitive to MMS than those expressing wild-type XRCC1 ($P < 0.05$) (Figure 6E). Taken together, these results indicate that BER capacity can be modulated by the phosphorylation status of XRCC1. Furthermore, though not essential, the effect of ATM-Chk2-dependent phosphorylation of XRCC1 on BER is apparently important.

Therefore, we examined whether such interaction between Chk2 and XRCC1 is of tumorigenic importance. If this interaction was important in determining cancer development, the relationship between cancer risk and susceptibility genotypes of Chk2 would be expected to be more significant in the subset of women with specific genotypes of XRCC1. As

Figure 5 Interaction between phosphorylated XRCC1 and glycosylase promotes the binding of XRCC1 to DNA. (A–D) Increased interaction of phosphorylation-mimicking XRCC1 mutant (T284D) with glycosylases, but not with downstream proteins of BER. *In vivo* association of glycosylases (MPG in (A) and UNG2 in (B)) or downstream BER proteins (polβ in (C) and PARP1 in (D)) with wild-type XRCC1 (XRCC1^{WT}), a phosphorylation-mimicking form (XRCC1^{T284D}), or a phosphorylation-deficient form (XRCC1^{T284A}). The 293T cells were co-transfected with His-tagged wild-type or mutant XRCC1 and Myc-tagged MPG, Myc-tagged UNG2, Myc-tagged polβ, or HA-tagged PARP1, then whole-cell lysates were subjected to immunoprecipitation (IP) with anti-His antibody and the immunoprecipitated proteins were analysed by immunoblotting (IB) with anti-His, anti-HA, or anti-Myc antibodies. (E) Chromatin fractionation analysis showing that decreased expression of MPG and Chk2 by siRNA blocks MMS-induced binding of XRCC1 to chromatin. 293T cells were transfected with siRNA specific for MPG and Chk2, then, after treatment with 0.3 mg/ml MMS for 1 h, whole-cell lysates were prepared, separated into soluble and chromatin-enriched fractions as described in Materials and methods, and analysed by IB using the indicated antibodies. Tubulin and histone H4 were protein controls present in the soluble and chromatin fractions, respectively. (F) *In vivo* association of chromatin with XRCC1^{WT}, XRCC1^{T284D}, or XRCC1^{T284A} showing that the XRCC1 mutant lacking the phosphorylation site (T284A) displays lower affinity for DNA after exposure to MMS. 293T cells were transfected with His-tagged wild-type (XRCC1^{WT}) or mutant XRCC1 (XRCC1^{T284D} or XRCC1^{T284A}), then whole-cell lysates were separated into soluble or chromatin-enriched fractions as in (E), and analysed by IB using the indicated antibodies.

suspected, a genotype-based case-control study of breast cancer (813 breast cancer patients and 818 healthy controls; considerations regarding methodological issues of this study have been described in detail in Ding *et al* (2007), and the validity of the study approach was also confirmed) found that cancer risk associated with genotypic polymorphisms of Chk2 was significantly modified by the XRCC1 genotypes (Supplementary Figure S9). Thus, the analysis gives future support to the importance of the functional interplay between Chk2 and XRCC1.

Discussion

It is now being recognized that the ATM-Chk2 and ATR-Chk1 pathways represent the two major safeguard mechanisms responsible for the DDR and have a vital function in the rapid and specific processing of DNA insults in eukaryotic cells. IR induces DNA DSBs, which, through chromatin alterations, are thought to trigger the activation of ATM-Chk2, leading to the induction of the p53 response. In contrast, induction of the DNA damage signalling pathway following the generation of bulky lesions, for example, by UV, is triggered by replication block, thus leading to ATR-Chk1 activation (Bartek and Lukas, 2003). A missing and interest-

ing piece of evidence needed for a more comprehensive view of the DDR is whether, and how, it is triggered by small base lesions, which do not seem to have a high potential to cause replication block. The present study addressed this important question by exposing cells to MMS or H₂O₂, which directly cause base modifications, such as N⁷-alkylguanine and 8-oxoguanine that rarely block the processing of DNA polymerase. In this study, we demonstrate that treatment of cells with MMS and H₂O₂ also triggered a rapid DDR of the ATM-Chk2 pathway. This DDR appeared to require initial processing of the lesions by glycosylases and had a function in XRCC1-mediated BER. As ATM-Chk2 response is widely accepted to be triggered by DSB, one concern is that whether the MMS-induced response is indeed triggered by damaged base or SSB, or it could be resulted from DSB as a consequence of stalled replication. Our result that MMS can activate the ATM-Chk2 DDR in G1 cells (Supplementary Figure S3) certainly precludes the involvement of DNA replication. However, it remains possible that APE- or glycosylase-mediated cleavage of neighbouring abasic sites might generate DSB even in G1 cells. Regardless, the requirement of processing by glycosylase for ATM-Chk2 activation is apparent. This ATM-Chk2 activation is still a DSB response, and so the question of whether and how ATM-Chk2 activation

Figure 6 Phosphorylation of XRCC1 Thr²⁸⁴ facilitates BER and promotes cell survival after MMS treatment. (A) Immunoblots showing expression of XRCC1 in *XRCC1*-defective EM9 cells stably transfected with wild-type (*XRCC1*^{WT}), the phosphorylation-mimicking form (*XRCC1*^{T284D}), the phosphorylation-deficient form (*XRCC1*^{T284A}), or vector alone. 'a' and 'b' represent two independent clones from each line. (B) The NAD(P)H depletion assay used to detect the accumulation of MMS-induced DNA single-strand breaks (SSBs). A DNA SSB activates PARP-1, resulting in the attachment of poly(ADP-ribose)-branched chains to the DNA using NAD. NAD is regenerated from NAD(P)H by electron transfer, which converts WST-8 to measurable formazan dye (orange colour). (C) Quantification of NAD(P)H depletion as an indicator of MMS-induced SSB accumulation in stable clones expressing the various *XRCC1*s treated with the indicated concentration of MMS for 4 h. **P* < 0.001; bar, standard error. (D) Quantification of the MMS-induced tail moments in stable clones expressing the various *XRCC1*s showed that cells harbouring the *XRCC1*^{T284A} mutant retained more unrepaired DNA (reflected by a higher tail moment) after treatment with 0.45 mg/ml of MMS for 15 min, followed by 0–150 min recovery. Bar, standard error. (E) Cells harbouring the *XRCC1*^{T284A} mutant are more sensitive to MMS. Colony survival of *XRCC1*-defective cells stably transfected with wild-type *XRCC1* (*XRCC1*^{WT}), phosphorylation-mimicking *XRCC1* (*XRCC1*^{T284D}), phosphorylation-deficient *XRCC1* (*XRCC1*^{T284A}), or vector alone in response to 1 h treatment with different concentrations of MMS. Survival is expressed as a percentage of the untreated control. Bar, standard error.

responds to MMS treatment without formation of DSB in G1 remains intriguing. Theoretically, the AP sites resulting from glycosylase-mediated base removal could attract undefined structural sensor proteins (Rinne *et al*, 2005), or alternatively, chromatin change resulting either from the action of glycosylases to flip out and excise damaged base or from SSBs generated by the subsequent action of APE1 could serve as a trigger (David *et al*, 2007). On a related note, ATM has been shown to be activated by chromatin-disrupting events other than DSB formation, indicating that the actual formation of DNA strand breaks may not be essential in the activation of ATM-Chk2 (Bakkenist and Kastan, 2003). We therefore suggest a novel mechanism by which the DDR operates on small base lesions, namely that BER recognition and processing of the lesion are required to trigger the ATM-Chk2 pathway. Interestingly, recent studies analysing checkpoint activation after UV irradiation also identified a nucleotide excision repair (NER)-dependent signal-transduction cascade leading to the phosphorylation of key checkpoint proteins, such as Chk1 and p53 (Bomgardner *et al*, 2006; Marini *et al*, 2006). Taken together, these findings point to the importance of the intermediates in the DNA repair process, rather than DNA damage itself, in linking the checkpoint machinery and the DNA repair pathway.

Details of the mechanism of the interplay between the DDR and DNA repair pathways are emerging. Though initiation of DNA repair can, in theory, be mediated by freely diffusing repair proteins finding lesions and forming repair complexes,

DDR activation would certainly aid DNA repair by both allowing more time for repair and promoting more efficient repair (Branzei and Foiani, 2008). The present study explored how BER is modulated by the activated ATM-Chk2 pathway in response to small base lesions, and we identified one specific consequence of this activated DDR as XRCC1 phosphorylation. On the basis of the differential affinity for glycosylases of the phosphorylation-mimicking and phosphorylation-deficient *XRCC1* mutants, we propose that, as XRCC1 is the scaffold protein that gathers downstream BER components to form a repair complex, XRCC1 phosphorylation may facilitate the recruitment of this repair complex to the initial lesion recognition/excision step mediated by specific glycosylases (Parsons *et al*, 2008). This would ensure that downstream BER proteins are brought together to continue the processing of BER intermediates following removal of the lesions by glycosylases, thus contributing to more efficient BER (Marsin *et al*, 2003). Consistent with this model, we found increased accumulation of BER intermediate (i.e. SSB), decreased DNA repair capacity following exposure to MMS, and, consequently, the associated hypersensitivity phenotype in cells harbouring a *XRCC1* mutant lacking the Chk2 phosphorylation site and with a lower affinity for glycosylases. Earlier, the relationship between Chk2 and XRCC1 has been defined through transcriptional regulation, in which Chk2 was found to phosphorylate and stabilize the transcription factor FOXM1, thereby promoting XRCC1 expression (Tan *et al*, 2007). The ATM-Chk2-XRCC1

pathway that we have demonstrated here represents yet another mechanism which operates directly through post-translational modifications and may provide more timely protection to the cells.

The role of post-translational modification in the DDR and DNA repair pathways has recently become more appreciated (Huen and Chen, 2008). In DSB repair, two of the latest notable results are that (a) both ubiquitination and phosphorylation are involved in promoting the recruitment of DSB repair complexes to DSB sites (Huen *et al*, 2007; Kolas *et al*, 2007; Mailand *et al*, 2007) and (b) in response to replication stress-induced DNA damage, Chk1-mediated phosphorylation of RAD51, a homologous recombination repair (HRR) protein, is required for efficient HRR (Sorensen *et al*, 2005). In NER, it has been shown that ATR can modulate NER through phosphorylation of XPA, which is responsible for damage recognition/confirmation (Wu *et al*, 2007). In BER, XRCC1 has been identified as the substrate of a protein kinase, CK2, and CK2-mediated phosphorylation of XRCC1 enables the assembly and activity of DNA SSB repair protein complexes (Loizou *et al*, 2004). These findings, together with our own, highlight the requirement for post-translational modification of DDR-DNA repair proteins to complete the processing of DNA lesions and increase the efficiency and fidelity of DNA repair. On the other hand, as our understanding of tumorigenesis is extended from single-gene mechanisms to multigenic networks, the consideration of whether there is a causal link between putative cancer-associated genes and tumour development might be extended to whole tumorigenic networks (Bau *et al*, 2004). The identification in the present study of highly coordinated processes linking DDR and BER should help our understanding of this tumorigenic network. We consider that our findings are of particular relevance and importance to tumorigenesis. As *ATM* and *Chk2* are two well-known cancer susceptibility genes (Kastan and Bartek, 2004), the involvement of the ATM-Chk2 pathway in BER lends further supports to the role of BER in cancer formation.

Materials and methods

Cell culture and treatments

HeLa (cervical cancer cells), MCF-7 (breast cancer cells), and 293T cells (embryonic kidney cells) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). U2OS cells (osteosarcoma cells) and Chinese hamster ovary EM9 (*XRCC1*-defective) cells were cultured, respectively, in McCoy's 5A medium or alpha minimum essential medium (both from Sigma-Aldrich) supplemented with 10% fetal bovine serum. To restore *XRCC1* in EM9 cells, *XRCC1*^{WT}, *XRCC1*^{T284A}, *XRCC1*^{T284D}, or control vector was introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), then the cells were continuously cultured and selected for 2 weeks in the presence of 1.5 mg/ml of G418. Each stable clone was maintained in a medium containing 0.5 mg/ml of G418.

Plasmid or siRNA transfection was performed as described earlier using either calcium phosphate precipitation (for 293T) or Lipofectamine 2000 (Wang *et al*, 2006).

MMS and H₂O₂ (both Sigma-Aldrich) were freshly diluted and added to culture medium for the indicated time before cell harvesting or further experiments. For IR treatment, the cells were irradiated as described earlier (Wang *et al*, 2006).

Constructs and small interference RNAs

The mammalian vector expressing Myc-tagged pXJ-Chk2 and the *Escherichia coli* expression vector pRSET-Chk2 have been described earlier (Wei *et al*, 2005). The full-length *XRCC1* cDNA, kindly

supplied by Dr Yung-chi Cheng, was cloned into the *SrfI* and *EcoRI* sites of the pCMV-2C vector (Stratagene, La Jolla, CA) for mammalian expression or into the *EcoRI* site of the pGEX4T-1 vector (GE Healthcare) for expression in *E. coli*. The full-length *MPG*, *UNG2*, *polβ*, and *PARP1* cDNAs were amplified from HeLa cells, and cloned into the Myc-tagged pXJ vector and the HA-tagged pcDNA3 vector (*PARP1* cDNA) for mammalian expression. The *XRCC1*-containing segments were generated using the PCR method and cloned into the *EcoRI* and *Sall* sites of pGEX4T-1. The *XRCC1* site-specific mutations and the *Chk2* kinase-dead or truncated mutations were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene).

The siRNA target sequences were: ATM, 5'-AAGCGCCTGATTCCG AGATCCT-3'; ATR, 5'-CCTCCGTGATGTTGCTTGATT-3'; Chk2, 5'-GT GTCACGAAGGATCAGATC-3'; DNA-PKcs, 5'-GATCGCACCTTACTCT GTTGA-3'; MPG, 5'-AAGAAGCAGCGACCAGCTAGA-3'; TTK, 5'-TGAACAAAGTGAGAGACAT-3'; XRCC1, 5'-AACTCGACTCACTGTGC AGAA-3'; control (random), 5'-AAGTCAATATGCGACTGATGG-3'. All siRNAs were synthesized by Sigma-Prolog.

Cell lysis, lysate fractionation, and immunoblotting

Cell lysates were prepared and western blotting was performed as described earlier (Wei *et al*, 2005).

Chromatin fractionation was performed using the HCl extraction method (Yu *et al*, 2006). Briefly, cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 100 mM NaCl, and 0.5% NP-40) containing 1 mM DTT and a mixture of protease inhibitors (Roche Applied Science) and the insoluble fraction was pelleted in a microcentrifuge. The chromatin-enriched pellet was further extracted for 5 min with lysis buffer containing 0.2 M HCl, then neutralized by adding 1.5 M Tris-HCl (pH 8.8).

The antibodies used for immunoblotting were: anti-GST (sc-138), anti-myc (sc-40), anti-Chk2 (sc-9064), anti-Chk2 (sc-5278), and anti-TTK (sc-540) from Santa Cruz; anti-Flag (F3165), anti-His (H1029), anti-HA (H9658), anti-actin (A2066), and anti- α -tubulin (T6199) from Sigma-Aldrich; anti-ATR (PC538) from Calbiochem (San Diego, CA); anti-DNA-PKcs (A300-516A) from Bethyl Laboratories (Montgomery, TX); anti-p53^{Ser15} (9286), anti-Chk2^{Thr68} (2661), and anti-ATM^{Ser1981} (4526) from Cell Signaling Technology (Beverly, MA); anti-XRCC1 (clone 33-2-5) from NeoMarkers (Fremont, CA); anti-ATM (clone 2C1) from GeneTex (San Antonio, TX); anti-histone H4 pan (05-858) from Upstate Biotechnology; and anti-MPG (clone 1E10) from Abnova (Taiwan). For the generation of anti-phospho-Thr²⁸⁴ XRCC1 antibody, KLH-conjugated XRCC1 phosphopeptide (APTRTPAP₂₇₇₋₂₉₁TAPVPPARA) derived from amino acids 277–291 was used for antiserum production in rabbits by Mimotopes (Australia).

Indirect immunofluorescence

Indirect immunofluorescence was performed according to a previous study (Loizou *et al*, 2004) with a minor modification. Briefly, cells grown on coverslips were pretreated with either DMSO or Chk2 inhibitor, and then treated with 2 mM H₂O₂ in PBS on ice for 10 min or with 0.3 mg/ml of MMS for 20 min at 37°C. The Chk2 inhibitor (i.e. Chk2 inhibitor II) used was from Calbiochem, and was dissolved in DMSO and stored in –20°C. The coverslips were washed and incubated for 10 min in drug-free medium at 37°C before fixation overnight with 2% paraformaldehyde at 4°C. The cells were then permeabilized at room temperature with 0.5% Triton X-100 in PBS and co-immunostained for 1 h at room temperature with a mixture of anti-Chk2^{Thr68} antibody (US Biological, Swampscott, MA; 1:50 dilution) and either anti-XRCC1 antibody (1:100 dilution) or anti-XRCC1^{T284} antibody (1:50 dilution). Bound antibodies were detected using a mixture of FITC-conjugated anti-rabbit IgG antibody and TRITC-conjugated anti-mouse IgG antibody (both from Jackson ImmunoResearch; both 1:100 dilution). The nuclei were stained with 0.0001% DAPI (Sigma-Aldrich). Images were analysed and captured by confocal microscopy scanning (Radiance 2100; Bio-Rad Laboratories, Hercules, CA) and Confocal Assistant software.

Measurement of intracellular NAD(P)H

Quantification of intracellular NAD(P)H levels was performed according to a previous study (Nakamura *et al*, 2003). Briefly, the cells were seeded into 96-well plates at 5000 cells per well. After overnight incubation, they were treated with MMS (at the indicated concentrations) and CCK-8 solution (Dojindo Molecular

Technology, Japan). The cells were then incubated for 4 h to allow formazan dye production, which was measured on a microplate spectrophotometer (SpectraMax 340PC³⁸⁴; Sunnyvale, CA) at 450 nm with 650 nm as the reference filter. The decrease in intracellular NAD(P)H levels were determined by comparing the absorbance of treated cells with that of the control. The means and standard errors for each cell line were calculated from at least three independent experiments, each in triplicate.

Comet assay

The alkaline comet assay was performed using a comet assay kit (4250-050-K; Trevigen, Gaithersburg, MD) according to the manufacturer's recommendations. The cells were seeded overnight and treated with MMS for 15 min to induce tail moments, then incubated in fresh medium for the indicated time to allow DNA repair. To assess the values of the tail moment, the cells were stained with YOYO-1 iodide (Invitrogen; 1:5000 dilution), visualized by fluorescence microscope, and analysed using CometScore (TriTek Corp.). Typically, more than 100 cells per sample were used

to calculate the tail moment, and three independent assays were performed.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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References

- Ahn JY, Schwarz JK, Piwnica-Worms H, Canman CE (2000) Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer Res* **60**: 5934–5936
- Almeida KH, Sobol RW (2007) A unified view of base excision repair: lesion-dependent protein complexes regulated by post-translational modification. *DNA Repair (Amst)* **6**: 695–711
- Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421**: 499–506
- Bartek J, Lukas J (2003) Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* **3**: 421–429
- Bau DT, Fu YP, Chen ST, Cheng TC, Yu JC, Wu PE, Shen CY (2004) Breast cancer risk and the DNA double-strand break end-joining capacity of nonhomologous end-joining genes are affected by BRCA1. *Cancer Res* **64**: 5013–5019
- Bomgardner RD, Lupardus PJ, Soni DV, Yee MC, Ford JM, Cimprich KA (2006) Opposing effects of the UV lesion repair protein XPA and UV bypass polymerase eta on ATR checkpoint signaling. *EMBO J* **25**: 2605–2614
- Branzei D, Foiani M (2008) Regulation of DNA repair throughout the cell cycle. *Nat Rev Mol Cell Biol* **9**: 297–308
- Brem R, Fernet M, Chapot B, Hall J (2008) The methyl methane-sulfonate induced S-phase delay in XRCC1-deficient cells requires ATM and ATR. *DNA Repair (Amst)* **7**: 849–857
- Caldecott KW (2003) XRCC1 and DNA strand break repair. *DNA Repair (Amst)* **2**: 955–969
- Caldecott KW (2007) Mammalian single-strand break repair: mechanisms and links with chromatin. *DNA Repair (Amst)* **6**: 443–453
- Campalans A, Marsin S, Nakabeppu Y, O'Connor TR, Boiteux S, Radicella JP (2005) XRCC1 interactions with multiple DNA glycosylases: a model for its recruitment to base excision repair. *DNA Repair (Amst)* **4**: 826–835
- Chaturvedi P, Eng WK, Zhu Y, Mattern MR, Mishra R, Hurle MR, Zhang X, Annan RS, Lu Q, Faucette LF, Scott GF, Li X, Carr SA, Johnson RK, Winkler JD, Zhou BB (1999) Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. *Oncogene* **18**: 4047–4054
- David SS, O'Shea VL, Kundu S (2007) Base-excision repair of oxidative DNA damage. *Nature* **447**: 941–950
- Ding SL, Yu JC, Chen ST, Hsu GC, Shen CY (2007) Genetic variation in the premature aging gene WRN: a case-control study on breast cancer susceptibility. *Cancer Epidemiol Biomarkers Prev* **16**: 263–269
- Fortini P, Dogliotti E (2007) Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways. *DNA Repair (Amst)* **6**: 398–409
- Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA, Ellenberger T (2006) *DNA Repair and Mutagenesis*. Washington, DC: ASM Press
- Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. *Nature* **411**: 366–374
- Huen MS, Chen J (2008) The DNA damage response pathways: at the crossroad of protein modifications. *Cell Res* **18**: 8–16
- Huen MS, Grant R, Manke I, Minn K, Yu X, Yaffe MB, Chen J (2007) RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. *Cell* **131**: 901–914
- Hung RJ, Hall J, Brennan P, Boffetta P (2005) Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. *Am J Epidemiol* **162**: 925–942
- Ismail IH, Nystrom S, Nygren J, Hammarsten O (2005) Activation of ataxia telangiectasia mutated by DNA strand break-inducing agents correlates closely with the number of DNA double strand breaks. *J Biol Chem* **280**: 4649–4655
- Jeggo P, Lobrich M (2006) Radiation-induced DNA damage responses. *Radiat Prot Dosimetry* **122**: 124–127
- Kastan MB, Bartek J (2004) Cell-cycle checkpoints and cancer. *Nature* **432**: 316–323
- Kennedy RD, D'Andrea AD (2005) The Fanconi anemia/BRCA pathway: new faces in the crowd. *Genes Dev* **19**: 2925–2940
- Kolas NK, Chapman JR, Nakada S, Ylanko J, Chahwan R, Sweeney FD, Panier S, Mendez M, Wildenhain J, Thomson TM, Pelletier L, Jackson SP, Durocher D (2007) Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase. *Science* **318**: 1637–1640
- Li J, Stern DF (2005) Regulation of CHK2 by DNA-dependent protein kinase. *J Biol Chem* **280**: 12041–12050
- Loizou JI, El-Khamisy SF, Zlatanou A, Moore DJ, Chan DW, Qin J, Sarno S, Meggio F, Pinna LA, Caldecott KW (2004) The protein kinase CK2 facilitates repair of chromosomal DNA single-strand breaks. *Cell* **117**: 17–28
- Mailand N, Bekker-Jensen S, Fastrup H, Melander F, Bartek J, Lukas C, Lukas J (2007) RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell* **131**: 887–900
- Manke IA, Lowery DM, Nguyen A, Yaffe MB (2003) BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science* **302**: 636–639
- Marini F, Nardo T, Giannattasio M, Minuzzo M, Stefanini M, Plevani P, Falconi MM (2006) DNA nucleotide excision repair-dependent signaling to checkpoint activation. *Proc Natl Acad Sci USA* **103**: 17325–17330
- Marsin S, Vidal AE, Sossou M, Menissier-de MJ, Le PF, Boiteux S, de MG, Radicella JP (2003) Role of XRCC1 in the coordination and stimulation of oxidative DNA damage repair initiated by the DNA glycosylase hOGG1. *J Biol Chem* **278**: 44068–44074
- Melchionna R, Chen XB, Blasina A, McGowan CH (2000) Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. *Nat Cell Biol* **2**: 762–765
- Nakamura J, Asakura S, Hester SD, de MG, Caldecott KW, Swenberg JA (2003) Quantitation of intracellular NAD(P)H can monitor an imbalance of DNA single strand break repair in base excision repair deficient cells in real time. *Nucleic Acids Res* **31**: e104

- O'Neill T, Giarratani L, Chen P, Iyer L, Lee CH, Bobiak M, Kanai F, Zhou BB, Chung JH, Rathbun GA (2002) Determination of substrate motifs for human Chk1 and hCds1/Chk2 by the oriented peptide library approach. *J Biol Chem* **277**: 16102–16115
- Parsons JL, Tait PS, Finch D, Dianova II, Allinson SL, Dianov GL (2008) CHIP-mediated degradation and DNA damage-dependent stabilization regulate base excision repair proteins. *Mol Cell* **29**: 477–487
- Petrini JH (2007) Cell signaling. A touching response to damage. *Science* **316**: 1138–1139
- Rinne ML, He Y, Pachkowski BF, Nakamura J, Kelley MR (2005) *N*-Methylpurine DNA glycosylase overexpression increases alkylation sensitivity by rapidly removing non-toxic 7-methylguanine adducts. *Nucleic Acids Res* **33**: 2859–2867
- Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* **73**: 39–85
- Slupphaug G, Kavli B, Krokan HE (2003) The interacting pathways for prevention and repair of oxidative DNA damage. *Mutat Res* **531**: 231–251
- 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
- Stiff T, Walker SA, Cerosaletti K, Goodarzi AA, Petermann E, Concannon P, O'Driscoll M, Jeggo PA (2006) ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. *EMBO J* **25**: 5775–5782
- Tan Y, Raychaudhuri P, Costa RH (2007) Chk2 mediates stabilization of the FoxM1 transcription factor to stimulate expression of DNA repair genes. *Mol Cell Biol* **27**: 1007–1016
- Wang HC, Chou WC, Shieh SY, Shen CY (2006) Ataxia telangiectasia mutated and checkpoint kinase 2 regulate BRCA1 to promote the fidelity of DNA end-joining. *Cancer Res* **66**: 1391–1400
- Wang W (2007) Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. *Nat Rev Genet* **8**: 735–748
- Wei JH, Chou YF, Ou YH, Yeh YH, Tyan SW, Sun TP, Shen CY, Shieh SY (2005) TTK/hMps1 participates in the regulation of DNA damage checkpoint response by phosphorylating CHK2 on threonine 68. *J Biol Chem* **280**: 7748–7757
- Wu X, Shell SM, Liu Y, Zou Y (2007) ATR-dependent checkpoint modulates XPA nuclear import in response to UV irradiation. *Oncogene* **26**: 757–764
- Yu X, Chini CC, He M, Mer G, Chen J (2003) The BRCT domain is a phospho-protein binding domain. *Science* **302**: 639–642
- Yu X, Fu S, Lai M, Baer R, Chen J (2006) BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. *Genes Dev* **20**: 1721–1726
- Zhang J, Willers H, Feng Z, Ghosh JC, Kim S, Weaver DT, Chung JH, Powell SN, Xia F (2004) Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair. *Mol Cell Biol* **24**: 708–718
- Zhuang J, Zhang J, Willers H, Wang H, Chung JH, van G, Hallahan DE, Powell SN, Xia F (2006) Checkpoint kinase 2-mediated phosphorylation of BRCA1 regulates the fidelity of nonhomologous end-joining. *Cancer Res* **66**: 1401–1408