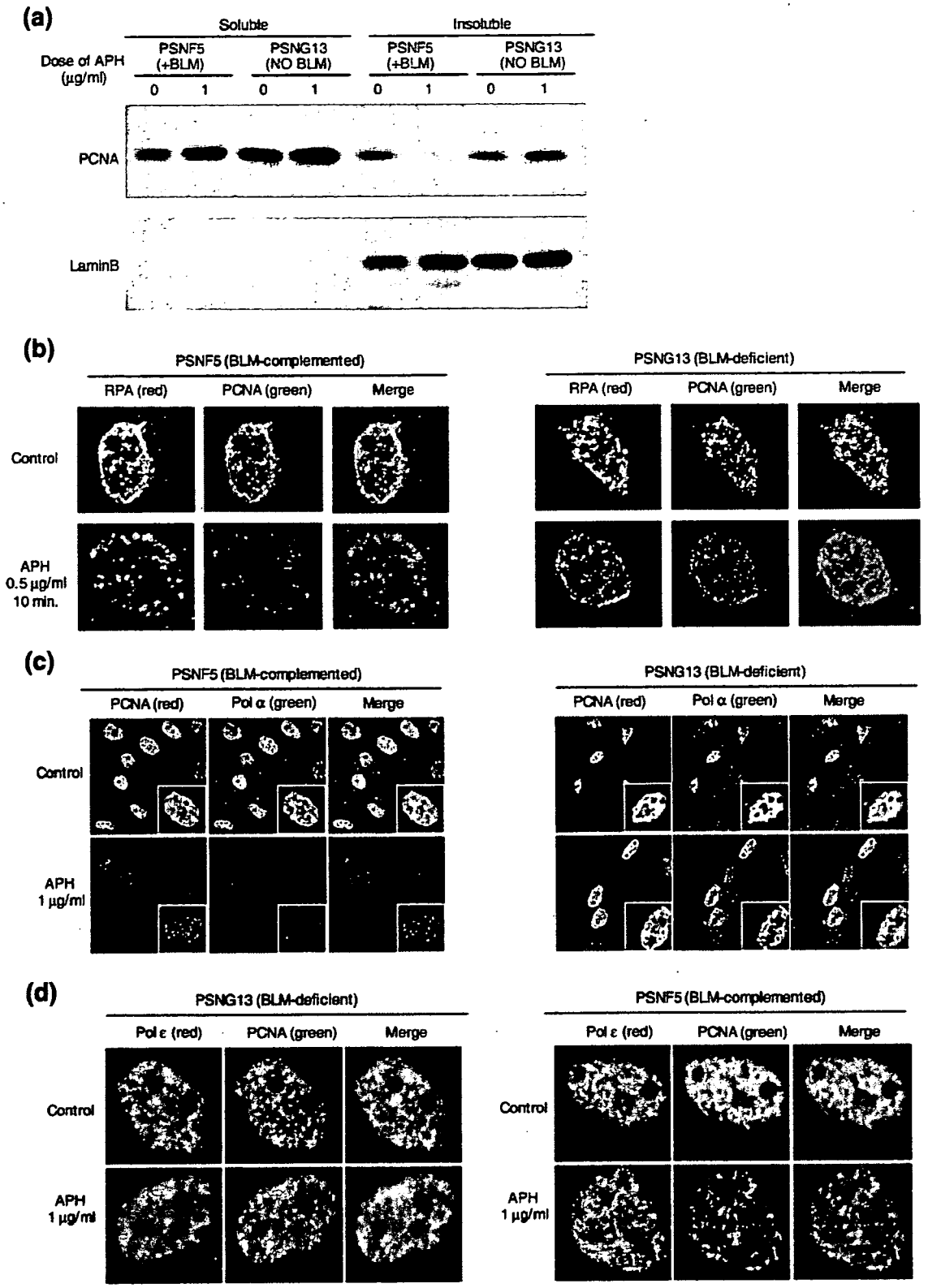


**Figure 6.** PCNA levels and distribution in response to inhibition of DNA replication. (a) Abundance of PCNA in soluble and insoluble fraction in BLM-deficient and BLM-complemented cells treated with APH. LaminB is for loading control. Cells were treated with 1 μg/ml of APH for 1 h and then immunoblotted with PCNA and LaminB as described in Methods. (b) Immunostaining for PCNA (green) and RPA (red). (c) Immunostaining for PCNA (red) and pol α (green). (d) Immunostaining for PCNA (green) and pol ε (red). Cells were treated with 1 μg/ml of APH as indicated. Insets show images at higher magnification.

proteins act in the same pathway, although the biochemical details of the interaction remain to be elucidated.

The studies presented here suggest that transient DSBs are formed in an active manner and that their rapid repair prevents the activation of the S-phase checkpoint and the accumulation of slower, stable DSBs. Our previous study[28] suggests that those transient DSBs are repaired *via* the NHEJ pathway, and that the repair process allows stalled replication forks to resume replication at a slow pace. The assumption that the NHEJ pathway is required to repair transient APH-induced breaks is based on the observation that APH-induced breaks persist in cells deficient in enzymes involved in the NHEJ pathway, such as DNAPK and XRCC4 ligase. We have also shown that cells that cannot form or process the transient breaks accumulate stable DNA breaks activate the S-phase checkpoint and recruit components of the homologous recombination pathway to resolve the permanent breaks. Together with the data presented here, our observations suggest that the S-phase checkpoint and homologous recombination are involved in the response to perturbed replication if DNA replication cannot resume, for example, in cells that exhibit inefficient repair (absence or inhibition of DNA-PK or XRCC1), inefficient formation of transient breaks (in cells that do not contain active forms of either BLM, Mus81 or ATR) or when cells are exposed to high doses of replication inhibitors. It is therefore hypothesized that the BLM and Mus81 depended transient breaks reported here are repaired by the non-homologous end-joining pathway to allow DNA replication to resume. The formation of irreversible DNA breaks in the absence of BLM was also reported in *Xenopus* cell extracts, in which BLM prevents the accumulation of DNA breaks during DNA replication.[29] A model summarizing the proposed cellular response to APH-induced replication stress is presented in Figure 7.

The suggestion that BLM/Mus81 trigger rather than resolve breaks following exposure to replication inhibitors is in line with experiments in mammalian cells proposing a role for BLM helicase in resolving replication lesions[2,15,30] and with data on yeast suggesting that Sgs1 represses homologous recombination after stalling of replication forks.[31–33] Mus81 converts intrastrand DNA crosslinks to DSBs[34] providing a precedent for such a role for Mus81 nuclease. BLM-dependent disassembly of replication forks, indicated by the removal of PCNA and pol α from replication foci, might play a role in the conversion of stalled replication forks to DNA breaks. Disassembly of replication forks was observed during the processing of other replication-dependent lesions, such as lesions induced by etoposide[35] and MMS-induced replication arrest in *Xenopus* egg extracts, in which replication arrest was accompanied by dissociation of PCNA, but not RPA or polymerase ε.[36]

The observations reported here suggest that ATR-dependent phosphorylation of BLM plays a role in the formation of transient APH-induced DNA breaks, consistent with the notion that phosphorylation of BLM by ATR plays a role in the resolution of APH-induced replication stress.[13,15,16] By contrast, cells treated with the topoisomerase inhibitor CPT exhibit ATM-mediated phosphorylation of BLM, which is required for rapid phosphorylation of γ–H2AX.[13,15,37] The involvement of different kinases in the response to those different drugs might reflect two different pathways for the phosphorylation of BLM. When a DNA replication fork

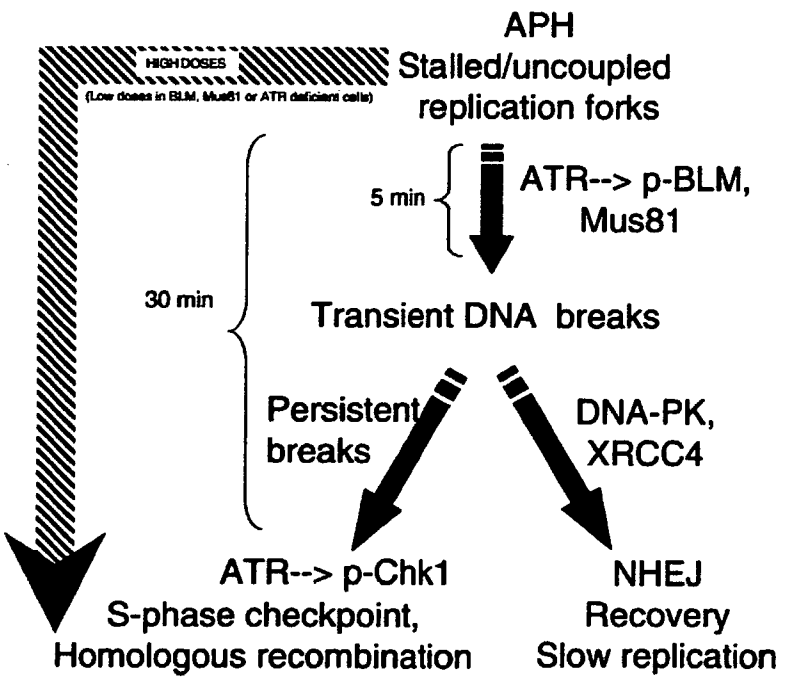


**Figure 7.** Schematic representation of the proposed events triggered by exposure to replication inhibitors such as APH. Low doses of inhibitors cause temporary inhibition of replication forks that is resolved by conversion of those forks to transient DNA breaks by the action of BLM helicase and Mus81 nuclease. This activity requires phosphorylation of BLM by ATR. The transient breaks are rapidly repaired by the non-homologous end-joining pathway (NHEJ); repair lead to recovery of replication at a slow rate even in the presence of the drug. In the absence of components of the NHEJ pathway, such as DNA-PK or XRCC4, the breaks persist and lead to the activation of the S-phase checkpoint. High doses of inhibitors, BLM, Mus81 or ATR deficiencies may also lead to persistent breaks and checkpoint activation.

encounters a topoisomerase: CPT cleavage complex, the encounter directly forms DSBs that trigger phosphorylation of BLM by ATM. In contrast, APH-induced replication stress might create a precursor lesion that is subsequently converted to a DSB *via* a process that requires ATR, BLM - and Mus81. It is likely that the precursor lesion contains stretches of ssDNA that are bound to RPA. *In vitro*, perturbation of DNA replication by APH forms ssDNA, possibly by uncoupling leading and lagging strand replication.[38] The resulting ssDNA is sufficient to activate ATR-mediated phosphorylation[39] by associating with RPA,[40] thereby recruiting ATR *via* ATRIP.[41] Consistent with this, RPA-ssDNA can function as a substrate for BLM helicase activity *in vitro*,[8] possibly facilitating the resolution of stalled replication forks leading to chicken-foot structures.[30] Thus, the transient BLM and Mus81-mediated DNA breaks reported here might play an intermediate role in both the detection of replication lesions and in their resolution.[6,7,13,30]

Previous studies have shown that purified yeast Mus81-Eme1 resolves Holliday junctions by a nick and counternick mechanism[42] and that human Mus81 cleaves Holliday junctions into linear duplexes,[43] supporting a role for Mus81 endonuclease in generating DNA lesion-induced DSBs. Although genetic defects in *mus81* are not lethal in mice, cells deficient in Mus81 constitutively activate components of the S-phase checkpoint pathway, suggesting that Mus81 might regulate S-phase progression in the absence of exogenous DNA damage. However, it is probable that many types of non-lethal lesions are generated during normal DNA replication and cell growth, and that BLM and Mus81 promote genomic stability by facilitating repair of these non-lethal lesions.

## Methods

### Cells and culture conditions

BLM-deficient (PSNG13) and BLM-complemented (PSNF5) fibroblasts[21] were a gift from Dr Ian Hickson (Oxford, UK) and were grown in Minimal Essential Alpha Medium (MEM) supplemented with 10%(v/v) heat-inactivated fetal calf serum containing 350 μg/ml of G418 (Invitrogen). GM00037 (normal human) and GM01492 (untransformed BS) were obtained from the Coriell Cell Repository (Camden, NJ) and were grown in DMEM supplemented with 10%(v/v) fetal bovine serum. ATRkd cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum and L-glutamine containing 400 μg/ml of G418. HCT116, HCT116 Mus81−/−, and Mus81−/− +Mus81 cells[44] were grown in McCoy's 5A medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum. Viability after exposure to drugs was measured as described.[28]

### Drugs

APH was purchased from Wako, USA, dissolved in $Me_2SO$ (1 mg/ml) and stored at −20 °C.

### Neutral comet assay

Neutral comet assay was performed using the CometAssay Kit (Trevigen) following the manufacturer's protocol. Cells were treated with APH or $H_2O_2$ for the indicated times. Cells were collected and suspended in low-melting-point agarose. The agarose was applied to CometSlides™ and allowed to set at 4 °C in the dark. After lysis of the agarose-embedded cells in lysis solution (2.5 M NaCl, 100 mM EDTA (pH 10), 10 mM Tris base, 1%(w/v) sodium lauryl sarcosinate, 0.01%(v/v) Triton X-100), the slides were electrophoresed in TBE (pH 8) (0.089 M Tris, 0.089 M boric acid, 0.003 M EDTA). The samples were then fixed in 70% (v/v) ethanol and dried overnight before staining with SyBr® Green (Molecular Probes, Eugene, OR) to visualize cellular DNA. Images of nuclei were captured using a CCD camera (Roter Scientific; Cool SNAP FX) with epifluorescence microscopy (Olympus; IX70) using a 20× objective lens. For each sample, 50 cells were scored for tail length. Tail length was manually measured using IPLab software. Two independent experiments were performed for each data set.

### DNA fiber analysis

DNA fiber analysis was performed as described.[27] Cells were labeled with 20 μM IdU for 10 min and then labeled with 20 μM CldU for 20 min. Cells were trypsinized and resuspended in phosphate-buffered saline (PBS) at $1 \times 10^6$ cells/ml. The cell suspension (2.5 μl) was mixed with 7.5 μl of lysis buffer (0.5%(w/v) SDS in 200 mM Tris-HCl (pH 7.4), 50 mM EDTA) on an uncoated glass slide (Daigger). After 8 min, DNA spreads were fixed in 3:1 methanol/acetic acid for 5 min and stored in 70% ethanol at 4 °C. Double immunostaining of CldU and IdU was performed according to Dimitrova and Gilbert.[45] The slides were incubated in 100% methanol at room temperature for 5 min and rehydrated with PBS. DNA was denatured with 2.5 M HCl at 37 °C for 30 min, then washed and incubated with primary antibodies. The anti-CldU (Accurate Chemical and Scientific Corporation) and anti-IdU (Becton Dickinson) antibodies were diluted in PBS with 0.5% bovine serum albumin (BSA). Cells were incubated with the antibodies for 1 h at 37 °C. The slides were then washed three times with 0.1% Triton X-100 in PBS and incubated for 1 h at 37 °C with secondary antibody conjugated with Alexa 488 (Molecular Probes for rat immunoglobin G) and Cy-3 (Jackson Immuno Research Laboratories, Inc. for mouse immunoglobin). The slides were washed three times with 0.1% Triton X-100 in PBS and counterstained for DNA with 4 μg/ml of 4′–6-diamino-2-phenylindole in aqueous mounting medium (Biomeda Corp.). Images of DNA fibers were captured by epifluorescence microscopy using 100× objective lens.

### Fluorescence-activated cell sorting (FACS) analysis

Cells were labeled with 20 μM BrdU and 0.25 μM fluorodeoxyuridine (FdU; Fluka), washed with PBS, and fixed in 70% ethanol overnight. DNA was denatured with 1 M HCl, 0.1% Triton X-100 on ice for 10 min followed by boiling for 10 min. Cells were incubated with fluorescein isothiocyanate-conjugated anti-BrdU antibody (Becton Dickinson) for 1 h, and DNA was stained with propidium iodide in the presence of RNase. BrdU-positive cells were detected and quantified by FACScan (Becton Dickinson).

### Immunofluorescence

Cells were grown on 18 mmX18 mm cover slips. After treatment with APH, cells were washed with PBS, treated with a hypotonic lysis solution (10 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% (v/v) Nonidet P-40) for 8 min on ice. Cells were fixed in 4% (w/v) paraformaldehyde in PBS for 10 min, washed in PBS, made permeable in 100% methanol at −20 °C for 15 min, and then washed and blocked with PBS containing 1% BSA and 0.1% Triton X-100 for 30 min. Cells were incubated with anti-PCNA (Santa Cruz), anti-γ-H2AX (Upstate), anti-T99p-BLM[13] and anti-phospho-Chk1-Serine-317 (Cell Signaling) antibodies. Antibodies were diluted in PBS with 0.5% BSA for 1 h at 37 °C. Slides were then washed three times with 0.1% Triton X-100 in PBS and incubated for 1 h at 37 °C with secondary antibody conjugated with Alexa 488 (Molecular Probes) or Cy-3 (Jackson Immuno Research Laboratories). Slides were washed three times with 0.1% Triton X-100 in PBS and counterstained for DNA with 4′–6-diamino-2-phenylindol. Images were captured by confocal microscopy (Nikon; PCM 2000) using 100× objective lens. For quantitative analyses, slides were imaged on a Pathway HT™ automated fluorescence imaging workstation (Atto Bioscience, Rockville, MD) equipped with a Hamamatsu ORCA-ER CCD camera. An air Olympus 40x/340UAPO 0.9 na (0.21 mm × 0.15 mm field of view) objective was used to capture images in the blue (DAPI – 360/10 nm excitation; 435 nm LP emission); green (Alexa 488 – 488/10 nm; 540/30 nm), and red (Cy3 – 548/20 nm; 570 nm LP) channels. Quantitative analyses of the intensity of each dye in each cell were performed IPlab for Pathway. To measure the intensity and distribution of PCNA, slides were imaged on a Pathway HT™ automated fluorescence imaging workstation (Atto Bioscience, Rockville, MD) equipped with a Hamamatsu ORCA-ER CCD camera. An air Olympus 40×/340UAPO 0.9 na (0.21 mm × 0.15 mm field of view) objective was used to capture images in the blue (DAPI – 360/10 nm excitation; 435 nm LP emission); green (Alexa 488 – 488/10 nm; 540/30 nm. The intensity of each dye in each cell was measured using the IPlab software.

### Western blot analysis

Western blot analysis was carried out as described[28] with minor variations. Membrane was blocked with 5% (w/v) non-fat milk for 1 h and incubated with rabbit anti-phopho-Chk1 (Ser317) (Cell Signaling) overnight in a cold room. Secondary incubation with peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz) was performed for 1 h and detection was achieved with Lumi-LightPLUS Western blotting substrate (Roche).

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## Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.11.006

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*Review Article*

# Clinical relevance of the homologous recombination machinery in cancer therapy

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**Cancer chemotherapy and radiotherapy kill cancer cells by inducing DNA damage, unless the lesions are repaired by intrinsic repair pathways. DNA double-strand breaks (DSB) are the most deleterious type of damage caused by cancer therapy. Homologous recombination (HR) is one of the major repair pathways for DSB and is thus a potential target of cancer therapy. Cells with a defect in HR have been shown to be sensitive to a variety of DNA-damaging agents, particularly interstrand crosslink (ICL)-inducing agents such as mitomycin C and cisplatin. These findings have recently been applied to clinical studies of cancer therapy. ERCC1, a structure-specific endonuclease involved in nucleotide excision repair (NER) and HR, confers resistance to cisplatin. Patients with ERCC1-negative non-small-cell lung cancer were shown to benefit from adjuvant cisplatin-based chemotherapy. Imatinib, an inhibitor of the c-Abl kinase, has been investigated as a sensitizer in DNA-damaging therapy, because c-Abl activates Rad51, which plays a key role in HR. Furthermore, proteins involved in HR have been shown to repair DNA damage induced by a variety of other chemotherapeutic agents, including camptothecin and gemcitabine. These findings highlight the importance of HR machinery in cancer therapy. (*Cancer Sci* 2008; 99: 187–194)**

DNA-damaging chemotherapeutic drugs and ionizing radiation induce a variety of DNA lesions in cancer cells as well as in normal cells. Among these lesions, DNA double-strand breaks (DSB) are the most serious, and they eventually lead to cell death unless properly repaired. DSB are repaired by non-homologous end-joining (NHEJ), homologous recombination (HR), and single-strand annealing (SSA).[1] NHEJ rejoins DSB by directly ligating the broken DNA ends, consequently generating small deletions or mutations. SSA repairs DSB by annealing complementary DNA on both sites of the broken DNA, resulting in the loss of a repeat and the DNA sequence between the repeats. HR repairs DSB by using the sister chromatid or homologous chromosome to ensure accurate repair for the maintenance of genome stability.

Non-homologous end-joining is a main pathway in the repair of DSB that are induced by ionizing radiation in mammals. DNA-dependent protein kinase (DNA-PK), consisting of the catalytic subunit DNA-PKcs and the DNA binding complex Ku70/80, plays a key role in NHEJ. The DNA ligase IV-XRCC4 complex re-ligates the broken DNA ends. Artemis processes complex DNA ends prior to repair. Cells deficient in the NHEJ pathway show extreme sensitivity to ionizing radiation, suggesting that specific inhibitors of NHEJ may be used as radio-sensitizers.

In contrast to NHEJ, the HR machinery consists of complex pathways.[2] The MRN complex (Mre11-Rad50-NBS1) recognizes DSB and resects the 5′ ends at break sites, followed by an ordered assembly with replication protein A (RPA), Rad52, Rad51, and Rad54. Rad51, the central player at early stages of HR, forms a nucleoprotein filament on single-strand DNA and catalyzes homologous DNA pairing and strand exchange. The complexity of the HR machinery in higher eukaryotes stems partly from the functional significance of Rad51 paralogs. Five members (Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3), sharing 20–30% sequence identity with Rad51, form two protein complexes, Rad51B-Rad51C-Rad51D-XRCC2 and Rad51C-XRCC3. The proteins' biochemical properties and biological functions reveal that they are involved in HR by assisting Rad51 function. In addition to its role in the early stages of HR, Rad51C and/or its associated proteins were shown to play a role at the late stages of HR by resolving Holliday junctions, which are four-way branched intermediates critical for crossover that are formed after strand exchange mediated by the Rad51 assembly. Like Rad51 paralogs, Rad54 is a multi-functional protein that is involved in several steps of HR by assisting Rad51 function, promoting Holliday junction migration, and remodeling chromatin. In addition to these critical proteins, many accessory proteins are involved in HR. Thus, HR is a complex process required for the repair of DSB after exposure to DNA-damaging agents.

## Nucleotide excision repair and homologous recombination act together to repair interstrand crosslinks

Interstrand crosslinking (ICL) agents such as mitomycin C and cisplatin form DNA adducts, which are repaired by a combination of nucleotide excision repair (NER) and HR.[3] Several NER proteins were identified from patients with xeroderma pigmentosum (XP), characterized by hypersensitivity to ultraviolet (UV) light and a high risk of skin cancer. Among these, XP complementation group F (XPF) forms a heterodimer with ERCC1, and the XPF/ERCC1 complex serves as a structure-specific endonuclease to remove 3′ ends (Fig. 1). Cells with ERCC1 mutation show hypersensitivity to ICL agents. Accordingly, high levels of ERCC1 in cancers confer resistance to platinum-based chemotherapy, whereas low levels of ERCC1 are well correlated with favorable responses of cancers to the therapy.[4–6] These findings led to a clinical study that investigated the association between ERCC1 expression levels in cancer tissues and the response to cisplatin-based adjuvant chemotherapy in surgically treated non-small-cell lung cancer (NSCLC) patients.[7] Patients with ERCC1-negative tumors were shown to benefit from cisplatin-based adjuvant chemotherapy, whereas patients with ERCC1-positive tumors did not. Furthermore, *ERCC1* polymorphisms, codon 118C/T and

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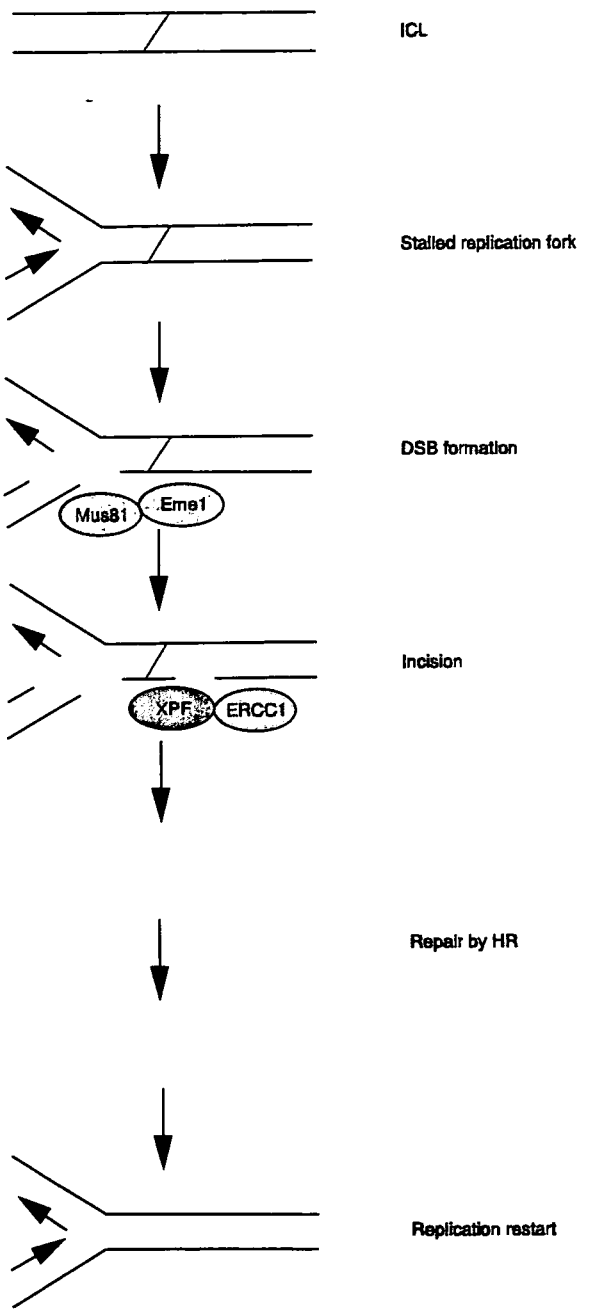


**Fig. 1.** Proposed model of interstrand crosslink (ICL) repair in mammalian cells. A replication fork, if it encounters ICL, is stalled, and a double-strand break (DSB) is generated by Mus81-Eme1.[31] Subsequently, a-3' end near the ICL is incised by XPF-ERCC1, resulting in the release of the ICL from one of the strands. The DNA lesion may be bypassed by translesional synthesis and is eventually repaired by homologous recombination (HR) after the ICL is excised.[3]

C8092A, were shown to be associated with a response to platinum-based chemotherapy.[8,9]

The significance of low levels of ERCC1 in sensitization to ICL agents also contributes to the combination therapy of ICL agents with drugs that reduce ERCC1 levels. Fludarabine, a purine nucleotide analog, is used for the treatment of chronic lymphocytic leukemia (CLL) and has been shown to reduce ERCC1 levels.[10] The reduction in ERCC1 levels was well correlated with the degree of synergy between fludarabine and the DNA minor groove ICL agent SJG-136, indicating the clinical use of the combination of these drugs in fludarabine-resistant CLL.

Although the clinical evaluation of ERCC1 as a biomarker of platinum resistance has been successful so far, it is apparent that platinum sensitivity can not be satisfactorily predicted by ERCC1 measurement alone. Potential candidate markers would emerge once the role of ERCC1 in ICL repair is understood. Targeted gene replacement was drastically impaired in ERCC1-null mouse embryonic stem (ES) cells, defining a novel role for ERCC1 in HR.[11] The DNA adducts formed by ICL agents are converted to DSB (Fig. 1), leading to a hypothesis that proteins involved in HR may be predictable markers of platinum resistance. Consistent with its role in the repair of ICL, Rad51 has been shown to be a good marker of cisplatin resistance in NSCLC.[12] This is supported by a function analysis showing that over-expression of Rad51 is well correlated with DNA damage resistance.[13]

To identify a gene responsible for regulating chemotherapeutic drug sensitivity, targeted disruption of genes of interest has proven to be a powerful tool. The roles of the genes involved in HR in the regulation of sensitivity to DNA-damaging agents have been extensively examined in the chicken B cell line DT40, which is highly proficient in gene targeting.[14,15] In accord with previous studies in knockout mouse demonstrating the early embryonic lethality of the Rad51 mutation, Rad51 is essential for cell viability in DT40 cells.[14] Subsequently, DT40 cells deficient in Rad51B, Rad51C, Rad51D, XRCC2, or XRCC3 were generated and exposed to several DNA-damaging agents.[15] The most dramatic hypersensitivity was found in cells treated with ICL agents, suggesting that Rad51 paralogs play a critical role in ICL repair. These findings were also supported by studies in Chinese hamster ovary (CHO) cells defective in XRCC2, XRCC3, or Rad51C.[16] Further evidence for the role of Rad51 paralogs in ICL repair came from the identification of XRCC3's role in melphalan resistance.[17] This drug's cytotoxic effects are thought to be exerted by alkylation and ICL.

The p53 protein is defective in DT40 and CHO cells, in which the functions of Rad51 paralogs were examined. In order to understand the roles of Rad51 paralogs in human cancer cells with intact p53, we have used the colon cancer cell line HCT116, because targeted recombination is more proficient in this cell line than in other human cells.[18,19] A defect in mismatch repair (MMR) due to MLH1 mutation in HCT116 emphasizes the importance of this cell line from the viewpoint of clinical oncology, because MMR is often defective in human colorectal cancers. In addition, because MMR status was shown to affect sensitivity to cisplatin, HCT116 provides an optimal tool for understanding the mechanisms underlying the drug sensitivity without the effect of the MMR pathways.[20] Consistent with studies in other species, mutations in XRCC3 or Rad51B caused hypersensitivity to mitomycin C and cisplatin, indicating that Rad51 paralogs play a critical role in ICL repair in human cancers with defective MMR even in the presence of intact p53.[18,19]

Like changes in expression levels, genetic polymorphisms are likely to affect sensitivity to ICL agents. XRCC2 R188H was shown to induce resistance to ICL agents in DT40 cells, suggesting that individuals with this variation may tolerate cisplatin treatment.[21] Unlike XRCC2 R188H, XRCC3 M241T does not affect sensitivity to DNA-damaging agents, although an association between this variation and cancer risk has been proposed. Instead, the XRCC3 variation seems to be associated with aneuploidy.[18] Thus, each genetic variation in the ICL repair genes is likely to have a distinct role.

In addition to proteins involved in the basal processes of HR, accessory proteins also play critical roles in the regulation



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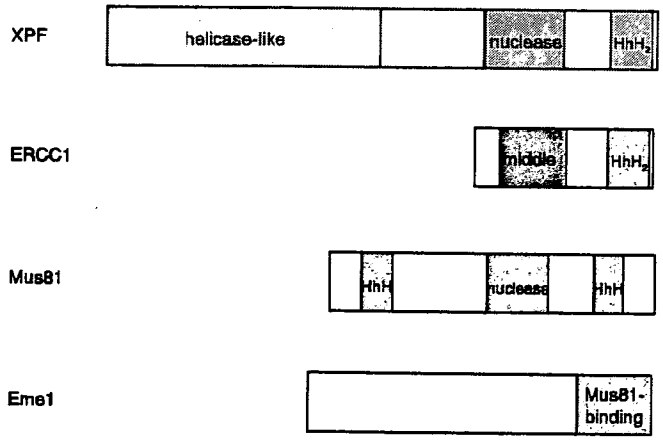


**Fig. 2.** Schematic representation of the protein structure of members of the xeroderma pigmentosum group F (XPF) nuclease family in mammals.[32,33] Interactions of the subunits are mediated by C-terminal helix-hairpin-helix (HhH) domains. Both XPF and ERCC1 contain C-terminal tandem HhH domains ($HhH_2$). XPF contains an N-terminal helicase-like domain, whereas Mus81 does an N-terminal HhH domain instead. Both XPF and Mus81 contain the nuclease domains harboring conserved metal-binding residues of a VERKxxxD motif. Remnants of nuclease domains are present in the middle domain of ERCC1.

of sensitivity to ICL agents. The BLM protein, whose mutation is responsible for Bloom syndrome, is also involved in HR by catalyzing branch migration of Holliday junctions. BLM expression is upregulated in chronic myelogenous leukemia (CML), in which BCR/ABL plays a causal role. The BCR/ABL protein was shown to promote the interaction between BLM and Rad51 to induce cisplatin resistance in CML.[22]

The physical interaction between BRCA2 and Rad51 suggests that a defect in HR is involved in the genesis of hereditary breast cancers.[23] This was supported by the findings that BRCA1 and BRCA2 play roles in HR. BRCA1 expression is upregulated in cisplatin-resistant breast cancer cell lines.[24] BRCA1 promotes cisplatin resistance in association with Rad51.[25] From a clinical point of view, BRCA1-negative ovarian cancer patients who received cisplatin-based chemotherapy after surgical resection have significantly longer median survival times and disease-free intervals than do age- and treatment-course-matched controls.[26] Similarly, lung cancer patients with low levels of BRCA1 benefit from cisplatin-based neoadjuvant chemotherapy more so than patients with high levels of BRCA1.[27] These data clearly indicate that BRCA1 expression can be used as a predictor of cisplatin resistance. Based on a similar hypothesis, a clinical trial comparing the efficacy between carboplatin and the microtubule poison docetaxel is underway to assess the benefits of ICL agents in patients with metastatic breast cancer harboring BRCA1 or BRCA2 mutations.[23]

The Fanconi anemia-BRCA (FA-BRCA) pathway is also involved in cisplatin resistance. A protein complex consisting of FANC proteins (A, B, C, E, F, G, L, M) monoubiquitinates FANCD2.[28] Subsequently, FANCD2 interacts with FANCD1/BRCA2, which is required for HR. FANCI is also monoubiquitinated and associates with FANCD2. FANCN/PALB2 is a binding partner of FANCD1/BRCA2 and stabilizes BRCA2. FANCJ/BRIP1 associates with BRCA1. In addition to BRCA1 and BRCA2, BRIP1 and PALB2 have been shown to be hereditary breast cancer susceptibility proteins. Furthermore, the FANC/BRCA pathway was shown to be defective in cisplatin-sensitive ovarian cancers.[29] These findings led to the idea that inhibitors of the FANC/BRCA pathway may sensitize cancer cells to cisplatin. Based on this idea, small-molecule inhibitors of the FANC/BRCA pathway are under investigation.[30]

Recent evidence suggests that ICL is converted to DSB by the Mus81-Eme1 complex (Fig. 1).[31] Mus81 was identified as a member of the XPF family of endonucleases, sharing an active motif, VERKxxxD (Fig. 2).[32] Like the XPF-ERCC1 complex, Mus81 forms a heterodimeric structure-specific endonuclease by interacting with Eme1.[33] Mus81-Eme1 resembles XPF-ERCC1, because only one partner possesses endonuclease activity. In yeast, *mus81* and *eme1* mutants demonstrate hypersensitivity to UV radiation, methylmethane sulfonate, hydroxyurea, and camptothecin, but not to ionizing radiation.[32,34] In contrast, Mus81- or Eme1-defective murine cells are hypersensitive to mitomycin C and cisplatin, suggesting that Mus81-Eme1 plays a role in ICL repair in mammals.[35] We observed that the haploinsufficiency of either Mus81 or Eme1 led to hypersensitivity to mitomycin C and cisplatin but not to other DNA-damaging agents in HCT116.[36] Thus, like XPF-ERCC1, Mus81-Eme1 might be a potential target of cisplatin-based chemotherapy.

The expression levels of the proteins described here have been extensively examined in primary cancer tissues (Table 1). To promote individualized platinum-based chemotherapy, an understanding of the biological basis of the response to ICL agents, together with information on the expression patterns of proteins associated with the HR machinery in cancer, will be greatly helpful.

## Tyrosine kinase inhibitors as sensitizers to chemotherapy and radiotherapy

After ATM is activated in response to DNA damage induced by ionizing radiation, Rad51 is phosphorylated by the c-Abl kinase.[37] A fusion tyrosine kinase BCR/ABL, arising from chromosomal translocation in CML, has been shown to enhance the Rad51 level through STAT5-dependent transcriptional control, although the precise molecular mechanism underlying this enhancement remains to be demonstrated (Fig. 3).[38] Similarly, other fusion tyrosine kinases, such as TEL/ABL, TEL/JAK2, TEL/PDGFβR, and NPM/ALK, enhance Rad51 levels in a STAT5-dependent manner.[39] Imatinib, an inhibitor of these tyrosine kinases, is now used in the treatment of BCR/ABL-positive leukemias and c-Kit-positive gastro-intestinal stromal tumors. Since BCR/ABL enhances resistance to cisplatin and

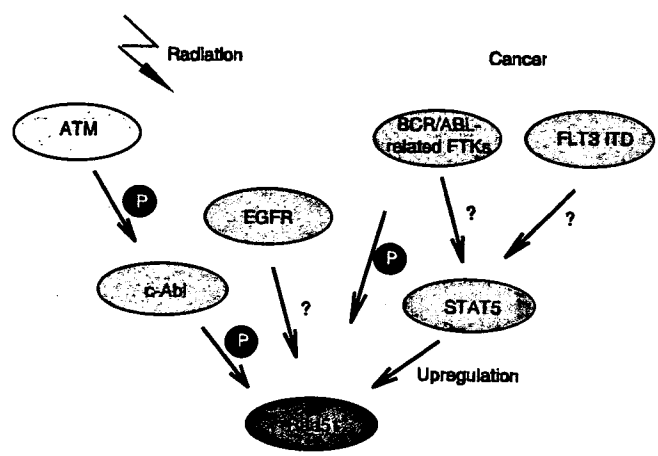


**Fig. 3.** Regulation of Rad51 by tyrosine kinases. When DNA is damaged by ionizing radiation, ATM activates c-Abl, whereby Rad51 is phosphorylated.[37] epidermal growth factor receptor (EGFR) upregulates Rad51 in response to ionizing radiation by an unknown mechanism.[45] BCR/ABL-related fusion tyrosine kinases (FTKs) arising from chromosomal translocation in cancer and FLT3 ITD mutations activate the transcriptional activator STAT5, which upregulates Rad51.[38,39,43,44] BCR/ABL also phosphorylates Rad51.[38]

Table 1. Altered expression levels of molecules involved in homologous recombination in primary sporadic cancer tissues

| Gene/protein | Increased expression | Decreased expression | References |
|---|---|---|---|
| Rad51 | Pancreatic cancer | Colorectal cancer | [12,13,56,70–73] |
| | Breast cancer | Breast cancer | |
| | Non-small-cell lung cancer | | |
| | Head and neck cancer | | |
| | Soft tissue sarcoma | | |
| Rad51C | Breast cancer | | [74] |
| Rad52 | | Colorectal cancer | [72] |
| Brca1 | Lung cancer | Breast cancer | [23,27,73] |
| | | Ovarian cancer | |
| | | Lung cancer | |
| Brca2 | | Ovarian cancer | [23,75] |
| Mre11 | | Breast cancer | [66,67] |
| | | Colorectal cancer | |
| Rad50 | | Breast cancer | [66] |
| NBS1 | Head and neck cancer | Breast cancer | [66,67,76,77] |
| | Melanoma | Colorectal cancer | |
| | | Melanoma | |
| BLM | Chronic myelogenous leukemia | | [22,78] |
| | Lymphoma | | |
| | Breast cancer | | |
| | Colon cancer | | |
| | Lung cancer | | |
| | Renal cell cancer | | |
| | Seminoma | | |
| WRN | Chronic myelogenous leukemia | Gastric cancer | [22,49] |
| | | Colorectal cancer | |
| FANCF | | Ovarian cancer | [79,80] |
| | | Cervical cancer | |
| ERCC1 | Non-small-cell lung cancer | Non-small-cell lung cancer | [4–7,12] |
| | Ovarian cancer | Gastric cancer | |
| | Colorectal cancer | Colorectal cancer | |

Expression has been confirmed at mRNA and/or protein levels. Studies using cultured cancer cells are excluded. Cancers in hereditary chromosome instability syndromes and familial cancers are also excluded. Only molecules whose main functions are associated with homologous recombination are listed.

mitomycin C by promoting the HR activity mediated by Rad51 and its associated proteins, inhibitors of c-Abl such as imatinib are expected to sensitize cancer cells to DNA-damaging agents. Consistent with this hypothesis, imatinib treatment enhanced sensitivity to cisplatin and mitomycin C in BCR/ABL-expressing myeloid cells.[38] From a mechanistic point of view, it is interesting to note that, in addition to Rad51, c-Abl also regulates Rad52 activity.[40] Thus, inhibitors of c-Abl can be used as sensitizers to cisplatin-based chemotherapy.

The HR pathway also regulates sensitivity to radiotherapy. When glioma cells were treated with imatinib, they became modestly sensitive to ionizing radiation, whereas normal fibroblasts did not.[41] The mechanism of this sensitization was explained by the finding that Rad51 levels were reduced by imatinib treatment. Similarly, imatinib treatment was shown to sensitize primary CLL cells to chlorambucil by reducing damage-induced Rad51 activation.[42] These data suggest that c-Abl inhibitors can sensitize tumor cells to DNA-damaging therapies whose sensitivity is regulated by the HR pathway.

FLT3 (fms-like tyrosine kinase 3) is activated in about 30% of acute myeloid leukemia (AML) cases. Internal tandem duplication (ITD) mutations in FLT3 are associated with the risk of relapse in AML.[43] Some patients with AML have dual mutations of ITD and the tyrosine kinase domain. The dual mutations induce resistance to FLT3 inhibitors and chemotherapeutic agents. The mechanism underlying the resistance was shown to be mediated by STAT5 activation, leading to upregulation of Bcl-x(L) and Rad51. Another study has shown that the FLT3 inhibitor PKC412 and the silencing of FLT3 by RNA interference repress Rad51 in cells with FLT3-ITD mutations but not in cells with intact FLT3. These data suggest that Rad51-mediated HR activity contributes to resistance to therapy in AML with FLT ITD mutations.[44]

Accumulating evidence suggests that concurrent radiotherapy with epidermal growth factor receptor (EGFR) inhibitors provides a survival benefit in a variety of cancers, such as those of the lung, head, and neck. The EGFR inhibitor Erlotinib was shown to inhibit radiation-dependent activation of Rad51, indicating that repressed Rad51 contributes to the effect of the concurrent therapy.[45]

Thus, some tyrosine kinase inhibitors may not only inhibit growth-promoting signals but also overcome resistance to chemotherapy and radiotherapy by downregulating the HR pathways mediated by Rad51 and its associated proteins.

## Werner protein regulates sensitivity to camptothecin

Topoisomerase I (Top1) is essential for unwinding supercoiled DNA structures for DNA metabolic functions, such as transcription, replication, and repair.[46] Camptothecin, a Top1 inhibitor, is frequently used in cancer therapy. However, symptoms caused by its toxicity, in particular diarrhea, are severe in some individuals, so a marker of sensitivity to the drug is needed. Single-strand breaks (SSB), which are normally transient, are stabilized by the interaction of camptothecin with Top1. These SSB are converted to DSB during replication,



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triggering the HR repair pathway. Furthermore, a recent study using single-molecule nanomanipulation revealed an unexpected novel role of topotecan, an analog of camptothecin, in impeding Top1-dependent uncoiling.[47] This finding suggests that topotecan induces DNA damage by the accumulation of positive supercoils during replication.

The Werner protein (WRN) is a member of the RecQ helicase superfamily with helicase and exonuclease activities. WRN mutations are responsible for the premature aging Werner syndrome, characterized by genome instability, atherosclerosis, myocardial infarction, and cancer predisposition. Cells derived from Werner syndrome patients and WRN-deficient murine cells are hypersensitive to DNA-damaging agents, particularly camptothecin.[48] In some human cancer cells, WRN expression is repressed by CpG island hypermethylation.[49] These cells are more sensitive to camptothecin than hypomethylated cells are. The introduction of the exogenous *WRN* gene in hypermethylated cells abolished the hypersensitivity. These findings led to a hypothesis that WRN may be a predictable marker of camptothecin-based chemotherapy. Irinotecan (CPT-11), a camptothecin analog, has been used in the treatment of colon cancers, in which CpG island hypermethylation frequently silences WRN. In one retrospective study assessing the survival of patients treated with irinotecan, the median survival time of patients with *WRN*-hypermethylated cancers was significantly longer than that of patients with unmethylated cancers.[49] This suggests the possibility that low levels of WRN in cancer cells may predict a good response to irinotecan-based therapy.

Since Top1 inhibition results in DSB, in addition to WRN, proteins regulating the HR repair pathway are likely to play a role in the regulation of sensitivity to camptothecin. This idea is also supported by the finding that WRN physically associates with Rad51, Rad54, and Rad54B.[50] However, the roles of these proteins in the regulation of sensitivity to camptothecin are complicated, unlike those to other DNA-damaging agents. Impairment of BLM, another member of the RecQ helicase superfamily, led to camptothecin resistance in mouse ES cells.[51] In contrast, deletion of BRCA2, like that of Rad54, led to hypersensitivity to camptothecin in the same mouse ES cells, suggesting that the role of BLM is different from those of BRCA2 and Rad54 in terms of sensitivity to camptothecin. Furthermore, there appear to be functional differences among Rad51 paralogs in the regulation of sensitivity to this drug. Each mutation of XRCC2, XRCC3, or Rad51C led to hypersensitivity to camptothecin in CHO cells. However, a mutation of XRCC3 exhibited the most drastic sensitivity to camptothecin without rapid induction of apoptosis, while a mutation of XRCC2 or Rad51C showed milder sensitivity to the drug, with rapid induction of apoptosis.[52] This finding suggests that XRCC3 plays a role in the induction of apoptosis in response to camptothecin-induced DNA damage. Thus, the mechanisms underlying sensitivity to camptothecin are regulated by complex pathways associated with the HR machinery.

### Roles of homologous recombination in regulation of sensitivity to topoisomerase II inhibitors

Etoposide, an inhibitor of topoisomerase II (Top2), is a key drug in the treatment of small-cell lung cancer (SCLC). However, sensitivity to etoposide is highly variable among SCLC cell lines. Because CHO mutant cells exhibiting defective HR were shown to be hypersensitive to etoposide, Rad51 levels were examined in SCLC cell lines.[53] Rad51 levels were well correlated with resistance to etoposide.[54] The introduction of the exogenous *Rad51* gene in etoposide-sensitive SCLC cells with a low level of Rad51 conferred resistance to etoposide. Conversely, the introduction of the antisense *Rad51* gene in etoposide-resistant SCLC cells with a high level of Rad51 conferred sensitivity to the drug. Thus, Rad51 appears to be involved in the regulation of sensitivity to etoposide in SCLC cells.

The role of the HR repair pathway in the regulation of sensitivity to etoposide was also revealed from studies in CHO mutant cells and DT40 cells. The genes that complement etoposide-hypersensitive CHO mutants turned out to be *XRCC2* and *XRCC3*.[53] Although other proteins involved in the HR machinery, such as Rad54 and BLM, do not appear to play a role in the regulation of etoposide sensitivity, Rad51 and its paralogs may be potential targets of etoposide-based chemotherapy.[51,53] Unlike yeast Rad52, vertebrate Rad52 was assumed to exhibit no distinct effects on sensitivity to DNA-damaging agents. However, the loss of Rad52 in DT40 cells increased sensitivity to etoposide, indicating a novel role for Rad52 in the repair of etoposide-induced DNA damage.[55]

Despite the successful development of new chemotherapeutic agents, doxorubicin, a Top2 inhibitor, still plays a key role in the treatment of cancers such as lymphoma, sarcoma and breast cancer. The Rad51 level is increased in response to DNA damage induced by doxorubicin in soft tissue sarcoma (STS) cells.[56] The silencing of Rad51 by RNA interference resulted in increased sensitivity to doxorubicin in STS cells. Rad51 overexpression has been found in a variety of cancers (Table 1). Overexpressed Rad51 may be therefore a potential target of doxorubicin-based chemotherapy. Because Rad51 expression was shown to be negatively regulated by p53, p53 mutations frequently found in cancers may induce resistance to doxorubicin by Rad51 overexpression.[56]

Although Rad51's role in the regulation of sensitivity to doxorubicin in STS cells was clearly demonstrated, that in breast cancer cells appears to be rather complicated.[57] Doxorubicin induced Rad51 expression in one breast cancer cell line and in normal breast cells, but reduced expression in another breast cancer cell line. The expression of Top2, the target of doxorubicin, was increased in all cell lines tested; suggesting that Rad51's role in the regulation of sensitivity to doxorubicin is dependent on the genetic background of cells. It is therefore highly likely that a complex pathway regulates sensitivity to the drug. Nevertheless, the HR repair pathway may be a potential target of doxorubicin in some cancer cells.

### Roles of homologous recombination in regulation of sensitivity to replication inhibitors

Gemcitabine, 2′,2′-difluorodeoxycytidine (dFdC), is a deoxycytidine analog that has antitumor activity against a broad spectrum of cancers by inhibiting DNA synthesis. Studies in cultured cells and mice have demonstrated cytotoxic synergy between gemcitabine and cisplatin, supporting the clinical use of the combination of these drugs.[58] Because ERCC1 was shown to be involved in the regulation of sensitivity to cisplatin, the possible involvement of ERCC1 in the synergy has been investigated. ERCC1 repression in MMR-deficient colon cancer cells abrogated the synergy, suggesting that gemcitabine-mediated inhibition of ERCC1 activity plays a role in the drug's synergy with cisplatin.[58] This finding supports the clinical evidence that the combination of gemcitabine with cisplatin was effective in some patients with ovarian cancers that had already been refractory to cisplatin-based chemotherapy.[59]

Genetic polymorphisms in DNA repair genes have been proposed to affect the clinical outcome of gemcitabine-based chemotherapy. The median survival time was significantly longer for NSCLC patients treated with gemcitabine and cisplatin harboring XRCC3 241MetMet than for patients with ThrMet or ThrThr.[60] Because this variation was shown to have no effect on sensitivity to cisplatin in CHO and HCT116 cells, the biological significance of XRCC3 T241M is likely to be associated with

the outcome of gemcitabine-based therapy.[18] Another study revealed that *RecQ1* A159C, *Rad54* C157T, *XRCC1* R194W, and *ATM* T77C genotypes affected the overall survival of patients with pancreatic cancer treated with neoadjuvant concurrent gemcitabine and radiotherapy.[61] It should be noted that *Rad54* C157T is a silent polymorphism, suggesting that this variation's effect is associated with linkage disequilibrium with other polymorphisms. The median survival time was significantly longer for patients with none of the adverse genotypes than for those with one or more at-risk alleles. Rad54 plays an essential role in HR. ATM functions as a sensor of DNA damages to promote DNA repair, cell-cycle regulation, and apoptosis. RecQ1, a member of the RecQ helicase superfamily, was shown to interact with MMR factors that regulate HR. XRCC1 plays a critical role in base excision repair. These findings strongly suggest that variations in DNA repair activity, including HR, affect the clinical outcome of patients treated with concurrent gemcitabine and radiotherapy.

Despite the development of new drugs effective for leukemias, hydroxyurea, which is an inhibitor of ribonucleotide reductase, is still used for the treatment of myeloproliferative disorders. Inhibition of this enzyme reduces a pool of deoxyribonucleotide triphosphates, leading to replication block. Stalled replication forks induced by hydroxyurea ultimately generate DSB, which can be repaired by HR. CHO cells overexpressing Rad51 are resistant to hydroxyurea, suggesting that Rad51-dependent HR plays a role in the repair of replication block-induced DSB.[53] Similarly, XRCC2 is involved in the repair of hydroxyurea-induced damages at stalled replication forks, suggesting that HR repairs replication block-induced DSB.[62] However, unexpectedly, BLM-deficient mouse ES cells retaining about 10% levels of endogenous BLM protein exhibited resistance to hydroxyurea, whereas sensitivity to hydroxyurea in human cells derived from Bloom syndrome patients is not different from that in normal cells.[51] Thus, the amount of BLM protein appears to play a critical role in the regulation of sensitivity to hydroxyurea.

## The MRN complex and radiotherapy

NBS1 is mutated in Nijmegen breakage syndrome, which is characterized by hypersensitivity to ionizing radiation, cancer predisposition, microcephaly, and immunodeficiency.[63] The hypomorphic mutation of Mre11 is responsible for ataxia-telangiectasia (AT)-like disorder (ATLD).[64] Patients with AT or ATLD are extremely sensitive to ionizing radiation. The murine ES cells harboring Rad50 mutation exhibit hypersensitivity to ionizing radiation.[65] These findings lead to a hypothesis that the MRN complex may be a potential target of radiotherapy. The expression levels of the complex are reduced in a variety of cancers (Table 1).[66,67] However, the association between a favorable outcome of radiotherapy and reduced levels of the MRN complex in cancer remains to be demonstrated. There is a paradoxical report showing that an intact level of the MRN complex is a predictable marker of good response to adjuvant radiotherapy in early breast cancer, inconsistent with the role of the complex in cultured cells.[68] Further studies are therefore needed to determine the role of the MRN complex in radiotherapy.

## Upregulation of Rad51 may be associated with the risk of therapy-related leukemia

Therapy-related acute myeloid leukemia (t-AML) is a devastating complication of chemotherapy and/or radiotherapy for a primary cancer. The risk of the development of t-AML was found to be associated with the G-to-C polymorphism at −135 of the 5′ untranslated region (135G/C-5′UTR) of *Rad51* and XRCC3 T241M.[69] The promoter activity of the *Rad51* gene is enhanced by the G-to-C substitution (135G/C-5′UTR), resulting in high levels of Rad51 expression in individuals with the variation. Rad51's role in t-AML was also supported by an indirect finding that Rad51 was upregulated in MMR-deficient murine ES cells. This process can be recapitulated by treatment with alkylating agents. MMR deficiency has been proposed to play an early role in therapy-related carcinogenesis. These data indicate that high levels of Rad51 not only confer resistance to DNA-damaging agents but also contribute to the development of therapy-related cancers. Unlike the *Rad51* polymorphism, XRCC3 T241M is unlikely to upregulate the protein level. Since we have shown that this variation promotes the formation of tetraploidy, aneuploidy may play a causal role in t-AML associated with XRCC3 T241M.[18]

## Conclusion

The HR machinery plays critical roles in the regulation of sensitivity to the majority of chemotherapeutic drugs currently used in cancer therapy. The multifunctional DNA repair protein ERCC1 has provided a paradigm for the clinical application of basic knowledge on the mechanisms of DNA repair. This success can be followed by the application of other information regarding the proteins discussed in this review as well as novel proteins characterized in the future. Furthermore, in addition to conventional therapeutics, a novel therapeutic strategy with the targeted inhibition of particular DNA repair pathways represents a new concept in cancer therapy. BRCA1 or BRCA2 dysfunction was shown to sensitize cells to the inhibition of poly(ADP-ribose) polymerase 1 (PARP1) activity.[23] PARP1 is involved in base excision repair, which plays a critical role in the repair of SSB. The inhibition of PARP1 increases the number of SSB, leading to DSB that can be repaired by HR mediated by BRCA1 and BRCA2. Based on these findings, inhibitors of PARP1 are in the early stages of clinical trials. Thus, knowledge gained from the study of DNA repair has considerable potential to impact the development of novel targeted cancer therapies.


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ORIGINAL ARTICLE

# Negative regulation of MEKK1/2 signaling by Serine-Threonine kinase 38 (STK38)

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**Mitogen-activated protein kinases (MAPKs) are activated through the kinase cascades of MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). MAPKKKs phosphorylate and activate their downstream MAPKKs, which in turn phosphorylate and activate their downstream MAPKs. MAPKKK proteins relay upstream signals through the MAPK cascades to induce cellular responses. However, the molecular mechanisms by which given MAPKKKs are regulated remain largely unknown. Here, we found that serine-threonine protein kinase 38, STK38, physically interacts with the MAPKKKs MEKK1 and MEKK2 (MEKK1/2). The carboxy terminus, including the catalytic domain, but not the amino terminus of MEKK1/2 was necessary for the interaction with STK38. STK38 inhibited MEKK1/2 activation without preventing MEKK1/2 binding to its substrate, SEK1. Importantly, STK38 suppressed the autophosphorylation of MEKK2 without interfering with MEKK2 dimer formation, and converted MEKK2 from its phosphorylated to its nonphosphorylated form. The negative regulation of MEKK1/2 was not due to its phosphorylation by STK38. On the other hand, *stk38* short hairpin RNA enhanced sorbitol-induced activation of MEKK2 and phosphorylation of the downstream MAPKKs, MKK3/6. Taken together, our results indicate that STK38 negatively regulates the activation of MEKK1/2 by direct interaction with the catalytic domain of MEKK1/2, suggesting a novel mechanism of MEKK1/2 regulation.**

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## Introduction

The mitogen-activated protein kinase (MAPK) cascades, in which the major components are MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK), are conserved in eukaryotic cells (Cano and Mahadevan, 1995; Herskowitz, 1995; Waskiewicz and Cooper, 1995; Chang and Karin, 2001). In mammals, three major groups of MAPKs have been characterized: the extracellular signal-regulated protein kinases (Cobb and Goldsmith, 1995; Marshall, 1995), the c-Jun $NH_2$-terminal kinases (JNK; Kyriakis *et al.*, 1994; Gupta *et al.*, 1996) and the p38 kinases (Han *et al.*, 1994; Enslen *et al.*, 1998). The extracellular signal-regulated protein kinases cascade is mostly activated by agonists of tyrosine kinase-encoded receptors and G protein-coupled receptors that induce mitogenesis or differentiation (Cobb and Goldsmith, 1995; Marshall, 1995), whereas the JNK and p38 cascades are strongly activated by proinflammatory cytokines or extracellular stresses (Davis, 2000; Kyriakis and Avruch, 2001).

MAPKKKs phosphorylate and activate MAPKKs, which in turn phosphorylate MAPKs. There is increasing evidence from biochemical and genetic analyses that the MAPKKKs link a variety of extracellular stimuli to cytoplasmic and nuclear effectors by activating downstream MAPK pathways. In addition, overexpression of many MAPKKKs could also activate the IκB kinase NF-kappaB pathway (Karin and Ben-Neriah, 2000; Yang *et al.*, 2001). MEKK1 is the first MAPKKK to be identified based on its homology with the *Saccharomyces cerevisiae* MAPKKK STE11 (Lange-Carter *et al.*, 1993). MEKK1 was initially discovered as an MAPKKK for the extracellular signal-regulated protein kinases pathway (Lange-Carter *et al.*, 1993). However, subsequent studies indicated that it preferentially activates the JNK pathway (Minden *et al.*, 1994; Yan *et al.*, 1994; Davis, 2000). The amino-terminal regulatory domain of MEKK1 has been suggested to interact with other proteins, such as 14-3-3 (Fanger *et al.*, 1998). The carboxy-terminal region of MEKK1 contains a catalytic domain that binds and phosphorylates SEK1 (Xia *et al.*, 1998). MEKK1

protects cells from apoptosis, and contributes to cell migration (Minamino *et al.*, 1999; Yujiri *et al.*, 2000). MEKK2 belongs to the MEKK/STE11 subfamily, which are widely expressed and potent activators of the NF-κB and MAPK pathways (Blank *et al.*, 1996). MEKK2 has a catalytic domain in its carboxyl-terminal region (Blank *et al.*, 1996), and is activated by forming a dimer (Cheng *et al.*, 2005a). MEKK2 has been suggested to be involved in T-cell-receptor signaling (Su *et al.*, 2001; Guo *et al.*, 2002) and was shown to regulate cytokine gene expression (Chayama *et al.*, 2001; Kesavan *et al.*, 2004). However, it is still largely unknown how MEKK1, MEKK2 and other MAPKKKs are activated.

Serine-threonine protein kinase 38 (STK38)/NDR1 (GenBank accession number NP009202) is a serine/threonine protein kinase belonging to a subclass of the AGC family of protein kinases (Manning *et al.*, 2002; Tamaskovic *et al.*, 2003; Hergovich *et al.*, 2006), which includes cyclic AMP-dependent kinase, protein kinase B and protein kinase C. The NDR family is highly conserved; it includes the mammalian STK38/NDR1 and STK38L/NDR2, *Drosophila melanogaster* TRC, *Schizosaccharomyces pombe* Orb6 and *Saccharomyces cerevisiae* Cbk1 and Dbf2 (Manning *et al.*, 2002; Tamaskovic *et al.*, 2003). Of these, Cbk1 and Orb6 are involved in regulating cell morphology (Verde *et al.*, 1998; Bidlingmaier *et al.*, 2001), and Dbf2 is a cell cycle-regulated kinase, the activity of which is required for progression through anaphase (Johnston *et al.*, 1990). Despite these intriguing observations, STK38/NDR1 has no known natural substrates and has not been implicated in the MAPK signal transduction pathway.

Previously, we reported the isolation of a radio-resistant mutant with low JNK activity from the human T-cell leukemia cell line MOLT-4 (Enomoto *et al.*, 2000). We then compared the gene expression profile of this mutant with that of the parental MOLT-4 cells using a DNA microarray system to elucidate the mechanism underlying the modulation of stress signaling. Our previous study demonstrated that X-irradiation induced downregulation of *c-myc* in MOLT-4 but did not in the radio-resistant mutant cells (Enomoto *et al.*, 2003). To further study, we constructed expression vectors encoding the genes that were significantly overexpressed in the radio-resistant cells, transfected them into 293T cells, and analysed their ability to modulate JNK signaling and to bind components of the JNK cascade. Among these gene products, we identified STK38 as an inhibitor of MEKK1 and MEKK2 (MEKK1/2), which are MAPKKKs that function in the MAPK cascades.

Here we show that STK38 physically interacts with MEKK1/2, and that overexpression of STK38 leads to reduced MEKK1/2 activity. In contrast, *stk38*-specific short hairpin RNA (shRNA) enhanced sorbitol-induced activation of MEKK2 and subsequent phosphorylation of the downstream MAPKKs, MKK3/6. Our findings indicate that STK38 functions as a negative regulator of MEKK1/2 *in vivo*, and does so, at least for MEKK2, by inhibiting its autophosphorylation.

## Results

### *Expression and localization of STK38*

We generated a polyclonal antibody against a portion of human STK38, and investigated STK38 expression in various cell lines by western blotting. STK38 was expressed in all the cell lines examined, including HeLa, 293T, MCF-7 and MOLT-4 (Figure 1a). Previously, we reported the isolation of a radio-resistant mutant cell line with lower than normal JNK activity, Rh-1a, derived from MOLT-4 cells (Enomoto *et al.*, 2000). We then found that the expression level of *stk38* mRNA was upregulated in Rh-1a cells as compared with the parental MOLT-4 cells by DNA microarray analysis (data not shown); the STK38 protein was also upregulated in the Rh-1a line (Figure 1a). Next, to determine the subcellular localization of STK38, we performed subcellular fractionation of 293T cells and subjected each fraction to western blotting with the anti-STK38 antibody. Figure 1b shows that STK38 localized to the cytoplasm.

### *STK38 interacts with MAPKKK but with neither MAPKK nor MAPK in the JNK cascade*

We then examined the possible involvement of STK38 in the JNK signaling pathway. First, the binding specificities

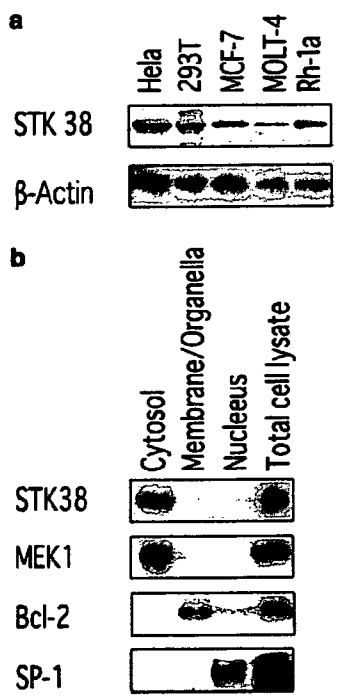


**Figure 1** Expression and subcellular localization of STK38. (**a**) The expression of STK38 protein examined by the western blotting of total proteins isolated from various cell lines probed with a polyclonal antibody to STK38. The Rh-1a cell line is a radio-resistant mutant derived from MOLT-4 cells. (**b**) Localization of STK38 in cells. Subcellular fractionation was performed using 293T cells, and each fraction was subjected to western blotting with the indicated antibodies. MEK1, Bcl-2 and Sp-1 were positive controls for cytosolic, mitochondrial and nuclear protein, respectively. STK38, serine-threonine kinase 38.

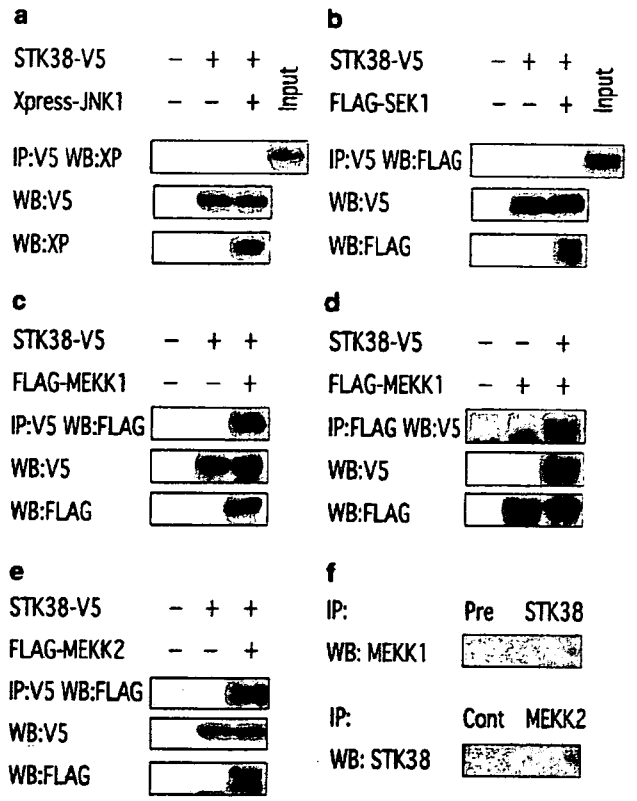


**Figure 2** STK38 interacts with MEKK1 or MEKK2 but not SEK1 or JNK1. (**a**, **b**) 293T cells were transfected with STK38-V5 alone or with Xpress-JNK1 (**a**) or FLAG-SEK1 (**b**) and subjected to immunoprecipitation (IP) using an anti-V5 antibody. The immunoprecipitates and cell lysates were analysed by western blotting (WB) with anti-Xpress, anti-FLAG or anti-V5 antibody, as indicated. (**c**–**e**) 293T cells were transfected with STK38-V5 alone or with FLAG-MEKK1 (**c**, **d**) or FLAG-MEKK2 (**e**) for immunoprecipitation using anti-V5 (**c**, **e**) or anti-FLAG antibody (**d**). The immunoprecipitates and cell lysates were analysed by western blotting with anti-FLAG or anti-V5 antibody, as indicated. (**f**) STK38 physically interacts with MEKK1 and MEKK2. Cell lysates from HeLa cells were subjected to immunoprecipitation with rabbit pre-immune serum (Pre), anti-STK38 serum, anti-rabbit immunoglobulin G or anti-MEKK2 antibody. The immunoprecipitates were analysed by western blotting with anti-MEKK1 (top) or anti-STK38 antibody (bottom). STK38, serine-threonine kinase 38.

of STK38 for various components of the JNK cascade were examined in co-transfection experiments. We transiently expressed V5-tagged full-length STK38 with Xpress epitope-tagged JNK1, FLAG-SEK1, or FLAG-MEKK1 in 293T cells. The STK38-V5 proteins were recovered from cell extracts by immunoprecipitation, and the precipitates were examined for JNK cascade components by western blotting with anti-Xpress or anti-FLAG antibody. The results showed that STK38 interacted with MEKK1, but no binding was detected with either JNK1 or SEK1 (Figures 2a–d). We further examined whether STK38 bound other MAPKKKs, and found that STK38 also interacted with MEKK2 (Figure 2e). Next, the physical interaction between STK38 and MEKK1/2 was evaluated. Endogenous STK38 was immunoprecipitated from HeLa cells using anti-STK38 serum. The immunoprecipitates were subjected to western blotting with anti-MEKK1 antibody. The results showed that STK38 physically interacted with MEKK1 (Figure 2f, top). Western blotting analysis of the MEKK2 immunoprecipitates using anti-STK38 antibody indicated that MEKK2 also physically associated with STK38 (Figure 2f, bottom). Moreover, we investigated the effects of extracellular stimuli on STK38 kinase activity and on the interaction between MEKK2 and STK38. Exposure of 293T cells to sorbitol or anisomycin did not modulate the STK38 kinase activity or the binding activity of MEKK2 to STK38 (Supplementary Figure 1 and data not shown).

*STK38 inhibits the activities of MEKK1 and MEKK2*

Expression of full-length MEKK1 or MEKK2 by transient transfection in cells leads to their auto-activation and the subsequent activation of the stress-activated protein kinase cascades, also known as the JNK and p38 cascades (Minden *et al.*, 1994; Yan *et al.*, 1994; Blank *et al.*, 1996; Cheng *et al.*, 2000). To investigate the possible effects of STK38 on the stress-activated protein kinase signaling pathway, we first examined whether MEKK1-induced SEK1 phosphorylation was influenced by the co-transfection of STK38 in 293T cells. Overexpression of full-length MEKK1 induced a marked increase in endogenous SEK1 phosphorylation, and the co-transfection of STK38 inhibited MEKK1-induced SEK1 phosphorylation in a dose-dependent manner (Figure 3a). We next examined the effects of a kinase-inactive mutant STK38 (K118A) on MEKK1-induced SEK1 phosphorylation; this construct, STK38 (K118A), had a point mutation in the catalytic site at residue Lys-118, and had no STK38 kinase activity (Figure 3b). The expression of kinase-inactive STK38 (K118A) did not fully inhibit MEKK1-induced phosphorylation of endogenous SEK1 (Figure 3c). To confirm that STK38 is a negative regulator of MEKK1, we examined the ability of MEKK1 to phosphorylate glutathione *S*-transferase (GST)-SEK1 (K129R) in the presence or absence of STK38. Consistent with the results for endogenous SEK1 phosphorylation, wild-type STK38 inhibited the MEKK1 activity but STK38 (K118A) did not (Figure 3c, bottom). Moreover, we examined the role of STK38 in MEKK2 signaling. Overexpression of STK38 significantly suppressed MEKK2 activity and MEKK2-mediated phosphorylation of endogenous SEK1 or MKK7, another downstream target of MEKK2. However, STK38 (K118A) failed to suppress MEKK2 activation (Figure 3d). We further investigated whether STK38 is involved in the p38 signaling pathway. Transfection of full-length MEKK1 into 293T cells caused the phosphorylation of p38, and this phosphorylation was weakly inhibited by STK38 (Figure 3f).

*STK38 interacts with the catalytic domain of MEKK1 and MEKK2, and inhibits ΔMEKK1-induced SEK1 phosphorylation*

To define the region of MEKK1 responsible for its interaction with STK38, a series of FLAG-tagged or

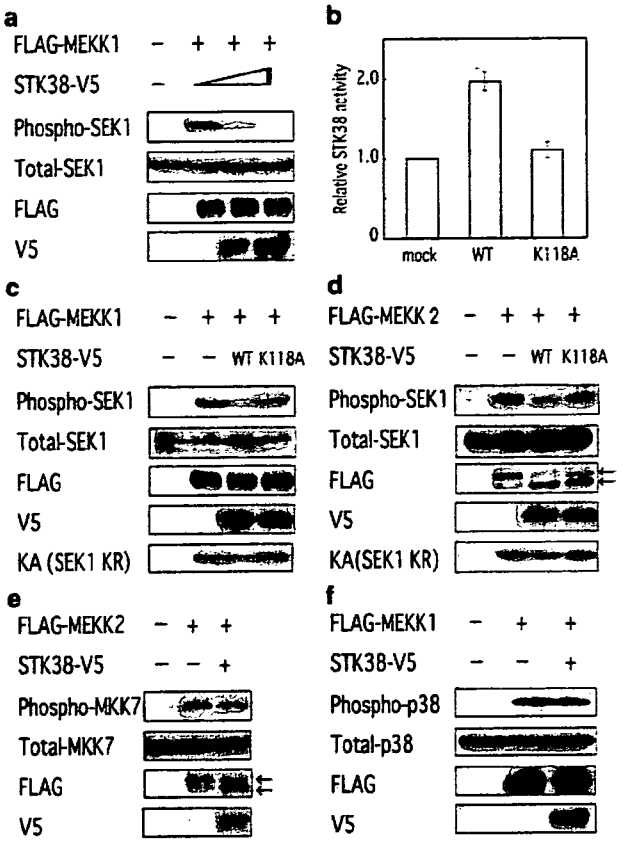


**Figure 3** STK38 inhibits the activation of MEKK1/2. (**a**, **c**–**f**) 293T cells were co-transfected with FLAG-MEKK1 (**a**, **c**, **e**) or FLAG-MEKK2 (**d**) alone or with STK38-V5 or STK38 (K118A)-V5. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody or western blotting with the indicated antibodies. The immunoprecipitates were analysed for MEKK1/2 activity by immune complex kinase assay (KA) with GST-SEK1 (KR) as the substrate. FLAG-MEKK2 showed two major bands: the faster and slower migrating bands are indicated by arrows. (**b**) STK38 kinase assay. 293T cells were transfected with the mock control, STK38-V5 or STK38 (K118A)-V5. The cell lysates were subjected to immunoprecipitation with anti-V5 antibody and the resultant immunoprecipitates were analysed for STK38 kinase activity by immune complex kinase assay with a synthetic peptide as the substrate. STK38 activity was measured with a liquid scintillation counter. The data are representative of three independent experiments. STK38, serine-threonine kinase 38.

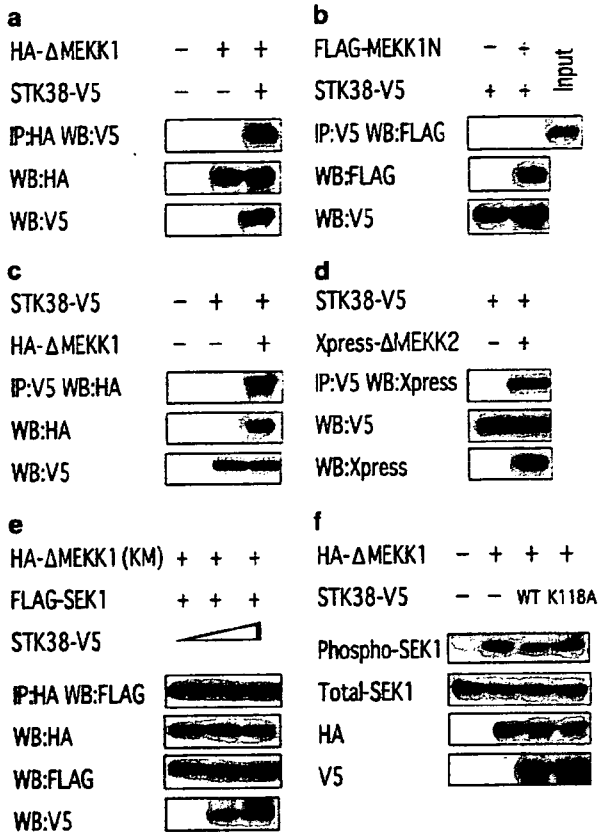


**Figure 4** STK38 interacts with the carboxy-terminal region containing the catalytic domain of MEKK1/2. (**a**–**d**) 293T cells were co-transfected with STK38-V5 and HA-ΔMEKK1 (1169 1488) (**a**, **c**), FLAG-MEKK1N (1 640) (**b**) or Xpress-ΔMEKK2 (342 619) (**d**), and subjected to immunoprecipitation (IP) using anti-HA (**a**) or anti-V5 (**b**–**d**) antibody. The immunoprecipitates and cell lysates were analysed by western blotting (WB) with the indicated antibodies. (**e**) STK38 does not disrupt the interaction between the catalytic domain of MEKK1 and SEK1. 293T cells were co-transfected with HA-ΔMEKK1 (1169 1488, KM) and FLAG-SEK1 in the absence or presence of STK38-V5, followed by immunoprecipitation with anti-HA antibody. The immunoprecipitates were analysed by western blotting with anti-FLAG antibody. (**f**) STK38 inhibits ΔMEKK1-mediated phosphorylation of SEK1. 293T cells were co-transfected with HA-ΔMEKK1 and STK38-V5 or STK (K118A)-V5. The cell lysates were analysed by western blotting with the indicated antibodies. STK38, serine-threonine kinase 38.

HA-tagged MEKK1 deletion mutants were co-transfected along with V5-tagged STK38 into 293T cells. A co-immunoprecipitation study showed that STK38 interacted with the catalytic domain in the carboxy terminus of MEKK1, termed ΔMEKK1, but not with the amino-terminal region (Figures 4a and b). The reciprocal experiment confirmed the interaction between STK38 and the catalytic domain of MEKK1 (Figure 4c). STK38 also associated with the carboxy-terminal region of MEKK2, termed ΔMEKK2 (Figure 4d). On the other hand, we investigated the binding region of STK38 involved in the interaction with MEKK1. We found that the region encompassing amino acids 87–465 of STK38, which encodes the catalytic domain, was co-immunoprecipitated with MEKK1 (Supplementary Figure 2b). Thus, the amino-terminal region of STK38 is not required for the interaction with MEKK1. The carboxy terminus of MEKK1 is necessary for its interaction with SEK1 (Xia *et al*., 1998). Therefore, we examined whether STK38 interfered with MEKK1–SEK1 complex formation. HA-tagged ΔMEKK1 (KM), a MEKK1 mutant in which the catalytic domain in the COOH terminus was inactive, was introduced into 293T cells along with FLAG-SEK1 with or without STK38-V5. A co-immunoprecipitation study showed that ΔMEKK1 (KM) interacted with SEK1 and this interaction was not disrupted by STK38 (Figure 4e).

Transfection of cell lines with ΔMEKK1, an active form of MEKK1, activated the JNK pathway (Yan *et al*., 1994).

We then examined the effects of STK38 on ΔMEKK1-induced SEK1 phosphorylation. As shown in Figure 4f, wild-type STK38 inhibited the phosphorylation of endogenous SEK1 by ΔMEKK1. The kinase-inactive mutant STK38 (K118A) partially suppressed ΔMEKK1-induced SEK1 phosphorylation, however, the effect was weak and was significantly different from that of wild-type STK38.

*STK38 converts MEKK2 from its phosphorylated to its nonphosphorylated form and inhibits MEKK2 autophosphorylation*

When transiently expressed, full-length MEKK2 displayed multiple protein bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 5a). Interestingly, co-transfection with STK38 significantly reduced the bands showing slower mobility and increased the faster migrating bands of MEKK2. Following treatment with a protein phosphatase or transfection with a kinase-inactive MEKK2 mutant such as MEKK2 (KM) alone or with STK38, only the faster migrating bands were observed. Therefore, the slower migrating bands of MEKK2 were phosphorylated species and the faster migrating bands were nonphosphorylated or hypo-phosphorylated species, suggesting that STK38 converts MEKK2 from its phosphorylated to its nonphosphorylated form. MEKK2 undergoes autophosphorylation, which is required for its activity (Cheng *et al.*, 2005a). Thus, we investigated the effect of STK38 on the autophosphorylation of MEKK2. GST-tagged MEKK2 or the MEKK2 (KM) mutant was mixed with an equivalent amount of immunopurified STK38 in the presence of [$\gamma$-$^{32}$P]ATP to quantify the kinase reaction. As shown in Figure 5b, wild-type MEKK2 underwent autophosphorylation, but MEKK2 (KM) did not. The $^{32}$P incorporated by wild-type MEKK2 was significantly decreased to 50% of the control levels by co-expression of wild-type STK38, indicating that STK38 significantly inhibited MEKK2 autophosphorylation activity. On the other hand, MEKK2 autophosphorylation was only weakly suppressed by the kinase-inactive mutant STK38 (K118A). These results indicate that STK38 inhibits MEKK2 activation by suppressing its autophosphorylation.

*MEKK2 can form dimers in the presence of STK38 but is not a substrate for STK38*

MEKK2 forms a dimer, leading to its activation through transphosphorylation (Cheng *et al.*, 2005a). As STK38 appeared to act as a negative regulator of MEKK2, one possibility was that STK38 interacted with the dimerization motif, preventing MEKK2 from forming a dimer, and hence inhibiting its activation. To assess this possibility, we co-transfected cells expressing FLAG-MEKK2 with Xpress-MEKK2 in the absence or presence of STK38-V5, and the cell lysates were immunoprecipitated with an anti-FLAG antibody. As shown in Figure 6a, Xpress-MEKK2 was co-precipitated by FLAG-MEKK2, indicating that MEKK2 formed dimers. The dimer formation of MEKK2 was not inhibited by co-expression

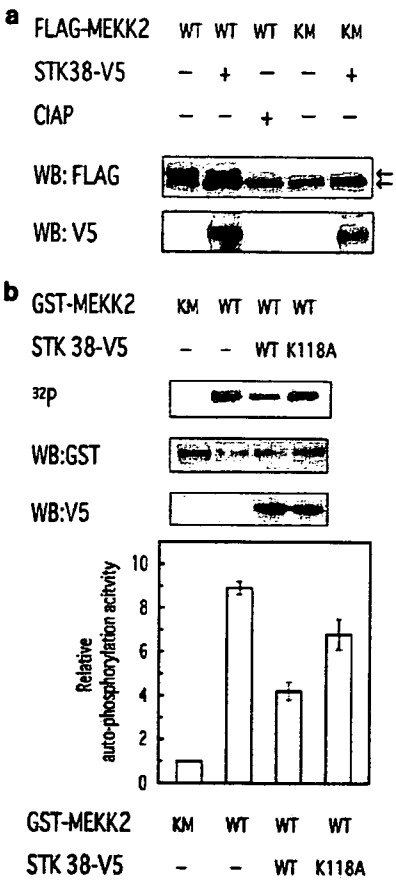


**Figure 5** STK38 converts MEKK2 from its phosphorylated to its nonphosphorylated form and inhibits the autophosphorylation of MEKK2. (**a**) STK38 converts MEKK2 from its phosphorylated to its nonphosphorylated form. 293T cells were transfected with FLAG-wild-type MEKK2 or FLAG-MEKK2 (KM) alone, or with STK38-V5. The cell lysates were subjected to western blotting analysis with anti-FLAG or anti-V5 antibody. For calf intestine phosphatase (CIAP) treatment, the cell lysates were incubated with or without CIAP at 30°C for 30 min before western blotting analysis. (**b**) STK38 inhibits MEKK2 autophosphorylation. *In vitro* kinase reaction was performed by incubating GST-MEKK2 alone or with the immunopurified wild-type STK38-V5 or STK38 (K118A)-V5 from the transfected 293T cells. The kinase reaction products were subjected to autoradiography (upper). The autophosphorylation activity of MEKK2 was measured using a phosphoimaging device and the results are shown as a *bar graph*. The data are representative of three independent experiments. Expression of GST-MEKK2 or STK38-V5 was confirmed by western blotting analysis using anti-GST or anti-V5 antibody, respectively. STK38, serine-threonine kinase 38; WB, western blotting; GST, glutathione *S*-transferase.

of STK38, indicating that the binding domain of MEKK2 for STK38 is different from the dimerization motif. Indeed, a co-immunoprecipitation assay indicated that MEKK2 with deletion of the dimerization motif (342–424) still interacted with STK38 (Supplementary Figure 2a). To determine whether the inhibitory action of STK38 on MEKK2 activity is mediated by STK38-catalysed MEKK2 phosphorylation, we immunopurified STK38-V5 from 293T cells and performed an *in vitro* STK38 kinase assay using GST-MEKK2 (KM) as a

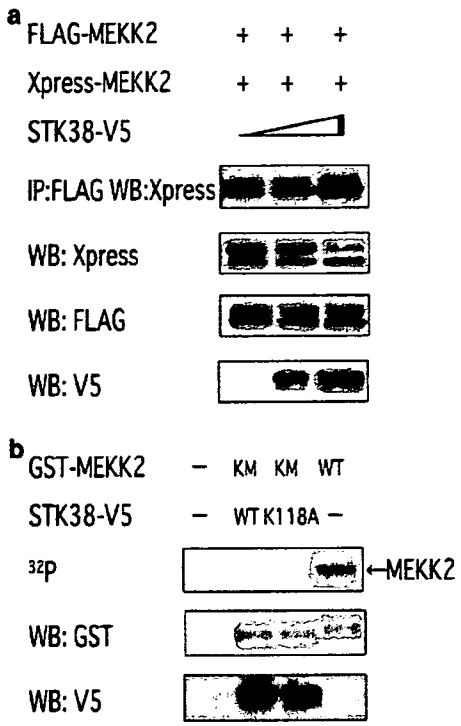


**Figure 6** MEKK2 forms dimers in the presence of STK38 but is not a substrate for STK38. (**a**) STK38 does not interfere with MEKK2 dimer formation. 293T cells were co-transfected with FLAG-MEKK2 and Xpress-MEKK2 in the absence or presence of STK38-V5. The cell lysates were subjected to immunoprecipitation using anti-FLAG antibody. MEKK2 dimer formation was evaluated by detecting Xpress-MEKK2 in the FLAG-immunoprecipitates by western blotting. (**b**) MEKK2 is not a substrate of STK38. 293T cells were transfected with STK38-V5 or STK38 (K118)-V5. The cell lysates were subjected to immunoprecipitation with anti-V5 antibody. The immunoprecipitates were analysed for STK38 kinase activity by immune complex kinase assay (KA) with GST-MEKK2 (KM) as the substrate. The kinase reaction products were subjected to SDS PAGE and analysed by autoradiography. STK38, serine-threonine kinase 38.

substrate. Autophosphorylation of GST-wild-type MEKK2 was observed in the absence of STK38. However, direct phosphorylation of GST-MEKK2 (KM) or GST-ΔMEKK1 (KM) by STK38 was not detected (Figure 6b and data not shown). These results indicate that MEKK1 or MEKK2 is not a substrate for STK38.

*Knockdown of STK38 enhances sorbitol-induced endogenous MEKK2 activation*

To confirm that STK38 acts as a negative regulator of MAPKKK, we constructed an *stk38* shRNA expression vector. As shown in Figure 7, transfection with the *stk38* shRNA, but not the control expression vector, specifically knocked down expression of the endogenous STK38 protein in HeLa cells (Figure 7, bottom). We next examined how STK38 knockdown affected the stress-induced activation of MAPKKK. Treatment with sorbitol resulted in the activation of various MAPKKKs, including MEKK1/2 (Chen *et al*., 2002; Zhang *et al*., 2006). The

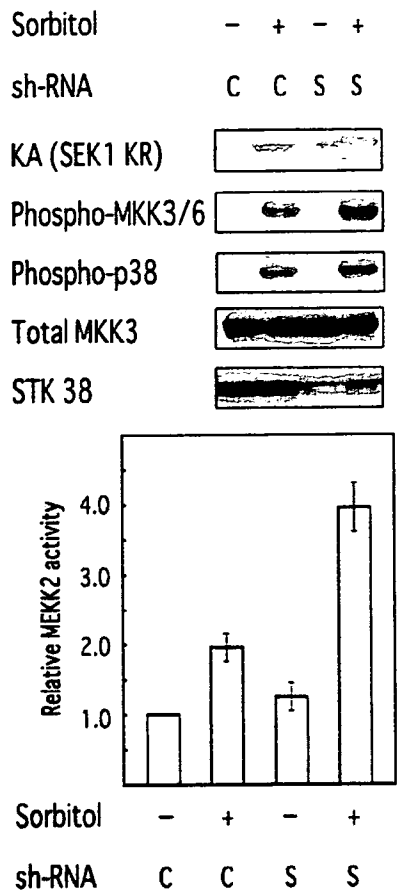


**Figure 7** Knockdown of STK38 enhances sorbitol-induced MEKK2 activation. HeLa cells were transfected with the negative control (C) or *stk38*-specific shRNA (S) expression vector. At 60 h after transfection, the cells were treated with 0.2 M sorbitol for 1 h or left untreated as controls and harvested. The cell lysates were subjected to immunoprecipitation with anti-MEKK2 antibody or western blotting with the indicated antibodies. The immunoprecipitates were analysed for MEKK2 activity by immune complex kinase assay (KA) with GST-SEK1 (KR) as the substrate. MEKK2 activity was measured using a phosphoimaging device and the results are shown as a *bar graph*. The data are representative of three independent experiments. STK38, serine-threonine kinase 38; shRNA, short hairpin RNA.

specific *stk38* shRNA enhanced sorbitol-induced activation of MEKK2 (Figure 7, top), subsequent phosphorylation of the downstream MAPKKs, MKK3/6, and of their targets p38 MAPK, as compared with those of the control. Exposure of HeLa cells to sorbitol or anisomycin preferentially activated the MKK3/6-p38 pathway, and sorbitol-induced phosphorylation of SEK1 or MKK7 was observed in neither control nor STK38 knockdown cells (data not shown).

## Discussion

MAPKKKs respond to various upstream stimuli and activate multiple MAPK signaling components. However,

it remains largely unclear how each MAPKKK is activated and which molecules regulate each MAPKKK. The overexpression of most MAPKKKs activates MAPK signaling pathways in mammalian cells (Yan *et al*., 1994; Blank *et al*., 1996). The results of these transfection studies suggest that MAPKKKs may normally be negatively regulated by inhibitors or intracellular compartmentalization.

The results of the present co-transfection studies demonstrated that the STK38 interacts with MEKK1/2, but not with either SEK1 or JNK1. We also showed that STK38 physically interacts with MEKK1/2 *in vivo*. The carboxy-terminal region, but not the amino-terminal region, of MEKK1 or MEKK2 was required for binding STK38. The carboxy terminus of MEKK1 is necessary for its interaction with SEK1 (Xia *et al*., 1998). A recent study demonstrated that GST M1-1 functions as a negative regulator of MEKK1 by interfering with the binding of MEKK1 to SEK1 (Ryoo *et al*., 2004). However, STK38 did not interfere with the MEKK1/SEK1 complex formation, suggesting that STK38 inhibits MEKK1 in a manner distinct from that of GST M1-1. The carboxy terminus of MEKK2 is necessary for the dimer formation (Cheng *et al*., 2005a). However, we showed that the dimerization motif of MEKK2 was not required for the interaction with STK38.

Full-length MEKK1 or MEKK2 was activated when overexpressed in 293T cells. Co-expression of MEKK1/2 with STK38 decreased the MEKK1/2 activity and inhibited phosphorylation of the downstream SEK1 or MKK7. MEKK1 also activates the MKK3/6-p38 signaling pathways (Lin *et al*., 1995). As expected, overexpression of STK38, at least partially, inhibited the MEKK1-induced phosphorylation of p38. Recent studies have indicated that MEKK2 can undergo autophosphorylation and activation through dimer formation (Cheng *et al*., 2005a) and that Mip1, a MEKK2-interacting protein, inhibits MEKK2 activation by preventing its dimer formation (Cheng *et al*., 2005b). However, we demonstrated that STK38 inhibits the autophosphorylation of MEKK2 *in vitro* and suppresses the activation of MEKK2 without interfering with its dimer formation in a transient transfection assay. In addition, MEKK1 activity is regulated by autophosphorylation (Deak and Templeton, 1997). Thus, STK38 may inhibit the activation of MEKK1 by interfering with its autophosphorylation.

STK38 is a serine-threonine protein kinase that belongs to a subfamily of the AGC family of kinases (Manning *et al*., 2002). However, natural substrates for STK38 have not been reported. It is possible that the inhibitory action of STK38 on MEKK2 activity is mediated by STK38-catalysed MEKK2 phosphorylation. However, our results indicated that MEKK2 is not a substrate for STK38 *in vitro*. These results suggest that MEKK1/2 activation is not inhibited through direct phosphorylation by STK38. On the other hand, the binding activity of STK38 (K118A) to MEKK1/2 was similar to that of wild-type STK38 (data not shown), suggesting that the kinase activity of STK38 may be necessary for inhibition of the activation of MEKK1/2. This raises the question of how STK38 regulates the activity of MEKK1/2. The kinase activity of STK38 may be necessary to recruit a negative regulator, such as a protein phosphatase, to MEKK1/2 and activate it, or STK38 may negatively regulate a putative activator of MEKK1/2, such as an MAPKKK kinase. Alternatively, it is possible that STK38 binds to the catalytic domain of MEKK1/2, disrupting its proper conformation for autophosphorylation.

Although the results of the present study established that STK38 functions as a negative regulator of MEKK1/2 signaling, it is likely that STK38 is also a critical regulator of MEKK3 as the STK38-binding motif in MEKK2 is conserved between MEKK2/3. Knockdown of STK38 by transfection with an STK38-specific shRNA expression vector enhanced sorbitol-induced activation of MEKK2 and subsequent phosphorylation of the downstream MAPKKs, MKK3/6. Taken together, our findings support the suggestion that STK38 is a natural negative regulator of MAPKKK, including at least MEKK1/2 and perhaps also MEKK3.

## Materials and methods

*Cell culture, transfection, and subcellular fractionation*

HEK293T, HeLa, MCF-7, and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA) and 1% penicillin/streptomycin (Sigma). MOLT-4 cells and the derivatives were grown in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For DNA transfection, the cells were plated, grown for 24 h, and transfected for 24–48 h with the appropriate expression vectors using FuGENE 6 (Roche, Mannheim, Germany). Empty pcD 3.1 vector was used to keep the total amount of DNA equivalent for each transfection. Subcellular fractionation was performed using a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany).

*Antibodies*

A polyclonal antibody against human STK38 was generated by injecting rabbits with a synthetic peptide (CEGLKDEEKRLRRSA) corresponding to amino-acid residues 55–68 of human STK38 and was purified by peptide affinity chromatography. Anti-SEK1/MKK4, anti-phospho SEK1/MKK4 (Thr261), anti-stress-activated protein kinase/JNK, anti-phospho stress-activated protein kinase/JNK (Thr183/Tyr185), anti-p38, anti-phospho p38 (Thr180/Tyr182), anti-MEK1, anti-MKK7, anti-phospho MKK7 (Ser271/Thr275) and anti-MKK3 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-V5, anti-HA and anti-S antibodies were from MBL (Nagoya, Japan). Anti-Xpress and anti-Bcl-2 antibodies were from Invitrogen (Carlsbad, CA, USA) and BD Bioscience Pharmingen (San Diego, CA, USA), respectively. Anti-FLAG (M2), anti-phospho-MKK3/6 (Ser189/207) and antiβ-actin antibodies were from Sigma. Anti-MEKK1, anti-MEKK2 and anti-Sp1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MEKK2 antibody was from Epitomics (Burlingame, CA, USA).

*Western blotting analysis*

The cells were washed twice with ice-cold phosphate-buffered saline, and the cell pellets were lysed with standard SDS–PAGE