

Supplemental Information

DNA Damage Activates a Spatially Distinct Late Cytoplasmic Cell-Cycle Checkpoint Network Controlled by MK2-Mediated RNA Stabilization

H. Christian Reinhardt, Pia Hasskamp, Ingolf Schmedding, Sandra Morandell, Marcel A.T.M. van Vugt, XiaoZhe Wang, Rune Linding, Shao-En Ong, David Weaver, Steven A. Carr, and Michael B. Yaffe

Supplemental Information Inventory

Supplemental Figures

- Figure S1. *RNAi-mediated knockdown efficiency, legend*
- Figure S2. *MK2 activity is essential to maintain a prolonged cell cycle arrest following DNA damage, legend*
- Figure S3. *MK2 undergoes p38MAPK- and Crm1-dependent translocation from the nucleus to the cytoplasm in response to genotoxic agents, while Chk1 remains in the nucleus, legend*
- Figure S4. *Nuclear and basophilic kinase activities are required for a functional cell cycle checkpoint response, legend*

Supplemental Experimental Procedures

- Chemicals and antibodies
- Cell culture and virus production
- Plasmids and RNAi
- Flow cytometry
- Immunohistochemistry
- Immunofluorescence
- Immunoprecipitation
- Immunoblotting
- In vitro kinase assays
- Mass spectrometric analyses

Supplemental Figures

Figure S1

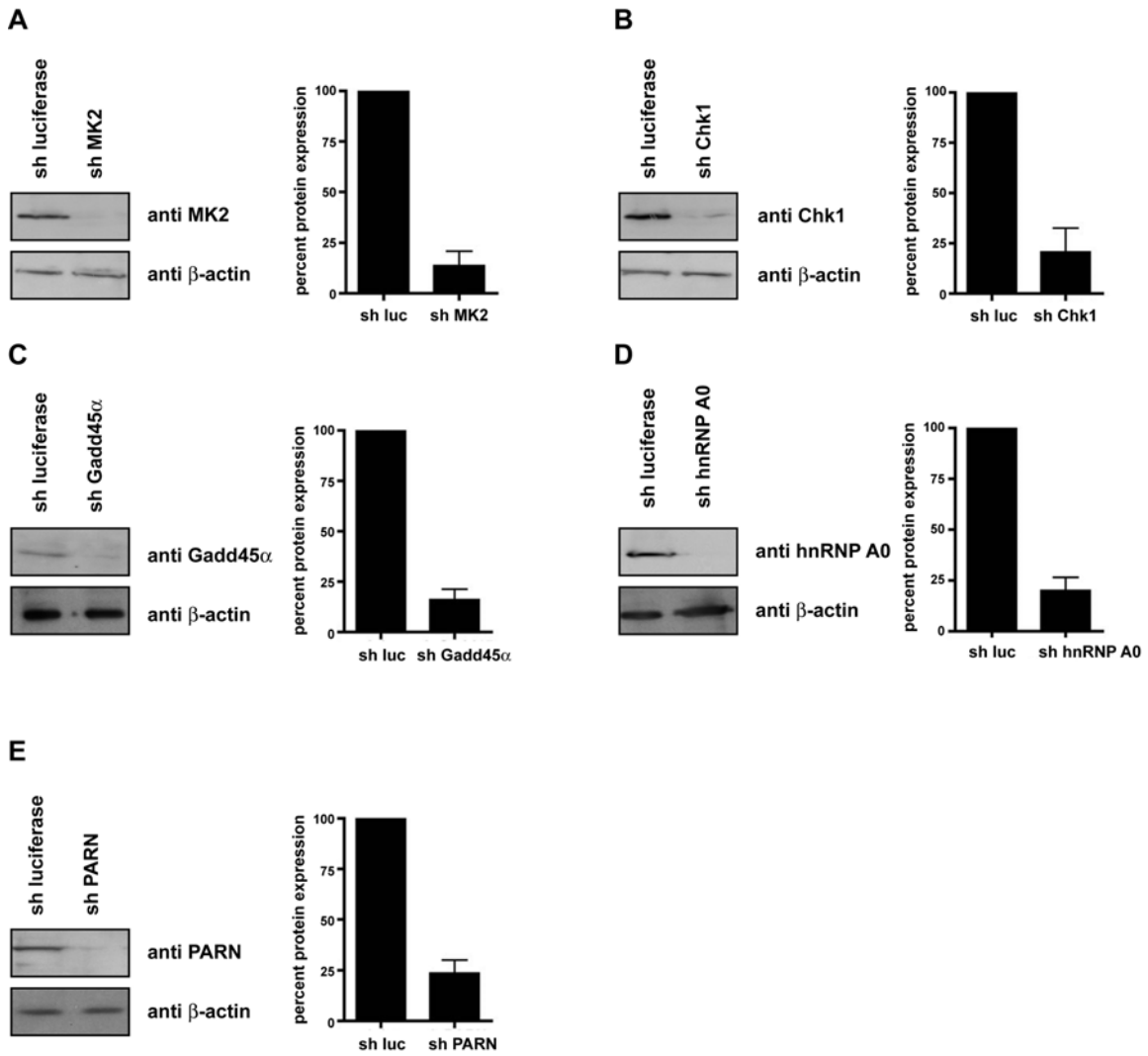


Figure S1. RNAi-mediated knockdown efficiency.

Cells were infected with lentiviruses expressing shRNAs targeting (A) MK2; (B) Chk1; (C) hnRNP A0; (D) Gadd45a or (E) PARN. Target cells were incubated with viral supernatants for 24 hr in the presence of 8 μ M polybrene. After three cycles of infection, cells were selected in puromycin for 48 hr before lysis and assessment of knockdown efficiency by SDS-PAGE. Knockdown efficiency was quantified in separate immunoblots from 3 independent experiments and normalized to β -actin expression using ImageQuant software. Mean values and standard deviations are shown. Panels A and B were performed in U2OS cells, panels C-E were performed in HeLa cells.

Figure S2

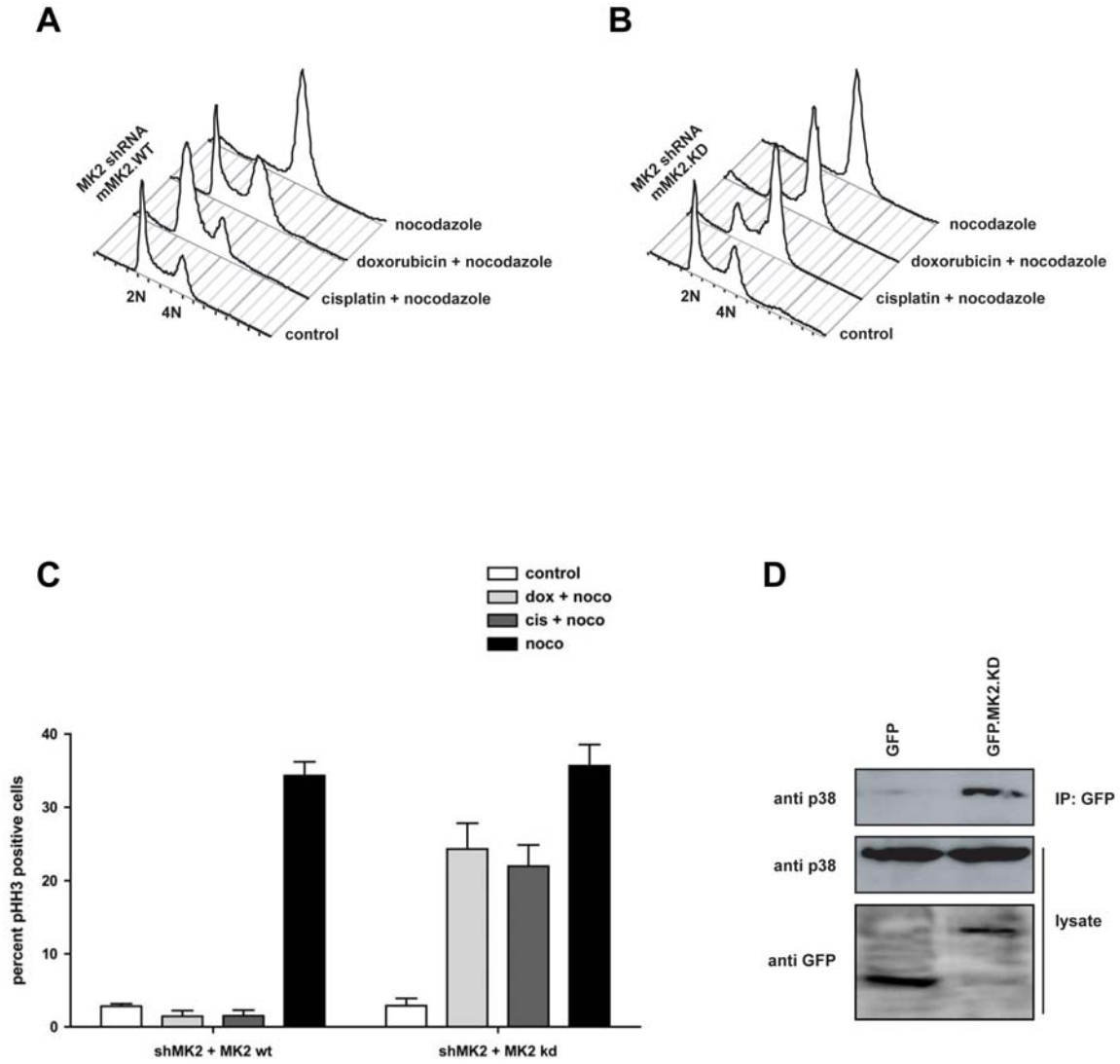


Figure S2. MK2 activity is essential to maintain a prolonged cell cycle arrest following DNA damage.

(A, B) Human U2OS cells were depleted of MK2 using shRNA, and complemented with either shRNA-resistant wildtype murine MK2 (panel A) or a kinase-dead isoform (panel B). Cells were either left untreated (control) or treated with 10 μ M of the indicated DNA damaging agents in a 30 hr nocodazole trap experiment, and analysed by flow cytometry. Cells that were complemented with wildtype MK2 were checkpoint competent, while cells that were complemented with the kinase-dead mutant were unable to maintain a functional cell cycle checkpoint as revealed by the accumulation of cells containing 4N DNA content.

(C) Quantification of the mitotic pHH3 staining of the samples shown in panels **A** and **B** (n=6 independent experiments. Mean values and standard deviations are shown.).

(D) Kinase-dead MK2 interacts with p38. Exogenous GFP-tagged kinase-dead MK2 was immunoprecipitated using anti-GFP antibodies. The immunoprecipitated material was resolved on SDS-PAGE gels, and immunoblotted using anti-p38 antibodies. Immunoprecipitation of exogenous GFP served as a control. A strong interaction between GFP.MK2.kd and endogenous p38 could be detected, while no interaction was seen between the GFP control and p38.

Figure S3

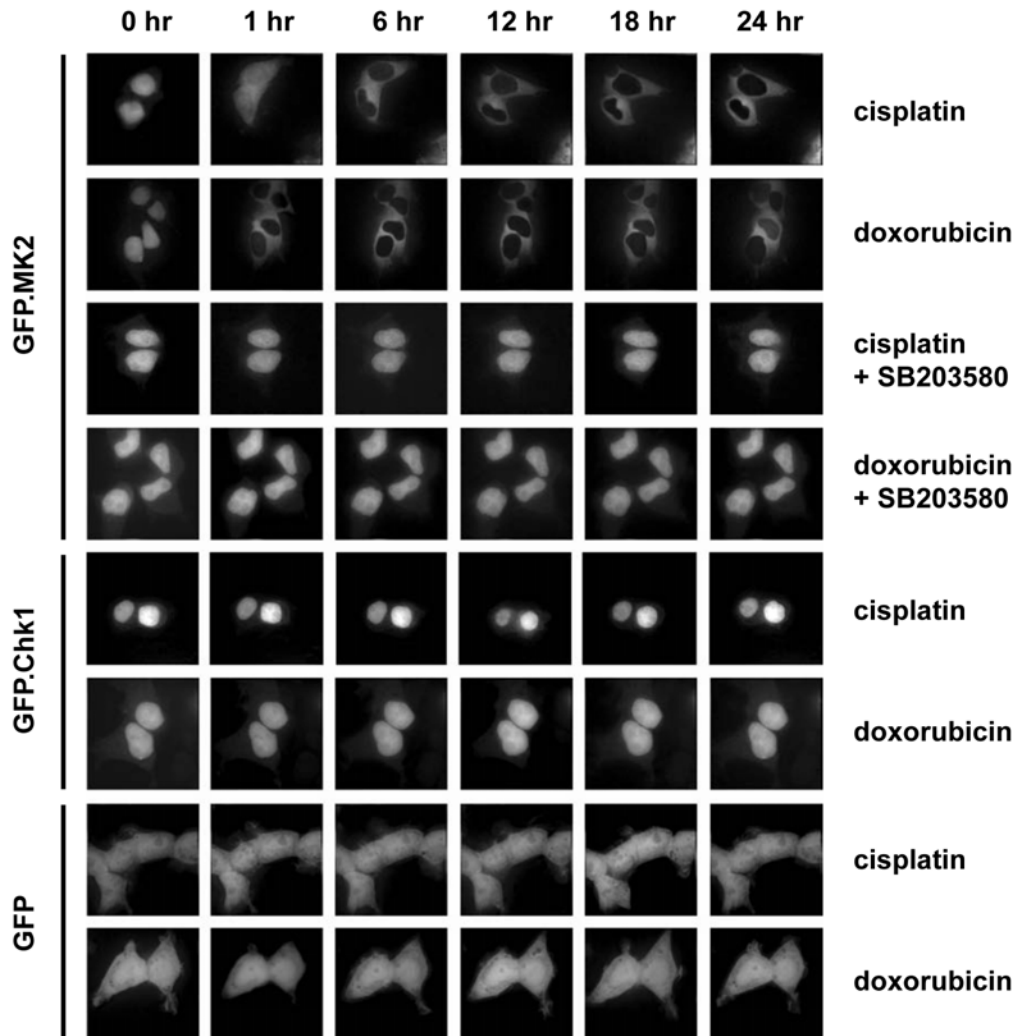


Figure S3. MK2 undergoes p38MAPK- and Crm1-dependent translocation from the nucleus to the cytoplasm in response to genotoxic agents, while Chk1 remains in the nucleus.

GFP.MK2 (top panels) and GFP.Chk1 (middle panels) fusion proteins were stably expressed at low levels in U2OS cells and their dynamic localization in response to the indicated treatments was analyzed at various times using live cell imaging as in Fig. 2. Unfused GFP served as a control (bottom panels). Treatment with 10 μ M cisplatin and doxorubicin induced a rapid relocalization of MK2 from the nucleus to the cytoplasm. This re-localization was completely abolished in the presence of 10 μ M of the p38 inhibitor SB203580. Chk1 remained predominantly nuclear following treatment with cisplatin or doxorubicin. Unfused GFP was dispersed throughout the nucleus and the cytoplasm and no obvious shifts in this localization pattern were seen following treatment with cisplatin or doxorubicin.

Figure S4

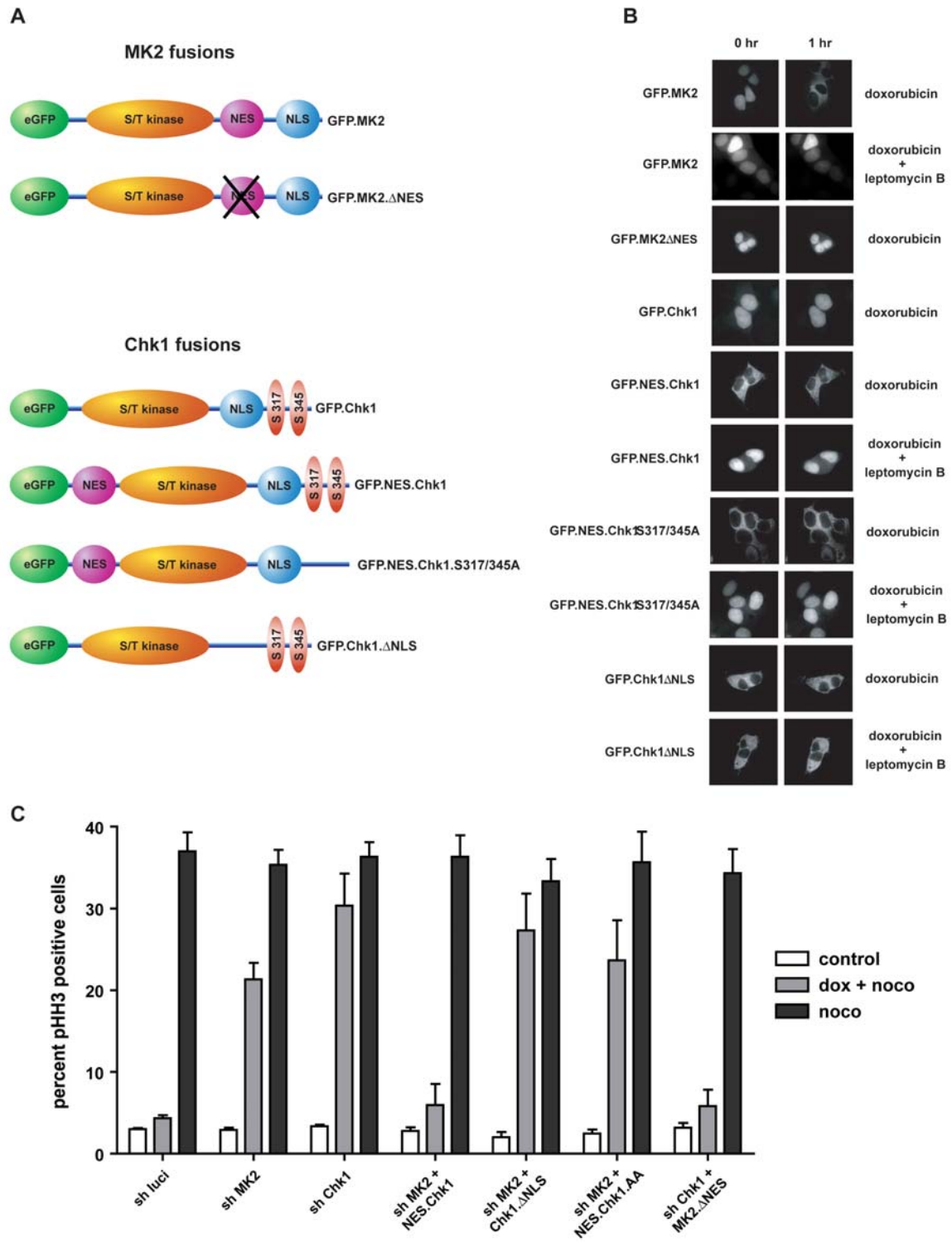


Figure S4. Nuclear and basophilic kinase activities are required for a functional cell cycle checkpoint response.

(A) Structural representation of the modular domain organization of the localization and kinase activation mutants that were used.

(B) Localization patterns of the MK2 and Chk1 mutants indicated in panel A. Wild-type and mutant constructs were transfected into U2OS cells, and live cell images obtained prior to, and 1 hr following, treatment with 10 μ M doxorubicin. Of note, inactivation of the NES in MK2 results in a mutant with abolished ability to localize to the cytoplasm following genotoxic stress (compare rows 1 and 3), similar to that seen after inhibition of Crm1-dependent nuclear export of wild-type MK2 with 20 nM leptomycin B (row 2). Fusion of the MK2 NES between GFP and Chk1 produces a mutant that is localized primarily to the cytoplasm (compare rows 4 and 5). This mutant retains the ability to shuttle between nucleus and cytoplasm, as evidenced by nuclear trapping following 12 hr treatment with 20 nM leptomycin B (row 6). A NES-fused Chk1 mutant in which the ATR phosphorylation sites Ser-317 and Ser-345 are mutated to Ala localizes with an identical pattern as the NES-fused wildtype counterpart (rows 7 and 8). Mutational inactivation of the Chk1 NLS produces a mutant that is exclusively localized to the cytoplasm (row 9). This mutant cannot be trapped in the nucleus with leptomycin treatment (row 10).

(C) Functional assessment of the ability of the localization mutants to establish and maintain cell cycle checkpoints. HeLa cells were infected with lentiviruses expressing luciferase control shRNA, MK2-specific shRNA or shRNA targeting

Chk1. Knockdown cells were complemented with the localization mutants as indicated. Cells were treated with doxorubicin in a 30 hr. nocodazole trap experiment and cell cycle profiles were assessed by FACS using phospho-histone H3 staining to monitor the percentage of cells that had escaped doxorubicin-induced cell cycle checkpoints. Loss of nuclear Chk1 could be functionally compensated by expression of the MK2 mutant that was re-targeted to the nucleus (MK2. Δ NES), while loss of cytoplasmic MK2 could be rescued by expression of the Chk1 mutant that was mis-localized to the cytoplasm but retained the ability to shuttle between nucleus and cytoplasm and (GFP.NES.Chk1). A non-activatable Chk1 mutant (GFP.NES.Chk1.AA) or a non-shuttling Chk1 mutant (GFP.Chk1.DNLS) failed to rescue the MK2 RNAi phenotype. Mean values and standard deviations are shown.

Supplemental Experimental Procedures

Chemicals and antibodies

Antibodies against pHH3 were from Upstate. Antibodies detecting Gadd45 α , GFP, histone H1, HuR, TIAR and TTP were from Santa Cruz. Anti-Flag (M2) and, β -actin antibodies and IgG were from Sigma. A second anti-GFP antibody and the anti- β -tubulin antibody were from Abcam. The PARN antibodies were purchased from Abnova and Abcam. p38, MK2 and Chk1 antibodies were from Cell Signaling Technology. The anti-HA antibody was purchased from Covance, the anti-6xHis antibody from Quiagen and the GST antibody from GE Healthcare.. Doxorubicin, cisplatin, acetonitrile (HPLC grade), formic acid (HPLC grade), leptomycin B and nocodazole were purchased from Sigma. Propidium iodide and SB203580 were purchased from Calbiochem.

Cell culture and virus production

HEK293T, U2OS and HeLa cells were cultured in DMEM supplemented with 10% FCS, L-Glutamin and penicillin/streptomycin at 37°C in a humidified incubator supplied with 5% CO₂. Amphotropic VSV-G pseudotyped lentiviruses encoding the indicated shRNAs were packaged in HEK293T cells using standard procedures (Rubinson et al., 2003), and all subsequent infections were performed in a BL2+ facility. Target cells were infected three times for 12 hr in the presence of 8 μ g/ml polybrene. Infected cells were selected in 8 μ g/ml puromycin for 2 days following the last infection.

Plasmids and RNAi

eGFP and eGFP.Gadd45 α -3'UTR were a kind gift from M. Gorospe (NIA-IRP, NIH). HA.hnRNP A0, HA.hnRNP A0.S84A, GST.hnRNP A0 and GST.hnRNP A0.S84A were kindly provided by P. Cohen (Dundee). His-tagged PARN in pET33 was kindly provided by A. Virtanen (Uppsala University). Retroviral packaging constructs pMDg and pMDg/p were a kind gift from T. Benzing (Cologne). Lentiviral packaging constructs pRSVrev, pMDg/pRRE and pVSV-G were provided by M. Hemann (MIT). shRNA constructs targeting MK2, Chk1 and luciferase have been described previously (Reinhardt et al., 2007). Gadd45 α 29mer shRNA constructs (in pRetroSuper) were obtained from Origene. MK2 and Chk1 eGFP fusion proteins were constructed in the pLNCX-2 (Clontech) retroviral backbone, using standard cloning procedures. GFP-fused MK2 and Chk1 localization constructs were assembled in the pLNCX-2 retroviral backbone. The NES in MK2 was disrupted through mutation of Leu-346 and Met-349 to Ala mutation. The NLS in Chk1 was disrupted through mutation of Arg-260/261/270/271 to Ala mutation. All mutations were generated using the Quickchange mutagenesis kit (Stratagene). N-terminally GFP-fused Cdc25B and C constructs were assembled in the pLNCX-2 backbone using standard cloning procedures. For mammalian expression PARN cDNA was subcloned into pLNCX-2 using standard procedures. TIAR and Hsp25 (AS 71-100, containing the MK2 phosphorylation motive) were subcloned into pGex-4T1 (GE Healthcare) using standard procedures. PARN shRNA was cloned into

pRetroSuper using the oligonucleotides 5'-GAT CCC **CTG GAG ATA ATC AGG**
AGC AAT TCA AGA GAT TGC TCC TGA TTA TCT CCA TTT TTG GAA A-3'
(forward) and 5'-AGC TTT TCC AAA AAT GGA GAT AAT CAG GAG CAA TCT
CTT GAA TTG CTC CTG ATT ATC TCC AGG G-3' (reverse). shRNA-resistant
PARN was generated in the pLNCX-2 backbone using the oligonucleotides 5'-
GGG GAC TAC AAG GAC GAC GAC GAC AAG ATG GAG ATT ATC AGG **TCA**
AAT TTT AAG AGT AAT CTT CAC AAA GTG TAC CAG GC-3' (forward) and 5'-
GCC TGG TAC ACT TTG TGA AGA TTA CTC TTA AAA TTT GAC CTG ATA
ATC TCC ATC TTG TCG TCG TCG TCC TTG TAG TCC CC-3' (reverse).
hnRNP A0 shRNA was cloned into pMLP using the oligonucleotides 5'-TGC TGT
TGA CAG TGA GCG AAA CGT TTA TGG ATA TCA CAA ATA GTG AAG CCA
CAG ATG TAT TTG TGA TAT CCA TAA ACG TTG TGC CTA CTG CCT CGG A-
3' (forward) and 5'-TCC GAG GCA GTA GGC ACA ACG TTT ATG GAT ATC
ACA AAT ACA TCT GTG GCT TCA CTA TTT GTG ATA TCC ATA AAC GTT
TCG CTC ACT GTC AAC AGC A-3' (reverse).

Flow cytometry

Flowcytometry experiments were performed as described earlier (Jiang et al., 2009). In brief, all transfections and treatments were conducted as indicated. Cells were then washed twice in ice-cold PBS, trypsinized and fixed in 70% ethanol overnight at -20°C, permeabilized with PBS containing 0.25% Triton X-100 for 20 min at 4°C, blocked with 2% FCS in PBS, and incubated with 1µg of anti-phospho-histone H3 per 10⁶ cells for 180 min at room temperature.

Following extensive washing, cells were incubated with Alexa488-conjugated secondary antibody (Invitrogen) (diluted 1:500 in PBS) for 90 min at room temperature, washed, and resuspended in PBS containing 50 µg/ml propidium iodide and RNase A prior to analysis on a BD FACScan flow cytometer.

Immunohistochemistry

Formalin-fixed, paraffin embedded sections of human head and neck tumor samples lacking any patient identifying information were obtained from previously evaluated pathological specimens following an IRB-approved protocol. Tissue sections were stained for immunohistochemistry using antibodies against phospho-MAPKAP Kinase2 (pT334-MK2; Cell Signaling Technology), phospho-HSP27 (pS78-HSP27; Epitomics) and γ -H2AX (Millipore). Tissue sections were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed and the tissues were stained with antibodies overnight at 4°C. The Super Sensitive™ IHC Detection System (BioGenex) was used for detection of pMK2, pHSP27 and γ -H2AX. Primary antibodies were omitted for negative control studies. Hematoxylin was used as nuclear counterstain.

Immunofluorescence

Cells were seeded onto 18mm² coverslips and either mock treated or treated with doxorubicin for 6 hr. Cells were then fixed in 3% PFA and 2% sucrose for 15 min at RT and permeabilized with 20mM Tris-HCl (pH7.8), 75mM NaCl, 300mM sucrose, 3mM MgCl₂, and 0.5% Triton-X-100 for 15min at RT. Cells were stained

with primary antibodies against MK2 or Chk1 at RT for 3 hr. After extensive washing, Alexa-488-conjugated secondary antibodies were used for 3 hrs at RT. Samples were then washed again and counterstained with Hoechst dye. Images were collected on an Axioplan2 microscope (Zeiss) using the Openlab software (Improvision).

Immunoprecipitation

U2OS cells were transfected with HA-tagged Gadd45 α or HA-tagged 14-3-3 ζ . 24 hr following transfection, cells were lysed in 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1.0% NP-40, 5 mM EDTA, 2 mM DTT, 8 μ g/ml pepstatin, 8 μ g/ml aprotinin, 8 μ g/ml leupeptin, 2 mM Na₃VO₄, 10 mM NaF, and 1 μ M microcystin for 15 min on ice. HA-tagged proteins were immunoprecipitated using protein G beads that were pre-coated with anti-HA antibodies. Immunoprecipitations were carried out for 2 hr at 4°C. Following washing, lysates and bead bound proteins were analyzed by SDS-PAGE, followed by transfer to PVDF membranes, and immunoblotting with the indicated antibodies.

Immunoblotting

Cells were lysed in 1ml of ice-cold lysis buffer (1% Triton X-100, 25mM CHAPS, 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 15 mM Na₄P₂O₇, 2 mM Na₃VO₄ and protease inhibitors) per 10-cm dish for 15 min on ice. Lysates were centrifuged at 4°C for 15 min at 14,000 rpm, and subsequently subjected to ultracentrifugation (100,000 g, 30 min, 4°C). 200 μ L of 6x Laemmli buffer was

added to the supernatants and protein concentrations were measured using the BCA Protein Assay Kit (Pierce). 100 µg of each lysate was run on 10% SDS-PAGE and transferred to PVDF (Millipore). The membranes were blocked with 5% BSA in 20 mM Tris- HCl (pH 7.5), 137 mM NaCl, 0.1% Tween-20 and stained with primary antibodies (1:1000 dilution, in TBS-T with 5% BSA) overnight at 4°C. Membranes were then washed in TBS-T and subsequently probed with HRP-conjugated secondary antibody (GE Healthcare) at a 1:5000 dilution and visualized by enhanced chemiluminescence (GE Healthcare). Anti β-actin or anti β-tubulin staining served as a loading control.

In vitro kinase assays

Recombinant 6xHis-tagged wildtype PARN or the SA mutant were expressed in BL21 cells from a pET33 vector, essentially as described previously (Nilsson and Virtanen, 2006). Recombinant GST-tagged hnRNP A0 or the SA mutant as well as recombinant GST, GST-tagged TIAR and Hsp25 (AS 71-100) were expressed from pGEX bacterial expression vectors. *In vitro* kinase assays were performed in identical 30 µl reactions containing 20 mM HEPES (pH 7.5), 10 mM MgCl₂, 3 mM β-mercaptoethanol, 100 µg/ml BSA, 50 µM ATP, 10 µCi ³²P-γ-ATP, and ~10µM His.PARN or GST.hnRNP A0 for the indicated times at 30°C. Recombinant MK2 (Millipore) was used at a concentration of 0.1 µM. *In vitro* kinase assays with GST, GST.TIAR and GST.Hsp25 (AS 71-100) were performed for 20 min in the presence of recombinant MK2 or p38MAPK (Millipore). Reactions were terminated by adding an equal volume of 0.5%

phosphoric acid to the reaction, and the entire reaction was analyzed by 10% SDS PAGE. Autoradiography was performed using Phosphorstorage plates and imaged on the Typhoon Variable Mode Imager station (GE Healthcare).

Mass spectrometric analyses

Immunoprecipitated protein was reduced and alkylated, on beads, in 2 mM DTT and 10 mM iodoacetamide respectively before adding sample buffer and heating at 70°C for 10 minutes. Proteins were resolved on a 4-12% gradient 1.5 mm thick Bis-Tris gel with MES running buffer (Nupage, Invitrogen) and Coomassie stained (Simply Blue, Invitrogen). The gel slices with the approximate molecular weight of PARN were excised from the four lanes, further cut into 1.5 mm cubes and placed into separate microfuge tubes. Protein was digested overnight with trypsin (approximately 0.1 µg enzyme) following standard protocols. Peptides from each gel slice were extracted with 0.1% TFA and cleaned up on C18 StageTips. Peptides were eluted in 50 µL of 80% acetonitrile/0.1% TFA and dried down in an evaporative centrifuge to remove organic solvents. The peptides were then resuspended by vortexing in 7 µL of 0.1% TFA and analyzed by nanoflow-LCMS with an Agilent 1100 with autosampler (HP, Palo Alto, CA) and a LTQ-Orbitrap (Thermo, Bremen Germany). Peptides were resolved on a 10 cm column, made in-house by packing a self-pulled 75 µm I.D. capillary, 15 µm tip (P-2000 laser based puller, Sutter Instruments) column with 3 µm Reprosil-C18-AQ beads (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) with an analytical flowrate of 200

nL/min and a 58 min linear gradient (~ 0.57 %B/min) from 0.1% formic acid in water to 0.1% formic acid/90% acetonitrile.

We used a data-dependent MS method with Orbitrap full scan (60,000 resolution), LTQ MS² and neutral loss-dependent MS³ scans for the top five most intense precursors from each preceding Orbitrap scan. Data-dependent settings were chosen to trigger an MS³ scan when a neutral loss of 98.0, 49.0, or 32.7 Da was detected among the three most intense fragment ions. Former target ions selected for MS² were dynamically excluded for 30 s. Total cycle time was approximately 3 secs. MS raw files were processed for protein identification and quantitation with Spectrum Mill (Agilent, VERSION) and IPI human ver.3.32 (<http://ebi.ac.uk>). Common contaminants like bovine serum albumin, trypsin etc. were also added to the database. Variable modifications used were oxidized methionine, pyroglutamic acid, deamidated asparagine, phosphoserine, phosphothreonine, and phosphotyrosine. Carbamidomethyl-cysteine was a fixed modification. The precursor mass tolerance used in the search was 0.05 Da and fragment mass tolerance was 0.7 Da. Peptides and proteins were autovalidated using default criteria (peptide score > 13, SPI% > 70%, proteins score > 25). This resulted with a FDR < 1% at the peptide level and < 0.01% at the protein level. Extracted ion chromatograms for the PARN peptide, NNSFTAPSTVGKR, in the phosphorylated and unphosphorylated forms were obtained using QualBrowser (Thermo) by defining a mass window +/- 0.01 m/z around the respective peptide m/z.