

Genotoxic stress causes the accumulation of the splicing regulator Sam68 in nuclear foci of transcriptionally active chromatin

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ABSTRACT

DNA-damaging agents cause a multifaceted cellular stress response. Cells set in motion either repair mechanisms or programmed cell death pathways, depending on the extent of the damage and on their ability to withstand it. The RNA-binding protein (RBP) Sam68, which is up-regulated in prostate carcinoma, promotes prostate cancer cell survival to genotoxic stress. Herein, we have investigated the function of Sam68 in this cellular response. Mitoxantrone (MTX), a topoisomerase II inhibitor, induced relocalization of Sam68 from the nucleoplasm to nuclear granules, together with several other RBPs involved in alternative splicing, such as TIA-1, hnRNP A1 and the SR proteins SC35 and ASF/SF2. Sam68 accumulation in nuclear stress granules was independent of signal transduction pathways activated by DNA damage. Using BrU labelling and immunofluorescence, we demonstrate that MTX-induced nuclear stress granules are transcriptionally active foci where Sam68 and the phosphorylated form of RNA polymerase II accumulate. Finally, we show that MTX-induced relocalization of Sam68 correlates with changes in alternative splicing of its mRNA target CD44, and that MTX-induced CD44 splicing depends on Sam68 expression. These results strongly suggest that Sam68 is part of a RNA-mediated stress response of the cell that modulates alternative splicing in response to DNA damage.

INTRODUCTION

Cells have developed several mechanisms to cope with external sources of stress, like heat shock and oxidative stress, or with insults that affect the integrity of the

genome, such as ultraviolet (UV) irradiation and DNA alkylating agents. Depending on the nature and the persistence of the stress, cells will adopt a ‘safety’ mechanism to limit and eventually overcome the damage, undergoing cell cycle arrest and DNA repair, or they will succumb by activating programmed cell death. A complex and well-studied stress response is that imposed by DNA damage (1), which has strong clinical implications in chemotherapy of human cancers. Most chemotherapeutic drugs induce breaks in the genome by targeting DNA processing enzymes, such as the topoisomerase inhibitors, or DNA directly, such as the alkylating agents. Although most cells are highly sensitive to these drugs and undergo apoptosis, cancer cells often escape this response and adopt mechanisms to withstand and repair the damage, thereby surviving to treatments. Thus, understanding the molecular mechanisms that allow cancer cells to survive to genotoxic stresses is a crucial step in the development of improved and more efficacious therapies.

Genotoxic stress causes a general suppression of the transcriptional activity, through degradation of the RNA polymerase II (RNAPII) (2), which allows to save energy and readapt the protein repertoire of the cell to the new tasks. In addition to changes in transcription, recent evidence demonstrated that genotoxic stress induces large spectrum modifications in alternative splicing (AS), thereby altering the isoforms produced by several genes (3). AS affects most human genes and allows to expand the cell proteome through differential assembly of exons in the mRNAs. AS is operated by the spliceosome, a macromolecular machinery composed by small nuclear ribonucleoprotein particles (snRNPs, U1, U2, U4, U5 and U6) and many constitutive and ancillary proteins that regulate the assembly of the spliceosome at the exon-intron junctions (4). The main regulators of constitutive and alternative splicing are RNA-binding proteins (RBPs) belonging to the serine–arginine (SR) rich proteins and the heterogeneous ribonucleoproteins (hnRNPs), which often play antagonistic roles (5).

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In addition to DNA damage, changes in AS have been reported in cellular responses to many other sources of stress (6), indicating that it is a crucial regulatory mechanism in cell adaptation to external insults. Moreover, recent observations have highlighted the specific differences in AS regulation in cancer cells (7–10), suggesting that this step of RNA processing plays a role also in cell transformation.

In line with its crucial role in the DNA damage response, several changes in AS of specific transcripts have been observed in cancer cells treated with cisplatin or etoposide. Remarkably, some of these transcripts encode for proteins regulating apoptosis, such as Caspase 2 (11) and Bcl-2 related genes (3), cell motility, like CD44 (12) and cell proliferation, like the p53 negative modulators MDM2 and MDM4 (13) or cyclin D1b, a splicing variant aberrantly expressed in prostate and breast cancer cells that confers resistance to therapies (14,15). Thus, it is likely that regulation of these AS events represents a novel mechanism by which cancer cells gain drug resistance and survive to chemotherapy.

The mechanisms underlying stress-induced changes in AS are just beginning to be understood (6). A recent report indicated that UV irradiation alters a substantial number of splicing events in hepatocarcinoma cells (3). The AS events were predominantly modulated by changes in the rate of pre-mRNA transcription, elicited through phosphorylation of the RNAPII (3). Nevertheless, additional mechanisms to modulate AS regulation exist. For instance, in p53-deficient cells, genotoxic stress caused up-regulation of SRp55, thereby promoting the inclusions of CD44 variable exons involved in tumorigenesis (12) and changes in AS of other cancer-related genes (16). DNA damage and other stresses can also alter the subcellular distribution of several RBPs involved in post-transcriptional events. Cisplatin and a mild heat shock caused accumulation in the nucleoli of the RNA recognition motif (RRM)-containing protein RDM1, which modulates the cellular response to genotoxic agents (17). In addition, various stresses triggered the cytoplasmic translocation of the splicing regulator hnRNP A1 (18), suggesting that they might alter nuclear RNA processing events regulated by this RBP (6). On the other hand, UV irradiation and heat shock induced accumulation of other RBPs in nuclear stress bodies (19,20), whose function is still largely unknown.

An interesting RBP involved in regulation of cell proliferation and survival is Sam68, which belongs to the signal transduction and activation of RNA (STAR) family (21). Sam68 is implicated in several steps of processing of target genes. In the cytoplasm, it regulates translation of viral and cellular mRNAs through its association with the polyribosomes or the stress granules (22–25). In the nucleus, Sam68 integrates signal transduction pathways with pre-mRNA AS in response to extracellular cues (26–28). Notably, we have previously demonstrated that Sam68 is up-regulated in prostate cancer (PCa) cells, where it sustains their resistance to treatments with chemotherapeutic agents (29). These results suggested that Sam68 plays a role in the cellular

response to genotoxic stress. Herein, we have investigated the function of Sam68 in PCa cells exposed to genotoxic stress induced by mitoxantrone (MTX), a topoisomerase II inhibitor used in chemotherapy. Our results demonstrate that Sam68 takes part to the cellular response to genotoxic stress by altering its subcellular localization and contributing to modulation of AS. These results suggest a novel role for Sam68 in the DNA damage response of cancer cells.

MATERIALS AND METHODS

Cell cultures and treatments

PC3 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL), LNCaP cells were cultured in RPMI 1640 medium (Lonza), media were supplemented with 10% fetal bovine serum (FBS) (Lonza), gentamycin, penicillin and streptomycin. For genotoxic treatments, cells at 80% of confluence were treated with 80 μ M cisplatin (Sigma-Aldrich) for 16 h, or with 5 μ M MTX (Sigma-Aldrich) for increasing times (2–24 h). At the end of the incubation, cells were fixed and stained for immunofluorescence analysis. For studies involving inhibitors, PC3 cells were pre-incubated for 15 min with 10 μ M JNK inhibitor 1 (JNK-inh.) (Alexis Biochemicals), 10 μ M SB202190 (Calbiochem) for p-38 kinase inhibition or 10 μ M MNK inhibitor 4-amino-5-(4-fluoroanilino)-pyrazolo[3,4-d]pyrimidine (MNK-inh.) (Calbiochem), or 1 h with 10 μ M 2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (ATM-inh.) (Calbiochem), and then incubated with 5 μ M MTX for 8–24 h. For RNASatIII induction experiments, PC3 cells were treated either with heat shock as previously described (30) or with 5 μ M MTX (15 min to 24 h).

Cell proliferation assay, cell viability and cell cycle analysis

The CellTiter A96 MTS (Promega) assay was used, as previously described (29), to monitor PC3 cell proliferation after treatment with MTX (0.5–5 μ M, Sigma-Aldrich) for increasing times (24–72 h) or for 2 h and released for 24–72 h after stress. For cell viability analyses, treated PC3 cells were incubated for 5 min at room temperature (RT) with 0.4% Trypan blue solution 1 : 1 (v/v) (Sigma-Aldrich) and analysed on a hemocytometer. The percentage of dead cells (stained) versus viable cells (unstained) was calculated. Cell cycle analysis with propidium iodide (10 μ g/ml) was performed with a FACSCalibur Flow Cytometer (Becton Dickinson) as described (29).

Stable knockdown of Sam68 in PC3 cells

PC3 cells were transfected either with pLKO.1puro (control pLKO) or PLKO.1-KHDRBS1_527 (pLKO-si-Sam68) (MISSION shRNA, Sigma Aldrich) in a 12 multi-well plate using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. The puromycin (Sigma-Aldrich) resistance marker was added at a concentration of 1 μ g/ml in fresh medium every 2 days for stable selection and maintenance of

PC3 clones. Sam68 knockdown was verified by RT-PCR and western blot analyses.

Plasmid vectors

For the pHsp70.2EGFP-Sam68 expression vector, the CMV promoter was excised from pEGFPC1-Sam68 (28), substituted with Hsp70.2 promoter, amplified with Pwo SuperYield DNA polymerase (Roche) from Hspa2-pblue (generous gift of Prof. E.M. Eddy), and introduced in pEGFP-C1-Sam68. The cDNA encoding murine Sam68 was amplified by PCR using cDNA from murine embryonic fibroblast (MEF) as template and Pfu polymerase (Roche), cloned in frame with myc into pCDNA3myc (28). The expression vector pEGFP-GSG (GFP-Sam68_{GSG}) was previously described (29). All expression vectors were analysed by sequencing.

Cell transfections and CD44v5-luciferase (v5-Luc) splicing assay

For GFP-tagged proteins expression, PC3 cells were seeded the day before transfection (3.5×10^5) in 35 mm plates and then transfected with 1 μ g of pEGFP_{hsp70.2}-Sam68 wt or pEGFP-GSG using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Twenty-four hours after transfection, cells were treated with 5 μ M MTX for 24 h, incubated 15 min at 37°C with 4',6-diamidino-2-phenylindole (DAPI), washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) 10 min on ice. Cells were then washed with PBS, mounted with Mowiol (Calbiochem) and analysed by confocal microscope (28). For Luciferase assays, control pLKO and pLKO-si-Sam68 PC3 cells were cultured in 12-well plates ($\sim 10 \times 10^5$ /well). Cells were treated with 5 μ M MTX for 2 h, rinsed with PBS and let recover with fresh medium for 2 h and then transfected with 0.4 μ g of minigene pETv5luc (31), 0.25 μ g of pCDNA3myc or pCDNA3mycSam68 (mycSam68) and the Renilla luciferase reporter gene (1 ng) as an internal control using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Twenty-four hours after transfection, cells were harvested, lysed and analysed using a biocounter luminometer using the dual-luciferase reporter assay system (Promega). Data from three experiments were normalized for transfection efficiency using the ratio between firefly and Renilla luciferase activity.

RNA extraction, real-time PCR and RT-PCR

For endogenous CD44 v5 AS analysis after genotoxic stress, control pLKO and pLKO-si-Sam68 PC3 stable clones were treated with 0.5 or 5 μ M MTX for 2 h, let recover after stress for 24 h and then harvested in TRIzol reagent (Invitrogen) for total RNA extraction according to the manufacturer's instructions. After digestion with RNase-free DNase (Roche), 1 μ g of total RNA was used for RT using M-MLV reverse transcriptase (Invitrogen). Quantitative real-time PCRs were carried out with Sybr Green mix (for Light-Cycler480 - Roche) according to manufacturer's instructions. Primers used for CD44 v5 were the followings: CD44 v5 Fw 5'TGTA

GACAGAAATGGCACCCTG3'; CD44 v5 Rev 5'TGG TGCTGTAGAAATGTGGGGTC3'. Samples were normalized on CD44 constitutive isoforms (with respect to the constitutive exons C6 and C7) using the following primers: CD44 c Fw 5'AGACACATTCCACCCCAG TG3'; CD44 c Rev 5'CCAGAGGTTGTGTTTGCTCCA CC3'. The value given for the amount of CD44 v5 PCR product present in untreated cells was set as 1. Data are reported as the mean \pm SD of three independent experiments. For SatIII RNAs expression experiments after heat shock or MTX treatments, RT-PCRs specific for G-rich RNA SatIII were performed as described (30). Samples were normalized on hypoxanthine-phosphoribosyl-transferase (HPRT) expression levels.

Western blot analysis

Western blot analysis of cell extracts was performed as previously described (29,32). Protein concentration was determined by using Bradford reagent (Bio-Rad). Primary antibodies used (at 1:1000 dilution; overnight at 4°C): rabbit anti-Sam68, anti-Erk2, anti-TFIIH and anti-cyclin A, goat anti-lamin b, and mouse anti-cyclin B1 (Santa Cruz Biotechnology); rabbit p-Chk2 and p-p38 (Cell Signalling); mouse c-Jun (BD biosciences); rabbit anti-pJNK1/2 and p-eIF4E (Biosource); mouse anti-hnRNP A1 (Sigma-Aldrich); rabbit anti-cyclin D1 and anti-Histone 3, unmodified (Abcam). Secondary anti-mouse, anti-rabbit or anti-goat IgGs conjugated to horseradish peroxidase (Amersham) were incubated for 1 h at RT (1:10000 dilution in PBS containing 0.1% Tween-20). Immunostained bands were detected by chemiluminescent method (Santa Cruz Biotechnology).

Nuclei isolation and chromatin fractionation

PC3 cells were treated with 0.5 or 5 μ M MTX for 24 h. Nuclei were isolated, resuspended in solution G (300 mM sucrose, 50 mM triethanolamine, 25 mM KCl, 4 mM MgCl₂, 1 mM CaCl₂ and 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and digested with micrococcal nuclease (Sigma-Aldrich) at 37°C as previously described (33). For chromatin fractionation, digested nuclei were cooled on ice for 10 min and centrifuged at 12 800 \times g for 10 min at 4°C. The supernatant (S1) was removed, the pellet was suspended in 2 mM EDTA pH 7.4 and centrifuged to separate supernatant S2 and pellet (P) as previously described (34). For protein analysis, samples were adjusted to 10 mM MgCl₂ and precipitated with two volumes of ethanol at -20°C. Protein samples were analysed by western blot.

Immunofluorescence and bromo-uridine incorporation

Cells were fixed and stained for immunofluorescence analysis as previously described (28). Primary antibodies were the followings: rabbit anti-Sam68 1:1000 and goat anti-TIA-1 1:200 (Santa Cruz, Biotechnology); mouse anti- γ H2AX 1:10000 (Cell signaling); mouse monoclonal antibody PG-M3 (anti-PML) 1:100 (generous gift from Prof. F. Lo Coco); mouse anti-hnRNP A1 1:500 and anti-SC35 1:500 (Sigma-Aldrich); mouse anti-SF2/ASF 1:200 (USBiological) and mouse monoclonal anti-RNA

polymerase II (phospho S2) H5 IgM 1:200 (Abcam). DAPI was used for DNA staining and the secondary antibodies were: Alexa Fluor 488, 568 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse (1:500) (Invitrogen); FITC-conjugated donkey anti-rabbit; cy3-conjugated donkey anti-goat 1:500 and cy3-conjugated donkey anti-mouse IgM 1:500 (Jackson Immunoresearch). For BrU incorporation, PC3 cells were incubated in the last 15 min to 1 h of culture with 1 mM BrU (Sigma-Aldrich). Cells treated with 5 μ M MTX (2–24 h) were incubated with BrU in the last hour of treatment. For MTX-release experiments, PC3 cells were treated with 5 μ M MTX for 2 h and let recover for 24 h and incubated with BrU in the last hour of recovery. Cells were fixed and stained as previously described (35). Briefly, cells were washed twice with sterile cold PBS supplemented with 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS*) and fixed in PFA 2% for 20 min on ice. After permeabilization with PBS* + 0.1% Triton X-100 (PBS*^T) for 5 min, samples were quenched with 50 mM NH₄Cl in PBS*^T 10 min, blocked with PBS*^T + 1% BSA for 1 h at RT, and incubated with the following primary antibodies: mouse anti-BrdU (1:40) (Becton-Dickinson) and rabbit anti-Sam68 (1:500) in PBS*^T + 0.2 % BSA 1 h at RT. Cells were washed three times in PBS*^T and incubated 1 h at RT with the following secondary antibodies diluted in PBS*^T + 0.2 % BSA: cy3-conjugated donkey anti-mouse 1:100 or cy3-conjugated donkey anti-mouse 1:100 and FITC donkey anti-rabbit 1:500 (Jackson Immunoresearch) for co-stainings. Finally, cells were washed, mounted with Mowiol (Calbiochem) and analysed by confocal microscope. All the solutions were prepared in RNAase-free water. Immunofluorescence analyses were performed using a Leica confocal microscope and a Plan-Neofluar HCX 40.0 \times /1.25 oil UV objective and acquired using IAS AF Lite software (Leica Microsystems). Images were saved as TIFF files, and Photoshop (Adobe) and PowerPoint (Microsoft) were used for composing the panels.

RESULTS

Genotoxic stress induces changes in the subcellular localization of Sam68

Depletion of Sam68 renders PCa cells more susceptible to apoptosis induced by genotoxic drugs (29). To further investigate this protective function of Sam68, we monitored the intracellular localization of the protein in response to MTX, a drug used in treatment of androgen-resistant PCa patients (36). Sam68 was diffusely localized in the nucleoplasm of control cells, whereas treatment with MTX for 2–24 h induced profound changes in the localization of the protein (Figure 1A and B). After 2–4 h, Sam68 accumulated in ring-shaped structures that surrounded the nucleoli, whereas at later times it was enriched in large granules that were faintly stained by DAPI (Figure 1A and B). Notably, treatment with MTX caused morphological changes in PC3 cells, which at

phase contrast appeared more flattened on the culture plate with larger nuclei than untreated cells (Figure 1C). Western blot analysis showed that MTX did not affect Sam68 protein levels during the time-course of the treatment (Figure 1D). Accumulation of Sam68 in nuclear granules was also observed in another PCa cell line, LNCaP, and in HeLa cells (Supplementary Figure S1), indicating that it is not a PC3 cell-specific response. Moreover, similar changes in the subcellular localization of Sam68 were also elicited by cisplatin (Figure 1E), an alkylating agent that causes DNA damage by a different mechanism than MTX, suggesting that various genotoxic stresses trigger this event in PCa cells.

To test whether the Sam68-containing granules were foci of DNA lesions induced by MTX, we co-stained PC3 cells with Sam68 and the phosphorylated form of H2AX (γ H2AX), a substrate of the ATM kinase that marks foci of double-strand breaks (37). However, confocal microscopy analysis showed that Sam68 nuclear granules and double-strand breaks foci are different structures (Supplementary Figure S2A). Other nuclear structures affected by genotoxic stress are the promyelocytic leukemia (PML) nuclear bodies, which are sites of post-transcriptional modifications of nuclear proteins such as HIPK2 and p53 (38). PML bodies are dynamic compartments that act as DNA damage sensors, they are regulated by ATM and its target Chk2, and their number in the cell increases under genotoxic stress (39). Nevertheless, also in this case confocal microscopy demonstrated that MTX-induced Sam68 nuclear granules and PML bodies are different entities (Supplementary Figure S2B).

Sam68 colocalizes with other stress-responsive RBPs both in nuclear and cytoplasmic granules

In addition to the nuclear relocalization of Sam68, 16–24 h of treatment with MTX (Figure 1F) and cisplatin (data not shown) caused the translocation of Sam68 in the cytoplasm in ~5–10% of cells, where it accumulated in cytoplasmic granules. These structures resembled the cytoplasmic stress granules (SGs) in which splicing factors, such as hnRNP A1 and TIA-1, re-localize as a stress-adaptation response of the cell (40). Thus, we tested whether genotoxic stress caused recruitment of Sam68 to the same granules marked by these stress-response RBPs. Confocal immunofluorescence analyses showed that MTX-induced genotoxic stress caused subcellular re-distribution of both hnRNP A1 and TIA-1 in the nucleus and in the cytoplasm similar to that observed for Sam68 (Figure 2). Moreover, Sam68 colocalized, with identical time-course, to the same structures as hnRNP A1 and TIA-1 both in the nucleus (Figure 2A and B, and Supplementary Figure S3A) and, albeit only in a small percentage of cells, in the cytoplasm (Supplementary Figure S3B and C).

Colocalization of Sam68 with TIA-1 in cytoplasmic stress granules has been recently reported in cells under oxidative stress (41). However, no colocalization of these

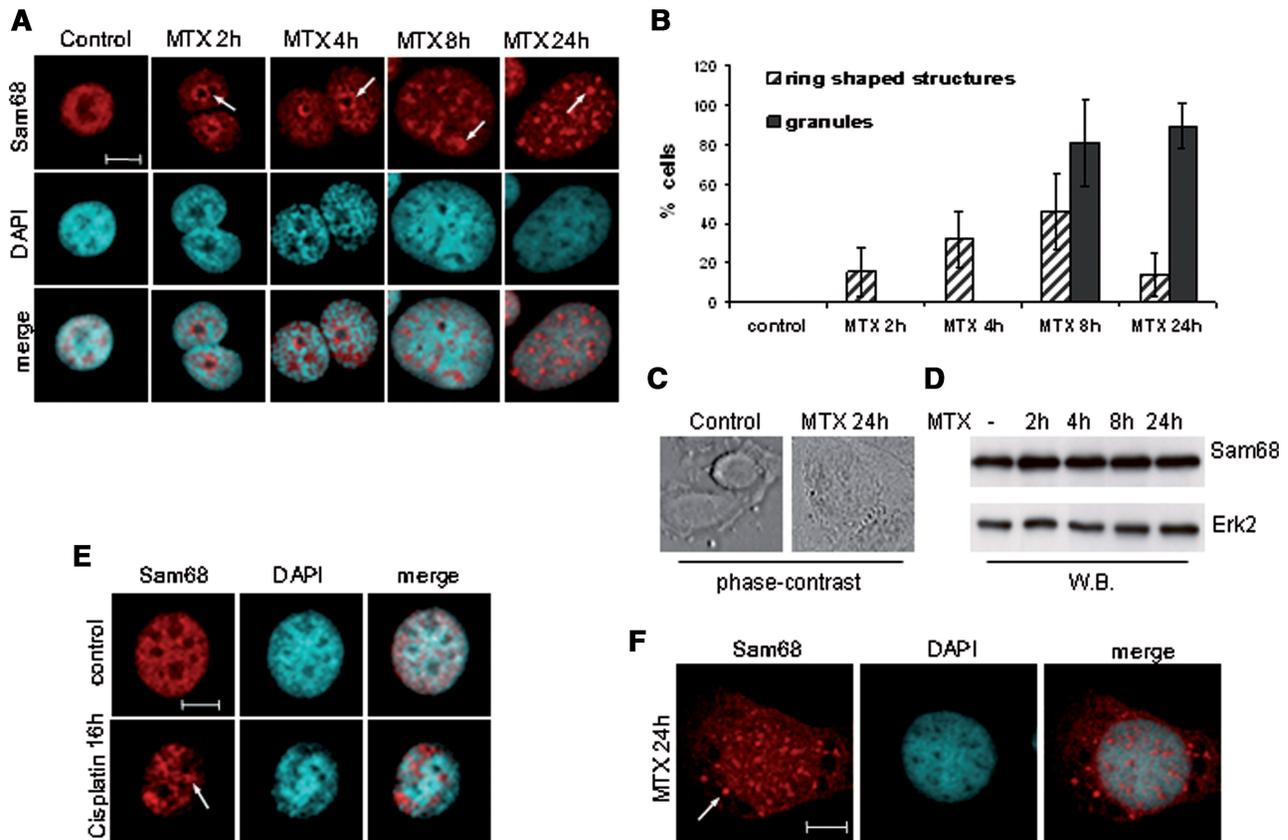


Figure 1. Sam68 relocalizes in subcellular compartments after genotoxic stress. (A) Time course treatment of PC3 cells with 5 μ M MTX. Treated cells were stained with an anti-Sam68-specific antibody and analysed by immunofluorescence by confocal microscopy. The localization of Sam68 around the nucleoli (2 h MTX) and in large nuclear granules (8–24 h MTX) is indicated by white arrows. Scale bar = 15 μ m. (B) Graph of the percentage of PC3 cells treated showing ring-shaped structures and granules after MTX treatment (5 μ M, 2–24 h). (C) Phase contrast images of control and MTX-treated cells (5 μ M). (D) Cell extracts (10 μ g) from PC3 treated as in panel A were analysed by SDS-PAGE and western blot with the indicated antibodies. (E) PC3 cells were treated for 16 h with 80 μ M cisplatin, fixed and stained with anti-Sam68 antibody. The white arrow indicates a Sam68 granule in treated cells. Scale bar = 15 μ m. (F) Confocal images of PC3 cells after prolonged treatment (24 h) with 5 μ M MTX indicating the translocation of Sam68 in the cytoplasm. Scale bar = 15 μ m.

proteins in nuclear granules was observed under these circumstances, suggesting that these structures are specifically induced by genotoxic stress. Nuclear stress bodies (nSBs) where RBPs are actively recruited have been previously described in response to heat shock. However, although these structures contained Sam68, SAFB and the SR protein ASF/SF2, other RBPs involved in splicing like hnRNP A1 or the SR protein SC35 were not recruited (20). To further characterize the nature of the MTX-induced stress granules, we analysed the subcellular localization of SC35 and ASF/SF2 in response to this genotoxic drug in PC3 cells. Confocal microscopy showed that SC35 and ASF/SF2 localized both to nuclear speckles and in the nucleoplasm in untreated cells, whereas MTX caused their accumulation in large granules in a time-dependent manner. Notably, Sam68, which is mostly diffused in the nucleoplasm of untreated PC3 cells, colocalized with these SR proteins in the large nuclear granules only after 8–24 h of MTX treatment (Figure 3A and B). These results indicate that MTX-induced nuclear stress granules are novel structures where both hnRNPs and SR proteins accumulate together with Sam68.

The GSG domain of Sam68 is sufficient for relocalization after genotoxic stress

We have previously demonstrated that Sam68 interacts with hnRNP A1 in a functional splicing complex (28). This interaction required the C-terminal 93 residues of Sam68, downstream of the GSG domain that mediates homodimerization and RNA binding. Similarly, it was recently shown that the region comprised by residues 269–321, mostly outside of the GSG, was required for the interaction with TIA-1 and the recruitment of Sam68 to cytoplasmic SGs (41). Since Sam68 colocalized with hnRNP A1 and TIA-1 in MTX-induced nuclear granules, we set out to test whether its relocalization required the interaction with these RBPs. PC3 cells were transiently transfected with full-length GFP-Sam68 or with GFP-Sam68_{GSG} (residues 91–276), which lacks the regions required for the interaction with hnRNP A1 and TIA-1. We observed that full-length GFP-Sam68 behaved like the endogenous protein and relocalized into nuclear granules after MTX-induced stress (Figure 4A). The GFP-Sam68_{GSG} protein showed a diffused localization both in the cytoplasm and in the nucleus in untreated cells. However, this deletion mutant lacking most of the

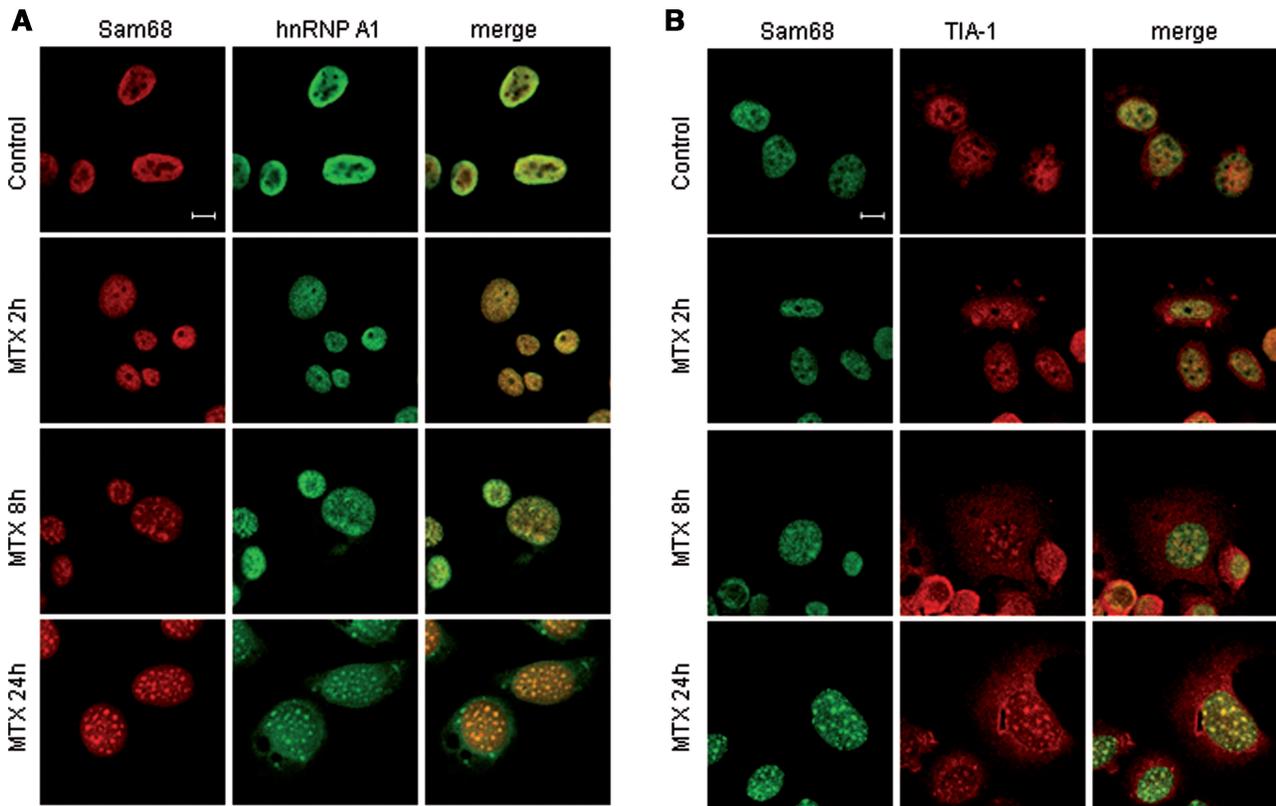


Figure 2. Sam68 colocalizes with stress-response RNA-binding proteins after genotoxic stress. PC3 cells were treated with 5 μ M MTX (2–24 h) and co-stained with Sam68 and hnRNP A1 (A) or TIA-1 (B) specific antibodies and analysed by confocal microscopy as in Figure 1. The merge panels show colocalization of Sam68 and hnRNP A1 or TIA-1 to the same nuclear granules. Scale bar = 10 μ m.

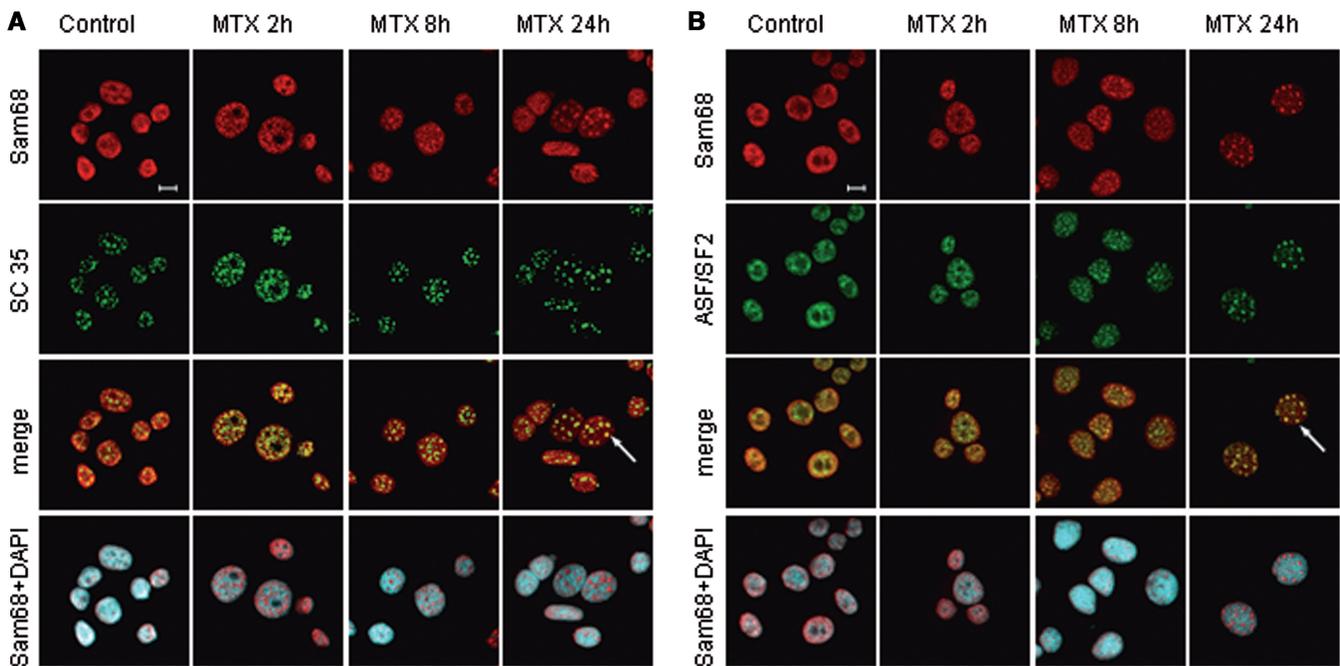


Figure 3. SR proteins colocalize with Sam68 in nuclear granules after genotoxic stress. Confocal analyses of PC3 cells treated with 5 μ M MTX (2–24 h) and co-stained with Sam68 and SC35 (A) or ASF/SF2 (B). Sam68 colocalized to the same stress-induced nuclear granules as SC35 and ASF/SF2 after 8–24 h of MTX treatment. Stress-induced nuclear granules are pointed by white arrows. Scale bar = 10 μ m.

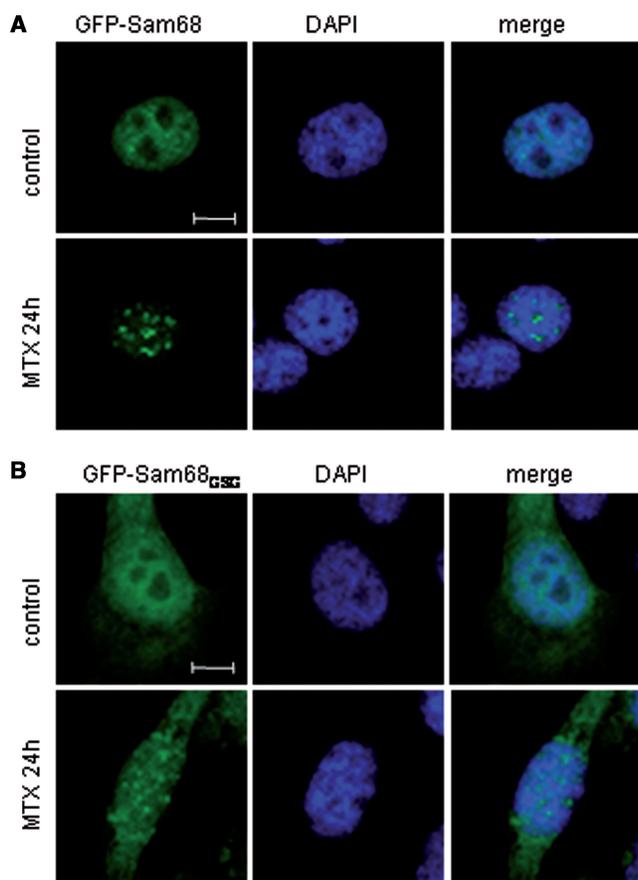


Figure 4. The Sam68 GSG homodimerization and RNA-binding domain is sufficient for relocalization after genotoxic stress. PC3 cells were transiently transfected with full-length GFP-Sam68 (A) or with GFP-Sam68^{GSG} (B) and treated with 5 μ M MTX. After 24h, the cells were fixed and analysed by confocal microscope. Scale bar = 10 μ m.

regions required for protein–protein interactions and post-translational modifications (21) was still capable to relocalize into nuclear granules after MTX treatment (Figure 4B), indicating that the homodimerization and RNA-binding activity of Sam68 were sufficient for this event. On the other hand, depletion of Sam68 by RNAi in PC3 cells did not affect the recruitment of hnRNP A1 and TIA-1 to the nuclear granules after MTX treatment (data not shown), indicating that these RBPs are independently recruited to these structures during genotoxic stress.

Signal transduction pathways activated after genotoxic stress in PCa cells do not affect Sam68 accumulation in nuclear granules

Sam68 is known to link signal transduction pathways to RNA metabolism (21). We asked whether the MTX-induced subcellular relocalization of Sam68 was determined by activation of specific signal transduction pathways. PC3 cells were treated with MTX in a time- and dose-dependent manner and extracts were analysed by western blot. The DNA-damage response involves activation of the ATM/Chk2 signal transduction pathway

(42), which recruits DNA repair proteins to the lesions, and of mitogen activated protein kinases (MAPKs) responsive to various stresses, such as JNK1/2 and p38 (43,44). We observed that MTX induced phosphorylation of the ATM substrate Chk2 at both doses, whereas full activation of JNK1/2 and p38 required the higher dose (Supplementary Figure S4A). Both doses of MTX were sufficient to elicit growth arrest, albeit at different cell cycle phases (Supplementary Figure S5), and to affect the localization of Sam68 in the nucleus. However, we observed that 0.5 μ M MTX, which was sufficient only for complete activation of the ATM/Chk2 (Supplementary Figure S4A), caused relocalization of Sam68 to the ring-shaped structures surrounding the nucleoli, typical of the early phase of the response to higher MTX doses. On the other hand, the accumulation of Sam68 in the nuclear granules was induced only by the higher dose of MTX (5 μ M) (Supplementary Figure S4B) and correlated more with the activation of MAPKs (Supplementary Figure S4A). Nevertheless, we tested whether any of these signal transduction pathways was required for Sam68 accumulation in nuclear granules. Cells were treated with inhibitors of ATM (ATM-inh.), the p38 downstream kinase MNK1 (MNK-inh.), which is known to regulate hnRNP A1 nucleocytoplasmic export after stresses (18), p38 (SB202190) and JNK1/2 (JNK-inh.). Remarkably, none of the inhibitors tested suppressed relocalization of Sam68 to nuclear granules induced by 5 μ M MTX (Supplementary Figure S6A and B). Western blot analysis confirmed the inhibitory effects of these drugs on the corresponding signal transduction pathways, Chk2 phosphorylation for the ATM-inh. (Supplementary Figure S6C), eIF4E phosphorylation for MNK-inh. and SB202190 (Supplementary Figure S6D and E), and c-Jun mobility shift for the JNK-inh. (Supplementary Figure S6E). Moreover, MTX treatment did not affect the levels of serine/threonine or tyrosine phosphorylation of Sam68 immunoprecipitated from PC3 cells (data not shown). These results indicate that post-translational modifications by stress-induced signal transduction pathways are not required for MTX-induced relocalization of Sam68 in nuclear stress granules.

Sam68 nuclear granules induced by genotoxic stress are transcriptionally active foci

It has been recently shown that heat shock induced the transcriptional activation of tandem arrays of repetitive Satellite III (SatIII) sequences, resulting in expression of non-coding RNA molecules of various length (45,46). SatIII RNAs remain associated with sites of transcription (47) and are bound by several RBPs, like SAFB and ASF/SF2, thus leading to the formation of nSBs (20). A milder induction of SatIII RNA transcription was also observed with other stresses, including that elicited by etoposide (30), a topoisomerase II inhibitors like MTX. However, we observed that, unlike heat shock (Supplementary Figure S7A), MTX did not stimulate transcription of SatIII RNAs in PC3 cells (Supplementary Figure S7B

and C), suggesting that MTX-induced nuclear granules were different structures than heat shock-induced nSBs.

Next, we hypothesized that MTX induced the recruitment of Sam68 to specific domains of chromatin structure and/or function. To test this possibility, we separated chromatin components using micrococcal nuclease treatment followed by divalent ions and EDTA extractions as previously described (33,34). This procedure yields fractions enriched (S1) or depleted (S2) in transcriptionally active genes, which also remain associated to the insoluble chromatin and nuclear matrix (P) (34). Chromatin separation from control or treated PC3 cells showed that MTX caused an enrichment of Sam68 and hnRNP A1 in the S1 fraction at both doses, similarly to what observed for the transcription factor TFIID p89 (Figure 5A and B). The effect of MTX appeared specific because it did not affect localization of Lamin B in the nuclear matrix fraction P (Figure 5A). This experiment suggested that genotoxic stress induces a change in distribution of chromatin components, with an enrichment of the splicing factors Sam68 and hnRNP A1 in the transcriptionally active fractions.

Since many splicing factors are recruited to the transcriptionally active chromatin by phosphorylated RNAPII, we investigated its localization in response to MTX treatment. We performed a time course treatment

with MTX and stained PC3 cells with a RNAPII antibody (H5). This antibody is specific for the phosphorylated serine2 in the carboxyterminal domain (CTD), which marks the elongating form of RNAPII engaged in transcription (48). We observed that genotoxic stress changed the subcellular distribution of RNAPII from a widely diffused localization in the nucleoplasm to discrete nuclear granules (Figure 6A). Moreover, co-staining of cells with H5 and Sam68 antibodies clearly showed that the two proteins colocalized to the same nuclear granules induced by MTX treatment (Figure 6B).

Although the hyperphosphorylated elongating form of RNAPII generally localizes into transcriptionally active foci throughout the nucleoplasm, it was previously shown that it can redistribute into transcriptionally inactive speckles under a general block of transcription induced by α -amanitin or heat shock (49). Thus, to test whether these stress-induced nuclear granules were still transcriptionally active, we performed a bromo-uridine (BrU) incorporation assay to follow RNA synthesis *in situ*. PC3 cells were treated with MTX for increasing times and incubated with BrU in the last hour of treatment. A time-course experiment indicated that 60 min of labelling with BrU were necessary to readily detect *de novo* transcription by immunofluorescence in PC3 cells (Figure S8A). Confocal analysis revealed that BrU

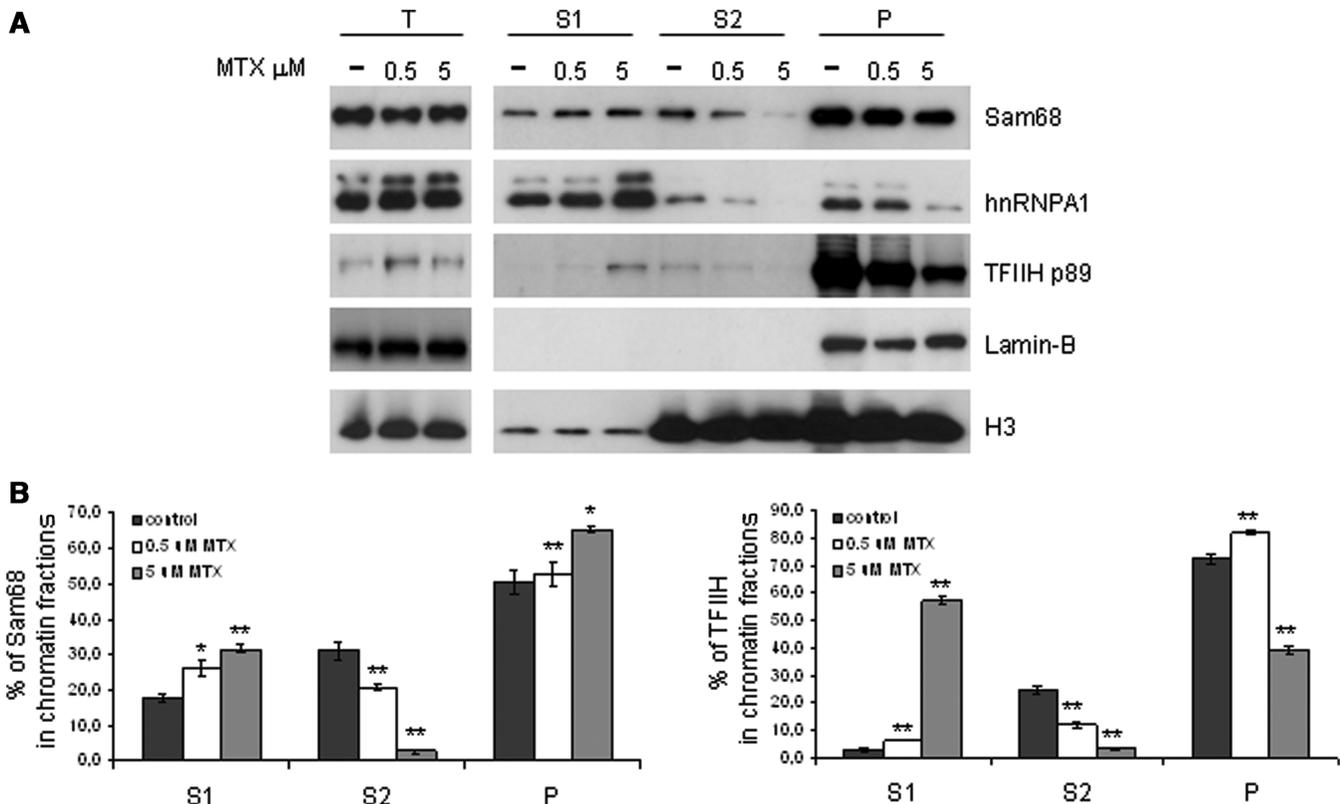


Figure 5. Genotoxic stress induced by MTX causes an enrichment of the splicing factors Sam68 and hnRNP A1 in the transcriptionally active fractions. (A) Western blot analysis of chromatin fractions obtained from PC3 cells treated with 0.5 or 5 μ M MTX. Distribution of proteins in total digested nuclei (T) and in chromatin fractions (supernatants S1–S2 and pellet, P) was analysed with the indicated antibodies. Lamin-B was used as nuclear matrix marker. Staining for histone H3 was performed as loading control. (B and C) Densitometric analysis of the distribution of Sam68 (B) and TFIID p89 (C) in S1–S2–P chromatin fractions from three experiments is expressed as percentage of the levels of these proteins in the total digested nuclei (T) (mean \pm SD; * P < 0.05; ** P < 0.01).

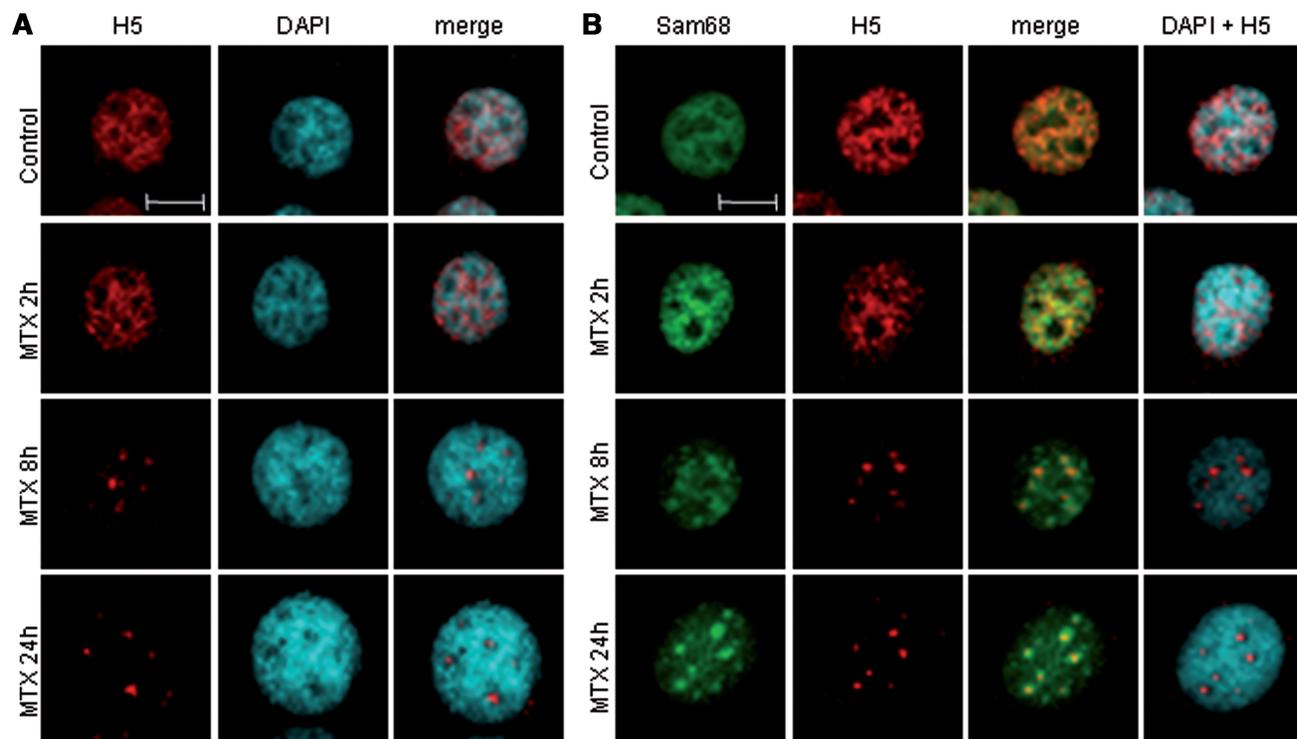


Figure 6. RNAPII and Sam68 colocalize in genotoxic stress-induced nuclear granules. (A) PC3 cells untreated (control) or treated with 5 μ M MTX (2–24 h), were stained with a RNAPII antibody (H5) specific for the phosphorylated serine2 and analysed by confocal microscopy. (B) Co-staining of Sam68 and phosphorylated RNAPII (H5) in PC3 cells treated as in panel A. Scale bar = 10 μ m.

staining, which was at first diffused into the nucleoplasm and partly in the cytoplasm, accumulated into specific nuclear foci under genotoxic stress (Supplementary Figure S8B). To understand if these foci were the same stress-induced sites of Sam68 accumulation, MTX-treated PC3 cells were stained for both BrU and Sam68. As clearly shown in Figure 7A, the stress-induced Sam68 nuclear granules incorporated BrU, indicating that they are transcriptionally active foci.

Genotoxic stress induced by MTX affects CD44 AS profile in a Sam68-dependent manner

Since we observed that Sam68 is recruited from the nucleoplasm to foci of residual transcription under genotoxic stress, we asked whether its function affects MTX-modulated AS of target mRNAs. A well described splicing event regulated by Sam68 is the inclusion of the variable v5 exon in CD44 (26,27,50), a membrane receptor involved in cell proliferation and migration (51). The CD44 gene contains 10 constitutive and variable exons that can be differentially assembled in the mature mRNA (52). Inclusion of several variable exons (v5, v6, v8 and v10) correlates with increased cell motility, invasion of neighboring tissues and malignancy and it has been correlated to poor prognosis in patients (50–53). Moreover, recent data have implicated a change in CD44 splicing isoforms as an adaptive response of some cancer cells to genotoxic stress (12). Thus, we investigated whether MTX caused Sam68-dependent modulation of the CD44 v5 exon inclusion. Control PC3 cells (pLKO),

or PC3 cells depleted of Sam68 by stable transfection of a siRNA (pLKO si-Sam68), were treated for 2 h with 0.5 or 5 μ M MTX. Cells were then allowed to recover after damage and harvested after 24 h (3). Under these conditions, Sam68 and BrU labelling accumulated in nuclear stress granules like under continuous MTX treatment (Figure 7B), and cells underwent a similar proliferation arrest (Supplementary Figure S5E and F). This protocol was used to allow cells to recover, thereby allowing higher transfection efficiency and transcription of new mRNAs without the interference of MTX, a genotoxic agent that could damage the transfected minigene. Real-time PCR analysis of CD44v5 endogenous levels, with respect to the constitutive exons c6 and c7, showed that MTX induced an increase in v5 inclusion in control PC3 cells (Figure 8A). Importantly, this effect required Sam68 expression, because its down-regulation by RNAi completely suppressed the effect of MTX (Figure 8A and B). In order to understand if the increase of CD44v5 isoform levels was directly due to AS modulation, we used a splice-reporter minigene in which inclusion of exon v5 leads to expression of firefly luciferase (pETv5luc) (26,31). pLKO and pLKO si-Sam68 PC3 cells were treated for 2 h with 5 μ M MTX. After recovery from the stress, cells were then transiently transfected with the splice-reporter minigene and analysed for luciferase activity 24 h later. Remarkably, also in this case, we observed that MTX treatment stimulated the inclusion of the variable CD44 exon v5 in a Sam68-dependent manner (Figure 8C). Moreover, inclusion of exon v5 in

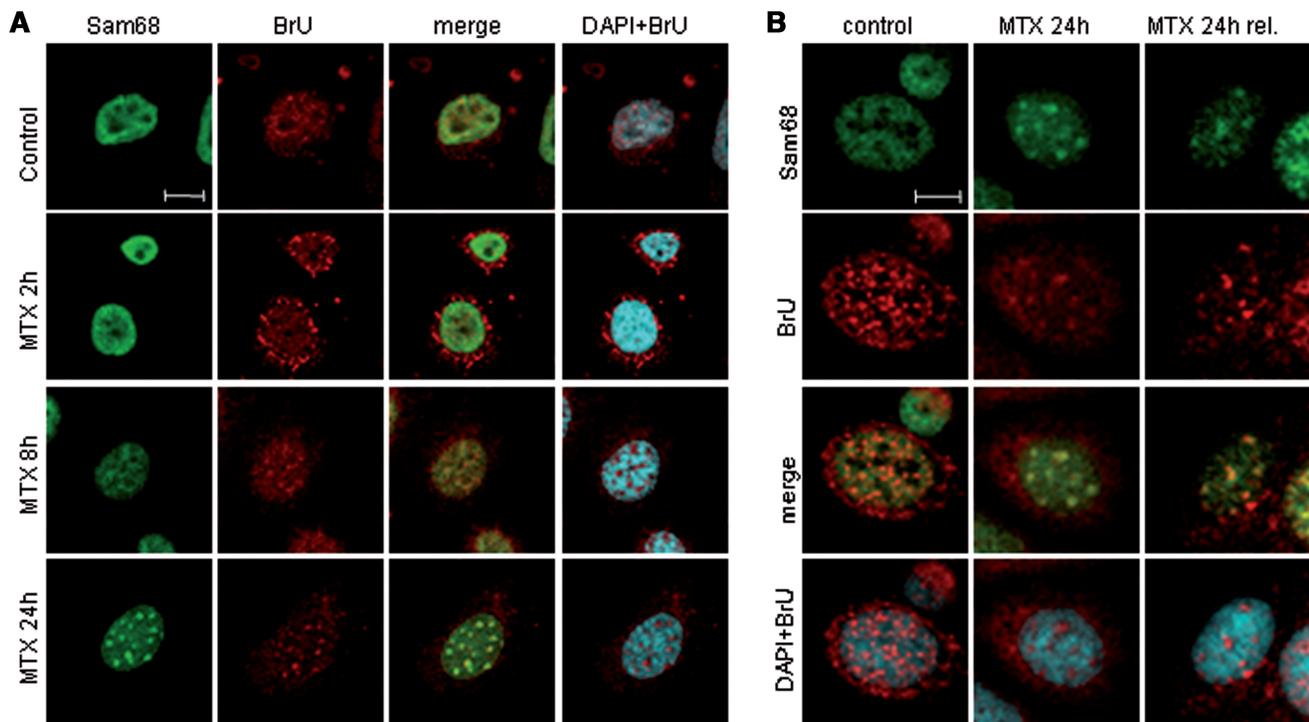


Figure 7. Sam68 nuclear granules induced by genotoxic stress are transcriptionally active foci. (A) BrU-incorporation assay in PC3 cells untreated (control) or treated with 5 μ M MTX (2–24h). PC3 cells were stained with anti-Sam68 and BrU specific antibodies. Confocal analysis revealed that Sam68 MTX-induced nuclear granules incorporate BrU. (B). PC3 cells were treated for 2h with 5 μ M MTX, and allowed to recover 24h after damage. At the end of incubation, cells were fixed and stained with anti-Sam68 and BrU specific antibodies. Scale bar = 10 μ m.

Sam68-depleted cells could be restored by overexpression of a non-degradable form of Sam68 (murine mycSam68 in Figure 8C and D). Myc-Sam68 similarly induced v5 inclusion also in pLKO control cells (data not shown), as previously shown for parental PC3 cells (29). Western blot analysis of cell extracts confirmed down-regulation of Sam68 levels in the pLKO si-Sam68 PC3 cells used for both experiments described above and the recovery in expression after transfection of the murine mycSam68 (Figure 8B and D).

DISCUSSION

In this report, we have investigated the subcellular localization and the function of Sam68 during the MTX-induced DNA damage response of PCa cells. These studies stemmed from our previous observation that Sam68 played a protective role in PCa cells subjected to genotoxic stress induced by chemotherapeutic drugs (29). We now show that genotoxic stress stimulates the accumulation of Sam68 in nuclear granules, together with other splicing regulators like TIA-1, SR proteins and hnRNP A1. Moreover, we provide evidence that these MTX-induced granules are transcriptionally active and contain the phosphorylated form of RNAPII. Finally, our results indicate that the subnuclear relocalization of Sam68 correlates with changes in AS of its mRNA target CD44 and that Sam68 function is necessary for such AS modulation. Thus, our study identifies Sam68 as a novel

stress-regulated RBP involved in the response to genotoxic stress in PCa cells.

As a first approach, we attempted a characterization of the Sam68 nuclear granules induced by MTX using markers of previously described nuclear structures affected by genotoxic stress. Topoisomerase inhibitors and alkylating agents cause double-strand breaks in the DNA, which set in motion a well-characterized DNA damage response (1,42). The ATM kinase is recruited to the lesions and phosphorylates H2AX, which acts as a signal to recruit proteins involved in DNA repair. Thus, the phosphorylated form of this histone (γ H2AX) is used as a marker of DNA repair foci. By using this marker, we were able to show that Sam68 is not recruited to double-strand break foci in the cell. By using a similar approach, we also demonstrated that Sam68 nuclear granules were different from the PML bodies, which also enlarge after DNA damage and participate to the genotoxic stress response (39). Moreover, inhibition of the ATM signalling pathway did not affect recruitment of Sam68 to the nuclear foci. Thus, although Sam68 relocalization is induced by lesions in the DNA, the sites of its accumulation are not chromatin foci involved in DNA repair.

The most characterized nuclear structures induced by stress are the nSBs that form in cells exposed to heat shock (20). These bodies present several features in common with the MTX-induced nuclear granules. First, both structures are characterized by accumulation of Sam68 (19 and this work). Moreover, nSBs also recruit

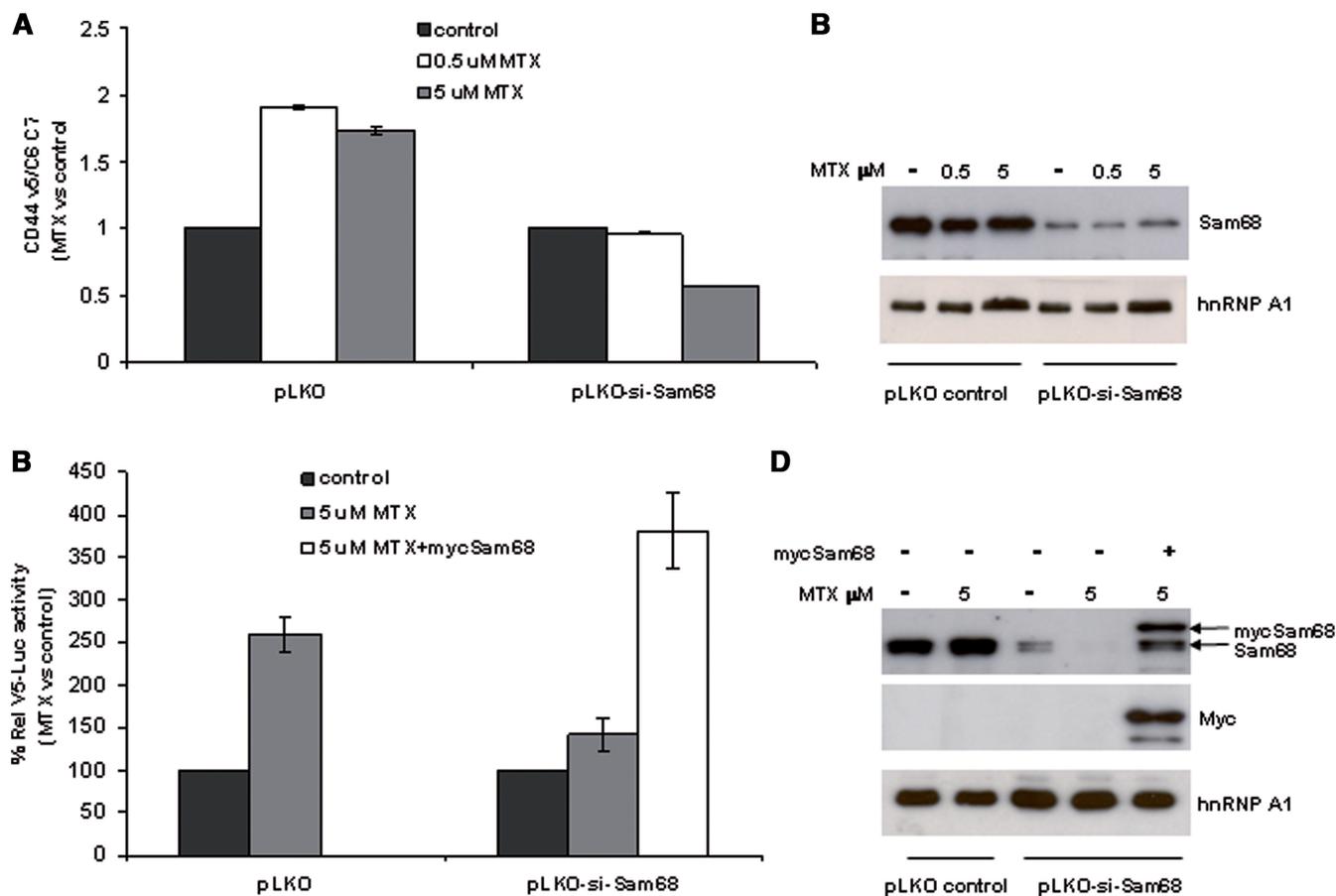


Figure 8. Genotoxic stress induced by MTX affects CD44 alternative splicing in a Sam68-dependent manner. (A) control pLKO and pLKO-si-Sam68 PC3 cells were treated for 2 h with 0.5 (white columns) or 5 μ M MTX (dim grey columns), allowed to recover from stress for 24 h and analysed for the levels of endogenous CD44 v5 exon inclusion by real-time PCR. CD44 v5 levels in untreated cells (dark grey columns) were set as 1 and MTX-induced v5 inclusion was calculated as fold increase. Samples were normalized for CD44 constitutive isoforms levels (see details under 'Materials and Methods' section). Data are expressed as mean-fold induction \pm SD from three separate experiments. (B) Western blot analysis of cell extracts (15 μ g) from the PC3 clones confirmed down-regulation of Sam68 levels in pLKO-si-Sam68 cells. (C) pLKO and pLKO-si-Sam68 PC3 cells were treated with 5 μ M MTX for 2 h, allowed to recover and transiently transfected with pETv5Luc minigene in the presence (+) or absence (-) of murine mycSam68. The v5-Luc activity in untreated cells was set as 100% (dark grey columns) and v5-Luc activity in MTX-treated cells without (dim grey columns) or with (white column) murine mycSam68 was expressed as percentage respect to untreated cells. Data are expressed as mean-fold induction \pm SD from three separate experiments. (D) Western blot analysis of the pLKO clones cell extracts (15 μ g) confirmed down-regulation of Sam68 levels in pLKO-si-Sam68 cells and the overexpression of mycSam68. Protein loading was normalized on hnRNP A1 levels both in panel B and D.

RNAPII and are transcriptionally active (54) like the stress granules induced by MTX. However, the activity of these structures appears somewhat different, because nSBs are engaged in transcription of SatIII repetitive sequences after heat shock (46), whereas these non-coding RNAs are not transcribed in response to MTX treatment. Moreover, the repertoire of RBPs recruited to nSBs and MTX-induced nuclear granules is only partially overlapping. For instance, the latter structures recruit also hnRNP A1 and SC35, which were excluded from nSBs (20). Thus, although they share some components and activities, they likely play different functions in the cell, which might be instructed by the differential needs imposed by heat shock and DNA lesions.

Another type of RNA-containing structure that resembles MTX-induced stress granules are the well-characterized cytoplasmic SGs (40). SGs are involved in the stress-adaptation response of the cell

induced by several poisoning treatments, such as oxidative stress, osmotic and heat shock. Cytoplasmic SGs are characterized by accumulation of the splicing regulators TIA-1 and hnRNP A1. We observed that Sam68 also accumulated in cytoplasmic granules in a subset of MTX-treated cells. Since these structures also contained hnRNP A1 and TIA-1, they can be regarded as *bona-fide* SGs. However, although Sam68 interacts with hnRNP A1 through its C-terminal region, a GFP-Sam68_{GSG} deletion mutant lacking the entire C-terminal domain was still capable to relocalize both in the nuclear and cytoplasmic stress granules after MTX treatment, suggesting that interaction with hnRNP A1 was not necessary for these events. Interestingly, our results are in line with the recently described accumulation of Sam68 in cytoplasmic SGs after oxidative stress (41), and suggest that genotoxic stress also causes formation of Sam68-containing SGs. These data indicate that Sam68 is part of a

RNA-mediated genotoxic stress response of the cell and that it may exert an active role through changes in its subcellular localization.

Sam68 is a prototype of the STAR proteins, which are known to link signal transduction pathways to changes in RNA metabolism (21). For instance, growth factors stimulate the MAPKs ERK1/2, which in turn phosphorylate Sam68 and stimulate its splicing activity (26). Since MTX triggered the activation of stress-response MAPKs, we asked whether these pathways were involved in Sam68 relocalization. However, our results, obtained with specific inhibitors of the p38 and JNK1/2 pathways, strongly suggest that phosphorylation of Sam68 by these kinases is not required for its recruitment to MTX-induced nuclear stress granules.

Chromatin fractionation experiments and colocalization with the phosphorylated RNAPII and nascent RNAs indicated that MTX-induced nuclear granules were transcriptionally active. We labelled the cells in the last hour of treatment, thus, the BrU-positive granules are likely sites of *de novo* synthesis of RNA rather than sites of accumulation of RNAs that had been synthesized before the MTX treatment. Since several RBPs involved in AS and other steps of post-transcriptional regulation of mRNAs were recruited to these MTX-induced nuclear granules, we asked whether Sam68 was involved in processing of target mRNAs during the genotoxic stress. A particularly interesting splicing target of Sam68 is CD44. This gene encodes for several isoforms that differ for the inclusion of variable exons (v1–v10), which confer oncogenic potential to the corresponding proteins (51–53). Remarkably, a recent study showed that CD44 undergoes profound changes in AS during genotoxic stress caused by cisplatin in cancer cells (12). Thus, we tested whether MTX induced changes in the inclusion of v5, the target of Sam68 activity (26), and whether or not these changes required Sam68. Remarkably, our experiments, which monitored both the endogenous CD44 mRNA and a reporter minigene, clearly showed that v5 exon inclusion correlated with formation of the nuclear stress granules and that this splicing event was dependent on Sam68 expression. Thus, it is possible that compartmentalization of Sam68, and of other splicing factors, in the transcriptionally active nuclear stress granules redirects the splicing machinery during the response to genotoxic stress, thereby affecting specific splicing events. This response might allow the cell to produce protein isoforms that allow to withstand the stress and to favor repair of the damage caused. Indeed, expression of CD44v5 isoforms was shown to have prognostic value in human thymic tumors (53). In this regard, it will be interesting to determine the genes affected by MTX at the level of AS on a large scale, similarly to what recently described for the response to UV irradiation (3). Since Sam68 protects PCa cells from genotoxic stress (29), it is possible that its splicing targets contribute to enhance cell survival in response to DNA damage. Thus, future studies will aim at clarifying how Sam68 modulates AS in response to genotoxic stress and at the identification of mRNAs whose AS is regulated in a Sam68-dependent manner after DNA damage in cancer cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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