Targeting Protein for Xenopus kinesin like protein 2 (TPX2) regulates gamma-H2AX levels upon ionizing radiation

Gernot Neumayer*◊, Angela Helfricht^~, Su Yeon Shim*◊~, Hoa Thi Le*, Cecilia Lundin♦, Camille Belzil*, Mathieu Chansard*, Yaping Yu◊, Susan P. Lees-Miller◊, Oliver Gruss#, Haico van Attikum^, Thomas Hellday♦ and Minh Dang Nguyen*◊

*Departments of Clinical Neurosciences and Cell Biology & Anatomy, Hotchkiss Brain Institute, ◊Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive NW, T2N4N1, Canada. ♦ Science for Life Laboratory, Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 21 Stockholm, Sweden. ^Leiden University Medical Center, Department of Toxicogenetics, Einthovenweg 20, 2333 ZC, Leiden, The Netherlands. # DKFZ-ZMBH Alliance, ZMBH, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany. ~ Equal contribution.

Running Title: A novel function for TPX2

To whom correspondence should be addressed: Dr. Minh Dang Nguyen; Tel.: 1-403-210-9626; Fax: 1-403-210-8802; E-mail: mdnguyen@ucalgary.ca

Keywords: TPX2, MAPs, Cytoskeleton, DNA damage response, Mitosis, Cell cycle, Neurons

Background: TPX2 is an essential protein for mitosis but its nuclear function is unknown.

Results: TPX2 goes to DNA double strand breaks and regulates γ-H2AX levels upon ionizing radiation.

Conclusion: We discover a novel and the first nuclear function for TPX2.

Significance: Our study provides new insights into the physiological and oncological roles of TPX2.

SUMMARY

The microtubule-associated protein TPX2 plays a key role in spindle assembly and is required for mitosis in human cells. In interphase, TPX2 is actively imported into the nucleus to prevent its premature activity in microtubule organization. To date, no function has been assigned to nuclear TPX2. We now report that TPX2 plays a role in the cellular response to DNA double strand breaks induced by ionizing radiation. Loss of TPX2 leads to inordinately strong and transient accumulation of ionizing radiation-dependent Ser139-phosphorylated Histone 2AX (γ-H2AX) at G0 and G1 phases of the cell cycle. This is accompanied by the formation of increased numbers of high intensity γ-H2AX ionizing radiation-induced foci. Conversely, cells overexpressing TPX2 have reduced levels of γ-H2AX after ionizing radiation. Consistent with a role for TPX2 in the DNA damage response, we found that the protein accumulates at DNA double strand breaks and associates with the Mediator of DNA damage Checkpoint 1 (MDC1) and the Ataxia Telangiectasia Mutated (ATM) kinase, both key regulators of γ-H2AX amplification. Pharmacologic inhibition or depletion of ATM or MDC1, but not of DNA-dependent protein kinase (DNA-PK), antagonizes the γ-H2AX phenotype caused by TPX2 depletion. Importantly, the regulation of γ-H2AX signals by TPX2 is not associated with apoptosis or the mitotic functions of TPX2. In sum, our study identifies a novel and the first nuclear function for TPX2 in the cellular responses to DNA damage.

Human TPX2, the ortholog of the Targeting Protein for Xenopus kinesin-like protein 2, was initially named and described as the Restricted Expression / Proliferation-associated protein 100 due to its high expression levels in proliferating cells and tissues (1). The current name of TPX2 is based on its function to regulate the Xenopus kinesin-like protein 2, a plus end-directed microtubule motor required for centrosome separation and maintenance of spindle
polarity during mitosis (2-8). During cell cycle progression TPX2 levels gradually increase with highest expression in mitosis and lowest levels in G1 phase (1-6). In mitotic human cells, TPX2 mediates the binding of the human ortholog of Xenopus kinesin-like protein 2 (i.e. kinesin family member 15) to microtubules, activates the mitotic serine-threonine Aurora A kinase, and nucleates microtubules (2-6,9). By virtue of its microtubules-associated functions, TPX2 is defined as a microtubules-associated protein and a critical factor for spindle assembly and mitosis in mammalian cells (2-6). During interphase, lasting up to 23h out of a 24h cell cycle (e.g. HeLa cells), TPX2 is actively transported into the nucleus via the importin α/β receptors (10,11). This nuclear import is thought to sequester TPX2 away from cytoplasmic tubulin to prevent premature spindle assembly (10,11). However, to date, no function has been associated with nuclear TPX2 despite the fact that TPX2 resides in the nucleus during the majority of the cell cycle.

Intriguingly, elevated levels of TPX2 have been detected in numerous cancers (ovary, lung, pancreas, bone, carcinoma, cervix, etc) and amplification of the TPX2 gene has been suggested to promote the progression of colorectal malignancies (12-19). Conversely, TPX2 haploinsufficiency, leading to decreased levels of TPX2, significantly increases the propensity for the development of tumors in mice (20). Together, these results suggest that deregulation of TPX2 levels and functions are associated with the etiology of cancers.

Many cancers arise from genomic instability caused by disturbed responses to DNA damage (21,22). Interestingly, TPX2 has been shown to associate with the Breast Cancer 1 protein (BRCA1) during cytoskeletal remodeling events (23,24) and was identified as a potential substrate of the ATM (Ataxia Telangiectasia Mutated) kinase, as suggested by a high throughput screen that displayed over 700 other hits (25). BRCA1 and ATM are key factors of the cellular DNA damage response to DNA double strand breaks (26-28). However, a function for TPX2 in DNA damage response has not been identified.

The DNA damage response consists of a complex network of signaling and repair pathways that maintain genomic integrity (26,29). Experimentally, the DNA damage response is frequently studied by examining the cellular response to ionizing radiation (26,29). Ionizing radiation induces multiple forms of DNA damage, the most lethal of which is the DNA double strand break (26,29). Upon ionizing radiation treatment the DNA damage response mediates cell cycle arrest (through checkpoints), facilitates DNA repair, promotes cell survival or triggers apoptosis if the damage is too severe. At the molecular level, the DNA damage sensor protein complex MRN (composed of MRE11, Rad50 and NBS1) is recruited to the DNA double strand breaks at early stages of DNA damage response. NBS1, the Nijmegen Breakage Syndrome protein 1, then recruits the ATM kinase to the DNA double strand break (30-37). Subsequently, the ATM kinase, which becomes activated by the presence of DNA double strand breaks (38), phosphorylates multiple DNA damage response proteins including the chromatin core component Histone 2AX (H2AX) and the Mediator of DNA damage Checkpoint 1 (MDC1) (30-37). However, phosphorylation of H2AX can also be carried out by the DNA-dependent Protein Kinase (DNA-PK) (36). Upon phosphorylation at serine 139, p-H2AX (also called γ-H2AX) binds to BRCA1 C-terminal (BRCT) domains in DNA damage response proteins such as MDC1, resulting in the accumulation of MDC1 at DNA double strand breaks-flanking chromatin regions. MDC1 then recruits more ATM kinase to the sites of DNA lesions, which induces further γ-H2AX formation. Together, this creates a positive feedback loop resulting in the amplification and spreading of H2AX phosphorylation over chromatin regions containing megabases of DNA (39-43). This DNA double strand breaks-flanking accumulation of γ-H2AX and MDC1 is essential to attract and retain numerous other DNA damage response-factors (e.g. RNF8, RNF168, BRCA1, 53BP1, etc) to the lesion sites (26-29), thereby facilitating DNA damage response. The accumulation of DNA damage response molecules at DNA double strand breaks manifests as so-called ionizing radiation-induced foci, which are microscopically visualized as bright spots (29).

In this study we aimed to determine whether alteration in TPX2 levels, as found in
numerous cancers exhibiting genomic instability, is associated with abnormal DNA damage response. Since phosphorylation of H2AX lies at the core of the DNA damage response we analyzed γ-H2AX levels in cells with altered expression of TPX2. Using four different RNAi approaches, we found that depletion of TPX2 in cells leads to a transient increase in γ-H2AX levels following treatment with ionizing radiation. Conversely, overexpression of TPX2 decreases ionizing radiation-induced MDC1 foci and γ-H2AX levels. Importantly, these phenomena are not correlated with apoptosis and occur independently of the mitotic functions of TPX2. Furthermore, TPX2 accumulates at DNA double strand breaks and associates with the machinery of DNA damage response that controls the amplification of γ-H2AX (i.e. MDC1 and ATM). Our findings identify a novel and the first nuclear function for TPX2.

EXPERIMENTAL PROCEDURES

Western blots – Protein extracts were isolated for specific purposes as described below. The protein concentration was estimated by the Bradford procedure (Bio-Rad Laboratories, Hercules, CA) or Bio-Rad Dc assay to ensure equal loading. Proteins were fractionated on SDS-PAGE and blotted on a PVDF membrane for western blot analysis. Membranes were incubated with antibodies against Actin (Chemicon), ATM (Upstate), p-ATM (S1981; Rockland; Epitomics), DNA-PKcs (18-2; Abcam), GAPDH (HRP conjugated abs) (Abcam), GFP (Santa Cruz Biotechnology), FLAG (Sigma), H2A (H-124; Santa Cruz Biotechnology), H2AX (Abcam), γ-H2AX (S139; JBW301; Millipore), p-H3 (S10; Millipore), H3 (Abcam), cleaved PARP1 (Millipore), NBS1 (1D7, Abcam), NdeI (home-made, (44)), MDC1 (Abcam) and TPX2 (184, Novus Biologicals). Quantification of band-signals was performed using Quantity-One software from Bio-Rad and signals were corrected with levels of actin, GAPDH or non-phosphorylated form of the protein of interest. For the statistical analysis, please refer to individual figure legends.

Co-immunoprecipitations – Total protein extracts of cells were obtained by lysis of cells in ice cold RIPA buffer (50mM Tris – HCl pH 7.4, 150mM NaCl, 10mM KCl, 1mM EDTA, 0.5% Tween20, 0.5% NP40, 1mM PMSF, 5mM sodium fluoride, 1 mM sodiumorthovandate, 1x protease inhibitor cocktail complete Mini, EDTA free (Roche)). Extracts were sonicated twice for 5 sec with a Sonic Dismembrator Model 100 at Level 4 and protein concentration was determined using the Bio-Rad Protein (Bradford) assay. Co-immunoprecipitations and nuclear fractionation were performed using standard laboratory procedures. Antibodies used for co-immunoprecipitations are MDC1 (Abcam) and TPX2 (184 from Novus Biologicals, TPX2 serum and KiS2). Commercially available TPX2 antibodies 184 are specific for the C-terminus (immunogen: a.a. 700-749) of TPX2 (specificity has been further confirmed by peptide competition- see Suppl. Fig.5 - and by depletion of TPX2 by RNAi, see Fig.4). TPX2 serum ((45), a kind gift of Dr. D. Compton) was obtained from rabbits immunized with recombinant full length TPX2. The monoclonal TPX2 KiS2 antibody was a kind gift of Dr. Heidebrecht (1).

GST-TPX2 pull-down assays – GST-TPX2 fusion proteins were produced by cloning of TPX2 into pGEX-6.1p, expression in E. coli and purification on glutathione sepharose beads using standard procedures. To couple proteins to beads, 5 µg of purified GST-TPX2 or GST were incubated with glutathione sepharose 4B beads (GE Healthcare) for 1h at 4°C in buffer containing 160 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, 2 mM DTT plus 0.2 mM PMSF, 0.2 µg/ml leupeptin and 0.2 µg/ml pepstatin. The beads were then washed with a buffer containing 50 mM Tris-HCl, pH7.5, 1 mM EDTA and 1% (v/v) NP40 plus 0.2 mM PMSF, 0.2 µg/ml leupeptin, 0.2 µg/ml pepstatin, 0.2 µg/ml aprotinin and 1 µM microcystin-LR. HeLa cells were lysed in the same buffer, extracts were diluted to contain 0.25% NP40 and 2 mg of extract was incubated with glutathione beads to which either GST-TPX2 or GST protein had been coupled. Extracts were incubated for 2 h at 4°C and washed. Samples were analyzed by western blot.

Chromatin fractionation for analysis of γ-H2AX levels – Cells were washed twice in PBS (37°C) and lysed in ice cold NETN buffer (150mM NaCl, 1mM EDTA, 50mM Tris-Cl pH 7.4, 1% NP40 and 1 x protease inhibitor cocktail complete Mini, EDTA free (Roche)). Extracts were
were sonicated once for 5 sec with a Sonic Dismembrator Model 100 at Level 4 and the unsoluble chromatin fraction was pelleted for 20 min at 4 °C at full speed in a table top centrifuge. The soluble NETN fraction (containing the nuclear lamins but no histones) was kept at -80°C for further analysis and the insoluble chromatin fraction (containing the histones but not nuclear lamins) was washed once in 1ml NETN buffer. The chromatin fraction was solubilized by addition of 1% SDS in PBS followed by 1 freeze and thaw cycle at -80°C, incubation at 95°C for 15 min and sonication for 15 sec. Protein concentrations were determined using the Bio-Rad Dc protein assay (chromatin fraction) or Bio-Rad Protein (Bradford) assay (NETN soluble fraction).

Ionizing radiation -induced DNA damage was generated using a source of Cs$^{137}$ from a MDS Nordion Gammacell 1000 (for all Figs. except Figs.1D-E) or a GSR D1 from Gamma-Service Medical GmbH (for Figs.1D-E).

AsiSI-ER cells – U2OS cells containing a stably integrated AsiSI-ER construct (a kind gift of Dr. Gaëlle Légube) were grown in selective medium (1 μg/ml pyromycin). To induce nuclear expression of AsiSI-ER and generate site-specific DNA double strand breaks, cells were treated with 300 nM 4-hydroxytamoxifen (4-OHT) for 4 hours.

Laser micro-irradiation – Laser micro-irradiation was carried out on a Leica SP5 confocal microscope equipped with an environmental chamber set to 37°C and 5% CO2. U2OS cells were grown on glass cover slips. DNA double strand breaks-containing tracks (1.5 x n μm) were generated with a Mira modelocked Ti:Sapphire laser (λ = 800 nm, pulse length = 200 fs, repetition rate = 76 MHz, output power on the cells for DNA damage induction = 80 mW). Cells were irradiated at 1 min time intervals and after 10 min immunostained with TPX2 (184, Novus Biologicals) and γH2AX (Millipore) antibodies.

Generation of constructs and RNAi sequences – To generate the bait-TPX2 vector (full length or a.a. 8-747) for Y2H, human TPX2 cDNA (bp 1 or 22-2241) of pQE-70-TPX2 (4) was cloned into pGBK7 (Clontech). The pGADT7-MDC1 was generated by cloning human MDC1 cDNA (bp 5049 of coding sequence – end of 3'UTR) of pACT2-MDC1 (from Y2H screen) into pGADT7 (Clontech). Human TPX2 cDNA (bp 4-2241 or 3-2241) was cloned into pcDNA4/HISMAX (Invitrogen) or pEGFP-C1 (Clontech) to generate the His-TPX2 or GFP-TPX2 vector. RNAi sequences were selected based on the criteria of Ambion, Inc. Complementary shRNA sequences were commercially synthesized and cloned into pSilencer 2.0 under promotor U6 (Ambion). The sequence for the mouse TPX2 shRNA is AACACTTACCACAAAGAGACA. Sequences for the human TPX2 siRNA, TPX2 miRNA, Ndel1 siRNA, NUF2 siRNA and MDC1 siRNA have been described previously [(4,17,39,46,47), see s37983 from Ambion for NUF2 siRNA]. The specific ATM and DNA-PKcs siRNAs were purchased from Dharmacon. The TPX2 3’UTR siRNA was purchased from Qiagen. A random sequence without homology to any known mRNA was used for control RNAi (44,48-50). RNAi constructs were tested in CAD cells, primary neuronal cultures, U2OS cells and HeLa cells by both western blots and immunofluorescence staining.

Cell cultures, transfection, induction and pharmacological treatments – HeLa (ATCC), HeLa EM2-11-TPX2 (17), MCF-7 (ATCC) and U2OS cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin – streptomycin. LAN1 cells (a kind gift of Dr. Valentiner Ursula) were cultured in RPMI media containing L-Glutamine, 10% FBS and 1% penicillin – streptomycin. Primary cortical and hippocampal cultured post-mitotic neurons were prepared from mice as per (44,49,50). The neurons were maintained in BME supplemented with Glucose (6mg/ml), Glutamine (2mM), HEPES (10mM), Na-pyruvate (1mM), FBS (2%), penicillin-streptomycin (2%) and B-27 (2%). Transfection of cell lines with constructs or oligonucleotides was performed using Lipofectamine™ 2000 Reagent (Invitrogen), HiPerFect Transfection Reagent (Qiagen) or DharmaFECT 1 (Dharmacon) in accordance with the supplier’s recommendations. Primary neurons were either transfected with Lipofectamine 2000 or electroporated as per (44,49). Expression of TPX2 targeting miRNA in HeLa EM2-11-TPX2 was induced by doxycycline (1μg/ml media). Of note, although the doxycycline inducible TPX2 RNAi originates from an exogenous miRNA, the mechanism of TPX2 knock-down is siRNA based
A novel function for TPX2

(17). Elements of miRNA precursors such as PolIII promotor, drosha processing and Exportin 5 recognition sequences are employed in this system to produce a siRNA double strand that is identical (apart from stabilizing modifications added to synthetic stealth siRNAs) to a synthetic siRNA duplex administered by transfection. However, the amount of siRNA that originates from this exogenous doxycycline inducible miRNA is lower than what is delivered to the cell by regular transfection of synthetic siRNAs and thus, maintains the cell in a more physiological context. Target proteins were knocked down for 24h or 48h (TPX2), 72h (MDC1) and 96h (ATM/DNA-PKcs) before sample processing. For the TPX2/MDC1 double knockdown, HeLa cells were transfected with MDC1 (39) or AllStars Negative control (Qiagen) siRNA (10nM) 24h post-seeding. 48h after the initial MDC1/control knockdown, cells were transfected again with combined TPX2-control, MDC1-control, TPX2-MDC1 or control RNAi mixes (20nM each) and cultured for 24h before harvesting. For the TPX2/ATM or TPX2/DNA-PKcs double knockdown, HeLa cells were transfected with ATM, DNA-PKcs or AllStars Negative control (Qiagen) siRNA (10nM) 24h post-seeding. 72h after the initial ATM, DNA-PKcs or control knockdown, cells were transfected again with TPX2 or control siRNA (20nM) and cultured for 24h before harvesting. For pharmacological inhibition of ATM and DNA-PK, KU55933 and NU7441 (Tocris Bioscience) were dissolved in DMSO and administered to cells 45 min prior to ionizing radiation at concentrations of 8μM and 5μM, respectively. For pharmacological inhibition of caspases, Z-VAD-FMK (Santa Cruz Biotechnology; dissolved in DMSO) was administered to cells prior to ionizing radiation at a concentration of 10μM (51).

Immunofluorescence staining and microscopy analysis – Cells were fixed, permeabilized and blocked as described in (44,48-50). Cells were stained with antibodies against γ-H2AX (S139; JBW301; Millipore), TPX2 (184, Novus Biologicals) or MDC1 (Abcam). Nuclei were stained with DAPI. Images were acquired using a NIKON D-Eclipse C1 confocal microscope. Total signal-intensity of individual γ-H2AX ionizing radiation-induced foci in U2OS cells, images were acquired using an IN Cell Analyzer 1000 (GE Healthcare) and analyzed with IN Cell Analyzer 1000 Workstation software using the Multi Target Analysis module (GE Healthcare). γ-H2AX foci with intensity above set threshold (2 standard deviations above mean foci intensity) were counted as high-intensity foci.

Cell cycle synchronization – For G1, S and G2 phases: HeLa EM2-11-TPX2 cells or U2OS cells were seeded 24h before treatment with 2mM Thymidine for 20h. Cells were then trypsinized and released in Thymidine free media for 15h followed by treatment with 2mM Thymidine for an additional 14h. Early S phase arrested cells were released into fresh media and monitored throughout the following synchronous cell cycle via flow cytometry based cell cycle profiling. Samples were taken at indicated cell cycle stages and used for ionizing radiation treatment and western blot analysis or ethanol-fixation, Propidium Iodide staining and flow cytometry based cell cycle profiling. For M phase: Hela cells were treated with 100ng/ml of nocodazole (Sigma) for 16-17h to arrest cells at the M phase. The M phase-synchronized cells were irradiated with 10 Gy and harvested for western blot analysis or ethanol-fixation, Propidium Iodide staining and flow cytometry based cell cycle profiling.

Yeast two hybrid screen and assay – Y2H screen in the Saccharomyces cerevisiae strain AH109 (Clontech) was carried out with the pGBK7-TPX2 (a.a. 8-747) bait vector and a human fetal brain MATCHMAKER cDNA library cloned in pACT2 plasmids (Clontech). Sequential transformation of the bait and cDNA library was performed as per references (52,53) and Y2H experiments employed combined ADE2 / HIS3 high stringency reporters. The putative TPX2-interacting proteins were identified via isolation of pATC2 plasmids from colonies growing on high stringency selection media and sequencing. For the independent Y2H assay, the AH109 yeast strain was co-transformed with pGBK7-TPX2 (a.a. 1-747 or 8-747) and pGADT7-MDC1 (a.a. 1683-STOP and 1809-STOP) and processed as described above.

Downloaded from http://www.jbc.org/ by guest on October 30, 2017
RESULTS

TPX2 regulates the levels of γ-H2AX upon treatment with ionizing radiation – TPX2 associates with DNA damage response proteins such as BRCA1 and may be a target of the ATM kinase (23-25). Moreover, TPX2 levels are altered in cancers associated with aberrant cellular responses to DNA damage and genomic instability (12-19). These observations raise the question as to whether TPX2 is involved in DNA damage response. To test this hypothesis, we analyzed the levels of γ-H2AX, a key marker of DNA damage response amplification, in HeLa cells depleted of TPX2 by small interfering RNA (siRNA) and in a HeLa stable cell line expressing a doxycycline-inducible exogenous microRNA (miRNA) targeting specifically TPX2 mRNA (see Experimental procedures for further details on this cell line and (17)) following ionizing radiation. The latter system presents the advantage to knockdown TPX2 in an entire population of cells. Specificities of the TPX2 siRNA and miRNA have been demonstrated previously (4,17), and were confirmed by western blot analysis (Fig.1). In absence of ionizing radiation, loss of TPX2 did not trigger an increase in γ-H2AX signal (Fig.1A-C, F and H) nor did it result in higher amounts of DNA double strand breaks as detected by the neutral comet assay (Suppl. Fig.1). Interestingly, we found that a 60 to 95% knockdown of TPX2 significantly enhanced the levels of γ-H2AX by ~3 to 9 fold, respectively, 1h post-10 Gray (Gy) when compared to irradiated control cells (Fig.1A). Both approaches targeting different sequences of TPX2 mRNAs generated the same effect. To further support the specificity of the observed phenotype we performed rescue experiments by expressing a GFP-TPX2 construct in HeLa cells depleted of endogenous TPX2 with a third siRNA sequence targeting the 3’ untranslated region of TPX2 mRNA. In these cells, endogenous TPX2 is depleted but GFP-TPX2 is still expressed (Fig.1B). While irradiated TPX2-depleted cells exhibit increased γ-H2AX, expression of GFP-TPX2 reduces such increase (Fig.1B) (see Fig.5 and Suppl. Fig.5 for similar results with an additional fourth RNAi sequence). Taken together, these results provide compelling evidence that TPX2 impacts γ-H2AX levels during DNA damage response.

To further understand the correlation between TPX2 depletion and increased γ-H2AX signals, we performed a time course analysis of ionizing radiation-triggered H2AX phosphorylation over a 6h period. We found that both TPX2-depleted cells and control cells exhibited similar time courses for γ-H2AX formation: Both groups showed 10 Gy-triggered phosphorylation of H2AX 15 min to 2h after ionizing radiation. Phosphorylation of H2AX started to decrease 3h after ionizing radiation and was almost reduced to background levels 4h post-ionizing radiation (Fig.1C). However, knockdown of TPX2 caused dramatic increases in γ-H2AX levels at 15 min to 2h after ionizing radiation when compared to control cells (Fig.1C). Of note, TPX2 gets enriched in the chromatin fraction during the course of DNA damage response (shown in Fig.1C, see Experimental Procedures).

We next analyzed γ-H2AX ionizing radiation-induced foci in TPX2-depleted U2OS cells and control cells using immunofluorescence microscopy. Ionizing radiation-induced foci were analyzed for their intensity and mean number per cell at different time points (15 min, 1h, 2h and 3h) post a non-lethal dose of 4 Gy. Note that a higher dose of 10 Gy cannot be used for this type of experiments as it interferes with the resolution of single γ-H2AX foci, thereby impeding the interpretation of the results. We found that 1h and 2h post 4Gy, the percentage of cells with high intensity γ-H2AX ionizing radiation-induced foci (i.e. foci intensity 2 times the standard deviation above mean foci intensity) was markedly increased in TPX2-depleted populations compared to control cultures (Fig.1D). 3h post-ionizing radiation, the percentage of TPX2-depleted cells with high intensity γ-H2AX ionizing radiation-induced foci decreased to the level of control cells (Fig.1D). These results are consistent with our western blot time course data of γ-H2AX signals at these time points (Fig.1C). However, we did not observe significant differences in the number of γ-H2AX ionizing radiation-induced foci between control and TPX2-depleted cells (Fig.1E). This result suggests that the number of DNA double strand breaks is similar between the two groups and that the increased γ-H2AX signal in irradiated TPX2-depleted cells is not associated with apoptotic DNA laddering / fragmentation (which
can also induce γ-H2AX but would lead to increased numbers of γ-H2AX foci). To further support the notion that the TPX2 depletion-dependent increase in ionizing radiation-triggered γ-H2AX is not linked to apoptosis, we employed caspase-3-deficient MCF7 breast cancer cells that do not undergo ionizing radiation-induced DNA fragmentation and apoptosis (54,55). These cells still display the ionizing radiation-dependent increase in γ-H2AX following TPX2 depletion by siRNA for 24h or 48h (Fig.1F). Furthermore, several cell types depleted of TPX2 (i.e HeLa, U2OS cells) that were treated with non-lethal doses of ionizing radiation (2 and 4 Gy; see Fig.1D, E, G) still display upregulated γ-H2AX signals, although they recover from these ionizing radiation doses and continue their cell cycle (Suppl. Fig.2). Finally, 1h after ionizing radiation, TPX2-depleted cells treated with the broad spectrum caspases inhibitor Z-VAD-FMK at a known effective dose (see (51) and the decreased levels of cleaved PARP1, a marker of apoptosis, in cells co-treated with the DNA damaging drug camptothecin and the inhibitor Z-VAD-FMK) still exhibited increased levels of γ-H2AX compared to control cells expressing TPX2 (Fig.1H). Taken together, these results indicate that the absence of TPX2 causes a hyperamplification of γ-H2AX signals at DNA double strand breaks but no increase in the number of γ-H2AX ionizing radiation-induced foci (Fig.1A-E). This elevation of γ-H2AX signals in TPX2-depleted cells after ionizing radiation-treatment is not associated with pronounced apoptosis (Fig.1E-H).

We next asked how an increase in TPX2 levels affects H2AX phosphorylation after ionizing radiation treatment. Conversely to the phenotype observed in irradiated TPX2-depleted cells, ectopic expression of a vector encoding His- or GFP-TPX2 reduced ionizing radiation-triggered phosphorylation of H2AX by ~ 80% when compared to irradiated control cells as detected by western blots (Fig.2A) and immunofluorescence microscopy (Fig.2B). We found that a transient transfection of 4 to 8 µg of vector in our experimental set-up (depending on the plasmid used), corresponding grosso modo to a 1.5 to 2 fold overexpression of ectopic TPX2 in an asynchronous cell culture, is required to abolish gamma-H2AX induction (Fig.2C). Compatible with a model of an inter-dependency between γ-H2AX formation and MDC1 ionizing radiation-induced foci formation (27,28,42), cells overexpressing His- or GFP-TPX2 also displayed impaired formation of MDC1 ionizing radiation-induced foci (Fig.2D). In sum, these results indicate that changing the levels of TPX2 dramatically impacts the levels of γ-H2AX, possibly through alterations in MDC1 ionizing radiation-induced foci formation.

TPX2 localizes to DNA double strand breaks – The impact of altered TPX2 levels on ionizing radiation-induced γ-H2AX formation (Fig.1-2) suggests a novel function for TPX2 in DNA damage response. Since many proteins involved in DNA damage response accumulate at DNA double strand breaks (28,30-37), we sought to determine the cellular distribution of TPX2 upon treatment with ionizing radiation. The hypothesis of an ionizing radiation-induced change in TPX2 localization was supported by data showing ionizing radiation-dependent enrichment of TPX2 in the chromatin fraction (Fig.1C). Strikingly, TPX2 formed ionizing radiation-induced foci that partially co-localized with γ-H2AX ionizing radiation-induced foci in undifferentiated neuroblastoma LAN1, a sub-population of cycling U2OS cells (Fig.3A-B) and primary post-mitotic mouse neurons (Suppl. Fig.5). It is noteworthy that in neuroblastoma LAN1 cells, TPX2 localizes, like in neurons, to the nucleus and cytoplasm (see below and Suppl. Fig.5 for further details on the expression of TPX2 in neuronal cells). The usage of G0 primary neurons in these experiments suggests that the herein reported function of TPX2 in DNA damage response is distinct from the mitotic functions of TPX2 since these cells do not divide (see below).

Furthermore, TPX2 also formed foci positive for γ-H2AX in a system that introduces AscI-generated DNA double strand breaks (56) (Fig.3C). Finally, TPX2 also accumulates at sites of DNA lesions generated via micro-irradiation with a multi-photon laser (57). Upon micro-irradiation, TPX2 was found to be enriched by ~1.6 fold in the resulting laser tracks which were also positive for γ-H2AX, indicating the presence of DNA double strand breaks (Fig.3D). Taken together, these results underscore the recruitment of TPX2 to DNA double strand breaks generated
A novel function for TPX2

TPX2 regulates γ-H2AX levels and forms ionization radiation-induced foci at G1 phase – TPX2 expression levels are regulated in a cell cycle-dependent manner (4). This raises the question as to whether γ-H2AX signal amplification by TPX2 occurs at specific cell cycle stage(s). Furthermore, not all cells in Fig. 3B exhibited TPX2 ionizing radiation-induced foci, indicating cell cycle dependent differences. To investigate this, we synchronized HeLa cells depleted of TPX2 using a double thymidine block (see Experimental procedures, Fig.4 and Suppl. Figs.3-4), and analyzed γ-H2AX signal levels following 10 Gy and 1h recovery at specific cell cycle stages. 11 and 12 hours after release from the thymidine-triggered early S phase block ~80% of control and TPX2-depleted cells were in G1 phase as determined by flow cytometry (Fig.4 see Suppl. Fig.3 for profile of non-synchronized populations displaying less than 50% G1 phase cells). Significantly, TPX2-depleted G1 cells exhibited a marked increase in γ-H2AX signals following ionizing radiation treatment when compared to control populations (Fig.4A). In populations enriched for S-phase cells (~95% 2h after release) or G2-phase cells (~80% 6h after release), no significant differences in ionizing radiation-dependent γ-H2AX signals were observed between control and TPX2-depleted cultures (Suppl.Fig.4A). To analyze a potential effect of TPX2-depletion on γ-H2AX levels during mitosis, cells were treated with nocodazole to obtain populations highly enriched for M phase (~90%). Note that duration of mitosis in these cells is very short (less than 2h), making it virtually impossible to capture synchronous M phase populations via release from a double thymidine block. No difference in ionizing radiation-triggered γ-H2AX was found between nocodazole treated control or TPX2-depleted cell cultures (Suppl.Fig.4B).

In agreement with these western blot results we found using confocal microscopy that U2OS cell cultures synchronized at G1 phase with a double thymidine block exhibited prominent TPX2 ionizing radiation-induced foci that partially co-localized with γ-H2AX foci. However, cell cultures enriched for S or G2 phase did not form such pronounced TPX2 ionizing radiation-induced foci (Fig.4B). It is noteworthy that a previous study suggested that the basal levels of γ-H2AX in unirradiated S and G2 phase cells are ~3 fold higher than in G1 phase cells (58). The slope of the ionizing radiation-induced γ-H2AX response curve for G1 phase cells is in fact 2.8 times steeper than the slope for S phase cells (58). Our results in Fig.4B are in agreement with these data (note the higher levels of γ-H2AX in unirradiated S and G2 phase cells vs G1 phase cells).

In sum, our data indicate that TPX2 regulates γ-H2AX signal amplification at G1. Of note, during mitosis TPX2 stays associated with the mitotic spindle even in the presence of DNA double strand breaks that are marked by γ-H2AX (Figure 4C). These results further support the idea that TPX2’s function in regulating γ-H2AX levels is distinct from its mitotic roles (see below).

Altered levels of γ-H2AX by TPX2 dysfunctions are not caused by mitotic anomalies – Cells overexpressing or lacking TPX2 display spindle abnormalities (2,4), raising the question as to whether the alterations in levels of γ-H2AX observed in these cells were due to spindle defects and/or mitotic arrest rather than disturbances of the DNA damage response. To prove that spindle and mitotic defects are not involved in the observed γ-H2AX phenotype arising from altered TPX2 levels, we knocked down in HeLa cells two additional proteins involved in spindle biology and mitosis by siRNA and analyzed their levels of γ-H2AX upon irradiation. First, depletion of the microtubule-associated protein Ndel1 (Nuclear distribution E homolog like 1) that binds TPX2 and is also involved in microtubule dynamics (59) and spindle orientation and integrity (60-62), did not increase the levels of ionizing radiation-dependent γ-H2AX (Fig.5A). Second, depletion of the kinetochore complex member NUF2, which interferes with the attachment of the spindle to the kinetochore and triggers a strong mitotic arrest reminiscent of TPX2 deficiency (63), did not affect the levels of γ-H2AX when compared to controls (Fig.5B). The mitotic arrest upon NUF2 or TPX2 depletion (Fig.5B and Suppl. Fig.2) was confirmed with the increased levels of the mitotic marker Histone 3 phosphorylated at Serine10 (H3S10p). Note that although irradiated TPX2-depleted and NUF2-depleted cells exhibit similar
levels of H3S10p, TPX2-depleted cells display strikingly elevated ionizing radiation-dependent γ-
H2AX levels compared to NUF2-depleted cells. In
sum, these data support the idea that TPX2’s
regulation of γ-H2AX levels in cycling cells is not
an indirect consequence of defective mitosis but
rather reflects a previously unknown function for
TPX2.

To further support the notion of a novel
TPX2 function distinct from its mitotic roles we
isolated and cultured primary cortical mouse
neurons. These post-mitotic neurons are at G0
phase, no longer cycle and do not form a mitotic
spindle. However, they still express TPX2 as
evidenced by protein, RNA and immunofluorescence analysis (Suppl. Fig.5 and (59)). In neurons, TPX2 is localized in neuronal
processes, the cytoplasm and nucleus (Suppl.Fig.5
and (59)). Specificity of TPX2 antibodies (184;
Novus Biologicals) used for immunostaining was
determined by peptide competition (Suppl. Fig.5)
and depletion of TPX2 by RNAi (see below).

To examine the effect of TPX2 on γ-
H2AX formation in mouse cortical neurons, we
generated a short hairpin RNA (shRNA)-construct
targeting mouse TPX2 mRNA. The efficacy of the
vector was first tested in neuroblastoma mouse
CAD cells: Endogenous TPX2 protein was
diminished by ~70% in cells transfected with the
TPX2 shRNA vector, as determined by western
blot analysis (Suppl.Fig.5). No decrease was
observed in CAD cells transfected with a control
plasmid exhibited levels of TPX2 shRNA and GFP demonstrated a significant
decrease of TPX2 as determined by confocal
microscopy (Fig.5C). In the absence of ionizing
radiation, these neurons did not display γ-H2AX
signals. 10 Gy-irradiated neurons transfected with
the control plasmid exhibited levels of γ-H2AX
similar to irradiated non-transfected surrounding
cells. Importantly, 1h after ionizing radiation
neurons depleted of TPX2 by specific shRNA
exhibited much higher levels of γ-H2AX than
surrounding control cells and control shRNA-
transfected cells (Fig.5D). The average ratio of
total nuclear γ-H2AX signals of TPX2 RNAi /
untransfected cells was ~ 4 times higher than the
ratio found in control RNAi / untransfected cells
(Fig.5D, F). Conversely, the ectopic expression of
GFP-TPX2 abolished the 10 Gy-induced phosphorylation of H2AX in post-mitotic neurons
by 50% when compared to irradiated surrounding
cells. However, cells expressing GFP exhibited γ-H2AX levels similar to surrounding
untransfected cells (Fig.5E, G). Using a dose of 3
Gy, we further confirmed that alterations in TPX2
levels in these neurons impact γ-H2AX amplification (Fig.5H-I). The average ratio of total
nuclear γ-H2AX signals of 3 Gy-treated TPX2
RNAi / untransfected cells was ~ 20% higher than
the ratio found in irradiated control RNAi /
untransfected cells (Fig.5H). Conversely, the
ectopic expression of GFP-TPX2 abolished by
~30% the 3 Gy-induced phosphorylation of H2AX
in post-mitotic neurons when compared to
irradiated surrounding control cells. However,
cells expressing GFP exhibited γ-H2AX levels
similar to surrounding untransfected cells (Fig.5I).
Taken together, using four different TPX2
 targeting RNAi sequences and a rescue approach
(Figs.1, 4-5), our results confirm that TPX2 levels
dictate the extent of γ-H2AX formation during
DNA damage response in a similar manner in G1
cycling cells and G0 post-mitotic neurons. In sum,
the observed effects of TPX2 on γ-H2AX
formation during DNA damage response are
independent of TPX2’s mitotic functions.

TPX2 associates with proteins of the DNA
damage response machinery – MDC1 and
activated ATM mediate the amplification of γ-
H2AX signals at the proximity of the DNA double
strand breaks (40-42). Since we establish herein
that TPX2 impacts the levels of γ-H2AX (Figs.1-2
and 4-5) and in light of data suggesting that TPX2
is a target of ATM (25) (ATM is known to be in
complex with MDC1), we sought to investigate a
potential association between TPX2 and proteins
of the DNA damage response machinery. This
hypothesis was supported by an independent
experimental approach in which we recovered
MDC1 as a potential novel binding partner for
TPX2 in a yeast two-hybrid (Y2H) screen that
employed a ~25 week-old human embryonic brain
cDNA library. During that period of brain
development neuronal precursors and newly-born
neurons are highly susceptible to DNA damage-
induced cell death and, therefore, require an
10
efficient DNA damage response (64-67). This may explain the expression of MDC1 in the library. Using TPX2 and fragments of MDC1, we confirmed the interaction in independent Y2H assays: TPX2 (a.a. 8-747 and full length) binds to the C-terminus of MDC1 (a.a. 1683-2089 and a.a. 1809-2089) (Fig.6A), which includes the BRCT tandem domain that interacts with γ-H2AX (68).

When expressed ectopically in HeLa cells, the Flag-tagged C-terminus of MDC1 (C-MDC1-Flag, a.a. 1807-2089) co-immunoprecipitated with endogenous TPX2 (Fig.6B). Moreover, ectopically expressed GFP-TPX2 was also co-immunoprecipitated with endogenous MDC1 in HeLa cells (Fig.6B). In addition, we performed pull-down assays of MDC1 from HeLa cell lysates using purified glutathione S-transferase (GST) and GST–TPX2 full length (GST-TPX2) fusion proteins. MDC1 and the positive control Aurora A (69) were pulled down by GST–TPX2 but not by GST (Fig.6C).

Next, we determined whether TPX2 forms a complex with MDC1 under physiological conditions. Co-immunoprecipitation experiments were performed on U2OS, HeLa and neuroblastoma LAN1 cell lysates using three different antibodies specific for TPX2 (TPX2 184, TPX2 KiS2 and TPX2 serum) and antibodies specific for MDC1 (for details on and specificity of antibodies, see Experimental procedures). As shown in Fig.6D, TPX2 was co-immunoprecipitated with MDC1 from U2OS cell lysates using MDC1 antibodies. The reverse co-immunoprecipitation using commercially available TPX2 antibodies 184 also recovered MDC1 in the immunoprecipitates. Furthermore, NBS1, a member of the tripartite MRN complex known to associate with MDC1 (30-37), and a slower-migrating MDC1 species (suggesting post-translational modification), were also co-immunoprecipitated with TPX2 using the TPX2 KiS2 antibodies (Fig.6D). As shown in Fig.6E, we also detected a TPX2-MDC1 complex in HeLa cell lysates or LAN1 nuclear lysates using co-immunoprecipitations with either TPX2 184, TPX2 serum or MDC1 antibodies. To analyze the TPX2-MDC1 association in context of DNA damage response, HeLa cells were irradiated prior to co-immunoprecipitations with TPX2 184 antibodies. Following ionizing radiation, TPX2 was also found to associate with MDC1, serine 1981-phosphorylated ATM (p-ATM) and its substrate γ-H2AX (Fig.6E).

It is noteworthy that in HeLa cells the MDC1 antibodies recognize at least three bands of distinct molecular weight that may correspond to MDC1 isoforms based on entries in the uniprot-database (www.uniprot.org). Experimentally, treatment of HeLa cells with 2 different MDC1 siRNAs abolishes all these bands (Fig.7), indicating that they are MDC1 species. However, in U2OS cells only the lower molecular weight isoforms (~196/198 kDa) co-immunoprecipitate with TPX2, indicating cell line specific differences. In sum, our Y2H, pull down and co-immunoprecipitation data from three different cell lines and obtained with three TPX2 and MDC1 antibodies provide compelling evidence that TPX2 forms a complex with MDC1 in cells. ATM and NBS1 may be part of this novel TPX2 complex.

The effects of TPX2 on γ-H2AX levels can be antagonized by MDC1 and ATM. It is known that MDC1 is an essential factor for the amplification of γ-H2AX during DNA damage response. Loss of MDC1 decreases ionizing radiation-dependent γ-H2AX levels ((40-42) and Fig.7A). Herein we establish that TPX2 associates with the H2AX phosphorylation machinery (i.e. MDC1 and ATM) at sites of lesions. Furthermore, the levels of TPX2 are inversely correlated with the levels of γ-H2AX and formation of MDC1 ionizing radiation-induced foci. In light of all these results, we hypothesized that the TPX2 RNAi phenotype (i.e. increased ionizing radiation-dependent γ-H2AX levels) should be ameliorated if MDC1 function is lost. To test this, we knocked down both MDC1 and TPX2 with RNAi and determined the levels of ionizing radiation-dependent γ-H2AX. To minimize the effects of TPX2 knockdown on mitosis, cells were treated with TPX2 RNAi for a maximum of 24h while efficient knockdown of MDC1 was achieved 72h post-transfection (see Experimental procedures for double knockdown protocol). We found that depletion of MDC1 in irradiated TPX2-depleted cells antagonized the increased levels of γ-H2AX (triggered by the loss of TPX2) (Fig.7A).

During DNA damage response, H2AX can be phosphorylated by ATM and DNA-PK. To determine which kinase(s) account(s) for the
increased γ-H2AX levels in irradiated TPX2-depleted cells, we treated control and TPX2 RNAi-treated cells with DMSO (control), the ATM inhibitor KU55933 or the DNA-PK inhibitor NU7441 prior to ionizing radiation. Inhibition of ATM but not DNA-PK efficiently reduced the increased γ-H2AX levels triggered by depletion of TPX2 to background levels (Fig.7B). Of note, treatment with NU7441 similarly increased the γ-H2AX signals in TPX2-depleted and control cells compared to cells treated with DMSO, which is in agreement with a recent study (70). To support these results, we performed loss of ATM or DNA-PK function experiments using specific siRNA. To minimize the effects of TPX2 knockdown on mitosis, cells were treated with TPX2 RNAi for a maximum of 24h while efficient knockdown of DNA-PK catalytic subunit (DNA-PKcs) and ATM was achieved 96h post-transfection (see Experimental procedures for double knockdown protocol). Depletion of ATM but not DNA-PKcs abrogated the elevated γ-H2AX levels in irradiated TPX2-depleted cells (Fig.7C-D). It is noteworthy that depletion of DNA-PKcs downregulated ATM levels (as previously reported (71)). However, the remaining p-ATM levels were still sufficient to trigger the TPX2-depletion-dependent γ-H2AX phenotype. Taken together, these results indicate that the upregulation of γ-H2AX in irradiated cells depleted of TPX2 depends on ATM and MDC1. Interestingly, we did not observe a difference in ionizing radiation-dependent activation of ATM between control and TPX2-depleted cells, as indicated by the unchanged levels of p-ATM (Figs.1C and 7C). These data suggest that ATM is implicated in the γ-H2AX increase of irradiated TPX2-depleted cells, although the mode of ATM activation unlikely contributes to the phenotype (see Discussion). In light of our findings, we conclude that MDC1 and ATM can antagonize the effects of TPX2 on the amplification of γ-H2AX signals. It remains unclear whether TPX2 impacts the levels of γ-H2AX directly through its association with MDC1 and / or ATM (see discussion).

DISCUSSION

In the present study, we found that the levels of TPX2 inversely correlate with the levels of γ-H2AX during DNA damage response (Figs.1-2,4-5,7). Using four different RNAi sequences, we found that cells lacking TPX2 exhibit a significant increase in ionizing radiation-dependent phosphorylation of H2AX (1-2h after ionizing radiation) that is accompanied by increased numbers of cells with high intensity γ-H2AX ionizing radiation-induced foci (Fig.1). This phenotype was rescued by expression of a siRNA-insensitive TPX2 construct (Fig.1). Conversely, cells overexpressing TPX2 display decreased ionizing radiation-dependent γ-H2AX levels and defective MDC1 ionizing radiation-induced foci formation (Fig.2). TPX2 appears to impact γ-H2AX formation in G0 and G1 phases of the cell cycle as indicated by the use of synchronized cell cultures and post-mitotic primary neurons (Figs.4-5). Furthermore, consistent with a role in DNA damage response, TPX2 localizes to DNA double strand breaks and associates with MDC1 and p-ATM (Figs.3-4,6), known key factors for γ-H2AX amplification. Finally, the TPX2-depletion and ionizing radiation-dependent increase in γ-H2AX can be antagonized by inhibition or knockdown of MDC1 or ATM (Fig.7).

The early γ-H2AX upregulation observed in TPX2-depleted cells 15 min to 2h after ionizing radiation is not linked to increased apoptosis, as indicated by unchanged amounts of DNA double strand breaks before and after ionizing radiation-treatment (detected by comet assay and the number of γ-H2AX ionizing radiation-induced foci; Suppl. Fig.1 and Fig.1E), the use of non-lethal doses of ionizing radiation, the use of caspase-3 deficient MCF-7 cells that do not undergo ionizing radiation-triggered apoptosis associated with DNA fragmentation and the use of a broad spectrum caspases inhibitor (Fig.1). Interestingly, knockdown of TPX2 sensitzes cells to ionizing radiation, leading ultimately to increased ionizing radiation-induced apoptosis at later time points (9-12h after IR; data not shown). Such perspective could be thoroughly examined by therapeutic strategies combining radiotherapy and modulation of TPX2 levels to eradicate different types of cancer cells.

Previous studies have shown that mitotic cells accumulate more γ-H2AX signals than interphase cells independently of ionizing radiation (72,73). A recent study also showed that mitotic cells exhibit an atypical DNA damage
A novel function for TPX2

response compared to interphase cells (70). Since TPX2 is essential for mitosis, it might be argued that the increase in γ-H2AX signals in irradiated TPX2-depleted cells is caused primarily by mitotic arrest. However, depletion of Ndel1 or NUF2, which, like depletion of TPX2, leads to mitotic arrest and spindle abnormalities (60-63), did not increase ionizing radiation-dependent γ-H2AX levels (Fig.5A-B). Furthermore, the molecular signatures of mitotic cells and TPX2-depleted cultures with DNA lesions differ. For instance, damaged cells synchronized at M phase exhibit 53BP1 that migrates slower on SDS-PAGE gels and is hypophosphorylated at Ser 25 when compared to unsynchronized cells (70). In contrast, in irradiated TPX2-depleted cultures 53BP1 migrates normally and does not exhibit decreased phosphorylation at Ser 25 compared to control populations (Suppl. Fig.6). Our data also clearly show that during G1 phase, there is a pronounced formation of TPX2 ionizing radiation-induced foci whereas during M phase TPX2 stays associated with the mitotic spindle apparatus in the presence of DNA damage (Fig. 4). Finally, we found that TPX2 controls the ionizing radiation-dependent levels of γ-H2AX similarly in both cycling cells (Figs.1-4) and post-mitotic neurons that no longer enter mitosis (Fig.5). Taken together, our results highlight a novel and unexpected role for TPX2 in DNA damage response that is distinct from its mitotic function. This is the first nuclear function assigned to TPX2.

Based on our data showing an association between TPX2 and MDC1/ATM (Figs.6-7), TPX2 could regulate γ-H2AX amplification via MDC1 and ATM. However, the accumulation of γ-H2AX signals during DNA damage response is also influenced by the chromatin structure (74,75). In light of TPX2’s accumulation at ionizing radiation-induced foci (Figs.3-4) and TPX2’s association with BRCA1 (76), which has recently been shown to function as chromatin remodeling factor (76), it is possible that TPX2 regulates γ-H2AX formation via DNA double strand breaks-flanking chromatin remodeling. In addition, it is also possible that TPX2 impacts global chromatin architecture independently of DNA damage, thereby having an indirect effect on MDC1 ionizing radiation-induced foci formation and γ-H2AX levels once DNA double strand breaks are induced.

The time course of H2AX phosphorylation in TPX2-depleted cells differs from those observed when specific γ-H2AX protein phosphatases are depleted in which cases prolonged γ-H2AX signals are observed (77-82). In contrast, TPX2-depleted cells silence their elevated γ-H2AX signals with the same time course as control cells (Fig.1C). An unidentified phosphatase modulating γ-H2AX amplification early after ionizing radiation could be mis-regulated in the absence of TPX2. In this particular context, TPX2 may impact the dephosphorylation of γ-H2AX and thus, would regulate its steady-state levels. Alternatively, TPX2 may control H2AX phosphorylation by competing with γ-H2AX for binding to the BRCT domain of MDC1, thereby antagonizing MDC1’s amplificatory function. In support of this hypothesis, TPX2 was found to associate with the C-terminus of MDC1 (a.a. 1809-2089) containing its BRCT domains (Fig.6A-B). The exact molecular mechanisms by which TPX2 impacts the ionizing radiation-dependent γ-H2AX levels remain to be determined.

TPX2 has been proposed as a biomarker and effector for cancer progression based on its elevated levels in numerous malignancies that are correlated with disease progression (12-17,19,83). So far, the suspected involvement of TPX2 in these diseases has been mostly linked to its functions in mitosis and activation of Aurora A (84). With the novel function of TPX2 described herein, we propose that TPX2 also contributes to cancer pathology by impacting DNA damage response. Since phosphorylation of H2AX is essential for the amplification of DNA damage response, it is conceivable that aberrant levels of γ-H2AX caused by abnormal expression of TPX2 (as detected in cancers) deregulate the DNA damage response. This mechanism could contribute to the genomic instability found in many cancers.
REFERENCES


A novel function for TPX2

56. Massip, L., Caron, P., Iacovoni, J. S., Trouche, D., and Legube, G. Cell Cycle 9, 2963-2972
A novel function for TPX2

70. Giunta, S., Belotserkovskaya, R., and Jackson, S. P. J Cell Biol 190, 197-207

Acknowledgements – We are grateful to the members of the Lees-Miller lab, Drs. Onder Kamil, James Wang, Laurie Kennedy and Hong Tran for technical help, Aaron Sheldon for help with the statistical analysis and Felix Baeren for regeneration of TPX2 miRNA-expressing cell line. This work was supported by a Canadian Institute of Health Research (CIHR) operating grant, an Alberta Innovates Health Sciences cancer grant (to MDN), and by the Medical Research Council (to TH). MDN held a Career Development Award from HFSPO, a New Investigator Award from the CIHR, a Scholarship from the Alberta Heritage Foundation for Medical Research (AHFMR). GN received a DOC-fellowship of the Austrian Academy of Sciences, an Achievers in Medical Sciences award from the University of Calgary and a scholarship from the Alberta Cancer Foundation. HvA receives grants from the Netherlands Organisation for Scientific Research (NWO-VIDI grant), HFSPO (HFSP-CDA grant), the Leiden University Medical Center and the LUF / Gratama fund.
A novel function for TPX2

Figure 1. Increased ionizing radiation-dependent phosphorylation of H2AX at DNA double strand breaks upon depletion of TPX2

(A) Transient transfection of TPX2 siRNA or doxycycline-induced expression of an exogenous miRNA specific for TPX2 significantly increases phosphorylation of H2AX in HeLa cells 1h after 10 Gy as indicated by western blot analysis. A: control siRNA +IR (100.0 +/- 13.2) vs. TPX2 siRNA +IR (279.0 +/- 17.6), p<0.001, n=4; -doxycycline +IR (100.0 +/- 20.9) vs. + doxycycline +IR (871.5 +/- 23.4), p<0.001, n=4; group (mean +/- SEM), unpaired t test. (B) GFP-TPX2 expression reduces the increased levels of γ-H2AX caused by depletion of TPX2 with a siRNA targeting the 3’ untranslated region of TPX2 mRNA. (C) Time-course analysis of H2AX phosphorylation in HeLa cells depleted of TPX2 by siRNA (n= 3-5). 15min: control siRNA (78.6 +/-31.7) vs. TPX2 siRNA (221.3 +/-25.9), p<0.05; 1h: control siRNA (90.4 +/-18.5) vs. TPX2 siRNA (354.1 +/-54.8), p<0.05; 2h: control siRNA (100.0 +/-0.0) vs. TPX2 siRNA (206.9 +/-38.4), p<0.05; group (mean +/- SEM), unpaired t test. Note the similar ATM activation in irradiated control and TPX2-depleted cells as indicated by the levels of p-ATM (S1981). (D) Increase in the number of U2OS cells with more than 5 high-intensity γ-H2AX ionizing radiation-induced foci (i.e intensity 2 standard deviations above mean foci intensity) following TPX2 depletion by siRNA 1h and 2h after 4 Gy. 1h: control siRNA (1.7% +/- 0.4%) vs TPX2 siRNA (6.7% +/- 1.1%), p<0.05, n=3; 2h: control siRNA (1.9% +/- 0.3%) vs TPX2 siRNA (5.2% +/- 0.9%); p<0.05, n=3; group (mean +/- SEM), unpaired t test. The number of cells with these high-intensity ionizing radiation-induced foci declines 3h post-IR. Representative pictures of γ-H2AX ionizing radiation-induced foci in control RNAi and TPX2 RNAi-treated cells 2h post-4 Gy. (E) TPX2 depletion in U2OS cells by siRNA does not cause a significant change in the mean number of γ-H2AX ionizing radiation-induced foci after 4 Gy (n=3). NS: non-significant; unpaired t test, SEM. (F) Increased phosphorylation of H2AX 1h after 10 Gy in caspase-3 deficient MCF-7 cells depleted of TPX2 for 24h or for 48h by siRNA (n=3) as detected by western blots. MCF-7 cells do not undergo ionizing radiation-induced apoptosis associated with DNA fragmentation (54,55). (G) Increased phosphorylation of H2AX in TPX2-depleted HeLa cells 2h after a non-lethal dose of 2 Gy (n=4). (H) Increased γ-H2AX levels in TPX2-depleted cells 1h after ionizing radiation are unaffected by the broad-spectrum caspases inhibitor Z-VAD-FMK. Z-VAD-FMK was applied at a known effective dose (51) and inhibited PARP1 cleavage (a marker for apoptosis) in the presence of the DNA damaging drug camptothecin. Relative quantifications of γ-H2AX signals from independent experiments are shown in bar charts (A, C, F-G). n= # of independent experiments; NS: non-significant, * p<0.05, ** p<0.01 and *** p<0.001. Bar (D): 10 µm. IR: ionizing radiation.

Figure 2. Effects of TPX2 overexpression on the levels of γ-H2AX and MDC1 ionizing radiation-induced foci

(A-B) Overexpression of His-TPX2 or GFP-TPX2 significantly decreases phosphorylation of H2AX in HeLa (A) and MCF-7 cells (B) 1h after ionizing radiation treatment as indicated by (A) western blot analysis (10 Gy) and (B) immunofluorescence microscopy (left panel: 2Gy; right panel: 4Gy). (A) Relative quantifications of γ-H2AX signals from independent experiments are shown in bar charts: control +IR (100.0 +/- 6.9) vs. His-TPX2 +IR (16.0 +/- 2.9), p<0.001, n=4 (independent experiments); group (mean +/- SEM), unpaired t test. Levels of actin and H2A were used as loading controls. NS: non-significant, *** p<0.001. (C) Dose-dependent effects of GFP-TPX2 or His-TPX2 on the levels of ionizing radiation-induced γ-H2AX. The amount of plasmid transiently transfected per 6cm cell culture dish is indicated. Proteins on western blots were visualized with the indicated antibodies. TPX2 antibodies recognize endogenous and exogenous TPX2 on the same western blot, thereby allowing comparison of absolute protein levels. (see text for details). (D) Overexpression of His-TPX2 or GFP-TPX2 inhibits MDC1 ionizing radiation-induced foci formation in MCF-7 cells post 2Gy or 4Gy (15 min recovery), respectively. Bar (B, D): 10 µm. Merged images include Dapi staining (B,D). IR: ionizing radiation.
A novel function for TPX2

Figure 3. TPX2 localizes to DNA double strand breaks

(A-B) TPX2 partially co-localizes with γ-H2AX-positive ionizing radiation-induced foci after 5 Gy in LAN1 (A) and U2OS cells (B), respectively (see also Suppl. Fig.5). TPX2 is found in the nucleus and cytosol in neuroblastoma LAN1 cells and neurons (see Text and Suppl. Fig.5 for details). (C) TPX2 co-localizes with γ-H2AX at 4-OHT (4-hydroxytamoxifen)/AsiSI-induced DNA double strand breaks. U2OS cells stably expressing AsiSI-Estrogen Receptor were left untreated or treated with 300nM 4-OHT for 4h and subsequently immunostained for TPX2 and γ-H2AX. (D) TPX2 accumulates in DNA double strand breaks-containing laser tracks marked by γ-H2AX. U2OS cells were either mock-treated (-MP) or micro-irradiated with a multi-photon laser (+MP). Representative image shows TPX2 accumulation at 10 min after irradiation. The intensity profiles of γ-H2AX and TPX2 immunofluorescence signals were measured in the yellow bars perpendicular to the laser tracks. Commercially available TPX2 antibodies 184 were used in all immunofluorescence images. See Suppl. Fig.5 and Fig.5 for specificity of TPX2 184 antibodies. Bars: 10 µm. IR: ionizing radiation.

Figure 4. TPX2 regulates the levels of ionizing radiation-dependent γ-H2AX and forms ionizing radiation-induced foci in G1 phase cells

(A) Enhanced levels of γ-H2AX 1h after 10 Gy in G1 phase HeLa cells depleted of TPX2 by doxycycline-induced TPX2 miRNA expression. Note that the γ-H2AX augmentation in these TPX2-depleted cells is ionizing radiation-dependent. Cells were synchronized using a double thymidine block, released into fresh media and then used at specified time points for ionizing radiation treatment as indicated. Unirradiated cells were used for flow cytometry-based cell cycle profiling (bar charts on the right). Relative quantifications of γ-H2AX signals from independent experiments are shown in bottom bar charts. 11h: -doxycycline +IR (229.0 +/-21.0) vs. +doxycycline +IR (748.6 +/-39.4), p<0.001; 12h: -doxycycline +IR (135.1 +/-34.2) vs. +doxycycline +IR (466.1 +/-98.6), p<0.05; group (mean +/- SEM), unpaired t test; n=3 (independent experiments); * p<0.05 and *** p<0.001. Note that 11h after release from the thymidine block, TPX2 depleted cultures contain slightly more G2/M phase cells (8.66%) than control cultures (3.25%). Thus, on the western blot, a 12h-11h loading-hierarchy is chosen to facilitate comparison between control 11h vs. +doxycycline 12h (3.96% G2/M cells). Although, the cell cycle profile between these two samples is highly similar, TPX2-depleted cells exhibit ~ twice the levels of γ-H2AX than control cells. (B) U2OS cell cultures synchronized with a double thymidine block as in (A; see flow cytometry-based cell cycle profiles in top histograms; NS: non-synchronized control) form TPX2 ionizing radiation-induced foci 1h after irradiation that partially co-localize with γ-H2AX during G1 phase. All images were taken under identical experimental and microscopic conditions. See text for details. (C) TPX2 maintains its association with the mitotic spindle in the presence of DNA double strand breaks marked by γ-H2AX. Early and late mitotic figures, as identified via DAPI and TPX2 – staining, with and without DNA damage are shown. Commercially available TPX2 antibodies 184 were used in all immunofluorescence images. See Suppl. Fig.5 and Fig.5 for specificity of TPX2 184 antibodies. IR: ionizing radiation

Figure 5. Regulation of γ-H2AX levels by TPX2 is distinct from its mitotic functions and also occurs in post-mitotic G0 primary neurons

(A) Depletion of Ndel1 does not increase γ-H2AX levels after ionizing radiation treatment. (B) Depletion of NUF2 does not increase γ-H2AX levels after ionizing radiation treatment. The mitotic arrest upon NUF2 depletion was confirmed with the increased levels of the mitotic marker Histone 3 phosphorylated at Serine10 (H3S10p) (see text for details). (C) Significant decrease in TPX2 levels in mouse primary cortical neurons co-transfected with a TPX2 shRNA-encoding construct and GFP (ratio 5:1) as detected by immunofluorescence and confocal microscopy using commercially available TPX2 antibodies 184. See Suppl. Fig.5 for expression pattern of TPX2 in brain tissues, cellular distribution of TPX2 in primary neurons and specificity of the TPX2 shRNA and antibodies 184. (D) Enhanced levels of γ-H2AX in G0...
A novel function for TPX2

post-mitotic primary neurons co-transfected with vectors encoding TPX2 shRNA and GFP (ratio 10:1) 1h post-10 Gy compared to surrounding untransfected cells or cells co-transfected with vectors encoding a control shRNA and GFP (ratio 10:1), as determined by immunofluorescence and confocal microscopy. (E) Decreased levels of γ-H2AX in G0 post-mitotic primary neurons transfected with GFP-TPX2 compared to surrounding untransfected cells or cells transfected with GFP 1h post-10 Gy, as determined by immunofluorescence and confocal microscopy. Note that the γ-H2AX signals in control shRNA and GFP-expressing neurons are of similar intensity to untransfected surrounding cells. (F-G) Quantification of the relative changes in γ-H2AX signals in neurons in (D) and (E) expressed by the average ratio of [total nuclear γ-H2AX signals of transfected neurons / average total nuclear γ-H2AX signals of non-transfected surrounding cells]. F: ratio control shRNA (n=10) / non-transfected (n=196)= 1.7 +/-0.4 (SEM) vs. ratio TPX2 shRNA (n=16) / non-transfected (n=459)= 6.4 +/- 0.9 (SEM); p<0.001, unpaired t test. G: ratio GFP (n=9) / non-transfected (n=37)= 1.0 +/- 0.1 (SEM) vs. ratio GFP-TPX2 (n=8) / non-transfected (n=33)= 0.5 +/- 0.1 (SEM); p<0.001, unpaired t test. (H-I) Quantification of the relative changes in γ-H2AX immunofluorescence signals in neurons co-transfected with vectors encoding a control or TPX2 shRNA and a GFP (ratio 10:1) (H), or a GFP or GFP-TPX2 construct (I) 1h after 3 Gy. H: ratio control shRNA (n=58) / non-transfected (n=300)= 1.1 +/-0.1 (SEM) vs. ratio TPX2 shRNA (n=75) / non-transfected (n=330)= 1.3 +/- 0.1 (SEM); p<0.001, unpaired t test. I: ratio GFP (n=50) / non-transfected (n=297) = 1.2 +/- 0.1 (SEM) vs. ratio GFP-TPX2 (n=47) / non-transfected (n=420) = 0.9 +/- 0.1 (SEM); p<0.01, unpaired t test. Values are calculated as in (F-G). NS: non-significant, * p<0.05, ** p<0.01, *** p<0.001. Bars: 20 µm. IR: ionizing radiation.

Figure 6. TPX2 associates with MDC1, pATM, NBS1 and γ-H2AX

(A) Y2H experiment using bait (B)-TPX2 (a.a. 8-747) and prey (P)-MDC1 (a.a. 1683-2089 or 1809-2089). Plasmids were co-transformed as indicated. -Leu/-Trp selection agar plates are controls for transformation efficiency. Colonies on -Ade/-His/-Leu/-Trp selection agar plates reveal an interaction between B-TPX2 and P-MDC1. Plates were incubated for 6 days. (B) An ectopic C-terminal fragment of MDC1 (C-MDC1-Flag, a.a. 1807-2089) associates with endogenous TPX2 in HeLa cells as indicated by co-immunoprecipitations with TPX2 antibodies 184 from total cell lysate (left panel). Ectopic GFP-TPX2 also co-immunoprecipitates with endogenous MDC1 (right panel). The input lane for the C-MDC1-Flag (left panel) is from shorter exposure of the same western blot. The input lane for the endogenous MDC1 (right panel) is from stronger exposure of the same western blot. (C) GST-TPX2 pulls down MDC1 and the positive control Aurora A from total HeLa cell lysate. For MDC1, the input is a lower exposure of the same blot. (D) Co-immunoprecipitations from U2OS cell lysates with specified antibodies (see text for details on antibodies). TPX2 was co-immunoprecipitated with MDC1 from these cells using MDC1 antibodies (left panel). MDC1 also co-immunoprecipitated with TPX2 antibodies184. TPX2 was also found in complex with NBS1 and MDC1 species that migrate slower on SDS-PAGE gels when the co-immunoprecipitations were performed with the TPX2 KiS2 antibodies (see text for further details). (E) TPX2 and MDC1 associate in HeLa and LAN1 cells as detected by co-immunoprecipitations with specified antibodies from total (left and middle panel) or nuclear (right panel) lysates. TPX2 from neuroblastoma LAN1 cells (and primary neurons) migrates as doublet on gels (see Suppl. Fig.5). TPX2 is also found in complex with p-ATM and γ-H2AX after ionizing radiation treatment. Beads alone or antibodies against c-Myc were used as negative controls as indicated. IR: ionizing radiation.

Figure 7. The ionizing radiation-dependent increase in γ-H2AX caused by TPX2-depletion is antagonized by inhibition or knockdown of MDC1 or ATM

(A) A siRNA-mediated knock down of MDC1 antagonizes the ionizing radiation-triggered γ-H2AX hyperamplification in HeLa cells caused by TPX2 depletion (lane 3 vs lane 4). Note that TPX2 depletion has the opposite effect on γ-H2AX levels than MDC1 depletion during DNA damage response (lane 1 vs lane 3). (B) Inhibition of ATM with KU55933 antagonizes the ionizing radiation-dependent increase in γ-H2AX caused by depletion of TPX2. Inhibition of DNA-PK with NU7441 does not rescue this γ-H2AX
hyperamplification phenotype. (C) siRNA-mediated loss of ATM abrogates the ionizing radiation-dependent increase in γ-H2AX caused by depletion of TPX2. siRNA-mediated loss of DNA-PKcs partially decreases ATM levels (as per (71)) but does not rescue the γ-H2AX hyperamplification caused by TPX2 depletion. (D) Quantification of γ-H2AX signals in (C). γ-H2AX signals of control siRNA-treated cells were considered as 100% and compared to the respective TPX2 siRNA-treated cells. n= 3 [independent experiments]; NS: non-significant, * p<0.05; unpaired t test, SEM. All cells were treated with 10 Gy and harvested after 1h recovery. IR: ionizing radiation.
**Figure 4**

### A

**h release from double Thymidine block**

<table>
<thead>
<tr>
<th>Cell Cycle Distribution (%)</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>12h</td>
<td></td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>12h</td>
<td></td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>11h</td>
<td></td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>11h</td>
<td></td>
<td>70</td>
<td>30</td>
</tr>
</tbody>
</table>

**Doxycycline**

IR (10 Gy) -

- - - -
- + + +
+ - - -
+ + + -

**TPX2** -

- - - -
- + + +
+ - - -
+ + + -

**γ-H2AX** -

- - - -
- + + +
+ - - -
+ + + -

**H2AX** -

- - - -
- + + +
+ - - -
+ + + -

**Right Panel**

- Control
- TPX2 miRNA

#### γ-H2AX signal (A.U.)

- 11h
- 12h

- **No IR**
- **5 Gy**

**B**

**release from double Thymidine block**

- NS
- S
- G2
- G1

**No IR**

- TPX2
- γ-H2AX
- Merge + DAPI

**5 Gy**

- TPX2
- γ-H2AX
- Merge + DAPI
Figure 4

C

<table>
<thead>
<tr>
<th>TPX2</th>
<th>γ-H2AX</th>
<th>DAPI</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>No IR</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>No IR</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>5 Gy</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>5 Gy</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>
FIGURE 7

A

IR (10Gy): + + + +
MDC1 siRNA: + - - +
Control siRNA: - + - -
TPX2 siRNA: - - + +

MDC1

TPX2

γ-H2AX

Actin

H2AX

B

TPX2 miRNA: - + - - - -
IR(10Gy): + + + + + +

TPX2

γ-H2AX

H2AX

DMSO  NU7441  KU55933

C

ATM siRNA: - - - - + +
DNA-PKcs siRNA: - - + + - -
TPX2 siRNA: - + - + - -
Control siRNA: + - + - + -

TPX2

ATM (upper Band)

pATM

DNA-PKcs

Actin

γ-H2AX

H2AX

D

γ-H2AX signal in %

Ctrl. siRNA

DNA-PKcs siRNA

ATM siRNA

Ctrl. siRNA = 100% in each group

* N.S.
Targeting Protein for Xenopus kinesin like protein 2 (TPX2) regulates gamma-H2AX levels upon ionizing radiation
Gernot Neumayer, Angela Helfricht, Su Yeon Shim, Hoa Thi Le, Cecilia Lundin, Camille Belzil, Mathieu Chansard, Yaping Yu, Susan P. Lees-Miller, Oliver Gruss, Haico van Attikum, Thomas Helleday and Minh Dang Nguyen

J. Biol. Chem. published online October 8, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.385674

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2012/10/08/M112.385674.DC1