Supplementary Manuscript Material

Materials and Methods

Nuclear GIT2 is an ATM substrate and promotes DNA repair

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RNAi and transfection

Sequences of siRNAs: GIT2-A: Sense: CCAAUAAAGCGG AAUUCAU; Antisense: AUGAAUUCCGCUUUAUUGG. GIT2-B: Sense: GUACUCAUCAA CACG AAAU; Antisense: AUUUCGUGUUGAUGAGUAC. GIT2-C: Sense: GCGUUGAGAG UCAAGACAA; Antisense: UUGUCUUGACUCUCAACGC. Control siRNA-A: Sense: UUCU CCGAACGUGUCACGU; Antisense: CGUGACACGUUCGGAGAA.H2AX: 5'-CAACAAGAA GACGCGAAUCdTdT; MDC1: 5'-UCCAGUGAAUCCUUGAGGUdTdT; RNF8: 5'-GAGAAGCUUACAGAUGUUU.

Laser-induced DSBs and immunocytochemistry in multiple cell types

Throughout the experiment, cells were maintained at 37 ℃, 5% CO2, and 80% humidity using a Live Cell environmental chamber. After laser treatment, the cells were incubated at 37 ℃ for 10 minutes and fixed immediately in freshly prepared 4% formaldehyde (in PBS) for 10 minutes at room temperature. Fixed cells were permeabilized with a PBS solution containing 0.5% Triton X-100, 1% BSA, 100mM glycine, and 0.2 mg/mL EDTA on ice for 10 minutes and subsequently digested with RNase A at 37 ℃. For immunofluorescence staining, cells were incubated at 37 ℃ for 1 hour with various primary antisera: anti-γH2AX (Upstate, Millipore); anti-GIT2 (Genetex, San Antonio, TX), anti-MDC1 (Bethyl, Montgomery, TX), anti-RNF8 (Abcam), anti-BRCA1 (Oncogene) and anti-53BP1 (Abcam). Cells were incubated with corresponding secondary antibodies (Alexa Fluor goat anti-mouse or Alexa Fluor goat anti-rabbit; Molecular Probes, Invitrogen). After washing, they were mounted using ProLong Gold antifade reagent with DAPI

(Molecular Probes, Invitrogen). The immunostained cells were visualized and imaged using a Hamamatsu EM-CCD digital camera attached to the Nikon Eclipse TE2000 confocal microscope.

Immunoblot and Immunoprecipitation.

SH-SY5Y cells were fractionated using the Qproteome^{TM} Cell Compartment kit, according to the manufacturer's instructions (Qiagen, Valencia CA). All protein extracts were quantified using BCA reagent (ThermoScientific, Rockford IL) before resolution with SDS-PAGE and electrotransfer to PVDF membranes (Perkin Elmer, Waltham MA). The primary antibody immune-reactive complexes were identified using alkaline phosphatase-conjugated secondary antisera (Sigma Aldrich) with enzyme-linked chemifluorescence (GE Healthcare, Piscataway NJ) and quantified with a Typhoon 9410 phosphorimager. For immunoprecipitation, cells were lysed by IP lysis buffer (Pierce, Rockford, IL) with benzonase nuclease (Sigma,St. Louis, MO) and protein samples were incubated with an antibody and 25µL 50% protein A/G plus agarose beads (Calbiochem), and Protein A/G beads were collected. Immunoprecipitates were resolved by SDS–PAGE and analyzed by immunoblotting. Primary antibodies used in this study were rabbit anti GIT2 (Genetex, San Antonio, TX) for immunocytochemistry, rabbit anti-GIT2 (Bethyl, Montgomery, TX) for immunoblot or immunoprecipitation, mouse anti-NBS1 (Thermo scientific, Rockford, IL), mouse anti-ATM and mouse anti-p53 (Santa Cruz, Santa Cruz, CA), mouse anti-ATM-pS1981, anti-phospho-Serine and PARP2 (Millipore, Billerica, MA), mouse anti-γH2AX, mouse anti-PADPR, rabbit anti-SPEN, rabbit anti-NOP2 and rabbit anti-PARP1 (Abcam, Cambridge, MA), mouse anti-MDC1 (Bethyl, Montgomery, TX), mouse anti-53BP1 (Calbiochem, Los Angeles, CA), mouse anti-MRE11, rabbit anti-HMGN1, p-ATM/ATR substrate and rabbit anti-phospho-Threonine (Cell Signaling, Danvers, MA) and mouse anti-RAD50 (Genetex, San Antonio, TX), mouse anti-RFC1, mouse anti-Histidine and Histone H1 (Sigma, St. Louis, MO).

SILAC labeling and mass spectrometry.

Cellular protein extraction was performed with a standardized lysis buffer: 8 M urea, 50 mM Tris pH 8.0, 75 mM NaCl, 1 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethanesulfonylfluoride and a protease inhibitor cocktail (Roche Diagnostics). Protein lysate concentrations were determined by BCA protein

assay kit (Thermo Fisher Scientific), equal amounts of proteins from medium or heavy conditions were mixed to prepare the SILAC doublet mix. Proteins were reduced in 10 mM DTT at room temperature (RT) for 30 min and alkylated with 25 mM iodoacetamide at RT for 20min in the dark. The reaction was quenched with an additional incubation in 15 mM DTT at RT for 15min. Protein extracts were diluted in 50 mM Tris pH 8.0 to a final concentration of 1.5 M urea and were digested at 37^{o}C using sequencing grade trypsin (Promega) at a 1:100 ratio(trypsin/protein, w/w). After overnight digestion, formic acid was added to a final concentration of 0.5% (v/v) and the peptide samples were desalted using Sep-Pak Vac C18 Cartridges. Cartridges were washed with ACN (acetonitrile) and equilibrated with 0.1% formic acid. After loading peptides, cartridges were washed with 0.1% formic acid. Peptides were then eluted with 70% ACN 0.1% formic acid, dried, but not completely, in a SpeedVac (Thermo Fisher Scientific), and stored at -80^{o}C until used. To resolve peptides for subsequent quantitative analysis, strong cation exchange separation was performed prior to MS injection.

LC-MS/MS analysis of trypsin-digested peptides was carried out using a Thermo Fisher Scientific linear ion trap (LTQ/Orbitrap XL). Peptides (1-2 µg) were first loaded onto a trap cartridge (Zorbax 300SB-C18, 5 µm, 0.3x5mm, Agilent) at a flow rate of 2 µL/min. Trapped peptides were then eluted onto a reversed-phase PicoFrit column (Betabasic 5 µm C18, 150 Å, 10 cm bed length, 360 od/75 id, New Objective) using a linear gradient of ACN (2-35%) containing 0.1% formic acid. Gradient duration was 80 min at a flow rate of 0.25 µL/min, followed by 80% ACN washing for 5 min. Eluted peptides were introduced, via nanospray, to the LTQ-Orbitrap. Spray voltage and ion transfer tube temperature were set at 1.8 kV and 180 ℃, respectively. Data-dependent acquisition mode was enabled, and each survey MS scan was followed by four MS/MS scans with dynamic exclusion option on. Full-scan MS survey spectra (m/z 300–2,000) in profile mode were acquired in the Orbitrap (resolution: 60,000; AGC target: 5E+05; max. fill time: 500 ms). The four most intense peptide ions from the survey scan were fragmented by collision-induced dissociation (normalized collision energy: 35%; activation Q: 0.250; and activation time: 30 ms) in the LTQ (AGC target: 1E+04; max. fill time: 200 ms). Precursor ion charge state screening was used to reject unassigned charge states. The dynamic exclusion list was restricted to a maximum of 500 entries with a maximum exclusion duration of 90 s and a relative mass tolerance window of ±10 ppm. Raw data was analyzed using MaxQuant

(version 1.1.1.6.). Retention time-dependent mass recalibration was applied and peak lists were searched against a database containing all 87,061 entries from the International Protein Index human protein database (http://www.ebi.ac.uk/IPI/IPIhelp.html) version 3.68 and 262 frequently observed contaminants as well as the reversed sequences of all entries. Database searches were performed with the following settings. Precursor and fragment ion peaks were searched with mass tolerance of 7 ppm and 0.5 Da, respectively. Enzyme specificity was set to trypsin/P. Up to two missed cleavages were allowed and only peptides with at least 6 amino acids in length were considered. Carbamidomethylcysteine and oxidation on methionine were set as fixed and variable modifications, respectively. Using a decoy database strategy, peptide identifications were accepted based on their posterior error probability (PEP), until less than 1% reverse hits were retained in the list. SILAC ratios from MaxQuant were converted to $\log_2$-scale and the dataset's mean and standard deviation were calculated with SigmaPlot v11.0. The correlation curve of measured vs. pre-defined mixed ratios and its coefficients were obtained by linear regression (SigmaPlot) and the dataset's standard deviations were used for error bar calculations for each data point.

Supplementary Figure Legends

Figure S1. GIT2 is sensitive to CDDP induced DNA damage. (**a**) SH-SY5Y cells were treated with 4μM cisplatin (CDDP) for the indicated time followed by subcellular fractionation of proteins and subsequent immunoblotting (IB) analysis. The histogram depicts the relative quantitation of GIT2 expression changes in diverse cellular compartments. (**b**) Overexpression of GIT2 protects DNA from excessive CDDP induced damage indicated by neutral comet assay (100 nuclei counted for each group: data were expressed as mean ± S.E.M.). (**c**) Knockdown of GIT2 exacerbates CDDP-mediated DNA damage. (**d**) GIT2 is phosphorylated in response to DNA damage. SH-SY5Y cells were harvested immediately after CDDP treatment and cell extracts were incubated with or without λ protein phosphatase (λ-PPase) and subjected to immunoblot gel-migration analysis using GIT2 antibody. λ-PPase incubation reversed CDDP induced GIT2 gel retardation. (**e**) GIT2 gel retardation is dependent upon ATM. CDDP-treated $ATM^{+/+}$ (GM0637) and $ATM^{-/-}$ (GM5849) cells were harvested at the indicated time point and cell extracts were subjected to immunoblot gel-migration analysis using GIT2 antibody. CDDP induced GIT2 gel retardation was lost in $ATM^{-/-}$ cells. (**f**) DNA protective role of GIT2 is dependent on ATM phosphorylation sites. SH-SY5Y cells were transfected with the T/S-A/A GIT2 mutant for 24 hours before CDDP treatment, comet assay was performed immediately after treatment. Overexpression of T/S-A/A GIT2 failed to confer protection to CDDP-mediated DNA damage. (**g**) Classical DDR proteins associating with immunoprecipitated GIT2 were identified by specific IBs. Input represents 1% of total protein used in IPs.

Figure S2. ATM phosphorylation sites of GIT2 is required for its interaction with DNA repair proteins and recruitment to DNA damage sites. T/S-AA GIT2 mutant is not associated with DNA damage response proteins. SH-SY5Y cells were transfected with plasmid expressing wild-type GIT2 or T/S-A/A GIT2 mutant 24 hours before IR (**a**) or CDDP (**b**) treatment. Co-immunoprecipitations (Co-IPs)/immunoblots (IBs) were performed using the antibodies indicated. T/S-A/A GIT2 fails to form nuclear foci in response to DNA damage. SH-SY5Y cells were transfected with plasmid expressing wild-type GIT2 or T/S-A/A GIT2 mutant 24 hours before IR (**c**) or CDDP (**d**) treatment. Exogenous GIT2 was detected (63x magnification) using specific primary antibody followed by an Alexa Fluor 488 (green)-conjugated secondary antibody, respectively. Nuclear DAPI stain was employed to visualize cellular nuclei.

Figure S3. GIT2 expression is associated with DNA repair protein modulation and long-term cell survival after DNA damage. SH-SY5Y cells were transfected with various levels of FL (Flag)-GIT2 (1-10μg) and the expression of DNA repair proteins was assessed 48hrs post-transfection (**a**, **b**-quantification from n>3 individual experiments). FL-GIT2 overexpression attenuates Olive tail movement at 24 and 48hrs after acute IR insults (10Gy) (**c**). FL-GIT2 overexpression antagonizes the reduction in SH-SY5Y cell viability (measured 0.5, 24 and 48hrs post-insult using trypan blue exclusion) induced by acute IR (10Gy) (**d**), etoposide (1μM) (**e**) or peroxide (10μM) (**f**). Using GIT2 siRNA to reduce GIT2 expression in SH-SY5Y cells, the reduction in cell viability induced by IR (**g**), etoposide (**h**) and peroxide (**i**) insults was significantly potentiated.

Figure S4. DNA damage induced GIT2 nuclear foci formation with classical DNA-damage response factors. SH-SY5Y cells were treated with CDDP, fixed and then the subcellular localizations and co-localization of endogenous GIT2 with active ATM-pS1981 (**a**, merge enlargement **b**), γ-H2AX (**c**, merge enlargement **d**), 53BP1 (**e**, merge enlargement **f**), MDC1 (**g**, merge enlargement **h**) and NBS1 (**i**, merge enlargement **j**) was assessed. Co-localizations are indicated with white arrows. GIT2 or the DNA damage complex proteins were detected (63× magnification) using specific primary antibodies followed by an Alexa Fluor 488 (green)-or Alexa Fluor 568 (red)-conjugated secondary antibody, respectively. Nuclear DAPI stain was employed to visualize cellular nuclei.

Figure S5. DNA damage fails to generate GIT2-positive nuclear foci in H2AX$^{-/-}$ cells. GIT2 forms nuclear foci (indicated with white arrows) that co-localize with γ-H2AX (**a**), active ATM (**b**) or MDC1 (**c**) in CDDP-treated H2AX$^{+/+}$ but not H2AX$^{-/-}$ cells. Immunocytochemical foci were detected (63x magnification) using antibodies against GIT2, γ-H2AX, ATM-pS1981 and MDC1 followed by an Alexa Fluor 488 (green)-or Alexa Fluor 568 (red)-conjugated secondary antibody, respectively. Nuclear DAPI stain was employed to visualize cellular nuclei.

Figure S6. GIT2 DSB recruitment is sensitive to modulation of ATM and H2AX activity status. GIT2 DSB initial recruitment requires the presence of ATM kinase but not kinase activity.

(**a**) IR-mediated DSBs induces GIT2 cellular re-distribution in $ATM^{-/-}$ (GM5849) cells. (**b**) Genomic deletion of ATM in GM5849 cells does not prevent IR-induced γ-H2AX foci formation. (**c**) GIT2 forms IR-dependent nuclear foci in $ATM^{+/+}$ cells (GM0637) but not in $ATM^{-/-}$ (GM5849) cells.

Figure S7. PARP activity is modulated by GIT2 in response to DNA damage. (a) Overexpression of WT GIT2 (His-tagged), but not R39A GIT2 (Flag-tagged), potentiates the increased synthesis of PARP1-associated PADPR in response to CDDP treatment. **(b)** Overexpression of T/S-A/A GIT2 (His-tagged) is not able to enhance the synthesis of PARP1-associated PADPR. **(c)** Knockdown of GIT2 by siRNA-inhibited synthesis of PARP1- associated PADPR in response to CDDP treatment.