Protein Phosphatase-1α Regulates Centrosome Splitting through Nek2

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Abstract

ATM is a central mediator of the cellular response to the DNA damage produced by ionizing radiation. We recently showed that protein phosphatase 1 (PP1) is activated by ATM. Because Nek2 is activated by autophosphorylation, and because its dephosphorylation is catalyzed by PP1, we asked if the radiation damage signal to Nek2 was mediated by PP1. Overexpression of Nek2 induces premature centrosome splitting probably by phosphorylating centrosome cohesion proteins C-Nap1 and Rootletin. In this study, we show isoform specificity of PP1 binding and regulation of Nek2. Although both PP1α and PP1γ coimmunoprecipitated with Nek2, only PP1α regulated Nek2 function. Ionizing radiation inhibited Nek2 activity, and this response was dependent on ATM and on PP1 binding to Nek2 and coincident with Thr328 dephosphorylation of PP1. Radiation-induced inhibition of centrosome splitting was abrogated in cells expressing Nek2 mutated in the PP1-binding motif outside the kinase domain. Conversely, cells depleted of PP1α by small interfering RNA showed enhanced centrosome splitting and loss of radiation-induced inhibition of centrosome splitting. The identification of a PP1-specific isoform mediating a checkpoint response opens up the possibility of selectively targeting phosphatases as novel radiation sensitizers. [Cancer Res 2007;67(3):1082–9]

Introduction

Cellular responses to DNA damage include activation of repair pathways, control of cell cycle checkpoints, and apoptosis. All of these processes are regulated through signal transduction pathways. ATM is a member of a family of phosphatidylinositol 3–like kinases, including DNA-PK and ATR, which transmit the damage signal to effector proteins. We have recently shown that protein phosphatase 1 (PP1) is activated by ionizing radiation in an ATM-dependent manner. Because PP1 and Nek2 are known to regulate centrosome splitting, we asked if the damage signal produced by ionizing radiation and transmitted to Nek2 was mediated by PP1. Centrosomes consist of two barrel-shaped centrioles and pericentriolar material and function to nucleate polarized microtubular arrays. This action is key for distribution of cytoplasmic organelles, for the mitotic spindle organization, and for cytokinesis. Following cytokinesis, cells have a single centrosome consisting of two centrioles that replicate during S phase, separate in G2-M, and form part of the spindle poles during mitosis. Centrosomes are thought to play an important role in facilitating accurate chromosome segregation. The molecular mechanism controlling centrosome splitting and duplication in mammalian cells is not well understood. Phosphorylation and dephosphorylation are thought to play a critical role in this process, and multiple kinases have been implicated, including cyclin-dependent kinase 2 (Cdk2)/cyclin E (1), the Polo-like kinases (2, 3), Aurora-A (4, 5), and Nek2 (6, 7).

Nek2 is a core component of centrosomes and a regulator of centrosome splitting. Nek2 is a cell cycle–dependent serine/threonine kinase, structurally related to NIMA (never in mitosis A) of the filamentous fungus Aspergillus nidulans. Nek2 protein levels and activity increase in S-G2 phase and are reduced following mitotic arrest. Overexpression of Nek2 induces premature centrosome splitting, which is thought to be achieved by phosphorylating centrosome cohesion proteins C-Nap1 and Rootletin (8, 9). These coiled-coil domain-containing proteins provide a docking site for a dynamic linker structure that tethers parental centrioles. Injection of anti-C-Nap1 antibodies in cells at any phase of the cell cycle promotes centrosome splitting. This observation suggests that C-Nap1 is the downstream effector of Nek2 to regulate centrosome splitting, and that interfering with C-Nap1 function results in premature centrosome splitting during any phase of the cell cycle (9). Nek2 overexpression not only promotes centrosome splitting but also induces multiple centrosomes in HBL100 cells (10). Nek2 itself is regulated by phosphorylation as its activity increases with autophosphorylation and the phosphatase inhibitors and decreases with overexpression of PP1. Yeast two-hybrid analysis and coimmunoprecipitation in mammalian cells showed that Nek2 can form a complex in vitro and in vivo with PP1 using a canonical recognition sequence KVHF (6, 11).

PP1 is an abundant serine/threonine phosphatase, which has diverse functions in cellular metabolism, transcription, and cell cycle progression. The specificity of its various function is achieved in large part through different subunits, which generally bind to PP1 using a (R/K)VXF motif. These include GM, MYPT1, NIPP1 (nuclear inhibitor of PP1), Nek2, and PNUTS (PP1 nuclear targeting subunit). PP1 is present as three isoforms in mammalian somatic cells. The isoforms show no significant difference in cellular distribution in interphase cells (12). However, at mitosis, PP1α localizes to the centrosome, whereas PP1β/δ and PP1γ localize in chromosomes and microtubules of the mitotic spindle, respectively. Premature centrosome splitting is triggered by both overexpression of Nek2 and inhibition of PP1 (13). Therefore, PP1 is believed to be the physiologic antagonist of Nek2. Although ionizing radiation–induced inhibition of centrosome splitting is thought to be mediated by Nek2 (14, 15), the involvement of PP1 in control of Nek2 in the presence and absence of radiation is unclear.

We have previously shown that PP1 is activated via dephosphorylation by ionizing radiation in an ATM-dependent manner (16), and because PP1 regulates Nek2, we used ionizing radiation to examine the relationship between PP1 and Nek2. We first asked...
if PP1 binding with Nek2 is isoform specific, and whether PP1 is essential for ionizing radiation inhibition of Nek2 activity. Our observations reveal that (a) both PP1α and PP1γ are coimmunoprecipitated with Nek2, (b) PP1α regulates not only Nek2 activity and centrosome splitting but also centrosome duplication in an isoform-specific manner, and (c) ionizing radiation regulates centrosome splitting through PP1α. These results show for the first time that ATM signals to Nek2 through PP1α.

Materials and Methods

Cell culture. HeLa and COS-7 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). The human retina cell line ARPE-19 were grown in DMEM/F12 (1:1; Invitrogen) supplemented with 10% FBS, FT/pEB77 and FT/pEB7-YZ5 were generously provided by Y. Shiloh (Tel Aviv University, Israel) and grown in DMEM with 100 μM/L nonessential amino acid, 10% FBS, and 100 μg/mL hygromycin B. Cells were incubated in a humidified incubator at 37°C with 5% CO2. All cells were in an exponential growth phase at the time of irradiation.

Plasmid and antibody. Full-length Nek2 was prepared by reverse transcription-PCR using mRNA prepared from HeLa cells and was subcloned into the BamHI/ EcoRI sites of the pBK-CMV plasmid expression vector, with an NH2-terminal triple-hemagglutinin (HA) epitope tag (sequence YPYDVPDYA), Double mutant Nek2- AA was prepared by mutation of Phe366 to alanine and Val357 to alanine at the PP1-binding motif of Nek2, K37F (6). Nek2KD was prepared by single mutagenesis of lysine 37 to arginine at the kinase domain of Nek2. The following commercial antibodies were used: anti-phospho-PP1α (Thr320) antibody (Cell Signaling Technology, Inc., Beverly, MA), anti-Ku86, anti-PP1, anti-HA monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Nek2, anti-PP1α (sc6104, Santa Cruz Biotechnology), anti-PP1β/δ (sc6106, Santa Cruz Biotechnology), anti-PP1γ polyclonal antibody (sc6108, Santa Cruz Biotechnology), and anti-γ-tubulin polyclonal antibody (Sigma, Inc., St. Louis, MO).

Radiation treatment. Cell cultures were irradiated with a Varian linear accelerator at a dose rate of 1.48 Gy per minute. During irradiation, the cultures were maintained in a container designed to mimic the conditions of the cell culture incubator (5% CO2 and 95% air at 37°C). Cultures were maintained in a container designed to mimic the conditions of the cell culture incubator (5% CO2 and 95% air at 37°C).

Immunoprecipitation and in vitro kinase assays. COS-7 cells or pairs of mutant and corrected AT cells (FT/pEB77 and FT/pYZ5) in 100-mm dishes were transfected using FuGENE (Roche Molecular Biochemicals, Indianapolis, IN) with 8 μg of plasmid for NH2-terminally triple HA-tagged wild-type and mutant Nek2 (referred to as Nek2 and Nek2-AA), respectively, as described in the manufacturer’s instructions (Roche Molecular Biochemicals). Cells were incubated for 16 h followed by irradiation with 10 Gy, harvested at indicated time points, and lysed in 1 mL PBS lysis buffer (17), 0.5% (v/v) Triton X-100, 0.5 mM/L EDTA, 1 mM/L DTT, 0.2 mM/L sodium vanadate, and protease inhibitors. Aliquots of 0.5 μg were mixed with 4 μg of monoclonal anti-HA antibody, and bound proteins were recovered by binding to 15 μL of protein-A agarose (Sigma). Nek2 activity was assayed in the immunoprecipitates in 20 μL of reaction mixture containing 25 mM/L MOPS (pH 7.2), 10 mM/L MgCl2, 1 mM/L DTT, and 0.4 mM/L Pefabloc plus 0.2 mg/mL myelin basic protein (Sigma) and 0.1 mM/L γ-32P-ATP (1 μCi/μmol) as substrates. After incubation at room temperature for 30 min, samples were separated by 12% SDS-PAGE gel and transferred to nitrocellulose or analyzed using ImageQuant software of PhosphorImager (Amersham Biosciences, Piscataway, NJ). Densitometry quantitative Western blot was analyzed by ImageJ software provided by NIH.

Western blot analysis. Whole-cell extract or proteins recovered from immunoprecipitation beads were separated by 10% SDS-polyacrylamide gels and transferred (with Tris/glycine/methanol buffer, 0.6 A for 6 h) to nitrocellulose. Immunoblot analysis was done with anti-HA antibody, anti-Nek2 antibody, anti-PP1α, anti-PP1β/δ, anti-PP1γ, anti-phospho-specific PP1 at T320, or anti-PP1α/β/γ isofoms. Proteins were detected with horseradish peroxidase-conjugated antibodies. Blots were developed using an enhanced chemiluminescence kit (Amersham Biosciences).

Centrosome-splitting assay. A 100-mm plate of COS-7 cells or HeLa cells was split 1:8 onto fibronectin-coated 22 × 22 mm coverslips seated in 35-mm tissue culture dishes. Dishes were incubated at 37°C for at least 6 h before transfection. Each 35-mm culture was transfected with 1 μg of either empty vector plasmid or plasmid encoding wild-type or mutant HA3-Nek2 using 3 μL of FuGENE 6 (Roche Molecular Biochemicals) in serum-free DMEM following the manufacturer’s instructions. Cells were incubated for 72 h, followed by irradiation with 10 Gy, then fixed as described below to preserve microtubule structure at indicated time points. Cells were stained with antibodies directed against either HA or endogenous γ-tubulin. A stage micrometer was used to calibrate the measurement tool in OpenLab software (at ×60, 48 pixels = 10 μm; Improvision, Coventry, United Kingdom). Centrosome distances in transfected cells were measured directly from contrast-enhanced images of γ-tubulin staining using the calibrated measuring tool. Only those cells with two clearly distinguished foci of γ-tubulin staining were included in the analyses.

Immunofluorescence microscopy. Cells were rinsed once with 1.2 × PEM (1 mol/L, PIPES, 50 mM/L EGTA, and 20 mM/L MgCl2) at 37°C, fixed with methanol at −20°C for 3 min, rinsed twice again with 1.2 × PEM, and permeabilized with 0.1% Triton X-100 in 1.2 × PEM for 5 min at room temperature. Cells were rinsed thrice with PBS and incubated in 3% bovine serum albumin (BSA) in PBS blocking solution for 1 h at room temperature. Mouse anti-HA monoclonal, rabbit anti-γ-tubulin polyclonal were diluted in 3% BSA-containing PBS and applied to the coverslips for at least 1 h at room temperature or overnight at 4°C. Cells were rinsed thrice with PBS for 5 min each before staining with the appropriate secondary antibodies, including rhodamine-conjugated goat anti-rabbit, Oregon Green 488-conjugated goat anti-mouse, diluted in 3% BSA-containing PBS and 1 μg/mL Hoechst 33342 nuclear stain for 1 h at room temperature. Coverslips were rinsed again five times with PBS as described above and mounted onto glass slides with 10 μL of Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Background staining was determined by preparing identical coverslips without primary antibody. Images of fixed cells were captured using a Nikon fluorescence microscope (Microphoto-SA) with Openlab software, which was equipped with a Nikon Plan Apo ×60/1.4 oil immersion objective; filter sets for FITC, Texas Red, and 4,6-diamidino-2-phenylindole fluorophores; and a Hammatsu Orca C4742-95 digital camera. Raw data images were converted to 8-bit tiff images in Openlab.

RNA interference assay. For RNA interference (RNAi) assay, 5 × 105 HeLa cells were seeded in each well of a six-well plate overnight. Cells were washed with PBS and serum-free medium one time each, followed by transfection with PP1 isoform-specific small interfering RNA (siRNA) oligonucleotides (smart pool, Dharmaco, Inc., Lafayette, CO) using TransIT-TKO transfection reagent (Mirus, Inc., Madison, WI) as described in the manufacturer’s instructions; non-target siRNA was taken as control. Briefly, 20 μL TransIT-TKO were diluted in 250 serum-free medium and incubated for 15 min at room temperature after complete mix. Then, 50 mM/L (final concentration) siRNA oligonucleotides were added in the diluted TransIT-TKO solution and incubated for additional 15 min, and the mixture was added to each well. Six hours after incubation with serum-free medium, 250 μL complete medium were added into each well and incubated for an additional 18 h.

Results

Isoform-specific association of PP1 with Nek2 in vivo. We analyzed the association of Nek2 with PP1 in HeLa cells using isoform-specific antibodies against PP1α/β/γ isofoms (Fig. 1). Both PP1α and PP1γ associated with endogenous Nek2 protein (Fig. L4 and C). However, Nek2 protein was not coimmunoprecipitated with PP1β/δ (Fig. 1B). In reciprocal experiments, PP1α and PP1γ (see Fig. 1A and C), but not PP1β/δ (see Fig. 1B), were coimmunoprecipitated with Nek2. Together, these results show

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that Nek2 selectively associates with PP1α and PP1γ but not with PP1β/δ.

**PP1α is necessary for ionizing radiation inhibition of Nek2 activity in vivo.** It is not known if ionizing radiation regulates PP1 bound to Nek2 as a means of controlling the kinase. To determine the effect of ionizing radiation on Nek2 kinase activity, triple HA-tagged wild-type Nek2 and Nek2-AA (a mutant deficient in binding to PP1) were transfected into COS-7 cells. Nek2 was immunoprecipitated from lysates of transfected cells using HA antibody at various times following 10 Gy irradiation. As shown in Fig. 2A, ionizing radiation inhibited Nek2 activity, measured using myelin basic protein as substrate, with maximal inhibition occurring at 60 to 90 min after irradiation. As a control, the activity of Nek2-AA was not inhibited by ionizing radiation. Note that PP1α immunoprecipitated with Nek2, and that the T320 site is dephosphorylated following ionizing radiation as detected with anti–phospho-T320 of PP1α antibody. This response of PP1 to ionizing radiation confirms our previous results that ionizing radiation signals activate PP1 by dephosphorylation of T320 (16). As expected, PP1α did not coimmunoprecipitate with Nek2-AA, confirming that the double AA mutation renders Nek2 incapable of binding to PP1. These results show that PP1 binding is necessary for the ionizing radiation inhibition of Nek2 activity and suggests that ionizing radiation activates PP1 to inhibit Nek2 activity by dephosphorylation.

**Ionizing radiation inhibition of Nek2 activity is ATM dependent.** ATM has been shown to be essential for PP1 dephosphorylation and activation after irradiation. To determine if ionizing radiation inhibition of Nek2 activity is ATM dependent, Nek2 was immunoprecipitated using HA antibody at various times following 10 Gy irradiation from mutant (ATM deficient, PEBS) and corrected (ATM proficient, YZ5) AT cells. Ionizing radiation inhibited Nek2 activity in corrected AT cells with maximal inhibition occurring at 60 to 90 min after ionizing radiation (Fig. 2B). However, activity of Nek2-AA was not inhibited in mutant AT (PEBS) cells. Note that PP1α coimmunoprecipitated with Nek2 (not Nek2-AA), and that PP1α was dephosphorylated at T320 following ionizing radiation as detected with anti–phospho-T320 of PP1α antibody. As expected, dephosphorylation of PP1α at T320 occurred only in corrected AT cells (YZ5) and not in ATM-deficient AT cells (PEBS). Ionizing radiation inhibition of Nek2 activity was only seen in corrected AT (YZ5) cells, not in ATM-deficient AT cells (PEBS). Thus, ATM is necessary for the ionizing radiation–induced inhibition of Nek2 activity. These results show that Nek2 is a downstream target in the ionizing radiation–activated ATM pathway, and that radiation-induced inhibition of Nek2 activity is likely mediated by PP1α activation due to dephosphorylation at T320.

**Ionizing radiation inhibition of centrosome splitting is dependent on Nek2 binding with PP1.** Ionizing radiation has been shown to inhibit centrosome splitting (15). To determine whether PP1 is essential for this response, COS-7 cells were transfected with empty vector, or plasmids encoding wild-type Nek2, or the mutant Nek2-AA. Cells expressing these proteins were analyzed by immunofluorescence microscopy at various times after ionizing radiation of 10 Gy. Figure 3A shows a typical immunofluorescent staining pattern of γ-tubulin as a centrosome marker at various time points after irradiation. Ionizing radiation transiently inhibited centrosome splitting at 1 h, and overexpression of wild-type Nek2 enhanced centrosome splitting initially but ionizing radiation produced significant inhibition (Fig. 3B). Moreover, cells expressing Nek2-AA showed enhanced centrosome splitting. Ionizing radiation–induced inhibition of centrosome splitting was both time dependent and reversible, with maximal inhibition occurring at 60 min in controls and at 60 to 120 min in cells overexpressing Nek2. Ionizing radiation failed to decrease centrosome splitting in the Nek2-AA cells (Fig. 3B). These data confirm that ionizing radiation inhibits centrosome splitting and show that the response is dependent on PP1 binding to Nek2.

**PP1α isoform regulates centrosome splitting.** To test whether a specific PP1 isoform regulates centrosome splitting in response to DNA damage, HeLa cells were transfected with synthetic siRNA smart pool, designed to selectively knock down the three PP1 isoforms one at a time. Figure 4A shows that the expression of each PP1 isoform was successfully (>90%) and specifically knocked down in HeLa cells. HeLa cells depleted of PP1α, PP1β/δ, and PP1γ or treated with non-target control RNA were irradiated with 10 Gy and centrosome splitting was measured at 0, 1, 2, and 4 h after ionizing radiation. Cells depleted of PP1α, but not of PP1β/δ or PP1γ, had higher basal levels of centrosome splitting (see Fig. 4B). This result establishes that only PP1α selectively regulates Nek2 in the absence of ionizing radiation. In response to ionizing radiation, cells depleted of β/δ or γ, as well as control cells, show significant inhibition of centrosome splitting (*, P < 0.01). Although in contrast, centrosome splitting was not inhibited by ionizing radiation in cells depleted of PP1α (*, P > 0.05). This result establishes that PP1α regulates centrosome splitting in response to DNA damage.

**Nek2 regulates centrosome number.** In addition to promoting centrosome splitting, elevated expression of Nek2 has been shown...
to be associated with multiple centrosomes per cell (14). To determine if centrosome number is regulated by Nek2 activity, the human epithelial ARPE 19 cells expressing wild-type Nek2, kinase-dead Nek2 (Nek2KD), or vector were stained and examined 5 days after transfection. Cells were transfected to express HA3-tagged wild-type Nek2 (A and B) or mutant Nek2-AA (A) that does not bind PP1, followed by irradiation with 10 Gy at 72 h after transfection. Cells were harvested at indicated time points (0, 30, 60, or 90 min after irradiation). Nek2 was immunoprecipitated with anti-HA monoclonal antibody. Immunoprecipitated proteins were split into two fractions: one was subjected to in vitro kinase assay using myelin basic protein as substrate, and the other was subjected to Western blot. The gel was analyzed using ImageQuant software by PhosphorImage. The blots were probed with anti-HA, anti-PP1, and anti-phospho-PP1 (Thr320), respectively.

Figure 2. Ionizing radiation (IR) inhibition of centrosome splitting is dependent on Nek2 binding with PP1. COS-7 cells in chamber slides were transfected to express HA3-tagged wild-type Nek2 or double mutant Nek2 (Nek2-AA). Cells were irradiated with 10 Gy at 72 h after transfection and fixed in 3% paraformaldehyde at the indicated time points (0, 1, 2, and 4 h). Cells were stained with anti-HA mouse monoclonal and anti-γ-tubulin rabbit polyclonal antibody, plus FITC-conjugated antirabbit and Texas Red–conjugated antimouse secondary antibodies, covered with mounting solution containing 1 ng/mL 4,6-diamidino-2-phenylindole, and analyzed by immunofluorescence microscopy. Centrosome distances were measured on separated centrosome pairs (>1 μm). Fifty cells were analyzed per time point, and two independent experiments were done. Points, mean (centrosome splitting); bars, SE. A, representative immunofluorescence images from transfected cells after irradiation at the indicated time points (0, 1, 2, and 4 h), γ-tubulin staining (yellow); see arrow at bottom left), HA staining (red), 4,6-diamidino-2-phenylindole (blue). B, dynamic changes in centrosome splitting of transfected cells after ionizing radiation.

Figure 3. Radiation inhibition of centrosome splitting is dependent on Nek2 binding with PP1. COS-7 cells in chamber slides were transfected to express HA3-tagged wild-type Nek2 or double mutant Nek2 (Nek2-AA). Cells were irradiated with 10 Gy at 72 h after transfection and fixed in 3% paraformaldehyde at the indicated time points (0, 1, 2, and 4 h). Cells were stained with anti-HA mouse monoclonal and anti-γ-tubulin rabbit polyclonal antibody, plus FITC-conjugated antirabbit and Texas Red–conjugated antimouse secondary antibodies, covered with mounting solution containing 1 ng/mL 4,6-diamidino-2-phenylindole, and analyzed by immunofluorescence microscopy. Centrosome distances were measured on separated centrosome pairs (>1 μm). Fifty cells were analyzed per time point, and two independent experiments were done. Points, mean (centrosome splitting); bars, SE. A, representative immunofluorescence images from transfected cells after irradiation at the indicated time points (0, 1, 2, and 4 h), γ-tubulin staining (yellow); see arrow at bottom left), HA staining (red), 4,6-diamidino-2-phenylindole (blue). B, dynamic changes in centrosome splitting of transfected cells after ionizing radiation.
Depletion of PP1α induces premature centrosome splitting in the presence and absence of ionizing radiation. HeLa cells in chamber slides were transfected to express PP1α-, PP1β-, or PP1γ-specific siRNA, respectively, and non-target siRNA as a negative control. Cells were fixed in 3% paraformaldehyde 72 h after transfection and stained by anti-γ-tubulin mouse monoclonal antibody, plus Texas Red–conjugated antimouse secondary antibody, and analyzed by immunofluorescence microscopy. Centrosome distances were measured on separated centrosome pairs (>1 μm). Fifty cells were analyzed per time point, and three independent experiments were done. A, Western blot using isoform-specific antibodies shows that PP1α, PP1β, or PP1γ isoforms were successfully and selectively depleted after PP1 isoform-specific siRNA transfection. B, dynamic changes in centrosome splitting of PP1 isoform knocked down cells after ionizing radiation at the indicated time points (0, 1, 2, and 4 h). *, P < 0.05, distance between pre-irradiation and post-irradiation (1 h) in PP1α-depleted cells is not statistically significant. #, P < 0.01, distance between pre-irradiation and post-irradiation (1 h) in PP1β/δ- and PP1γ-depleted and control cells is statistically significant.

Discussion

Centrosomes provide an important microtubule organizing function and are required for microtubule nucleation and other microtubule-based cellular processes, such as polarized secretion, migration formation of a primary cilium, and spindle poles in cell division. Centrosomes duplicate during S phase and separate during G2-M. Centrosome number and splitting are regulated by protein kinases, including Cdk (18, 19), Aurora-related kinases (4, 5), Polo-like kinases (2, 3), and the NIMA-related kinases (6, 7), like Nek2. PP1 is a serine/threonine phosphatase that regulates these kinases and therefore is expected to affect the centrosome cycle and centrosome functions.

The most abundant centrosome associated protein phosphatase is PP1. PP1 exists in three isoforms that are 90% identical with only variation in a few amino acids, mostly near the end of COOH terminus. PP1α, PP1γ, and PP1β/δ are products of distinct genes (20, 21). This variation in sequence among isoforms is known to affect the binding specificity of PP1 to its targets. Examples of isoform-specific binding subunits of PP1 include MYPT1 (22, 23), Neurabin (24), and Repo-Man (25). The three PP1 isoforms are differentially distributed in mammalian cells during both interphase and mitosis (12). During mitosis, PP1α predominately localizes to the centrosome, whereas PP1γ associates with microtubules of the mitotic spindle. During interphase, PP1α is localized to nuclear matrix and centrosome, whereas PP1γ is mainly distributed to nucleol. One means by which PP1 isoforms may achieve specificity is by binding to different proteins, thereby becoming targeted to different sites within the cell. Moreover, although PP1γ is associated with Nek2 and PP1α (26), our data show that only PP1α regulates centrosome number and splitting. Nek2 may now be considered a centrosome targeting subunit for PP1α. Previous results show that ionizing radiation causes dephosphorylation of all isoforms of PP1 (16); thus, it is unlikely that the phosphorylation status of the T320 site influences binding to different sites within the cell. Moreover, although PP1γ is associated with Nek2 and PP1α (26), our data show that only PP1α regulates centrosome number and splitting. Nek2 may now be considered a centrosome targeting subunit for PP1α. Previous results show that ionizing radiation causes dephosphorylation of all isoforms of PP1 (16); thus, it is unlikely that the phosphorylation status of the T320 site influences binding of the other PP1 isoforms (α and γ) to Nek2 (27). Based on the different intracellular distribution of PP1 isoforms, it is not surprising that they have distinct functions.

Centrosome splitting is thought to be regulated by phosphorylation of cohesion proteins. This involves tipping the balance between kinase and phosphatase activity. The present study was aimed at...
Overexpression of Nek2 or depletion of PP1α causes centrosome number. Human retinal ARPE 19 cells in chamber slides were transfected with HA3-tagged wild-type Nek2 or mutant kinase-dead Nek2 (Nek2KD; B). HeLa cells in chamber slides were transfected with PP1α-, PP1β/γ-, or PP1γ-specific siRNA, respectively, or non-target siRNA as a negative control. For PP1 isoform-specific knockdown efficiency, see (A). Cells were fixed in 3% paraformaldehyde 5 d after transfection and stained by anti-γ-tubulin mouse monoclonal antibody, plus antimouse Texas Red–conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. Cells with multiple centrosomes (>2) were counted in each experiment. Centrosomes in 500 cells were analyzed, and two independent experiments were done. Columns, mean percentage of cells with multiple centrosomes; bars, SE. A, representative immunofluorescent images from transfected HeLa cells with normal or abnormal centrosomes, γ-tubulin staining (red), 4,6-diamidino-2-phenylindole (blue). B, histogram indicates percentage of cells with abnormal centrosomes in Nek2 overexpressed ARPE19 cells. C, histogram shows percentage of cells with abnormal centrosomes in HeLa cells depleted of the indicated PP1 isoforms.
expanding the role of PP1 in regulating centrosome splitting and number. PP1 forms a complex with Nek2 and at least one of its substrates C-Nap1. C-Nap1 is thought to interact with Rootin physically linking the two daughter centrioles (9, 28). Phosphorylation of C-Nap1 results in the dissimer of the dimer and therefore gives centrosome splitting. Our results show that PP1α regulates Nek2 function in terms of kinase activity and centrosome number and splitting, both in the presence and absence of radiation-induced DNA damage. The role of ATM in regulating centrosome splitting is not limited to PP1 because ATM also regulates Plk1, which interacts with Nek2 in vivo and phosphorylates Nek2 in vitro. Interestingly, Plk1-mediated changes in Nek2 activity were PP1-dependent (14). However, the role of PP1 in regulation of centrosome function is not likely limited to Nek2. PP1 also regulates Aurora-A [29]. PP1 binds Aurora-A and Aurora-A binds Inhibitor-2. Inhibitor-2 activates Aurora-A kinase in a bifunctional manner not only through inhibition of PP1 activity but also by allosterically activating Aurora-A. Following radiation-induced dephosphorylation of the inhibitory site at T320, PP1 would be activated to dephosphorylate multiple centrosome substrates, such as Nek2, Aurora-A, Plk1, and C-Nap1, thereby inhibiting centrosome splitting (15).

Our data clearly show that PP1 regulates centrosome splitting and number in an isoform-specific manner. The question of how the ionizing radiation damage signal is transmitted from ATM to PP1 remains unanswered. The simplest explanation is that inhibition inhibits the activity of CDKs, which phosphorylate the Thr205 site of PP1. Dephosphorylation of this site occurs by an intramolecular reaction that increases the activity of PP1; thus, decrease in CDK activity removes tonic inhibition leading to increase PP1 activity. Because PP1 regulates cell cycle progression as well as centrosome number, and because radiation is known to produce cell cycle blocks, the question arises whether observed PP1α effects on centrosome splitting and duplication are secondary to changes in the cell cycle. The fact that centrosome splitting was observed at a dose of 10 Gy and was reversible within 90 min argues strongly that this effect is not secondary to normal cell cycle arrest because 10 Gy produces a G2 arrest in several hours. The effect of PP1 on centrosome duplication is more complicated. Multiple centrosome phenotypes could be due to a direct effect of PP1α on Nek2 activity. However, an indirect effect of PP1α on cytokinesis could also explain the multiple centrosomes per cell phenotype.

Interestingly, radiation has been shown to lead to increased centrosome numbers in a variety of cell lines derived from human solid tumors (30, 31). However, it is unclear which protein(s) or pathway mediates this phenotype, although overexpression of p21 blocked the radiation-induced increase in centrosome number. Under certain circumstances, the centrosome cycle can be coupled to the cell cycle. For example, depletion of the replication licensing inhibitor geminin has been noted to cause overduplication of centrosome as well as mitotic abnormalities (32). For PP1α to regulate centrosome number indirectly through a cell cycle block would mean that PP1α depletion produces different cell cycle perturbations than PP1β and PP1γ because the depletion of the later two isoforms did not produce increased number of centrosomes. Other proteins have also been identified as critical for centrosome duplication, including Plk4, Pin1, BRCA1, nucleophosmin/B23, mMP51/Esk, CP110, etc. (33–38) as well as transcription factor E2F and Rb (39).

In summary, Nek2 is a downstream target of PP1α, and our results show this operates both in the presence and absence of DNA damage. Ionizing radiation activates ATM through autophosphorylation (40), and activated ATM inhibits CDKs that phosphorylate PP1 at T320 (16). PP1 is therefore activated after irradiation by reduction in the inhibitory T320 phosphorylation. The mechanism by which activated PP1 inhibits centrosome splitting is unknown, but we propose that PP1α keeps Nek2, Aurora-A, and C-Nap1 dephosphorylated to prevent centrosome splitting. The long-term implications of our findings are 2-fold. First, the link between radiation, Nek2, and PP1 in terms of centrosome splitting raises the possibility that PP1 may play an important role in maintaining genome stability, given the observations that centrosome overduplication has been associated with aneuploidy. Second, the observation that specific isoforms of PP1 have different functions in the damage response opens the possibility of targeting various isoforms or isoform-protein interactions using small-molecule inhibitors as radiation sensitizers.

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