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Mice Lacking Protein Phosphatase 5 Are Defective in Ataxia Telangiectasia Mutated (ATM)-mediated Cell Cycle Arrest*

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Protein phosphatase 5 (Ppp5), a tetratricopeptide repeat domain protein, has been implicated in multiple cellular functions, including cellular proliferation, migration, differentiation and survival, and cell cycle checkpoint regulation via the ataxia telangiectasia mutated/ATM and Rad3-related (ATM/ATR) signal pathway. However, the physiological functions of Ppp5 have not been reported. To confirm the role of Ppp5 in cell cycle checkpoint regulation, we generated Ppp5-deficient mice and isolated mouse embryonic fibroblast (MEF) cells from Ppp5-deficient and littermate control embryos. Although Ppp5-deficient mice can survive through embryonic development and postnatal life and MEF cells from the Ppp5-deficient mice maintain normal replication checkpoint induced by hydroxyurea, Ppp5-deficient MEF cells display a significant defect in G_2/M DNA damage checkpoint in response to ionizing radiation (IR). To determine whether this defect in IR-induced G_2/M checkpoint is due to altered ATM-mediated signaling, we measured ATM kinase activity and ATM-mediated downstream events. Our data demonstrated that IR-induced ATM kinase activity is attenuated in Ppp5-deficient MEFs. Phosphorylation levels of two known ATM substrates, Rad17 and Chk2, were significantly reduced in Ppp5-deficient MEFs in response to IR. Furthermore, DNA damage-induced Rad17 nuclear foci were dramatically reduced in Ppp5-deficient MEFs. These results demonstrate a direct regulatory linkage between Ppp5 and activation of the ATM-mediated G_2/M DNA damage checkpoint pathway *in vivo*.

In eukaryotic cells, DNA repair and cell cycle checkpoints are highly integrated processes that are critical for maintaining genomic stability and integrity (1). In the event of DNA damage, two closely related checkpoint kinases, ataxia telangiectasia mutated (ATM)[3] and ATM and Rad3-related (ATR), are thought to be master controllers of cell cycle checkpoint signaling that coordinate multiple cellular processes, such as cell cycle arrest, G_2/M checkpoint, intra-S-phase checkpoint, DNA repair, and cellular apoptosis (2). Defects in cell cycle checkpoints will result in genetic mutations, chromosomal abnormalities, and aneuploidy, all of which contribute to tumorigenesis (3). Although ATM and ATR appeared to have an overlapped biological function, ATM was thought to be more relevant in sensing DNA double-stranded breaks induced by ionizing radiation (4), and ATR was responsible for the checkpoint activation in response to DNA damage induced by a variety of broader stressors, such as UV light, hydroxyurea (HU), and other types of genotoxic insults (2). Interestingly, ataxia telangiectasia (AT) cells or ATM-deficient cells often display ionizing radiation (IR)-resistant DNA synthesis in S-phase (2), suggesting that ATM may also function in DNA replication checkpoint in S-phase.

Protein phosphatase 5 (Ppp5, also known as PP5) is a serine/threonine phosphatase involved in several important cellular functions. Ppp5 is unique among phosphatases in that it contains a series of 34-amino acid tetratricopeptide repeat (TPR) motifs that serve as a protein-protein interaction domain (5). Ppp5 is ubiquitously expressed and is able to form heterocomplexes with glucocorticoid receptors via heat shock protein 90 (Hsp90) (6). In addition to steroid signaling, Ppp5 has been implicated in cellular growth, proliferation, differentiation, migration, and survival via several intracellular signaling complexes, including CDC16- and CDC27- (7), apoptosis signal-regulating kinase 1- (ASK1) (8), $G\alpha_{12}/G\alpha_{13}$- (9), Rac GTPase- (10), and Raf-MEK-ERK-mediated pathways (11). Recently, several lines of evidence have suggested a critical role for Ppp5 in both the ATM/ATR and the DNA-dependent protein kinase (DNA-PK) pathways controlling cell cycle checkpoints and DNA double-strand break repair (12–14). Down-regulation of Ppp5 via small interfering RNA or overexpression of a catalytically inactive Ppp5 mutant in cells inhibits ATM activation and ATM autophosphorylation on serine 1981, leading to a checkpoint defect in DNA-damaged cells (14). Another report suggests that Ppp5 is also required for ATR or ATM activation in response to HU-induced replication block (12). Taken as a whole, these studies strongly hint at the important contribution of Ppp5 to various basic cellular functions.

To confirm the above activities of Ppp5 in a physiological context, we undertook a loss-of-function study by generating

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[3] The abbreviations used are: ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3-related; Ppp5, protein phosphatase 5; MEF, mouse embryonic fibroblast; IR, ionizing radiation; TPR, tetratricopeptide repeat; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; HU, hydroxyurea; Gy, grays; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; RFC, replication factor C; RT, reverse transcription.

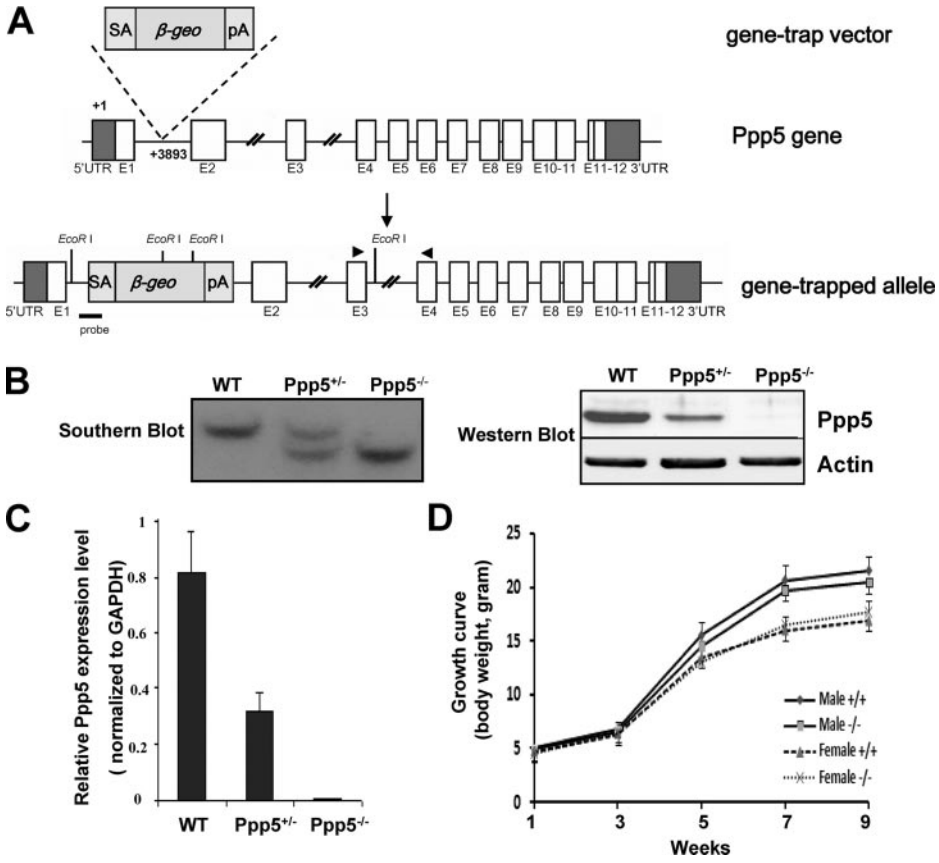

FIGURE 1. **Generation of Ppp5-deficient mice.** *A*, genomic structure of the mouse Ppp5 gene, gene trap vector, and Ppp5 mutant allele. *UTR*, untranslated region; SA, splice acceptor site; *pA*, poly(A) site. *B* and *C*, Southern blot and Western blot (*B*) and quantitative RT-PCR analyses confirm the Ppp5 mutant allele to be null (*C*). For Southern blot, the genomic DNA was digested by EcoRI (New England Biolabs). *WT*, wild type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *D*, growth curves of wild-type and Ppp5-deficient mice. The probe indicated in *A* reveals a 11.4-kb fragment from wild-type allele and a 7.1-kb fragment from Ppp5 mutant allele. Real-time PCR primers are indicated in *A*.

Ppp5-deficient mice. Surprisingly, Ppp5 deficiency did not give rise to dramatic growth defects during embryonic development and postnatal life. To investigate the potential function of Ppp5 in ATM-mediated cell cycle checkpoint signaling induced by double-strand breaks, we examined the checkpoint response to IR in mouse embryonic fibroblasts (MEFs) isolated from wild-type and Ppp5-deficient embryos. We demonstrate that Ppp5 is essential for the activation of G_2/M checkpoint induced by IR exposure but not the S-phase replication checkpoint that can be triggered by HU treatment. Our results support a regulatory linkage between Ppp5 and ATM kinase-mediated G_2/M checkpoint activation in response to radiation-induced DNA damage. Together, these data strongly suggest that Ppp5 is a crucial regulator in the DNA damage checkpoint pathway.

MATERIALS AND METHODS

Generation of Ppp5-deficient Mice—A promoter-trapped embryonic stem cell line XG029 containing an insertional mutation in the mouse *ppp5* gene was obtained from BayGenomics. The gene trap vector (pGT1Lxf) contains a splice acceptor sequence upstream of the reporter gene *β-geo*. Using the PCR mapping approach, Southern blot, and sequencing analyses, we confirmed and identified the location of a single genomic insertion in the first intron of the mouse *ppp5* gene. The genotyping primer sequences and probes for Southern blot analysis were designed based on this insertion site at +3893 from the transcription initiation site (Fig. 1*A*). Chimeric male mice were then generated and were further bred to C57BL/6J females to generate F1 offspring. Primers for genotyping are: Ppp5 geno forward, 5′-TACAGAGCAGGGAAGTGGGGTCAG3′; Ppp5 geno reverse, 5′-AGGTTGGAGGACCATGTGCCAG-3′; Ppp5 geo reverse, 5′-TTCAGTCTTCCTTGGTGGCCTGTC-3′.

Quantitative RT-PCR—Total RNA was extracted from various tissues using TRIzol (Invitrogen). First strand cDNA was synthesized by the iScript cDNA synthesis kit (Bio-Rad) using 1 μg of total RNA as a template according to the protocol provided by the manufacturer. Real-time PCR was performed using iCycle iQ (Bio-Rad). The relative expression was normalized to the reference gene ribosomal protein L7 (RPL7). Sequences of specific primers are as follows: q-Ppp5 forward, 5′-CACAGACGCTCTGTCGTGGACTCTC-3′; q-Ppp5 reverse, 5′-GCACTTCCGGTGCAGTTTCTTCTG-3′; RPL7 forward, 5′-AGTTGAAGGTGAAGCGCCTGAGG-3′; RPL 7 reverse, 5′-TGCCATCCTAGCCATCCGAATC-3′.

Cell Culture, IR Treatments, and Fluorescence-activated Cell Sorting Analysis—The Ppp5 wild-type, Ppp5 heterozygous (+/−) mutant, and Ppp5 homozygous (−/−) mutant mouse embryonic fibroblasts were isolated from an embryonic day 13.5 embryo and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells were irradiated with 3 Gy of ionizing radiation in the AGFA X-RAD320 irradiation system. 24 h after IR (3 Gy), IR-treated or untreated MEF cells were fixed with 70% ethanol and incubated for 30 min with RNase A (100 μg/ml) and propidium iodide (50 μg/ml) at 37 C. Cell cycle distributions were analyzed by flow cytometry. Mitotic indexes in wild-type and Ppp5-deficient MEF cells before and after IR treatment were determined as described previously (15).

Western Blot Analysis and in Vitro ATM Kinase Assay—Irradiated MEF cells were harvested at 1 h after IR or as indicated. Western blot analysis of phospho-Rad17 (pRad17), total Rad17, phospho-Chk2 (pChk2), total Chk2, and Ppp5 was performed as described previously (14). Phospho-specific antibody for Rad17 Ser-645 (α-pS645-Rad17) was obtained from GeneTex (San Antonio, TX), and the phospho-specific antibody for

Rad17 Ser-635 (α-pS635-Rad17) has been previously characterized (12, 15). The phospho-specific antibody for Chk2 and Chk1 and the antibody for total Chk2 and Chk1 were from Cell Signaling Inc. Antibody for total Rad17 was purchased from Santa Cruz Biotechnology, and antibody for Ppp5 was a generous gift from Dr. Michael Chinker. The ATM complex was immunoprecipitated from untreated or irradiated wild-type and Ppp5-decifient MEF cells, and the *in vitro* ATM kinase assays were performed as described previously (15). ATM kinase activities were assessed using 1 μg of GST-hRad17 fusion protein containing the C-terminal 185 amino acids of hRad17 as substrate.

Immunofluorescent Staining—MEF cells isolated from wild-type or Ppp5-deficient mice were cultured on coverslips in Dulbecco's modified Eagle's medium with 10% fetal bovine serum followed by irradiation. Cells were fixed at 3 h after IR with 4% paraformaldehyde and permeabilized with 0.1% Triton-100 in PBS. After blocking with 3% bovine serum albumin, cells were incubated with the α-pS645-Rad17 antibody (diluted according to the manufacturer's instructions) at 4 °C overnight. Following three washes with PBS, cells were incubated with rhodamine-conjugated anti-rabbit IgG secondary antibody for 1 h. After washing with PBS, cell nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma). Pictures were taken under a fluorescent microscope (Axiovert 200, Zeiss).

RESULTS AND DISCUSSION

Ppp5 heterozygous mutant mice were viable and fertile and were intercrossed to obtain Ppp5 homozygous mutants. Genotyping analysis of 153 F2 offspring at 2 weeks of age demonstrated normal Mendelian 1:2:1 distribution of all three genotypes (+/+, 42; +/−, 74; −/−, 37), indicating that Ppp5-deficient mice are viable *in utero* through the course of embryonic development. Western blotting and real-time quantitative RT-PCR analysis of Ppp5 expression in wild-type, Ppp5 heterozygous, and Ppp5 homozygous mutant mice demonstrated a 50% reduction in Ppp5 heterozygous and a complete absence of Ppp5 protein and mRNA in Ppp5 homozygous mutant (Fig. 1, *B* and *C*). There was no leaky Ppp5 expression found in this Ppp5 mutant mouse strain. Therefore, this promoter-trapped allele is a Ppp5 null allele. Both Ppp5-deficient male and Ppp5-deficient female mice were fertile and showed a rate of growth equivalent to that of wild-type (Fig. 1*D*). Analysis of potentially compromised glucocorticoid receptor activity in Ppp5-deficient mice is on the way. Here, as our first report on the generation of Ppp5-deficient mice, we focus on our initial analysis of the role of Ppp5 in ATM-mediated cell cycle checkpoint pathway.

ATM is important in cell cycle checkpoint activation triggered by ionizing radiation and radiomimetic agents (15). Recently, several reports using small interfering RNA have suggested a role for Ppp5 in ATM kinase activity and its subsequent checkpoint activation (12, 14). To investigate whether Ppp5 is essential to cell cycle checkpoints, we isolated mouse embryonic fibroblasts from wild-type and Ppp5-deficient embryonic day 3.5 embryos. The MEF cells were exposed to ionizing radiation (3 Gy) and HU, respectively. In the case of IR, cell cycle profiles were analyzed 24 h after irradiation using flow cytometry (fluorescence-activated cell sorter). Our analysis revealed that wild-type MEF cells displayed a typical and distinct G_2/M cell cycle arrest after IR exposure (43% arrest at G_2/M-phase) (Fig. 2). In contrast, Ppp5-deficient MEF cells significantly failed to accumulate in G_2/M-phase after IR exposure (23% arrest at G_2/M). These data strongly indicate a defect in the IR-induced G_2/M checkpoint in Ppp5-deficient cells. This conclusion was further confirmed by determining the mitotic indexes in wild-type and Ppp5 null MEF cells before and after IR treatment. As shown in Fig. 2*C*, at 24 h after IR, Ppp5 null MEF cells display a significant higher mitotic index than the wild-type cells, indicating that Ppp5 null cells have a defect in G_2 arrest in response to IR, which further demonstrates that Ppp5 null cells are defective in the IR-induced G_2 checkpoint. In the case of HU treatment, however, Ppp5-deficient MEF cell did not appear significantly different when compared with wild-type MEF cells (data not shown).

In previous studies (12, 14), Ppp5 was found in a complex with ATM. Ppp5 expression was also increased after the cells were exposed to the radiomimetic agent neocarzinostatin. To determine whether ablation of Ppp5 affects ATM activation

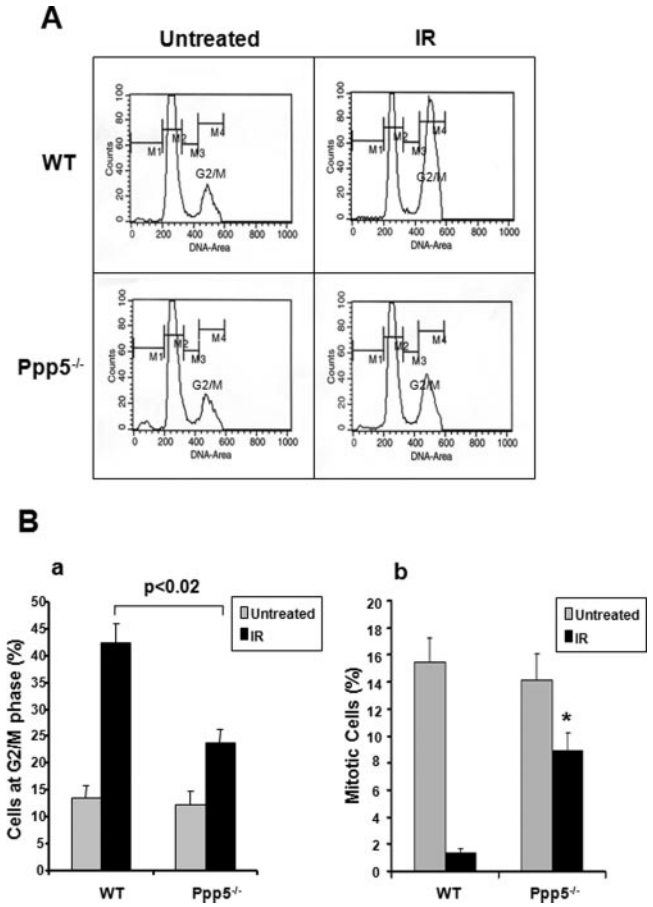

FIGURE 2. **Ppp5-deficient MEF cells display a defect of ionizing radiation-induced G_2/M checkpoint activation.** *A*, cell cycle profiles of Ppp5-deficient and wild-type (*WT*) cells with or without ionizing radiation exposure (3 Gy). Cell cycle distributions were determined by flow cytometry at 24 h after IR treatment. *B*, *panel a*, statistical analysis of G_2/M-phase arrest in Ppp5-deficient and wild-type cells with or without IR exposure. The IR-induced G_2/M arrest in Ppp5-deficient cells is significantly less than in wild-type cells. *B*, *panel b*, mitotic index confirmed that Ppp5-deficient cells displayed significantly greater fraction of mitotic cells after IR treatment.

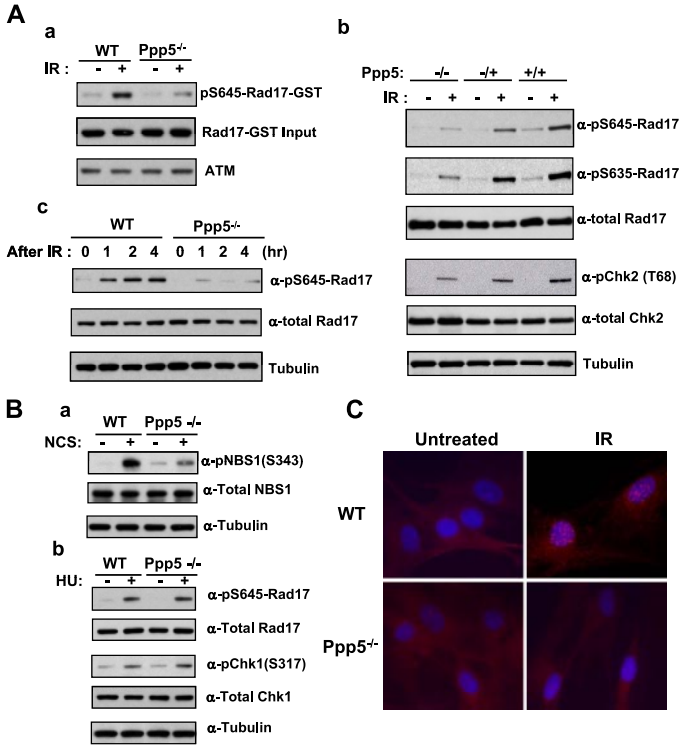

FIGURE 3. **Ablation of Ppp5 decreased ATM-mediated signaling.** *A*, *panel a*, ATM kinase activity is reduced in Ppp5-deficient MEF cells. Wild-type and Ppp5-deficient cells were exposed to IR (3 Gy) followed by immunoprecipitation of ATM complexes and *in vitro* ATM kinase assay, as described previously (15). GST-hRad17 fusion protein was used as substrate, and detection of phosphorylation was performed with antibody specific to pS645-Rad17. *A*, *panel b*, IR-induced phosphorylation of Rad17 and Chk2 is attenuated in Ppp5-deficient cells. Wild-type (+/+), Ppp5 heterozygous (+/−), and homozygous (−/−) cells were exposed to 3 Gy ionizing radiation and harvested at 1 h after IR. Levels of phosphorylated Rad17 (pS635-Rad17 and pS645-Rad17), phosphorylated Chk2 (pChk2-T68), and total Rad17 and Chk2 were determined by immunoblotting with specific antibodies. *A*, *panel c*, time course of Rad17 phosphorylation induced by IR exposure in wild-type and Ppp5-deficient MEF cells. MEF cells were exposed to IR (3 Gy). Cellular extracts were collected at 0, 1, 2, and 4 h after IR and were immunoblotted with specific antibodies against pS645-Rad17, total Rad17, or tubulin (used as internal loading control). *B*, Western blot analysis of wild-type and Ppp5-deficient cells treated with HU (10 mM, 20 h) and NCS (100 ng/ml, 2 h). *B*, *panel a*, phosphorylated NBS1 was reduced in Ppp5-deficient cells when compared with wild-type controls after treating with NCS. *B*, *panel b*, there is no significant difference in Rad17 and Chk1 phosphorylation between wild-type and Ppp5-deficient MEF cell before and after HU treatments. *C*, pS645-Rad17 immunofluorescent staining of wild-type and Ppp5-deficient cells was exposed to IR (3 Gy). At 3 h after IR, cells were fixed and immunostained with a phospho-specific Rad17 antibody (a-pS645-Rad17). The Rad17 nuclear foci (in *red*) were examined under a fluorescence microscope. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (in *blue*). IR-induced Rad17 nuclear foci appeared in wild-type MEF cells but not in the Ppp5-deficient MEF cells.

induced by IR exposure, *in vitro* kinase assays were performed using the ATM-specific substrate Rad17 (15). A GST fusion protein containing a 185-amino acid C-terminal peptide of human Rad17 was incubated with ATM immunoprecipitates derived from wild-type and mutant Ppp5 MEF cells. ATM kinase activity was assessed by Western blot using a specific antibody against phosphorylated Rad17 at serine 635 (12, 15). As expected, ATM kinase activity was greatly increased after IR treatment in wild-type MEF cells (Fig. 3*A*, *panel a*). In contrast, ATM kinase activity was dramatically reduced in Ppp5-deficient cells after IR treatment, pointing to Ppp5 as a key regulator of ATM kinase activity.

To further investigate the ATM pathway in Ppp5-deficient cells, we measured phosphorylation of two ATM targets, Rad17 and Chk2, in intact cells. Rad17 is a checkpoint protein required for cell cycle arrest and DNA repair. This protein binds to chromatin prior to DNA damage and is phosphorylated by ATM after damage (16). Phosphorylation of Rad17 is required for DNA damage-induced cell cycle arrest and is thought to be a critical early event during checkpoint signaling (15). The protein kinase Chk2 is also phosphorylated and activated in response to DNA damage by ionizing radiation, with both events dependent on ATM activity (17). Chk2-deficient embryonic stem cells fail to maintain radiation-induced arrest in the G_2-phase of the cell cycle (18). Thus, phosphorylation levels of Rad17 and Chk2 are key indicators of endogenous ATM activity. As shown in Fig. 3*A*, *panel b*, ionizing radiation induced phosphorylation of Rad17 at both Ser-645 and Ser-635 in wild-type MEF cells. Induction of phosphorylation was detected 1 h after IR exposure. Similarly, Chk2 phosphorylation was enhanced in wild-type MEF cells after irradiation. In contrast, IR-induced phosphorylation of both Rad17 and Chk2 was significantly reduced in Ppp5-deficient cells, although levels of each protein remained normal. A time course study further revealed that ATM activity remained dramatically reduced up to 4 h after IR (Fig. 3*A*, *panel c*). Furthermore, phosphorylated NBS1 was also significantly reduced in Ppp5-deficient cells when treating with a radiomimetic drug neocarzinostatin (NCS) (Fig. 3*B*, *panel a*). Consistent with normal cellular response to replication blocker HU, wild-type and Ppp5-deficient MEF cells did not display a significant difference in the phosphorylation of Rad17 and Chk1 in response to HU treatment (Fig. 3*B*, *panel b*).

In addition to biochemical alterations, we also found that the biological function of Rad17 was defective in Ppp5-deficient MEF cells. Rad17 shares strong similarity with DNA replication factor C (RFC) and can form a complex with RFCs (19). The Rad17-RFC complex, along with another DNA repair complex (Rad9-Rad1-Hus1), have both been implicated in the early phase of cell cycle checkpoint control (20). They function as sensors for DNA damage and genome replication errors (21). Phosphorylation of Rad17 is required to form this complex (15), and a critical feature required for activity appears to be formation of Rad17-containing nuclear foci after IR (15, 22). To determine whether Ppp5 activity is required for complex formation, we examined nuclear foci formation in Ppp5-deficient MEF cells after IR exposure. Wild-type and Ppp5-deficient MEF cells were fixed and stained with a specific antibody against phospho-Rad17-Ser-645 at 3 h after IR. As shown in Fig. 3*C*, wild-type MEF cells displayed a typical punctuate pattern of nuclear staining after IR. In contrast, this nuclear staining pattern was almost completely missing in Ppp5-deficient MEF cells (Fig. 3*C*), suggesting that reduced phosphorylation of Rad17 in Ppp5-deficient cells may decrease nuclear foci (checkpoint complex) formation.

Taken as a whole, our data provide the first conclusive evidence that Ppp5 is an important regulator of ATM-mediated signaling. We have shown that Ppp5 is essential to the ability of ATM to phosphorylate key substrates, Rad17, Chk2, and NBS1, and that these phosphorylation events are critical to checkpoint control of

cell cycle arrest following DNA damage. One enigma from our initial characterization of Ppp5-deficient mice is that, despite significant alteration of ATM-mediated signaling, Ppp5 is not critical to mouse survival and fertility. In contrast, ATM-deficient mice display growth retardation, male and female infertility due to meiotic failure and abnormal chromosomal synapsis, defects in lymphocyte maturation, and extreme sensitivity to radiation. Moreover, most ATM-deficient mice die before 4 months of age from thymic lymphomas (23). Although we have not yet thoroughly investigated most of these parameters in the Ppp5-deficient mouse, it is clear that fertility appears to be unaffected and that lethality is much reduced when compared with the ATM-deficient mouse. This suggests that ATM is involved in a broader checkpoint function than Ppp5. Our data demonstrate that Ppp5 is likely involved in regulating ATM kinase activity only in response to a subset of genotoxic stresses, such as IR. When we examined the HU-induced replication checkpoint activation in Ppp5-deficient and wild-type MEF cells, we found that the Rad17 and Chk1 phosphorylations were not significantly different between wild-type and Ppp5-deficient MEF cells, suggesting that Ppp5 was not involved in the intra-S-phase replication checkpoint. On the other hand, it was demonstrated that AT cells or ATM-deficient cells displayed IR-resistant DNA synthesis in S-phase (2), suggesting that ATM was also associated with S-phase DNA replication checkpoint. Alternatively, other protein phosphatases may partially compensate for the loss of Ppp5 in the Ppp5-deficient cells. Together, these findings may help to explain the phenotypic difference between ATM-deficient and Ppp5-deficient mice. Given the high incidence of lymphomas in ATM null mice, it will be interesting to see whether Ppp5-deficient mice require a longer period of time to develop lymphomas or other tumors.

Also consistent with our hypothesis is the fact that Ppp5 appears to be a tightly regulated phosphatase. Several studies have shown extremely low basal phosphatase activity for Ppp5, most likely due to an auto-inhibitory function of the TPR domain of the protein (24–26). It has been demonstrated that the TPR domain cooperates with the final 13 residues of the C terminus to maintain the phosphatase in an inactive state. Removal of either the TPR domain or the C-terminal 13 residues by limited proteolysis or deletion mutagenesis greatly increases the Ppp5 enzymatic activity (25, 27–29). Interestingly, several reports now exist that both Hsp90 and fatty acids, such as arachidonic acid, can stimulate Ppp5 phosphatase activity by a direct interaction with the TPR domain (29, 30). Although it is far from clear whether fatty acids are true physiological regulators of Ppp5, it is likely that other, as yet unknown, upstream factors will be found to regulate the phosphatase. Given the role uncovered here, it would not be surprising to find activation of Ppp5 either by radiation-induced signaling molecules or via byproducts of the DNA damage process itself.

Clearly, the exact mechanisms that activate Ppp5 during DNA damage stress or the direct targets of Ppp5 in the ATM survival mechanism remain unknown and will be the subject of much investigation. The Ppp5-deficient mice and cells we have generated may not only help to identify these missing factors but may also contribute to the understanding of how genomic instability leads to tumorigenesis.

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