Pir51, a Rad51-interacting protein with high expression in aggressive lymphoma, controls mitomycin C sensitivity and prevents chromosomal breaks

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Abstract

Pir51, a protein of unknown function that interacts with Rad51, was identified in a screen for genes that were highly expressed in aggressive mantle cell lymphoma (MCL) versus indolent small lymphocytic lymphoma (SLL) patient samples. We show that Pir51 is a nuclear protein expressed in a variety of cell types and that its expression is regulated during the cell cycle in a pattern nearly identical to Rad51. Also similar to Rad51, Pir51 levels did not change in response to a variety of DNA damaging agents. siRNA depletion of Pir51 did not reduce homologous recombination repair (HRR), but sensitized cells to mitomycin C (MMC)-induced DNA crosslinking and resulted in elevated levels of double-strand breaks (DSBs) in metaphase chromosome spreads and reduced colony formation. Therefore, Pir51 maintains genomic integrity and potentially connects the early response to DNA crosslinks, orchestrated by the ATR kinase and Fanconi Anemia (FA) proteins, to later stages of Rad51-dependent repair. Our results provide the first example of a Rad51-binding protein that influences DNA crosslink repair without affecting homologous recombination repair.

Keywords: Pir51; Rad51; Cancer; DNA repair; Cell cycle

1. Introduction

Homologous recombination repair (HRR) is a major pathway for the repair of DNA double-strand breaks (DSBs) [1]. A key protein in the HRR pathway is Rad51, the eukaryotic homolog of bacterial recA [1]. Mammalian Rad51 is implicated in the repair of DSBs that
may arise at stalled DNA replication forks, or by exposure to γ-irradiation, alkylating agents or UV light [2]. Increased Rad51 expression is reported for a variety of tumor cell lines [1] and elevated Rad51 levels promote genome instability [3]. Conversely, the disruption of Rad51 function in CHO cells also promotes genome instability and tumor formation in nude mice [4], while loss of Rad51 in DT40 cells induces chromosome breaks prior to cell death [1]. Rad51 interacts with many accessory proteins during HRR [1] and is involved in the repair of DNA crosslinks that are caused by agents such as mitomycin C (MMC). The MMC repair pathway involves the checkpoint kinase ATR, the Fanconi Anemia (FA) proteins and BRCA1, and is often referred to as the FA/BRCA pathway [5]. Furthermore, Rad51 interacts with the tumor suppressor/cell cycle checkpoint proteins ATM and p53 [1]. Collectively, these findings suggest that the level of Rad51 expression influences DNA damage repair, tumorigenesis and apoptosis and that the HRR pathway works in concert with a complex array of signaling, tumor suppressor and cell cycle checkpoint proteins in the maintenance of genomic integrity.

Pir51 was first identified in 1997 in a yeast two-hybrid screen for proteins that interact with human Rad51 [6]. In biochemical assays, Pir51 binds single-stranded DNA, double-stranded DNA and RNA, and promotes DNA aggregation [6]. A murine Pir51 homolog, Rab22, also interacts with human Rad51, while exogenously expressed Rad51 and Rab22 interact in vivo and co-localize in nuclear foci in untreated interphase cells [7]. Although the ability of Pir51 to bind DNA and for exogenous Pir51 to co-localize with Rad51 in nuclear foci suggests a role for Pir51 in DNA repair, the physiologic function(s) of native Pir51 remains unknown.

Several primary human tumor types display enhanced Pir51 expression, including hepatocellular carcinoma [8] and acute myeloid leukemia with complex karyotypic abnormalities [9]. Conversely, Pir51 expression is reduced and its promoter CpG methylated in prostate cancer [10], suggesting that, like Rad51, alterations in the level of Pir51 expression may have a context-specific role in cancer. A link may also exist between Pir51 expression and the cell cycle, since most tumor cell types with high replication rates show elevated Pir51 expression. Interestingly, slowing the growth of aggressive breast cancer cells with progesterone reduces Pir51 levels [11], while arresting mouse lymphoma cell growth in S phase with hydroxyurea results in a marked increase in Pir51 expression [12]. These paradoxical findings could be resolved if Pir51 is expressed at specific stages in the cell cycle, such as in S phase. Although cell cycle fluctuations in Pir51 abundance have not been reported, the Pir51 promoter is repressed by the transcription factor E2F1 in G0/G1 arrested cells [13]. Since many genes under the control of E2F1 are cell cycle-regulated and a subset of these are involved in the DNA damage response [14–17], Pir51 may be an additional member of this group, although this idea requires investigation.

In the current study, we identified Pir51 in a suppression subtractive hybridization (SSH) screen as a gene that is more highly expressed in cycling, aggressive mantle cell lymphoma (MCL) patient samples compared to quiescent, indolent non-Hodgkin lymphoma samples. Because Pir51 interacts with Rad51, Pir51 was tested for functions associated with known features of Rad51, including changes in expression during the cell cycle, subcellular distribution, response to DNA damaging agents, and for a role in DNA repair. Our results predict a functional role for Pir51 in regulating genome integrity.

2. Materials and methods

2.1. Patient samples and cell lines

Fresh-frozen lymphoma samples were kindly provided by J. Said (UCLA, Los Angeles, CA) and T. Grogan (University of Arizona Cancer Center, Tucson, AZ). Tissue blocks were placed into RNA STAT-60 (Tel-Test, Friendswood, TX), and total RNA was extracted according to the manufacturer’s instructions. Granta 519, NCEB1 and Rec1 MCL lines were kindly provided by M. Dyer (University of Leicester, Leicester, UK). All other lines were obtained from ATCC (Rockville, MD). Cells were maintained at 37 °C in 5% CO2. Ramos, Granta 519, NCEB1, Rec1, Jurkat and HEK293T cells were grown in RPMI 1640 medium with 10% FBS. K562 was grown in Iscoves, HeLa was grown in DMEM, and T24 and HCT116 lines were grown in McCoy’s 5 A Modified medium, all with 10% FBS added.

2.2. Suppression subtractive hybridization

cDNA was synthesized from one mantle cell lymphoma and one small lymphocytic lymphoma (SLL) using the SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA). Bidirectional SSH was performed using the PCR-Select cDNA Subtraction Kit (Clontech) and subtracted cDNA fragments were cloned into pCR2.1 using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced.

2.3. “Virtual” Northern analysis

cDNA was synthesized from lymphoma sample RNA using the SMART PCR cDNA Synthesis Kit. A 0.5 μg/sample cDNA was fractionated in a 1% agarose gel, denatured, neutralized, transferred and fixed to a Nytran nylon membrane (Schleicher & Schuell, Keene, NH). A Pir51 cDNA probe was labeled
with biotinylated CTP using the SpotLight Random Primer Labeling Kit (Clontech). Hybridization was performed with the SpotLight Chemiluminescent Hybridization Kit (Clontech).

2.4. Northern analysis

RNA was prepared from cell lines using the RNeasy Midiprep Kit (Qiagen, Carlsbad, CA). A 15 µg/sample RNA was fractionated in a 1% agarose, 0.67 M formaldehyde denaturing gel, transferred and fixed to Nytran nylon membranes. [α-32P]ATP (NE-NEN, Boston, MA) labeled Pir51 and GAPDH probes were generated using the Prime-It II Random Primer Labeling Kit (Stratagene, San Diego, CA). Hybridizations were performed in ExpressHyb solution (Clontech) followed by autoradiography.

2.5. Pir51 antiserum

A Pir51 cDNA was obtained by RT-PCR of Ramos B cell RNA using the SuperScript One-Step RT-PCR System (Invitrogen). The Pir51 cDNA was cloned into pCR2.1, sequenced, and then subcloned into the GST expression vector pGEX-6P (Amersham Biosciences, Piscataway, NJ). pGEX/Pir51 was transformed into One Shot BL21(DE3)pLysE cells (Invitrogen) and expression induced with 0.5 mM IPTG. Protein lysates were incubated with a 50% slurry of Glutathione Sepharose 4B (Pharmacia Biotech, Piscataway, NJ) and the GST tag was removed by digestion with PreScission Protease (Amersham Biosciences). Recovered Pir51 protein was dialyzed into 1 x PBS, pH 7.4, and two New Zealand white rabbits were immunized (Covance Research, Denver, PA).

2.6. Cell fractionation and Western analysis

Whole cell extracts were prepared from cell lines using a triple detergent lysis buffer (50 mM Tris pH8, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) with protease inhibitors. Nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). Proteins were separated by SDS-PAGE, transferred to NitroBind nitrocellulose membranes (Osmonics, Minnetonka, MN), and blocked with 5% non-fat milk in TBST (20 mM Tris pH7.4, 150 mM NaCl, 0.05% Tween-20). Primary and secondary antibody incubations were performed in 2% non-fat milk in TBST. Proteins were detected using the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA). Primary reagents included: rabbit anti-Pir51 antiserum (see above), rabbit anti-ERK1/2, rabbit anti-Bob1, rabbit anti-calnexin, rabbit anti-Cdk2, mouse anti-Cyclin-A, mouse anti-p53 and goat anti-Rad51 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Mouse anti-Rad51 (Oncogene Research Products, San Diego, CA) and mouse anti-HDAC1 (Upstate, Charlottesville, VA) antibodies were also used. Donkey anti-rabbit IgG-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA), goat anti-mouse IgG-HRP (Jackson) and rabbit anti-goat IgG-HRP (Sigma, St. Louis, MO) were used as the secondary antibodies.

2.7. Cell cycle analysis

T24 cells were arrested in G0 by contact inhibition on 150 mm plates, as described [18]. After 5 days of confluence, cells were placed on 100 mm plates at a concentration of 1 x 10^6 cells per dish. Sixteen plates were harvested at each time-point for FACS, Northern and Western analyses. For FACS, cells were fixed in cold 70% ethanol, resuspended in hypotonic DNA staining solution (3.4 mM sodium citrate, 0.3% Triton X-100, 0.15 mM propidium iodide, 325 units RNase A), and analyzed on a FACSscan using CellQuest v3.3 and ModFit LT v3.0 (Becton Dickinson, San Jose, CA). RNA was prepared from each time-point using the RNeasy Miniprep Kit (Qiagen). Whole cell extracts were prepared from each time-point and analyzed as described above.

2.8. DNA damage treatments

1 x 10^6 HCT116 cells were placed on 100 mm plates. Cells were incubated with 0.2 µg/ml doxorubicin (Sigma) for 24 h before harvest, or cells were exposed to 10 Gy from a 137Cs source and recovered for 24 h before harvest, or cells were exposed to 100 J/m² UV light in a Stratalinker (Stratagene) and recovered for 24 h before harvest. Whole cell extracts were prepared and proteins separated by SDS-PAGE for Western analyses. Also, HeLa cells were exposed to 2 mM hydroxyurea (Sigma) for 24 h, or exposed to MMC (Roche, Indianapolis, IN) dissolved in 1 x PBS, pH 7.4, for 24 h. Whole cell extracts were separated by SDS-PAGE for Western analyses.

2.9. siRNA

Oligonucleotides for human Pir51 (ggatggctttagatgacaagc) or oligos for human Rad51 (gcagtgatgtcctggataatg) were cloned into a siRNA expression vector driven by the mouse U6 promoter. For knockdown, cells were transfected with 1 µg of a siRNA expression or control vector and were selected with 2.5 µg/ml blasticidin for 24 h post-transfection, followed by washout and incubation for another 48 h.

2.10. Homologous recombination repair assay

Briefly, HeLa cells were transfected with the plasmid p59xDR-GFP6 [19] and selected for puromycin resistance. Cells were transiently transfected with the I-Sce1 expression construct and Pir51 siRNA expression or control vectors. Three days after transfection, cells were harvested and GFP positive cells determined by flow cytometry. Homologous recombination efficiency was determined by dividing the number of GFP positive cells resulting from dual exposure to siRNA and I-Sce1 by the number of GFP positive cells resulting from exposure to I-Sce1 alone.
2.11. Colony formation

Three days after siRNA transfection, HeLa cells were seeded at $1 \times 10^3$ cells per 100-mm dish for each MMC dose tested. After a 6-h incubation at 37 °C, cells were treated with graded concentrations (0–0.3 μM) of MMC for 1 h. The MMC-containing medium was then removed and replaced with 10 ml of complete DMEM, and cells incubated at 37 °C for colony formation. After 14 days, colonies were counted by staining with 0.05% Giemsa containing 50% methanol.

2.12. Analysis of chromosomal aberrations

Three days after siRNA transfection, log-phase HeLa cells were incubated in medium containing 50 nM MMC for 30 m. Colcemid was added to 0.05 μg/ml for the last 1-h before trypsinization. Cells were washed and resuspended in 75 mM KCl for 18 m at 37 °C. 12 ml of ice-cold 3:1 methanol:acetic acid was added dropwise to resuspend cell pellets, followed by incubation on ice for 20 m. The cells were centrifuged and resuspended in 300 μl of ice-cold 3:1 methanol:acetic acid solution. Cells were dropped onto glass slides to obtain metaphase chromosome spreads. Spreads were stained with DAPI.

3. Results

3.1. Preferential expression of Pir51 in aggressive MCLs

Bi-directional SSH was performed between an aggressive MCL and a small lymphocytic lymphoma to identify genes involved in aggressive human lymphoma. Results showed that Pir51 was more highly expressed in a MCL patient sample than a SLL sample (data not shown). Northern analyses for confirmatory and extension studies were not possible due to limited sample RNAs. Therefore, “virtual” Northern analyses, in which RNA was converted into cDNA and linearly amplified [20], was used to determine Pir51 expression levels in a panel of patient lymphoma samples. As shown in Fig. 1A, Pir51 was differentially expressed between the MCL and SLL samples that were analyzed by SSH (lanes 10 and 5). Overall, Pir51 exhibited low expression in only one MCL sample and moderate to high expression in four MCL samples. In contrast, Pir51 showed low expression in the SLL sample, and was undetectable in the remaining indolent lymphoma samples that included three follicular lymphoma (FL) and one marginal zone lymphoma (MZL) cases. These results indicate that Pir51 expression is preferentially increased in primary aggressive MCL.

3.2. Pir51 levels in distinct cancer cell types

Pir51 expression was evaluated in multiple transformed cell types to determine the breadth of its expression. Lines including Ramos Burkitt lymphoma, Granta 519, NCEB1 and Rec1 MCL, Jurkat acute T cell leukemia, K562 chronic myelogenous leukemia, HEK293T embryonal kidney and T24 bladder carcinoma lines expressed Pir51 at low to robust levels (Fig. 1B). Pir51 expression was also detected in B cell lines representing different stages of B cell development,
including Nalm-6, BLIN-1, 729,BL41, OCl-LY10, SU-DHL-6, AF10 and HS-Sultan (data not shown). To evaluate protein levels, a rabbit antiserum specific for Pir51 was developed that detected a 41.5 kDa recombinant protein band (Fig. 1C) that was 1.5 kDa larger than full length Pir51 due to a retained linker sequence. In whole cell extracts the antiserum detected a 40 kDa Pir51 protein in 9 of 10 cell lines tested, including HeLa cervical and HCT116 colon carcinoma cells (Fig. 1C and data not shown). Overall, Pir51 mRNA and protein expression is present in a variety of cell types, including multiple B, T and epithelial lines.

3.3. Nuclear localization of Pir51

We confirmed the association of Pir51 with Rad51 by endogenous co-immunoprecipitation from HeLa cells (Fig. 1D) [1]. The interaction of Pir51 with Rad51, a protein that traffics between the cytosol and the nucleus [1], suggests that Pir51 may also be present in both the nucleus and the cytoplasm. However, an exogenous murine Pir51-GFP fusion protein homologue, Rab22-GFP, localizes to the nucleus in CHO cells [7]. Inspection of the amino acid sequence revealed the presence of multiple consensus nuclear localization signals within Pir51 (Fig. 2A). Endogenous Pir51 was detected in nuclear extracts from Ramos and Granta 519 cells, albeit in lower quantities than in whole cell extracts from the same cells (Fig. 2B). Rad51 and Bob1, a B cell-specific nuclear protein [21], displayed reduced abundance in nuclear extracts (Fig. 2B) indicating that loss of nuclear material had occurred during extract preparation when compared to whole cell extracts. Calnexin [22] and ERK1/2 [23] are membrane and cytosolic proteins, respectively, that were absent from the nuclear fractions, as expected (Fig. 2B). To determine whether Pir51, like Rad51 [1], also existed in the cytoplasm, asynchronous HeLa cells were subjected to subcellular fractionation [24]. Pir51 was exclusively detected in the nuclear fraction while Rad51 was in both the cytosolic and nuclear fractions (Fig. 2C). Control calnexin and ERK2 proteins were primarily in the cytosolic fraction, as expected (Fig. 2C). These results indicate that Pir51 localizes to the nucleus and, unlike Rad51, does not exist in the cytoplasm at detectable concentrations.

3.4. Similar cell cycle regulation of Pir51 and Rad51 expression levels

Since Rad51 expression is cell cycle regulated, peaking during and after DNA replication [1], we determined whether Pir51 expression was similarly regulated. The T24 bladder carcinoma cell line was chosen for study (Fig. 1C), arrested in G0 by contact inhibition as described [18], and plated at low density for synchronized re-entry into the cell cycle. Cells were harvested at various times after plating and flow cytometry was used to determine the cell cycle distribution profile at each time-point (Fig. 2D). Pir51 transcripts were essentially undetectable in G0/G1 cells (Fig. 2D). As cells traversed the G1/S boundary ~12 h after release from cycle arrest, Pir51 transcription increased and remained high throughout S, G2 and M. The highest level of Pir51 expression was detected after 16 h, when approximately 50% of the cells were in S phase. This pattern of Pir51 expression was similar to Rad51, although Rad51 expression peaked a few hours earlier than the Pir51. As previously described, Cyclin-A transcripts were detectable in resting cells, but were detected throughout S, G2 and M [25] while GAPDH transcripts were detected at all times throughout the cell cycle [26] (Fig. 2D). Pir51, Rad51 and Cyclin-A [25] proteins followed the same expression patterns as their mRNAs but in each case appeared slightly later in the cell cycle (Fig. 2D). The cell cycle fluctuations of Pir51 mRNA and protein therefore follow the pattern of Rad51, but lag slightly behind.

3.5. Pir51 levels remain stable in response to DNA damage

DNA damage does not cause a change in Rad51 mRNA or protein levels [27-29]. UV light does not cause a change in the level of the Rad51-binding protein RPA [30] and UV light or γ-irradiation does not cause a change in c-Abl protein levels [31,32]. In contrast, the levels of the Rad51-binding protein BRCA1 increase with UV light [33], p53 levels increase with various forms of DNA damage [34], and BRCA2 protein levels decrease with MMC exposure [35]. To determine whether Pir51 protein expression is altered by DNA damage, HCT116, a human colon carcinoma line with wild type p53 [36], was exposed to multiple DNA damaging agents. Pir51 and Rad51 protein levels were unchanged in cells treated with doxorubicin, UV light and γ-irradiation, while p53 protein levels increased dramatically in each case, as expected [34] (Fig. 3A). Pir51 protein levels also did not change in HeLa cells exposed to UV light or MMC (Fig. 3B), but did increase upon exposure to hydroxyurea (Fig. 3B), similar to an increase in Pir51 expression seen for hydroxyurea treated mouse lymphoma cells [12]. We attributed the increase in Pir51 protein expression with hydroxyurea exposure to cells arresting in S phase, when Pir51 levels were highest (Fig. 2D). These results indicate that Pir51 protein
levels remain unchanged in response to a variety of DNA damaging agents.

3.6. Pir51 does not alter HRR

Rad51 plays a central role in HRR [37]. Direct measurements of DSB HRR using I-SceI nuclease-based assays reveal that deficiencies in Rad51-interacting proteins vary tremendously in their effects on HRR. Deficiencies in the Rad51 paralogs Xrcc2 and Xrcc3 produce severe (>25-fold) reductions in HRR [38,39]. BRCA2-deficient cells exhibit a 6–100-fold decrease, depending on the cell line [19], while BRCA1-deficient cells only reduce HRR five to six-fold [40] and disruption of ATM function reduces HRR approximately two-fold [41]. In contrast, p53-deficiency stimulates HRR [42,43]. To determine whether Pir51-deficiency alters the efficiency of HRR, we depleted Pir51 using siRNA.
in HeLa cells stably transfected with a reporter construct that expressed a functional GFP gene via the HRR of a DSB generated using I-SceI [19]. Pir51 protein levels were reduced with Pir51 siRNA in both native HeLa cells (Fig. 4A) and the HeLa cells containing the HRR GFP-reporter (Fig. 4B). In HeLa cells with a normal capacity for HRR, DSBs were repaired, resulting in GFP expressing cells that were readily identified using flow cytometry (Fig. 4C, top left panels; Fig. 4D). The increased number of GFP-positive cells contrasted sharply with the lack of GFP-expressing cells seen with vector control transfectants that lacked the DSB-inducing enzyme I-SceI (Fig. 4C, top left panels). siRNA directed against Pir51 had little or no effect on HRR (Fig. 4C, bottom left panels; Fig. 4D) and in this regard resembled control siRNA directed against histone deacetylase 1 (Fig. 4C, top right panels; Fig. 4D). In contrast, siRNA directed against Rad51 significantly reduced HRR, as expected [37] (Fig. 4C, bottom right panels; Fig. 4D).

3.7. Pir51 regulates MMC sensitivity and DNA crosslink repair

Although the repair of DNA crosslinks involves HRR proteins, deficiencies in a number of proteins that are critical for crosslink repair cause only modest reductions in HRR, including the FA proteins [44–46] and BRCA1 [40]. BRCA1 interacts with Rad51 [47] and the FA proteins FancD2 [48] and FancG [49] co-localize with Rad51 in foci during S phase and/or in response to DNA damage. Since BRCA1-deficient cells and FA cells display MMC sensitivity [47], we determined whether Pir51-deficient cells displayed increased sensitivity to MMC. HeLa cells exposed to siRNA against Pir51 were subjected to escalating doses of MMC. Pir51-deficient cells were significantly more sensitive to MMC and formed fewer colonies than control cells at all MMC concentrations that did not result in total cell death (Fig. 5A and B). Increased MMC sensitivity indicates that the repair of DNA crosslinks is compromised in Pir51-deficient cells.

MMC treatment of FA cells not only increases cell death but also increases the frequency of chromosome breaks in metaphase chromosome spreads [50]. This raised the question of whether MMC-treatment of Pir51-deficient cells also caused an increase in chromosome breaks. Treatment of HeLa cells with MMC alone, or with siRNA directed against Pir51 alone, did not increase the frequency of chromosome breaks in metaphase chromosome spreads over untreated cells (Fig. 6C). In contrast, exposure of Pir51 siRNA treated cells to MMC resulted in a significant increase in chromosome breaks per chromosome spread (Fig. 6B and C). Pir51 therefore protects cells against chromosome breakage induced by the DNA crosslinking agent MMC.

4. Discussion

We identified Pir51 based on its higher expression in aggressive versus indolent lymphoma patient sam-

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Fig. 3. Stable expression and localization of Pir51 with DNA damage. (A) Immunoblots of whole cell extracts of treated and untreated HCT116 cells. Conditions were 0.2 µg/µl doxorubicin treatment for 24 h (lanes 1 and 2), 100 J/m² UV light followed by 24 h recovery (lanes 3 and 4), or 10 Gy γ-irradiation followed by 24 h recovery (lanes 5 and 6). ERK1/2 served as a loading control. (B) Immunoblots from nuclear extracts of treated and untreated HeLa cells. Conditions were 2 mM hydroxyurea for 24 h (lane 2), 40 ng/ml MMC for 16 h (lane 3), 100 J/m² UV light (2 h recovery, lane 4) or 100 J/m² UV light (4 h recovery, lane 5). β-actin served as a loading control. Note that Pir51 protein expression increased only in response to hydroxyurea (lane 2).
Fig. 5. Pir51 deficiency sensitizes HeLa cells to MMC. (A) HeLa cell colony formation assay. Colony formation was reduced in cultures simultaneously treated with siRNA against Pir51 and MMC compared to individual treatments or no treatment. (B) Dose titration for MMC sensitivity of control and Pir51 siRNA transfected HeLa cells. Stable HeLa cell transfectants with Pir51 knockdown or a control, scrambled siRNA were treated with MMC at the indicated concentrations. Each treatment was done in triplicate and averages and standard deviations for three independent experiments are shown.

Pir51 is known for its interaction with Rad51 [6,7] but to date no function has been shown for this protein. Our strategy was to evaluate Pir51 expression patterns, localization and functions in comparison with Rad51 properties. Pir51 mRNA and protein were detected in multiple transformed cell lines with expression levels regulated during the cell cycle in a nearly identical pattern to Rad51, peaking in S phase. Also similar to Rad51, Pir51 protein levels remained unchanged following treatment of cells with a variety of DNA damaging agents, including doxorubicin, UV light, γ-irradiation and MMC. In contrast to Rad51, which traffics between the cytosol and nucleus [1,2] and plays a central role in HRR [37], Pir51 protein was restricted to the nucleus. Pir51-deficiency also did not impact HRR. However, reduction in Pir51 protein levels using siRNA rendered HeLa cells hypersensitive to MMC and led to reduced colony formation and an increase in MMC-induced gaps or breaks in metaphase chromosome spreads.

Pir51 is the first Rad51-binding protein to be identified that influences DNA crosslink repair but not HRR. This finding suggests that Pir51 functions in the early phase of DNA repair that involves the ATR and FA proteins and, from its interaction with Rad51, may provide a crucial link to resolution stages of Rad51-mediated HRR. Although the reduction of Pir51 by siRNA had no significant effect on HRR, we cannot exclude the possibility that Pir51-null cells will display a mild HRR defect reminiscent of cells that are null for one of the FA proteins [44–46]. The only FA protein whose loss has a strong effect on HRR repair is BRCA2/FancD1 [19], which appears to have a role in HRR that is independent of the upstream functions of other FA proteins in the crosslink repair pathway [51]. BRCA1 [48] and ATR [52] also have additional functions that are independent of this DNA repair pathway.

Our findings strongly suggest that Pir51 mRNA and protein levels are most directly related to the proliferation activity of cells. Pir51 was more highly expressed in an aggressive human lymphoma, MCL, than in multiple indolent lymphoma subtypes, including FL, MZL and SLL. Many studies have shown that MCL has increased expression of the Ki-67 cell cycle marker compared to FL, MZL and SLL, indicating that MCL tumors have...
a higher proliferative activity than FL, MZL and SLL tumors [53]. We determined that Pir51 was most highly expressed in the late G1, S and early G2 phases of the cell cycle and down regulated in cells arrested in G0 by contact inhibition. We also did not detect Pir51 expression in nuclear extracts derived from bulk normal human spleen in which the vast majority of cells are non-cycling (data not shown), further suggesting that Pir51 expression is low or absent in normal cells with low proliferation. These findings are consistent with a report that Pir51 [13] joins Rad51 [14,17] and a number of Rad51-associated proteins including RPA [16,17], BRCA1 [16,17], FancA [15] and Rad54 [17] in having their expression controlled by the transcription factor E2F1 that is thought to integrate the cell cycle with DNA repair. The increased expression of Pir51 in cycling, aggressive hepatocellular carcinomas [8] and MCL, and peaked protein expression in cells with replicating and replicated DNA, strongly suggest a role for Pir51 in correcting DNA damage by non-HRR mechanisms in multiple cell lineages.

It may seem paradoxical that Pir51, which we have shown functions in protecting the genome, displays elevated expression in aggressive cancer cells with DNA damage and genome instability. One possible resolution to this paradox is that transforming mutations overwhelm non-cycling cells because their genome defense is limited by a low abundance of Pir51 and other genome maintenance proteins. In this scenario, the activation of Pir51 expression may help to suppress further DNA damage in the non-cycling tumor cells and counteract therapeutic strategies aimed at increasing DNA damage to cause cell death. Another possible resolution is that both over-expression and under-expression of Pir51 promotes genome instability as has been reported for Rad51 [3]. For example, high expression of Pir51 may disrupt DNA repair complexes that normally do not include Pir51 but contain protein(s) that bind Pir51 in other repair complex(es). Either way, reduced Pir51 expression in aggressive prostate cancer cell lines from aberrant promoter DNA methylation [10] should mimic our observa-

Fig. 6. MMC-treated HeLa cells display increased chromosomal fragility with reduced Pir51. (A) Metaphase chromosome spread of control HeLa cells. Chromosome gaps and breaks were not detected in this untreated HeLa cell. (B) Metaphase chromosome spread of HeLa cells stably transfected with Pir51 siRNA and treated with MMC. A break or gap in one sister chromatid (arrow) in Pir51 knockdown HeLa cells exposed to 50 nM MMC for 1 h is shown. (C) Proportion of metaphase chromosome spreads displaying chromosome breaks or gaps in HeLa cells that were untreated, treated with 50 nM MMC for 1 h or siRNA against Pir51 alone, or simultaneously subjected to the MMC and Pir51 siRNA treatments.
tions with siRNA and impair crosslink repair and genome stability.

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