ARTICLE
Journal of Cellular Biochemistry 117:671–683 (2016)

Journal of Cellular Biochemistry

Nuclear α Spectrin Differentially Affects Monoubiquitinated Versus Non-Ubiquitinated FANCD2 Function After DNA Interstrand Cross-Link Damage

Pan Zhang, Deepa Sridharan, and Muriel W. Lambert*

Department of Pathology and Laboratory Medicine, Rutgers New Jersey Medical School, 185 South Orange Avenue, Newark, New Jersey 07103, USA

ABSTRACT

Nonerythroid α spectrin (αIISp) and the Fanconi anemia (FA) protein, FANCD2, play critical roles in DNA interstrand cross-link (ICL) repair during S phase. Both are needed for recruitment of repair proteins, such as XPF, to sites of damage and repair of ICLs. However, the relationship between them in ICL repair and whether αIISp is involved in FANCD2′s function in repair is unclear. The present studies show that, after ICL formation, FANCD2 disassociates from αIISp and localizes, before αIISp, at sites of damage in nuclear foci. αIISp and FANCD2 foci do not co-localize, in contrast to our previous finding that αIISp and the ICL repair protein, XPF, co-localize and follow a similar time course for formation. Knock-down of αIISp has no effect on monoubiquitination of FANCD2 (FANCD2-Ub) or its localization to chromatin or foci, though it leads to decreased ICL repair. Studies using cells from FA patients, defective in ICL repair and αIISp, have elucidated an important role for αIISp in the function of non-Ub FANCD2. In FA complementation group A (FA-A) cells, in which FANCD2 is not monoubiquitinated and does not form damage-induced foci, we demonstrate that restoration of αIISp levels to normal, by knocking down the protease μ-calpain, leads to formation of non-Ub FANCD2 foci after ICL damage. Since restoration of αIISp levels in FA-A cells restores DNA repair and cell survival, we propose that αIISp is critical for recruitment of non-Ub FANCD2 to sites of damage, which has an important role in the repair response and ICL repair. J. Cell. Biochem. 117: 671–683, 2016.

KEY WORDS: NONERYTHROID ALPHA SPECTRIN; NUCLEAR SPECTRIN; FANCD2; NON-UBIQUITINATED-FANCD2; DNA REPAIR; DNA DAMAGE; DNA INTERSTRAND CROSS-LINKS; XPF; FANCONI ANEMIA

Repair of DNA ICLs is a complex process. The primary mechanism occurs in cells in S phase when a replication fork encounters an ICL, while a secondary mechanism acts outside of S phase [Akkari et al., 2000; Raschle et al., 2008; Knipscheer et al., 2009; Legerski, 2010]. Both nonerythroid α spectrin (αIISp) and the FA protein, FANCD2, have been shown to be important in ICL repair during S phase [Knipscheer et al., 2009; Moldovan and D′Andrea, 2009; Thompson and Hinz, 2009; Sareen et al., 2012; Zhang et al., 2013]. A key event in repair of DNA ICLs at replication forks stalled in S phase of the cell cycle is the monoubiquitination of FANCD2 by a core complex of eight FA proteins [Wang, 2007; de Winter and Joenje, 2009; Moldovan and D′Andrea, 2009; Walden and Deans, 2014]. This is followed by dual incisions on either side of the ICL that unhook the ICL from one DNA strand; lesion bypass then takes place and fully repaired DNA is generated by homologous recombination mediated repair [Raschle et al., 2008; Legerski, 2010; Zhang and Walter, 2014; Klein Douwel et al., 2014]. We have shown that αIISp also plays a critical role in the ICL repair process where it localizes to sites of damage in nuclear foci specifically in S phase [Sridharan et al., 2003; McMahon et al., 2009; Zhang et al., 2010; Zhang et al., 2013; Lambert, 2015]. αIISp binds directly to DNA containing ICLs and is involved in the incision steps of the repair process where it is needed for recruitment of repair proteins, such as XPF (FANCQ), to sites of damage [McMahon et al., 2001; Lambert, 2015]. Loss of αIISp leads to cellular hypersensitivity to DNA ICL agents, decreased cell survival and increased chromosomal aberrations after ICL exposure [McMahon et al., 2009; Zhang et al., 2010].

We have demonstrated that there is a deficiency in αIISp in cells from patients with the inherited bone marrow failure disorder, FA,

Present address of Pan Zhang is Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, Cornell University, 1300 York Avenue, New York, NY 10065.
Present address of Deepa Sridharan is Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720.
Grant sponsor: NHLBI; Grant sponsor: National Institutes of Health; Grant number: RO1 HL054860.
*Correspondence to: Muriel W. Lambert, PhD, Department of Pathology and Laboratory Medicine, Rutgers New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103.
E-mail: mlambert@njms.rutgers.edu
Manuscript Received: 18 August 2015; Manuscript Accepted: 19 August 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 22 August 2015
DOI 10.1002/jcb.25352 • © 2015 Wiley Periodicals, Inc.

which is characterized by congenital abnormalities, genomic instability and an increased predisposition for development of cancer [McMahon et al., 1999; Sridharan et al., 2003; Mathew, 2006; de Winter and Joenje, 2009; Moldovan and D′Andrea, 2009]. Cells from FA patients have a marked hypersensitivity to DNA interstrand cross-linking agents and a defect in ability to repair DNA ICLs produced by these agents [Kumaresan and Lambert, 2000; Kumaresan et al., 2007; de Winter and Joenje, 2009; Kim and D′Andrea, 2012; Thompson and Hinz, 2009]. The deficiency of αIISp in FA cells correlates with increased sensitivity to ICL agents and decreased levels of DNA repair and is due to decreased stability or increased breakdown of αIISp in these cells [Kumaresan et al., 2007; Zhang et al., 2010; Lambert, 2015]. We propose that FA proteins play an important role in maintaining αIISp stability in the cell and that a deficiency of these proteins in FA cells leads to the loss of αIISp observed [Lefferts and Lambert, 2003; Lefferts et al., 2009; Zhang et al., 2010; Lambert, 2015]. Since αIISp is essential for recruitment of repair proteins such as XPF to sites of ICLs and for production of the incisions produced by XPF at these sites, we have proposed a model for the role of αIISp in ICL repair: αIISp binds to DNA at sites of ICLs and acts as a scaffold for recruitment of repair proteins (i.e., XPF) to sites of damage, enhancing the repair process. A deficiency in αIISp, such as occurs in FA cells, leads to loss of recruitment of repair proteins to damage sites and loss of DNA repair [McMahon et al., 2001; Lefferts et al., 2009; Zhang et al., 2010; Lambert, 2015]. Though both αIISp and ubiquitylation of FANCD2 are needed for the recruitment of XPF to sites of damage and the repair of ICLs, the relationship between αIISp and FANCD2 in this process is unclear [Sridharan et al., 2003; Moldovan and D′Andrea, 2009; Thompson and Hinz, 2009;]. It has recently been shown, using *Xenobus* egg extracts, that XPF acts downstream of FANCD2 producing incisions at sites of ICLs, and that XPF-ERCC1 does not affect the monoubiquitination of FANCD2 or its loading onto ICL damaged DNA [Klein Douwel et al., 2014]. Since αIISp is needed for recruitment of XPF to sites of ICLs, co-localizes with it at these sites and follows a similar time course for localization to these sites, it is important to determine whether αIISp also interacts with FANCD2 and is important in its function in the repair process [Sridharan et al., 2003; Zhang et al., 2013; Lambert, 2015].

The present studies were thus undertaken to determine the relationship between αIISp and FANCD2 in the repair process and whether αIISp interacts with FANCD2 after ICL damage and is important in its monoubiquitination and localization to chromatin and sites of damage. The results demonstrate that in normal human cells FANCD2 associates with αIISp but that, after ICL damage, FANCD2 dissociates from αIISp and does not co-localize with it in nuclear foci. Formation of FANCD2 and αIISp foci follow a different time course for formation, with FANCD2 foci forming before αIISp foci, suggesting that αIISp acts downstream of FANCD2, similar to XPF. Knock-down of αIISp has no effect on monoubiquitination of FANCD2 or its localization to chromatin or nuclear foci after ICL damage, indicating that αIISp, like XPF, is not needed for the functioning of FANCD2-Ub. However, our studies using FA-A cells have helped elucidate an important role for αIISp in the functioning of non-ubiquitinated FANCD2 (non-Ub FANCD2). In FA-A cells, FANCD2 is present but not monoubiquitinated and does not form nuclear foci in response to ICL damage [Garcia-Higuera et al., 2001, Moldovan and D′Andrea, 2009]. Our present results demonstrate, however, that restoration of αIISp levels to normal in FA-A cells, by knocking down μ-calpain, a protease which cleaves αIISp, leads to formation of non-Ub FANCD2 nuclear foci after ICL damage. This shows that αIISp is needed for recruitment of non-Ub FANCD2 to sites of damage. Since studies indicate that non-Ub FANCD2 can promote efficient replication fork restart at replication forks that are blocked or that break upon encountering DNA damage [Wilson et al., 2008, 2010; Raghunandan et al., 2015], it is possible that recruitment of non-Ub FANCD2 to sites of damage is an important factor in the repair response and in correction of the repair defect in FA-A cells when levels of αIISp are restored to normal. Our studies thus suggest that αIISp, like XPF, acts downstream of Ub-FANCD2 and is not required for its monoubiquitination or localization to sites of damage. αIISp, however, is needed in recruitment of non-Ub FANCD2 to damage sites, which in turn may be important in the damage response, just as it is needed for recruitment of XPF to these sites [Sridharan et al., 2003; Zhang et al., 2010].

MATERIALS AND METHODS

CELL CULTURE

Normal human lymphoblastoid cells (GM3299) were obtained from the Coriell Institute for Medical Research (Camden, NJ). FA complementation group A (FA-A) cells (HSC 72) were a gift from Dr. Manuel Buchwald, Hospital for Sick Children, Toronto, Canada). Lymphoblastoid cells were grown in RPMI 1640 medium (Hyclone, Thermo Scientific) as previously described [McMahon et al., 2009; Zhang et al., 2010].

PROTEIN EXTRACTION AND CELL FRACTIONATION

Whole cell and nuclear extracts of normal and FA-A cells were prepared as previously described [McMahon et al., 2009; Zhang et al., 2010]. For analysis of localization of monoubiquitinated FANCD2 to chromatin, cells were fractionated and soluble nuclear and chromatin fractions isolated using a subcellular protein fractionation kit (Pierce Biotech) following the manufacturer′s protocol.

DEPLETION OF αIISP AND μ-CALPAIN BY SIRNA

siRNA against αIISp nucleotides (AAGAUUCCUAUCGAUUCCA-GUUU) and μ-calpain nucleotides (GUGAAGGAGUUGCGGACAA) were purchased from Dharmacon and a control non-target siRNA from Qiagen. FA-A cells and normal cells were transfected with μ-calpain siRNA (300 pmol), αIISp siRNA (600 pmol), or non-target siRNA using Lipofectamine 2000 Transfection Reagent (Invitrogen, Inc.) as previously described [McMahon et al., 2009; Zhang et al., 2010]. Cells were harvested at 0, 24, 48, 72, and 96 h after transfection and whole cell protein lysates prepared.

TREATMENT OF CELLS WITH DNA INTERSTRAND CROSS-LINKING AGENTS

Normal and FA-A cells in culture were treated with 3.5 μM 8-methoxypsoralen (8-MOP) (Sigma–Aldrich, Corp) plus two doses of UVA light (principally 366 nm) as previously described [Lambert

et al., 1992, 1997; Sridharan et al., 2003]. Normal and FA-A cells were also treated with 400 nM mitomycin C (MMC) (Sigma–Aldrich, Corp) [McMahon et al., 2009; Zhang et al., 2010]. Cells were collected and/or examined at various times after treatment. Normal and FA-A cells transfected with either μ-calpain siRNA, αIISp siRNA or non-target (Nt) siRNA were similarly treated with 400 nM MMC 24 h after transfection. Cells were collected and/or examined at various time points after treatment.

CO-IMMUNOPRECIPITATION OF PROTEINS

For examination of co-immunoprecipitation of FANCD2 with αIISp, nuclear extracts from normal cells were prepared and FANCD2 co-immunoprecipitated with anti-αIISp antibody or anti-mouse IgG. For this, anti-αIISp antibody (Chemicon), or mouse IgG (Sigma–Aldrich, Corp.) was bound to protein G-coated agarose beads (Sigma–Aldrich, Corp.) and immunoprecipitations carried out as previously described [McMahon et al., 1999; Sridharan et al., 2003, 2006]. For co-immunoprecipitation of αIISp with FANCD2, anti-FANCD2 antibody (custom made, Bethyl Laboratories, or Novus Biologicals) or rabbit IgG (Sigma–Aldrich, Corp.) was bound to protein A-coated agarose beads (Sigma–Aldrich, Corp.) and the binding reactions and immunoprecipitations were carried out as previously described [McMahon et al., 1999; Sridharan et al., 2003, 2006]. The immunoprecipitates were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted as previously described [McMahon et al., 1999; Sridharan et al., 2003, 2006].

IMMUNOBLOT ANALYSIS

Western blot analysis was carried out as previously described [McMahon et al., 1999; Sridharan et al., 2003, 2006]. Immunoblots were probed with anti-FANCD2 (Bethyl Laboratories; Novus Biologicals), anti α-spectrin, anti-tubulin (Santa Cruz Biotechnology), or anti-ORC2 antibody (Santa Cruz Biotechnology). Immunoblots were developed using Pierce Ultra chemiluminescent substrate (Pierce, Thermo Scientific) and then exposed to X-ray film [McMahon et al., 1999; Sridharan et al., 2003, 2006]. Images were scanned using a Hewlett-Packard ScanJet 4c/T scanner and analyzed with ImageQuant (Molecular Dynamics). Determination of FANCD2-Ub and non-Ub FANCD2 on western blots was based on their migration as two distinct bands of different molecular weight upon gel electrophoresis.

INDIRECT IMMUNOFLUORESCENCE

Normal and FA-A cells treated with 8-MOP plus UVA light were harvested at various times post damage and examined for nuclear localization of FANCD2, aIISp, and γH2AX foci using indirect immunofluorescence as previously described [Sridharan et al., 2003; McMahon et al., 2009; Zhang et al., 2010]. Normal and FA-A cells either not transfected or transfected with Nt siRNA, aIISp siRNA, or μ-calpain siRNA were treated with MMC and harvested 16 h post MMC and examined for nuclear localization of FANCD2 and aIISp foci using indirect immunofluorescence [Sridharan et al., 2003; McMahon et al., 2009; Zhang et al., 2010]. Primary antibodies used were anti-α-spectrin (Chemicon), or anti-FANCD2 (Novus Biologicals). Secondary antibodies were Alexafluor 488 goat anti-mouse IgG conjugate or Alexafluor 594 goat anti-rabbit IgG conjugate (Molecular Probes, Invitrogen). Cells were viewed with a Leitz DMRB microscope (Leica) and images captured using a DEI-750 analog camera or with a Zeiss AXIOVERT 200 M (Carl Zeiss international) microscope. Images were imported into a computerized imaging system using Image Pro-Plus 6.0 or AxioVision software and the number of nuclei containing multiple foci counted. Cells containing five or greater foci were counted as positive. The number of nuclear foci per cell was also quantitated as previously described [Sridharan et al., 2003; Zhang et al., 2010]. Three hundred cells were counted in each group for each experiment. Each experiment was independently repeated 3–5 times.

RESULTS

AFTER DNA ICL DAMAGE, FANCD2 FOCI FORM BEFORE THOSE OF αIISP AND XPF AND FOLLOW A DIFFERENT TIME COURSE

We have previously shown that in normal human cells αIISp localizes to nuclear foci after DNA ICL damage (8-MOP plus UVA light, or MMC) and is involved in repair of DNA ICLs [Sridharan et al., 2003; McMahon et al., 2009; Zhang et al., 2010]. FANCD2 has similarly been shown to form nuclear foci after ICL damage and to be critical for DNA ICL repair [Garcia-Higuera et al., 2001; Taniguchi and D′Andrea, 2006]. The important question we now asked is whether αIISp and FANCD2 localize to sites of damage in a similar or different time course. The results showed that FANCD2 foci formed earlier than αIISp foci, forming at 2 h after damage with 8-MOP plus UVA light (Fig. 1). Foci formation increased at 8 h after damage, plateaued at 16 h, and remained elevated until 48 h post treatment after which they decreased but were still present at 72 h. In contrast, formation of αIISp foci was not observed until 10 h after ICL damage, peaked at 16 h, and was gone by 24 h after damage (Fig. 1). Since FANCD2 is monoubiquitinated after ICL damage [Garcia-Higuera et al., 2001; Taniguchi and D′Andrea, 2006; de Winter and Joenje, 2009; Moldovan and D′Andrea, 2009], levels of FANCD2-Ub after damage were examined and found to follow the same time course as that for formation of FANCD2 nuclear foci (Supplementary Figure S1). Non-ubiquitinated FANCD2 was present during the entire time course. The ratio of FANCD2-Ub:FANCD2 was 0.1, 0.3, 0.8, 1.0, and 0.47 at 2, 8, 16, 24 and 72 h after damage, respectively (Supplementary Figure S1).

Since γH2AX has been shown to be needed for proper accumulation of FANCD2 at sites of damage [Bogliolo et al., 2007], the time course for formation of γH2AX foci after ICL damage was also examined. The time course for γH2AX foci was found to be similar to that for FANCD2 except that the γH2AX foci remained elevated through 72 h post treatment (Fig. 1). Thus the time courses for the formation of FANCD2 and γH2AX foci are similar to each other but are different from that of αIISp foci.

In contrast, we have previously shown that XPF localizes to sites of damage over the same time course as αIISp, with foci appearing at 10 h after damage and disappearing by 24 h after ICL damage (Fig. 1) [Sridharan et al., 2003]. Since formation of both αIISp and XPX foci occurs after formation of FANCD2 foci, this suggests that FANCD2 loads onto sites of damage upstream of these proteins. The results of these studies are similar to those in which *Xenopus* egg extracts were

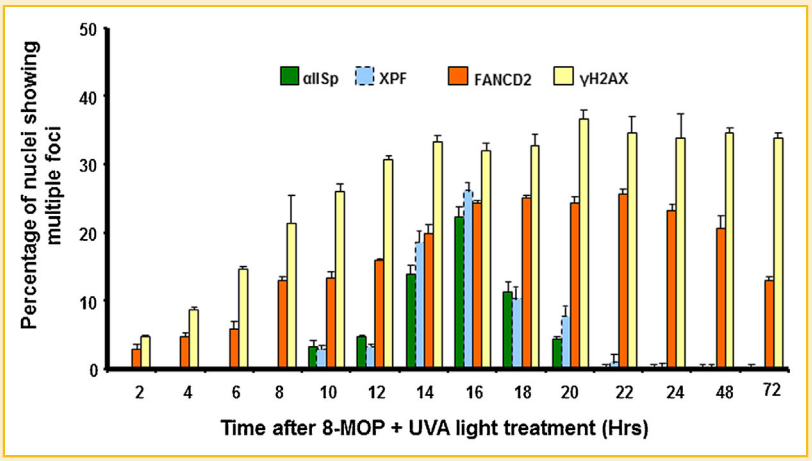

Fig. 1. Time course for appearance of αIISp, XPF, FANCD2, and γH2AX nuclear foci after DNA ICL damage. Normal human cells were treated with 8-MOP plus UVA light and the percentage of nuclei showing multiple αIISp, XPF, FANCD2, and γ H2AX foci determined at the indicated times post treatment. Notation of XPF foci is from Sridharan et al. [2003]. Nuclei containing four or more foci were counted as positive. Nuclear foci for 100 cells were counted for each time point after treatment. These experiments were repeated 3 times. Error bars represent SEM.

used and which indicated that FANCD2 loads on to sites of ICLs upstream of XPF-ERCC1 [Klein Douwel et al., 2014]. Thus, though both αIISp and FANCD2-Ub are critical for recruitment of XPF to sites of ICL damage and for the repair process, they associate with these sites over a different time course.

FANCD2 DISSOCIATES FROM αIISP AFTER DNA ICL DAMAGE AND DOES NOT CO-LOCALIZE WITH IT IN NUCLEAR FOCI

In order to determine whether αIISp and FANCD2 interact with each other in the nucleus in damaged and undamaged normal cells, co-immunoprecipitations (IP) were carried out using nuclear extracts from normal cells. Anti-FANCD2 IP and immunoblotting with anti-αIISp antibody demonstrated, that in undamaged normal cells, αIISp co-immunoprecipitated with non-Ub FANCD2 and the low levels of FANCD2-Ub present in undamaged cells (Fig. 2A). Similarly, anti-αIISp IP and immunoblotting with anti-FANCD2 antibody demonstrated that mainly non-Ub FANCD2 and low levels of FANCD2-Ub co-immunoprecipitated with αIISp in these cells (Fig. 2B). Thus, FANCD2 associates with αIISp in undamaged cells. However, after the cells were damaged with 8-MOP plus UVA light, FANCD2 dissociated from αIISp. This is demonstrated in co-immunoprecipitation studies using anti-FANCD2 antibody which showed that, after ICL damage, αIISp did not co-immunoprecipitate with either non-Ub FANCD2 or FANCD2-Ub (Fig. 2A). Similarly, studies using anti-αIISp IP, demonstrated that after ICL damage neither non-Ub FANCD2 nor FANCD2-Ub co-immunoprecipitated with αIISp (Fig. 2B). These results thus demonstrate FANCD2 associates with αIISp in undamaged cells, but that after ICL damage, it dissociates from αIISp.

Studies were carried out to determine whether αIISp foci co-localized with FANCD2 foci after DNA ICL damage. The results showed that 2 h after treatment of the cells with MMC, FANCD2 foci were observed; however, only a few αIISp foci formed in the nuclei of these cells at this time period (Fig. 3A). The staining for FANCD2 and αIISp overlaid the fluorescent signal for DAPI, which showed that these areas were in the nucleus. The average number of FANCD2 foci per nucleus was 29 compared to 4 for αIISp (Fig. 3B). The percentage of nuclei showing FANCD2 foci at this time point was 25% compared to only 5% of the nuclei showing αIISp foci (Supplementary Figure S2). By 16 h post damage, αIISp had formed foci in the nucleus (Fig. 3A). At this point, the average number of FANCD2 and αIISp foci (36 and 34, respectively) was

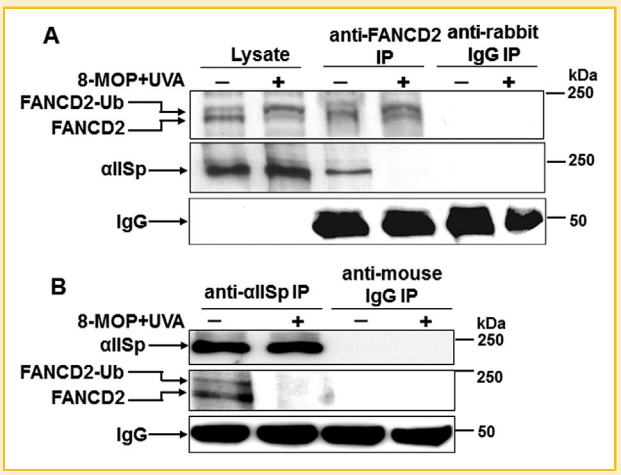

Fig. 2. After DNA ICL damage, FANCD2 dissociates from αIISp. Normal cells were either undamaged or treated with 8-MOP plus UVA light. Nuclear extracts were prepared from the cells 15 h after treatment. Co-immunoprecipitations were carried out using (A) anti-FANCD2 antibody or (B) anti-αIISp antibody and the interaction of αIISp and FANCD2 examined by western blot analysis. FANCD2-Ub is the monoubiquitinated form and FANCD2 is the non-monoubiquitinated form. The IgG_1 heavy chain was used as a loading control. Molecular weight markers are indicated on the right.

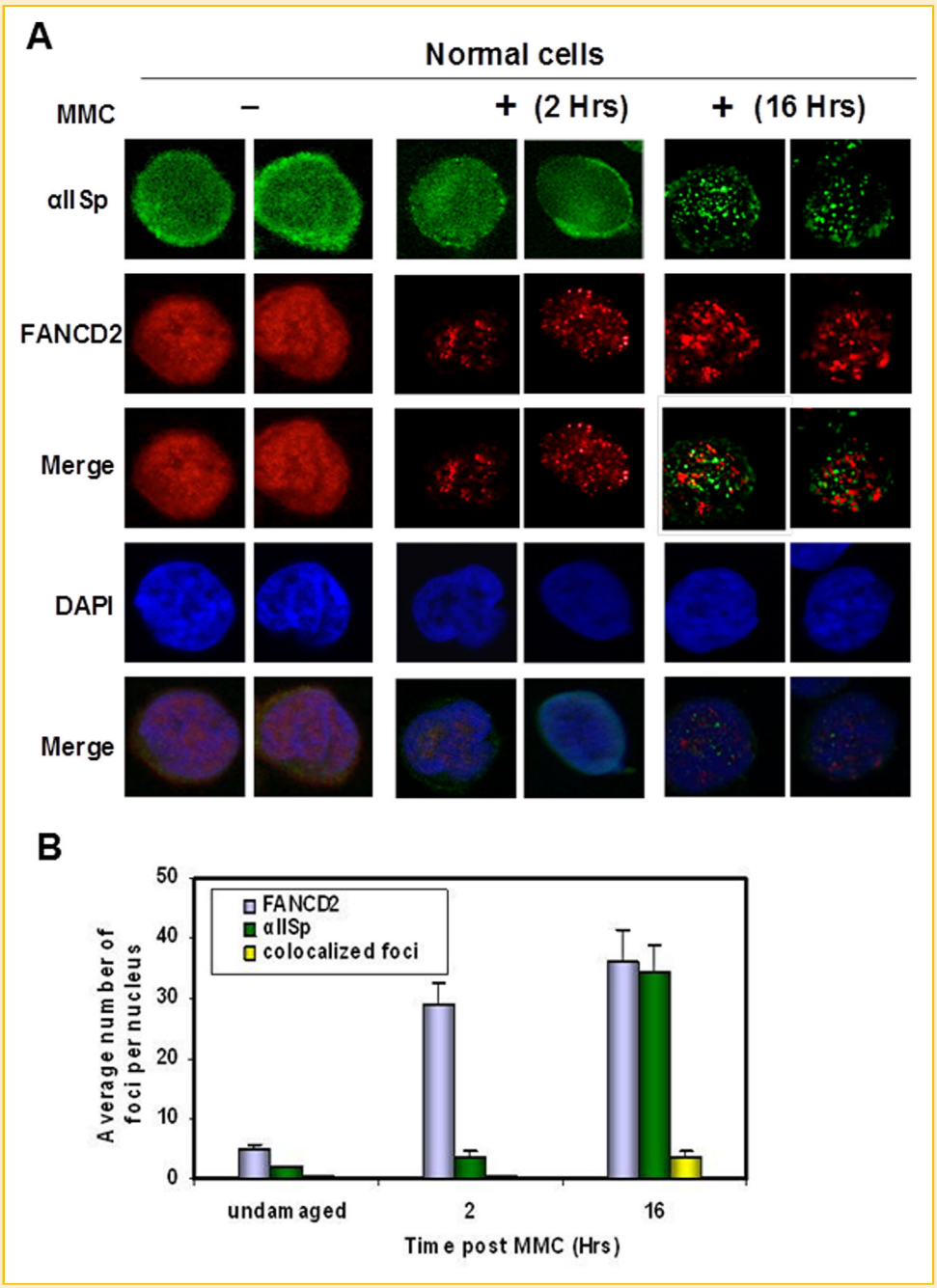

Fig. 3. After DNA ICL damage, FANCD2 nuclear foci form in normal cells before formation of αIISp foci and do not co-localize with αIISp foci. Normal cells were untreated or treated with MMC (400 nM). A: Formation of FANCD2 and αIISp nuclear foci was examined 2 and 16 h after treatment using indirect immunofluorescence and staining with anti-FANCD2 or anti-αIISp. Cells were also counter stained for the DNA-specific DAPI. The images were merged to examine co-localization of FANCD2 and αIISp foci. The images were also merged with the DAPI stained nuclei to show that these foci were present in the nucleus. B: The average number of FANCD2 and αIISp foci per nucleus and the average number of FANCD2 and αIISp nuclear foci co-localizing were quantitated in cells 2 and 16 h after MMC treatment. Nuclear foci were counted for 300 cells for each time point after exposure to MMC. Each of these experiments was repeated 5 times. Error bars represent SEM.

similar (Fig. 3B) as was the percentage of nuclei showing FANCD2 (52%) and αIISp (47%) foci (Supplementary Figure S2). FANCD2 foci, however, did not co-localize to any extent with αIISp foci at any time point after damage with MMC as is shown in Figure 3A and B. At 16 h after damage, the average number of FANCD2 and αIISp foci co-localizing per nucleus was only 2.5% (Fig. 3B). These studies were carried out using two different sources of FANCD2 antibody with similar results. These results demonstrate that FANCD2 and αIISp do not co-localize after DNA ICL damage and are recruited to different nuclear foci.

αIISP IS NOT REQUIRED FOR THE MONOUBIQUITINATION OF FANCD2 OR ITS LOCALIZATION TO CHROMATIN OR TO NUCLEAR FOCI AFTER ICL DAMAGE

In order to determine whether αIISp is important for the monoubiquitination of FANCD2 and for its localization to chromatin and nuclear foci after ICL damage, αIISp was knocked down by siRNA to levels that were approximately 35–40% of those of normal cells, since these are the levels of αIISp present in FA cells (Fig. 4A). In undamaged normal cells, knockdown of αIISp had no effect on the levels of non-Ub FANCD2 (Fig. 4A). The levels of non-Ub FANCD2 were similar to those in the nontarget (Nt) siRNA transfected cells (Fig. 4A). The effect of knockdown of αIISp on formation of FANCD2-Ub after ICL damage was then examined. The results showed that, in cells transfected with αIISp siRNA, the ratio of FANCD2-Ub to non-Ub FANCD2, over a time course of 2–72 h after MMC damage (Fig. 4B), was similar to that found for cells transfected with Nt siRNA and treated with MMC (Fig. 4C) and for untransfected normal cells treated with MMC (Supplementary Figure S1). At 16 h, for example, when the percentage of nuclei showing multiple foci had plateaued, the ratio of FANCD2-Ub to non-Ub FANCD2 was 0.9. Thus knocking down expression of αIISp had no effect on the monoubiquitination of FANCD2 after ICL damage.

FANCD2-Ub has been shown to localize to chromatin after DNA ICL damage [de Oca et al., 2000; Wang et al., 2004; Moldovan and D'Andrea, 2009]. Whether knocking down αIISp had any effect on the localization of FANCD2 to chromatin or to nuclear foci after ICL damage was also examined. After αIISp knockdown and 16 h post MMC treatment, cells were examined for localization of FANCD2-Ub to chromatin. The results showed that FANCD2-Ub localized to the chromatin in cells transfected with αIISp siRNA and treated with MMC just as in MMC treated cells transfected with Nt siRNA (Fig. 5). It was not found in the soluble nuclear extract. Non-Ub FANCD2 was also found in the chromatin extract of cells transfected with αIISp or Nt siRNA (Fig. 5). These results indicate that knocking down αIISp had no effect on the localization of FANCD2-Ub or non-Ub FANCD2 to chromatin in cells after ICL damage.

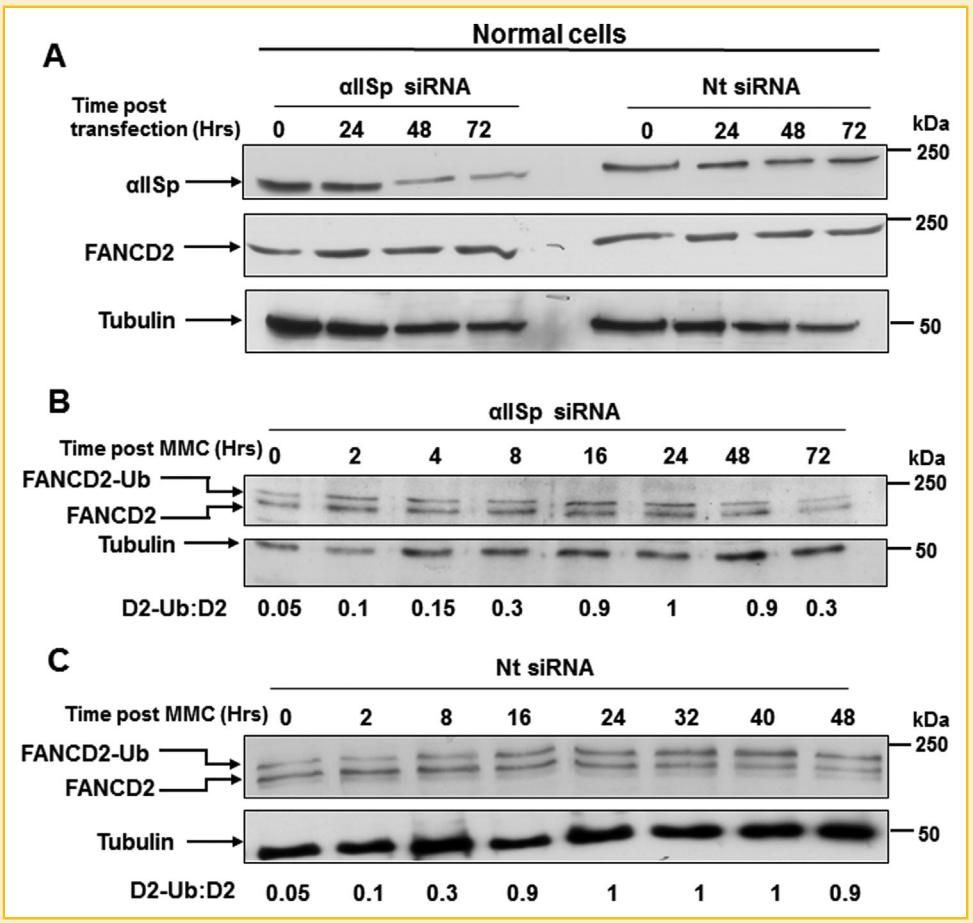

Fig. 4. In normal cells, knockdown of αIISp has no effect on monoubiquitination of FANCD2 after ICL damage. A: Normal human cells were transfected with either nontarget (Nt) siRNA or αIISp siRNA. Cells were not damaged. Whole cell extracts from these undamaged cells were collected at the indicated time points after transfection. Levels of αIISp and FANCD2 were examined by western blotting. B: Normal human cells were transfected with αIISp siRNA and then 24 h. after transfection they were damaged with MMC (400 nM). Monoubiquitination of FANCD2 was examined at the indicated time points after exposure to MMC. Ratios of FANCD2-Ub to FANCD2 (D2-Ub:D2) at each time point are indicated and are the average of three separate experiments. C: Normal human cells were transfected with Nt siRNA and then damaged with MMC (400 nm). Monoubiquitination of FANCD2 was examined at the indicated time points after exposure to MMC. Notations are as above. Tubulin was used as a loading control. Molecular weight markers are indicated on the right.

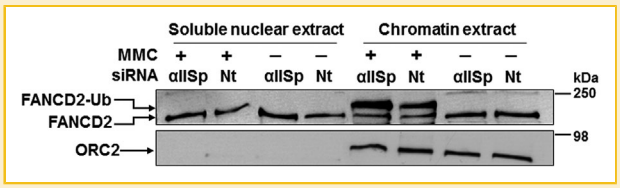

Fig. 5. In normal cells, knocking down αIISp had no effect on the localization of FANCD2-Ub to the chromatin after ICL damage. Normal human cells were transfected with either Nt siRNA or αIISp siRNA and then treated with of MMC (400 nM) for 16 h. Cells were fractioned into the soluble nuclear extract and chromatin extract. Levels of FANCD2-Ub were examined by western blotting. Immunoblots were probed with anti-FANCD2 antibody to detect both FANCD2-Ub and non-Ub FANCD2. ORC2 was used as a loading control for the chromatin extracts. Molecular weight markers are indicated on the right.

The effects of knocking down αIISp expression on formation of FANCD2 nuclear foci was also examined. Sixteen hours after MMC treatment, FANCD2 nuclear foci were observed in normal cell transfected with αIISp siRNA (Fig. 6A). There was a small reduction in the average number of FANCD2 foci per nucleus in cells in which αIISp had been knocked down compared to Nt siRNA transfected cells, but this reduction was not significant (Fig. 6B). Thus, after knock-down of αIISp, FANCD2 foci were still observed in the nucleus after ICL damage.

IN FA-A CELLS, IN WHICH LEVELS OF αIISP HAVE BEEN RESTORED TO NORMAL, NON-UB FANCD2 NUCLEAR FOCI FORM AFTER DNA ICL DAMAGE

To further examine the relationship between αIISp and FANCD2 nuclear foci formation after ICL damage, FA-A cells were examined. We have previously shown that in FA-A cells levels of αIISp are reduced to 35–40% of normal [McMahon et al., 1999]. In FA-A cells, FANCD2 is present but it has been shown that after ICL damage it is not monoubiquitinated and it does not form nuclear foci [Garcia-Higuera et al., 2001; Taniguchi and D′Andrea, 2006; Moldovan and D′Andrea, 2009]. Studies were therefore undertaken to determine whether restoration of levels of αIISp to normal in FA-A cells, by knocking down μ-calpain, had any influence on the ability of these cells to form FANCD2 foci after ICL damage.

We first examined the effects of restoring levels of αIISp to normal in FA-A cells on the levels of FANCD2 in these cells. We have previously shown that the levels of μ-calpain siRNA used in the present studies lead to a reduction in levels of μ-calpain to 44% of those in FA-A cells transfected with Nt siRNA and resulted in an increase in αIISp levels to those found in normal cells [Zhang et al., 2010]. The results of the present study showed that in undamaged FA-A cells only non-Ub FANCD2 was present (Fig. 7A) and that knocking down μ-calpain had no effect on levels of non-Ub FANCD2 in these cells, as determined by western blot analysis (Fig. 7A). Non-Ub FANCD2 levels in undamaged μ-calpain siRNA transfected FA-A cells were similar to those in undamaged Nt siRNA transfected FA-A cells and μ-calpain and Nt siRNA transfected normal cells (Fig. 7A). After μ-calpain siRNA transfected FA-A cells were damaged with MMC, no FANCD2-Ub was observed over the 72 h. time course examined after damage (Fig. 7B). Only non-Ub FANCD2 was present. Levels of non-Ub FANCD2 were similar to those observed in ICL damaged Nt siRNA transfected FA-A cells (Fig. 7C). This is in keeping with studies by others which have shown that in FA-A cells, after ICL treatment, FANCD2 is not mono-ubiquitinated [Garcia-Higuera et al., 2001; Taniguchi and D′Andrea, 2006; Moldovan and D′Andrea, 2009]. Thus, restoring levels of αIISp to normal in FA-A cells did not lead to monoubiquitination of FANCD2 after MMC damage, and had no effect on the levels of non-Ub FANCD2 present in these cells.

Studies were then carried out to determine whether restoration of αIISp levels to normal in FA-A cells had any influence on formation of FANCD2 foci after ICL damage. The results showed that, in Nt siRNA transfected FA-A cells, FANCD2 foci did not form after MMC treatment (Fig. 8A), similar to previously reported studies [Garcia-Higuera et al., 2001; Taniguchi and D′Andrea, 2006; Moldovan and D′Andrea, 2009]. However, importantly, after transfection of FA-A cells with μ-calpain siRNA and restoration of αIISp levels to normal, formation of FANCD2 foci was observed when cells were damaged with MMC (Fig. 8A). These studies were carried out using two different sources of anti-FANCD2 antibody. The percentage of nuclei in FA-A cells showing FANCD2 foci 16 h after MMC damage was approximately 80% of that of normal cells showing FANCD2 nuclear foci 16 h after MMC treatment (Fig. 8B). FANCD2 nuclear foci in the μ-calpain siRNA transfected FA-A cells were observed at 2 h post MMC damage (Supplementary Figure S3A). The percentage of nuclei showing FANCD2 had increased by 20 h after MMC damage and were still present at 24 h (Supplementary Figure S3A). Since FANCD2 was not monoubiquitinated in these MMC treated FA-A cells (Fig. 7B), this indicates that FANCD2 in these foci was not monoubiquininated. As an additional control to show that knock-down of μ-calpain, by itself, was not directly leading to an increase in FANCD2 foci, knockdown of μ-calpain by siRNA was also examined in normal cells. In these cells, after μ-calpain knockdown the percentage of nuclei, in either undamaged (15%) or MMC treated (58%) cells, showing FANCD2 foci was similar to the percentage of nuclei in Nt siRNA transfected undamaged (18%) or MMC treated (54%) cells showing FANCD2 foci (Fig. 8B).

After μ-calpain knockdown in FA-A cells, the average number of αIISp and FANCD2 foci formed per nucleus in FA-A cells after MMC was similar, 29% and 27%, respectively (Supplementary Figure S3B). However, only approximately 2.5% of these nuclei co-localized (Supplementary Figure S3B). These results thus importantly show that αIISp is needed for the formation of non-Ub FANCD2 nuclear foci in FA-A cells after ICL damage; αIISp's role in this process may or may not be direct.

DISCUSSION

We have previously shown that αIISp in the nucleus is critical for repair of DNA ICLs in S phase of the cell cycle [Sridharan et al., 2003; McMahon et al., 2009; Zhang et al., 2010, 2013]. Monoubiquitination of FANCD2 is also a key event in repair of ICLs present at replication forks stalled in S phase [Gregory et al., 2003; Seki et al., 2007; Alpi and Patel, 2009; Kim and D′Andrea, 2012]. Resolution of these ICLs is thought to involve a number of distinct steps which

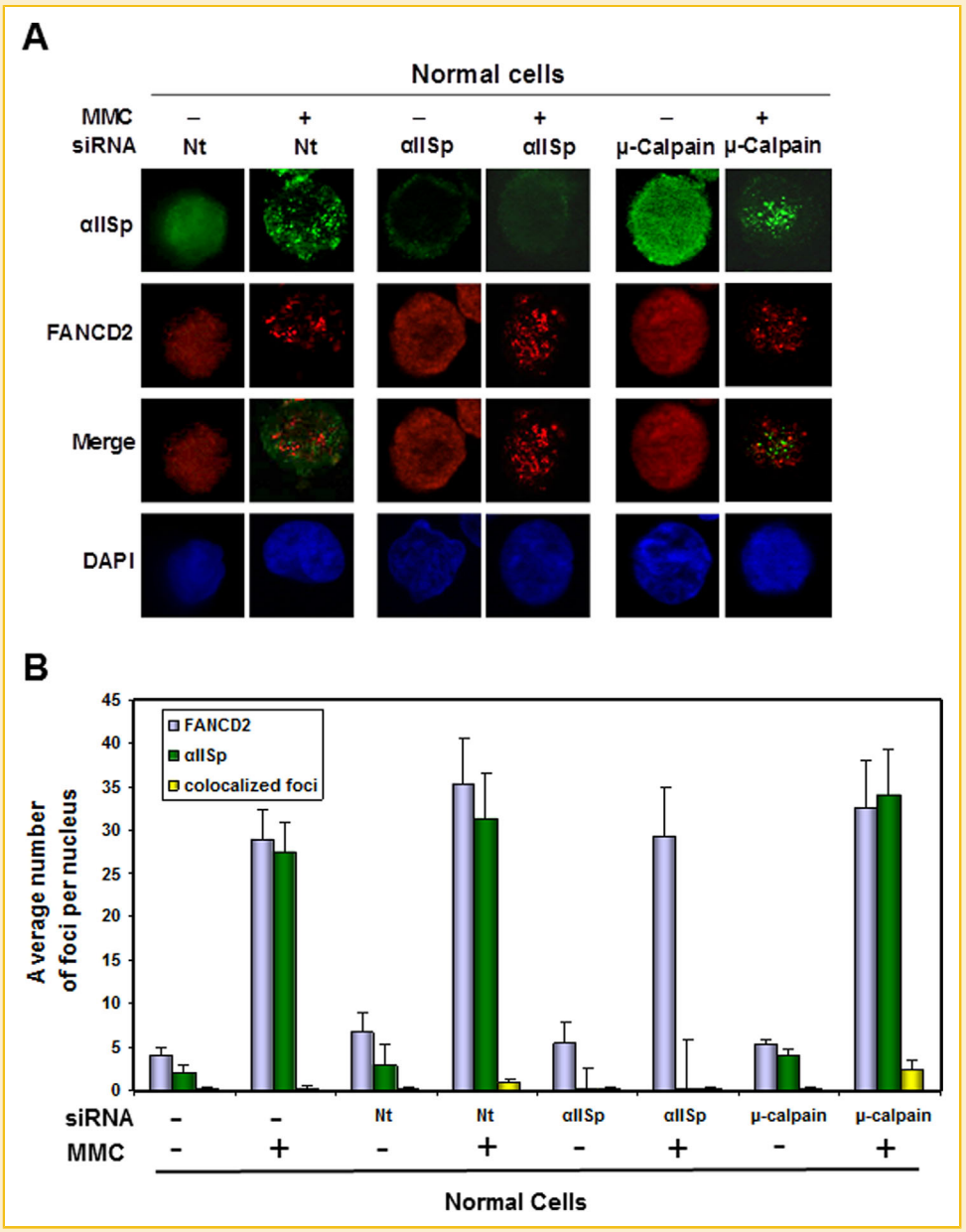

Fig. 6. In normal cells, knocking down αIISp had no effect on localization of FANCD2 to nuclear foci after ICL damage. A: Normal cells were transfected with either Nt siRNA or αIISp siRNA and then treated with of MMC (400 nM) for 16 h. Cells were also transfected with μ-calpain siRNA and either treated or not treated with MMC. Formation of FANCD2 and αIISp nuclear foci was examined using indirect immunofluorescence and staining with anti-FANCD2 or anti-αIISp. Cells were also counter stained with DAPI. The images were merged to examine co-localization of FANCD2 and αIISp foci. B: Normal cells were transfected with either Nt siRNA, αIISp siRNA or μ-calpain siRNA, and then treated with MMC (400 nM) for 16 h. The average number of FANCD2 and αIISp foci per nucleus and the average number of FANCD2 and αIISp nuclear foci co-localizing were quantitated. Nuclear foci were counted for 300 cells. These experiments were repeated 5 times. Error bars represent SEM.

include: recognition of the damage, nucleolytic incision and unhooking of the cross-linked DNA in which XPF is involved, translesion DNA synthesis and homologous recombination [Raschle et al., 2008; Al-Minawi et al., 2009; Bhagwat et al., 2009; Ho and Scharer, 2010; Legerski, 2010]. Loss of either αIISp or FANCD2 from normal cells leads to a deficiency in repair of DNA ICLs [Seki et al., 2007; Alpi and Patel, 2009; McMahon et al., 2009; Zhang et al., 2010; Kim and D′Andrea, 2012]. However, the relationship between αIISp and FANCD2 in the repair process is unclear, as is whether αIISp interacts with FANCD2 after ICL damage and is important in its monoubiquitination and localization to chromatin and sites of damage. These key questions are addressed in the present study.

Our results show that FANCD2 interacts with αIISp in undamaged normal cells, however, after ICL damage it dissociates from αIISp and forms nuclear foci. FANCD2 nuclear foci form before those of αIISp, at 2 h instead of 8–10 h after ICL damage. They also form before those

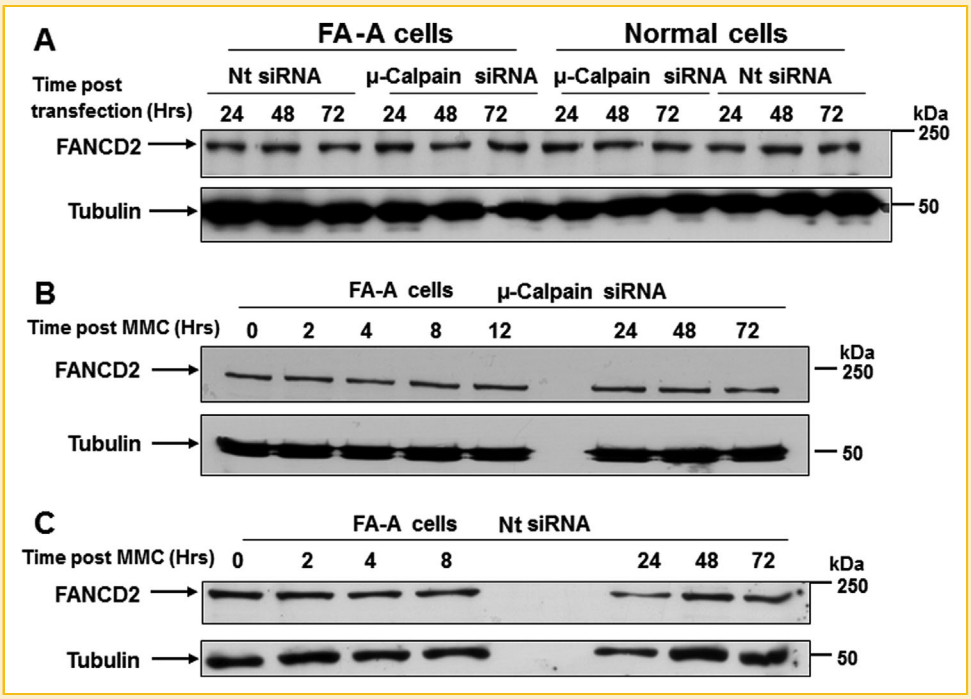

Fig. 7. In FA-A cells, after μ-calpain knockdown and DNA ICL damage, only non-Ub FANCD2 is present. FA-A or normal cells were transfected with either Nt siRNA or μ-calpain siRNA. A: Whole cell extracts from FA-A and normal cells were collected at multiple time points after transfection as indicated. Western blot analysis of undamaged FA-A and normal cells 24–72 h after siRNA transfection showed that only non-Ub FANCD2 was present. B: FA-A cells were transfected with μ-calpain siRNA and then treated with MMC (400 nM). Whole cell extracts were collected at multiple time points after MMC treatment. Western blots analysis showed that at time points from 0 to 72 h after MMC only non-Ub FANCD2 is present. C: FA-A cells were transfected with Nt siRNA and then treated with MMC (400 nM). Whole cell extracts were collected at multiple time points after MMC. Western blots analysis showed that at time points from 0 to 72 h after MMC only non-Ub FANCD2 is present. Immunoblots were probed with anti-FANCD2 antibody. Tubulin was used as a loading control. Molecular weight markers are indicated to the right.

of XPF, which form at 8–10 h [Sridharan et al., 2003]. This suggests that FANCD2 is recruited to sites of ICL damage upstream of αIISp and XPF. These results are similar to those obtained using a *Xenopus* egg extract system [Klein Douwel et al., 2014]. These latter studies, which employed a plasmid-based ICL repair assay using *Xenopus* egg extracts, showed that FANCD2 is recruited to sites of ICLs before XPF, indicating that FANCD2 is loaded at these sites upstream of XPF [Klein Douwel et al., 2014]. Our present studies show that formation of FANCD2 foci plateaus at 16 h and starts decreasing by 24 h, but that FANCD2 foci are still present at 72 h. This extended time period in which FANCD2 foci are present suggests that FANCD2 could be involved in subsequent steps in the ICL repair process, as has been proposed [Klein Douwel et al., 2014]. In contrast, we have shown in the present as well as in a previous study that, after ICL damage, formation of αIISp foci peaks at 16 h and αIISp foci are no longer observed 24 h post damage [Sridharan et al., 2003]. This same time course is observed for formation and disappearance of XPF foci [Sridharan et al., 2003]. We propose that the incision steps in the ICL repair process occur during this period since localization of XPF to sites of ICLs is involved in production of incisions at these sites [Kumaresan et al., 1995; Kumaresan and Lambert, 2000; Klein Douwel et al., 2014]. In support of this, we have shown that XPF is needed for the incisions produced at the site of an ICL on a DNA substrate containing a site-specific psoralen ICL [Kumaresan et al., 1995; Kumaresan and Lambert, 2000]. Klein Douwel et al. [2014] have shown that XPF is recruited to the site of an ICL with delayed kinetics compared to FANCD2; XPF is recruited after FANCD2 and its recruitment is coincident with production of incisions in an assay system using *Xenopus* egg extracts and a plasmid template. These studies collectively suggest that after DNA ICL damage both αIISp and XPF are recruited to sites of ICLs downstream of FANCD2.

Since αIISp and FANCD2 both form nuclear foci after ICL damage, an important question is whether they localize to the same or different foci. Our results show that αIISp and FANCD2 do not co-localize to any extent in the same foci over the time course examined. We have demonstrated, however, that αIISp does co-localize with XPF in foci after ICL damage [Sridharan et al., 2003]. These results thus indicate that, after ICL damage, αIISp localizes in the same nuclear foci as XPF but in different nuclear foci than FANCD2.

Whether αIISp has any effect on the ability of FANCD2 to form nuclear foci after ICL damage was another question addressed. Our present studies on knocking down expression of αIISp in normal cells demonstrate that αIISp is not needed for localization of FANCD2 to nuclear foci after ICL damage. In contrast, however, αIISp is required for the recruitment of XPF to nuclear foci and sites of ICLs [Sridharan et al., 2003; McMahon et al., 2009; Zhang et al., 2010]. We have shown that knockdown of αIISp in normal cells leads to loss of XPF nuclear foci after damage and that a monoclonal

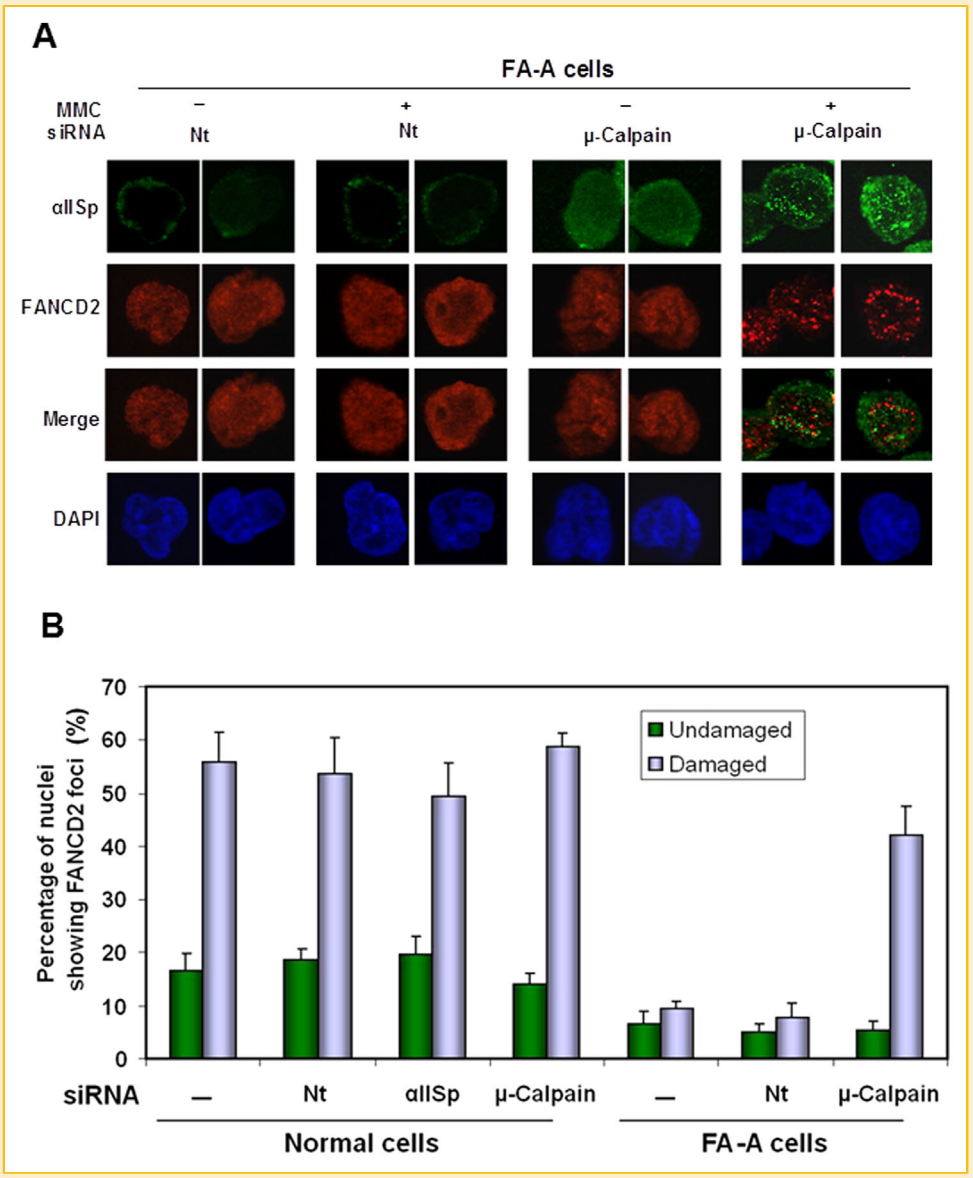

Fig. 8. In FA-A cells, knocking down μ-calpain leads to the formation of αIISp and FANCD2 nuclear foci after DNA ICL damage. FA-A cells were transfected with either Nt siRNA or μ-calpain siRNA, and then treated with MMC (400 nM) for 16 h. A: Formation of αIISp and FANCD2 nuclear foci was examined using indirect immunofluorescence and staining with anti-αIISp or anti-FANCD2 antibodies. Cells were also counter stained with DAPI. The images were merged to examine co-localization of FANCD2 and αIISp foci. B: Normal cells were transfected with either Nt siRNA or μ-calpain siRNA, and then treated with MMC (400 nM) for 16 h. FA-A cells were transfected with either Nt siRNA or μ-calpain siRNA and treated with MMC for 16 h. The percentage of the nuclei showing FANCD2 foci was quantitated in both normal and FA-A cells. Nuclei containing five or more FANCD2 foci were counted as positive. Nuclear foci were counted for 300 cells. Error bars represent SEM.

antibody against αIISp inhibits incisions produced by XPF at the site of a psoralen ICL on a DNA substrate [McMahon et al., 2001, 2003]. Thus after ICL damage, recruitment of FANCD2 to sites of damage and to nuclear foci is independent of αIISp, however, αIISp is critical for recruitment of XPF to sites of ICLs.

Since monoubiquitination of FANCD2 and its localization to chromatin are key steps in DNA ICL repair, we carried out studies to determine whether αIISp plays a role in this process. Our data on knocking down expression of αIISp in normal cells demonstrate that αIISp is not needed for monoubiquitination of FANCD2. It is also not needed for the localization of FANCD2 to chromatin after ICL damage. XPF, another key protein in the ICL repair process, is similarly not needed for the ubiquitination of FANCD2 after ICL damage as shown in a system using *Xenopus* egg extracts [Klein Douwel et al., 2014]. Thus two proteins involved in ICL repair, αIISp and XPF, which both show similarities in their targeting to sites of ICLs, are not involved in the monoubiquitination of FANCD2 after ICL damage.

Utilization of FA-A cells has aided these studies on the role of αIISp in FANCD2 function after ICL damage. In FA-A cells, levels of αIISp are 35–40% of normal [McMahon et al., 1999; Zhang et al., 2010]; FANCD2 is present but does not form nuclear foci after ICL

damage [Garcia-Higuera et al., 2001; Moldovan and D′Andrea, 2009]. We found that, when levels of αIISp are returned to normal in FA-A cells by knocking down μ-calpain, FANCD2 nuclear foci form after ICL damage and at 16 h post damage are at 80% of normal levels. Since FANCD2 is not monoubiquitinated in FA-A cells after ICL damage, as shown by our current data and that of others [Garcia-Higuera et al., 2001; Moldovan and D′Andrea, 2009], this indicates that the FANCD2 in these foci is non-monoubiquitinated. We have previously demonstrated that when levels of αIISp are returned to normal in FA-A cells by knocking down μ-calpain, a number of the phenotypic deficiencies observed after ICL damage are corrected: survival of these cells is increased from 36% to 85% of normal levels; XPF foci are now observed which co-localize with αIISp foci; and chromosomal aberrations such as interchromatid exchanges, fusions/radials, and breaks are corrected [Zhang et al., 2010]. We have proposed that αIISp acts as a scaffold to recruit specific repair proteins to sites of ICL damage in S phase; we have shown that it interacts with XPF and recruits it to sites of damage [Sridharan et al., 2003, 2006; Lambert, 2015]. Our present studies indicate that αIISp is also needed for localization of non-Ub FANCD2 to sites of damage in FA-A cells when μ-calpain is knocked down so as to express αIISp. It is thus possible that non-Ub FANCD2 may play an important role in the repair process. In support of this, in normal cells after ICL damage and when peak levels of FANCD2-Ub are observed, non-Ub FANCD2 is also present, as is shown in the present study and also by others [Garcia-Higuera et al., 2001; Bogliolo et al., 2007; Seki et al., 2007]. Since αIISp does not co-localize with non-Ub FANCD2 in these damage-induced foci, this indicates that αIISp′s role in this process may or may not be direct. We further propose that the FANCD2 foci observed in normal cells after ICL damage represent both FANCD2-Ub and non-Ub FANCD2. Since the FANCD2 antibody used recognizes both non-Ub FANCD2 and FANCD2-Ub, it is not possible to distinguish between localization of these two forms of FANCD2. This factor could also potentially explain why, when αIISp is knocked down in normal cells and these cells are then damaged with an ICL agent, the average number of FANCD2 foci observed per nucleus does not significantly decrease, which would be expected if αIISp is needed for formation of non-Ub FANCD2 foci. However, if non-Ub FANCD2 and FANCD2-Ub localize to the same foci, then one could postulate that a decrease in molecules of non-Ub FANCD2 in these foci while FANCD2-Ub molecules are still present could not be accurately determined since a decrease in fluorescence of individual foci, which we project would occur, could not be detected by the methods used.

Our studies which show that non-Ub FANCD2 can form nuclear foci after ICL damage in FA-A cells when αIISp is expressed would appear to be contradicted by studies in which a ubiquitination resistant mutant of FANCD2 (K5612 mutation) was expressed in FA-D2 cells [Garcia-Higuera et al., 2001; Seki et al., 2007; Kachnic et al., 2010]. In these studies, expression of this mutant FANCD2, which could not be monoubiquitinated, in FA-D2 cells did not lead to formation of FANCD2 nuclear foci after ICL damage. It was concluded that FANCD2 has to be monoubiquitinated in order to localize to nuclear foci after ICL damage. However, a very important point that has not been considered in analysis of these results is that in FA-D2 cells there is a marked deficiency in αIISp, as we have shown in human FA-D2 cells, and αIISp nuclear foci do not form in these cells after ICL damage [McMahon et al., 1999]. We, therefore, propose an alternate interpretation for the studies examining FA-D2 cells which express the mutant non-Ub FANCD2. We propose that non-Ub FANCD2 foci do not form in these cells after ICL damage because there is loss of αIISp, which we have demonstrated in the present studies is needed for formation of non-Ub FANCD2 foci. Studies to further examine this, in which these FA-D2 cells are transfected with an αIISp cDNA so as to determine if the presence of αIISp leads to formation of non-Ub FANCD2 foci, have not yet been feasible due to low transfection efficiency of αIISp cDNA owing to its large size.

There is evidence to support the view that non-Ub FANCD2 is important in ICL repair and in replication fork recovery. Non-Ub FANCD2 forms a complex with FANCD1/BRCA2, FANCG and the RAD51 paralog, XRCC3 [Hussain et al., 2006; Wilson et al., 2008, 2010]. Formation of this complex occurs independently of FANCD2 monoubiquitination and the FA core complex [Wilson et al., 2008, 2010; Sareen et al., 2012]. It has been proposed that this non-Ub FANCD2 complex may serve as a replication restart complex and promote or modulate the homologous recombination component of DNA ICL repair and cell survival in response to replication forks that are blocked or that break upon encountering DNA damage [Wilson et al., 2008, 2010]. Additionally, there is recent evidence that non-Ub FANCD2, in cooperation with FANCJ and BRCA2, also promotes replication fork recovery independently of the FA core complex [Raghunandan et al., 2015]. Thus our proposal that non-Ub FANCD2 can function in ICL repair in FA-A cells in the absence of FANCA, but in the presence of αIISp, is supported by several other reported studies. Non-Ub FANCD2 has been shown to associate with DNA structures that mimic DNA intermediates occurring in the ICL repair process [Park et al., 2005; Sobeck et al., 2007; Yuan et al., 2009]. Binding of FANCD2 to one or more of these structures may stabilize stalled replication forks and facilitate the DNA damage response [Park et al., 2005; Yuan et al., 2009]. Based on the above studies, we propose that non-Ub FANCD2 plays a role in the ICL repair process in FA-A cells when levels of αIISp have been restored to normal, even though there is a deficiency in levels of FANCA, a core complex protein.

The present studies indicate that αIISp is not involved in the functioning of FANCD2-Ub in repair of DNA ICLs but it is involved in the functioning of non-Ub FANCD2 after ICL damage. Both αIISp and FANCD2-Ub, nevertheless, are critical for ICL repair and the recruitment of XPF to sites of ICLs [Kim and D′Andrea, 2012; Kottemann and Smogorzewska, 2013; Lambert, 2015]. Evidence for the importance of αIISp, in addition to FANCD2, in the repair process is seen in our studies using normal cells in which αIISp has been knocked down. In these cells, FANCD2 is present and even though it is monoubiquitinated and localizes to nuclear foci and chromatin after ICL damage, without the presence of αIISp in these cells, XPF is not recruited to nuclear foci and chromosome stability and cell survival are deficient [McMahon et al., 2009; Zhang et al., 2010]. The mechanism utilized by FANCD2-Ub in the recruitment of XPF to sites of ICLs is not known. Based on our studies, however, we propose a mechanism by which αIISp could recruit XPF-ERCC1 to sites of ICLs: (1) αIISp binds to DNA at sites of ICLs. Evidence for this is that we have shown that purified αIISp binds preferentially to a DNA substrate containing psoralen ICLs [McMahon et al., 2001]; (2) αIISp

localizes to foci down steam from FANCD2. Evidence to support this is that αIISp foci form after those of FANCD2; (3) αIISp recruits XPF-ERCC1 to these sites of damage. Evidence for this is that XPF co-localizes with αIISp at damage sites in normal cells and knock-down of αIISp inhibits this [Sridharan et al., 2003; McMahon et al., 2009]; (4) Recruitment of XPF-ERCC1 by αIISp to these sites could be aided by FANCG. We have shown that FANCG binds to the SH3 domain of αIISp via a motif with a consequence sequence that specifically binds to SH3 domains [Lefferts et al., 2009]. We have shown that FANCG also binds to the central domain of ERCC1 via one of its TPR repeats [Wang and Lambert, 2010]. ERCC1 binds to XPF via an XPF-binding domain in its C-terminus [Manadhar et al., 2015]. αIISp could thus aid in the targeting of XPF-ERCC1 to sites of DNA ICLs via its binding to FANCG, which in turn binds to XPF-ERCC1; (5) XPF-ERCC1 then takes part in the incision process and ICL repair proceeds. In FA-A cells, when levels of αIISp are returned to normal, XPF foci form in the nucleus after ICL damage [Zhang et al., 2010] as do non-Ub FANCD2 foci. DNA repair, cell survival and chromosome stability are restored [Zhang et al., 2010]. The precise role of non-Ub FANCD2 in this process is not yet clear. We propose, however, that both FANCD2-Ub, and non-Ub FANCD2 have important roles in ICL repair, but that unlike FANCD2-Ub, non-Ub FANCD2 requires αIISp to function in the ICL repair process just as does XPF.

REFERENCES

Akkari YM, Bateman RL, Reifsteck C, Olson SB, Grompe M. 2000. DNA replication is required to elicit cellular responses to psoralen-induced DNA interstrand cross-links. Mol Cell Biol 20:8283–8289.

Al-Minawi AZ, Lee Y-F, Hakansson D, Johansson F, Lundin C, Saleh-Gohari N, Schultz N, Jenssen D, Bryant HE, Meuth M, Hinz JM, Helleday T. 2009. The ERCC1/XPF endonuclease is required for completion of homologous recombination at DNA replication forks stalled by inter-strand cross-links. Nucleic Acids Res 37:6400–6413.

Alpi AF, Patel KJ. 2009. Monoubiquitylation in the Fanconi anemia DNA damage response pathway. DNA Repair 8:430–435.

Bhagwat N, Olsen AL, Wang AT, Hanada K, Stuckert P, Kanaar R, D'Andrea A, Niedernhofer LJ, McHugh PJ. 2009. XPF-ERCC1 participates in the Fanconi anemia pathway of cross-link repair. Mol Cel Biol 29:6427–6437.

Bogliolo M, Lyakhovich A, Callen E, Castella M, Cappelli E, Ramirez MJ, Creus A, Marcos R, Kalb R, Neveling K, Schindler D, Surralles J. 2007. Histone H2AX and Fanconi anemia FANCD2 function in the same pathway to maintain chromosome stability. EMBO J 26:1340–1351.

de Oca RM, Andreassen PR, Margossian SP, Gregory RC, Taniguchi T, Wang X, Houghtaling S, Grompe M, D'Andrea AD. 2000. Regulated interaction of the Fanconi anemia protein, FANCD2, with chromatin. Blood 105:1003–1009.

de Winter JP, Joenje H. 2009. The genetic and molecular basis of Fanconi anemia. Mutat Res 668:11–19.

Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M, d'Andrea AD. 2001. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell 7:249–262.

Gregory RC, Tankguchi T, D'Andrea AD. 2003. Regulation of the Fanconi anemia pathway by monoubiquitination. Semin Cancer Biol 13:77–82.

Ho ATV, Scharer OD. 2010. Translesion DNA synthesis polymerases in DNA interstrand crosslink repair. Environ Mol Mutatgen 51:552–566.

Hussain S, Wilson JB, Blom W, Thompson LH, Sung P, Gordon SM, Kupfer GM, Joenje H, Mathew CG, Jones NJ. 2006. Tetratricopeptide-motif-mediated interaction of FANCG with recombination proteins XRCC3 and BR CA2. DNA Repair 5:629–640.

Kachnic LA, Li L, Fournier L, Willers H. 2010. Fanconi anemia pathway revealed by cisplatin and oxaliplatin treatments. Cancer Lett 292:73–79.

Kim H, D'Andrea AD. 2012. Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway. Genes Dev 26:1393–1408.

Klein Douwel D, Boon RACM, Long DT, Szypowska AA, Raschle M, Walter JC, Knipscheer P. 2014. XPF-ERCC1 acts in unhooking DNA interstrand crosslinks in cooperation with FANCD2 and FANCP/S LX4. Mol Cell 54:460–471.

Knipscheer P, Raschle M, Smogorzewska A, Enoiu M, Ho TV, Scharer OD, Elledge SJ, Walter JC. 2009. The Fanconi anemia pathway promotes replication-dependent DNA interstrand cross-link repair. Science 326:1698–1701.

Kottemann MC, Smogorzewska A. 2013. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. Nature 493:356–363.

Kumaresan KR, Hang B, Lambert MW. 1995. Human endonucleolytic incision of DNA 3′ and 5′ to a site-directed psoralen monoadduct and interstrand cross-link. J Biol Chem 270:30709–30716.

Kumaresan KR, Lambert MW. 2000. Fanconi anemia, complementation group A, cells are defective in ability to produce incisions at sites of psoralen interstrand cross-links. Carcinogenesis 21:741–751.

Kumaresan K, Sridharan D, McMahon L. Lambert MW 2007. Deficiency in incisions produced by XPF at the site of a DNA interstrand cross-link in Fanconi anemia cells. Biochemistry 46:14359–14368.

Lambert MW. 2015. Functional significance of nuclear α spectrin. J Cell Biochem 116:1816–1830.

Lambert MW, Tsongalis GJ, Lambert W, Hang B, Parrish DD. 1992. Defective DNA endonuclease activities in Fanconi′s anemia cells, complementation groups A and B. Mutat Res 273:57–71.

Lambert MW, Tsongalis JT, Lambert WC, Parrish DD. 1997. Correction of the DNA repair defect in Fanconi anemia complementation groups A and D cells. Biochem Biophys Res Commun 230:687–591.

Lefferts JA, Lambert MW. 2003. Fanconi anemia cell lines deficient in αII spectrin express normal levels of αII spectrin mRNA. Biochem Biophys Res Comm 307:510–515.

Lefferts JA, Wang C, Sridharan D, Baralt M, Lambert MW. 2009. The SH3 domain of αII spectrin is a target for the Fanconi anemia protein, FANCG. Biochemistry 48:254–263.

Legerski RJ. 2010. Repair of DNA interstrand cross-links during S phase of the mammalian cell cycle. Environ Mol Mutagen 51:540–551.

Manadhar M, Voulware KS, Wood RD. 2015. The ERCC1 and ERCC4 (XPF) genes and gene products. Gene 569:153–161.

Mathew CG. 2006. Fanconi anaemia genes and susceptibility to cancer. Oncogene 25:5875–5884.

McMahon LW, Sangerman J, Goodman SR, Kumaresan K, Lambert MW. 2001. Human alpha spectrin II and the FANCA, FANCC, and FANCG proteins bind to DNA containing psoralen interstrand cross-links. Biochemistry 40:7025–7034.

McMahon LW, Walsh CE, Lambert MW. 1999. Human α spectrin II and the Fanconi anemia proteins FANCA and FANCC interact to form a nuclear complex. J Biol Chem 274:32904–32980.

McMahon LW, Zhang P, Sridharan DM, Lefferts JA, Lambert MW. 2009. Knockdown of αII spectrin in normal human cells by siRNA leads to chromosomal instability and decreased DNA interstrand cross-link repair. Biochem Biophys Res Commun 381:288–293.

Moldovan G-L, D'Andrea A. 2009. How the Fanconi anemia pathway guards the genome. Ann Rev Genet 43:223–249.

Park W-H, Margossian S, Horwitz AA, Simons AM, D'Andrea AD, Parvin JD. 2005. Direct DNA binding activity of the Fanconi anemia D2 protein. J Biol Chem 280:23593–23598.

Raghunandan M, Chaudhury I, Kellich SL, Hanenberg H, Soebeck A. 2015. FANCD2, FANCJ and BRCA2 cooperate to promote replication fork recovery independently of the Fanconi anemia complex. Cell Cycle 143:342–353.

Raschle M, Knipsheer P, Enoiu M, Angelov T, Sun J, Griffith JD, Ellenberger TE, Scharer OD, Walter JC. 2008. Mechanism of replication-coupled DNA interstrand crosslink repair. Cell 134:969–980.

Sareen A, Chaudhury I, Adams N, Sobeck A. 2012. Fanconi anemia proteins FANCD2 and FANCI exhibit different DNA damage responses during S-phase. Nucleic Acids Res 40:8425–8439.

Seki S, Ohzeki M, Uchiada A, Hirano S, Matsushita N, Kitao H, Oda T, Yamashita T, Kashihara N, Tsubahara A, Takata M, Ishiai M. 2007. A requirement of FancL and FancD2 monoubiquitination in DNA repair. Genes to Cells 12:299–310.

Sobeck A, Stone S, Hoatlin ME. 2007. DNA structure-induced recruitment and activation of the Fanconi anemia pathway protein FAN CD2. Mol Cell Biol 27:4283–4292.

Sridharan D, Brown M, Lambert WC, McMahon LW, Lambert MW. 2003. Nonerythroid alpha II spectrin is required for recruitment of FANCA and XPF to nuclear foci induced by DNA interstrand cross-links. J Cell Sci 116:823–835.

Sridharan DM, McMahon LW, Lambert MW. 2006. αII-Spectrin interacts with five groups of functionally important proteins in the nucleus. Cell Biol Intl 30:866–878.

Taniguchi T, D′Andrea AD. 2006. Molecular pathogenesis of Fanconi anemia: Recent progress. Blood 107:4223–4233.

Thompson LH, Hinz JM. 2009. Cellular and molecular consequences of defective Fanconi anemia proteins in replication-coupled DNA repair: Mechanistic insights. Mutat Res 668:54–72.

Walden H, Deans AJ. 2014. The Fanconi anemia DNA repair pathway: Structural and functinal insights into a complex disorder. Ann Rev Biophys 43:257–278.

Wang W. 2007. Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat Rev Genet 8:735–748.

Wang X, Andreassen PR, D′Andrea AD. 2004. Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. Mol Cell Biol 24:5850–5862.

Wang C, Lambert MW. 2010. The Fanconi anemia protein, FANCG, binds to the ERCC1-XPF endonuclease via its tetratricopeptide repeats and the central domain of ER CC1. Biochemistry 49:5560–5569.

Wilson JB, Blom E, Cunningham R, Xiao Y, Kupfer GM, Jones NJ. 2010. Several tetratricopeptide repeat (TPR) motifs of FANCG are required for assembly of the BRCA2/D1-D2-G-X3 complex, FANCD2 monoubiquitylation and phleomycin resistance. Mutat Res 689:12–20.

Wilson JB, Yamamoto K, Marriott AS, Hussain S, Sung P, Hoatlin ME, Mathew CG, Takata M, Thompson LH, Kupfer GM, Jones NJ. 2008. FANCG promotes formation of a newly identified protein complex containing BRCA2, FANCD2 and XR CC3. Oncogene 27:3641–3652.

Yuan F, Hokayem JE, Zhou W, Zhang Y. 2009. FANCI protein binds to DNA and interacts with FANCD2 to recognize branched structures. J Biol Chem 284:24443–24452.

Zhang P, Herbig U, Coffman F, Lambert MW. 2013. Non-erythroid alpha spectrin prevents telomere dysfunction after DNA interstrand cross-link damage. Nucleic Acids Res 41:5321–5340.

Zhang P, Sridharan D, Lambert MW. 2010. Knockdown of μ-calpain in Fanconi anemia, FA-A, cells by siRNA restores αII spectrin levels and corrects chromosomal instability and defective DNA interstrand cross-link repair. Biochemistry 49:5570–5581.

Zhang J, Walter JC. 2014. Mechanism and regulation of incisions during DNA interstrand cross-link repair. DNA Repair 19:135–142.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher′s web-site.