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Functional Significance of Nuclear α Spectrin

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ABSTRACT

Nonerythroid alpha spectrin (αIISp) interacts in the nucleus with an array of different proteins indicating its involvement in a number of diverse functions. However, the significance of these interactions and their functional importance has been a relatively unexplored area. The best documented role of nuclear αIISp is in DNA repair where it is critical for repair of DNA interstrand cross-links (ICLs), acting as a scaffold recruiting proteins to sites of damage in genomic and telomeric DNA. A deficiency in αIISp can importantly impact DNA ICL repair as is seen in cells from patients with the genetic disorder, Fanconi anemia (FA), where loss of αIISp leads to not only defects in repair of both genomic and telomeric DNA but also to telomere dysfunction and chromosome instability. This previously unexplored link between αIISp and telomere function is important in developing an understanding of maintenance of genomic stability after ICL damage. In FA cells, these defects in chromosome instability after ICL damage can be corrected when levels of αIISp are returned to normal by knocking down μ-calpain, a protease which cleaves αIISp. These studies suggest a new direction for correcting a number of the phenotypic defects in FA and could serve as a basis for therapeutic intervention. More in depth, examination of the interactions of αIISp with other proteins in the nucleus is of major importance in development of insights into the interacting key elements involved in the diverse processes occurring in the nucleus and the consequences loss of αIISp has on them. J. Cell. Biochem. 116: 1816–1830, 2015.

KEY WORDS: NONERYTHROID ALPHA SPECTRIN; NUCLEAR SPECTRIN; DNA REPAIR; DNA DAMAGE; DNA INTERSTRAND CROSS-LINKS; XPF; SPECTRIN CLEAVAGE; SH3 DOMAINS; μ-CALPAIN; TELOMERES; TELOMERE DYSFUNCTION; TRF1 AND TRF2; TELOMERE SIGNAL-FREE ENDS; CHROMOSOME STABILITY; CHROMOSOME INSTABILITY; FANCONI ANEMIA; FANCONI ANEMIA PROTEINS; FANCG

Spectrins are long flexible molecules that were first identified as an integral component of the red blood cell (RBC) membrane skeleton. They are composed of various isoforms of two subunits, α and β, which can form heterodimers [Winkelmann and Forget, 1993; Goodman et al., 1995; de Matteis and Morrow, 2000; Bennet and Baines, 2001]. These heterodimers interact with actin to create a scaffold important in structural support and flexibility of the RBC membrane [de Matteis and Morrow, 2000; Bennet and Baines, 2001]. In addition, this spectrin cytoskeletal network has been identified in all nonerythroid mammalian cells, where it also provides structural support for the cell membrane and maintenance of cell shape [Winkelmann and Forget, 1993; Goodman et al., 1995; de Matteis and Morrow, 2000; Bennet and Baines, 2001; Machnicka et al., 2012]. Nonerythroid alpha spectrin (αIISp) is the major α-spectrin expressed in nonerythroid cells; several isoforms of αIISp have been identified in nonerythroid cells. Five β-spectrins are expressed (βI–V). These α- and β-spectrins have additional known functions in the cytoplasm, such as protein sorting, organelle and vesicle trafficking, synaptic transmission in neurons, cell cycle progression, signal transduction, and cell growth and differentiation [Winkelmann and Forget, 1993; Goodman et al., 1995; de Matteis and Morrow, 2000; Metral et al., 2009; Stankewich et al., 2011; Machnicka et al., 2012]. Though αIISp and a truncated form of β-IV spectrin have been identified in the nucleus, the presence of these proteins and their functional importance in the nucleus has been a relatively unexplored area.

αIISp has been shown, using immunocytochemical methods, to be associated with the nuclear matrix and nuclear envelope in rat liver cells; its function, though, is unknown [Bachs et al., 1990]. We have identified αIISp, via sequence analysis of this protein, in human cell nuclei and demonstrated that it plays an important role in DNA repair and chromosome stability after DNA damage [Brois et al., 1999; McMahon et al., 1999]. Tse et al. [2001] have subsequently shown that a truncated isoform of βIV-spectrin (βIVΣ5) is present in the nucleus of human and various mammalian cell lines where it associates with promyelocytic leukemia protein (PML) bodies and the nuclear matrix; its function is unknown. We have demonstrated that αIISp binds to the truncated βIV-spectrin in the nucleus but the

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significance of this interaction and whether it has a role in the formation of a structural framework in the nucleus is unclear [Sridharan et al., 2006]. αIISp is essential for cell function. Deletion of an ortholog to the αIISp gene from *Drosophilia melanogaster* and *Caenorhabditis elegans* leads to embryonic lethality [Lee et al., 1993; Norman and Moerman, 2002]. Knockdown of αIISp in cultured human lymphoblastoid cells leads to cell death [McMahon et al., 2009] and in cultured melanoma cells leads to inhibition of cell growth [Metral et al., 2009]. In addition, loss of αIISp is embryonic lethal in mice [Stankewich et al., 2011].

Of particular interest, we have found that there is a severe deficiency in αIISp in cells from patients with the hereditary bone marrow failure disorder, Fanconi anemia (FA), [Brois et al., 1999; McMahon et al., 1999] which is characterized by chromosome instability, congenital abnormalities, a marked predisposition to develop cancer, and a striking cellular hypersensitivity to DNA interstrand cross-linking (ICL) agents [de Winter and Joenje, 2009; Moldovan and D'Andrea, 2009; Kottemann and Smogorzewska, 2013]. Sixteen complementation groups of FA have been identified, each characterized by mutations in a different FA gene and thus a different FA protein [de Winter and Joenje, 2009; Moldovan and D'Andrea, 2009; Kottemann and Smogorzewska, 2013]. However, cells in all FA groups examined to date have a common defect: a marked deficiency of αIISp [McMahon et al., 1999]. This deficiency in αIISp correlates with a defect in ability to repair DNA interstrand cross-links, which is considered to be an underlying basis for this disorder. Restoring levels of αIISp in FA cells restores DNA repair and chromosome stability after ICL damage, indicating that αIISp is critical in the repair process and in maintenance of genomic stability after DNA damage [Zhang et al., 2010]. This review will concentrate on the functional importance of αIISp in the nucleus, particularly its role in DNA repair and chromosome stability and our view that a deficiency in αIISp in FA cells is an important component in the development of many of the phenotypic changes characteristic of this disorder.

IDENTIFICATION OF A ROLE FOR αIISP IN THE NUCLEUS

Our studies on repair of DNA ICLs have led to the isolation of a complex of chromatin-associated proteins from the nuclei of normal human lymphoblastoid cells which is involved in repair of DNA ICLs [Lambert et al., 1997; Brois et al., 1999]. This protein complex has an isoelectric point of 4.6. We developed monoclonal antibodies (mAbs) against proteins in this complex and found that one of the mAbs, against an approximately 230 kDa protein, inhibited ability of this complex to incise DNA containing ICLs [Brois et al., 1999]. Sequence analysis of this protein showed that it was αIISp [McMahon et al., 1999], which has an isoelectric point of 5.4 [Harris et al., 1989]. This indicated a potential role for αIISp in the nucleus in DNA ICL repair.

Studies using cells from patients with FA, which are defective in ICL repair, have helped in the elucidation of whether αIISp is important in the repair process. Analysis of αIISp in the corresponding protein complex isolated from cells from all of the FA complementation groups tested (FA-A, -B, -C, -D1, -D2, -F, -G) showed that levels of αIISp were only 35–40% of normal, depending on the complementation group [Brois et al., 1999; McMahon et al., 1999]. Levels of αIISp mRNA in these FA cell lines were similar to normal, indicating that decreased levels of αIISp in these FA cells are not due to reduced expression of αIISp but rather to its reduced stability [Lefferts and Lambert, 2003]. This will be discussed later in this review. This deficiency corresponded to reduced levels of DNA ICL repair (34–43% of normal) in these FA cells, as determined by measuring levels of unscheduled DNA synthesis (UDS) [Lambert et al., 1997; Kumaresan et al., 2007].

These studies have led to our subsequent investigations into the role of nuclear αIISp in repair of ICLs in both genomic (non-telomeric) and telomeric DNA in human cells and the consequences of decreased αIISp on chromosomal stability. This has been a totally unexplored area which could have significant impact on our understanding of some of the important factors underlying genomic stability and the serious consequences that loss of αIISp can have.

αIISP IS CRITICAL FOR REPAIR OF GENOMIC (NON-TELOMERIC) DNA

Numerous lines of evidence have led to our conclusion that αIISp is critical in the processing of damaged DNA in both genomic and telomeric DNA. Our finding that αIISp is involved in DNA ICL repair represents a new role for this structural protein. Based on our studies, we have proposed a model for the role of αIISp in the repair process (Fig. 1). In this model, αIISp binds to DNA at sites of damage (ICLs) and acts as a scaffold important in the recruitment of proteins involved in repair of DNA ICLs to sites of damage, aiding in their alignment and interaction with each other, and thus enhancing the repair process [McMahon et al., 2001]. In its absence, such as in FA cells, there is a deficiency in binding of αIISp to damaged DNA and in recruitment of repair proteins to sites of damage. This in turn leads to decreased levels of DNA repair (Fig. 1) [McMahon et al., 2001]. The evidence for this is presented below.

Analysis of binding of αIISp to damaged DNA has shown that purified bovine brain αIISp binds directly to a DNA substrate containing 4,5′,8-trimethylpsoralen (TMP) ICLs, as does αIISp from HeLa cell nuclei [McMahon et al., 2001]. This spectrin has no affinity for a DNA substrate containing a TMP monoadduct, indicating its preference for sites of ICLs [McMahon et al., 2001]. This was the first report of interaction of αIISp with DNA. In addition, αIISp is important in production of incisions in DNA produced at the site of an ICL by XPF, a protein involved in ICL repair [McMahon et al., 2001]. A mAb specifically against αIISp inhibits the production of these incisions produced by XPF in an in vitro system containing a DNA substrate with a site-directed TMP ICL and chromatin-associated protein extracts from HeLa cells [McMahon et al., 2001]. Purified αIISp also enhances incisions produced by XPF in this in vitro system [McMahon et al., 2001]. These studies provide support for our model that αIISp binds to cross-linked DNA and is important in the incision steps in the repair process where it aids in recruitment of repair proteins to the site of damage. In further support of this model are our studies which show that in FA cells (FA-A, -B, -C, -D2, -F, and -G), in which αIISp levels are reduced to 35–40% of normal, there is a deficiency in production

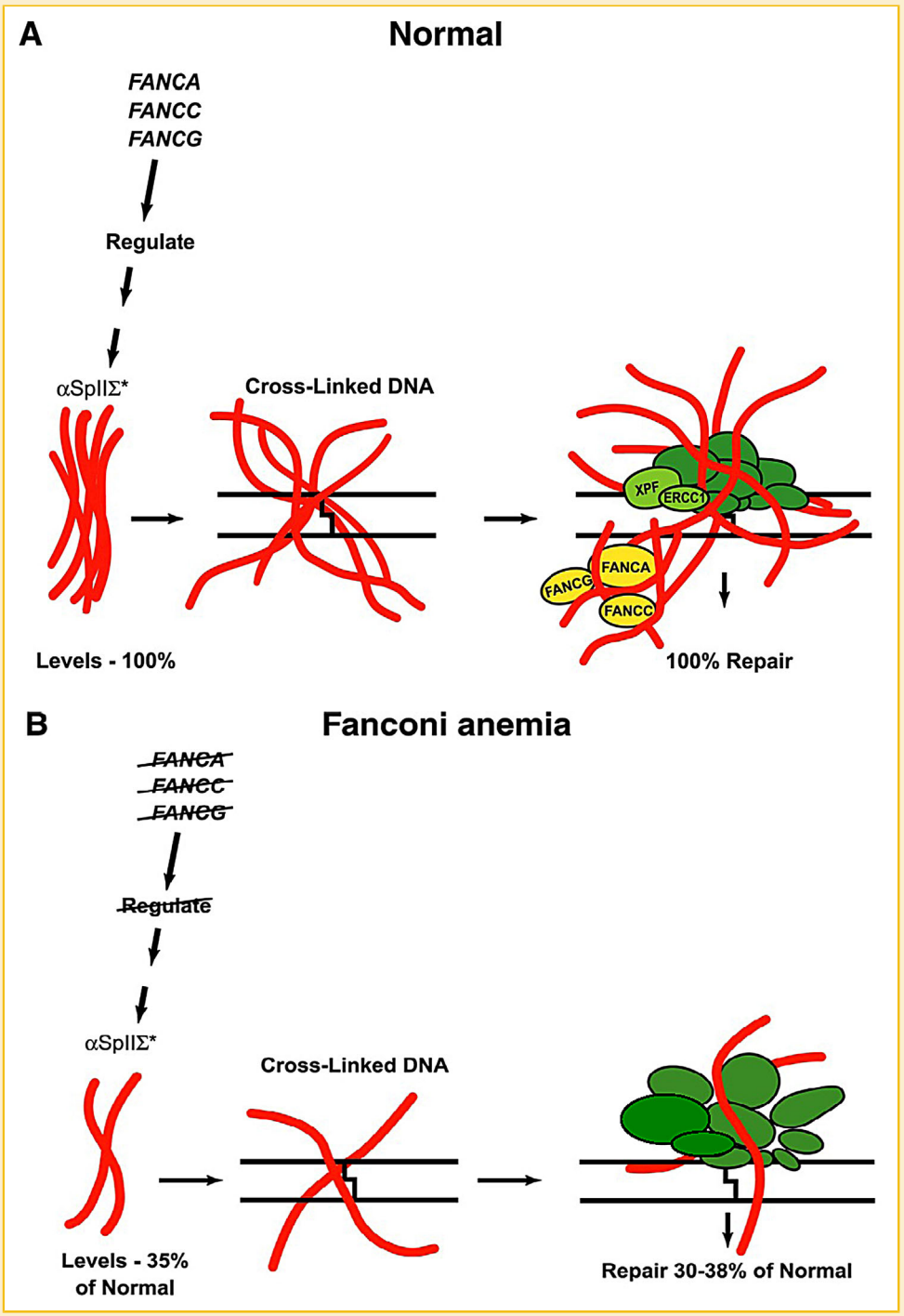

Fig. 1. Model proposing a role for αIISp in the repair of DNA ICLs in normal cells and in the repair defect in FA cells. (A) In normal cells FA proteins, for example FANCA, FANCC, or FANCG, regulate the stability of αIISp. αIISp binds to cross-linked DNA and acts as a scaffold in the recruitment of proteins involved in ICL repair (e.g., FANCA, FANCC, FANCG, and XPF/ERCC1) to sites of ICLs, enhancing the repair process and resulting in normal levels of DNA repair. (B) In FA cells, deficiencies in specific FA proteins (e.g., FANCA, FANCC, FANCG) lead to decreased levels of αIISp in the FA cells (35–40% of normal) due to increased cleavage by μ-calpain. FA proteins, in some manner, regulate cleavage of αIISp by μ-calpain; deficiencies in these FA proteins lead to increased μ-calpain activity and cleavage of αIISp. The reduced levels of αIISp ultimately lead to decreased recruitment of repair proteins to sites of damage and decreased DNA ICL repair. (Adapted from McMahon et al., 2001. Biochemistry 40:7025–7034 with permission from the American Chemical Society).

of incisions produced by XPF at sites of ICLs even though the levels of XPF in these cells are normal [Kumaresan et al., 1995, 2007; Kumaresan and Lambert, 2000].

Further evidence that αIISp is involved in ICL repair comes from our investigations on localization of αIISp to nuclear foci following ICL damage [Sridharan et al., 2003]. Immunofluorescence studies show that in undamaged normal human cells αIISp is present diffusely throughout the nucleus (Fig. 2A–C) [Sridharan et al., 2003; Zhang et al., 2010]. After damage of the cells with an ICL agent, 8-MOP plus UVA light or mitomycin C, αIISp localizes to discrete foci in the nucleus (Fig. 2A and B). The ICL repair protein, XPF, co-localizes with αIISp at these foci as well as does the FA protein, FANCA, which may also be involved in the repair process, thus indicating an involvement of αIISp and FANCA in ICL repair with XPF (Fig. 2) [Sridharan et al., 2003]. This view is strengthened by studies which show that these three proteins co-immunoprecipitate with each other, indicating that they interact in the cell [McMahon et al., 1999; McMahon et al., 2001; Sridharan et al., 2003]. Another strong indication that αIISp is involved with these proteins in ICL repair is demonstrated in time course studies. Formation of αIISp, XPF, and FANCA foci in the nucleus is first seen at 8 hours after ICL damage by 8-MOP plus UVA; the number of these foci and the percentage of nuclei showing multiple foci peaks at 16 h after damage, and by 24 h these foci are no longer observed (Fig. 3) [Sridharan et al., 2003]. We have found that this pattern is in contrast to that of other proteins proposed to be involved in this repair process, which co-localize to different sites and have a different time course for formation and dissolution.

Our model for the role of αIISp in ICL repair is further supported by studies using cells in which there is a loss of αIISp: either FA cells or normal cells in which αIISp has been knocked down. In FA-A cells, where levels of αIISp are approximately 35–40% of normal, few αIISp foci form in the nucleus after ICL damage; XPF does not localize to nuclear foci after ICL damage, even though levels of XPF in these cells are similar to those in normal cells [Sridharan et al., 2003; Zhang et al., 2010]. In normal cells in which αIISp has been knocked down to levels found in FA cells using αIISp siRNA, there is a significant decrease in formation of damage-induced αIISp and XPF nuclear foci after ICL damage [McMahon et al., 2009]. This decrease in αIISp leads to cellular changes similar to those observed in FA cells after ICL damage, such as failure of XPF to form nuclear foci and decreased cellular survival. These changes were not due to reduced levels of FA proteins or XPF in the cells since knockdown of αIISp in normal cells had no effect on the levels of these proteins [McMahon et al., 2009; Zhang et al., 2013]. Together, these studies show that αIISp is involved in targeting repair proteins to sites of damage and that, when levels of αIISp are significantly reduced, so is the recruitment of repair proteins to these damage sites.

LOSS OF αIISP IN FA CELLS IS DUE TO ITS INCREASED BREAKDOWN BY μ-CALPAIN

Since we have shown that, in all FA complementation groups analyzed, loss of αIISp is not due to decreased expression of αIISp, we hypothesized that it could be due to its enhanced breakdown. Structurally αIISp is composed of 20 triple α helical repeats (just like βII-spectrin). It most notably differs from βII-spectrin in that: it has an SH3 domain within repeat 9; repeat 10 contains a μ-calpain cleavage site, a m-calpain cleavage site, a binding site for calmodulin, and cleavage sites for caspases; and it has EF hand motifs at the C-terminal [Huh et al., 2001; Nicolas et al., 2002; Machnicka et al., 2012]. The stability of αIISp in cells is dependent upon a number of factors. Among these is the susceptibility of αIISp to cleavage by the protease, μ-calpain. μ-Calpain cleaves αIISp into distinct cleavage products and this process plays an important role in the regulation of essential cellular functions mediated by αIISp [Huh et al., 2001; Nicolas et al., 2002; Machnicka et al., 2012].

Examination of FA cells (FA-A, -C, -D2, -F, and -G) showed that there is a three to fourfold increase in μ-calpain activity in these cells, but not in levels of μ-calpain [Zhang et al., 2010]. This increase in activity correlated with increased levels of a 150 kDa μ-calpain αIISp breakdown product in FA cells and reduced levels of full-length αIISp [Zhang et al., 2010]. These studies indicate that in FA cells, loss of αIISp is due to its increased breakdown by μ-calpain.

As discussed above, αIISp loss, such as that which occurs in FA cells and normal cells in which αIISp has been knocked down, correlates with the DNA repair defect observed in these cells after ICL damage and indicates an important role for αIISp in the repair process. Increased cleavage of αIISp in cells by μ-calpain, such as occurs in FA cells, would thus be expected to have an important effect on regulation of essential cellular functions mediated by αIISp. The potential role of FA proteins in this process will be discussed below.

αIISP IS CRITICAL FOR CHROMOSOME STABILITY AFTER ICL DAMAGE

Defects in repair of DNA ICLs have been hypothesized to be a major cause for chromosome instability, which is a hallmark of FA. Since αIISp is critical for ICL repair, we have hypothesized that a loss of αIISp in FA cells could have a significant effect on chromosome stability. Analysis of the effects of knocking down levels of αIISp in normal human cells to those found in FA cells, by αIISp siRNA, showed that there was a significant increase in chromosomal aberrations such as intrachromatid exchanges, radials, fusions and breaks, similar to levels observed in FA cells after ICL damage [McMahon et al., 2009]. This is a strong indication that αIISp plays a significant role in maintaining chromosome stability, particularly after DNA ICL damage.

In support of this view are our studies in which levels of αIISp have been restored to normal in FA-A cells by knocking down μ-calpain using siRNA. In these cells, there was a reduction in levels of chromosomal aberrations (i.e., interchromatid exchanges, fusions/radials, and breaks) after ICL damage to levels that were similar to those found in similarly treated normal cells (Fig. 4) [Zhang et al., 2010]. Additionally, restoration of αIISp levels in these FA-A cells corrected a number of the other cellular defects observed which included decreasing sensitivity to DNA ICL agents and increasing DNA repair and cell viability after ICL damage [Zhang et al., 2010]. Thus, restoring levels of αIISp to normal in FA in cells by knocking down μ-calpain corrects a number of the

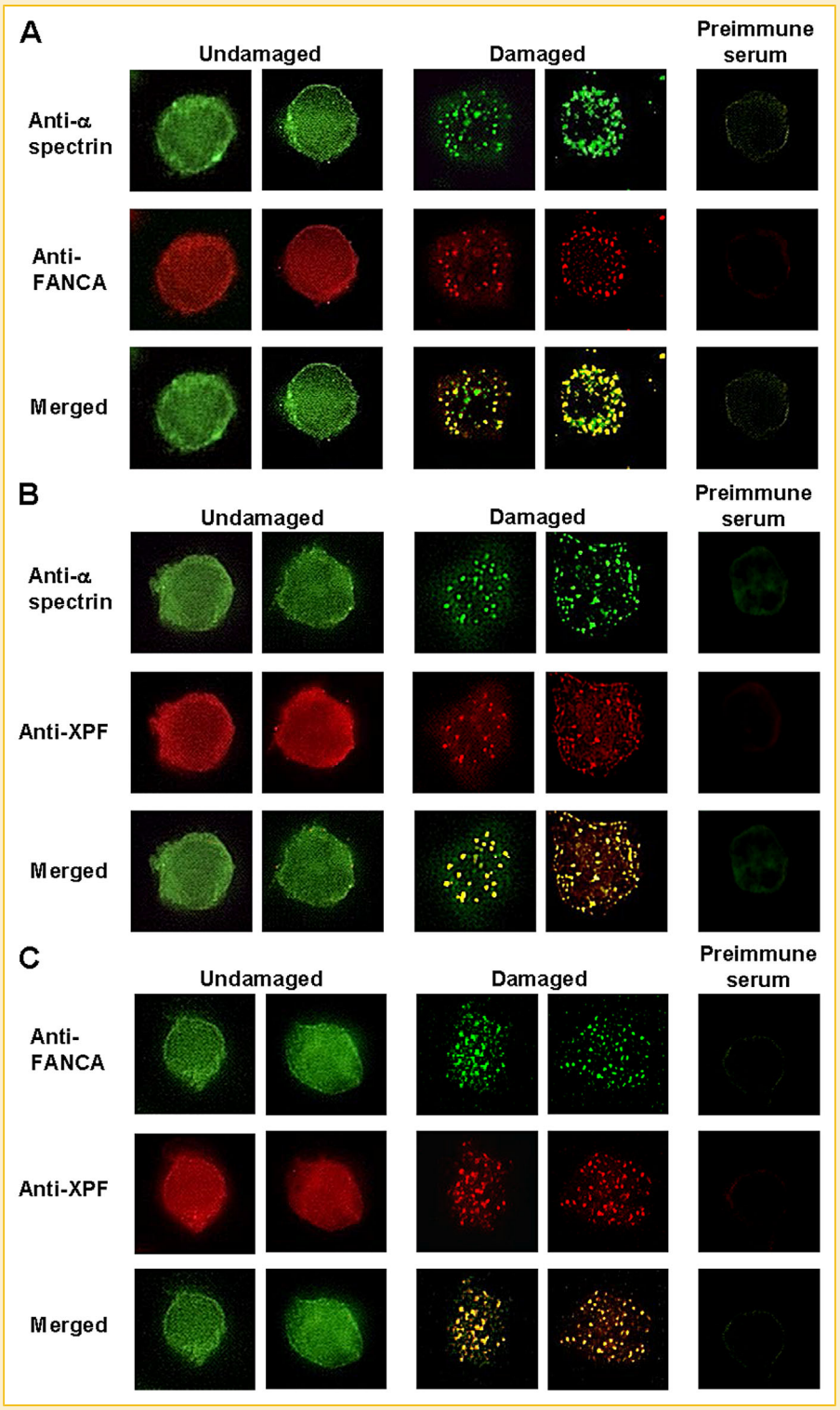

Fig. 2. Localization of αIISp, FANCA, and XPF to sites of DNA damage after treatment with a DNA ICL agent. Normal human lymphoblastoid cells were either undamaged or treated with 8-MOP plus UVA light (6 kJ m^{-2}) and the localization of αIISp, FANCA, and XPF in the nucleus examined 15 h after treatment. (A) Dual staining was carried out using purified monoclonal anti-α-spectrin antibody and affinity-purified polyclonal anti-FANCA antibody. Stained cells were analyzed by immunofluorescence. When the fluorescent signals for αIISp (green) and FANCA (red) were merged, the overlapping foci are yellow, indicating co-localization of the these two proteins. (B) An analysis similar to that in (A) was carried out using anti-α-spectrin antibody (green) and affinity-purified polyconoal anti-XPF antibody (red). Fluorescence signals for both proteins were merged and overlapping foci appear yellow indicating co-localization of these proteins. (C) Dual staining was also carried out using anti-FANCA (green) and anti-XPF (red) antibodies. The fluorescence signals were merged and overlapping foci are yellow indicating co-localization of these two proteins. In all of the above experiments, cells were also stained with the appropriate preimmune sera. (Reproduced from Sridharan et al., 2003. J Cell Sci 116:823–835 with permission from The Company of Biologists Ltd.).

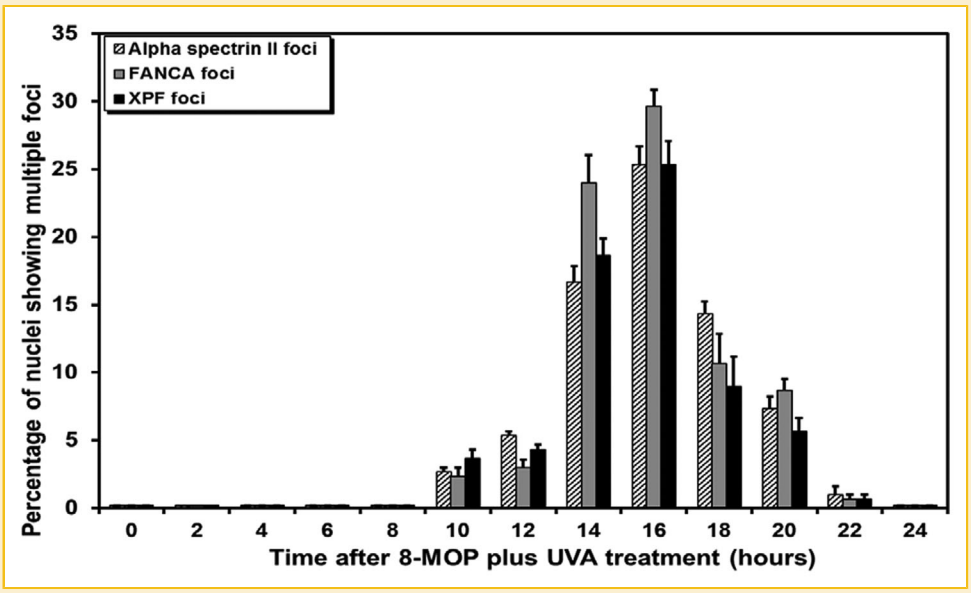

Fig. 3. Time course for formation of αIISp, FANCA, and XPF foci in the nucleus after treatment with 8-MOP plus UVA light. Normal human lymphoblastoid cells were treated with 8-MOP plus UVA light (6 kJ m^{-2}), fixed at the indicated time points post-treatment and stained independently using either anti-α-spectrin, anti-FANCA, or anti-XPF antibodies. The percentage of nuclei showing multiple αIISp, FANCA, and XPF foci was counted over a period of 1–24 h after treatment. Nuclei in 100 cells were counted at each time point. Vertical lines indicate ±S.E.M. (Reproduced from Sridharan et al., 2003. J Cell Sci 116:823–835 with permission from The Company of Biologists Ltd.).

phenotypic deficiencies observed, providing further strength to our model in which αIISp plays a critical role in both DNA repair and chromosome stability.

αIISP IS ESSENTIAL FOR TELOMERE MAINTENANCE AND FUNCTION AFTER ICL DAMAGE

Chromosome stability is dependent upon the integrity of telomeres, which are specialized nucleoprotein structures at the ends of chromosomes. They are critical for preserving genomic stability by preventing chromosome ends from being recognized and treated by the cell as double-strand breaks (DSBs), thus preventing end-to-end fusions [Palm and de Lange, 2008; Murnane, 2012]. Telomere dysfunction can be an important driving factor behind chromosome instability [Palm and de Lange, 2008; Murnane 2012]. Human telomeres are bound by a telomere-specific multiprotein complex, shelterin, which helps protect telomeres and prevents telomere dysfunction [Palm and de Lange, 2008; Murnane 2012; Sfeir and de Lange, 2012]. Since αIISp is critical for both repair of DNA ICLs and chromosome stability after ICL damage, an extremely important question is whether it is also critical for maintenance of telomere function. Our studies have shown that αIISp is, indeed, critical for maintenance of telomere function after DNA ICL damage [Zhang et al., 2013]. This link between αIISp and telomere function is a previously unexplored area, which is highly relevant to the role αIISp plays in genomic stability; the evidence for this is presented below.

Immunofluorescent staining of αIISp in conjunction with fluorescent in situ hybridization using a telomeric peptide-nucleic acid (PNA) probe (immunoFISH) has shown that, in undamaged cells, αIISp is present diffusely throughout the nucleus [Zhang et al., 2013]. However, after ICL damage with either 8-MOP plus UVA or mitomycin C, a portion of αIISp (49%) co-localizes with the PNA probe and also with two of the proteins of the shelterin complex bound to telomeres, TRF1 and TRF2 [Zhang et al., 2013] (Fig. 5). These results demonstrate that αIISp associates with telomeric DNA and with TRF1 and TRF2 at telomeres after ICL damage. Since TRF1 and TRF2 interact with or recruit specific proteins important for telomere function [Smogorzewska and de Lange, 2004; Blackburn, 2005], this suggests that, after ICL damage, TRF1 and TRF2 recruit αIISp to telomeres. This view is supported by immumoprecipitation data which show that there is an enhanced association of αIISp with TRF1 and TRF2 after ICL formation [Zhang et al., 2013].

ImmunoFISH studies have also shown that, after ICL damage, αIISp localizes to telomeres specifically in S phase [Zhang et al., 2013]. Since telomeres undergo DNA replication in S phase [Palm and de Lange, 2008], this indicates that, after ICL damage, αIISp associates with telomeres when they are replicating. Since ICLs can lead to blocking of DNA replication and production of stalled replication forks in S phase, it can be hypothesized that αIISp is recruited to ICLs at stalled replication forks at telomeres and is needed for recruitment of DNA damage response factors which aid re-initiation of these stalled replication forks [Zhang et al., 2013].

The importance of αIISp in maintenance of telomere function after ICL damage is further demonstrated by studies examining cells in which there is loss of αIISp. In both normal cells, in which αIISp has been selectively knocked down, and FA-A cells, in which αIISp levels are 35–40% of normal, there is lack of localization of αIISp to telomeres after ICL damage (Fig. 5) [Zhang et al., 2013]. There is also a significant increase in telomere dysfunction-induced foci (TIF) in these cells, as measured by examining the presence of γH2AX foci at

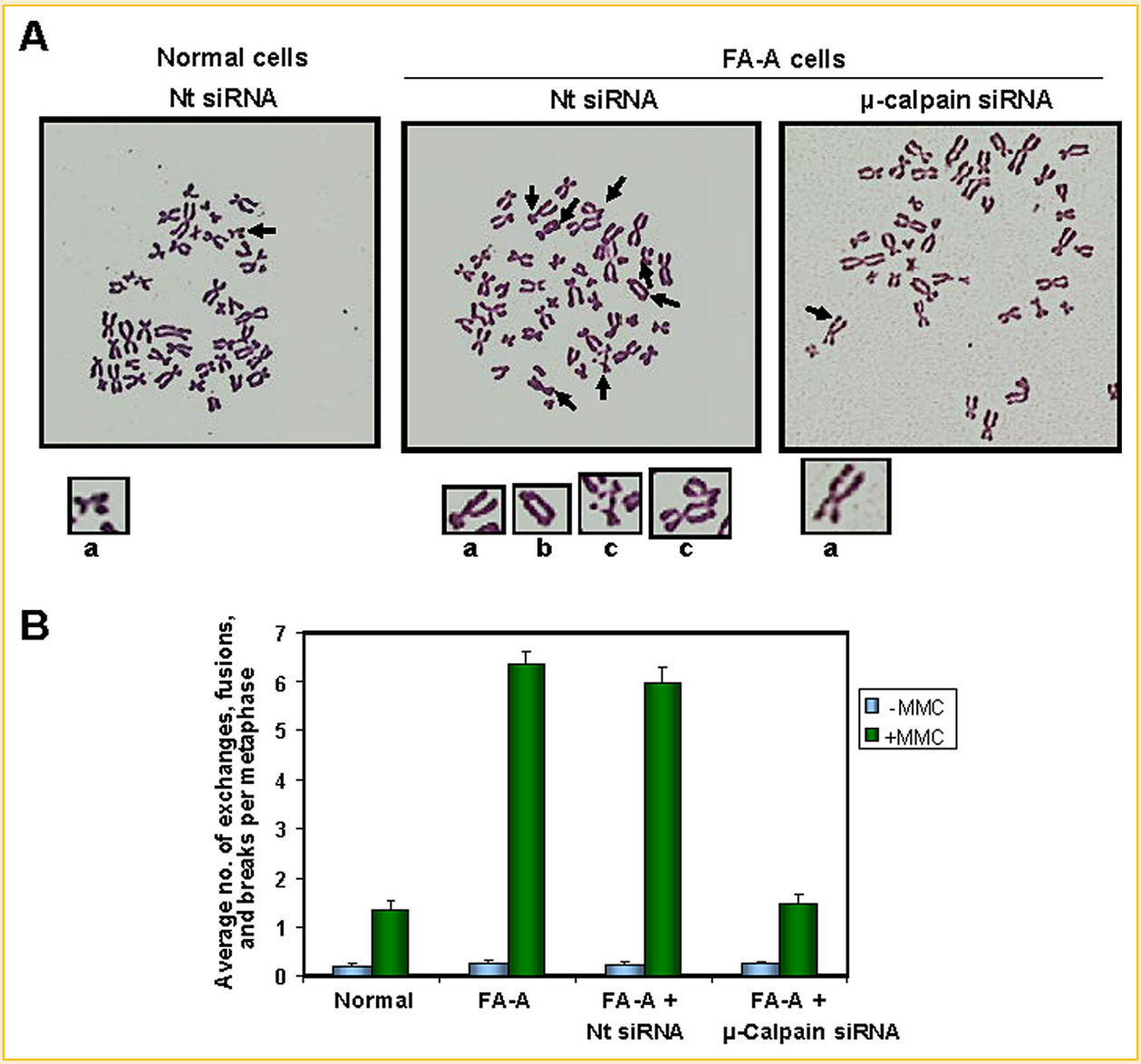

Fig. 4. Knockdown of μ-calpain in FA-A cells by siRNA restores chromosomal stability. (A) Normal and FA-A cells were transfected with non-target (Nt) siRNA and FA-A cells were also transfected with μ-calpain siRNA (300 pM). Cells were subsequently treated (24 h post-transfection) with MMC (30 nM) for 24 h. Metaphase spreads were prepared and examined for chromosomal aberrations (interchromatid exchanges, fusions/radials, and breaks). Arrows indicate these aberrations. Several of the chromosome aberrations indicated with arrows are magnified below each metaphase spread: a. chromatid breaks, b. interchromatid exchange, c. fusion/radial. (B) One hundred metaphase spreads were scored for interchromatid exchanges, fusions/radials, and breaks and the average number of these aberrations per metaphase was quantitated. Vertical lines represent ±S.E.M. (Reproduced from Zhang et al., 2010. Biochemistry 49:5570–5581 with permission from the American Chemical Society).

telomeres, which are markers for DNA DSBs and have been used as an indicator of dysfunctional telomeres [Takai et al., 2003]. In addition, there are five to tenfold increased chromosomal aberrations, in particular sister chromatid end-to-end fusions, after ICL damage (Fig. 4). Significantly, in FA-A cells, telomere dysfunction can be corrected, after ICL damage, when αIISp levels are returned to normal by knocking down μ-calpain; the number of TIF positive cells and chromosome aberrations are also reduced to normal levels when μ-calpain is knocked down (Fig. 4) [Zhang et al., 2013]. These studies show that αIISp is critical in preventing TIF formation and telomere dysfunction after ICLs.

Along with the presence of TIFs and chromatid end-to-end fusions, another indicator of telomere dysfunction is loss of telomeres or signal-free ends (SFEs). An important mechanism proposed for this is that stalled replication forks in telomeres result in replication fork collapse, which leads to formation of DNA DSBs and to subsequent telomere breakage and loss [Palm and de Lange, 2008; Murnane, 2012]. We have shown that loss of αIISp leads to a catastrophic loss of telomeres after DNA ICL damage [Zhang et al., 2013]. In both FA-A cells and normal cells, in which αIISp was knocked down, examination of metaphase chromosomes by FISH using a PNA probe showed that the frequency of SFEs per

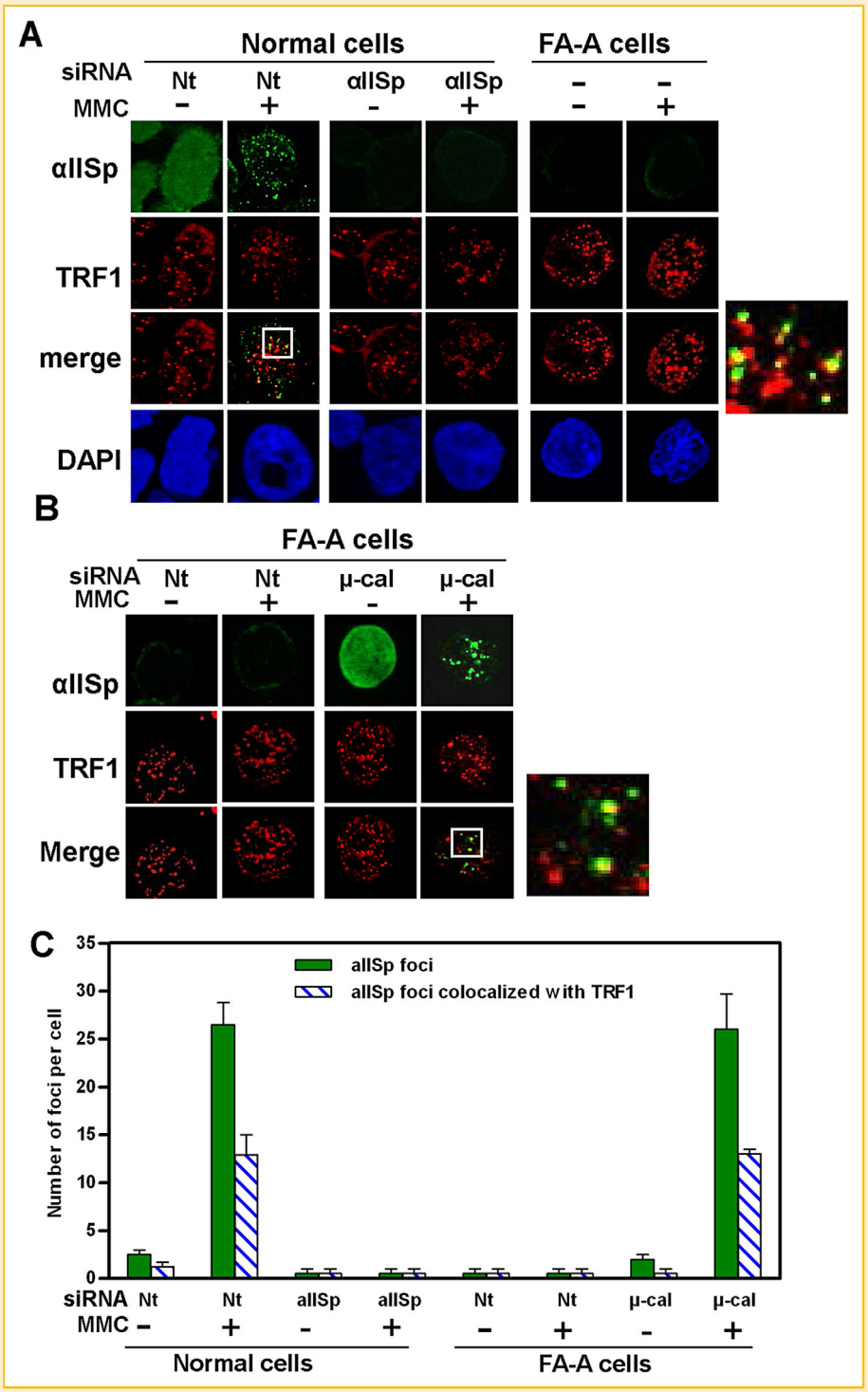

Fig. 5. Knockdown of αIISp in normal cells leads to loss of localization of αIISp to telomeres similar to that observed in FA-A cells. (A) Normal cells, transfected with either Nt siRNA or αIISp siRNA, and FA-A cells were treated with MMC (400 nM). Co-localization of αIISp with TRF1 was examined 16 h after MMC treatment using immunoFISH and staining with anti-αIISp (green) and anti-TRF1 (red) antibodies. Nuclear DNA was counterstained with DAPI (blue). Pictures were taken by z-stack. Only one optical slice is displayed. A magnified image of co-localization of αIISp with TRF1 in MMC treated Nt siRNA transfected normal cells is shown to the right. (B) Knocking down μ-calpain (μ-cal) in FA-A cells restores levels of αIISp and localization of αIISp to telomeres after MMC treatment. FA-A cells were transfected with either Nt siRNA or μ-calpain siRNA and subsequently treated with MMC (400 nM). Co-localization of αIISp with TRF1 nuclear foci at telomeres was examined as above 16 h after MMC treatment. A magnified image of co-localization of αIISp with TRF1 in MMC treated μ-calpain siRNA transfected FA-A cells is shown to the right. (C) The number of αIISp nuclear foci per cell and αIISp nuclear foci that co-localized with TRF1 foci before and after MMC treatment in normal and FA-A cells was quantitated. Bar diagrams represent mean values of five independent experiments. Three hundred cells were counted in each group in each experiment. Error bars represent S.E.M. (Reproduced from Zhang et al., 2013. Nucleic Acids Res 41: 5321–5340 with permission from Oxford University Press).

chromosome increases approximately threefold after ICL damage (Figs. 6 and 7). We have hypothesized that after ICL damage, reduced levels of αIISp prevent efficient repair of telomeric ICLs during S phase resulting in replication fork stalling and formation of telomeric DSBs, which promotes a dramatic loss of telomeres [Zhang et al., 2013]. This catastrophic loss of telomeres in FA-A cells, after ICL damage, can be reversed when αIISp levels are returned to normal by knocking down μ-calpain (Fig. 7) [Zhang et al., 2013]. These studies demonstrate the importance of αIISp for telomere maintenance after ICL damage.

We have proposed that αIISp also acts as a scaffold in recruitment of repair proteins to sites of damage at telomeres [Zhang et al., 2013]. The DNA repair protein heterodimer, XPF/ERCC1, has been demonstrated to play a role in ICL repair of genomic (non-telomeric) DNA [Kuraoka et al., 2000] and we have shown that αIISp is needed for localization of XPF to sites of damage [Sridharan et al., 2003; Zhang et al., 2010]. XPF has additionally been shown to be important in regulation of telomere function and integrity in undamaged DNA; approximately 1% of nuclear XPF associates with TRF2 at telomeres; the rest is dispersed throughout the nucleus [Wu et al., 2008]. Our studies demonstrated that, after ICL damage, αIISp is also needed for the localization of XPF to damage-induced foci at telomeres. After ICL damage, there is increased (more than 10-fold) co-localization of XPF with the PNA probe as well as with TRF1 and TRF2 [Zhang et al., 2013]. The importance of αIISp in the localization of XPF/ERCC1 to telomeres after ICL damage is seen in cells in which there is loss of αIISp. In FA-A cells and normal cells, in which αIISp has been knocked down, XPF fails to localize to telomeres after ICL damage [Zhang et al., 2013]. Restoration of levels of αIISp to normal in FA-A cells, by knocking down μ-calpain, reverses this [Zhang et al., 2013]. These studies indicate that αIISp plays an important role in the repair process at telomeres through recruitment of DNA repair factors such as XPF to telomeres after ICL damage.

These studies were carried out in cells which express telomerase. In human cells, telomeres are maintained and chromosome ends extended during DNA replication either by a pathway in which there is expression of telomerase (a ribonucleoprotein enzyme complex) or by activation of telomerase-independent pathways [Smogorzewska and de Lange, 2004; Blackburn, 2005]. Somatic cells which express little telomerase are deficient in ability to repair DNA damage at telomeres; in contrast, highly proliferating cells, bone marrow, peripheral blood cells, stem cells, and 85–90% of cancer cells express telomerase [Kim et al., 1994]. Thus, the role of αIISp in maintaining telomere and chromosome stability after ICL damage, in telomerase expressing cells, could extend to a wide range of cells and this could be particularly important in disorders, such as FA, in which there is bone marrow cell failure and cancer development. Whether αIISp is also involved in ICL repair in telomerase-negative cells is unknown but will be of interest to examine.

A PROPOSED ROLE FOR FA PROTEINS IN αIISP STABILITY AND THE REPAIR PROCESS

We have shown that reduced levels of αIISp in FA cells are not due to reduced expression of this protein and have proposed that it is due to its reduced stability, in particular its enhanced breakdown by μ-calpain [Lefferts and Lambert, 2003; Zhang et al., 2010]. In FA cells, there is a significant (three to fourfold) increase in μ-calpain activity and this correlates with increased αIISp breakdown and levels of αIISp μ-calpain breakdown product in these cells [Zhang et al., 2010]. Since transfection of cells from at least three FA complementation groups, FA-A, FA-C, FA-G, with the corresponding FA cDNAs restores levels of αIISp to normal, this suggests that FA proteins play a role in maintaining αIISp stability in the cell [Brois et al., 1999; McMahon et al., 1999; Zhang et al., 2010]. We propose that they accomplish this by regulating μ-calpain cleavage of αIISp. There are a number of ways we propose in which this could be carried out by the cell.

αIISp in cells is cleaved by μ-calpain at Tyr^{1176} in repeat 10 and this cleavage is controlled by phosphorylation of this residue by c-Src, a kinase that binds to a flanking scr-homology 3 (SH3) domain in the 9th repeat of αIISp [Nicolas et al., 2002; Nedrelow et al., 2003]. SH3 domains are modular domains that are important in mediating protein–protein interactions and formation of protein networks [Mayer, 2001]. When Tyr^{1176} is phosphorylated, αIISp becomes resistant to μ-calpain cleavage [Nicolas et al., 2002; Nedrelow et al., 2003]. Tyr^{1176} can be dephosphorylated by low-molecular-weight phosphotyrosine phosphatase (LMW-PTP) when it binds to the SH3 domain of αIISp [Nicolas et al., 2002; Nedrelow et al., 2003]. This in turn leads to cleavage of αIISp by μ-calpain. Based on our studies, as well as these of other investigators, we have developed a model for maintance of αIISp stability in normal human cells and the increased breakdown of αIISp in FA cells (Fig. 8) [Zhang et al., 2010]. In this model, a FA protein (e.g., FANCG) binds to the SH3 domain of αIISp. We have shown, using yeast two-hybrid analysis and site-directed mutagenesis, that FANCG binds directly to the SH3 domain of αIISp via a motif which contains a consensus sequence with preference for the SH3 domain of αIISp [Lefferts et al., 2009]. In addition, nine other FA proteins (FANCA, FANCD1, FANCD2, FANCI, FANCL, FANCM, FANCN, FANCP, and FANCQ) contain similar SH3 domain binding motifs and could also potentially bind to αIISp via them [Lefferts et al., 2009]. We propose that in normal cells an equilibrium exists between specific FA proteins, LMW-PTP and c-Src for binding to the SH3 domain of αIISp. When a FA protein (e.g., FANCG) binds to the SH3 domain of αIISp, this prevents binding of LMW-PTP to this domain, thus inhibiting dephosphorylation of Tyr^{1176} and preventing cleavage of αIISp by μ-calpain. In addition, FA proteins (e.g., FANCG and FANCA) could directly bind to μ-calpain, as has been shown using yeast two-hybrid analysis, and in this way inhibit its ability to cleave αIISp [Lefferts et al., 2009; Zhang et al., 2010]. Thus, in normal human cells, one or more FA proteins could play an important role in maintaining normal levels of full length αIISp in one or more ways: an FA protein could bind to the SH3 domain of αIISp, inhibiting the ability of μ-calpain to cleave αIISp, and/or it could bind directly to μ-calpain, inhibiting its activity and ability to cleave αIISp.

According to this model, in FA cells, a deficiency in a specific FA protein, for example FANCG in FA-G cells, would lead to a deficiency in binding of this protein to the SH3 domain of αIISp (Fig. 8) [Zhang et al., 2010]. Of particular interest, there are at least 18 patient-derived mutations in this motif in FANCG which could

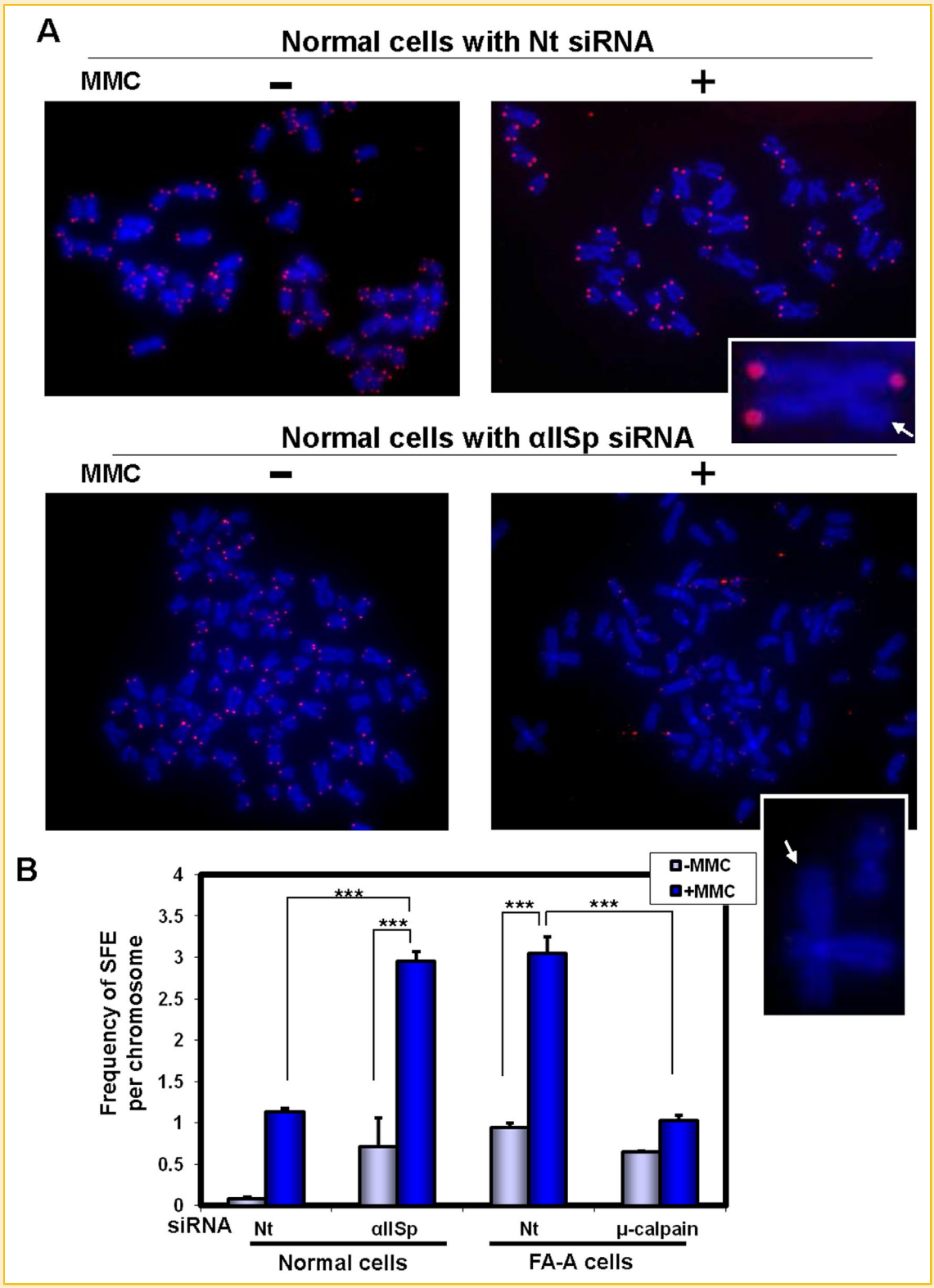

Fig. 6. αIISp deficiency leads to enhanced loss of telomeres after ICL damage. (A) Normal cells were transfected with either Nt siRNA or αIISp siRNA and subsequently treated with MMC (400 nM) for 24 h. Metaphase spreads were prepared and chromosomes stained with DAPI (blue). Telomeric DNA was detected by FISH with a Cy3-labeled telomere specific PNA probe (red). Inserted panels show magnified images of metaphase chromosomes. Arrowheads point to telomere signal free ends (SFEs). (B) Frequency of SFEs per chromosome was quantitated. Averages are shown of five independent experiments in which 4,600 chromosomes were counted per experiment. Student's *t*-test was used to calculate statistical significance. Error bars: S.E.M. ***$P < 0.0001$. (Reproduced from Zhang et al., 2013. Nucleic Acids Res 41:5321–5340 with permission from Oxford University Press).

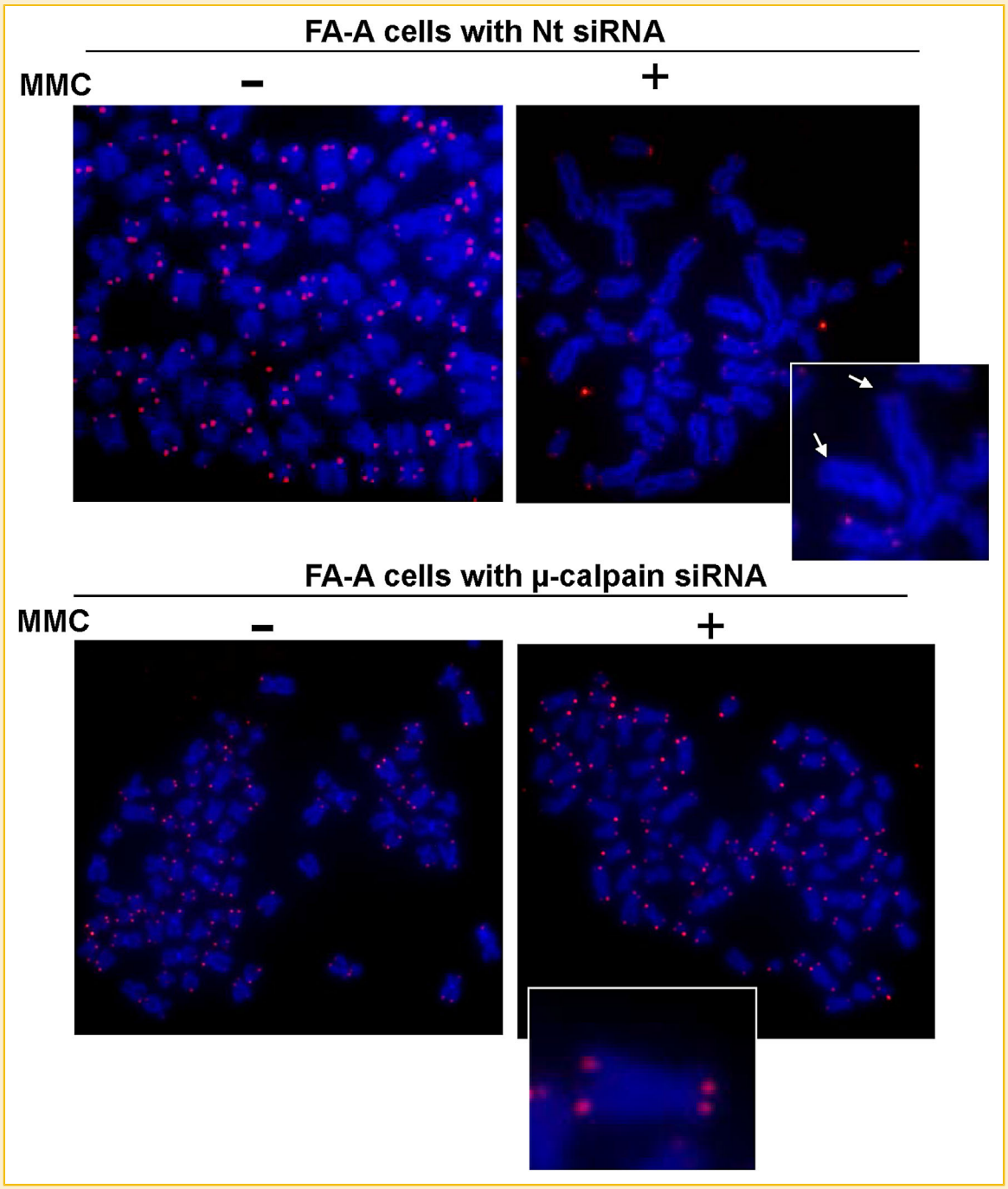

Fig. 7. In FA-A cells, loss of telomeres is enhanced after damage with MMC and this loss is corrected after knockdown of μ-calpain. FA-A cells were transfected with Nt siRNA or μ-calpain siRNA and subsequently treated with MMC (400 nM) for 24 h. Metaphase spreads were prepared and chromosomes stained with DAPI (blue). Telomeric DNA was detected by FISH with a Cy3-labeled telomere specific PNA probe (red). Inserted panels show magnified images of metaphase chromosomes. Arrowheads point to telomere signal free ends (SFEs). (Reproduced from Zhang et al., 2013. Nucleic Acids Res 41:5321–5340 with permission from Oxford University Press).

affect its ability to bind to the SH3 domain in αIISp [Lefferts et al., 2009]. Loss of binding of FANCG to the SH3 domain would allow LMW-PTP to bind to αIISp without interference and dephosphorylate Tyr^{1176}, permitting μ-calpain to cleave αIISp. This would lead to increased breakdown of αIISp in these cells. FANCG would also not be present or not be able to bind to μ-calpain and inhibit its activity, which could lead to increased breakdown of αIISp. It is possible that other FA proteins may be similarly involved in this process, either directly or indirectly. Calpain cleavage of αIISp can also be enhanced by binding of calmodulin to αIISp at its site adjacent to the calpain cleavage site [Harris et al., 1989]. It is possible that one or more of the FA proteins may regulate αIISp stability by modulating calmodulin binding to αIISp. Thus, increased breakdown of αIISp in FA cells could be due to loss

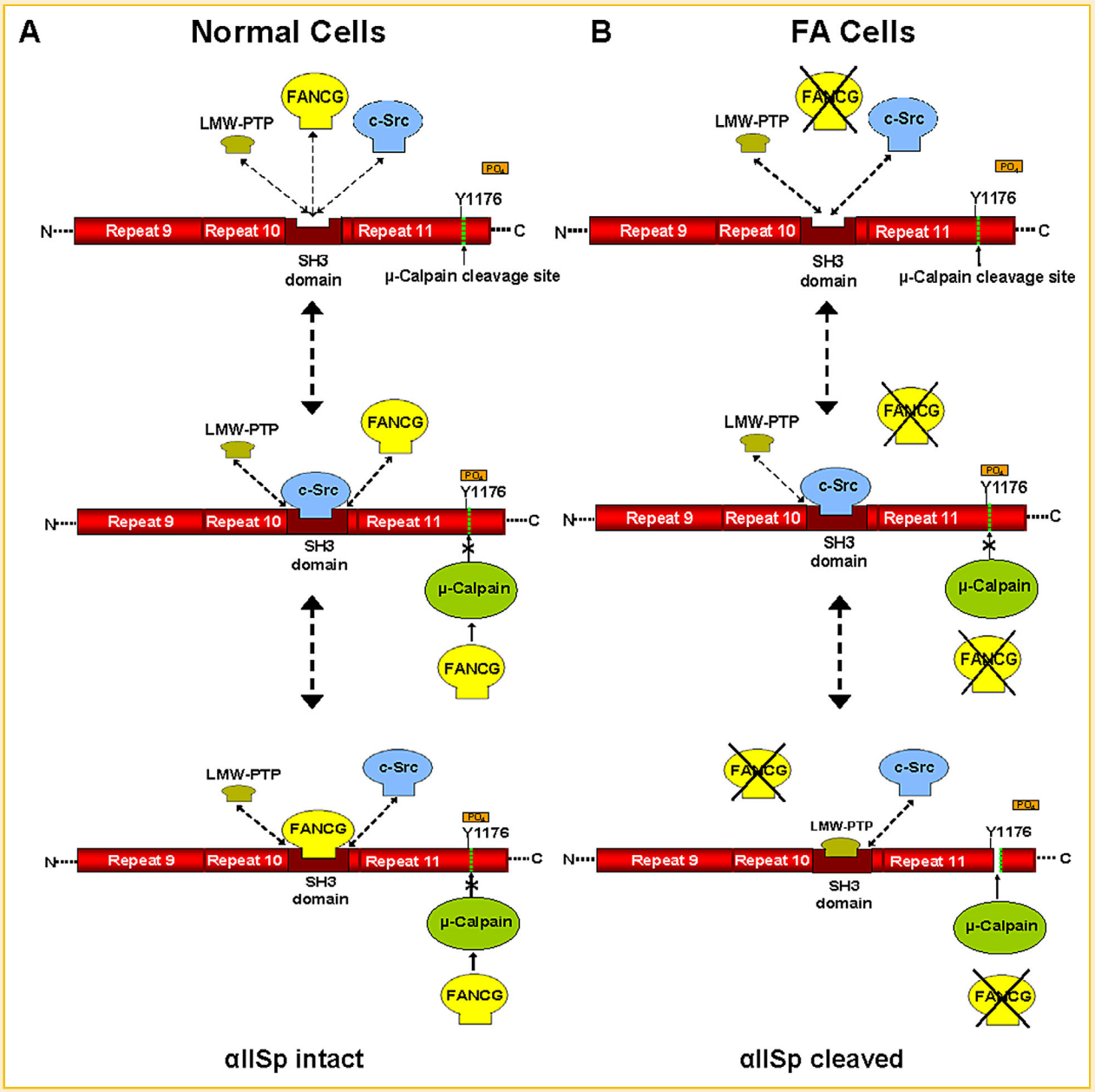

Fig. 8. Proposed model for involvement of FA proteins in cleavage of αIISp by μ-calpain. FANCG is used as an example in this figure; other FA proteins, such as FANCA, may be substituted into this figure. A portion of αIISp is shown containing repeats 9–11. (In some publications, including the present paper, these repeats have been renumbered 8–10). (A) In normal cells an equilibrium exists between LMW-PTP, FANCG, and c-Src for binding to the SH3 domain of αIISp. When c-Src binds to the SH3 domain of αIISp, it phosphorylates Tyr^{1176} (Y1176) and prevents cleavage of αIISp by μ-calpain. However, when MW-PTP binds to the SH3 domain of αIISp, this leads to the dephosphorylation of Y1176, which allows μ-calpain to cleave αIISp at its cleavage site. When FANCG binds to the SH3 domain, this prevents the binding of LMW-PTP to this site. This inhibits the ability of μ-calpain to cleave αIISp at this site, thus preventing the cleavage of αIISp. FANCG (or another FA protein) may also separately bind to calpain and inhibit its ability to cleave αIISp as shown. (B) In FA cells (FA-G cells are used here as an example), there is absence of the FANCG protein and thus there is no binding of FANCG to the SH3 domain of αIISp. LMW-PTP can then bind to the SH3 domain and dephosphorylate Tyr^{1176}, allowing μ-calpain to cleave αIISp at its cleavage site. There is also no FANCG to bind separately to μ-calpain and inhibit its ability to cleave αIISp. This results in μ-calpain breakdown of αIISp in FA-G cells. Similar events may occur in different FA complementation groups (e.g., FA-A). (Reproduced from Zhang et al., 2010. Biochemistry 49:5570–5580 with permission from the American Chemical Society).

of functional FA proteins needed to maintain its stability, which may be a critical factor in the increased chromosomal instability and defective DNA repair observed.

We, therefore, propose a new and critical role for FA proteins: maintenance of αIISp stability in the cell [Lefferts et al., 2009; Zhang et al., 2010, 2013]. This could be an endpoint for FA protein function. When this endpoint is achieved by alternate means, such as occurs when αIISp levels are restored to normal in FA cells by knocking down μ-calpain, then those functions depending on this endpoint (i.e., recruitment of repair proteins to the site of an ICL, and

telomere and chromosome stability after ICL damage) can be carried out. The presence of a specific FA protein would not be as critical. This could explain why phenotypic defects observed in these FA cells, after ICL damage, can be corrected in the absence of a specific functional FA protein when αIISp levels are restored to normal by knocking down μ-calpain. This view is further supported by our studies which show that in normal cells, after knockdown of αIISp, XPF foci do not localize to sites of ICL damage and DNA repair is not carried out, even though levels of FA proteins are normal [McMahon et al., 2009].

CLINICAL RELEVANCE OF LOSS OF αIISP

There is growing evidence implicating spectrin in the pathogenesis of neoplastic bone marrow (BM) disorders and leukemogenesis, in particular acute myeloid leukemia (AML) [Gorman et al., 2007; Wolqust et al., 2011]. A recent study of BM from AML patients found that in 44% of the AML BMs examined there was a loss of αIISp; it was postulated that αIISp could play a role in leukemogenesis [Wolgust et al., 2011]. This is of interest since FA patients have a strong predilation to develop AML. These studies, along with ours in which we show that there is a deficiency in αIISp in FA cells, indicate that loss of αIISp could be an important factor in the etiopathogenesis of a number of BM disorders.

Our studies on restoring levels of αIISp to normal in FA cells by knocking down μ-calpain suggest a new direction for restoration of genomic stability in these cells [Zhang et al., 2010]. μ-calpain is a cysteine protease which is composed of two subunits: an 80 kDa subunit, which has the catalytic activity and is specific for μ-calpain, and a 28 kDa regulatory subunit, which is the same as that of m-calpain [Goll et al., 2003]. The siRNA used in our studies was specific for the 80 kDa subunit. Knocking down μ-calpain in FA-A cells to levels that lead to restoration of αIISp to normal levels had little effect on the viability of these cells [Zhang et al., 2010]. It had no effect on levels of any of the FA or DNA repair proteins examined. Studies using a mouse model have shown that knockdown of μ-calpain has no effect on development [Goll et al., 2003]. Thus approaches targeting reduction of the increased μ-calpain activity present in FA cells, by knocking down μ-calpain, for example, may be an effective way to prevent the increased αIISp cleavage that occurs in FA cells and correct a number of the phenotypic defects that occur after ICL damage. This could serve as a basis for therapeutic intervention in FA.

OTHER POTENTIAL FUNCTIONS OF αIISP IN THE NUCLEUS

We have shown that αIISp interacts with five groups of functionally important proteins in the nucleus of normal human cells: structural proteins, proteins involved in DNA repair, chromatin remodeling proteins, FA proteins, and transcription and RNA processing factors [Sridharan et al., 2006]. αIISp could thus play a role in a number of diverse and important processes in the nucleus. The functional significance of these protein interactions is not yet clear. The association of αIISp with DNA repair proteins and FA proteins, as described above, is the one that has been most clearly elucidated and best documented. The direct binding of αIISp to FANCG, via its SH3 domain, is an excellent example of this. FANCG's interaction with αIISp could be critical for maintenance of αIISp stability and also serve as a mechanism for association, with αIISp, of other proteins involved in the repair process, enabling them to be targeted to sites of damage. We have shown for example, that FANCG in addition to binding to the SH3 domain of αIISp also binds directly, via several of its tetratricopeptide repeat (TPR) motifs, to the central domain of ERCC1 [Wang and Lambert, 2010]. TPR motifs are involved in mediating protein–protein interactions [Hussain et al., 2006]. FANCG thus has two different types of motifs that are important in protein–protein interactions: TPR motifs, several of which interact with ERCC1, and an SH3 binding motif that interacts with αIISp. The SH3 binding motif is located between two of the TPR repeats [Wang and Lambert, 2010]. αIISp could thus serve as a platform for interaction of FANCG with ERCC1/XPF and aid in targeting these proteins, and potentially other proteins, to sites of damage in genomic or telomeric DNA.

αIISp is also important in cellular morphology and its loss can lead to changes in cell shape, cell proliferation capabilities, and cell adhesion and spreading. We have shown, for example, that normal human lymphoblastoid cells in culture are very pleiomorphic, with numerous pseudopodia. However, after αIISp siRNA, the cells are smaller and rounded with few if any pseudopodia and resemble FA-A lymphoblastoid cells, which are also smaller and rounded [McMahon et al., 2009]. Similar results have been observed in a human melanoma cell line, where siRNA knockdown of αII-spectrin leads to the cells becoming rounded and decreased in cell size [Metral et al., 2009]. These cells, in addition, show cell cycle arrest at G_1, have defects in cell proliferation, impaired cell adhesion and spreading, and disorganization of the actin skeleton accompanied by loss of actin stress fibers. It has been suggested that αII-spectrin could play a role in regulation of the actin machinery [Metral et al., 2009]. Interestingly, it has been reported that macrophages in Fancc-/- mice show impaired adhesion, migration, and phagocytosis, and are round in shape rather than having multiple protrusions as do wild type macrophages [Liu et al., 2012]. In addition, dysregulated F-actin rearrangements are observed in these cells [Liu et al., 2012]. Since we have found that there is a reduction in levels of αIISp in bone marrow cells from Fancc-/- mice (unpublished results), it would be interesting to speculate that impaired actin rearrangements and the associated cellular changes occurring in these Fancc-/- cells could be due to loss of αIISp. Collectively, these studies suggest a role for αIISp in organization of the actin skeleton in the cytoplasm. Since αIISp has also been shown to interact with actin in the nucleus [Sridharan et al., 2006; Holaska and Wilson, 2007], it is possible that this interaction could be of similar importance for actin function in the nucleus.

A number of the phenotypic changes observed after depletion of αIISp thus suggest that αIISp is critical for functions associated with both the cytoskeleton and the nucleoskeleton, particularly since it interacts with a number of structural proteins in the nucleus (i.e., lamin A, actin, emerin, protein 4.1, and βSpIVΣ5) [Sridharan et al., 2006; Holaska and Wilson, 2007; Zhong et al., 2010]. It has been suggested that, just as the spectrin-actin-protein 4.1 network is responsible for elasticity and mechanical recovery

after deformation in erythrocytes, their combined presence in the nucleus may contribute to nuclear elasticity as well [Zhong et al., 2010]. Another structural protein, emerin, which binds to A- and B-type lamins in the nucleus, has been shown to interact directly with actin and also associates with αIISp [Holaska and Wilson, 2007]. These studies, collectively, combined with ours suggest that αIISp and its interactions with a number of structural proteins in the nucleus are involved in maintaining the structural integrity of the nucleus, which may, in turn, influence diverse nuclear processes.

CONCLUSIONS

The interaction of αIISp, in the nucleus of mammalian cells, with an array of different proteins indicates that it has an involvement in a number of diverse functions in the nucleus and could serve as a platform for promoting these interactions. However, the nature of these interactions and their importance to nuclear function has been a relatively unexplored area. The best documented role of αIISp in the nucleus is in DNA repair. We have shown that it binds directly to DNA containing ICLs and, after DNA ICL formation, co-localizes at sites of damage, in both genomic and telomeric DNA, with proteins involved in ICL repair. In the hematological, cancer prone genetic disorder, FA, we have shown that there is a deficiency in αIISp in the nucleus and that this is an important factor in the ICL repair defect characteristically observed in these cells. We have proposed a model for the role of αIISp in the repair process in which αIISp binds to DNA at sites of ICLs and acts as a scaffold in recruitment of proteins involved in ICL repair to sites of damage, enhancing the repair process. In FA cells, a deficiency in αIISp leads to a reduction in recruitment of repair proteins to sites of damage, resulting in decreased levels of DNA repair in genomic and telomeric DNA. Thus, reduced levels of αIISp in cells, such as FA, can have an important impact on DNA repair which needs to be considered when examining phenotypic changes occurring after ICL damage.

Our studies show that reduced levels of αIISp in FA cells are due to its increased breakdown by the protease, μ-calpain, which has three to fourfold increased activity in FA cells. We propose that FA proteins play an important role in maintaining αIISp stability in cells by regulating μ-calpain cleavage of αIISp. This could occur in a number of different ways, such a direct binding to the SH3 domain of αIISp via specific motifs which bind to SH3 domains or by direct interaction with μ-calpain. Both of these actions could lead to inhibition of μ-calpain cleavage of αIISp. This represents a new and critical role for FA proteins and could be an endpoint for FA protein function. If this endpoint is achieved by alternate means, in the absence of a specific FA protein, such as when αIISp levels are restored to normal, then DNA repair can proceed. This could explain why phenotypic defects observed in FA cells after ICL damage can be corrected, in the absence of a FA protein, when μ-calpain is knocked down and αIISp levels restored to normal.

Association of αIISp with telomeres is critical for maintenance of telomere function after ICL damage. We have demonstrated that a deficiency in αIISp can lead to loss of telomeres, after ICL damage, in FA-A cells and in cells in which αIISp has been knocked down by siRNA. This in turn leads to telomere dysfunction. When αIISp levels are returned to normal, telomere function is restored. This link between αIISp and telomere function after ICL damage is a previously unexplored area likely to be highly relevant in development of an understanding of factors important in maintenance of chromosome stability after ICL damage.

We have shown that a deficiency in αIISp, which results in DNA ICL repair defects in both genomic and telomeric DNA, can lead to chromosome instability (i.e., intrachromatid exchanges, breaks, and fusions) and is of particular importance in FA. Loss of αIISp has also been noted in BM cells from AML patients and is part of growing evidence implicating αIISp in the pathogeneis of BM disorders and leukemogenesis. Importantly, in FA cells these defects in chromosome instability after ICL damage can be corrected by knocking down μ-calpain, which restores levels of αIISp to normal. These studies, which target knockdown of μ-calpain, suggest a new direction for correcting a number of the phenotypic defects in FA cells after ICL damage and could serve as a basis for therapeutic intervention in FA.

In conclusion, our studies demonstrate that αIISp plays a critical role in maintaining chromosome stability in cells after DNA ICL damage by repairing damage that occurs in both genomic and telomeric DNA. A more in depth examination of the interactions of αIISp with other structural and non-structural proteins in the nucleus is of major importance in the development of insights into the interacting key elements involved in the diverse processes occurring in the nucleus and the consequences loss of αIISp can have on these processes.

ACKNOWLEDGMENTS

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